

Dynamics of natural competence in *Streptococcus salivarius*

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sciences

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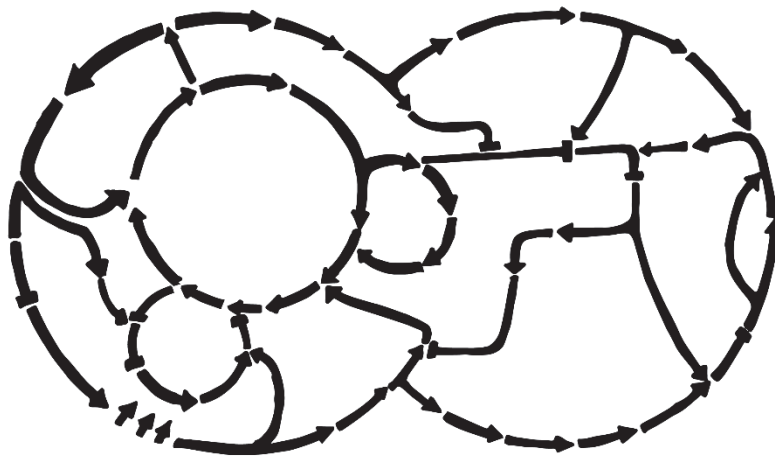
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Abstract

Competence is one of the most successful survival strategies in bacteria. This powerful tactic synchronizes the production of antimicrobial compounds which eliminates competitors, providing genomic material availability for subsequent DNA uptake. Yet, competence regulation in streptococci is only partially understood. Particularly, the molecular mechanisms explaining how activation is restricted to a subpopulation (bimodality) and to a limited window of time (shut-off) are unknown.

In this thesis, we aimed to decipher those complex mechanisms in the major commensal species *Streptococcus salivarius*. Combining molecular biology, biochemistry, microscopy and mathematical simulations, we unveil new regulatory pathways mastering ComRS, the major pheromone-pathway controlling competence in streptococci. In a first set of experiments, we show that the proportion of cells activating competence is directly linked to the concentration of the intracellular receptor ComR. We next demonstrate that this bimodal initiation requires a positive feedback loop on the pheromone *comS*, and identify the environmental sensor CovRS to control this process through *comR* regulation. We next describe a new degradation machinery of the pheromone controlled by competence activation, thereby ensuring ComRS shut-off. Finally, we uncover a link between bacterial cell-wall integrity and competence activation thanks to a genome-wide screen by CRISPR-interference.

Altogether, those results help us understand how bacteria sense their environment and transmit it to complex regulatory pathways in order to produce collaborative and timed responses.

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List of abbreviations

ABC transporter	ATP-Binding Cassette transporter
Acetyl-CoA	Acetyl-Coenzyme A
ADP	Adenine Diphosphate
Ala	Alanine
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
CA	Catalytic ATP-binding domain
CDM	Chemically-Defined Medium
CDP	Cytidine Diphosphate
CMP	Cytidine Monophosphate
CRISPR-I	Clustered Regulatory Interspaced Short Palindromic Repeats Interference
CSP	Competence Stimulating Peptide
CTP	Cytidine Triphosphate
DHP	Dimerization and Histidine phosphotransfer domain
DNA	Desoxyribonucleic Acid
dsDNA	Double-stranded DNA
dTDP	desoxythymidine Diphosphate
GAS	Group A streptococci
GBS	Group B streptococci
GlcNAc	N-Acetyl Glucosamine
Glif	Galacto-furanose
Glu	Glutamic acid
GroP	Glycerol-Phosphate
HAMP	Histidine kinases, Adenylyl cyclases, Methyl binding proteins, Phosphatases domain
HK	Histidine Kinase
HTH	Helix-Turn-Helix domain
LTA	Lipoteichoic Acid
LUCA	Last Universal Common Ancestor
Lys	Lysine
ManNAc	N-Acetylmannosamine
m-DAP	meso-Diaminopimelic acid
MIP	Mutacin Inducing Peptide
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
MurNAc	N-Acetyl Muramic acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NGS	Next-Generation Sequencing

NOD-like	Nucleotide Oligomerization Domain receptor-like
ODE	Ordinary Differential Equations
PASTA	Penicillin-binding protein And Serine/Threonine-kinase Associated domains
PBP	Penicillin Binding Protein
PG	Peptidoglycan
QS	Quorum-Sensing
RboP	Ribitol-Phosphate
REC	Phosphate Receiver motif
Rgg	Regulator Gene of Glucosyltransferase transcription factors family
Rha	Rhamnose
RNA	Ribonucleic Acid
RR	Response Regulator
RRNPP	Rap, Rgg, NprR, PlcR, and PrgX family
Ser	Serine
SsDNA	Single-stranded DNA
STK	Serine Threonine Kinase
STKP	Serine/Threonine Kinase Protein
TCS	Two-Components System
TPR	Tetratricopeptide domain
UDP	Uridyl-Diphosphate
UMP	Uridyl-Monophosphate
UTP	Uridyl-Triphosphate
WTA	Wall Teichoic Acid
XIP	ComX Inducing Peptide

INTRODUCTION

1 Foreword

One of the first question the FRIA jury asked me at the proposal presentation to get the grant I would work on was “Why do we care about *Streptococcus salivarius*?”. This question highlights not only the fact that this species is still mostly unfamiliar in the microbiology field but also that even in fundamental research, spotlights remain mainly on human pathogenic species, which *Streptococcus salivarius* is not.

So, let me present you this bug and convince you that the study of this species will bring forward our understanding of microbial collective behavior, intercellular communication, predation, evolution, adaptation and biochemical network regulation.

S. salivarius is a Gram-positive bacterium belonging to the *Firmicutes* clade, *Bacillae* class, *Lactobacillales* order, *Streptococcus* genus. Species from this genus display an ovococcal shape (ovococcus) with a propensity to form chains by division and are facultative anaerobes [1]. More precisely, *S. salivarius* is part of the salivarius group encompassing only three close-related species including *S. thermophilus*, a dairy food starter and *S. vestibularis*, another inhabitant of the oral cavity. In the mostly pathogenic streptococcal genus, *S. salivarius* stands as an exception since its presence has been positively correlated with human healthy oral cavity and digestive tract [2]. This latter property has driven companies to commercialize *S. salivarius* as a probiotic and recent studies suggest that pathogenic species depletion would occur through small antimicrobial compounds production known as bacteriocins [3,4]. Moreover, the study of those compounds has revealed their wide spectrum including major human pathogens such as the methicillin-resistant *Staphylococcus aureus* (MRSA) [4].

Aside from medical applications, *S. salivarius* is a particularly relevant species to study microbial survival strategies. Indeed, this bacterium lives in the human oral and gastro-intestinal tract, one of the most challenging bacterial environments. Huge microbial density and diversity combined to inconstant physico-chemical conditions have driven evolution towards efficient survival tactics [5–7]. Not only the tactics show high diversity but their implementation can take various modalities, from individual to collective, from intra to interspecies and from competition to cooperation [8,9].

Orchestrating concomitant bacteriocin production and natural transformation, competence in streptococci is a glaring illustration of such ploy.

While the production of antimicrobial compounds eliminates competitors, rival cell lysis guarantees genomic material availability for subsequent DNA uptake [4,10,11]. While genomic data accumulation together with the emergence of antibiotic multi-resistant strains have underlined the importance of this strategy for bacterial long-term adaptation and evolution, the activation of this peculiar process remains costly from an energetic point of view [12,13]. This is presumably the reason why bacteria have evolved complex regulation circuitry to tightly control competence activation-deactivation. These specific networks schemes allow bacteria to restrict competence activation to only a subpopulation (bimodality) and to a limited time window (shut-off) [14–17].

In this thesis, we sought to investigate those two processes in the ComRS system, the competence signaling pathway present in *S. salivarius* and in most streptococci. Using classical genetic and biochemical tools together with mathematical simulations and genome-wide genetic screens, we tried to identify and understand how biochemical networks and interactions can give rise to population differentiation (bimodality) and temporal limitation (shut-off).

To understand the experimental work and the investigations conducted during this thesis, I will first introduce the competence process and describe the transformosome necessary for natural transformation. I will then summarize the known regulatory pathways controlling competence in streptococci. The next section will focus on discussing phenotypic heterogeneity in the particular case of competence development. As we report a link between competence and cell-wall assembly, I will shortly describe current literature about cell wall biogenesis. Finally, I will discuss the most relevant streptococcal environmental sensors regarding the experimental work of this thesis and conclude with a brief discussion on genome-wide screens.

2 Competence

As previously mentioned, competence is a particular physiological state during which bacteria can activate natural DNA transformation and bacteriocin production. Bacteriocins secretion will trigger competitors cell death and release of genetic material, and concomitant natural transformation will allow DNA uptake and chromosomal integration of foreign DNA [10,18]. While the natural transformation process is mostly conserved, competence and bacteriocin regulation is most often species-specific and differs greatly according to the lifestyle of the bacteria [19,20].

2.1 Natural transformation

Natural transformation refers to the process involving the uptake and the genomic integration of foreign DNA. It has been shown that this mechanism requires 14 genes in *Streptococcus pneumoniae* [21,22]. Those genes are strongly conserved in other competent bacterial species and even in Gram-negative species, where some other proteins are necessary to facilitate the DNA entry through the outer membrane [23].

Natural transformation has been mostly described in the models *Bacillus subtilis* and *S. pneumoniae* [21,24,25]. Little work has been performed in *S. salivarius* to study the transformation process, but since proteins with high sequence homology are found in those three species, most probably they share the same transformation mechanisms. For this reason, I will describe in this section the natural transformation process based on *B. subtilis* and *S. pneumoniae* knowledge.

2.1.1 DNA uptake

DNA uptake starts with the production of a pseudopilus, a structure evolutionary related to type IV pili. This structure is made of a major pilin-like protein ComGC and involves six other proteins necessary for transformability [26,27]. Among those six players, we find four minor pilin-like proteins (ComGD, ComGE, comGF, ComGG) of unclear function, an ATPase (comGA) and a platform protein (ComGB) associated to ComGA (Fig. 1). The translocation of the pilus across the cell wall involves also ComC, a prepilin protease that will help to process and translocate comGC across the cell envelope [28,29]. The pseudopilus produced can reach 2-3 μm long as shown in *S. pneumoniae* and can directly bind dsDNA. It is not yet clear how the structure retracts and brings DNA to the cell membrane, but Lam and colleagues were recently successful to image the pilus retraction in *S. pneumoniae* and hypothesized that the ComGA ATPase necessary for pilus assembly could also be involved in the pilus retraction [30,31].

Next, double-stranded DNA (dsDNA) is conveyed to ComEA, which delivers it to EndA, a nuclease with competent-independent constitutive expression that will produce single-stranded DNA (ssDNA). This degradation seems necessary for the translocation into the cytosol through a transmembrane channel formed by ComEC, coupled to ComFA, an ATP-dependent translocase (Fig. 1) [21,24].

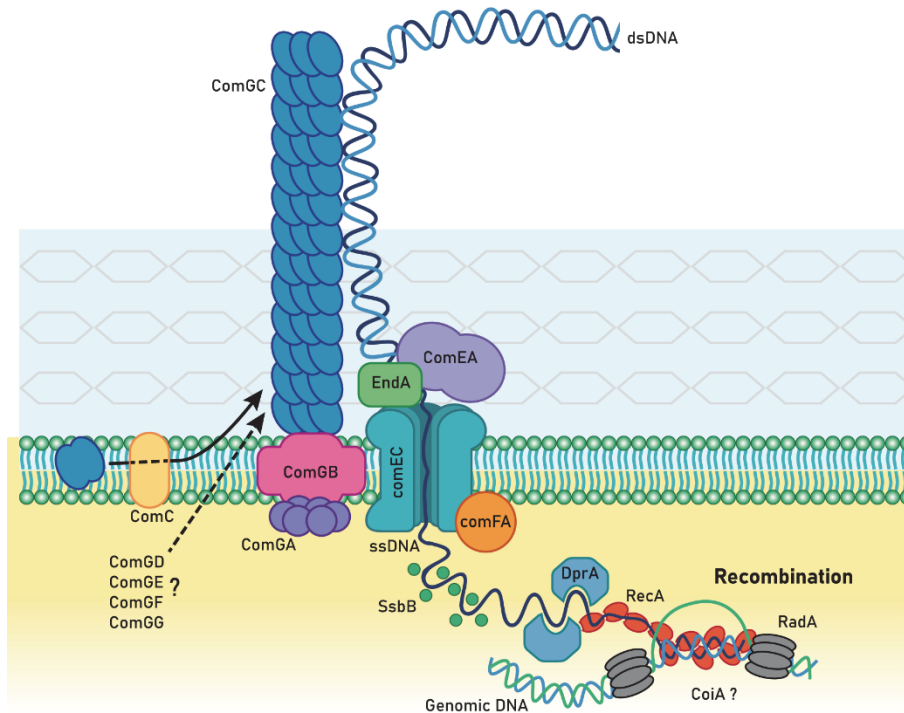


Figure 1. Schematic view of the transformasome. dsDNA binds to the ComGC pseudopilus constituted by ComGC monomers. Maturation and translocation through the membrane occurs thanks to ComC, ComGB and the ATPase ComGA. Supplemental prepilin ComGD-E-F-G are involved in the process with unclear functions. Once DNA binds the pseudopilus, it is most probably depolymerized thanks to ComGA-B and is driven to ComEA, which in turn will bring dsDNA to the nuclease EndA. This latter produces ssDNA which is translocated across the membrane through the ComEC translocase with the help of the ComFA ATPase. ssDNA is then protected by SsbB from degradation, and DprA will then bind DNA and recruit RecA, which will scan for homology and perform recombination with the help of RadA, a DnaB-type helicase. CoiA is involved in this process by an unknown mechanism.

2.1.2 Chromosomal integration

Once ssDNA enters the cytosol, it is rapidly coated by SsbB, protecting DNA from degradation [32]. SsbB is then displaced by DprA, the RecA loader. RecA will subsequently scan the genome for homology with the ssDNA and catalyze the strand exchange reaction that integrates ssDNA into the recipient's genome [33–35]. This step requires RadA, a DnaB-type helicase, which will facilitate the RecA-driven ssDNA recombination by unwinding dsDNA on the edges of the recombination site and stabilizing the D-loop structure [36]. A supplemental protein called CoiA has been shown to be dispensary for ssDNA uptake but necessary for chromosomal integration, suggesting a role in DNA processing or recombination [37].

Of note, extensive data have been collected on the interactions between all the proteins involved in the uptake, the processing and the integration of DNA, which suggest a strong interconnection between all the players. Those evidences made emerge the concept of the transformasome, a complex machinery involving all the proteins previously described [24].

Recently, important work has been achieved, mainly in the human pathogenic species *Vibrio cholerae*, to characterize the transformasome machinery in Gram-negative bacteria [25,38]. While several common players with Gram-positive bacteria were shown for the inner membrane translocation and ssDNA recombination, the diderm structure requires another type of pilus. Indeed, it was shown that a type IV pilus consisting of PilA subunits matured by the prepilin peptidase PilD can elongate or retract with the help of the PilB or PilT ATPase, respectively [39,40]. The complete structure of the pilus comprises many other subunits including PilCFMNOPQ [38].

2.2 Regulation

As previously mentioned, while the transformasome is broadly conserved, competence regulation is rather species-specific and is tightly dependent on the bacteria lifestyle [19]. In *B. subtilis*, competence has been shown to be triggered upon entry into stationary phase and depends on the cell density and the sensing of a signaling peptide. Other *Bacillae* display the same general scheme but the activation of competence seems to be rather link to nutrient exhaustion than cell density [41]. *Vibrio cholerae*, on the other hand, triggers competence upon chitin binding, a polymer found on many arthropods where the bacterium lives [42]. Finally, the *Streptococcus* genus relies on two distinct pheromone-based systems operable during early exponential phase in laboratory conditions. The trigger of those pathways remains elusive but several Two-Components System (TCS) sensors were shown to simultaneously control the regulation, suggesting a multifactorial activation [19,43,44].

The two pheromone-based systems identified in streptococci are the so-called ComRS and ComCDE pathways. The ComRS system is found in the groups pyogenes, bovis, mutans and salivarius and the ComCDE in the anginosus and mitis groups, the latter comprising the best studied organism of the genus: *S. pneumoniae*. The Figure 2 shows the phylogenetic distribution of the systems in streptococci [43].

While those two regulation systems share common features (positive feedback loop, pheromone production), they also differ in many aspects such as

the pheromone recognition, occurring intracellularly for the ComRS system, and extracellularly for the ComCDE system. Nonetheless, both systems will ultimately lead to the transcription of a gene encoding an alternative sigma factor (ComX) which will guide RNA polymerase on specific promoters controlling genes coding for the transformosome machinery [19].

2.2.1 ComCDE

Initially discovered in *S. pneumoniae* [45], the ComCDE system relies on the activation of the *comCDE* operon that produces ComC, a small hydrophobic peptide which is matured and exported thanks to the ABC transporter ComAB. The extracellular mature peptide called CSP (Competence stimulating peptide) is then able to bind the histidine kinase ComD, which in turn will transfer a phosphate to its cognate response regulator ComE (Fig. 3). The phosphorylated ComE (ComE~P) can then bind a conserved DNA motif found in the promoter of *comX*, the alternative sigma factor. In parallel, ComE~P will bind the same conserved motif on at least 17 other promoters, such as P_{comAB} and P_{comCDE} , resulting in a positive feedback-loop [44]. The genes activated by ComE~P are defined as the early genes in opposition to the late genes activated by ComX. The early regulon comprises several key players such as ComW, a stabilizer of ComX and BlpAB, a bacteriocin ABC transporter (ATP binding cassette transporter). This latter system allow concomitant bacteriocin production with natural transformation [10]. Once the alternative sigma factor ComX is produced, it will lead RNA polymerase on specific target promoters and trigger the late competence genes. Those genes are much more numerous than the early ones and encompass about 80 different players among which only 14 are necessary for natural transformation [22].

In addition to its transcriptional control by ComCDE, ComX is also regulated at the post-translational level by the ClpEP proteolytic machinery [46]. This control acts as a locking device, preventing basal level of ComX to activate spontaneously competence late genes. Interestingly, in *S. pneumoniae*, a competence essential early gene called *comW* plays a role in ComX stabilization and activity. This protein has been shown to stabilize ComX by preventing ClpEP degradation and is suggested to bind aspecifically DNA and promote ComX-RNA polymerase association [47–49].

Aside from the main TCS regulator of competence (ComDE), another distal regulator, CiaRH, has been shown to regulate competence [50–53]. Puzzling preliminary results showed that while CiaRH negatively impact ComCDE production, CiaR did not repress *comCDE* transcription [54]. Since then, two

main mechanisms have been proposed to explain CiaRH repression of competence in *S. pneumoniae*. The first one involves the activation of the virulence factor HtrA by CiaRH, which would ultimately lead to extracellular CSP degradation and subsequent competence disruption [55]. The other mechanism involves the activation of a small interference RNA, which binds the *comC* transcript, impairing translation [56].

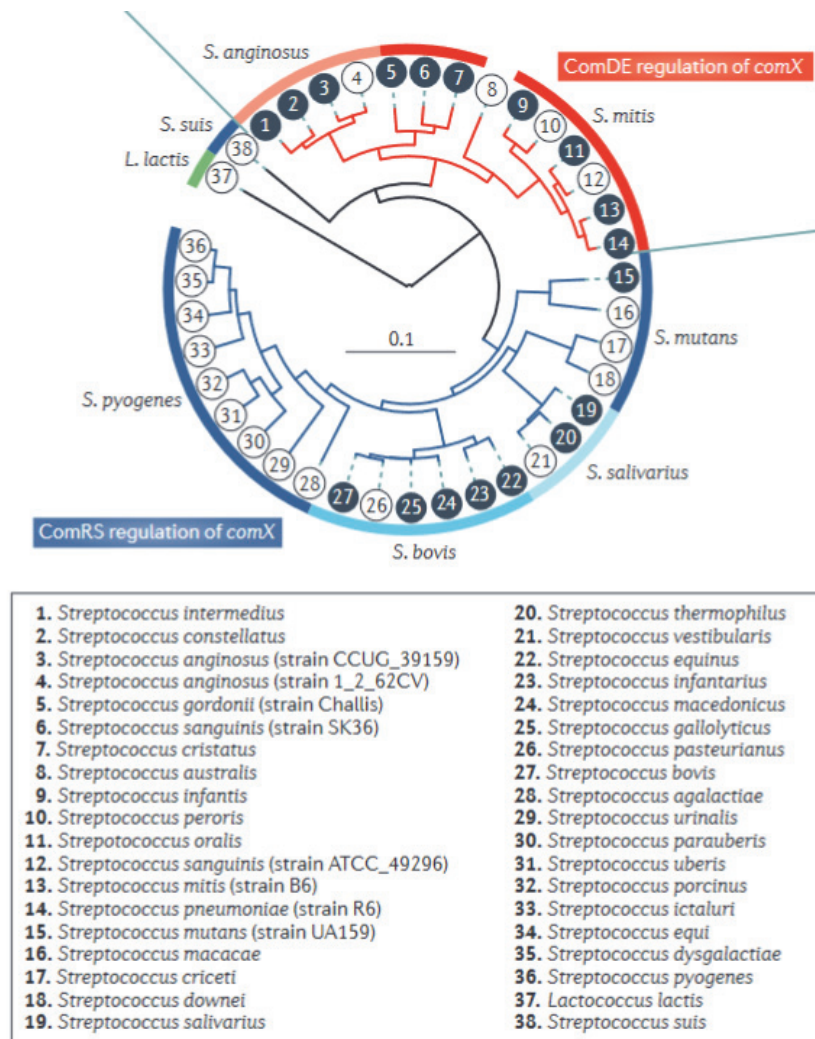


Figure 2. Distribution of the two regulatory pathways of competence in streptococci. Filled circles with white digits indicates species that have been shown to be naturally transformable. Scale bar represent phylogenetic distances. Figure from [43] modified from [14].

Another TCS, LiaSR (or VraSR) has been shown to sense cell-wall damages produced by autolysin activity during competence activation [57]. The activation of this system leads to the production of stress proteins helping the cell to resist to autolysis (also known as fratricide) [57]. Finally, another TCS, VicRK (or WalRK) has also been hypothesized to regulate competence in *S. pneumoniae*. Although some authors showed that VicRK mediates oxygen sensing to competence, more recent studies did not confirm this link [58–60]. The VicRK controversial regulation has also been suggested to be activated by the Serine-threonine kinase StkP [61]. This muropeptide sensor (see section 4.2 for details) has been shown to upregulate *comCDE* competence genes and *blpRH* bacteriocin regulatory genes in *S. pneumoniae* and *Streptococcus mutans*, through phosphorylation of the VicR response regulator [62–66].

Aside from the serine-threonine kinase (StkP) and TCS direct (ComDE) or distal (CiaRH, VicRK) regulation of competence, another completely different mechanism for competence activation has been described in *S. pneumoniae*. Indeed, Martin and co-workers showed that competence can be activated upon DNA replication impairment [67]. This mechanism has been later characterized and relies on the genomic position of the *comCDE* and *comAB* operon, close from the origin of replication. This key position results in a copy number increase of those operons upon replication disruption and therefore increase their transcription, ultimately leading to competence activation [68].

While the molecular mechanisms involved in competence activation have been identified for long in *S. pneumoniae*, specific exit of competence through a negative feedback loop has been only recently uncovered. To date, only one clear mechanism has been described. This mechanism involves DprA, a late competence gene product necessary for RecA loading onto DNA during homologous recombination for natural transformation [69]. Mirouze and colleagues showed that this protein has a second key role in natural transformation by shutting-off competence. Indeed, they showed that DprA can inhibit ComE phosphorylation, therefore ending activation of early genes and ComX production [14]. More recently, the same team has shown that the DprA-mediated shut-off takes place at one cell-pole thanks to ComX conveying DprA next to ComD [70]. Although this is the only shut-off mechanism described in *S. pneumoniae*, supplemental layer of shut-off have been hypothesized (see section 2.2.6) [15].

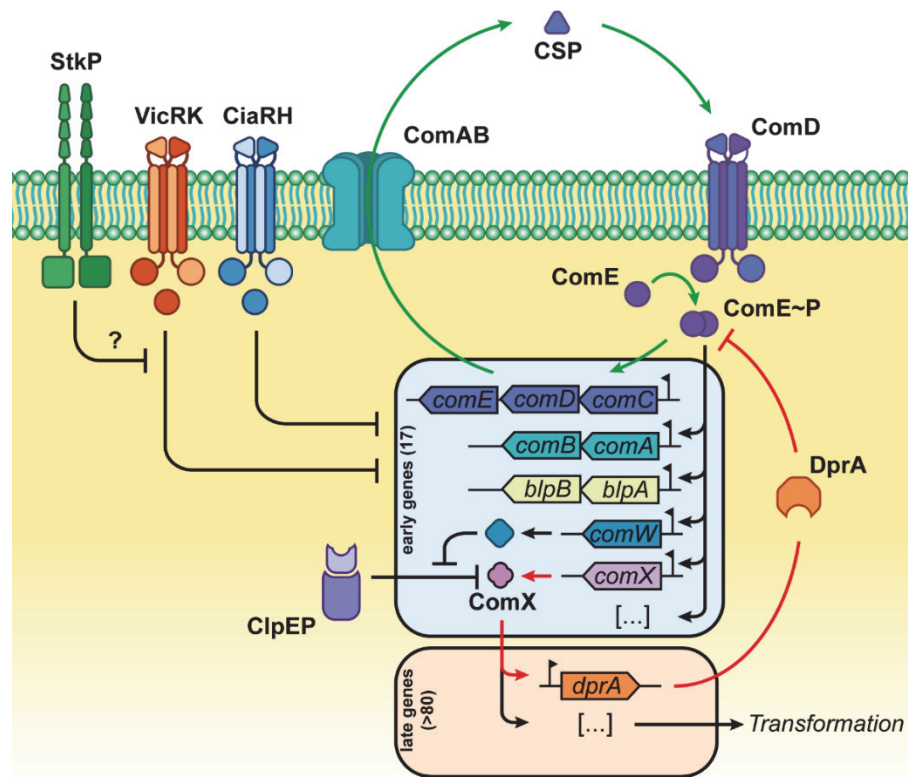


Figure 3. ComCDE competence regulation. In the ComCDE system, competence is triggered upon ComC production, which is matured and exported by the ComAB transporter to produce the CSP pheromone. This pheromone can then bind ComD and trigger a phosphorylation cascade ultimately leading to the response regulator ComE phosphorylation (ComE~P). ComE~P activates about 17 promoters of the so-called early competence genes among which the ComAB and ComCDE operons, resulting in a positive feedback loop. ComE~P will also trigger ComX production which will activate about 80 promoters associated to competence late genes, triggering natural transformation and other physiological responses. Among the late genes, DprA is produced and will impair ComE~P phosphorylation, resulting in a negative feedback loop. Of note, ComX is controlled at the post-translational level by the ClpEP protease machinery. This activity is inhibited by ComW. Aside from the proximal regulation, distal regulators such as, CiaRH, VicRK and StkP seem responsible to couple environmental stimuli and proximal regulation.

2.2.2 ComRS

Discovered by P. Hols' team in 2010, 15 years after ComCDE, the ComRS system is the most widely spread competence regulation scheme in streptococci [43,71]. It shares many common features with the ComCDE system but also displays several key divergences such as the pheromone intracellular

importation and a positioning of the pheromone receptor *comR* gene outside of the positive feedback loop (Fig. 4).

2.2.2.1 Peptide secretion and maturation

Competence activation starts by the production of the peptide ComS, which is exported through the PptAB ABC transporter and matured by Eep, a membrane protease [72]. Those processes would occur simultaneously, since the catalytic site of Eep is predicted to be located at the transmembrane domain and PptAB was shown to only secrete the mature form of the pheromone [73]. Of note, PptAB has been described as the main ComS exporter in *Streptococcus thermophilus* but results obtained in *S. mutans* suggest that a supplemental mechanism of secretion is taking place, since PptAB disruption does not impair completely ComS secretion [74]. Interestingly, streptococcal ComS peptides sequences do not share common secretion signatures [19], suggesting either that PptAB has a wide secretion spectrum or that other exporters are involved.

From the maturation point of view, all the identified ComS were shown to be matured into their C-terminal domain called XIP (ComX inducing peptide). Mass spectrometry analysis of a *S. thermophilus* supernatant showed XIP pheromone size of 9, 10 and 11 amino-acids ((I)(A)ILPYFAGCL) but synthetic peptides as short as 8 amino acids were shown to be sufficient to trigger the system [72,75,76]. XIP sequences can be classified into three classes. The first and second groups are characterized by a double aromatic residues ((Y/F)F or WW, respectively). The first class comprises the salivarius group while the second encompasses all the other species of the ComRS streptococci. A third class, specific only to some *Streptococcus suis* strains, is characterized by a WG(K/T)W motif [19].

Once in the extracellular medium, the peptide can be reimported through the generic Ami/Opp oligopeptide transporter (Fig. 4). Of note, the free diffusion of the peptide in the medium is still a controversial question. While XIP has been shown to freely diffuse extracellularly when produced in *S. mutans*, XIP produced by *S. thermophilus* seems to remain associated to the cell wall [72,77]. The same disagreement exists for the CSP pheromone of *S. pneumoniae* [78,79], suggesting that the type of communication could be strain-specific.

2.2.2.2 ComR structure and ComR·XIP formation

Once the peptide has been internalized by the Ami/Opp transporter, it can bind to the intracellular sensor ComR. This regulator is part of the widely-spread RRNPP family (Rgg, Rap, NprR, PlcR, PrgX) in *Firmicutes*, which are intracellular receptors able to bind small peptides and perform transcriptional regulation upon binding. Particularly, ComR is part of the Rgg subfamily largely represented in streptococci and lactococci [80]. From a structural point of view, ComR is made of two main domains. The first one, a tetratrico peptide-like domain (TPR) is responsible for the peptide binding. A small hydrophobic pocket is able to bind key hydrophobic residues of XIP which will ultimately lead to the release of the second domain, a helix-turn-helix (HTH). Once this domain is released, the ComR·XIP complex can dimerize and can bind a conserved palindromic motif called a ComR-box [76,81].

Transcriptomic analysis by RNA sequencing of the ComR·XIP regulon performed in *S. salivarius* allowed the identification of a ComR-box consensus sequence present in 17 promoters [4]. Genes regulated by ComRS include *comS*, *comX* and several bacteriocin-related promoters such as the *blpK* or *slvX* operon. The activation of *comS* through the ComR·XIP is of particular interest since it will trigger a positive feedback loop strengthening activation. In parallel, *comX* activation will result in the production of the alternative sigma factor ComX, which will lead RNA polymerase on promoters harboring a specific motif signature [4]. As reported in *S. pneumoniae*, those genes are called the late competence genes, and will lead to the transformosome assembly and subsequent natural transformation (see section 2.1.1).

Similarly to *S. pneumoniae*, ComX undergoes a post-translational control by the proteolytic machinery ClpCP. This proteolytic apparatus is associated to MecA, an adaptor protein that will specifically bind ComX. This machinery acts as a locking device, preventing spontaneous competence activation [82,83].

How the ComRS system stops once the pheromone positive feedback loop has been activated is still an open question (see section 2.2.6). Several negative players have been described, but none of them was shown to constitute a true shut-off mechanism *i. e.* being under competence control (negative feedback loop) and resulting in longer and more intense activation when inactivated.

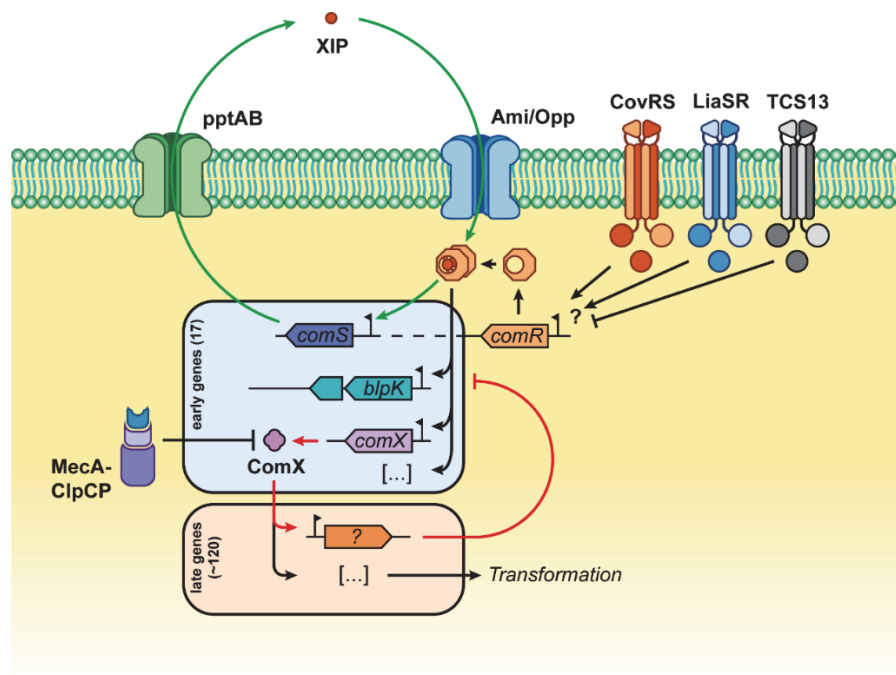


Figure 4. ComRS competence regulation in *S. salivarius*. Competence is induced upon ComS production, which is exported by the PptAB exporter, matured into XIP and reimported through the Ami/Opp importer. XIP can then bind ComR to form the complex ComR·XIP which can dimerize and trigger transcription of the early competence genes, among which *comS*, *comX* and bacteriocin genes such as *bfpK*. Activation of *comS* results in a positive feedback loop boosting competence (green arrows). In parallel, ComX will lead RNA polymerase on late competence genes promoters which will trigger the assembly of the transformasome and subsequent natural transformation. Of note, ComX is controlled at the post-translational level by the MecA-clpCP proteolytic complex. In *S. salivarius*, CovRS, LiaSR and TCS13 are suspected to control *comR* transcription (FVC data). On the other hand, mathematical and experimental data obtained in *S. thermophilus* suggest an unknown late competence gene to be involved in a negative feedback loop.

2.2.2.3 Distal regulators

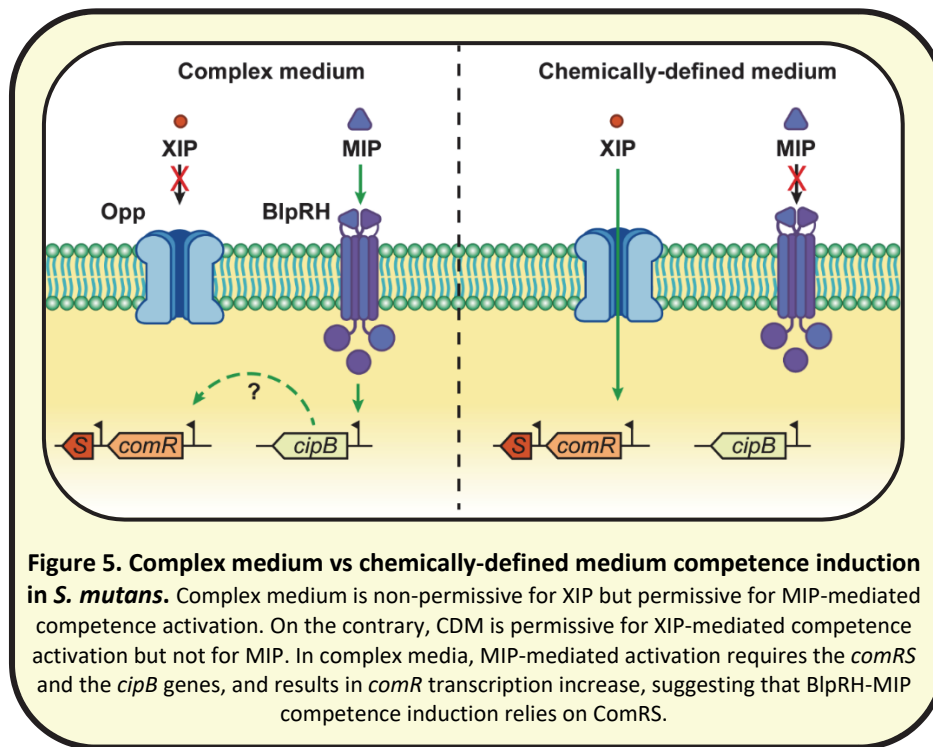
Several distal regulators have been identified in ComRS streptococci and shown to integrate environmental cues and transmit the signal to enhance or reduce ComRS activation [19]. Identification of such interactions have been particularly addressed in the *S. mutans* species, but molecular details of those interactions are still poorly understood. For instance, inactivation of the pheromone peptide *scnC* associated to the ScnRK two-components system (oxidative/acidic stress and biofilm formation) results in a 10-fold reduction of competence [84].

BOX 1: The particular case of *S. mutans*

Streptococcus mutans is a major dental pathogen responsible for cariogenic dental plaque. In this well-studied species, a Quorum-Sensing (QS) system similar to the *S. pneumoniae* system has been firstly described as the main competence regulatory apparatus and therefore named ComCDE [85]. The discovery of the ComRS system in *S. thermophilus* in 2010 led to the identification of an homolog system regulating competence in *S. mutans*, suggesting the presence of the two regulatory pathways in the same species. Indeed, competence could be activated with the two specific peptides of the systems: CSP and XIP [86,87].

Since then, the identification of a new TCS controlling bacteriocin production in streptococci (BlpRH) has shown to share more homology with the previously named “ComCDE” system [43,44]. Moreover, “ComCDE” activation of competence through ComX production has been shown to require ComRS in this species, suggesting that the “ComCDE” system of *S. mutans* is not the proximal regulator [88]. It is now clear for most of the competence scientists that the “ComCDE” system of *S. mutans* is a BlpRH homolog and activates competence through an indirect mechanism [19,89]. This is the reason why we will favor the terms BlpRH, and MIP (Mutacin inducing peptide) to describe this QS system triggering competence in *S. mutans*.

Importantly, several studies have shown that XIP can trigger competence in *S. mutans* by promoting the ComRS activation, but only in chemically-defined medium (CDM) [88–90]. In parallel, complex medium is not permissive for XIP-competence induction, most probably because of Opp peptide saturation [91]. On the other hand, MIP can induce competence only in complex medium (Fig. 5). This intriguing result is still not fully understood but some key transcriptomic and mutational data suggest that BlpRH and MIP are able to up-regulate *comR* only in complex medium. Of note, this activation requires an intact copy of *cipB*, encoding a bacteriocin under the control of BlpRH [92,93].



Similarly to *S. pneumoniae*, the CiaH sensor was also shown to regulate competence in *S. mutans*. But conversely to the pneumococcus, the CiaH sensor was suggested to positively regulate competence, together with mutacin and biofilm production [94]. Other distal controls have been highlighted such as the HdrRM and the BsrRM systems, which consist of a membrane bound inhibitor protein (HdrM and BsrM) that inhibit the activity of their associated transcription factor (HtrR and BsrR). Cross-talk would occur between those two systems, as BsrM inactivation or HtrR overexpression both trigger ComR up-regulation [95–97]. More recently, BsrR was also shown to protect ComX from the ClpCP degradation machinery, mimicking the ComW function in *S. pneumoniae* [98].

The most characterized distal regulator in *S. mutans* is the BlpRH bacteriocin TCS (Box 1). Upon MIP addition, this TCS will trigger mutacin (CipB) production which upregulates *comR* in complex media by an unknown mechanism [99]. Since complex media is not permissive for XIP importation, another mechanism of ComR-XIP formation must be present. Strikingly, experimental evidences have shown that ComR can be activated by the unmaturred form

of ComS in *S. mutans* and trigger competence, bypassing the exportation of the pheromone signal [88,100].

In the salivarius group, literature is scarce regarding distal control of competence. Recent work conducted in our lab allowed the identification of 14 TCS in *S. salivarius*. Systematic knock-out of those TCS were performed and their effect on *comR* and *comX* transcription monitored (F. Vande Capelle data). This study suggests that CovS and LiaSR have a positive impact on *comR* transcription while a newly described TCS (named TCS13) represses *comR* expression. This analysis shows several differences with the previously identified competence-related TCS of *S. mutans*, suggesting that distal regulators are species-specific. This assumption makes sense regarding the fact that different species have diverse lifestyles and must trigger competence upon distinct stimuli.

2.2.3 Bacteriocins

In streptococci, natural transformation is always triggered during competence by the alternative sigma factor [19]. On the other hand, the control of the production of bacteriocins can be species-specific. Indeed, the general paradigm in streptococci to activate antimicrobial compounds during competence involves the pheromone-based TCS BlpRH [44]. While different activators of this system exist during competence (ComR-XIP for *S. thermophilus*, ComE~P for *S. pneumoniae*, and ComX for *S. mutans*), all those species use the BlpRH system as the central controller for bacteriocin production [4].

By contrast, it was recently shown in our lab that *S. salivarius* has developed a shortcut to activate bacteriocins directly via ComRS activation [4]. In this species, there is no functional BlpRH and this shortcut might provide to *S. salivarius* a way to kill other bacteria earlier during competence activation. This would provide an advantage over competitors and explain the prevalence of this species in the human digestive tract [4].

2.2.4 Signal propagation

In streptococci, both regulation systems (ComRS and ComCDE) lead to the production of a small peptide which is exported extracellularly. This signal could therefore be sensed by other bacteria displaying the appropriate receptor. This type of communication acting as a quorum-sensing module could play a role to synchronize a response across a population and was investigated in various streptococci [77–79]. In *S. pneumoniae*, contradictory results were obtained showing either signal propagation via free diffusion of

the CSP pheromone [78] or requiring cell-to-cell contact for CSP-induction of competence [79]. In the ComRS species, early studies in *S. thermophilus* suggested the retention of the secreted XIP pheromone in the cell wall, implying a cell contact to transmit the signal [72]. Contrary results were latter obtained in *S. mutans* where it was shown that the XIP pheromone can freely diffuse in the extracellular media [77]. Together, those results suggest that the competence signal propagation among a population could be species and even strain-specific.

2.2.5 Peptidases

Recently, several pheromone-degrading enzymes have been described in streptococci. Those peptidases not only degrade competence-related pheromones, but also other communication peptides associated to Rgg regulators involved in various functions. In *S. pyogenes*, PepO has been shown to degrade at least two pheromones associated to Rgg2 and Rgg3, two transcriptional factors involved in biofilm development and lysozyme resistance [101–103]. Moreover, a PepO homolog was found to degrade XIP in *S. mutans* and the peptidase knock-out resulted in a ComRS activation increase [104]. The specificity of the cleavage has not been investigated, but those studies highlight a supplemental layer of control in pheromone-based communication.

2.2.6 Shut-off

Since competence is energy-consuming and shifts the metabolism to such an extent that regular cell cycle is impaired, limiting its activation to a sharp window of time is crucial [12–14]. This process, known as the shut-off, has been mostly studied in the ComCDE system of *S. pneumoniae* where DprA inhibits ComE phosphorylation, ultimately leading to early genes extinction [14,15,70]. This mechanism showcases a perfect shut-off apparatus, where competence induction triggers a negative feedback loop which will decrease both the timing and the intensity of the activation of the competence genes (Fig. 6A, mechanism 1).

Other mechanisms have been suggested for the shut-off in *S. pneumoniae*. Indeed, the same team showed that a $\Delta comX$ strain, therefore lacking the DprA shut-off, was still able to shut-down competence [105]. They provided an explanation for this observation by demonstrating that the unphosphorylated form of ComE was able to bind the promoter of the *comCDE* operon without activating it. This strongly suggest that upon competence activation, ComE outcompetes ComE~P for P_{comCDE} binding, ultimately leading to

comCDE inhibition (Fig. 6A, mechanism 2). Another layer of control was proposed by Weng and colleagues [15]. By building a *comX-comW* inducible strain, they were able to activate late competence genes independently from the early ones. They next demonstrated that this strain was still able to shut-down competence, suggesting a mechanism excluding ComE. They showed that the ClpCP or ClpEP machineries were not involved in this regulation and that the late genes transcripts levels were dropping before ComX or ComW concentrations. Those observations ruled out any degradation mechanisms and suggested that a late competence gene would inhibit ComX/ComW transcriptional activity (Fig. 6A, mechanism 3). Nevertheless, they were unsuccessful to identify such player.

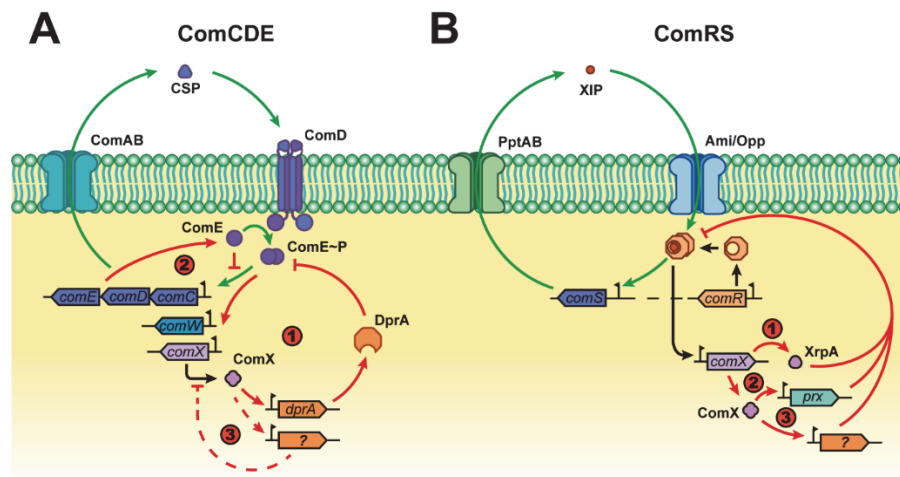


Figure 6. Shut-off in ComCDE and ComRS species. A. In the *S. pneumoniae* ComCDE species, three distinct mechanisms take place. The first one involves a negative feedback loop triggered by ComX and acting through DprA to inhibit the ComE~P transcriptional activity (①). The second one is mediated by the excess of unphosphorylated ComE produced by the *comCDE* operon activation. The unphosphorylated form of ComE binds the promoter of *comCDE*, competing with ComE~P (②). The third hypothetical mechanism involves an unknown actor under the control of ComXW and inhibiting ComX transcriptional activity (③). **B.** In the ComRS species, three mechanisms also exist but were reported in three different species. The first one observed in *S. mutans*, implies the production of a small protein, XrpA, which is produced in an alternative open reading frame within the *comX* gene (①). The second one reported in *S. pyogenes* involves the ComX-regulated *paratox* gene, which can prevent ComR dimerization (②). Finally, in *S. thermophilus*, a third mechanism was suggested to act through an unknown actor under the control of ComX (③).

In the ComRS species, several negative players of competence have been described, but none of them displayed the proper features of a shut-off apparatus. One of those mechanism described in *S. mutans* relies on the

production of a peptide called XrpA (ComX regulatory peptide A) [106]. Strikingly, this peptide is encoded within the *comX* gene but in another reading frame. The transcriptional activation of *comX* results in the production of two distinct mRNA, one encoding *comX* and the other *xrpA*. XrpA biochemical characterization has shown that it can interact with ComR, preventing XIP binding and subsequent transcriptional activation [107]. Although this player activity is embedded within a negative feedback loop, *xrpA* inactivation did not result in longer competence activation, ruling out its role in ComRS shut-off (Fig. 6B, mechanism 1).

In the same species, an intriguing post-translational mechanism was recently shown by Qin and colleagues [98]. They highlighted the role of BsrR, a LytTR transcriptional regulator associated to the membrane component BsrM, as a ComX protector of the ClpP proteolytic activity. This mechanism was shown to be sufficient to trigger late competence gene activation without stimulating the early ones. This represents an alternative pathway to ComRS to trigger natural transformation. From a shut-off point of view, BsrR is able to bind several late competence proteins, which will shift equilibrium and results in ComX release from the BsrR-ComX complex upon late gene activation. This will lead to ComX susceptibility to the ClpP machinery, finally shutting down late competence genes activation. Nevertheless, this system appears restricted to the BsrRM activation of competence and seems not functional for the classical ComRS activation.

In *Streptococcus pyogenes*, a XrpA similar mechanism involving another player has been identified. In this species, the mosaic prophage paratox gene termed *prx* was shown to be upregulated upon ComRS activation, thanks to a ComX-box located in the promoter of the gene [108]. Biochemical and structural characterization of Prx inhibition showed that this protein can bind ComR and stabilizes its apo-form even when the ComR-XIP complex is already formed. This mechanism thus prevents dimerization and subsequent DNA binding (Fig. 6B, mechanism 2) [109]. Interestingly, this mechanism sheds light on the interplay between phages and competence inhibition. Since competence provides a way to cure any acquired prophage by integrating DNA material from close-relative species, competence inhibition can be an efficient survival strategy to cure integrated prophages. Those ecological aspects aside, paratox mutants did not exhibit longer competence activation, suggesting that this mechanism is not a proper shut-off.

Competence shut-off has also been investigated in *S. thermophilus*. A mathematical model developed by Haustenne and co-workers suggested the presence of a ComX-activated protein involved in competence exit (Fig. 6B, mechanism 3). To test if a *S. pneumoniae* similar shut-off mechanism could occur in *S. thermophilus*, a *dprA* mutant was generated, but did not result in ComRS longer activation [110].

2.3 Bimodality

2.3.1 Phenotypic heterogeneity

Asexual mode of proliferation is one of the most successful strategies in living organisms but comes with a cost. Low genetic diversity reduces survival options among populations, therefore decreasing overall fitness of a community. To overcome this deficiency, prokaryotes have evolved alternative mating systems such as conjugation or natural transformation. In parallel, another successful strategy consist to produce phenotypic heterogeneity in a clonal population [111].

Phenotypic heterogeneity is a process that will allow the emergence of a sub-population with a distinct phenotype. Those phenotypes can have diverse functions such as sporulation, competence, toxin production, exopolysaccharide and biofilms production, or bioluminescence [112]. Ecological advantages of heterogeneous activation at the community level have been studied in various systems and mainly two types of benefits were identified: division of labor and bet-hedging [112].

The first strategy implies two or more different pathways both having a positive effect on bacterial fitness. Yet, the two pathways are somehow more interesting to express independently. Because the population will profit from both strategies, benefits are shared even though sub-populations specialize into one or the other process. Division of labor is particularly well exemplified by the surfactin-matrix production in *B. subtilis* biofilms [113]. In those structures, cells specialize into surfactin producer or matrix producer. The surfactin producers seem to signal to other cells to trigger matrix production although not activating matrix production themselves. Despite molecular details of this interaction are still unclear, co-culture of engineering strains specialized in one or the other process has shown to be more efficient than the natural strain, underlining the power of division of labor [114].

Phenotypic heterogeneity can also serve in the context of a bet-hedging strategy. This tactic differs from labour division in the sense that the two (or

more) physiological functions co-activated do not synergize in a positive effect on fitness. Generally, one of the activated systems is not suited to the current condition but might be more effective in a future situation. If a change of favorable environmental circumstances occurs, the sub-population having activated this system will be advantaged. This bet on a future change is a risky game and limiting it to a sub-population enables the community to choose multiple tactics at the same time. This approach is also particularly relevant in the context of natural transformation, when DNA uptake can result in genetic benefit or disadvantage [111].

Molecular mechanisms underlying heterogeneous activation in a clonal population have been challenging to understand. Early 2000's development of single-cell tools has allowed the emergence of important concepts necessary to decipher it. Using single-cell fluorescent reporters, Elowitz and co-workers were the first to show that homogeneous conditions and clonal population are permissive for protein number heterogeneity between cells [115]. Moreover, they showed that lowly expressed genes are the noisiest (most stochastic), while highly expressed genes display more homogenous distribution across a population. This observation must be linked to the limited actor number in transcription and translation. As the number of partner drops, the probability of their encounter decreases and the randomness of the protein production increases, enhancing protein distribution heterogeneity within the population [112]. Nevertheless, this process known as noise or stochasticity is inherent to low number of actors which are often not sufficient to trigger a physiologically significant action. This is the reason why bacteria have evolved various manners to amplify signal noise and enhance heterogeneity. For instance, strong ribosome binding sites associated to low mRNA copy number maintain heterogeneous mRNA distribution while providing a high number of proteins for cells expressing the gene [116–118]. On the other hand, topological gene circuitry such as mutual repression or positive feedback loops can also amplify heterogeneity [111,119,120].

Interestingly, heterogeneous activation has been extensively described for QS systems. Those sensing mechanisms rely on the detection of a small molecule called a pheromone, which is transmitted into a signal to trigger various function. Initially thought to be only a cell density mechanism, increasing evidences showing links between environmental conditions and the QS efficiency have modified the QS opinion [112]. Not strictly limited to a communication device, they also collect multiple inputs which will regulate their

efficiency, as exemplified by the streptococcal ComRS/ComCDE pH dependency [78,131].

BOX 2: Genetic circuitry modeling

As molecular biology moved forward, challenges did not lie in gene characterization anymore, but rather on the description of interwired genetic circuitry [121]. New complex pathways were described and it was soon clear that mathematical modeling would help to understand how those systems would react. Indeed, their intricacy made difficult any human-brain rational prediction. Since then, several models have helped to answer fundamental biological questions, in complementation to wet-lab traditional work [16,122–125].

To describe biochemical processes, two main types of model exist. The first one, called determinist, describes biochemical reactions by ordinary differential equations (ODEs). This type of model portrays homogeneous systems with continuous variable, which are based on the phenomenological law of mass action [126]. Since ODEs are intensively used in many other scientific fields, numerous tools are available to construct such models and are therefore common. On the other hand, stochastic modeling can describe biological systems based on discrete variables [127]. Those models, rather than considering constant reactions, are built on the probability of reaction events, which are function of the number of players modeled in the system. Much more uncommon, this method is however most suited to describe biological processes involving low number of actors, such as lowly expressed bacterial genes [126]. Especially, bimodal activation through noisy gene amplification by positive feedback loop were successfully described by those simulations [16,122].

Yet, stochastic model design remains complicated, and a prevalent method consist to design a determinist model describing the biological processes at work, which is further translated into stochastic equations by dedicated algorithm [128,129]. This method has proven to be efficient for low number of variables, but has a high computational cost for systems involving numerous variables [126].

Recent breakthrough in artificial intelligences solving complex problems might be the next step in genetic circuitry simulation. The evolution of this technology that requires large data set to train and improve together with the current burst of biological data (genomic, transcriptomic and proteomic) might provide a way to solve biological circuitry complexity. Future will tell us how this technology can help describe and understand sophisticated biochemical regulation and design new synthetic circuits [130].

2.3.2 A case-study: competence in *B. subtilis*

One of the best studied natural phenotypic heterogeneity is the competence activation in *B. subtilis* [111]. Early work in this species has shown that competence arises only in a subpopulation, which displays high level of ComK, the central regulator of competence (Fig. 7) [132–136]. The segregation between the two populations occurs in stationary phase and has been referred to bistability since those two states are relatively stable in time. In this species, the competence regulatory pathway has been extensively studied and comprises about 30 different players. Nevertheless, bistability relies only onto one regulatory feature of the system. Indeed, it has been shown that ComK upregulates its own expression. This positive feedback loop was proven experimentally and theoretically to be sufficient to trigger bistability [16,137,138]. Mathematical stochastic models (Box 2) have suggested that noise on ComK basal expression was amplified by the positive feedback loop, ultimately leading to different cell fates [138,139]. This assumption is confirmed by experimentally manipulating the level of noise, showing that a two-fold noise decrease of *comK* leads to a ten-fold reduction of the probability to switch to the competent state [16]. Furthermore, another study focused on reducing noise while keeping the same population-wide ComK concentration. To this aim, they engineered a strain displaying *comK* higher transcriptional activity while lowering translational efficiency. Following this approach, it was shown that significantly less cells were able to enter the competent state [140].

Another interesting feature of the competence regulation in *B. subtilis* is the involvement of a proteolytic machinery to control ComK abundance. Indeed, it was shown that the MecA-ClpCP complex can degrade ComK, therefore preventing entry into competence [141,142]. Yet, this locking device can be inhibited upon entry into stationary phase. Once a critical cell density is reached, a QS system involving the pheromone ComX, the exporter ComQ and the TCS ComPA triggers the production of a small protein termed ComS, which will bind MecA (Fig. 7) [143]. This binding allows ComK to escape from the degradation complex and generates permissive conditions for the positive ComK feedback loop ultimately leading to bistability [144]. Altogether, those investigations highlight how bacteria can take advantage of genetic circuitry to amplify intrinsic noise and generate heterogeneous activation.

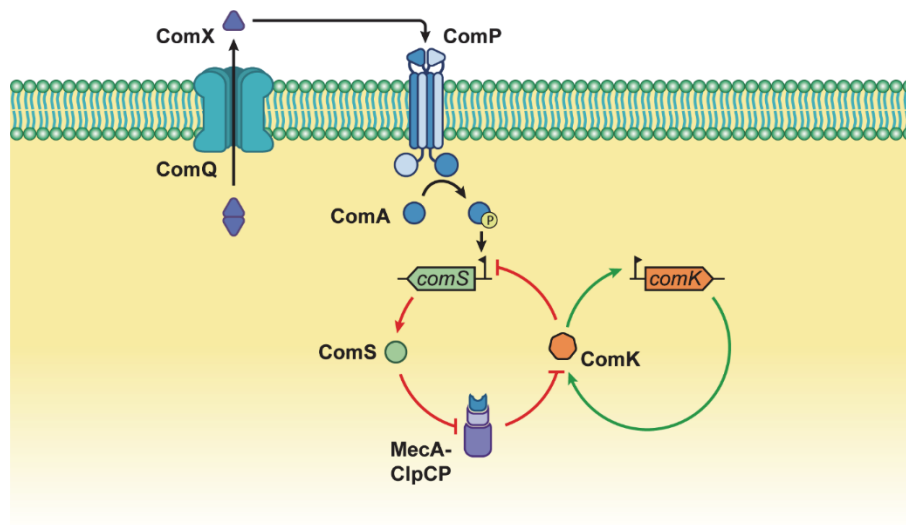


Figure 7. *Bacillus subtilis* competence regulation. Upon entry into stationary phase, the extracellular concentration of the ComX pheromone exported by ComQ reaches a critical concentration sufficient to trigger a phosphorelay mediated by ComPA. The phosphorylated form of ComA then triggers the transcription of *comS*, which will bind MecA, thereby preventing ComK degradation through the MecA-ClpCP machinery. Subsequently, ComK availability will increase and ComK will activate its own transcription. This positive feedback loop is the central mechanism to explain bistability of competence in *B. subtilis*. This figure is a simplification and does not contain all the known regulators of competence for this species.

2.3.3 ComRS

Bistability has also been observed for competence in streptococci [78,88]. Although mostly present in literature, the idea of bistability for the heterogeneous activation of competence is not completely accurate for streptococci. Indeed, ComRS competence is a transient phenomenon, and bimodality describes more precisely the transitory duality of competence activation. Bimodality in ComRS streptococcal species has been mostly studied in the pathogenic species *S. mutans*. As previously mentioned (Box 1), identification of a TCS able to activate competence and sharing homologies with the ComCDE system of *S. pneumoniae* led to name it ambiguously “ComCDE” [85,145]. Further identification of the ComRS system in this species together with the fact that this TCS shares more homology with the BlpRH system of *S. pneumoniae* led the scientific community to consider it as a BlpRH homolog [20].

BlpRH and its interaction with the ComRS system have been thoroughly investigated in *S. mutans* [44]. This is probably because BlpRH activation results in a bimodal activation of competence. Interestingly, bimodality is only

triggered in a complex medium upon BlpRH activation by MIP, while MIP cannot trigger competence in a chemically-defined medium (*i.e.* peptide-free medium) [90]. Conversely, XIP can induce competence only in a chemically-defined medium, in a unimodal fashion [88]. To explain such intriguing results, considerable experimental work has been achieved the last decade. A first clue to explain bimodality origin was given by the fact that bimodal MIP induction requires a functional ComRS system [88]. This suggested that bimodality could arise from the ComRS system itself and was not due to the BlpRH activation of ComX. Since then, several studies have shown that BlpRH controls *comR* transcription in a complex medium which requires an intact copy of the *cipB* gene encoding a bacteriocin [89,90,92,93,99]. The exact molecular details of this link are still missing and to know if ComR is upregulated because of CipB activity or another mechanism (*e.g.* cell envelope stress) is still an open question (Fig. 8) [44].

While the activation of the ComRS system by the MIP-induced BlpRH through CipB was clear, several other observations were still confusing. In particular, the observation of a unimodal activation of the BlpRH-controlled CipB bacteriocin together with the bimodal activation of BlpR was senseless [90]. An explanation was provided by Reck and colleagues, who showed that ComX controls BlpRH transcription [89]. This result confirmed by another team argues again for ComRS being responsible for bimodality generation [146]. Indeed, unimodal activation of CipB through MIP-BlpRH could then be transmitted to the ComRS system, which would generate and transmit bimodality to *comX*, ultimately activating BlpR in a bimodal fashion (Fig. 8).

Because ComRS bimodality generation most probably arises from a positive feedback loop as similarly observed in *B. subtilis*, it was intriguing that this phenomenon could take place in a complex medium. Indeed, the positive feedback loop relies on the externalization and maturation of the signaling peptide which is then reinternalized by the Ami/Opp importer. Since XIP importation has been shown to be impaired in a complex medium, how to conciliate those two models? An answer was brought by Underhill and colleagues [100]. They showed that intracellular ComS is able to activate ComR and trigger an intracellular positive feedback loop, which could be responsible for bimodality (Figure 8). This explains also the observation that bimodality can be triggered by MIP in a Ami/Opp mutant lacking the XIP importer [88].

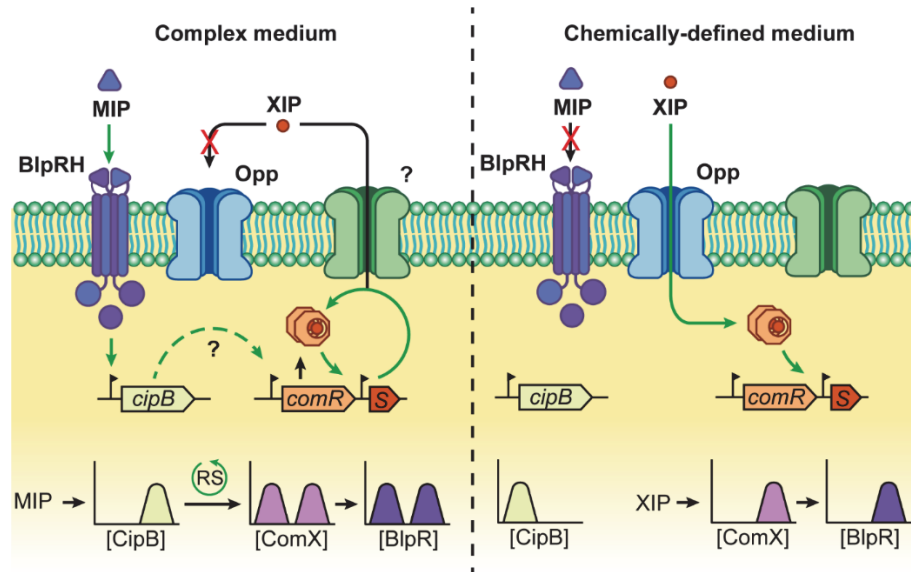


Figure 8. Current model explaining bimodality in *Streptococcus mutans*. In complex medium, MIP can trigger unimodal *cipB* activation. CipB then upregulates ComR by an unknown mechanism, which triggers a ComRS positive feedback loop involving the intracellular non-mature form ComS. The loop generates bimodality on *comX* which in turn activates BlpR in a bimodal fashion. In chemically-defined medium, MIP cannot induce *cipB* but XIP can trigger unimodal activation of *comX* which in turn activates BlpR in a unimodal fashion.

Regarding environmental factors triggering bimodality, several studies have highlighted key determinants such as pH, carbon source and oxidative stress [131,147,148]. In parallel, the endopeptidase PepO was shown to directly regulate XIP concentration and its absence was associated to unimodal competence activation, presumably via an increase in XIP availability [104]. Nevertheless, a direct proof of the bimodality generation by the ComRS system is still lacking.

3 Gram-positive cell wall

Several results obtained during this thesis suggest a link between cell-wall integrity and competence activation. In particular, a CRISPR-interference (CRISPRi) screen uncovered a link between the inhibition of several key enzymes involved in the cell-wall assembly and competence. This connection has been previously suggested by a Tn-Seq screen in *S. mutans* but the mechanism of this link is still unknown [149]. In parallel, teichoic acid have been shown to play a crucial role for foreign DNA binding during natural transformation in *B. subtilis*, but apart from this functional link, no effect on the regulation has been reported in this species [150]. This section will describe the

main envelope biogenesis pathways with a focus on the enzymes involved, which are of main importance to understand further results.

The cell-wall is one the most outstanding and important feature of bacteria. This structure protects cells from turgor bursting, external stresses and since it represents the boundary with the environment, it includes numerous proteins involved in communication, secretion and nutriment uptake [151]. In prokaryotes, two main architectures exist. On one hand, Gram-positive bacteria have developed a monoderm cell-wall with one phospholipid membrane and a thick peptidoglycan (PG) layer anchored to the membrane via lipoteichoic acids (LTA) and strengthened by wall teichoic acids (WTA). On the other hand, Gram-negative bacteria have evolved a diderm structure comprising an inner phospholipid membrane and an outer glycolipid membrane separated by a periplasmic space filled with a thin PG layer. This PG layer is anchored to the outer membrane thanks to (lipo)proteins [151].

3.1 Peptidoglycan composition

Even though cell-wall diversity is observed in prokaryotes, they all share the same exoskeleton made of PG. Since this structure is only found in bacteria, numerous antibiotics targeting this polymer have been developed. This is probably the reason explaining early scientific interest in this structure [152,153]. The PG is an arrangement comprising glycan chains made of repeated units of N-acetylmuramic acid (MurNAc) and N-acetylglucosamine (GlcNAc). The MurNAc residue is linked to a pentapeptide which can differ in between species. The most common is the L-Ala-D-Glu-m-DAP-D-Ala-D-Ala, found in both Gram-positive and negative, as exemplified by the two models *Escherichia coli* and *B. subtilis*. Another pentapeptide only found in Gram-positive is the L-Ala-D-Glu-L-Lys-D-Ala-D-Ala which can be found in staphylococcus and streptococcus genera [153,154]. The glycan chains formed of GlcNAc-MurNAc-pentapeptide repeating units can be further interlinked with other glycan chains through transpeptidation between pentapeptides. Once again, the nature and the residues from the pentapeptide involved in the peptide bond can greatly differ from one species to another [154]. While pentaglycine bridges between L-Lys and D-Ala is observed in *S. aureus*, direct binding between m-DAP (*meso*-diaminopimelate) and D-Ala is observed in *L. plantarum*, and D-Ala₂₋₃ bridges between L-Lys and D-Ala are observed in *S. salivarius* [155,156].

3.2 Cytosolic peptidoglycan precursors synthesis

The most basic building block of PG synthesis is fructose-6-P, which can be obtained from various pathways (from glucose-6-P through glucose-6-P epimerase, mannose-6-P through mannose-6-P epimerase, Glucosamine-6-P through Glucosamine-6-P deaminase, or simply from fructose through a fructokinase) [157]. An amino group from a glutamine is then transferred by the GlmS enzyme onto fructose-6-P to generate glucosamine-6-P and the phosphoglucosamine mutase GlmM transfers the phosphate group from the position six to one (Fig. 9). GlmU, an enzyme with uridyl and acetyl transferase functions, catalyzes subsequently acetylation of the amino group from acetyl-CoA and urydilation from UTP at the position one to yield UDP-N-acetylglucosamine (UDP-GlcNAc). A pyruvate group is then added from phosphoenolpyruvate at position six thanks to MurA, a carboxyvinyltransferase. The pyruvate group is next reduced to a muramate group thanks to NADPH and the reductase MurB, generating the final glycosyl group UDP-MurNAc (UDP-N-Acetylmuramic acid, Fig. 9). This glycosyl group can then be further functionalized with a peptidyl chain [154,157].

As previously mentioned, while the glycosyl groups used for PG synthesis are common to most bacteria, the peptides used for transpeptidation between glycosyl polymers can differ greatly. Therefore, we will describe here the known steps and structure for streptococci. The first step involves an L-Alanine ligase (MurC) that will functionalize UDP-MurNAc with L-Alanine at the muramate function. The precursor D-Glutamate previously epimerized by MurI will then be added to L-alanine by transpeptidation via MurD (Fig. 9). L-lysine will then be added to D-Glutamate by MurE, but specifically at the carboxylic acid function of glutamate instead of the usual peptide carboxylic function. MurF will then bring to the L-Lys amino acid a double D-Alanine peptide previously epimerized and assembled by alanine racemase (Alr) and D-Ala-D-Ala ligase (Ddl), respectively.

The generated UDP-MurNAc-pentapeptide is then fused to undecaprenol-phosphate at the UDP-carbon position by the transferase MraY. This reaction generates free UTP and a double phosphate bond for undecaprenol functionalization (Fig. 9). The glycosyl-lipido-pentapeptide is called Lipid I and will be anchored to the membrane thanks to the hydrophobic properties of the undecaprenyl function. Then, the enzyme MurG will catalyze the fusion of the

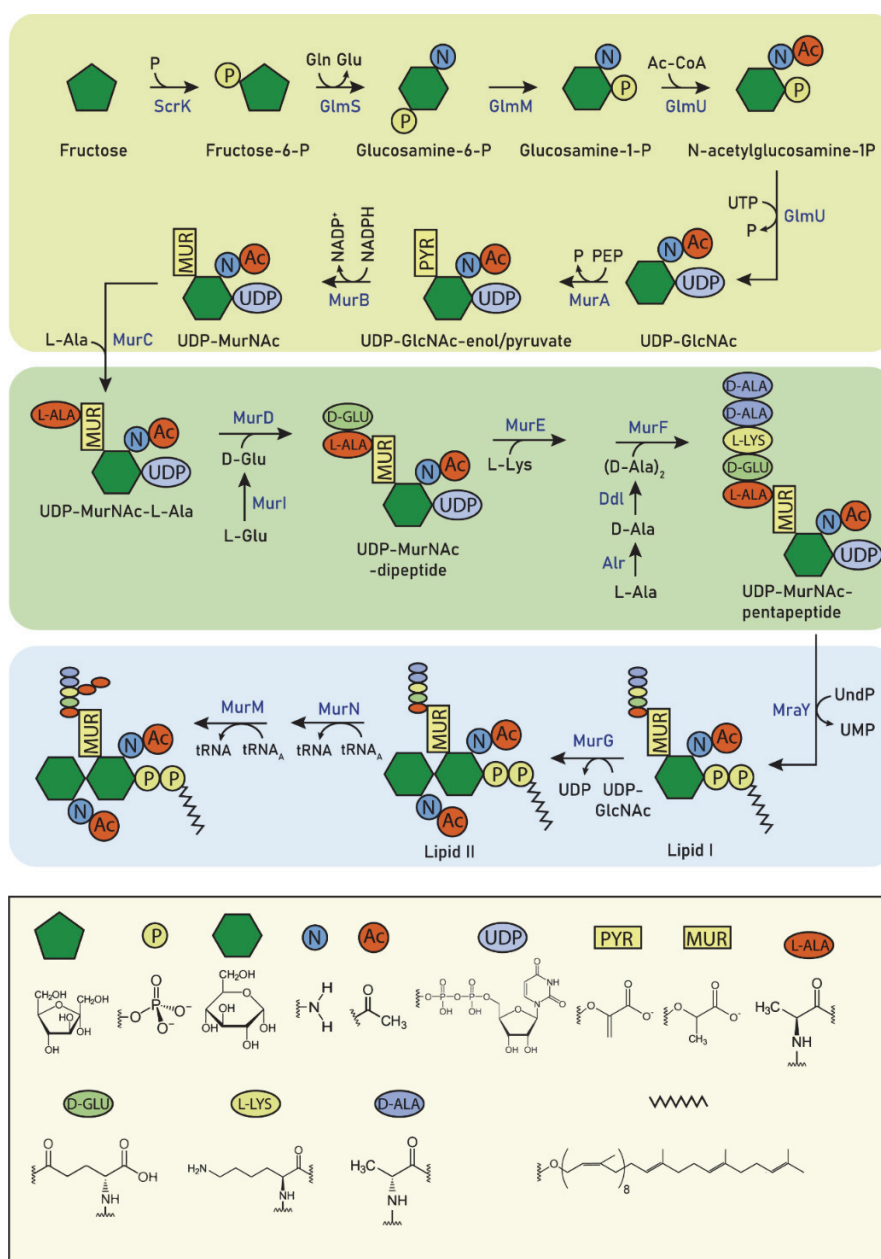


Figure 9. Cytosolic peptidoglycan precursors assembly pathway in streptococci.

First panel depicts the sugar functionalization to obtain N-acetyl-glucosamine and N-acetyl-muramic acid, second panel depicts the different transpeptidation steps and last panel depicts the transformation into lipid I and II and the lateral transpeptidation. (P: phosphate, N: amine, Ac-CoA: Acetyl-Coenzyme A, PEP: Phosphoenolpyruvate, Ac: Acetyl, UTP: Uridyl-triphosphate, UDP: Uridyl-diphosphate, PYR: enol-pyruvate, MUR: muramic acid, L/D-ALA: L/D-alanine, D-GLU: D-γ-glutamate, L-LYS: L-lysine, GlcNAc: N-acetylglucosamine, MurNAc: N-acetylmuramic acid, UndP: Undecaprenyl-phosphate).

Lipid I with UDP-GlcNAc, generating a double-glycosyl molecule called Lipid II which can be seen as the monomer of the PG polymer structure [152,153]. Finally, the glycosyl-pentapeptide will be functionalized by the streptococcal specific ligases MurM and MurN (Fig. 9). The first enzyme will transfer to the L-Lysine a D-Alanine residue, with the peculiarity that the amino acid transferred comes from a tRNA_{Ala}. MurN will then catalyze the functionalization of D-Ala to the D-Ala amino acid previously added using a tRNA_{Ala} like MurM [154,158–160].

How the Lipid II is flipped across the cell membrane in bacteria has remained controversial during many years. Some investigations have provided insights into the mechanisms involved in Gram-negative bacteria. While it was first shown *in vitro* that FtsW can act as a flippase [161], recent *in vivo* evidences show that MurJ is the flippase of Lipid II in *E. coli* [162,163]. In addition, FtsW and its homolog RodA were shown to be glycosyltransferases involved in polymerization of PG glycan chains [164–166]. In Gram-positive bacteria, similar enzymes are at work, as *B. subtilis* was shown to use YtgP as a flippase (the MurJ ortholog of Gram-positive bacteria) and another player (Amj) that can replace YtgP under stress conditions [167].

3.3 Extracellular peptidoglycan synthesis

Previously thought to be carry out only by PBP (Penicillin binding proteins), the polymerization of the lipid II precursor to produce PG chains was recently shown to involve other transmembrane proteins such as RodA and FtsW [168]. While those two transmembrane proteins are responsible for glycan polymerization activity, their associated class B PBPs are responsible for transpeptidation [168]. More precisely, recent work has shown that those two complexes (RodA-PBP2B and FtsW-PBP2X) are specifically involved in peptidoglycan polymerization during cell elongation and division, respectively [164–166,169]. This discovery questions which activity carry the PBP of class A, previously thoughts to be the glycan polymerization enzymes. Those bifunctional enzymes with transpeptidation and transglycosylation activities were recently shown to work autonomously from the elongasome and divisome complexes [170]. Also, the peptidoglycan produced by their activity exhibits different properties from the RodA-PBP2B/FtsW-PBP2X-based peptidoglycan, suggesting a different composition/architecture [170]. This latter feature could explain why peptidoglycan structures of different strengths can be found in the cell envelope (discussed in [168]).

Because PG must evolve with cell growth, some enzymes are necessary for remodeling its structure concomitantly with cell shape evolution. Many types of PG hydrolases exist and can be classified in three families, depending on the bond they cleave [171]. Glycosidases cleave the glycoside bonds in the glycan chain, amidases hydrolyze the amide bonds between the first amino-acid of the peptide and the MurNAc residue, and peptidases cleave the peptide bonds between amino-acids. Within glycosidases, we can distinct two types of enzymes. The first type, called N-acetyl- β -glucosaminidase hydrolyzes the bond between a GlcNAc and the adjacent MurNAc, as exemplified by LytB [172–174]. A second type, called muramidase, cleave the bond between sequential MurNAc and GlcNAc residues (Fig. 10).

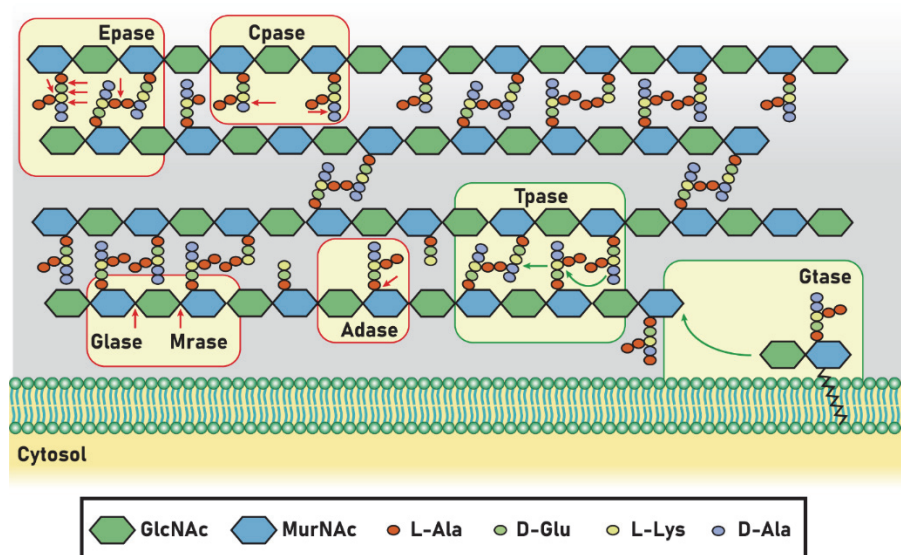


Figure 10. Extracellular synthesis of peptidoglycan from Lipid II precursor. Lipid II is first anchored to the pre-existing PG thanks to glycosyltransferase enzymes (Gtase: FtsW/RodA and class A PBP1a, PBP1b, PBP2a) and then branched to other peptides thanks to transpeptidase enzymes (Tpase: class B PBP2b/PBP2x and class A PBP1a, PBP1b, PBP2a). The PG can then be matured by peptidases: carboxypeptidases (Cpase: PBP3, DacB, LdcB) or endopeptidases (Epsase: PcsB). Amidase (Adase) can also cleave the bond between MurNAc and the pentapeptide. In parallel, the glycan chain can be degraded by glycosidases. Muramidases (Mrase) cleave the GlcNAc-MurNAc-GlcNAc bond and are either Lytic transglycosidase (MltG) or lysozymes. N-acetyl- β -glucosaminidase (Glase: LytB) cleave the bond between two disaccharides monomers. Green arrows and green panels depict bond formation and red arrows and red panels bond hydrolyzation. (L/D-Ala : L/D-Alanine, D-GLU: D- γ -Glutamate, L-Lys: L-Lysine, GlcNAc: N-acetylglucosamine, MurNAc: N-acetylmuramic acid, Und: Undecaprenyl function).

Within muramidase, a supplemental segregation is made between enzymes reducing the MurNAc end generated (lysozymes, as exemplified by LytC in *S. pneumoniae* [175,176]) and enzymes generating an intramolecular rearrangement of MurNAc leading to the production of anhydromuropeptides (lytic transglycosylases, as exemplified by MltG in streptococci) [177]. Finally, the peptidase family comprises two type of hydrolases, the endopeptidases capable of cleaving internal peptide bonds such as PcsB in *S. pneumoniae* and the carboxypeptidases only able to cleave peptides residues at the C-terminus [171,178]. In streptococci, it has been demonstrated that PBP3, catalyzes the trimming of the pentapeptide to a tetrapeptide which is further process to a tripeptide by DacB, another carboxypeptidase [179,180]. Of note, another peptidase, LdcB, has been shown to help trimming the peptide in *S. pneumoniae* but its precise role in the process is still unclear [181].

3.4 Teichoic acids

The cell wall of most Gram-positive bacteria contains teichoic acids, which are negatively charged polymers made of alditol-phosphate repeating units [182]. Two main groups of those components exist: wall-teichoic acids (WTA) and Lipo-teichoic acids (LTA). While the first group is covalently attached to the PG, the second is anchored to the membrane thanks to a glycolipid moiety [183,184]. Those components are of main importance for cell-wall robustness and can represent up to 50% of the cell wall dry weight [185].

3.4.1 Structure

There are four types of WTA and five types of LTA in Gram-positive bacteria. In this section, we will only focus on the type I of both components. Indeed, their structure and synthesis has been thoroughly described in the *S. aureus* and *B. subtilis* while other types of teichoic acids are less common and the literature regarding their synthesis is scarce [153]. Especially, the best streptococcal teichoic acid described is the one of *S. pneumoniae*, but its cell wall decoration displays several odd features that seem restricted to this species [153].

Wall teichoic acids of type I are made of polyglycerol-phosphate or polyribitol-phosphate while lipoteichoic acids of type I are only made of polyglycerol-phosphate (Fig. 11). WTA are covalently linked to a disaccharide made of *N*-acetylmannosamine-(β 1 $\rightarrow$ 4)-*N*-acetylglucosamine-1-phosphate which is covalently linked to the PG thanks to a phosphodiester bond to the C6 hydroxyl group of MurNAc [183]. On the other hand, the polyglycerol-phosphate chain of LTA is covalently linked to a disaccharide, itself covalently linked to a

diacylglycerol group. This latter group allows the anchoring of the LTA to the cell membrane [184]. Both structures can be decorated with D-alanine and GlcNAc or glucose, being linked to the free alcohol functions of the polymers [186].

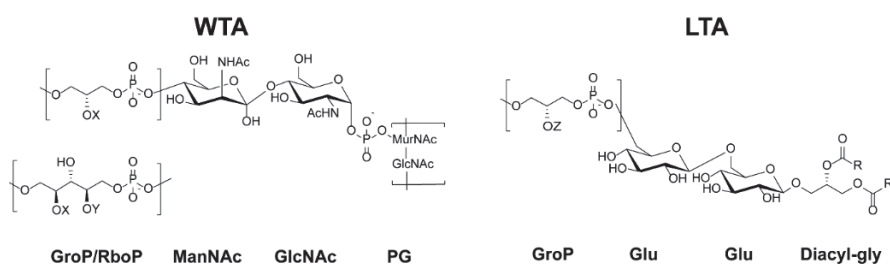


Figure 11. Structure of type I wall teichoic acid and lipoteichoic acid. X, Y, Z stand for: D-alanylation (X, Z), N-acetyl glucosamination (Y, Z) or glucosylation (X, Y). R stands for lipid anchor. GroP: glycerol-phosphate, RboP: ribitol-phosphate, ManNAc: N-acetylmannosamine, GlcNAc: N-acetylglucosamine, PG: peptidoglycan, MurNAc: N-acetyl muramic acid, Glu: Glucose, Diacyl-gly: Diacyl-glycerol.

3.4.2 Synthesis

The synthesis of WTA and LTA is partially characterized. Even though central to the bacterial physiology, this lack of knowledge is explained by the difficulty to study essential genes and highly hydrophobic lipid-anchored precursors [183]. Nevertheless, some data were generated in *S. aureus* and *B. subtilis*, allowing a partial understanding of those crucial pathways (Fig. 12). Unlike LTA, WTA synthesis starts intracellularly [187]. TagO (RgpG) catalyzes the first step by transferring GlcNAc on the undecaprenyl-phosphate lipid carrier, a shared intermediate with PG synthesis [188]. Then, TarA catalyzes the transfer of ManNAc (produced by MnaA from GlcNAc) onto the GlcNAc-undecaprenyl molecule (Figure 12) [189,190]. The nascent glycolipid is then fused to a first glycerol-phosphate by TagB, using CDP-glycerol as precursor [191]. This is the last step of a common pathway shared by *B. subtilis* and *S. aureus* species. From now on, several divergences exist and we will only describe the best studied and most simple *B. subtilis* 168 strain pathway [183]. After the first glycerol-phosphate (GroP) addition by TagB, TagF adds 45 to 60 GroP units to produce the GroP polymer [192,193]. Of note, this polymerization reaction uses CDP-glycerol precursors, which are produced by TagD [194]. It is also interesting to mention that *S. aureus* and some *B. subtilis* strains such as W23 produce polyribitol-phosphate instead of polyglycerol-phosphate polymers. Therefore, TagF polymerization is replaced by the TarIJKL activity, allowing the ribitol-phosphate polymerization (for review see

[183]). In parallel, several glycosyl-transferases have been identified to decorate polyols polymers with different sugars. In *B. subtilis* 168, the TagE glycosyltransferase adds glucose to the free alcohol function of the polyphosphate polymer [195]. The role of the WTA glycosylation is still not completely understood but several studies have shown its importance for antibiotic resistance, virulence and even DNA binding during competence for natural transformation [150,196–198].

Finally, the glycosylated polymer is exported thanks to the ABC transporter TagGH and finally fused to the MurNAc subunit of the PG thanks to the TagTUV enzymes [199–201]. The polymer produced and anchored to the PG is then decorated with D-alanylation by the DltABCD proteins [202–204].

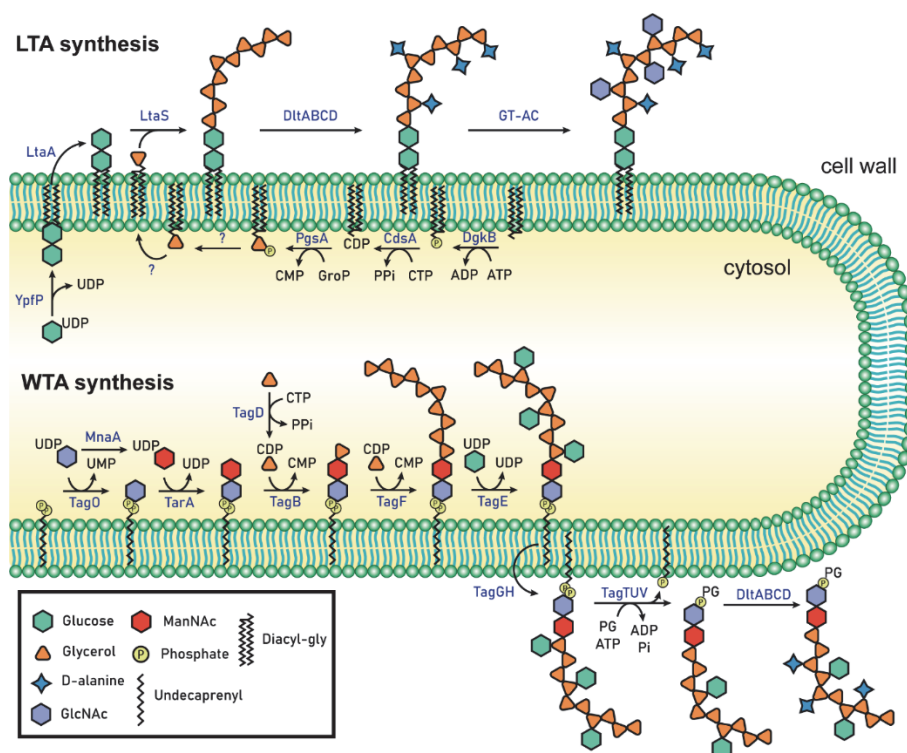


Figure 12. Lipoteichoic acid and wall teichoic acid biosynthesis. LTA synthesis pathway is described from the *S. aureus* model and the WTA synthesis from *B. subtilis* 168 model strain. UMP/UDP: Uridyl-mono/diphosphate, CMP/CDP/CTP: cytidine mono/di/triphosphate, Pi: phosphate, PPi: pyrophosphate, ATP/ADP: adenosine tri/biphosphate, GroP: glycerol-phosphate, PG: peptidoglycan, GlcNAc: N-acetyl-glucosamine, ManNAc: N-acetyl-mannosamine, Diacyl-gly: diacyl-glycerol.

Unlike WTA synthesis, LTA production occurs mainly extracellularly [184]. LTA synthesis has been best characterized in *S. aureus* where UDP-glucose

can be linked to a diacyl-glycerol molecule by YpfP (Fig. 12) [205–208]. The WTA precursor is then flipped across the membrane most probably by the flippase LtaA prior to glycerol-phosphate polymerization [209]. This latter reaction uses a glycerol-phosphate moiety coming from the phosphatidylglycerol membrane lipid and is catalyzed extracellularly by the LtaS enzyme [210–212]. The polymer can then be decorated with D-alanylation or glycosylation by the DltABCD machinery and GT-AC (Glycosyltransferases A and C) proteins, respectively [213,214]. Similarly to WTA, the glycosylation can diverge importantly even within the same species, as exemplified by *B. subtilis* strains [215,216].

An important feature of the LTA assembly is the fact that every GroP added to the polymer result in one flipping movement across the membrane of the lipid phosphatidylglycerol. Therefore, assembly of the LTA requires a high level of undecaprenyl carrier recycling [211]. This task is performed by DgkB, CdsA, PgsA and other unknown enzymes [217–219].

3.5 Extracellular polysaccharides

High variety of extracellular polysaccharides exist in bacteria and some of them can be anchored to the cell-wall directly on the peptidoglycan. Those cell-wall polysaccharides can be highly abundant and cover the bacterium with a layer of glycan directly exposed to the environment [220]. Those polysaccharides can be so thick they result in a capsule structure that has major importance for virulence in diverse species [221]. Of note, the structure of those glycans is highly diverse, strain-specific and represents therefore key antigens for the development of bacterial vaccines [222].

Particularly, rhamnose polymers were one of the first extracellular polysaccharides discovered (in 1933 by Lancefield), and were named Lancefield antigens since then [223]. Those polymers were shown to play crucial role for cell-wall biogenesis and virulence in group A (GAS, *S. pyogenes*) and group B (GBS, *S. agalactiae*) streptococci, and the characterization of the genes involved in their synthesis have been only recently uncovered in GBS, GAS and other species such as *Lactococcus lactis* [224–227]. Historically, the serotyping of this antigen was used to classify *Lactobacillales*. Nevertheless, evidences accumulation of shared antigens between different species or different serotypes within the same species have led to favor the use of genomic data to classify the family, ultimately leading to the division of the streptococcal family into *Enterococcus*, *Lactococcus* and *Streptococcus* [1,228]. Those phylogenic considerations aside, rhamnose extracellular

polysaccharides are of main importance for *Lactobacillales* and are hypothesized to be partial to complete substitutes for WTA in the PG structure [220].

The structure of rhamnose extracellular polysaccharides can be divided in two parts. The first part, common to most species is called the rhamnan stem and consists of a repeat of L-rhamnose monomers anchored to the cell wall via a link to the MurNAc subunit of the peptidoglycan [220]. This stem rhamnan is linked to oligosaccharides with highly divergent structures which are strains-specific. Those oligosaccharides can polymerize and reticulate, ultimately generating a multi-branched structure comprising numerous oligosaccharides repeats [220].

Recently, the team of Chapot-Charpentier uncovered in *L. lactis* the first complete pathway and genetic loci involved in biogenesis of rhamnose (Rha) polysaccharides [226,227]. The synthesis starts with the production of the dTDP-L-rhamnose precursor synthesized by the RmlABCD enzymes from D-glucose-1-P [229]. The enzymes TagO/RgpG, RgpABFE are then responsible to produce the stem rhamnan onto undecaprenyl-phosphate, which consists of a GlcNAc-(Rha)_n-GlcNAc polysaccharide. The polysaccharide is next flipped across the membrane by a putative ABC transporter and anchored to the MurNAc moiety of the peptidoglycan by LcpA (Fig. 13) [227]. In parallel, the Glf enzyme produces UDP-galactofuranose (Glf) from UDP-galactose to feed the oligosaccharide repeat unit synthesis on undecaprenyl-phosphate by the WpsABCDEF enzymes, generating a GlcNAc-Rha-GlcNAc-Glc-Galf oligosaccharide. After flipping across the membrane through the WpsG flippase, the oligosaccharide is side-functionalized with a glucose moiety by an unknown enzyme to generate the final monomer form. The polymerization of the oligosaccharide is next performed by the two WpsHI enzymes and finally anchored to the stem rhamnan by WpsJ (Fig. 13) [227].

3.6 Muropeptides communication

During growth and maturation, PG is digested by specific enzymes (Fig. 10) in a process called PG turnover. This controlled degradation produces PG fragments called muropeptides which can represent up to 50% of the total PG upon one generation of growth in *E. coli* [171,230].

To avoid massive loss of energy into PG synthesis, some recycling systems exist. Initially, Gram-positive bacteria were thought to be exempt of such system because of the large amount of PG by-products present in the culture medium [231]. However, recent evidences have shown the presence of recycling enzymes in most of the Gram-positive which are orthologs of those

identified in Gram-negative bacteria [232]. Furthermore, other studies have proven the functionality of those systems and their importance for survival in *Streptomyces coelicolor*, *S. aureus* and *B. subtilis* [233,234]. This suggests that both Gram-positive and Gram-negative bacteria recycle their cell-wall components, even though more by-products are released by monoderm species, presumably due to their thicker layer of PG [171].

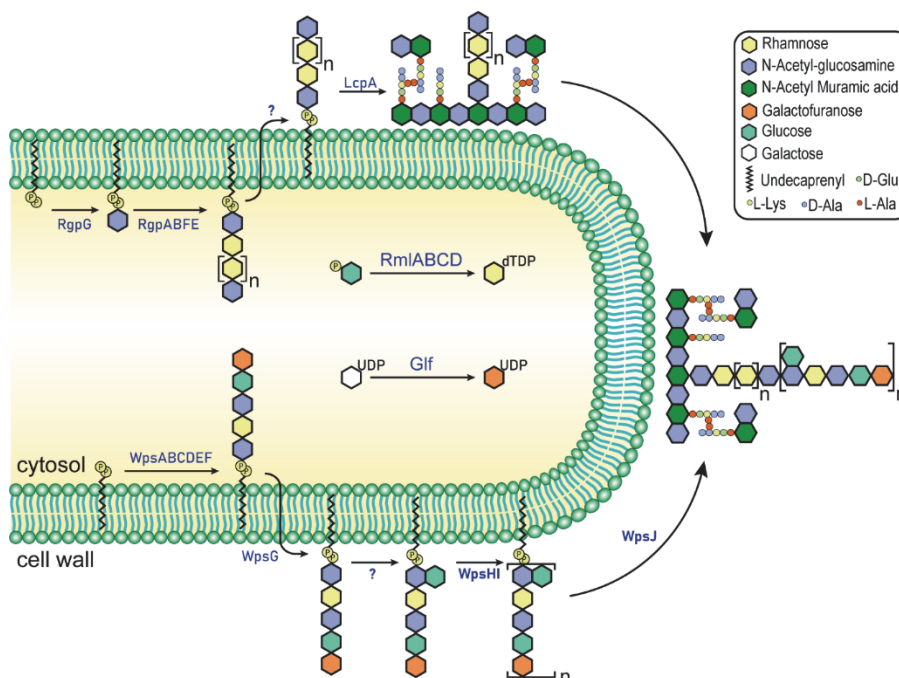


Figure 13. Rhamnose extracellular biosynthesis. In *L. lactis*, the rhamnose extracellular synthesis pathway has been mostly elucidated. Two glycosidic polymers are synthesized in parallel (a poly-rhamnose chain and an oligosaccharide repeat). The synthesis of the stem polysaccharide starts with the intracellular polymerization of a GlcNAc-(Rha)_n polymer that ends with a GlcNAc. The glycosidic chain is flipped across the membrane by an unknown flippase and is anchored to the peptidoglycan at a MurNAc moiety by LcpA. In parallel, an oligosaccharide consisting of a GlcNAc-Rha-GlcNAc-Glc-Glf is synthesized intracellularly by the enzymes WpsABCDEF. After flipping across the membrane by WpsG, a glucose moiety is added to the first glycosidic monomer of the chain and is polymerized by WpsHI. The generated polymer is next fused onto the stem polysaccharide by WpsJ. Of note, the precursors dTDP-Rhamnose and UDP-galactofuranose are produced from Glucose-1-P by the enzymes RmlABCD and from UDP-Galactose by the Glf enzyme, respectively. Figure inspired from [227].

The nature of the released fragments produced by PG remodeling during growth, cell division or incorporation and assembly of protein complexes into the cell wall can be highly heterogeneous depending on the type of the processing-enzymes involved. As many types of PG hydrolases exist (see section

3.3 for details), bacteria and even eukaryotes have evolved multiple sensors with different PG fragments specificities. Those sensors are particularly useful to sense the presence of bacterial proliferation or cell-wall degradation. Different protein domains were identified for PG sensing among which the generic GlcNAc glycan sensor motif LysM, found in many bacterial PG remodeling enzymes. Other domains such as the NOD-like (Nucleotide binding and oligomerization domain) domains are able to recognize D-Glu-mDAP or MurNAc-D-Ala-D-Glu in mammalian cells where they trigger a regulation cascade ultimately leading to inflammatory response and antimicrobial activity [171].

In bacteria, one of the most widespread PG sensor domain is the PASTA (Penicillin-binding and Ser/Thr kinase associated) domain. This motif (often repeated) is usually associated with a transmembrane and an eukaryotic-like Serine/Threonine kinase (Stk) domain. The protein formed by those three domains allows bacteria to sense and respond to environmental triggers such as bacterial growth or cell-wall targeting antibiotics. The signal is then transmitted by a phosphorelay to various physiological pathways such as cell-wall homeostasis, cell division, and dormancy [235–238]. Of note, the PASTA domain is able to bind muropeptides, and is more sensitive to anhydro-MurNAc with longer glycans chains [236]. Moreover, the fourth amino-acid of the peptide stem (D-Ala) is crucial for recognition and the PASTA domain from species with m-DAP or L-Lys were shown to bind specifically to their cognate muropeptides [236,238].

4 Environmental sensors

Adaptation to changing environmental conditions such as temperature, physico-chemical or ecological conditions is a key feature for bacterial survival. To survey those changes, bacteria have evolved environmental sensors, triggering the appropriate physiological pathway in response to a specific stimulus [239,240]. The most widespread system in bacteria are the Two-components systems (TCS) but other systems such as the eukaryotic-like Serine/Threonine kinase exist [241,242]. Investigations conducted during this thesis suggested or established several links between environmental sensors and competence. Therefore, this section will describe the most relevant systems in relation with the data obtained.

4.1 Two-components systems

TCS are widespread phosphorelay signal transducers found in bacteria and archaea. While some TCS have been found in eukaryotic organisms, the animal kingdom seems to be devoided of any TCS orthologs [243,244]. Those

environmental sensors consist of two partners. While a transmembrane protein called the histidine kinase (HK) senses and transmits the signal across the cytoplasmic membrane, a so-called response regulator (RR) captures the transmitted phosphorelay and performs most often specific transcriptional activation [239,240].

Several types of HKs have been described but the most common consists of a sensing extracellular domain followed by a transmembrane helix and the well conserved HAMP domain (found in histidine kinase, adenyl cyclases, methyl-accepting proteins and phosphatases). Those motifs are followed by a histidine transfer domain (DHP, Dimeric helix with Histidine Phosphorylated), which is followed by a catalytic-ATP binding domain (CA) (Fig. 14). On the other hand, the RRs are made of a phosphate receiver motif (REC) and an effector domain that will most often showcase DNA binding properties [244]. While the DHP, CA, HAMP and REC domains are mostly conserved in TCS, sensors and effectors are highly variables [245].

Signals perception by the HK sensing domain can be quite diverse. Metal ions, pH, small signaling molecules or envelope stresses can bind or activate the sensing domain of the HK. Several mechanisms have been proposed for the signal transfer from the sensing domain to the histidine autophosphorylation [246–251]. One of the best studied models is probably NarQ, a nitrite-nitrate sensor of *E. coli*. The structure of this HK has been recently solved in the apo- and holo-form and show that the transmembrane helix undergoes a “piston-like” movement upon nitrate binding, which will force the HAMP domain to twist on itself and transmit a rotation movement to the DHP domain [252]. The rotation is further transferred to the double alpha helix of the DHP domain and will bring closer a conserved histidine residue and the CA domain. Since HK are homodimers, the autophosphorylation can occur within the same molecule (*cis*) or can be performed by the CA of the other subunit (*trans*) and both systems have been observed [253–255]. Another important feature of the kinase-activated HK is its asymmetry. It has been mostly observed that the rotation motion brings asymmetry and that only one of the histidine of the dimer is phosphorylated [244,256,257]. Once in its activated form, the histidine residue can transfer the phosphate to an aspartate residue from its cognate RR [258]. It is also important to note that in addition to autophosphorylation and phospho-transfer to the RR, HKs are also able to perform dephosphorylation of the RR. This activity occurs when the sensing domain is not activated and autophosphorylation of the His residue is not present (Fig. 14). The catalytic site performing this activity is

generally very close from the key histidine residue and involves sometimes the same residues as the kinase activity [240].

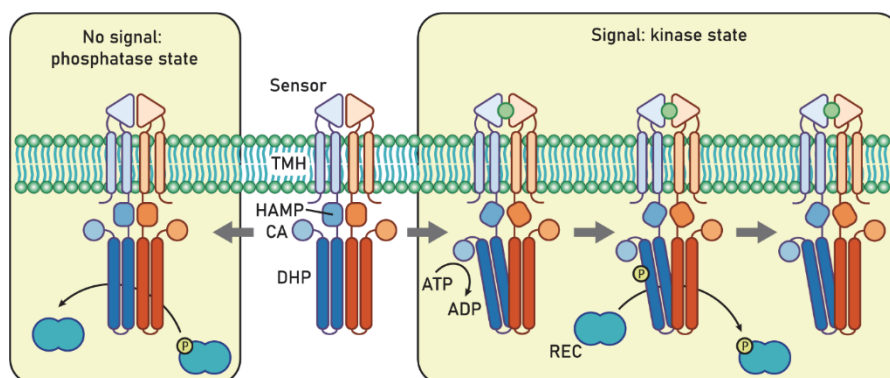


Figure 14. General mechanism of TCS phosphorylation relay in bacteria. The sensor domain of the Histidine kinase (HK) can bind a ligand which will trigger HAMP domain twist and DHP rotation. This will lead to an asymmetrical shape that will bring closer the catalytic ATP binding domain (CA) and the key histidine of the DHP domain, resulting in the histidine phosphorylation. The phosphate can then be transferred to the receiver domain (REC) of the response regulator (RR) that will perform a specific task thanks to its effector domain. On the other hand, when inactivated, some histidine kinase can perform phosphatase activity through a phosphatase catalytic site present on the DHP domain.

Determining the specificity of HK for its cognate RR from a biochemical point of view is nothing trivial. Genetic location is often a better method, since most of the HK-RR pairs are organized in chromosomally adjacent operons. Nevertheless, the existence of some genetic orphan HK or RR and cross-talk complexifies the genetic-correlation analysis [244,259]. Upon phosphorylation, most of the RRs will undergo homo-dimerization or multimerization but some heteromerization have also been observed [260]. Once the RR is in its multimer activated form, it can execute various functions. Those can be classified in 5 main types: DNA-binding, RNA-binding, enzymatic activity, protein-binding and single-domain RR which display only a REC domain [244]. Most of the RRs are part of the DNA-binding class and can trigger activation/repression of diverse physiological functions such as oxidative stress resistance, competence, heat-shock response, bacteriocin production, sporulation or virulence [53,261].

4.1.1 The CovRS system

One of the best studied TCS in streptococci is CovRS (Control of virulence), controlling virulence in GBS and GAS. Initially discovered in GAS [262] and later in GBS [263], this TCS is made of the transmembrane histidine kinase CovS which responds to diverse stimuli such as Mg^{2+} , the human

antimicrobial peptide LL-37, iron starvation, pH and high glucose concentration [264–270]. When deactivated, CovS performs a phosphatase activity, using the key residue T284 [271]. On the other hand, upon activation, CovS undergoes H280 autophosphorylation ultimately leading to a phospho-transfer operated by the catalytic site comprising the E281 kinase key residue [272]. The phosphate group is next transferred to the D53 aspartate residue of CovR which initiates dimerization and triggers CovR transcriptional repression [273,274]. Of note, this simple TCS scheme is complicated by the observation that even in absence of CovS, CovR is phosphorylated in GAS [275]. This suggests the presence of another CovR phosphate donor and underlines the phosphatase main role of CovS.

Aside from its trigger, CovRS interplay with other regulators can influence the phosphorylation state and the transcription of this TCS. For instance, RocA, an orphan pseudo kinase present in GBS and GAS has been shown to bind CovS and promotes its kinase activity [276–279]. In addition, since the regulons of *covS* and *rocA* mutants do not completely overlap, RocA must have other targets [280]. As CovS has its RocA partner, CovR is also regulated at the post-translational level by StkP, a Serine/Threonine kinase protein. This kinase was shown to phosphorylate CovR at the position T65, a residue mutually exclusive with the D53 phosphorylated residue of CovR [274,281,282]. This supplemental phosphorylation site suggests that CovR has multiple activators/repressors and that its wide regulon is modulated by the fine-tune of multiple phosphorylation sites.

Determining the exact binding motif of CovR has been a problematic topic for long. While early DNase footprinting experiments have identified that repeated ATTARA motifs were associated to CovR DNA-binding [283–285], another type of motif consisting of TTA repetitions spaced with ~11 nucleotides is also able to bind CovR [286]. In parallel, contradictory results on the cooperativity of CovR for DNA binding have suggested that this RR could have different behavior depending on the promoter architecture [286–288]. As ATTARA sequences seem not to involve cooperativity, TTA repetitions display a highly cooperative mechanism for CovR binding [286].

CovRS microarrays experiments identified the CovRS regulon in GAS and GBS which correspond approximatively to 15% of the total genes of the species [264,289,290]. This high proportion showcases the importance of CovRS as a pleiotropic regulator and proves that multiple virulence genes are targeted. Those transcriptomics evidences have been later confirmed by the isolation

of GBS and GAS hypervirulent strains associated with CovS mutations [291]. It is also important to note that the CovR regulon comprises *covR* itself and experiments have shown that CovR represses or activates its own transcription in GAS and GBS, respectively [285,292].

CovRS homologs from GBS and GAS exist in most streptococci but their regulons can differ greatly. For instance, microarrays data obtained in *S. mutans* have shown that, unlike GAS and GBS, competence is downregulated in a $\Delta covR$ mutant. This suggests that CovR phosphorylation would promote competence activation in this species [293].

4.2 Serine-Threonine kinases

Serine/threonine kinases proteins (STKP) are a superfamily of proteins sharing a S/T kinase highly conserved domain [294,295]. Initially thought to be restricted to eukaryotes, it is now clear that homologs exist in prokaryotes, and display high diversity [296]. Although an horizontal transfer from eukaryotes to prokaryotes has been hypothesized, recent genome-wide analysis suggest that this superfamily arose early in the tree of life, and was probably present in LUCA [297]. Evolution would then have favored the TCS system in prokaryotes while keeping STKP system for other functions, presumably because TCS labile phosphorylation fits better prokaryotic lifetime [242].

4.2.1 Structure

The serine/threonine kinase well-conserved catalytic domain folds in a two-lobed catalytic core structure. The smaller N-ter lobe is mainly involved in ATP molecule binding and the larger C-ter lobe performs substrate recognition and phosphate transfer initiation. The catalytic site is located at the interface between the two lobes and imply residues from both regions [294,298]. This interface comprises a Mg^{2+} binding loop, an activation loop, a P+1 loop and an α -C-helix. Upon activation by ligand binding, the activation loop is autophosphorylated or transphosphorylated on a serine/threonine residue, which will bring closer the α -C-helix. This will bring the two lobes closer, resulting in a closed, catalytically activated conformation [295,299]. Of note, the p+1 loop is also important for substrate recognition and has a different structure in tyrosine kinase [295].

This general scheme can be translated to the particular case of PknB, the first prokaryotic STKP described and structure-solved (Fig. 15) [300,301]. Discovered in *Mycobacterium tuberculosis*, this protein displays a catalytic loop with a DXKPXN consensus including the catalytic base D138 for the

phosphorylation reaction [295]. It also exhibits a conserved salt bridge in the activated form between residues K40 and E59, that will help closing the conformation and bridging ATP with the α -C-helix [302]. The activated form of this enzyme has been crystallized as a homodimer with the interface between the two dimers located at the external N-ter lobe. The dimerization has been shown to stabilize the active enzyme conformation, but is not necessary for its activity [303,304].

Aside from its kinase subdomain, extracellular domains of STKP can be quite diverse as exemplified by the four available crystal structure of different *M. tuberculosis* STKP. While PknD exhibits a six-bladed β -propeller shape, PknH and PknI display completely different structures, allowing ligand specificity [305–307]. The fourth available structure, PknB, displays four repeated structure known as PASTA domains. Those domains consisting of one α -helix and 3 β -strands linearly arranged [308,309], are able to bind PG fragments [235,238] or Lipid II [310,311], but their specificity is still not completely clear. Also controversial is the recent evidences of the role of PknB homologs in cell constriction and separation after division [235,311,312]. This suggests that PASTA motifs binding to mucopeptides could ensure proper localization at the site of PG production and turnover, and challenges the classical ligand-induced view of STKP [236,308].

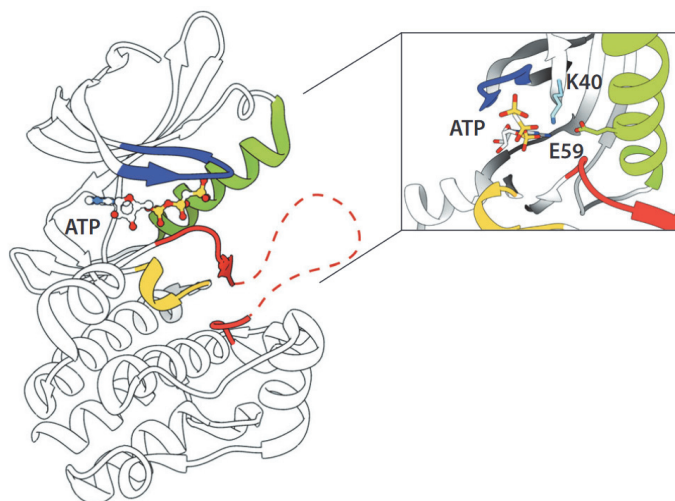


Figure 15. Structure of the kinase domain of PknB from *M. tuberculosis* in complex with ATP. Catalytic loop = yellow, activation loop = red, α -C-helix = green, P+1-loop = blue. Upper lobe is N-ter, lower lobe is C-ter. Figure taken from [313].

Histidine and aspartate phosphorylation is relatively labile and therefore, phosphatases are not useful to inactivate phosphorylated TCS. On the other hand, threonine and serine phosphorylation are strong and require enzymatic hydrolyzation [295]. This is the reason why STKP are most often genetically associated with a cognate phosphatase. In some species, the deletion of the STKP-associated phosphatase is not viable while both partners deletion does not trigger cell death [314,315]. This is probably because the phosphatases counterbalance the effect of their cognate kinase by dephosphorylation of the STKP targets but also by a control of the autophosphorylation level of the kinase [316–319].

4.2.2 Functions

Although mostly present in eukaryotes, a lot of different serine/threonine kinases have been described in bacteria. Phosphoproteomes of those kinases have been investigated in various species and have underlined the diversity of the functions controlled by those sensors, as well as their pleiotropic effects. STKs have been described to control various pathways involved in developmental processes such as dormancy in *B. subtilis*, secondary metabolism, virulence, central metabolism or cell division and cell wall synthesis [295]. We will only cover in this section the different functions found for the *pknB* STK, firstly described in *M. tuberculosis*, and later called *stkP* in *S. pneumoniae*, which displays a four-repeated PASTA domain.

In *B. subtilis*, it has been reported that dormant cells could germinate in response to muropeptides addition. This physiological response relies on the PASTA domain recognition of muropeptides of a PknB/StkP homolog, PrkC [236]. Less specific pathways have also been identified to be under the control of PrkC in *B. subtilis*. General metabolism enzymes involved in the Krebs cycle, sugar transport, catabolic repression or the pentose phosphate pathways were indeed shown to be phosphorylated by PrkC [295,320].

As cited before, the serine/threonine kinase PknB/StkP controls virulence through cross-talk with the RR CovR in GAS and GBS. This control occurs through the phosphorylation of the residue T65 which is mutually exclusive with the key phosphorylation site at the position D53 [274,281,282]. Similarly, lower pathogenicity associated to *stk* mutants have been observed in species such as *S. aureus* and *S. suis* but the authors hypothesized that this reduction would be an effect of the general metabolism disorder [321,322]. Nevertheless, other studies have shown in *S. aureus* that Stk directly

phosphorylates SarA, a global regulator involved in activation of virulence genes [323–325].

Although PknB/StkP homologs have been shown to control various pathways in different species, the most common function found in bacteria for this kinase is the regulation of cell wall synthesis and division. The link between PknB/StkP and those functions has been initially hypothesized because of the genomic context of the kinase-phosphatase system. Indeed, *pknB/stkP* homologs are generally found in operon with key genes involved in cell-wall synthesis or division (*divIA*, *pbpA*, *rodA*). This link has been strengthened by experimental evidences of STK defective strains displaying cell shape abnormality and lower resistance to antibiotics targeting cell-wall synthesis [63,324,326–330]. Furthermore, colocalization studies have highlighted the importance of PASTA domains repeats for septal localization of StkP, and its interaction with the PG hydrolase LytB to promote cell separation after division [312,331–333]. Moreover, multiple cell-wall or division-related phospho-targets of StkP/PknB have been identified among which the penicillin-binding protein A (PbpA) [334], the essential cell shape/division DivIVA [326], the major septum division components FtsZ and MapZ [318,335–337] and the PG precursor epimerase GlmM [318,338]. StkP was also shown to phosphorylate the major cell-wall sensor TCS WalRK (VicRK) in *B. subtilis* [61]. Although other targets have been suggested by proteomics or other *in vitro* experiments, those results are subject to high false-positive rates and *in vivo* confirmation should be carried out before premature conclusions are drawn [295,339].

In addition, to its key role in division and cell wall remodeling, an unexpected link between competence and StkP/PknB has been highlighted in *S. pneumoniae* and *S. mutans* [63,64]. In *S. mutans*, transcriptomics data in mutants deficient for *pknB* or its cognate phosphatase *pppL* have shown that those kinase/phosphatase control competence, bacteriocin production, cell wall metabolism and biofilm production [63]. In *S. pneumoniae*, the link between StkP and competence is still unclear. Indeed, two independent studies showed that *stkP* deletion leads to competence impairment [64] or overexpression [65]. Besides, a biochemical characterization study has shown that StkP can phosphorylate ComE at the T128 residue which triggers a ComCDE distinct regulon involved in acidic stress induced lysis [340]. This finding suggests that different regulons can be activated by ComE in *S. pneumoniae*, depending on the residue phosphorylated (T128 for StkP and D58 for ComD). This mechanism showcases another example of crosstalk between TCS and STK in streptococci.

4.3 Environmental sensors and cell-wall regulation

Since a link between cell-wall impairment, environmental sensors and competence activation is underlined in this thesis, we will briefly describe here the literature regarding the different cell-wall stress responses in Gram-positive bacteria.

Three main pathways in response to cell-wall stresses have been described: serine/threonine kinase, two-components systems or Spx responses [341] (Fig. 16). As different species showcase increasing susceptibility to antibiotics in the absence of StkP, this sensor has been suggested [328,329,342] and recently shown [66] to sense cell-wall integrity through its PASTA domains. This sensor subsequently triggers the activation of key envelope proteins by phosphorylation. In parallel, many TCS have been described to sense cell-wall integrity such as the VicRK and the LiaFSR systems. Particularly, VicRK (also called WalRK) is essential in many bacteria and seem to play a major role to control cell-wall related proteins activation [343]. On the other hand, LiaFSR (also called VraFSR) has also a major role in envelope biogenesis regulation and was shown to sense cell-wall degradation produced by murein hydrolases and Lipid II antibiotics [57,344]. Finally, a last pathway involving the cell-wall and the disulfide stress response Spx was deciphered in *B. subtilis* [345,346]. Upon cell-wall stress, several sigma proteins can trigger Spx transcription, which will subsequently activate key cell-wall genes [346]. In parallel, another control can take place by a TCS named CssRS, that senses cell-wall damages and trigger the production of a ClpXP antagonist, preventing Spx from degradation [345]. In *B. subtilis*, the oxidation state of the Spx regulator dictates its activity toward redox or cell-wall stress response [346].

In streptococci, two different *spx* genes are present in the genome (*spxA1* and *spxA2*) [347]. While the activity of Spx towards cell-wall or oxidative stress response is fine-tuned in *B. subtilis* thanks to the Spx redox state, streptococci seem to have evolved two specific proteins for each pathway. In *S. mutans*, SpxA1 and SpxA2 were suggested to be involved in oxidative stress and cell-wall rescue, respectively [347]. On the contrary, recent data generated in *S. pneumoniae* showed that the SpxA1 homolog is involved in the cell-wall response [66]. As those two studies are newfinds, further work will help to establish SpxA1/A2 roles in streptococci. Notably, the *spxA2* gene is regulated by LiaFSR in *S. mutans* [347]. This crosstalk is a glaring illustration of communication between the three cell-wall stress pathways as shown in many other species such as the CssRS-Spx link in *B. subtilis* [345], the StkP-VicRK crosstalk in *S. mutans*, *S. pneumoniae*, and *B. subtilis* [61,63,66], the

StkP-CovRS connection in GAS [274] or the StkP-LiaFSR crosstalk shown in *S. aureus* [348] (Fig. 16).

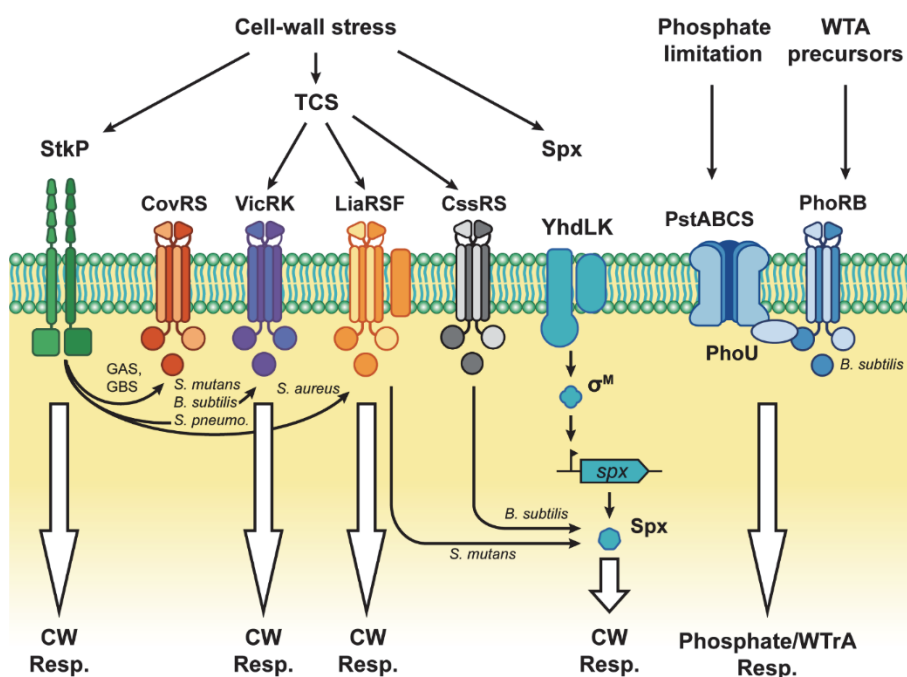


Figure 16. Environmental sensors and cell-wall regulation. Main pathways in response to cell-wall stress encounter in Gram-positive bacteria. Name of the species where the cross-talk or the pathway were described are depicted. *S. pneumo*: *S. pneumoniae*, WTA: Wall Teichoic acids, CW Resp.: Cell-wall response, Phosphate/WTrA Resp.: Phosphate and Wall-teichuronic acid response.

In parallel to cell-wall stress mostly encountered by antibiotic treatment, bacteria can face phosphate scarcity with dramatic effects on the synthesis of teichoic acids with high phosphate content [349]. To face the phosphate stress, *B. subtilis* evolved a regulation cascade involving the phosphate transporter PstABCS. Upon phosphate limitation, the PsABCS transporter undergoes a conformational change that is transmitted to the protein PhoU, which frees the histidine kinase PhoR, ultimately leading to the response regulator PhoB phosphorylation and transcriptional activity (Figure 16). PhoB~P can then activate several genes involved in recycling and uptake of phosphate and switches the metabolism from teichoic acid to teichuronic acid synthesis, which is less phosphate-consuming [349]. Finally, the PhoR activity can be fine-tuned by the accumulation of WTA intermediates [350].

5 Genome-wide screens

Genome-wide screens have been extensively used the last two decades to discover new genes involved in various pathways [351]. The most common method is based on the production of a random transposition library which is later screened or NGS-analyzed for the absence of transposition events associated to essential genes or other phenotypes (Tn-Seq). In this thesis, we conducted a genome-wide screen based on the recent CRISPR-interference (CRISPRi) technology. This technique is derived from the CRISPR immune system, initially discovered in *S. thermophilus* [352]. Among the countless applications derived from this system [353], CRISPRi is used to specifically repress genes transcription using a Cas9 mutant (dead-Cas9 or dCas9) inactive for endonuclease activity (D10A, H840A) [354].

This technique offers several advantages for genetic inactivation studies [355]. First, gene repression can be finely-tuned by using the *dcas9* under the control of an inducible promoter or by introducing mismatches into the complementary sequence of the guide RNA [356,357]. Second, this system allows multiple targets to be repressed at the same time by using different guides. Third, the silencing is reversible once the dCas9 inducer is washed away from the culture medium [357].

On the other side, the technology has several drawbacks. A “bad-seed” effect has been described with a 5-nucleotides sequence present in RNA guides that produces toxicity regardless of the targeted gene [358]. Also, dCas9-mediated repression has a strong polar effect since all the downstream genes from an operon will be transcriptionally inactivated together with the targeted gene [358]. More unexpected is the reverse polar effect, resulting in the down-regulation of the upstream genes from the same operon, probably due to the destabilization of the interrupted transcript [355,358]. Like Cas9, strong overexpression of the dCas9 produces toxicity in some species, regardless of the guide associated [359,360]. Finally, off-targets were reported for as low as 9-nucleotides common sequences, underlining the needs to carefully design the guides [358].

In comparison to the previous silencing RNA (sRNA) technology, the CRISPRi exhibits some key differences. The most important divergence is probably the process targeted. While CRISPRi targets gene expression, sRNA inhibits translation [355]. Also, the sRNA requires RNA chaperones to produce stable RNA complexes while no other proteins are required for dCas9-guide RNA complex binding [361]. Furthermore, since the 20-nucleotides gRNA design

only requires the NGG protospacer adjacent motif (PAM) sequence vicinity and the sRNA design involves much longer sequences, the CRISPRi technology appears as a new easy tool for gene silencing.

Also, CRISPRi screens offer a new solution to overcome some Tn-seq drawbacks. Indeed, size-library requirement can be smaller for CRISPRi and only a subset of the genome can be screened [355]. Furthermore, interesting essential genes activity can be analyzed by interference while transposon inactivation of the gene produces non-viable strains. Moreover, Tn-seq offers poor statistical significance for small genes. Although the CRISPRi technology showcases several drawbacks, its advantages and easy-to-use features will probably expand its popularity in genetic screens and essential genes functional studies.

OBJECTIVES

Competence has been shown to play a key role in bacterial adaptation and evolution. This strategy is of main importance to survive in a competitive ecological niche and its comprehension is particularly relevant in the global context of antibiotic-resistance emergence. For those reasons, important efforts have been made to understand bacterial competence those last years but several key processes concerning its regulation are still missing.

The commensal bacterium *Streptococcus salivarius* is of main interest to study competence. Indeed, this species uses the ComRS signaling pathway to tune competence activation. While this system is the most shared among streptococcal species, it is also the less characterized. Therefore, the goal of this thesis is to understand and decipher competence dynamics in the *S. salivarius* HSISS4 strain. In particular, the heterogeneous activation of competence known as bimodality is well-documented in other species but the key regulatory mechanisms are still lacking to explain such intriguing phenomenon. Also, the exit (shut-off) from competence has been well described in the ComCDE streptococci but remains unknown for the ComRS system. This thesis will investigate those two mechanisms (bimodality and shut-off) and will be divided in three main objectives.

- 1. Study of ComRS bimodality in *S. salivarius*.** To decipher the molecular mechanism governing heterogeneous activation in the salivarius group of streptococci, we will combine genetic overexpression and design fluorescent tools to investigate at the single-cell level competence initiation. Once the molecular mechanisms uncovered, we will focus on identifying environmental sensors controlling the emergence of a competent sub-population.
- 2. Shut-off investigations in *S. salivarius*.** Previous work in the close-relative *Streptococcus thermophilus* had suggested the presence of a negative player under the control of ComX acting as a shut-off mechanism. The objective of this section is to confirm this negative

feedback loop in *S. salivarius* and subsequently identify and characterize the late competence gene responsible for this deactivation.

3. **Design of a conditional-mutant screen to identify novel ComRS antagonists.** To broaden our search for shut-off players in the genome of *S. salivarius*, we will set up a screening method. Since competence overactivation is toxic and the shut-off impairment could therefore lead to lethality, we will design a conditional mutant screen based on the CRISPR-interference technology. This screening method will then be used to identify unknown ComRS (de)activation pathways.

RESULTS

CHAPTER I

The CovRS environmental sensor directly controls the ComRS signaling system to orchestrate competence bimodality in *Salivarius streptococci*

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AK, LF, PG, JMi, LLG and PH conceived and designed the study. The laboratory work was carried out by AK (Fig. 1.1-3, 1.4B, 1.5, 1.6DE, 1.7, 1.8, S1.1-3, S1.5, S1.6), FVC (Fig. 1.4A), LF (Fig. 1.6ABC), MV (pre-data), JMi (pre-data), and LLG (pre-data). The data were analyzed and interpreted by AK (table S1.2), FVC (table S1.1), LF, AS (table S1.2), LLG and PH (table S1.1-2). AK, FVC, JMi, LF, PG, JMa, AS, TC, LLG and PH wrote or revised the manuscript.

Phenotypic heterogeneity is an efficient strategy to rescue low genetic diversity created by bacterial asexual mode of proliferation. How such intriguing phenomenon occurs has been thoroughly described in *Bacillus subtilis* but remains unclear for streptococci. In particular, bimodal competence activation through the ComRS pathway has been studied in *Streptococcus mutans* but the existence of an additional Quorum Sensing (QS) system controlling competence has complexified investigations. In this chapter, we examined bimodality in the species *Streptococcus salivarius* harboring no supplemental QS competence control.

By overexpressing the proximal actors involved in ComRS regulation, we show that ComR represents the bottleneck for competence activation in *S. salivarius*. Using *salivarius*-optimized fluorescent tools, we studied the single-cell initiation of competence and show that the number of activated cells is directly linked to the intracellular concentration of ComR. Particularly, we proved that ComRS bimodal activation relies on the positive feedback loop acting on the pheromone *comS* gene.

Because *comR* is not under the control of the ComRS positive feedback loop, we next sought to investigate what were the determinants governing ComR concentration and indirectly the proportion of the competent subpopulation. Searching for a two-component system playing this role, we identified CovRS, a virulence control system in pathogenic streptococci. We next demonstrated that the response regulator CovR represses *comR* by direct binding to its promoter and characterized its binding region.

We finally assessed if CovRS would transmit environmental stimuli to the ComRS system by fine tuning the level of ComR and studied its impact on the percentage of competent cells. Since CovR depletion was toxic in *S. salivarius*, we tested this assumption in *Streptococcus thermophilus* LMD-9, a strain spontaneously competent thanks to a CovR mutation. By increasing the level of wild-type CovR, we were able to generate bimodality, underlining the importance of the CovRS sensor in bimodal activation. Together, those results help us to understand how environmental sensors are able to transmit signals to QS systems such as the ComRS. This transmission finally activates a response in a fraction of the population, which size depends on the initial input intensity.

ABSTRACT

In bacteria, phenotypic heterogeneity in an isogenic population compensates for the lack of genetic diversity and allows concomitant multiple survival strategies when choosing only one is too risky. This powerful tactic is exploited for competence development in streptococci where only a subset of the community triggers the pheromone-signaling system ComR-ComS, resulting in a bimodal activation. Deciphering the mechanisms underlying this puzzling behavior has remained challenging, especially when competence is controlled by more than one cell-to-cell communication system. In the present study, we identify CovRS, a well-described virulence regulator in streptococci of groups A and B, to control bimodality in the *salivarius* group via the direct transcriptional control of *comR*. Investigating this process using single-cell analysis of fluorescent reporter strains together with regulatory mutants, we show that the intracellular concentration of ComR determines the proportion of competent cells in the population. This bimodal activation requires a functional positive feedback loop acting on ComS production, as well as its exportation and reinternalization via dedicated permeases. Since CovRS integrates environmental stimuli that control ComR intracellular concentration, we suggest that it is the missing piece of the puzzle that connects environmental conditions and (bimodal) competence activation in *salivarius* streptococci.

Asexual mode of proliferation in bacteria is one of the most successful strategy in living organisms but comes with the cost of low genetic diversity [1]. To overcome this drawback, bacteria have evolved complex horizontal gene transfer mechanisms such as conjugation and natural transformation. Those processes are extremely efficient to generate genomic diversity and allow integration of DNA derived not only from closely-related species, but also from distant clades [1,2]. Recently, accumulation of genomic sequencing data has underlined the importance of those mechanisms for bacterial adaptation and evolution [3].

In bacteria, natural DNA transformation is triggered upon activation of a master transcriptional regulator and subsequent entry into a transient physiological state called competence [3]. Mechanisms of competence activation and regulation can drastically differ, even between closely-related bacterial species. However, the cost of this strategy in terms of energy and metabolism-shifting is significant [4,5] and as a consequence, activating this process can be fateful. To share responsibility at the population scale, initiating the system only in a subset of the community (bet-hedging) is a useful strategy and this is presumably why bacteria have evolved complex regulatory pathways to control competence development. Optimally benefiting from biological noise, these regulatory systems can stochastically activate the switch from non-competent to competent state [6]. Stochastic cellular decision making processes hidden behind competence acquisition have been thoroughly studied in *Bacillus subtilis* [7,8]. It has been determined in this species that the main factor for competence development relies on the master regulator ComK [9]. By experimentally manipulating the level of noise, it has been shown that a two-fold decrease of noise in *comK* expression led to a ten-fold reduction of the probability for switching to the competence state [7]. A critical aspect is the temporal modulation of the basal level of ComK that transiently pushes individual cells to reversibly switch from the non-competent to the competent state. Indeed, bimodal cell-to-cell distribution of molecules like ComK, is often referred as a bistable system. However, competence development should be considered as an excitable process rather than a classical bistable system as it stochastically switches on in a sub-population for a short period of time [8].

In streptococci, two main cell-to-cell communication systems for the control of competence have been described, ComCDE for the mitis and anginosus groups and ComRS for all other groups. While both systems show common features such as a transcriptional positive feedback on pheromone production, heterogeneous

activation of competence has been mostly investigated for ComRS-containing species [10–15]. This latter system involves two early competence (*com*) genes responsible for the induction of the master regulator ComX (alternative σ^X factor) and subsequent activation of late *com* genes required for the transformasome assembly (Fig. 1.1A) [16–19]. More precisely, ComX induction is triggered by the small peptide XIP (*comX*-inducing peptide) acting as a signaling pheromone. This oligopeptide is the mature form of an intracellular precursor (ComS), which can be exported by the PptAB transporter and processed by the Eep peptidase as shown in *Streptococcus thermophilus* [17,20] (Fig. 1.1A). Subsequently, XIP is reimported through the Ami/Opp transporter and binds to the transcriptional sensor ComR. This binding triggers ComR dimerization, resulting in the formation of the ComR:XIP complex that amplifies the early competence stage by enhancing *comS* transcription through a positive feedback loop [19,21]. Above a specific XIP concentration, ComR:XIP also induces ComX accumulation; ComX subsequently binds to the RNA polymerase and triggers the transcription of late *com* genes (Fig. 1.1A). In *Streptococcus salivarius*, the ComRS system additionally couples the activation of competence to predatory mechanisms [22], presumably to ensure the release of free DNA from bacteriocin-sensitive preys.

Understanding phenotypic heterogeneity and answering the question of how the ComRS system is able to generate a bimodal activation of competence in streptococci has remained challenging. This phenomenon has been mostly studied in *Streptococcus mutans*, where competence is regulated by both ComRS and the transcriptional regulation system BlpRH, ambiguously named ComCDE [10,11]. In this species, bimodality arises only via the activation of BlpRH through its cognate peptide MIP (Mutacin Inducing Peptide) but requires the ComRS system, suggesting a ComRS-triggering role for BlpRH [11,12,14].

In the present study, we investigated the origin of the ComRS-related bimodality in competence activation. We used *S. salivarius*, a model that does not contain a second competence-associated pheromone system such as the ComCDE/BlpRH found in *S. mutans* [22]. We showed that the intracellular concentration of the sensor protein ComR is the key factor that controls the bimodal activation of competence in *S. salivarius*. Searching for a two-component system (TCS) controlling *comR* expression, we unexpectedly revealed that CovRS, a well-described virulence controlling system in streptococci, fine-tunes the amount of ComR and thus competence bimodality in *salivarius* streptococci.

RESULTS

Competence activation is ComR-sensitive

Recently, we showed that the human gut-isolated *S. salivarius* strain HSISS4 strain was able to activate natural transformation through the ComRS system upon addition of the synthetic mature form of ComS (sXIP) [22]. To unveil activation bottlenecks in the ComRS pathway, we overexpressed all known components of the system (Fig. 1.1A).

For this purpose, we first transformed the *comX* promoter to luciferase reporter genes (P_{comX} -*luxAB*) into an ectopic locus in HSISS4. We then used this competence reporter strain to construct transcriptional fusions of the different known components of the ComRS system under the control of inducible or strong constitutive promoters (Fig. 1.1B). In line with previous data [22,23], we observed a high sensitivity of the signaling pathway regarding the amount of ComR (P_{xyl2} -*comR*, ~100-fold increase). In addition, we constructed *comR* overexpression systems in tandem with *comS* (full length pheromone, P_{xyl2} -*comR-comS*) or *xip* (an artificial gene encoding a 12-aa intracellular form of ComS, P_{xyl2} -*comR-xip*). Those strains showed high reactivity upon transcriptional induction (220- and 380-fold increase, respectively), presumably through bypassing *comS* transcriptional activation by ComRS and pheromone export-import, respectively (Fig. 1.1B). Although their functionality was validated (Fig. S1.1A and B), upregulation of the PptAB pheromone exporter [20] (P_{lac} -*pptAB*) or importer [16] (AmiA3, P_{32} -*amiA3* or AmiACDEF, P_{xyl2} -*amiACDEF*) showed no increase in competence activation. Unexpectedly, *comS* (P_{xyl2} -*comS*) or *xip* (P_{xyl2} -*xip*) overexpression had no effect on P_{comX} while exogenous sXIP addition elicited a marked response (260-fold increase) (Fig. 1.1B). Because functionality of the two constructs was confirmed (Fig. S1.1C), we concluded that the system was not sensitive to endogenous pheromone increase at native ComR concentration. Together, these results demonstrate that competence acquisition in *S. salivarius* depends mainly on the amount of ComR.

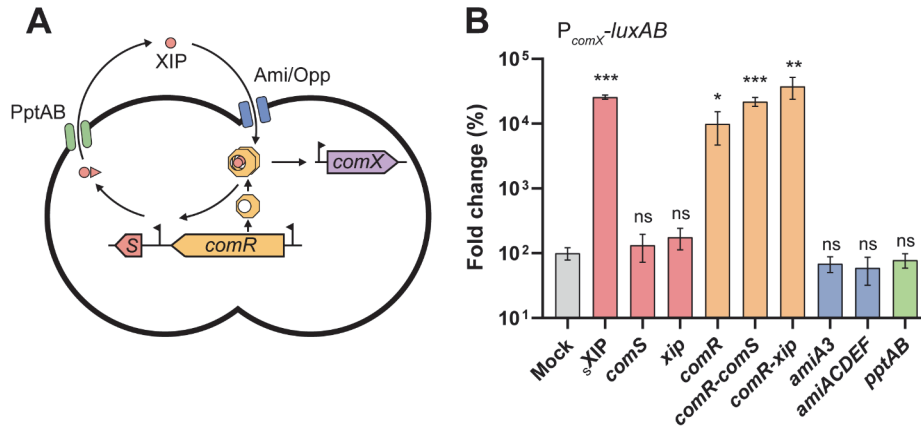


Figure 1.1 ComRS sensitivity analysis. **A.** Scheme of the ComRS system showing all components overexpressed for the sensitivity analysis. **B.** Fold change of maximum specific luciferase activity (RLU/OD₆₀₀) for the overexpression of the different ComRS components compared to mock (P_{comX} -*luxAB* reporter strain). For induction, 1% xylose and 1 mM IPTG was added for the xylose-inducible promoter (*comS*, *xip*, *comR*, *comR-comS*, *comR-xip*, *amiACDEF*) and the lactose-inducible promoter (*pptAB*), respectively. Mean values ($n = 3$) $\pm$ standard deviation. One-way ANOVA with Dunett's test was performed for each overexpressed component in comparison to mock (ns, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

ComR levels dictate bimodality

Since ComR is the most sensitive parameter for competence activation, we sought to determine its importance for ComRS-mediated competence bimodality. We therefore designed a transcriptional fusion between P_{comX} and a 5'-UTR- and codon-optimized *gfp*⁺ gene (P_{comX} -*gfp*⁺). We transferred the xylose-inducible *comR* cassette (P_{xyl2} -*comR*) into this background and performed microscopic analysis with populations incubated with various xylose concentrations (Fig. 1.2A). Results showed the emergence of a high *gfp*⁺-expressing subpopulation proportional to xylose increase. We observed a bimodal activation of P_{comX} from 0.1 to 0.2% xylose concentration, with 0.5% displaying unimodal P_{comX} activation across the population (Fig. 1.2A). To validate that bimodality was not an artefact due to the xylose-inducible promoter itself, we constructed a P_{xyl2} -*gfp*⁺ strain. Cell populations of this control strain incubated with the same range of xylose concentrations exhibited no bimodal activation (Fig. S1.2), supporting the idea that bimodality arises from the ComRS system itself. To measure the amount of ComR protein added to the native system through the P_{xyl2} -*comR* construct, we performed western blot analyses on crude cell extracts from xylose-induced samples (Fig. 1.2B). Comparison with a non-

induced condition showed that a small increase in the amount of ComR (< 1.5-fold at the population level) was sufficient to activate P_{comX} in a subpopulation.

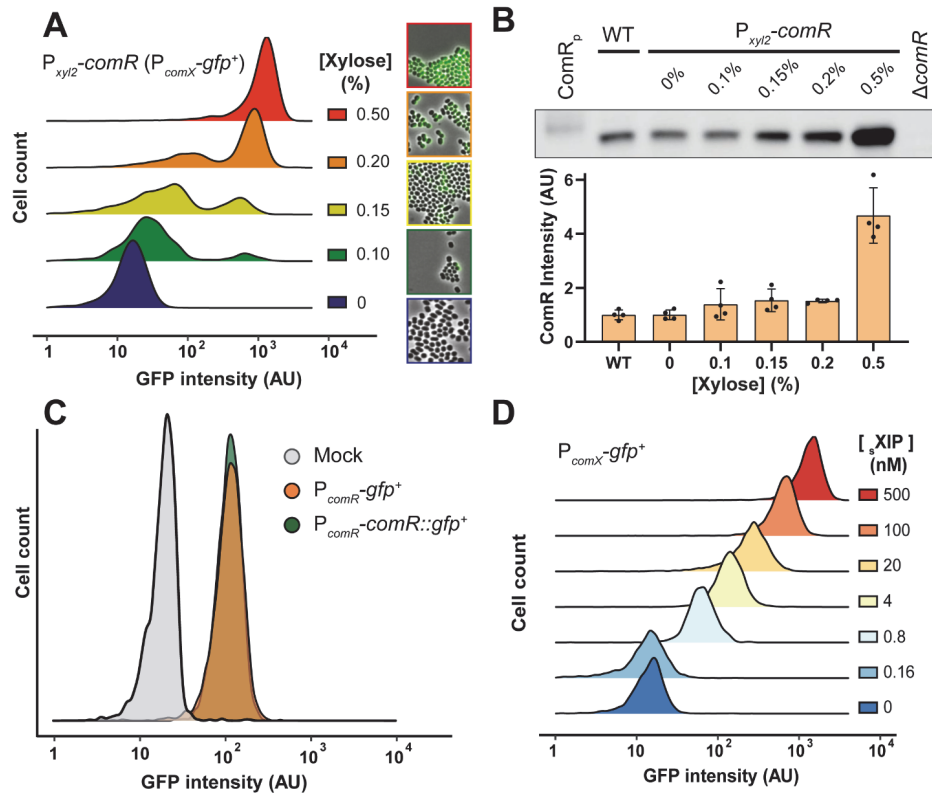


Figure 1.2. Control of competence bimodality by ComR. **A.** Density plot of single-cell GFP intensity (arbitrary unit (AU), log scale) from a gradient of ComR levels. The overexpression of $comR$ (P_{xyl2} - $comR$) was monitored with a P_{comX} - gfp^+ reporter strain in presence of increasing xylose concentrations (%). Pictures on the right are representative cell samples of each xylose concentration tested. **B.** Semi-quantification of ComR by immunoblotting. ComR western blot with crude extracts (technical replicates, $n = 4$) from WT (strain HSISS4), $\Delta comR$ mutant (negative control), and $comR$ overexpressing strain (P_{xyl2} - $comR$ P_{comX} - gfp^+) incubated 2 hours with increasing xylose concentrations (%). $ComR_p$ denotes recombinant ComR-StrepTag used as positive control. Gel bands intensities were quantified and normalized using total protein quantification from Coomassie blue staining. **C.** Density plot of mock (gray), P_{comR} activation (orange), and ComR levels (green) from microscopic analysis of HSISS4 WT, P_{comR} - gfp^+ , and P_{comR} - $comR::gfp^+$ fluorescence intensities, respectively. **D.** Density plot of a sXIP dose-response (nM) from microscopic analysis of P_{comX} - gfp^+ fluorescence intensities. For panels A, C, and D, the fluorescence of individual cells was examined from biological triplicates ($n > 500$ cells in each experiment).

Finally, to assess that bimodality was arising from the topology of the ComRS regulatory circuitry and not from an upstream bimodal transcription of *comR*, we analyzed its expression at the single cell level using a $P_{comR}\text{-}gfp^+$ reporter strain. Microscopic analysis showed unimodal *gfp*⁺ expression for the whole population, suggesting a low-noise *comR* transcription (Fig. 1.2C). To verify ComR unimodality at the translational level, we performed a population-wide microscopic analysis of a ComR-GFP⁺ fusion ($P_{comR}\text{-}comR::gfp^+$) (Fig. 1.2C) and a sXIP dose-response on $P_{comX}\text{-}gfp^+$ expression (Fig. 1.2D). A unimodal response was observed in all tested conditions, revealing that the distribution of ComR over the cells in the population does not phenocopy the bimodal distribution of *comX* expression.

Altogether, those results show that the level of ComR is important for the activation of ComRS into a hypersensitive mode and is the key factor for competence bimodal activation; this latter feature does not arise from heterogenous ComR distribution in the population.

A positive feedback loop is required for bimodal activation

While we showed that ComR levels played a central role in the bimodal activation of competence, we had no information on mechanisms involved in this phenomenon. Since positive feedback loop-based networks have been shown to generate heterogeneity in other bacterial systems [24,25], we investigated the role of the ComRS feedback loop for the bimodal activation of the system (Fig. 1.1A). For this purpose, we inactivated the loop by constructing mutants of its key players (*i.e.* $\Delta comS$, $\Delta amiACDEF$, $\Delta pptAB$) into a $P_{comX}\text{-}gfp^+$ background harboring the $P_{xyI2}\text{-}comR$ overexpression cassette. ComR overproduction through xylose induction in those strains showed no P_{comX} activation, underlining the necessity of the pheromone, and its export and import for ComR-mediated activation (Fig. 1.3A and B).

We then focused on the origin of bimodality and its link to the positive feedback loop. For this purpose, we compared a WT strain to $\Delta comS$ and $\Delta comS P_{32}\text{-}comS$ mutants (Fig. 1.3C). The first mutant is deficient in pheromone production while the second expresses the pheromone under the control of a strong constitutive promoter unplugged from the feedback loop. Of note, those three strains possess the $P_{xyI2}\text{-}comR$ overexpression cassette in a $P_{comX}\text{-}gfp^+$ background. We applied various xylose concentrations and observed no competence activation for the pheromone deficient strain ($\Delta comS$) while the reference strain showed

bimodal activation for mild xylose induction (Fig. 1.3C). In contrast, the mutant producing the pheromone unwired from the loop showed a unimodal activation, gradually increasing in line with *comR* overexpression (Fig. 1.3C). Together, these results show that the ComRS positive feedback loop is necessary for ComR-mediated competence activation and its bimodal behavior.

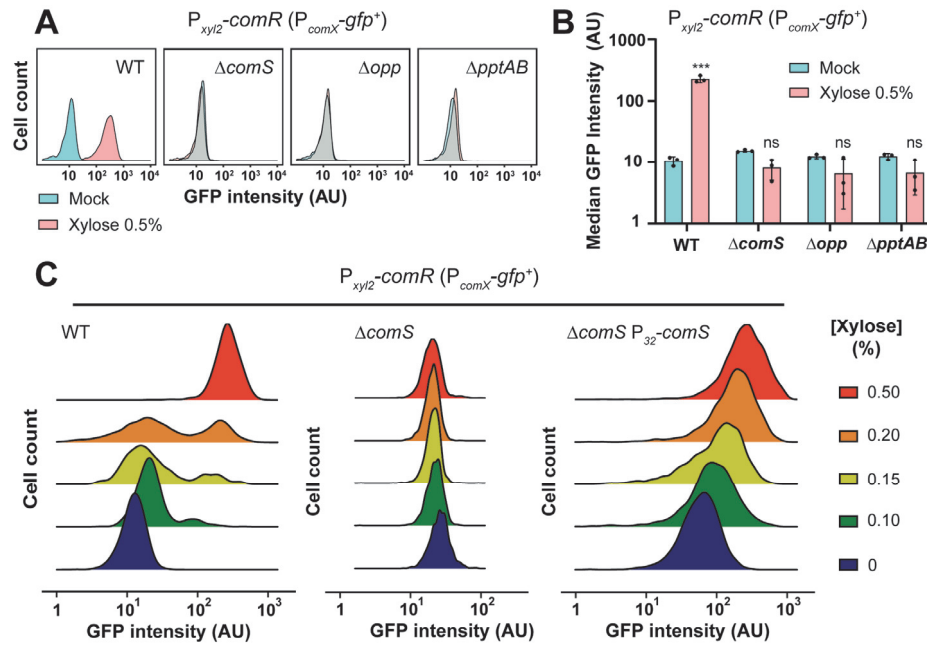


Figure 1.3. Implication of the positive loop in competence bimodality. **A.** Representative density plots of loop inactivation from microscopic analysis of a $P_{comX}-gfp^+$ $P_{xyI2}-comR$ strain harboring either a native loop (WT), or an inactivated loop through deletion of *comS* (pheromone precursor; $\Delta comS$), *amiACDEF* (XIP importer; Δopp) or *pptAB* (XIP exporter; $\Delta pptAB$). Each sub-panel depicts a population fluorescence analysis for each strain either without (mock) or with strong xylose induction (0.5%). **B.** Bar chart depicting biological triplicates of the representative loop-inactivation experiments shown in A. Mean of median values ($n = 3$) $\pm$ standard deviation. Statistical *t*-tests were performed for each inactivated component in comparison to its related mock (ns, non-significant; ***, $P < 0.001$). **C.** Representative density plot of single-cell GFP intensity (arbitrary unit (AU), log scale) from a gradient of ComR levels. The overexpression of *comR* ($P_{xyI2}-comR$) was monitored with a $P_{comX}-gfp^+$ reporter strain in presence of increasing xylose concentrations (%). The same conditions were applied to three different genetic backgrounds: a mock strain ($P_{comX}-gfp^+$ $P_{xyI2}-comR$), and the same strain deficient for the pheromone ($\Delta comS$) and with the pheromone under the control of a strong constitutive promoter, unplugged from the positive feedback loop ($\Delta comS P_{32-comS}$). The fluorescence of individual cells was examined from biological triplicates ($n > 500$ cells in each experiment).

The two-component system CovRS controls comR expression

As we identified that the amount of ComR is central in the bimodal activation of competence, we investigated how ComR production and subsequent bimodality were controlled. Various two-component systems (TCSs) that sense environmental signals were previously shown to modulate competence in streptococci [26–28], and we decided to investigate their role in *comR* regulation. To this end, we conducted an *in silico* search in the HSISS4 genome and identified 13 putative TCSs harboring a response regulator in tandem with a histidine kinase (Table S1.1). To investigate their role in *comR* regulation, we successfully inactivated 10 complete TCS in the P_{comR} -*luxAB* reporter strain (Fig. 1.4A). Because we were not able to generate three mutants ($\Delta covRS$, $\Delta fasAB$, $\Delta vicRK$), presumably due to their essential functions, we generated viable single gene deletions of their histidine kinase ($\Delta covS$, $\Delta fasB$, $\Delta vicK$). Since the $\Delta covS$ mutant showed the strongest and most significant effect on *comR* expression (Fig. 1.4A), we decided to investigate the importance of the CovRS system in more depth.

The CovRS system (Control of virulence) has been well characterized in *Streptococcus pyogenes* and *Streptococcus agalactiae* where it regulates the expression of a high proportion of the genome, including several virulence genes [29,30]. Mechanistic studies have shown that upon activation through a stimulus (e.g. human defensin, divalent cations or osmotic shock), CovS is able to trigger either phosphorylation (via a glutamate residue) or dephosphorylation (via a threonine residue) of an aspartate on its cognate response regulator CovR [31–34]. As a result of phosphorylation, CovR forms dimers and binds DNA, mainly as a repressor [35]. Because we identified a conserved motif in CovS at the phosphatase site, we decided to perform a point mutation and substitute the key T287 residue for alanine. In parallel, we produced two other point mutations of the phosphorylation site of CovR, D53A and D53E, mimicking the unphosphorylated and constitutive phosphorylated state, respectively. We transferred those mutations into a P_{comR} -*luxAB* background and monitored the fold change in expression (Fig. 1.4B). Because CovR_{D53E} and CovR_{T287A} over-repressed *comR* expression while CovR_{D53A} relaxed repression, we concluded that the phosphorylated form of CovR (CovR~P) is responsible for *comR* repression. In addition, RNAseq experiments (Table S1.2) showed that CovS_{T287A} and CovR_{D53E} mutants over-repressed most of the genes of the ComR regulon that was previously established, as well as a large set of genes directly controlled by ComX which are essential for natural transformation [22].

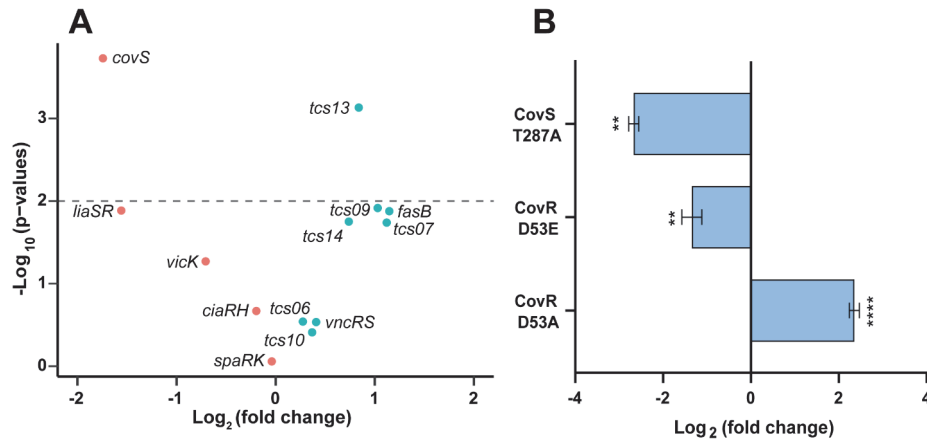


Figure 1.4. The two-component system CovRS controls *comR* expression. **A.** Volcano plot showing the inactivation effect of complete TCS or their histidine kinase (*covS*, *vicK*, *fasB*) on P_{comR} activation. Dashed line denotes threshold corresponding to $P = 0.01$, green and red dots show *comR* upregulation and downregulation, respectively. **B.** Analysis of key residues of CovRS activity on *comR* expression. Phosphorylated residue of CovR was mutated either in alanine (D53A) or glutamate (D53E) to mimic unphosphorylated or phosphorylated state, respectively. CovS residue for phosphatase activity was mutated in alanine (T287A). For A. and B., P_{comR} activation was monitored with the P_{comR} -*luxAB* reporter strain. Maximum specific luciferase activity (RLU/OD₆₀₀, Log₂) were computed from biological triplicates and mean values were used to calculate fold changes ($\pm$ standard deviation in B.) in comparison to mock. One-way ANOVA with Dunett's test was performed on log₂ fold change data to generate p-values (**, $P < 0.01$; ****, $P < 0.0001$ in B.).

Altogether, these data demonstrate that the CovRS system is pivotal in the control of competence development in *S. salivarius* through modulation of *comR* expression.

CovR inhibits comR expression through direct transcriptional control

As the CovRS system had shown strong control over the *comR* promoter *in vivo*, we characterized *in vitro* interactions between CovR and P_{comR} . To this end, we purified three variants of CovR (CovR_{WT}, CovR_{D53A}, and CovR_{D53E}, Fig. S1.3) and performed electrophoretic mobility shift assays (EMSA) on Cy3-labelled DNA corresponding to P_{comR} (150 bp upstream of the start codon) (Fig. 1.5A and B). The results showed a binding of purified CovR_{WT} to P_{comR} , which is lower and higher for the CovR_{D53A} variant and the phosphomimetic CovR_{D53E}, respectively. The ~2-fold difference in binding efficiency between the two CovR mutants is similar to previously reported data between unphosphorylated CovR and CovR~P from other streptococci [36]. Although this difference seems low, it has been

demonstrated that the presence of the RNA polymerase enhances the CovR~P repression, explaining higher *in vivo* repression [37]. To demonstrate the specificity of this binding, we performed EMSA with the promoter and an internal CDS fragment of the physiologically unrelated gene *dnaE* (alpha subunit of PolIII, P_{dnaE} and CDS_{dnaE} , respectively), as well as the promoter of CovR (P_{covR}) that has previously been reported to be bound by CovR in other streptococci [38,39]. While CovR showed a binding to P_{covR} , weak unspecific binding at very high CovR concentration and no binding were observed for P_{dnaE} and CDS_{dnaE} , respectively (Fig. S1.4A). Furthermore, competition assays with unlabeled probes (P_{comR} , P_{dnaE} and CDS_{dnaE}) showed a decrease in CovR- P_{comR} interaction for the P_{comR} competing probe but no effect for P_{dnaE} and CDS_{dnaE} , underlining the specificity of these interactions (Fig. S1.4B).

Previous work in *S. pyogenes* had identified the DNA motif responsible for CovR binding (ATTARA motif) [36,38], and we screened the *comR* promoter for this motif (Fig. 1.5C). We found many motifs with one mismatch but no consensus sequences. Particularly, the region just upstream of the predicted -10 box showed a high density of directly repeated motifs on both DNA strands, which in most cases are separated by five nucleotides (Fig. 1.5C). To determine whether that region could bind CovR, we performed EMSA with a rational restriction of the P_{comR} probe labelled with Cy3 and Cy5 at the 5' and 3' end, respectively (Fig. 1.5C). While unrestricted P_{comR} showed an orange bandshift upon CovR binding due to red and green fluorophore co-labelling, the restriction mapping based on the mobility shift of 5'-end (green) and 3'-end (red) labelled fragments showed that only the region upstream of the -10 box with the mapped motifs is able to bind CovR (Fig. 1.5C). Particularly, we observed a shift for both 5' and 3' fragments when restriction was performed in the middle of the binding region (AseI), suggesting interaction of CovR with multiple sites. In addition, a double restriction surrounding this region (PstI-NsiI) showed no band-shift, which excludes the presence of other binding sites in the 150-bp fragment upstream of the start codon of *comR*.

Altogether, these results show that the CovRS system controls competence activation in *S. salivarius* by directly repressing *comR* transcription through binding of CovR to multiple sites in its promoter region.

CovR represses competence in the salivarius group

We next investigated if the CovRS-mediated competence repression could be broadened to other streptococci of the salivarius group. Since inactivation of the

A

P_{comR}

CovR WT CovR D53A CovR D53E

B

K_d value (μM)

WT D53A D53E

C

P_{comR}

/ NsiI AseI PstI DraI PstI-NsiI

CovR_{WT} - + - + - + - + - + - +

CovR-binding site
ATTARA =
ATTAAA or ATTAGA

5/6 (with TT) 4/6 (with ATT)

Direct repeats
Strand + (N=5)
GT TAAA AATGC ATTAA C
ATTAA C TGTAT ATTAAT
ATTATA TTATA ATTAT C
Strand - (N=5)
ATTATA TCATA ATTATA
ATTATA ATATA ATTAAT

Cy3

5' - [N]₆₄ GTTAAAAATGCATTAACTGTATATTAATTATATTATATATGATATAATGTTTTTTAAAA [N]₂₅ - 3'

3' - [N]₆₄ ACAATTTTTCGTAATTGACATATAATTAATATAATTAATACTATATTACAAAAATTTT [N]₂₅ - 5'

NsiI AseI PstI DraI

NsiI
AseI
PstI
DraI
PstI-NsiI

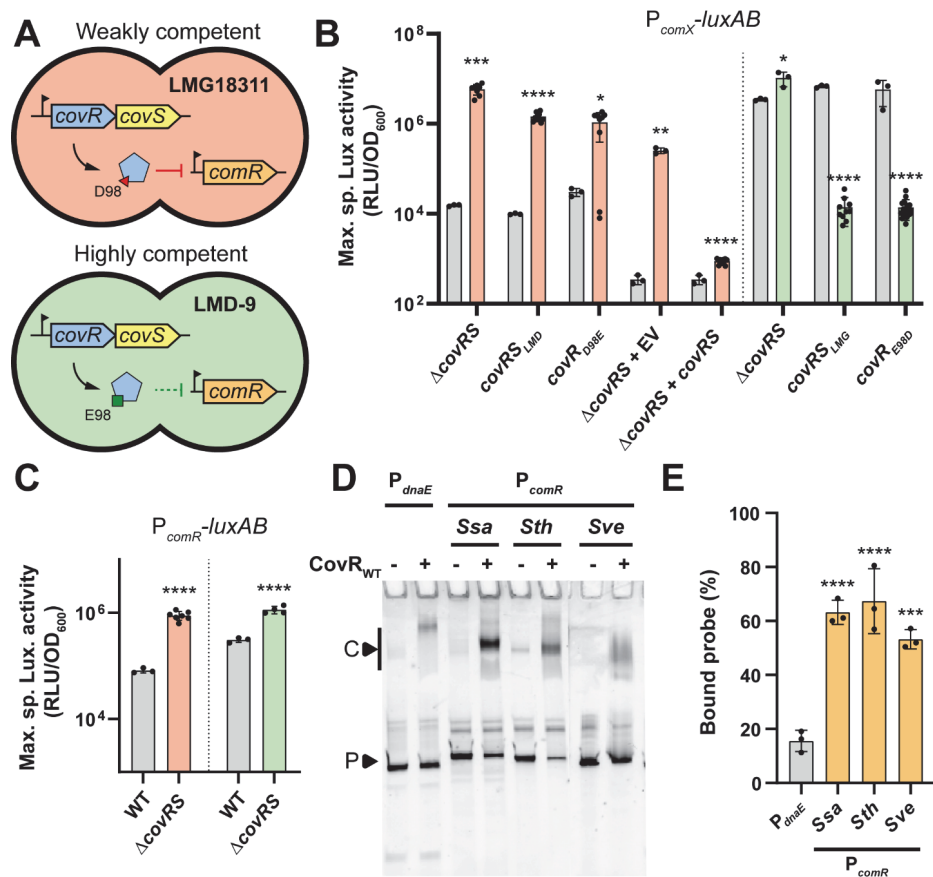
Cy5

← **Figure 1.5. Characterization of CovR binding to P_{comR} .** **A.** Representative EMSA (upper panels) performed with the P_{comR} probe (150 bp, 0.05 μ M) and a gradient of purified CovR_{WT}, CovR_{D53A} or CovR_{D53E} (triangles, maximum of 1.4 μ M with serial 1.4:1.4 dilutions). Minus sign depicts lanes with no CovR. P and C denote the unbound probe and the CovR-DNA complex, respectively. Gels displayed are representative of technical triplicates. Non-linear regressions of percentage of bound probes (black dots show triplicates values) were implemented using a Hill equation ($n = 4$) (lower panels). **B.** Comparison of estimated K_d values ($\pm$ standard deviation) between CovR variants. These values were extrapolated from non-linear regressions shown in A. One-way ANOVA with Dunett's test was performed for the K_d values of CovR variants in comparison to WT (*, $P < 0.05$; **, $P < 0.01$, $n=3$). **C.** Mapping of CovR binding motifs by restriction of the P_{comR} probe. EMSA experimental data and schematic view of P_{comR} restriction pattern are displayed on left and right, respectively. P_{comR} probe (150 bp) was double labelled with Cy3 and Cy5 fluorophores and restricted by NsiI, AseI, PstI, DraI, and PstI-NsiI for the fine mapping of CovR binding motifs. The resulting products (0.05 μ M or 0.1 μ M for unrestricted and restricted DNA, respectively) were incubated with (+) or without (-) CovR_{WT} (0.43 μ M). The depicted double stranded DNA sequence shows positions 65 to 125 of the P_{comR} probe. Putative CovR-binding sites (ATTARA) upstream of the -10 box (purple) of P_{comR} are highlighted by colored boxes (light blue, one mismatch with TT conservation; gray, two mismatches with ATT conservation). Direct repeats separated by 5 nucleotides are highlighted in a separate box. Green and red sun icons represent 5'-end Cy3 and 3'-end Cy5 labelling, respectively. Positions of scissors denote cleavage sites of restriction enzymes and green/red horizontal lines display restriction products with color code corresponding to their labelled fluorophore. Continuous and dashed lines denote presence and absence of a shift on the gel, respectively.

Notably, the impact of CovRS inactivation was much more pronounced in the LMG18311 background than in the spontaneously transformable LMD-9 strain (Fig. 1.6B and C), suggesting a differential repression effect of the CovRS system. By comparing *comR* promoter sequences, we observed a high sequence conservation and a similar distribution of ATTARA motifs between the two strains (Fig. S1.5A). We then aligned CovR and CovS sequences from representative strains of the salivarius group and found a specific mutation (D98E) at a conserved position in CovR-LMD-9 compared to CovR-LMG18311 (Fig. S1.5B). To investigate the role of this mutation in spontaneous competence activation of LMD-9, we exchanged the *covRS* operon and the point mutation between the two strains. As shown in Fig. 1.6B, the reciprocal exchange of CovRS or D98E mutation alone inverted the level of P_{comX} activation between the two genetic backgrounds. Notably, due to the D98E mutation, the *comX* expression in LMG18311 increased to a sufficient level to unleash natural transformation. The measured transformation rate of various clones was $\sim 10^{-4}$, which is similar to strain LMD-9 [41]. These results show an altered functionality of CovR_{D98E} that is responsible for a spontaneous transformation in strain LMD-9.

Finally, as CovR proteins and *comR* promoters of *S. salivarius*, *S. thermophilus* and *S. vestibularis* show a high level of conservation (Fig. S1.5), we performed EMSAs with the purified CovR_{H515S4} and the different promoter sequences. We observed a significant shift of the three promoter probes that validated direct interactions between partners (Fig. 1.6D and E).

Together, those data show that CovRS-mediated competence repression is a common feature in the salivarius group of streptococci. Moreover, a point mutation that partially abrogates CovR functionality could explain the spontaneous competence behavior of some *S. thermophilus* strains under laboratory conditions.



← **Figure 1.6. Distal regulation of ComR by CovRS in salivarius streptococci.** **A.** Schematic view of the two CovRS modules present in strains LMG18311 (orange) and LMD-9 (green) of *S. thermophilus*. Red and green lines illustrate strong and weak repression effect of CovRS on P_{comR} , respectively. The weak repression effect in LMD-9 is due to CovR_{D98E} mutation, which correlates with its high spontaneous transformability. **B** and **C.** Effect of CovRS modulation on P_{comX}/P_{comR} activity (B/C) in LMG18311 (orange) and LMD-9 (green). Data show maximum specific luciferase activity (RLU/OD₆₀₀) for $\Delta covRS$ mutants, $covRS$ reciprocal swap (LMG/LMD), $covR$ point mutation exchange (D98E/E98D), plasmid complementation of LMG18311 $\Delta covRS$ ($\Delta covRS + covRS$) and its control ($\Delta covRS +$ empty vector [EV]) in comparison to their related WT strain (grey). **D.** Representative EMSA performed with the negative control P_{dnaE} or P_{comR} probes of *S. salivarius* (Ssa) HSISS4, *S. thermophilus* (Sth) LMD-9, and *S. vestibularis* (Sve) NCTC12167 (150 bp, 0.05 μ M) in presence (+) or absence (-) of CovR_{WT} from Ssa HSISS4 (0.4 μ M). **E.** Bar chart showing the percentage of bound probe for each promoter shown in C. Dots show biological replicates values (B. and C., $n \geq 3$) or technical triplicates values (E.) $\pm$ standard deviation. Statistical *t*-test was performed for each condition in comparison to related mock (WT: mock, B. and C.) and one-way ANOVA with Dunett's test was performed for multiple comparison (P_{dnaE} : mock, E.) (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

CovR controls bimodal competence activation in the salivarius group

CovR is a major repressor of *comR* expression and we subsequently examined if we could generate bimodal competence activation through CovRS fine-tuning. For this purpose, we set-up a conditional mutation system based on a CRISPR-interference (CRISPRi) module targeting *covRS* expression. Using an optimized LacI repressor together with a dead-Cas9 (dCas9) under the control of a P_{lac} promoter [42], we first validated the system in *S. salivarius* (Fig. S1.6A and B) and used it to repress the transcription of the *covRS* operon using a guide RNA (gRNA) targeting the *covR* promoter (Fig. S1.6C). The conditional repression of *covRS* increased P_{comR} and P_{comX} activities to a sufficient level to trigger competence (Fig. S1.6D). However, a strong growth defect associated with abnormal cell morphology and cell aggregation was also observed (Fig. S1.6E and F). Although we were able to show bimodal competence activation through CovR depletion in *S. salivarius* (Fig. S1.6F), determining if toxicity contributes to heterogeneity remained challenging.

Taking the problem the other way around, we investigated if we could turn a unimodal activated system due to a lack of CovR functionality, as expected in *S. thermophilus* LMD-9 (CovR_{E98}, *covR*_{LMD}), into a bimodal system by overexpressing *covR* from strain LMG18311 (CovR_{D98}, *covR*_{LMG}). To this aim, we inserted the *covR*_{LMG} allele under the control of P_{xyl2} ($P_{xyl2^-} covR_{LMG}$) at an ectopic locus together with a P_{comX} -*gfp*⁺ reporter fusion in the LMD-9 background (Fig. 1.7A).

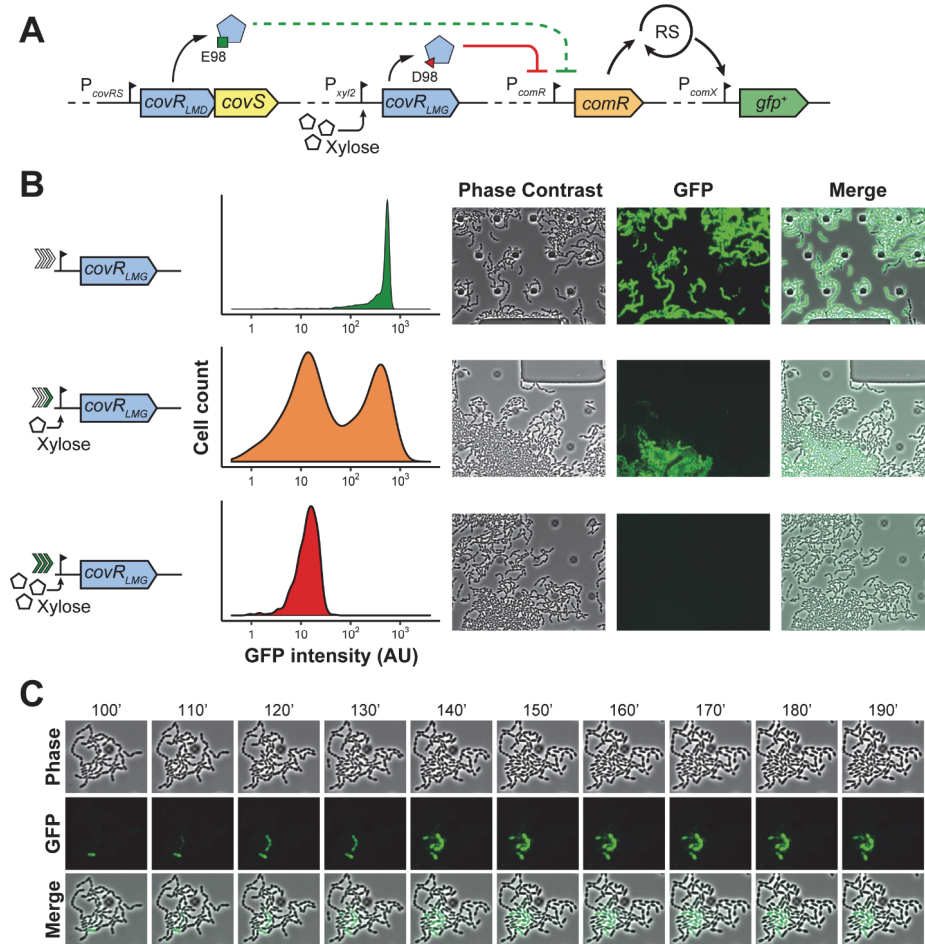


Figure 1.7. CovR controls competence bimodality. **A.** Schematic view of the merodiploid LMD-9 strain (*covR_{LMD}*, *CovR_{E98}*) expressing the xylose-inducible wild-type *covR* allele from LMG18311 (*covR_{LMG}*, *CovR_{D98}*) used in panel B and C. RS, signal amplification through ComRS. **B.** Gradual overexpression of *covR_{LMG}* fused to *P_{xyI2}* in LMD-9 strain harboring the *covR_{LMD}* mutated gene. The strain was grown 4 hours in M17G, washed and incubated 1 hour in CDMG prior to loading into a microfluidic device fed with CDMG. Cells with no/mild/high-induction (increasing green arrows) were preincubated with xylose 0/0.5/0.5% in M17G, 0/0.5/0.5% in CDMG and with 0/0/0.5% in the microfluidic device fed with CDMG, respectively. Density plots show the distribution ($n > 600$) of fluorescence, and representative pictures are displayed on the right side, taken after 150 min of growth. **C.** Timelapse showing bimodal activation of competence with a *covR_{LMG}* mild-induced condition.

Microscopic observations in a microfluidic device of the merodiploid strain showed 100% of P_{comX} activation in the non-induced cell population and no activation with the fully xylose-induced $covR_{LMG}$ gene (Fig. 1.7B). Interestingly, we were able to produce a bimodal activation by fully inducing $covR_{LMG}$ in a preculture, which was further cultivated in a xylose-free medium to generate $CovR_{LMG}$ depletion by cell division and subsequent dilution (Fig. 1.7B). Time-lapse experiments performed in the same condition illustrate heterogeneous initiation of competence (Fig. 1.7C).

Altogether, these results strongly suggest that controlling CovRS activity allows to reproduce the effect of ComR on the bimodal activation of competence in *salivarius* streptococci.

DISCUSSION

Investigating how environmental sensors influence individual cell fates and elicit bet-hedging strategies in the context of competence is of prime importance to understand how bacteria face different stresses collectively. In the present study, we selected *S. salivarius* as a representative species of ComRS-containing streptococci based on the presence of a single competence signaling system (*i.e.* ComRS) and absence of spontaneous transformation under laboratory conditions. Our results showed that intracellular levels of the transcriptional sensor ComR and noise amplification through the production of the pheromone ComS/XIP (feedback loop) are the key factors determining the bimodal activation of natural competence in *S. salivarius*. Notably, we revealed the direct transcriptional repression of *comR* by the CovRS system, a TCS controlling virulence in pathogenic streptococci and reported here for the first time as a key player in competence regulation.

Altogether, those data suggest a new model for the *salivarius* group where CovRS fine-tunes the amount of ComR which plays a pivotal role in bimodal competence activation (Fig. 1.8). Particularly, our results suggest two non-exclusive mechanisms that could be at work to explain bimodality in ComRS streptococci: an increase of ComR that would result in (i) a Gaussian distribution of ComR levels, with a subfraction of the population producing enough ComR to exceed the critical concentration threshold that activates competence and (ii) a decrease of the ComS threshold necessary for non-linear activation, ultimately generating bimodality from ComS noisy concentration amplification. These mechanisms might be extended to other species and explain why bimodality can be observed only in complex media upon BlpRH activation in *S. mutans*. Indeed,

transcriptomics data indicated that BlpRH is able to upregulate ComR only in complex media in this species [10,14]. Those results suggest that, similarly to the *salivarius* group, an increase of ComR concentration could account for bimodality. Besides, the control of ComR levels by CovRS in *S. salivarius* mirrors the control of ComRS by BlpRH in *S. mutans*. The first one is known as an environmental sensor while the second is a pheromone communication device. The use of differing distal regulators in both species is probably linked to their distinct lifestyles and specific needs to react toward different competence stimuli.

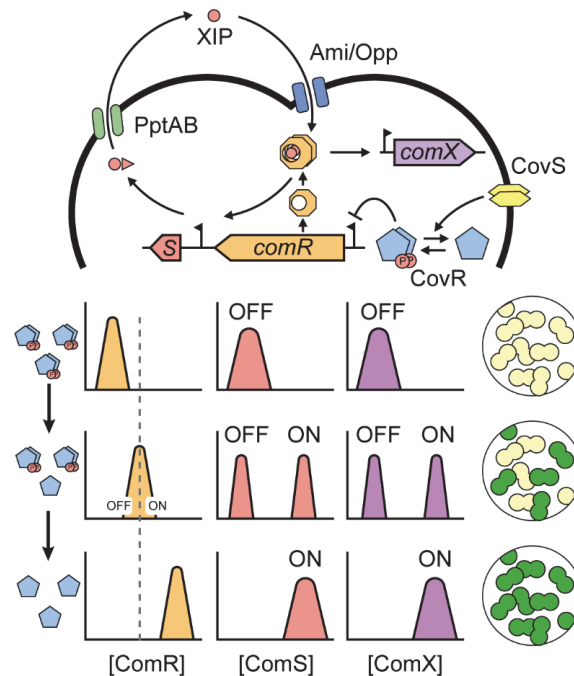


Figure 1.8. Model for competence bimodal activation in salivarius streptococci. The environmental sensor CovS interacts with CovR through its phosphatase/kinase activity to tune the phosphorylation state of CovR at position D53. Phosphorylated CovR will dimerize and bind P_{comR} to repress *comR* transcription while unphosphorylated CovR will not. Low levels of phosphorylated CovR will trigger an increase of ComR concentration to a level sufficient to launch the positive feedback loop driven by *comS* (S). The loop will be activated in part of the population generating bimodality either because an increase of ComR concentration (i) will lower the ComS threshold necessary for activation and noise of ComS concentration will be amplified or (ii) will shift ComR distribution and noise of ComR concentration will be amplified. Both scenarios will lead to the emergence of two subpopulations (ON/OFF) for *comX* and *comS* activation, but *comR* expression will remain unimodal.

In addition to a dissimilar distal control of ComRS, the pheromone-based induction system of competence behaves differently between *S. salivarius* and *S. mutans*. In this work, we have shown the necessity for a pheromone exporter (PptAB) and a peptide importer (Ami/Opp) for competence activation through ComR overproduction (Fig. 1.3).

This suggests that the pheromone ComS must be exported, matured, and reimported in order to generate a functional positive feedback loop in *S. salivarius*. Conversely, PptAB does not seem to be essential for ComS export in *S. mutans* [43] and its Opp system was shown to be dispensable for bimodal activation, presumably because of non-matured ComS intracellular activity [11,44].

Furthermore, the pheromone overproduction does not trigger competence in *S. salivarius* (Fig. 1.1), in contrast with the *S. mutans* model [44]. Those data underline the specific low sensitivity of the ComRS system toward subtle-to-mild ComS increase at native ComR levels in *S. salivarius*. This suggests that this species has evolved a negative player locking the entry into competence through ComS limitation, which is counter-intuitive regarding the fact that ComRS is sensitive to addition of low exogenous sXIP concentrations (Fig. 1.2D). A negative player such as a pheromone specific peptidase described in other streptococci could explain this discrepancy [45,46]. While peptidase processive activity could impair the progressive intrinsic peptide production, exogenous sXIP addition would rapidly saturate the degradation apparatus.

To our knowledge, the inhibition of competence through CovRS has never been reported in streptococci. While transcriptomics data suggest a positive correlation between the standalone CovR and competence activation in *S. mutans* [47], no link between CovRS and competence was reported in GAS (Group A Streptococci) or GBS (Group B Streptococci), despite extensive transcriptomics analyses [29,33,48–50]. Notably, we show that a natural mutation altering CovR function (*i.e.* CovR_{D98E}) is associated with spontaneous transformation in salivarius streptococci while CovRS mutations generate hyper-virulent strains in GAS [51]. Analogously, natural mutations in competence repression systems of bacilli were previously reported to explain spontaneous transformation of specific isolates under laboratory conditions [52]. Our mutagenesis results obtained with the non-phosphorylatable (D53A) and the phosphomimetic (D53E) alleles of CovR are in accordance with previous reports in GAS and GBS, showing an increase of transcriptional repression for the

phosphorylated form of CovR [39,53]. As expected, the phosphatase mutant (T287A) of CovS showed a decrease in *comR* expression since more abundant CovR~P is predicted. Whether CovS in GAS has mostly a phosphatase or a kinase activity is still unclear [34,54–56]. In *S. salivarius*, we showed that a $\Delta covS$ mutant displays a strong *comR* repression (Fig. 1.4A), suggesting a high CovR~P level and consequently the existence of alternative phosphorylation mechanism(s) (e.g. TCS crosstalk or small phospho-donor molecules) as hypothesized for GAS and GBS [34,57]. While this implies that CovS has mostly a phosphatase role in our experimental conditions, the environmental trigger(s) of this activity remains challenging to discover. In GAS and GBS, the CovS triggering stimuli have been extensively studied. While CovS of GAS reacts to pH, human defensin LL-37, osmotic shock, amino-acid starvation, temperature and iron starvation [31,33,58–61], the GBS CovS has been reported to only react to pH and glucose availability [62,63]. Environmental trigger identification performed in this work confirmed previous data [40], suggesting a pH dependency of the CovRS activity in the salivarius group (Fig. S1.7). However, we were unable to generate a response for the stimuli reported in GAS (Fig. S1.7). Because competence activation is extremely species-specific and depends on the bacterial lifestyle [3], it is not surprising to find that commensals belonging to the salivarius group do not respond to the same stimuli as GAS/GBS pathogens.

This study of the molecular mechanisms hidden behind competence bimodal activation in *S. salivarius* helps us to understand how bacteria can integrate external stimuli and translate them into a quantitative subpopulation activation. This collective survival strategy is of major importance for subsisting in highly competitive environments such as the human digestive tract and its study broadens our understanding of its dynamics in bacterial communities.

MATERIAL & METHODS

Bacterial strains, plasmids, and oligonucleotides

Bacterial strains, plasmids, and oligonucleotides used in this study are listed and described in supplementary information (Tables S1.3-1.5).

Growth conditions

S. salivarius HSISS4 [64], *S. thermophilus* LMG 18311 and *S. thermophilus* LMD-9 derivatives were grown at 37°C without shaking in M17 (Difco Laboratories, Detroit, MI) or in chemically-defined medium (CDM) [65] supplemented with 1% (w/v) glucose (M17G, CDMG, respectively). *Escherichia coli* TOP10 (Invitrogen) was cultivated with shaking at 37°C in LB (Lysogeny Broth). Chromosomal genetic constructions were introduced in *S. salivarius* and *S. thermophilus* via natural transformation [41]. Electrotransformation of *E. coli* was performed as previously described [66]. We added D-xylose (0.1 to 1 %; w/v), IPTG (1mM), ampicillin (250 µg/ml), spectinomycin (200 µg/ml), chloramphenicol (5 µg/ml), erythromycin (10 µg/ml) and kanamycin (500µg/ml) as required. The synthetic peptide XIP (sXIP, purity of 95%), was supplied by Peptide 2.0 Inc. (Chantilly, VA) and resuspended in water. Solid plates inoculated with streptococci cells were incubated anaerobically (BBL GasPak systems, Becton Dickinson, Franklin lakes, NJ) at 37°C. When stated, the peptide LL-37 supplied by Proteogenix (Schiltigheim, FR) (1 µM, purity of 95%), NaCl (300 mM), sorbitol (7% v/v), H₂O₂ (0.01% v/v) or branched chain amino acids (valine, leucine and isoleucine at final concentration of 7.5 mM, 9.7 mM and 4.3 mM, respectively) were supplemented to CDMG. A metal depletion solution was prepared by adding all the CDM components except MgCl₂·(H₂O)₆, CaCl₂·(H₂O)₄ and Mn(II)SO₄·H₂O.

Competence induction, donor DNA, and validation of mutants

To induce competence, overnight CDMG precultures were diluted to a final OD₆₀₀ of 0.05 in 500 µl of fresh CDMG and incubated 100 min at 37°C. Then, the pheromone sXIP and DNA (overlapping PCR products or plasmids) were added and cells were incubated for 3 h at 37°C before plating on M17G agar supplemented with antibiotics when required. All the details used to generate overlapping PCR for the construction of mutants reported below are available in Table S1.6. The integrity of all the constructed mutants was validated by Sanger DNA sequencing.

Construction of deletion mutants

Null-mutants were constructed by exchanging (double homologous recombination) the coding sequences (CDS) of target genes (sequence between start and stop codons) for either chloramphenicol or erythromycin resistance cassette (*cat* or *erm*). If stated, mutants were cleaned for the *lox* site-flanked resistance cassette using the Cre-*lox* system as previously described [41]. Integration of the antibiotic resistance cassette at the right location was subsequently checked by PCR.

Construction of overexpression mutants and reporter systems

Gene overexpression systems were designed by ATG fusion either to a mild xylose-inducible promoter (P_{xyl2} , associated to the *xylR* regulator gene), to a strong constitutive promoter (P_{32}) or to a strongly locked *S. pneumoniae* improved version of an IPTG-inducible promoter (P_{lac} , associated to a $P_{F6-lacI}$ repressor [42]). Those constructs were linked to a *cat* cassette, and integrated at the permissive tRNA serine locus (*HSISS4_r00062* for HSISS4 and *STER_t1568* for LMD-9) or fused to the native gene by double homologous recombination. To overexpress *comS* or *xip* fused to P_{xyl2} associated to a *cat* cassette, we used the permissive locus *tnpII* (downstream of *HSISS4_01854*).

The competence promoters were fused to the *luxAB* or *gfp*⁺ reporter genes and inserted with a spectinomycin resistance cassette (*spc*) at the permissive tRNA threonine locus (*HSISS4_r00061*) for HSISS4, at the *blp* locus (*stu_1673*) for LMG18311, at the *suc* locus (*ster_1709*) for LMD-9 or directly downstream of *comR* by double homologous recombination. For GFP constructs $P_{comX-gfp}^+$, $P_{xyl2-gfp}^+$, and $P_{comR-gfp}^+$, a codon-optimized sequence for GFP was specifically designed for use with an optimized 5'-UTR sequence, in order to maximize translation efficiency and improve GFP expression (designed by MeKaGene, Belgium). Synthetic constructs were ordered as Gblocks at IDT (Leuven, Belgium). They were then jointed to an antibiotic resistance cassette and homologous chromosomal regions via overlapping PCR. For CovR mutants, residue mutations in the protein sequence were introduced with nucleotide-mismatch primers. A *cat* cassette and homologous genomic regions for double recombination were associated to the construction by overlapping PCR.

Construction of CRISPRi conditional mutants

For dCas9-mediated (Cas9 catalytic mutant) transcriptional inhibition of *covRS* and $P_{32-luxAB}$ (validation tool), *lacI* was amplified from plasmid pPEPY- $P_{F6-lacI}$ [42], Gibson-assembled to HSISS4 recombination arms together with a *spc*

cassette and inserted at the tRNA threonine locus by natural transformation. The same technique was used to transfer *dcas9* under the control of a P_{lac} promoter (amplified from JWV102- P_{lac} -*dcas9*_{sp}, [42]) with a *cat* cassette into the tRNA serine locus. The two resistance cassettes were concomitantly excised as previously described [41] and a P_{comX} -*luxAB* or P_{32} -*luxAB* reporter system fused to *spc* were introduced into the *tnpII* locus. For *covR* inhibition, a P_{xyl2} -*comR*-*cat* construct was introduced at the *suc* locus (upstream of *HSISS4_01641*) and both resistance cassettes at the *tnpII* and *suc* loci were excised. Finally, a constitutive promoter (P_3) and a *dcas9* handle followed by a *S. pyogenes* terminator were amplified from plasmid pPEPX- P_3 -sgRNA_{luc} [42], fused to a specific gRNA targeting P_{covR} , and introduced with an *erm* cassette into a fifth permissive locus (*gor*, downstream of *HSISS4_00325*). Since P_{covR} has no PAM sequences, we introduced a point mutation into the promoter (spacer between -35 and -10 boxes) to generate the NGG sequence necessary for dCas9 binding.

Luciferase activity

Overnight precultures were diluted to a final OD₆₀₀ of 0.05. A volume of 300 µl of culture was transferred into the wells of a sterile covered white microplate with a transparent bottom (Greiner, Alphen a/d Rijn, The Netherlands). These culture samples were supplemented with D-xylose, IPTG, LL-37, NaCl, sorbitol, H₂O₂ or branched chain amino acids (BCAA) if stated or further incubated for 100 min at 37°C before supplementation with sXIP (1 µM). Growth (OD₆₀₀) and luciferase (Lux) activity (expressed in relative light units, RLU) were monitored at 10 min intervals during 8 h in a Hidex Sense microplate reader (Hidex, Lemminkäisenkatu, Finland). Maximum specific Lux activity was obtained by dividing Lux activity by the OD₆₀₀ over time and selecting the maximum value obtained. Biological or technical triplicates were then averaged. Statistical analyses of simple and multiple comparisons to the control mean were performed with *t*-test (unilateral distribution, heteroscedastic) and one-way ANOVA with Dunnett's test, respectively. For both, standard deviations and *P* values were calculated.

CovR and ComR purification

First, the PCR-amplified *covR*_{WT}, *covR*_{D53A} or *covR*_{D53E} genes (from strains HSISS4, AK0011, and AK0012, respectively) were cloned into the pBAD-comR-ST vector [22]. Because the DNA-binding domain of CovR is predicted at the C-terminus, the StrepTag was placed at the N-terminus by Gibson assembly and the final construct was verified by DNA sequencing. The ComR-StrepTag or StrepTag-CovR

recombinant proteins were overproduced *in E. coli* and purified in standard native conditions on Strep-Tactin agarose beads (IBA, Germany) as previously described [18].

Electrophoretic Mobility shift Assays (EMSA)

All double-stranded DNA fragments (150 bp) were obtained by PCR with genomic or synthetic DNA as template. PCR were performed with either Cy3-labelled (at 5' end) and unlabeled oligonucleotides or forward Cy3-labelled and reverse Cy5-labelled primers for double fluorescence. Primers used are listed in Table S1.6. Typically, a gel shift reaction (20 µl) was performed in a binding buffer (20 mM Tris, 1 mM CaCl₂, 1 mM DTT, 10 µg/ml polydIdC and 100 µg/ml BSA, pH 7.5) [36] and contained 100 ng of labelled probe and 1.4 µM with 1.4 × serial dilutions of purified StrepTagged CovR_{WT/D53A/D53E}. The reaction was incubated at 37°C for 5 min without the probe to get rid of any unspecific binding and for 10 min after adding the probe prior to the loading of the samples onto a native 4-20% gradient gel (iD PAGE Gel; Eurogentec, Belgium). The gel was run at 100 V for 100 min in MOPS buffer (Tris-base 50 mM pH 7.7, MOPS 50 mM, EDTA 1 mM). DNA complexes were detected by fluorescence on an Ettan DIGE Imager (GE Healthcare, Waukesha, WI) with bandpass excitation filters (nm): 540/25 (Cy3) or 635/30 (Cy5) and bandpass emission filters: 595/25 (Cy3) or 680/30 (Cy5). Gel fluorescence intensity quantification, background subtraction and normalization were performed with ImageQuant array (GE Healthcare). Those quantified intensities were then used to compute the percentage of bound probes with the formula $P_B = 100 - \frac{P_U - b}{ctrl} \times 100$ where P_U stands for unbound probe, b stands for background and $ctrl$ for the probe without CovR addition.

For experiments with restriction fragments of the *comR* promoter, P_{comR} was PCR-amplified using primers AK304-CY3 and AK326-CY5, and purified using ethanol extraction. The fragment was then digested with NsiI, PstI, DraI, AseI or PstI-NsiI and purified overnight by ethanol extraction. 100 ng of digested DNA was used for the experiment as previously described.

For the binding assay of CovR_{WT} to P_{comR} from various streptococci, synthetic DNAs corresponding to P_{comR} (150 pb) of three salivarius streptococci (*S. salivarius* HSISS4, *S. thermophilus* LMD-9 and *S. vestibularis* NTC1267) and one negative control (P_{dnaE} from HSISS4) were ordered at IDT (Leuven, Belgium). Flanking sequence of 20 pb were chosen arbitrary to amplify all the sequences with primers AK354-CY3 and AK355. Statistical analyses were performed as reported for luminescence assays.

Immunoblotting

Protein samples were separated by SDS-PAGE on 12% acrylamide pre-cast gel (iD PAGE Gel; Eurogentec) and washed in Tris-glycine-SDS solution (0.6% (w/v) Tris-HCl, 0.3% (w/v) glycine, 0.02% (w/v) SDS in 8:2 water/methanol solution) for 30 min. Gels were then blotted onto 0.2 μ m nitrocellulose membrane with TransBlot Turbo transfer system (BioRad) and subsequently blocked for 1 h with Tris-buffered saline (20 mM Tris-base; 150 mM NaCl), 0.05% (v/v) Tween 20 (TBST), and 5% (w/v) dry milk. Membrane were then then incubated for an additional hour with the primary antibodies diluted in TBST-milk. The membranes were washed 4 times for 5 minutes in TBST and incubated for 1 hour with the secondary antibody diluted in TBST-milk. The membranes were finally washed again 4 times for 5 min in TBST, revealed with Immobilon Western Blotting Chemoluminescence HRP substrate (Merck Millipore) and visualized with an Amersham Imager 600 (GE Healthcare). Rabbit antisera against ComR (anti-ComR) were used at a 1:5,000 dilution. HRP-conjugated Anti-rabbit secondary antibody was used at a 1:20,000 dilution (Jackson ImmunoResearch, West Grove, PA). ComR amount was quantified and normalized using total protein stained with coomassie using an Amersham Imager 600 (GE Healthcare).

Microscopy

CDMG overnight precultures were diluted in fresh CDMG at OD₆₀₀ of 0.05 and incubated at 37°C for 4 hours. For CRISPRi depletion of CovRS, cells were incubated in the same conditions, but were diluted at OD₆₀₀ of 0.01 and supplemented with IPTG 1 mM. Cells were then centrifuged, resuspended in fresh CDMG at OD₆₀₀ of 0.05 with D-xylose, and incubated for 2 hours at 37°C. When stated, sXIP was added after 30 min of incubation. Cells were then centrifuged, resuspended in 50 μ l of Phosphate Buffer Saline (PBS), and observed on agarose pads composed of 1% agarose and PBS buffer. For LMD-9 competence bimodality observation, competence non-permissive M17G medium was used for overnight preculture. Cells were then diluted in fresh M17G at OD₆₀₀ of 0.05 with or without xylose induction (0.5%) and incubated during 4 hours at 37°C. Cells were washed in fresh CDMG, diluted to OD₆₀₀ of 0.05 in CDMG, and incubated one hour at 37°C with or without xylose prior to microfluidic device loading (Cellulasic Onix2, Merck Millipore). Flow pressure was set to 10 kPa and pictures were taken every 10 min. Images were obtained using an Axio I inverted microscope (Zeiss) equipped with a Plan-Apochromat objective (100 $\times$ /1.46 Oil DIC M27) (Zeiss), a HXP 120 C lighting unit (Zeiss) and a C10600 ORCA-R2 camera (Hamamatsu). GFP fluorescence was detected with

filter set 38 HE, displaying bandpass excitation 470/40 nm and bandpass emission 525/50 nm (Zeiss). Images were analyzed using ZenPro software (Zeiss) and MicrobeJ [67].

Deep sequencing (RNAseq) and data processing

S. salivarius WT, *covS*_{T287A} or *covR*_{D53E} strains were pre-cultured overnight in CDMG at 37°C. Pre-cultures were then diluted at an OD₆₀₀ of 0.05 in CDMG, incubated 4 hours at 37°C, concentrated by centrifugation (2 min 4000×g) and OD₆₀₀ was measured. The strains were diluted again at an OD₆₀₀ of 0.2 in 10 ml of CDMG, grown for 25 minutes and the supernatants were discarded. The cell pellets were then frozen in liquid nitrogen. RNA was extracted the same day using the RNeasy plus bacteria kit (Qiagen) using the protocol provided by the manufacturer. Total RNA was checked for integrity with a RNA Nano chip (Agilent Technologies). rRNA depletion was performed on 2 µg total RNA with the Ribo-Zero rRNA removal kit for Gram-positive bacteria (Illumina) according to manufacturer's instructions. Total stranded mRNA libraries were prepped with the NEBNext Ultra Directional RNA Library Prep kit for Illumina (New England Biolabs). Library PCR was executed for 15 cycles. Quality of the libraries was evaluated with the use of a High sensitivity DNA chip (Agilent Technologies) and concentrations were determined by qPCR according to Illumina protocol. Libraries were pooled equimolarly for sequencing on a NextSeq 500 high-throughput run for generating 76 bp single reads. 2.3 pM of the library pool was loaded on the flowcell with a Phix spike-in of 5%. Sequenced mRNAs generated several millions reads that were mapped on the *S. salivarius* HSISS4 chromosome and processed on the Galaxy server (use.galaxy.org) using Bowtie2 algorithm to yield BAM files containing the read coordinates and Featurecount to count the number of reads per coding sequence (CDS). The dataset was exported into an excel file for further analyses. First, the dataset was standardized to CDS-mapped reads per million overall reads. Then, we estimated a ratio of CDS-mapped reads in mutants vs WT.

Data availability

All RNAseq data were deposited in the GEO database under accession number GSE158512.

SUPPLEMENTARY INFORMATION

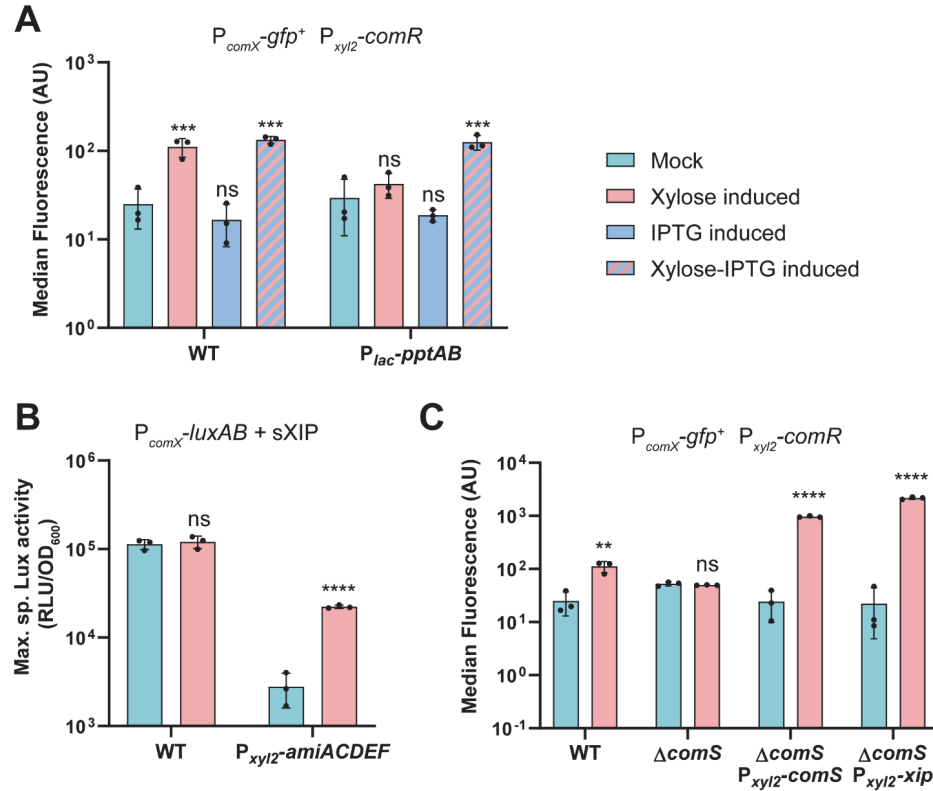


Figure S1.1. Validation of overexpression fusions. Luciferase and GFP-microscope assays were performed in order to show the functionality of different overexpression constructs unable to activate competence (Figure 1.1). **A.** Functionality of a $P_{lac}-pptAB$ ($P_{comX}-gfp^+$ as proxy) conditional mutant (native locus) through its rescue by IPTG induction of P_{lac} . Competence was activated by xylose addition (0.5%) thanks to the $P_{xyl2}-comR$ overexpression construct. **B.** Functionality of $P_{xyl2}-amiACDEF$ through xylose induction (1%) in a WT $P_{comX}-luxAB$ reporter strain. Competence was activated by $sXIP$ addition (500 nM). Results are displayed as maximum specific luminescence (RLU/OD₆₀₀). **C.** Functionality of $P_{xyl2}-comS$ and $P_{xyl2}-xip$ through complementation of a $\Delta comS$ ($P_{comX}-gfp^+$ as proxy) strain. Competence was activated through the $P_{xyl2}-comR$ overexpression construct. Overexpression fusions were induced by xylose addition (0.5%). For GFP assays (A. and C.), three populations from biological replicates were analyzed and their median fluorescence (arbitrary unit, AU) was calculated. The mean median values of the three populations together with standard deviations are displayed. For the luciferase assay (B.), biological triplicates were used. One-way ANOVA with Dunett's test was performed for multiple comparison to the mock (A.) and t -test was performed for simple comparison to related mock (B. and C.) (ns, non-significant; **, $P < 0.01$; ***, $P < 0.001$; **** $P < 0.0001$).

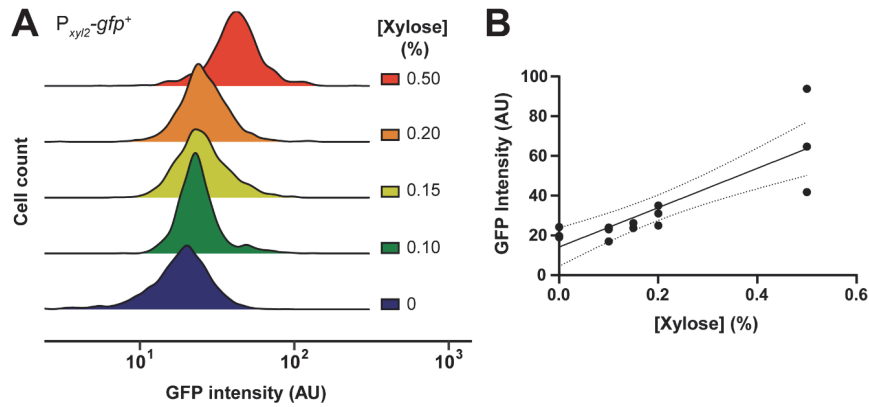


Figure S1.2. Absence of P_{xylZ} intrinsic bimodality. **A.** Density plot of single-cell GFP intensity (arbitrary unit (AU), log scale) from microscopic analysis of the $P_{xylZ}\text{-}gfp^+$ strain incubated with 0, 0.1, 0.15, 0.2 and 0.5 % of xylose. The fluorescence of individual cells was examined from biological triplicates ($n > 500$ cells in each experiment). **B.** Linear regression performed on median GFP intensities of cell populations (black dots denote median of distribution of biological replicates). Dashed lines denote confidence intervals (0.05) of the regression.

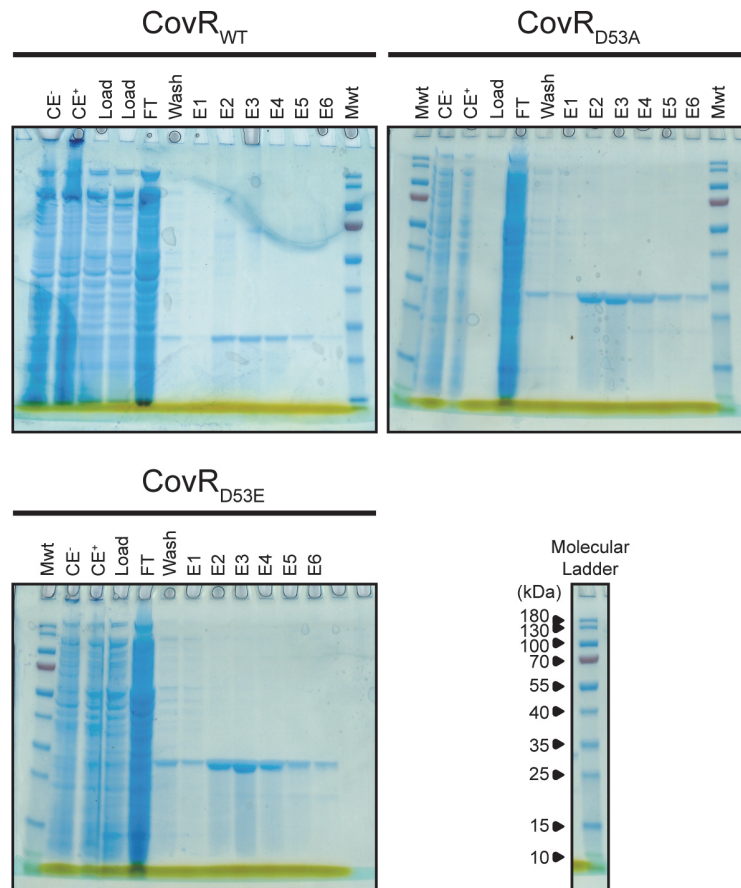


Figure S1.3. SDS-PAGE of CovR purification. Purification of HSISS4 CovR_{WT} , $\text{CovR}_{\text{D53A}}$ and $\text{CovR}_{\text{D53E}}$ are displayed. Mwt: Molecular weight, CE: Crude extract with (+) or without arabinose induction (-), FT: Flow-through, E1-6: elution fractions 1 to 6, kDa: kiloDalton.

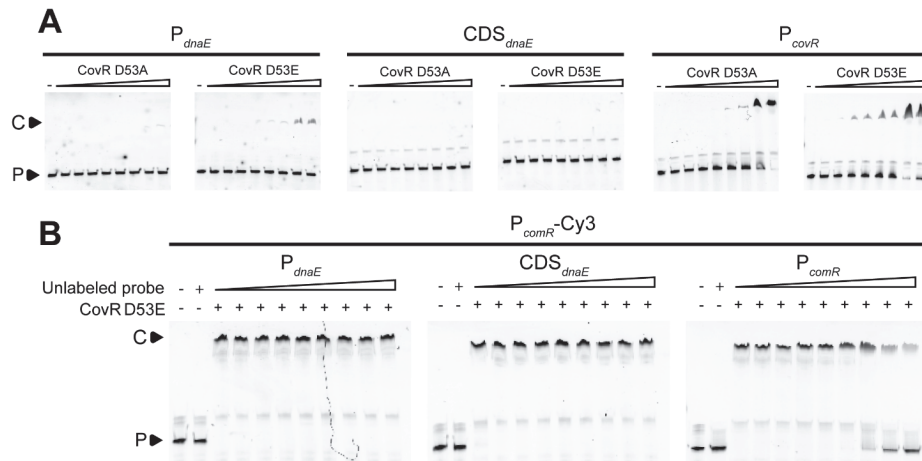
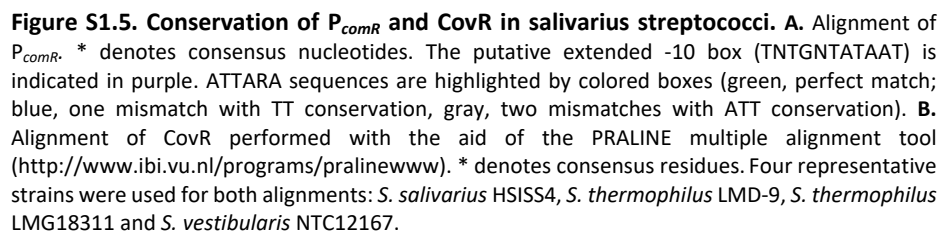


Figure S1.4. Specificity controls for P_{comR} -CovR interaction. **A.** EMSA with non-specific (P_{dnaE} and CDS_{dnaE}) and specific (P_{covR}) probes (150 bp, 0.05 μ M) and a gradient of purified CovR_{D53A} or CovR_{D53E} (triangles, maximum of 1.4 μ M with 1.4 $\times$ serial dilutions). We chose the promoter (P_{dnaE}) and CDS of *dnaE* (CDS_{dnaE} , alpha subunit of PolIII) for its unrelated physiological role regarding CovR regulation. The promoter of *covR* (P_{covR}) was used as specific probe since its interaction with CovR was reported in several streptococci [38,39]. **B.** EMSA competition assays performed between the P_{comR} -Cy3 (150 bp, 0.05 μ M)-CovR_{D53E} (0.7 μ M) complex and a gradient of unlabeled non-specific probes (P_{dnaE} and CDS_{dnaE}) or specific probe (P_{comR}) (triangles, maximum of 2 μ M with 1:2 serial dilutions). In A. and B., minus and plus signs depict absence and presence of unlabeled probe or protein, respectively. P and C denote the unbound probe and the CovR-DNA complex, respectively.



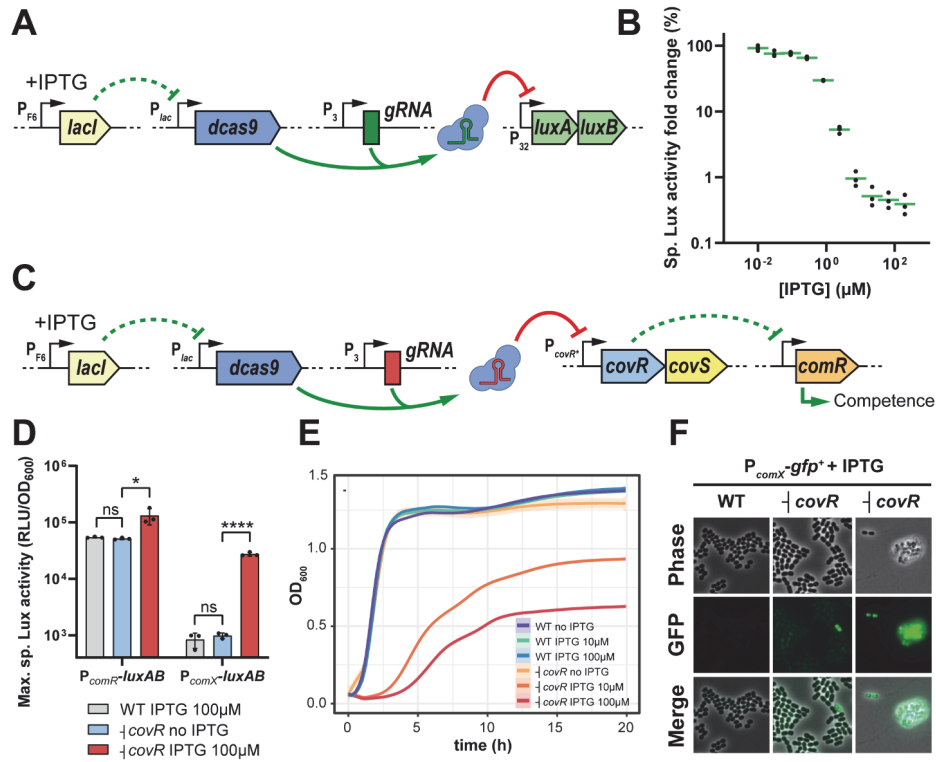


Figure S1.6. CovR depletion is toxic in *S. salivarius*. **A.** Strategy used to validate the CRISPR-interference system by targeting a constitutive promoter (P_{32}) (strategy used in B). Together with the reporter system (P_{32} -*luxAB*), the engineered strain carries an optimized *lacI* repressor under the control of a constitutive promoter (P_{F6} -*lacI*), a nuclease mutant of Cas9 (*dcas9*) under the control of a lactose-inducible promoter (P_{lac} -*dcas9*) and a guide RNA targeting the P_{32} promoter under the control of a constitutive promoter (P_3 -*gRNA_3*). **B.** Fold change (%) of specific luciferase activity (RLU/OD₆₀₀) for increasing concentration of IPTG in a constitutively-expressed luciferase strain (P_{32} -*luxAB*). Points show biological triplicates ($n = 3$), the green bar shows the mean. **C.** CRISPRi strategy for *covRS* repression (strategy used in D-F). **D.** CovR depletion increase *comR* and *comX* transcription. A strain harboring the *dcas9* module reported in C together with no guide RNA (WT) or with a guide targeting a single nucleotide modified promoter of *covRS* (Δ -*covR*) harboring a P_{comR} -*luxAB* or a P_{comX} -*luxAB* reporter system were incubated with no IPTG or 100 μ M IPTG and maximum specific luciferase activity was recorded. One-way ANOVA with Dunett's test was performed for technical triplicates (ns, non-significant; *, $P < 0.05$, ****, $P < 0.0001$). **E.** Growth defect upon *covRS* inhibition. The strain harboring the *dcas9* module with (Δ -*covR*) or without (WT) a guide targeting P_{covRS} reported in C were incubated in presence of 0, 10 or 100 μ M IPTG. Solid lines represent average measurements and shaded lines the standard errors. **F.** Microscopic single-

cell *covRS* repression. The same constructs as described in C were used, except for the reporter system being P_{comX} -*gfp*⁺. First vertical set of pictures show the WT, and second and third sets show the *covRS*-repressed strain with 100 μ M IPTG. The second and third vertical sets highlight heterogeneous *comX* activation and aggregation phenotype, respectively.

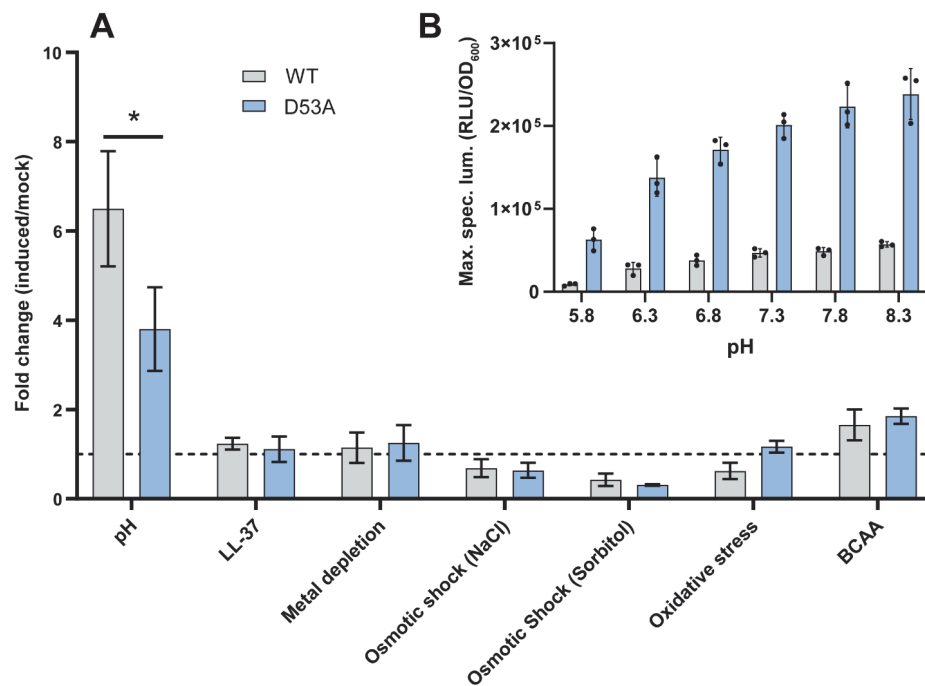


Figure S1.7. CovRS triggering conditions. **A.** Investigations of putative environmental triggers of P_{comR} activation by CovR. We compared the P_{comR} -*luxAB* reporter strain (WT) and the mutant strain impaired for its CovRS pathway (*covR*_{D53A}, P_{comR} -*luxAB*). Fold changes for each condition were calculated by dividing values of maximum specific luciferase activity (RLU/OD₆₀₀) obtained for stimulus induction with non-induced mock values. (BCAA, Branched Chained Amino Acid). For details on stimuli features, see Materials and Methods section. **B.** Influence of pH on P_{comR} activation by CovR. Maximum specific luciferase activity (RLU/OD₆₀₀) of P_{comR} -*luxAB* fusion in WT compared to the *covR*_{D53A} mutant at various initial pH (buffered CDM). Mean values of technical triplicates $\pm$ standard deviation. In A., a statistical *t*-test was performed for each condition compared to their relative mock (*, $P < 0.05$).

Table S1.1. Annotation of TCSs from *S. salivarius* HSISS4

S. salivarius HSISS4 ^a			S. thermophilus LMD-9		S. pneumoniae R6		S. pyogenes M1		S. mutans UA159	
Locus_tag	Gene name	Product	Locus_tag / gene name %QC / %Id ^b	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id	Locus_tag / gene name %QC / %Id
HSISS4_00246	covR	RR CovR	STER_RS01725 / rr01 100 / 96	SPR_RS01745 / csrR 100 / 48	SPY_RS01535 / csrR 100 / 82	SMU_RS08745 / gcrR 99 / 78				
HSISS4_00247	covS	HK CovS	STER_RS01730 / hk01 96 / 89	ND ^d	SPY_RS01540 / csrS 95 / 43	ND				
HSISS4_01097	ciaR	RR CiaR	ψ^e	SPR_RS03560 / ciaR 99 / 87	SPY_RS05180 / ciaR 100 / 84	SMU_RS05200 / ciaR 100 / 86				
HSISS4_01096	ciaH	HK CiaH	ψ	SPR_RS03565 / ciaH 99 / 48	SPY_RS05175 / ciaH 98 / 52	SMU_RS05195 / ciaH 97 / 54				
HSISS4_01036	spaR	RR	STER_RS04580 / rr04 100 / 86	SPR_RS09650 / pnpR 99 / 31	SPY_RS04515 / strR 99 / 37	SMU_RS03120 / spaR 100 / 58				
HSISS4_01035	spaK	HK	STER_RS04585 / hk04 100 / 85	SPR_RS00420 / pnpS 65 / 25	SPY_RS04520 / strK 73 / 29	SMU_RS03125 / spaK 90 / 48				
HSISS4_00836	vicR	RR vicR	STER_RS05520 / rr05 100 / 95	SPR_RS05525 / vicR 99 / 80	SPY_RS02235 / vicR 100 / 84	SMU_RS06885 / vicR 100 / 83				
HSISS4_00837	vicK	HK vicK	STER_RS05515 / hk05 100 / 92	SPR_RS05520 / vicK 90 / 72	SPY_RS02240 / vicK 93 / 74	SMU_RS06880 / vicK 92 / 74				
HSISS4_01178	hk06	HK	STER_RS06365 / hk06 100 / 85	SPR_RS09115 / hk11 98 / 35	ND	SMU_RS07025 / hk12 99 / 57				
HSISS4_01177	rr06	RR	STER_RS06360 / rr06 100 / 94	SPR_RS09110 / rr11 100 / 68	ND	SMU_RS07020 / rr12 99 / 74				
HSISS4_01193	rr07	RR	STER_RS06460 / rr07 100 / 98	SPR_RS07330 / rr01 100 / 85	ND	SMU_RS04640 / rr08 100 / 46				
HSISS4_01192	hk07	HK	STER_RS06455 / hk07 100 / 88	SPR_RS07325 / hk01 81 / 65	ND	SMU_RS04645 / hk08 79 / 33				
HSISS4_01345	liaS	HK LiaS	ψ	SPR_RS01780 / hk03 91 / 48	SPY_RS06770 / liaS 99 / 51	SMU_RS02335 / hk11 91 / 57				

HSISS4_01344	<i>liaR</i>	RR LiaR	STER_RS06830 / rr08 100 / 99	SPR_RS01785 / rr03 99 / 71	SPY_RS06765 / liar 99 / 84	SMU_RS02340 / rr11 99 / 84
HSISS4_00378	<i>rr09</i>	RR	STER_RS08080 / rr09 97 / 36	SPR_RS02375 / rr13 99 / 48	ND	SMU_RS08710 / come 99 / 51
HSISS4_00379	<i>hk09</i>	HK	STER_RS08085 / hk09 95 / 30	SPR_RS02380 / hk13 96 / 33	SPY_RS06555 / hk 84 / 26	SMU_RS08705 / comD 92 / 38
HSISS4_00352	<i>rr10</i>	RR	ND	ND	SPY_RS03640 / sptR 99 / 60	SMU_RS04280 / rr04 100 / 60
HSISS4_00353	<i>hk10</i>	HK	ND	ND	SPY_RS03645 / sptS 100 / 56	SMU_RS04285 / hk04 100 / 53
HSISS4_01248	<i>vncR</i>	RR VncR	ND	SPR_RS02675 / vncR 100 / 71	SPY_RS08395 / irr 99 / 38	ND
HSISS4_01247	<i>vncS</i>	HK VncS	ND	SPR_RS02680 / vncS 99 / 53	SPY_RS08390 / ihk 96 / 26	ND
HSISS4_01715	<i>fasA</i>	RR	ND	ND	SPY_RS01155 / fasA 99 / 56	ND
HSISS4_01716	<i>fasB</i>	HK	ND	ND	SPY_RS01150 / fasB 98 / 25	ND
HSISS4_01231	<i>rr13</i>	RR	ND	SPR_RS10180 / rr06 / 87 / 33	ND	SMU_RS04780 / rr07 100 / 74
HSISS4_01230	<i>hk13</i>	HK	ND	SPR_RS10175 / hk06 72 / 25	ND	SMU_RS04775 / hk07 91 / 69
HSISS4_01447	<i>rr14</i>	RR	ND	SPR_RS10400 / come / 96 / 33	ND	ND
HSISS4_01446	<i>hk14</i>	HK	ND	SPR_RS10405 / comD / 56 / 31	ND	ND

^a The TCS number 01 to 09 assigned to *S. salivarius* HSISS4 was based on previous TCS annotation from *S. thermophilus* LMD-9 [68]. TCS annotation is based on gene names assigned to orthologous systems in *S. pneumoniae* R6 [69], *S. pyogenes* M1 [70] and *S. mutans* UA159 [71]

^b %QC, percentage of query covering, %Id, percentage of identity.

^c *ψ*, pseudogene.

^d ND, not detected.

Table S1.2. Down-regulated genes in CovS_{T287A} and CovR_{D53E} mutants belonging to the ComR regulon

Locus tag	Strand	Gene	Description	Regulon ComR ^a	Regulon ComX ^b	Normalized ratio ^c	Normalized ratio ^c	ComR/X box ^d
				$\frac{\Delta comR}{WT}$	$\frac{\Delta comX}{WT}$	T287A $\frac{WT}{WT}$	D53E $\frac{WT}{WT}$	
HSISS4_00217	+	<i>comR</i>	Pheromone-responsive transcriptional regulator ComR			0.47	0.52	
Core regulon of ComR								
<i>Locus comX</i>								
HSISS4_00102	+	<i>comX</i>	Competence-specific sigma factor ComX	+		0.32	0.63	ComR
<i>Locus 00176-00180</i>								
HSISS4_00176	+		ABC transporter, ATP- binding protein	+		0.12	0.07	ComR
HSISS4_00177	+		ABC transporter permease protein	+		0.11	0.09	
HSISS4_00178	+	<i>pncB</i>	Nicotinate phosphoribosyltransferase	+		0.75	1.49	
HSISS4_00179	+	<i>nadE</i>	NAD synthetase	+		0.81	1.43	
HSISS4_00180	+		Transporter	+		0.46	1.57	
<i>Locus comA</i>								
HSISS4_00218	+	<i>comA</i>	Bacteriocin ABC transporter	+		0.04	0.03	ComR
<i>Locus bacteriocin BlpK</i>								
HSISS4_01581	-	<i>blpI</i>	Immunity protein	+		0.45	0.43	
HSISS4_01582	-		hypothetical protein	+		0.11	0.32	

HSISS4_01584	-	Biogenesis protein DsbD, protein-disulfide reductase	+	0.09	0.07	ComR
HSISS4_01585	-	hypothetical protein	+	0.19	0.04	
HSISS4_01586	-	<i>blpK</i> Bacteriocin BlpU	+	0.07	0.07	ComR
<i>Locus bacteriocin SlvV</i>						
HSISS4_01594	-	<i>slvV</i> putative bacteriocin SlvV	+	0.08	0.12	ComR
<i>Locus bacteriocin SlvW</i>						
HSISS4_01647	-	<i>comEB</i> dCMP deaminase/Late competence protein ComEB	+	0.89	0.98	
HSISS4_01648	-	<i>pepP</i> Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP-specific)	+	0.88	1.18	
HSISS4_01649	-	hypothetical protein	+	0.12	0.14	
HSISS4_01650	-	peptide ABC transporter ATP binding protein	+	0.19	0.24	
HSISS4_01651	-	<i>blpG</i> hypothetical protein	+	0.14	0.12	
HSISS4_01652	-	hypothetical protein	+	0.08	0.41	
HSISS4_01653	-	<i>slvW</i> putative bacteriocin SlvW	+	0.10	0.39	ComR
<i>Locus bacteriocin SlvX</i>						
HSISS4_01664	-	hypothetical protein	+	0.11	0.06	
HSISS4_01665	-	<i>slvX</i> putative bacteriocin SlvX	+	0.04	0.05	ComR
<i>Locus bacteriocin SlvYZ</i>						
HSISS4_01742	+	<i>slvY</i> putative bacteriocin SlvY	+	0.12	0.10	ComR
HSISS4_01743	+	<i>slvZ</i> putative bacteriocin SlvZ	+	0.09	0.17	
HSISS4_01744	+	hypothetical protein	+	0.11	0.12	

Accessory regulon of ComR
Core regulon of ComX

HSISS4_00029	-	<i>cbpD1</i>	putative peptidoglycan hydrolase	+	+	0.44	0.34	ComX
HSISS4_00295	+	<i>comFA</i>	Late competence protein ComFA, DNA transporter ATPase	+	+	0.31	0.35	ComX
HSISS4_00296	+	<i>comFC</i>	Late competence protein ComFC, phosphoribosyltransferase domain	+	+	0.30	0.21	
HSISS4_00676	-	<i>pilD</i>	Late competence protein ComC, processing protease	+	+	0.36	0.78	ComX
HSISS4_01041	-	<i>dprA</i>	DNA recombination-mediator protein A	+	+	0.46	0.63	ComX
HSISS4_01377	-	<i>radC</i>	DNA repair protein RadC	+	+	0.43	0.36	ComX
HSISS4_01466	-	<i>comeC</i>	Late competence protein ComEC, DNA transport	+	+	0.30	0.20	
HSISS4_01467	-	<i>comeA</i>	Late competence protein ComEA, DNA receptor	+	+	0.35	0.20	ComX
HSISS4_01746	-	<i>ssbA</i>	ssDNA binding protein	+	+	0.29	0.22	ComX
HSISS4_01759	-	<i>ackA</i>	Acetate kinase	+	+	1.43	1.81	
HSISS4_01760	-		Adenine-specific methyltransferase	+	+	0.79	1.33	
HSISS4_01761	-	<i>comGG</i>	Late competence protein ComGG	+	+	0.33	0.49	
HSISS4_01762	-	<i>comGF</i>	Late competence protein ComGF, access of DNA to ComEA	+	+	0.24	0.18	

HSISS4_01763	-	<i>comGE</i>	Late competence protein ComGE	+	+	0.32	0.18
HSISS4_01764	-	<i>comGD</i>	Late competence protein ComGD, access of DNA to ComEA	+	+	0.29	0.20
HSISS4_01765	-	<i>comGC</i>	Late competence protein ComGC, access of DNA to ComEA	+	+	0.29	0.26
HSISS4_01766	-	<i>comGB</i>	Late competence protein ComGB, access of DNA to ComEA	+	+	0.27	0.18
HSISS4_01767	-	<i>comGA</i>	Late competence protein ComGA, access of DNA to ComEA	+	+	0.17	0.06
ComX							
Others - regulon ComX							
HSISS4_00633	+		bacterial seryl-tRNA synthetase related	+	+	0.92	2.35
HSISS4_00661	+		hypothetical protein	+	+	0.77	0.70
HSISS4_00746	-		hypothetical protein	+	+	0.62	1.84
HSISS4_00747	-		Polyposphate(polyP) polymerase domain protein	+	+	0.55	2.03
HSISS4_00855	+	<i>mur1B</i>	N-acetyl/muramoyl-L-alanine amidase	+	+	0.69	0.37
HSISS4_01277	+		Choline binding protein A	+	+	0.42	0.91
HSISS4_01524	-		YoeB toxin protein	+	+	0.89	2.40
HSISS4_01525	-		YefM protein (antitoxin to YoeB)	+	+	0.37	2.05
Others - regulon ComR							
HSISS4_00104	+		hypothetical protein	+		0.16	0.05

HSISS4_00301	-	<i>recX</i>	Regulatory protein recX	+	0.25	1.82
HSISS4_00697	+	<i>citB</i>	Aconitate hydratase	+	1.62	0.89
HSISS4_00698	+	<i>gltA</i>	Citrate synthase	+	1.74	0.88

^a Down-regulated genes (red, ≥ 5 fold) in the $\Delta comR$ mutant ($\Delta comR/WT$) that were previously reported [22]. + denotes activation by ComR.

^b Down-regulated genes (red, ≥ 5 fold) in the $\Delta comX$ mutant ($\Delta comX/WT$) that are belonging to the ComR regulon [22]. + denotes activation by ComX.

^c Ratio of normalized $CovS_{T287A}$ reads vs normalized reads of WT (T287A/WT) or $CovR_{D53E}$ vs WT (D53E/WT).

Red, down-regulation > 5 -fold, dark pink, between 2 and 5-fold, and light pink, between 1.5 and 2 fold.

^d Mapping of upstream ComR (R)- and ComX (X)-boxes. Blue denotes genes belonging to the core regulon of ComR (8 loci) [22]. Yellow denotes genes belonging to the core regulon of ComX that are indirectly controlled by ComR

Table S1.3. List of bacterial strains used in this study

Strain	Characteristics	Reference/source
<i>E. coli</i>		
TOP10	<i>mcrA</i> , $\Delta(mrr-hsdRMS-mcrBC)$, Φ 80/ <i>lacZ(del)</i> M15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , $\Delta(ara-leu)$ 7697, <i>galU</i> , <i>galK</i> , <i>rpsL(SmR)</i> , <i>endA1</i> , <i>nupG</i>	Invitrogen, CA
<i>Streptococcus thermophilus</i>		
LMG 18311	Wild-type milky product isolate	BCCM ^b
LMD-9	Wild-type milky product isolate	ATCC ^c
LF146	LMG18311 (<i>blpU-blpX</i>)::P _{comR} - <i>luxAB</i>	[23]
LF150	LMG18311 (<i>blpU-blpX</i>)::P _{comR} - <i>luxAB</i> Δ <i>covRS::lox72</i>	This work
CB009	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i>	[23]
LF151	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> <i>covRS:kan</i>	This work
LF152	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> <i>covRS:lox72</i>	This work
LF153	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> pMG36ET	This work
LF154	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> Δ <i>covRS::lox72</i> pMG36ET	This work
LF155	LMG18311 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> Δ <i>covRS::lox72</i> pMG36ET- <i>covRS</i>	This work
CB001	LMD-9 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i>	[16]
LF123	LMD-9 (<i>blpU-blpX</i>)::P _{comR} - <i>luxAB</i>	[18]
LF156	LMD-9 (<i>blpU-blpX</i>)::P _{comX} - <i>luxAB</i> <i>covRS:kan</i>	This work

LF157	LMD-9 (<i>blpU-blpX</i>):: <i>P_{comX}-luxAB</i> <i>covRS:lox72</i>	This work
LF158	LMD-9 (<i>blpU-blpX</i>):: <i>P_{comR}-luxAB</i> <i>ΔcovRS::lox72</i>	This work
LF159	LMG18311 (<i>blpU-blpX</i>):: <i>P_{comX}-luxAB</i> <i>ΔcovRS::covRS_{LMD-9}</i>	This work
LF160	LMD-9 (<i>blpU-blpX</i>):: <i>P_{comX}-luxAB</i> <i>ΔcovRS::covRS_{LMG18311}</i>	This work
LF161	LMG18311 (<i>blpU-blpX</i>):: <i>P_{comX}-luxAB</i> <i>covR_{D98E}-kan</i>	This work
LF162	LMD-9 (<i>blpU-blpX</i>):: <i>P_{comX}-luxAB</i> <i>covR_{E98D}-kan</i>	This work
AK0042	LMD-9 <i>SUC::P_{comX}-opt-gfp-spc</i> <i>tRNA_{ser}::P_{xyI2}-covR_{E98D}-cat</i>	This work
<i>Streptococcus salivarius</i>		
HSISS4	Wild-type gastro-intestinal tract isolate	[72]
AK0001	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i>	This work
AK0002	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P_{xyI2}-comS-cat</i>	This work
AK0003	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P_{xyI2}-xip-cat</i>	This work
AK0004	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P_{xyI2}-comR-cat</i>	This work
AK0005	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P_{xyI2}-comR-P_{comS}-comS-cat</i>	This work
AK0006	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P_{xyI2}-comR-P_{comS}-xip-cat</i>	This work
AK0007	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>tRNA_{ser}::P₃₂-cat-amiA3</i>	This work

AK0008	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>amiACDEF::P_{xyl2}-amiACDEF-cat</i>	This work
AK0009	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spc</i> <i>pptAB::P_{F6}-lacI-P_{lac}-pptAB-cat</i>	This work
AK0010	HSISS4 <i>tRNA_{thr}::P_{comR}-luxAB-spc</i>	This work
AK0011	HSISS4 <i>tRNA_{thr}::P_{comR}-luxAB-spc</i> <i>covR::covR_{D53A}-cat</i>	This work
AK0012	HSISS4 <i>tRNA_{thr}::P_{comR}-luxAB-spc</i> <i>covR::covR_{D53E}-cat</i>	This work
AK0013	HSISS4 <i>tRNA_{thr}::P_{comR}-luxAB-spc</i> <i>covS::covS_{T287A}-cat</i>	This work
AK0014	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-spc</i>	This work
AK0015	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-spc</i> <i>tRNA_{ser}::P_{xyl2}-comR-cat</i>	This work
AK0016	HSISS4 <i>tRNA_{ser}::P_{xyl2}-opt-gfp-spc</i>	This work
AK0017	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i>	This work
AK0018	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔcomS::cat</i>	This work
AK0019	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>Δopp::cat</i>	This work
AK0020	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔpptAB::erm</i>	This work
AK0021	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>pptAB::P_{F6}-lacI-P_{lac}-pptAB-cat</i>	This work

AK0022	HSISS4 <i>tRNA_{thr}::P_{comR}-opt-gfp-spc</i>	This work
AK0023	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔcomS::cat</i> <i>tnpII::P_{xyl2}-comS-spc</i>	This work
AK0024	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔcomS::cat</i> <i>tnpII::P_{xyl2}-xip-spc</i>	This work
AK0025	HSISS4 <i>tRNA_{thr}::P_{comX}-opt-gfp-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔcomS::cat</i> <i>tnpII::P₃₂-comS-spc</i>	This work
AK0026	HSISS4 <i>P_{comR}-comR-opt-gfp⁺-spc</i>	This work
AK0027	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-spc</i>	This work
AK0028	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-spc</i> <i>tRNA_{ser}::P_{lac}-dcas9-cat</i>	This work
AK0029	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i>	This work
AK0030	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-spc</i>	This work
AK0031	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-spc</i>	This work
AK0032	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-spc</i>	This work
AK0033	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i>	This work

AK0034	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-lox72</i>	This work
AK0035	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-opt-gfp-lox72</i>	This work
AK0036	HSISS4 <i>GOR::P_{3-g_1}(luc)</i>	This work
AK0037	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P₃₂-luxAB-spc</i> <i>GOR::P_{3-g_3}(P₃₂)-erm</i>	This work
AK0038	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>GOR::P_{3-g_19}(P_{covRS*})-erm</i> <i>P_{covRS}::P_{covRS*}-cat</i>	This work
AK0039	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-lox72</i> <i>GOR::P_{3-g_19}(P_{covRS*})-erm</i> <i>P_{covRS}::P_{covRS*}-cat</i>	This work
AK0040	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-opt-gfp-lox72</i> <i>GOR::P_{3-g_19}(P_{covRS*})-erm</i> <i>P_{covRS}::P_{covRS*}-cat</i>	This work
FV0001	HSISS4 <i>P_{comR}-comR::luxAB-lox72</i>	This work
FV0002	HSISS4 <i>P_{comR}-comR::luxAB-lox72</i> <i>Δcovs::cat</i>	This work
FV0003	HSISS4 <i>P_{comR}-comR::luxAB-lox72</i> <i>ΔciaRH::cat</i>	This work

FV0004	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta spaRK::cat$	This work
FV0005	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta vick::cat$	This work
FV0006	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta TCS06::cat$	This work
FV0007	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta BceRS::cat$	This work
FV0008	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta LiaSR::cat$	This work
FV0009	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta TCS09::cat$	This work
FV0010	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta TCS10::cat$	This work
FV0011	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta vncRS::cat$	This work
FV0012	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta fasB::cat$	This work
FV0013	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta TCS13::cat$	This work
FV0014	HSISS4 P_{comR} - $comR$: $luxAB$ - $lox72$ $\Delta TCS14::cat$	This work
JM0001	HSISS4 $\Delta comR$	[22]
JM1125	HSISS4 $tRNA_{ser}::P_{xyl2}$ - gfp^+ - spc	[73]
JM1016	HSISS4 $tRNA_{ser}::P_{xyl2}$ - $comR$ - spc	[22]
JM1019	HSISS4 $tRNA_{thr}::P_{comS}$ - $luxAB$ - cat	[22]
JM1020	HSISS4 $tRNA_{thr}::P_{comX}$ - $luxAB$ - cat	[22]

^a *cat*, *spc*, *erm*, *kan*, indicate resistance to chloramphenicol, spectinomycin, erythromycin, kanamycin resistance and *lox72* stands for the lox scar.

^b BCCM, Belgian Coordinated Collections of Microorganisms, LMG Laboratory of Microbiology and Genetics, Ghent, Belgium.

^c ATCC, American Type Culture Collection, Rockville, MD.

Table S1.4. List of plasmids used in this study

Plasmid	Characteristics	Reference/so
pBAD-comR-ST	pBAD <i>hisA</i> derivative encoding ComR _{HSISS4} fused to a C-terminal StreptagII	[22]
pBAD-covR _{WT} -ST _{N-ter}	pBAD-comR-ST derivative encoding HSIS4 CovR _{WT} fused to a N-terminal StreptagII	This work
pBAD-covR _{D53A} -ST _{N-ter}	pBAD-comR-ST derivative encoding CovR _{D53A} fused to a N-terminal StreptagII	This work
pBAD-covR _{D53E} -ST _{N-ter}	pBAD-comR-ST derivative encoding CovR _{D53E} fused to a N-terminal StreptagII	This work
pGhostcre	Thermosensitive replication origin vector in <i>S. salivarius</i> , encoding the Cre recombinase; <i>erm</i>	[41]
pGIUD0855erm	pUC18 derivative containing the <i>erm</i> gene	[41]
pJUDspecmut1-gfp+ter	Terminator associated- <i>gfp</i> ORF	[22]
pJIMcat	pJIM4900 derivative with a <i>cat</i> cassette	[22]
pMG36ET	Expression vector; <i>erm</i>	[74]
pMG36ET-covRS	pMG36ET derivative encoding the <i>covRS</i> operon under the P ₃₂ constitutive promoter; <i>erm</i>	This work
pJWV102-PL-dcas9	Plasmid encoding a dead Cas9 under the control of a lactose inducible promoter	[42]
pPEPY-PF6-lacI	Plasmid harboring a <i>lacI</i> repressor under the control of the P _{F6} promoter, optimized for <i>S. pneumoniae</i>	[42]
pPEPX-P3-sgRNA _{luc}	Plasmid harboring a P ₃ constitutive promoter fused to a guide targeting the <i>luc</i> firefly luciferase gene together with a <i>dcas9</i> -handle and a terminator, <i>spc</i>	[42]

Table S1.5 List of oligonucleotides used in this study

Name	Sequence (5'-3')
AK44	ATGAAAAAAGCAAATGG
AK50	TGGGATTATCTTCCTTAAGTTATAGGGGTAACAC
AK52	TAACTAACAGTTAAAAGGAGTTTTATTTGATCACTATCTTGCCTTATTTG
AK55	AAATTGAGAGGTATAAATCAAGATTCCAAAGTAAACAGAAA
AK56	GTATGTAAGCAAAAAGTTTCCAAATTCATGAGACACTCCTTTATTTT
AK57	CTGAAGAGTGAATCATCAAG
AK58	ATGAAAAAAGTAAATGGTTGG
AK59	GCCAACCATTTACTTTTTTCATATTTACCTCCTTGATTAAAGTG
AK60	AAAGATCTTGAGAAACACGTTAAATAATTCACGTTACTAAAGGGA
AK61	TTATTTAACGTGTTTCTCAAGAT
AK62	ATTATCATCTGATGACATACAAAATG
AK69	CTGTTAAAACCATCTTCTTTTAATTACTTAAGGAAGATAAATCCATAAG
AK70	GCTAACCATTTGCTTTTTTTCATTTACGTTACTAAAGGGAA
AK71	GTAATTAAAAAGAAGATGGTTTTAACAGCTTTTATTTAACGTGTTTCTCAA GATC
AK87	CTTTTGAAAAATTGAGAGGTATAAATCAAAGTATAGATTTTCATTGCTGG
AK88	CTTATGGGATTTATCTTCCTTATAAATAATAAAAAAGCCGGATTAATAAT
AK91	CCTTATGGGATTTATCTTCCTTACTATTAGGTATATTCCATGTG
AK105	CTTTTGAAAAATTGAGAGGTATAAATCAAATTAATAGTTTTAGCTATTAAT CTTTT
AK106	CTTTTGAAAAATTGAGAGGTATAAATCAACTGCAGAAAATTACAATAAGG
AK107	GTGGTTATTATTCAAATTGCAG
AK108	CTTGATCTGCAATTTGAATAATAACCACTCCTTTGTTTATCCTCCTC
AK112	CTTTTGAAAAATTGAGAGGTATAAATCAAGAATGGCGATTCCAAAG
AK121	TGAACAATTGTTGCAATTTT
AK122	CACCTATTAAATAAGTTAAAATAGAAAC
AK123	CCGTCACCTTTTCTAATTTAAG
AK124	GATATTTTCTCGTGATTGG
AK133	CTATACTGAACCAAGACAAAG
AK134	TACATTCCTTTAGTAACGTGAATATCTTTTCTCTTTTGGTATG
AK147	GAAGGGACACTTTGTCTATTTCTGACTTATAGGGGTAACACTTAAAAAAG
AK148	CTCCTCTCCTTTTCAAGAAATAGACAAATTTTACGTTACTAAAGGGAATGT A
AK151	ATGAAAAAAGTAAATTTTACAC

AK152	AAGACAACCTGCAAAATAAG
AK153	CTTGCCTTATTTTGCAGGTTGTCTTTAAGGAAGATAAAATCCCATAAGG
AK169	CGTTTCTATTTTAACTTATTTAATAGGTGCTACACTACCTAATAATTTATC
AK170	GCTTAAATTAGAAAAGGTGACGGAGTGCAAAAAAGATATAATGG
AK184	GTTTCAGAAACCTTTATACAAG
AK187	CCTTATGGGATTTATCTTCCTTATTAAAGCTCAAGTTGAGGCG
AK188	TACATTCCCTTAGTAACGTGAAATCAATTTATAAGAGACTTAGAGTG
AK189	CGTACAGTTTTGGTGATTTT
AK194	CAACATAAGTTCCAAGAGAATAAGATCGAAATC
AK195	CTCTTGGA ^A CTTATGTTGCCTGAGATGGAC
AK222	GTGAGAATAGTGTAATAATTTTAGTTTTTCAATTGATATGCCTCCTAAA TTTTTATC
AK245	CATAAGGGCCAAGAGAATAAGATCGAAATC
AK246	CTCTTGG ^C CCTTATGTTGCCTGAGATGG
AK251	CGGCG ^C ACGCAATTCATGACTGACGTC
AK252	GAATTGCGT ^C CGCCGATTGCGGTTATCAAG
AK262	CATTTTGACTGCTATCTTTG
AK263	CAAATACTAATCAAATGATATTTATC
AK264	TTAAGTAGGAGTGCTTAATTC
AK265	AAATGAGTTATCCTTTCTCC
AK266	TTAAATATAGATAAATATCATTTGATTAGTATTTGTTACGTTACTAAA GGAATGTA
AK267	GTTTGAATTAAGCACTCCTACTTAATAAGGAAGATAAAATCCCATAAGG
AK269	CAGTATTTCTTACACTAGG
AK270	CCTATGACCTATACGAAATC
AK273	GTTTGAATTAAGCACTCCTACTTAATCTATAAAATGCATACTGTTATTG
AK287	GAACTGCGGGTGACTCCACATGGTTAATTCCTCCTGTTAG
AK288	CTAACAGGAGGAATTAACCATGTGGAGTCACCCGCAGTTCGAGAAAGGT GCTGCTGGTAGCAAACGCATTTTGATTGTTG
AK290	GCCAAGCTTCTAGAATTCTCATTTTTACGGATAACGTAACC
AK291	GGTTACGTTATCCGTGAAAAATGAGAATTCTAGAAGCTTGGC
AK298	ACAATTTTAAAAATCTATATTATTATATC
AK299	CATAATAAGTATTTACCACTTTC
AK300	AAAAATAATGAGCACTTAAAGAATG
AK301	CATGAAGGCCTCCTTCTATC
AK302	CCTGTAGTTCCTTACATAC
AK303	TTCCATTTCTTGAGGCGAG

AK304	TTGATACAAAGGAAGGTCTTTC
AK305	CAAGAGACACTCCTTTATTTTC
AK326	CAAGAGACACTCCTTTATTTTC
AK336	CTTTTGAAAAATTGAGAGGTATAAATCAATAGATAGTAGCGGTGAATCTA G
AK337	CCTTATGGGATTTATCTTCCTTATTATTATTATTGTCCACTTTCC
AK337	CCTTATGGGATTTATCTTCCTTATTATTATTATTGTCCACTTTCC
AK340	CTGTTAAAACCATCTCTTTTAACTGAACGCTCTCTACTAGAGTC
AK341	CCTTATGGGATTTATCTTCCTTATTAGTCACCTCCTAGCTGAC
AK347	TCTCCTTTACCAGCAGCACCTAAACCATCTGCCGCTTTAC
AK348	GGTGCTGCTGGTAAAGGAGAAGAATTGTTTACTG
AK349	AAAAATAATGAGCACTTAAAGAATG
AK350	CCTGTAGTTCCTTACATAC
AK351	TTGATACAAAGGAAGGTCTTTC
AK354	TGCATGATCTACGTGCGTCAC
AK355	CAACAGCGGTCTACTGAATCTG
AK361	TTGATATGCCTCCTAAATTTTATC
AK362	CGTTTGAATTAAGCACTCCTACTTAAGTCCTCGGGATATGATAAG
AK362	CGTTTGAATTAAGCACTCCTACTTAAGTCCTCGGGATATGATAAG
AK364	GGTAGATAAAAAATTTAGGAGGCATATCAAATGAAATTTGGAAACTTTTTG C
AK365	CGTTTGAATTAAGCACTCCTACTTAAAGTATAGATTTTCATTGCTGG
AK448	CGTTTGAATTAAGCACTCCTACTTAACTTATAGGGGTAACACTTAAAAAA G
AK452	TGAGTGAATGGTTTCAATTG
AK453	AACGGGTTTCAAGAATTTG
AK455	CTGGCTTCAATTGAAACCATTCACTCATTACGTTACTAAAGGGAATGTA
AK458	GTAGCAACACTCTTGTTTAAAG
AK459	GTGGCTGAATTATCAAAATAAATC
AK462	GAACAAAACTCATGGATCCCTCCTAAGGAAGATAAATCCCATAAGG
AK463	GAGGGATCCATGAGTTTTTG
AK464	CGTCACAAATCTTCTGAACCGTTGGTCGACAGATCTCCATTC
AK472	AAGTTTAAATAAGGCTAGTCCGTTATCAACTTG
AK473	TATAGTTATTATACCAGGGGGAC
AK474	CGGACTAGCCTTATTTAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAA CAAATTTTATCTACCTAGTATATAGTTATTATACCAGGGGGAC
AK475	CGGACTAGCCTTATTTAAAC

AK476	GTCCCCCTGGTATAATAACTATA
AK505	GTTTGAATTAAGCACTCCTACTTAAGATTCAAAGTAAACAGAAATAAAA AAAC
AK509	CAAAAGATCTAAAGAGGAGAAAGGATCTATGCTTAAATTAGAAAAGGTG AC
AK510	AGATCCTTTCTCCTCTTTAG
AK511	GAACGCTCTCTACTAGAGTC
AK512	GTGACTTAGTAGAGAGCGTTCTAGATAGTAGCGGTGAATC
AK534	CGGACTAGCCTTATTTAACTTGCTATGCTGTTCCAGCATAGCTCTTAAA CAACTAAAAATGTTTGACAATTATAGTTATTATACCAGGGGGAC
AK537	TTAATATGTCCTTGGAGAAC
AK538	TTTTAGTTTGGTTTTTTGAATTAATTTTCATCAAAAAGAC
AK539	ATTCAAAAAACCAAACTAAAAATGTTTGACAATTTTAAAAATC
AK544	GAAACTGCTTAAATCAAACG
AK565	GGCGAATCTAAAAATAAAGG
AK566	TGGTAGCAGCTTAAATATGC
AK567	CCAACTGGCTTCATATCAC
AK568	CTTGAAGCTAACCTTATTTT
AK569	CTAAATAAAAGCATATTTAAGCTGCTACCAAGTATAGATTTTCATTGCTGG
AK570	GAAGAGTGATATGAAGCCAAGTTGGTTCACGTTACTAAAGGGAATG
AK575	ATGAGCAAACGCATTTTTATTGTTGAAGATGAGAGAAACC
AK576	CAACAATTAATGCGTTTGCTCATATTTACCTCCTTTGATTTAAGTG
AK612	CCTTATGGGATTTATCTTCCTTATCATCTTTCACGGATAACG
AR901	AACTATCATCCGTCATCCCAG
DD_DNDele ovRS-1	TACATTCCCTTTAGTAACGTGAAACACCTCAAATTGAAGGT
DD_DNDele ovRS-2	GGTTTTAAAGCATCCACTGCTGGCTCACC
DD_UpDele vRS-1	TTGGAAATCCAGCTCCAGCACCGATATCC
DD_UpDele vRS-2	CCTTATGGGATTTATCTTCCTTACAAAATGCGTTTGCTCAT
DF_comS	TACATTCCCTTTAGTAACGTGAATAATAAGGAGTCACCATGTC
DF_SER	AAGCTGTAAAACCATCTTC
Dn.Rv.lox71	TTCACGTTACTAAAGGGAATGTA
DN_F_bceRS _lox71	TACATTCCCTTTAGTAACGTGAAGTTGGCGATTGATTAATAAC

DN_F_ciaRH_lox71	TACATTCCCTTTAGTAACGTGAATAGATAAGGCTAGAACCAGAC
DN_F_covS_lox71	TACATTCCCTTTAGTAACGTGAAGCCTCAACTTGAGCTTTAAATC
DN_F_fasB_lox71	TACATTCCCTTTAGTAACGTGAAGGCCCTAATATGAATATATAC
DN_F_liaSR_lox71	TACATTCCCTTTAGTAACGTGAACGCAGGATGATGAATAAAGC
DN_F_spaRK_lox71	TACATTCCCTTTAGTAACGTGAACTTTAAATACAGATGACTCC
DN_F_TCS06_lox71	TACATTCCCTTTAGTAACGTGAATGAGGAGAAGTAGATAAATTTTC
DN_F_TCS09_lox71	TACATTCCCTTTAGTAACGTGAAGATGTGGTTTGATTATGACG
DN_F_TCS10_lox71	TACATTCCCTTTAGTAACGTGAATTTAAATATTTGACTAGGTG
DN_F_TCS13_lox71	TACATTCCCTTTAGTAACGTGAACAGTGAGATTATAAAAAACATG
DN_F_TCS14_lox71	TACATTCCCTTTAGTAACGTGAAACACCAAAAGAAGATTAATG
DN_F_vicK_lox71	TACATTCCCTTTAGTAACGTGAAGAGATGTAATAGAAATGACC
DN_F_vncRS_lox71	TACATTCCCTTTAGTAACGTGAAGGTCTTGCCCCAAAAGTAAAAAG
Dn_R SS1-4	TCATCACAATGGTCACATCT
DN_R_bceRS	CTACTTGTGTCATCATTTGC
DN_R_ciaRH	GCCTAAAATAACGTATAATGAC
DN_R_covS	TGAAAGAACGGTAGACACTG
DN_R_diag_del_covS	CAAATTCTTCAATAGGTCTC
DN_R_diag_del_fasB	CCAAATTAATAAAAAAGGAGCG
DN_R_diag_del_vicK	ATCCGCTCCATCTTCATTAC
DN_R_diagdel_bceRS	GTATTTCTGGTTTAGTGATG
DN_R_diagdel_ciaRH	CTGGCGTGATATCAATTTGG
DN_R_diagdel_liaSR	ACAGCCATCACAAAACAAAG

DN_R_diagd el_spaRK	CTTAAGGTAGACTTTGTTCA
DN_R_diagd el_TCS06	GTTTCACTTCTATCATTGCC
DN_R_diagd el_TCS09	ATATCAGAAGCAATTGTTCC
DN_R_diagd el_TCS10	GCAAAGATAACAATACGACC
DN_R_diagd el_TCS13	CAGAGATTGTATCTGGATTC
DN_R_diagd el_TCS14	TCTTAGCTGCTTTTCTTCC
DN_R_diagd el_vncRS	GATATTTAACCTGTCTACTTG
DN_R_fasB	TTTAGTAATCAGCATCACC
DN_R_liaSR	TAGCTGGTACTTATAGTCG
DN_R_spaR K	GAAGCAATAACTGTAATGGC
DN_R_TCS0 6	TCTCCTAAAATCTCCCTTC
DN_R_TCS0 9	CCAGCGATTGAACTATAATC
DN_R_TCS1 3	GGTTCATACTCTGTTTTATC
DN_R_TCS1 4	GAATGATTTCCTGCATCTGG
DN_R_vicK	ATTGGACTCAATCAGATAGC
DN_R_vncRS	AATGGTTTCCCTTCAGC
DN_R_vncRS _2	ACCACCTTTGCTAGCTG
DN-covRS-2	GGTTTTAAAGCATCCACTGCTGGCTCACC
Dn- insertcovRS KAN-F	GGGAAATATTCATTCTAATTGGTCAATCATGAGCACGTC
DR_SER	TTGGATAAGGTCTTGACTTC
DR_THR	TTGATTTATACCTCTCAATTT
F_lux_ATG	ATGAAATTTGGAACTTTTTGC
JB546	GGTGTTGTATTCATCTTCAC
Kan OVL1	TTGATTTTCGTTCTGAATAC
Kan OVL2	CCAATTAGAATGAATATTTCCC

LF-DN_LMGCovRD98E_3'A	GTTTGGATCGTGGAGCTGATGAGTATATTATAAAACCATTTGCG
LF-pMGCovRD9XbaI-F	AAGCTCTAGATTTATAAAAGAGGGTAAATACTTATTATGAGC
LF-pMGCovSLMGPstI-R	AAACTGCAGATAAATCTCTTGTAATTGACTTAACC
LF-UpD9CovR_E98D-5'B	GTCATCAGCTCCACGATCCAAACCAGCAACAATATC
LF-UpLMGCovR_D98E-5'B	CTCATCAGCTCCACGATCCAAACCAGCAACAATATC
LL61	CATGGGAGTTCTCATCTATAAATCAACCTCTTTGAACATA
LL150	TACATTCCCTTTAGTAACGTGAA
MV13	CACTTAAATCAAAGGAGGTAAATATGCCGACATTAGAAATAGCACAAAAAAAC
MV23	CACTTAAATCAAAGGAGGTAAATATGAAAAAACTAAAATTATTACACTATTC
MV24	CACTTAAATCAAAGGAGGTAAATATGATCACTATCTTGCCTTATTTTG
MV25	CTTATGGGATTTATCTTCCTTAAATATATGGATATTTTGACATGG
MV29	CACTTAAATCAAAGGAGGTAAATATGAACATAAAAGACAGCATTGGAC
MV30	CTTATGGGATTTATCTTCCTTACCATTAAAAAAGTAGTGACATTTATG
ML38	CTTTATCTACGTCAGTATAAATC
ML40	TACATTCCCTTTAGTAACGTGAAAAATGGTGGTGACATAAATG
ML41	CTTACGACGAAACAAAATTG
ML45	TAAGGAAGATAAATCCCATAAGGTAAACATAAACATGCAAGGAG
ML46	TTACGTTACTAAAGGGAATGTAGAAAAGGCAATTGCGTTACC
Opp_S4_1	TGTAGCACGTGCACAATCTT
Opp_S4_2ca t	CCTTATGGGATTTATCTTCCTTATCAAGGCCGTCACGACAAATA
Opp_S4_3ca t	TACATTCCCTTTAGTAACGTGAAAAAGAACAAAGTAAATAAGTCT
Opp_S4_6B	TCACACTGATGGTTTTTGCT
PB11	CAGAAATAGACAAAGTGTCCTTCCAGCATAG
PB12	AATTTGTCTATTCTGAAAAGGAGAGGAGGGG

R_spec	ATAGGATGAGAACTCCCATG
RpxZ9_ATG	CATATTTACCTCCTTTGATTTA
SeqCovRS-F5	TCCTTGAAGAAAGTCTTGAAGCTGC
UF_R-lux	TAATTGAGGAGGTCTATGAG
UF_R-lux-ATG	ATGAAATTTGGAACTTTTTGC
UF_SER	CAAGATTAACCATGACCTTC
UF_THR	TGTCAAAGGATTAGGAAAAC
Up.Fw.lox66	TAAGGAAGATAAATCCCATAGG
Up.insertCovRSkan-R	GTATTCACGAACGAAAATCAAGAGTTAGTATAAATCTCTTG
Up_F_bceRS	ACTCTGACAAAGATCAAACC
Up_F_ciaRH	GTTACTGAAATCTATGGTGG
Up_F_covS	CTCTTGGACCTTATGTTGCC
Up_F_diagdel_covS	CTCTCTTGAACCTCAACACG
Up_F_diagdel_fasB	GTCTATAATAAACATATTGAGG
Up_F_diagdel_vicK	TGTTACGGCTTTTGATGGTC
Up_F_diagdel_bceRS	TGCGGTCTTCCAGTAATTC
Up_F_diagdel_ciaRH	GATGGTGGTATTGAACTTGC
Up_F_diagdel_liaSR	TTTTGACTCTGGGCCTGATC
Up_F_diagdel_spaRK	CAATTCCAATGTATGTGAGC
Up_F_diagdel_TCS06	TTCAGGTATGGAAATGAGTC
Up_F_diagdel_TCS09	TAGATGGCAGCCCAATCTTG
Up_F_diagdel_TCS10	TCTTAGCTTTAGAGTCCTTC
Up_F_diagdel_TCS13	CATGCAACCTTGGCGTTTTG
Up_F_diagdel_TCS14	TGGTTTTCTACAGCCTTATG

Up_F_diagdel_vncRS	CTCTACGATCTTGTGAAGAG
Up_F_fasB	AGAGGTTTTATCCGTTTATC
Up_F_liaSR	TTGACCATCTGTTTGATATC
Up_F_spaRK	AATATCGAGTTACCCTAAGC
Up_F_TCS06	ATGCTTAGAGAGGACCAAAC
Up_F_TCS09	GTTCTTTCTCGTATGGGGTG
Up_F_TCS10	TACAAGAAATAACAGACCCG
Up_F_TCS13	GATTTGAAGAGCCAAGAAAG
Up_F_TCS14	AAAAGTTGGAGTGTTGTGG
Up_F_vicK	CAAGTCACACACCAATTATC
Up_F_vncRS	GTTTATTGCTAACCCCTCTG
Up_R_bceRS_lox66	CCTTATGGGATTTATCTTCCTTATCCATGAAATCTTCAACAAG
Up_R_ciaRH_lox66	CCTTATGGGATTTATCTTCCTTACTAGTAGTATTTTAATCATAGTTATC
Up_R_covS_lox66	CCTTATGGGATTTATCTTCCTTAGTAATTTACTCATTTTTCACGG
Up_R_fasB_lox66	CCTTATGGGATTTATCTTCCTTATTTGTCCACCAATACGTAAG
Up_R_liaSR_lox66	CCTTATGGGATTTATCTTCCTTACTGTTTCTTCATTTGCGACG
Up_R_spaRK_lox66	CCTTATGGGATTTATCTTCCTTAGAGCCATAACCTTTCCTTTC
Up_R_TCS06_lox66	CCTTATGGGATTTATCTTCCTTACTAAACATGGTCACTTCTC
Up_R_TCS09_lox66	CCTTATGGGATTTATCTTCCTTAGCTCGGTAATAGACTCTAAC
Up_R_TCS10_lox66	CCTTATGGGATTTATCTTCCTTAAATTTCAAAGCCATTCTAG
Up_R_TCS13_lox66	CCTTATGGGATTTATCTTCCTTACATGTCTTAGTCCTCCAATG
Up_R_TCS14_lox66	CCTTATGGGATTTATCTTCCTTATAGAAAGCATCTTATTTCCC
Up_R_vicK_lox66	CCTTATGGGATTTATCTTCCTTAGCCAATACTAGTCATAATTC
Up_R_vncRS_lox66	CCTTATGGGATTTATCTTCCTTAAGTATCTTCATACATTATCC
Updelsterco vRS-A	TTGGAAATCCAGCTCCAGCACCGATATCC

UR_comS	CCTTATGGGATTTATCTTCCTTATAAACTCCTTTAACTGTTAG
UR_SER	AGTAATTAAAAAGAAGATGG
UR_THR	TTGATTATACCTCTCAATT

Table S1.6. List of (overlapping) PCR fragments, synthetic DNA constructs, and EMSA probes

Strain/ Plasmid	PCR fragment	Template DNA	Primer 1	Primer 2
AK0001	Upstream homologous region (Up HR) of <i>tRNA_{thr}</i> locus fused to <i>P_{comX}-luxAB</i>	JM1020	UF_THR	AK91
	<i>spc</i> , spectinomycin resistance cassette	pJUDspecm ut1-gfp+ter	Up.fw.lox 66	R_spec
	Downstream homologous region (Dw HR) of <i>tRNA_{thr}</i> locus	HSISS4	LL61	UR_THR
AK0002	Up HR of <i>tRNA_{ser}</i> fused to <i>xyIR-P_{xyI2}</i>	JM1125	UF_SER	RpxZ9_AT G
AK0003	<i>cat</i> , chloramphenicol resistance cassette	pJIMcat	Up.fw.lox 66	Dn.rv.lox 71
AK0004				
AK0005	Dw HR of <i>tRNA_{ser}</i>	HSISS4	LL150	DR_SER
AK0002	<i>comS</i> (HSISS4_10217)	HSISS4	MV23	MV25
AK0003	<i>xip</i> , mature C-terminal form of ComS (11aa)	HSISS4	MV24	MV25
AK0004	<i>comR</i> (HSISS4_00217)	HSISS4	MV29	MV30
AK0005	<i>comR-P_{comS}-comS</i>	HSISS4	MV29	MV25
AK0006	Up HR of <i>tRNA_{ser}</i> fused to <i>xyIR-xip-cat</i> fused to Dw HR of	AK0005	UF_SER	MV13
		AK0003	AK52	DR_SER
AK0007	Up HR of <i>tRNA_{ser}</i>	HSISS4	UF_SER	UR_SER
	<i>cat</i> , chloramphenicol resistance	pJIMcat	AK69	AK70
	<i>amiA3</i> (HSISS4_01366),	HSISS4	AK44	AK71
	Dw HR of <i>tRNA_{ser}</i>	HSISS4	DF_SER	DR_SER

AK0008	Up HR of <i>amiACDEF</i> (HSISS4_01361-01365)	HSISS4	AK57	AK58
	<i>xylR</i> -P _{<i>xyl2</i>}	pZX10	AK59	AK50
	<i>cat</i> , chloramphenicol resistance cassette	pJIMcat	Up.fw.lox66	AK60
	Dw HR of <i>amiACDEF</i> (HSISS4_01361-01365)	HSISS4	AK61	AK62
AK0009	Up HR of <i>pptAB</i> (HSISS4_01518-01519)	HSISS4	AK133	AK509
	<i>S. pneumoniae</i> optimized P _{<i>lac</i>} promoter	pJWV102-PL-dcas9	AK510	AK511
	<i>S. pneumoniae</i> optimized P _{<i>F6</i>} promoter fused to the <i>lacI</i> repressor gene	pPEPY-PF6-lacI	AK512	AK337
	<i>cat</i> , chloramphenicol resistance cassette	pJIMcat	Up.fw.lox66	Dn.rv.lox71
	Dw HR of <i>pptAB</i> (HSISS4_01518-01519)	HSISS4	AK134	AK124
AK0010	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF_THR	UR_THR
	Promoter of <i>comR</i> (P _{<i>comR</i>})	HSISS4	AK55	AK56
	<i>luxAB</i> genes fused to <i>spc</i> -Dw HR <i>tRNA_{thr}</i>	AK0001	F_lux_ATG	DR_THR
AK0011	<i>cat</i> , chloramphenicol resistance cassette	pJIMcat	Up.fw.lox66	Dn.rv.lox71
AK0012	Dw HR of <i>covRS</i> (HSISS4_00246-00247)	HSISS4	AK188	AK189
AK0011	Up HR of <i>covR</i> and <i>covR_{D53A}</i>	HSISS4	AK184	AK246
	Dw HR of <i>covR_{D53A}</i> and <i>covS</i> (HSISS4_00246-00247)	HSISS4	AK245	AK187
AK0012	Up HR of <i>covR</i> and <i>covR_{D53E}</i>	HSISS4	AK184	AK195
	Dw HR of <i>covR_{D53E}</i> and <i>covS</i> (HSISS4_00246-00247)	HSISS4	AK194	AK187
AK0013	<i>covR</i> and Up HR of <i>covS_{T287A}</i>	HSISS4	AK184	AK252
	Dw HR of <i>covS_{T287A}</i> (HSISS4_00247)	HSISS4	AK251	AK187

	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF_THR	UR_THR
AK0014	<i>P_{comX}-opt-gfp⁺</i> , <i>comX</i> promoter with 5'UTR optimized sequence fused to codon-optimized <i>afp⁺</i> <i>spc</i> fused to Dw HR of <i>tRNA_{thr}</i>	<i>P_{comX}-gfp⁺</i> (gBlocks DNA ^a) AK0001	AK87 Up.fw.lox	AK88 DR_THR
AK0015	<i>P_{xyl2}-comR-cat</i> at <i>tRNA_{ser}</i>	AK0004	UF_SER	DR_SER
	Up HR of <i>tRNA_{ser}</i> with upstream region of <i>xylR</i>	AK0004	UF_SER	AK107
AK0016	<i>P_{xyl2}-opt-gfp⁺</i> , <i>P_{xyl2}</i> promoter with 5'UTR optimized sequence fused to codon-optimized <i>afp⁺</i> <i>spc</i> fused to Dw HR of <i>tRNA_{ser}</i>	<i>P_{xyl2}-gfp⁺</i> (gBlocks DNA ^a) JM1016	AK108 Up.fw.lox	AK88 DR_SER
AK0017	<i>P_{xyl2}-comR-cat</i> at <i>tRNA_{thr}</i>	AK0004	UF_SER	DR_SER
	Up HR of <i>comS</i> (HSISS4_00217)	HSISS4	UF_R-lux	UR_comS
AK0018	Dw HR of <i>comS</i> (HSISS4_00217)	HSISS4	DF_comS	Dn_R SS1-4
	<i>cat</i> , chloramphenicol resistance	pJIMcat	Up.fw.lox	Dn.rv.lox7
	Up HR of <i>amiACDEF</i> (HSISS4_01361-01365)	HSISS4	Opp_S4_6B	Opp_S4_3cat
AK0019	Dw HR of <i>amiACDEF</i> (HSISS4_01361-01365)	HSISS4	Opp_S4_2cat	Opp_S4_1
	<i>cat</i> , chloramphenicol resistance	pJIMcat	Up.fw.lox	Dn.rv.lox7
	Up HR of <i>pptAB</i> (HSISS4_01518-01519)	HSISS4	AK121	AK122
AK0020	Dw HR of <i>pptAB</i> (HSISS4_01518-01519)	HSISS4	AK123	AK124
	<i>erm</i> , erythromycin resistance	pGIUD08	AK169	AK170
AK0021	IPTG-inducible system of <i>pptAB</i> expression (<i>P_{F6}-lacI</i> <i>P_{lac}-pptAB</i>)	AK0009	AK133	AK124
	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF_THR	UR_THR
AK0022	<i>P_{comR}-opt-gfp⁺</i> , <i>comR</i> promoter with 5'UTR optimized sequence fused to codon-optimized <i>gfp⁺</i> <i>spc</i> fused Dw HR of <i>tRNA_{thr}</i>	<i>P_{comR}-gfp⁺</i> (gBlocks DNA ^a) AK0001	AK112 Up.fw.lox 66	AK88 DR_THR

	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF_THR	UR_THR
AK0023	<i>P_{comS}-opt-gfp⁺</i> , <i>comS</i> promoter with 5'UTR optimized sequence fused to <i>Spc</i> fused-Dw HR of <i>tRNA_{thr}</i>	<i>P_{comX}-gfp⁺</i> (gBlocks) AK0001	AK106 Up.fw.lox66	AK88 DR_THR
	Up HR of <i>tRNA_{thr}</i> locus fused to <i>P_{comS}-luxAB</i>	JM1019	UF_THR	AK91
AK0024	<i>spc</i> , spectinomycin resistance cassette	pJUDspec mut1-gfp+ter	Up.fw.lox66	R_spec
	Dw HR of <i>tRNA_{thr}</i> locus	HSISS4	LL61	DR_THR
AK0025	<i>P_{xyI2}-comR</i> overexpression system fused to a chloramphenicol resistance cassette at the <i>tRNA_{ser}</i> locus	AK0004	UF_SER	DR_SER
	Up HR of <i>tnpII</i> locus	HSISS4	AK262	AK263
AK0023, AK0024	<i>spc</i> , spectinomycin resistance cassette	pJUDspec mut1-gfp+ter	AK266	AK267
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265
AK0023	<i>comS</i> under the xylose-inducible promoter <i>P_{xyI2}</i>	AK0002	AK88	AK448
AK0024	<i>xip</i> under the xylose-inducible promoter <i>P_{xyI2}</i>	AK0003	AK88	AK448
	Up HR of <i>tnpII</i> locus fused to a spectinomycin resistance cassette, <i>spc</i>	AK0023	AK262	AK153
AK0025	<i>comS</i>	HSISS4	AK151	AK152
	The constitutive promoter <i>P₃₂</i>	pJIMcat	AK362	AK222
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265

AK0026	Up HR of <i>comR</i> together P_{comR} and <i>comR</i> without stop codon	HSISS4	ML38	AK347
	<i>gfp</i> ⁺ codon-optimized gene followed by the <i>spc</i> resistance	AK0014	AK348	Dn.rv.lox 71
	Dw HR of <i>comR</i> locus	HSISS4	ML40	ML41
AK0027	Up HR of <i>tRNA</i> _{thr}	HSISS4	UF_THR	UR_THR
	P_{F6} synthetic constitutive promoter fused to the optimized <i>lacI</i> gene	pPEPY-PF6-lacI	AK336	AK337
	Dw HR of <i>tRNA</i> _{thr} locus	HSISS4	Up.fw.lox 66	DR_THR
AK0028	Up HR of <i>tRNA</i> _{thr}	HSISS4	UF_SER	UR_SER
	P_{lac} optimized promoter fused to a dead-cas9 (D10A H841A)	pJWV10 2-PL-	AK340	AK341
	Dw HR of <i>tRNA</i> _{thr} locus	HSISS4	Up.fw.lox 66	DR_SER
AK0030	Up HR of <i>tnpII</i> locus	HSISS4	AK262	AK263
	P_{comX} promoter fused to the <i>luxAB</i> genes together with a	AK0001	AK266	AK365
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265
AK0031	Up HR of <i>tnpII</i> locus	HSISS4	AK262	AK263
	P_{comR} promoter fused to the <i>luxAB</i> genes together with a spectinomycin resistance	AK0010	AK266	AK505
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265
AK0032	Up HR of <i>tnpII</i> locus	HSISS4	AK262	AK263
	P_{comX} promoter fused to the optimized <i>gfp</i> ⁺ gene together with a spectinomycin resistance	AK0014	AK266	AK365
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265

AK0036	<i>erm</i> resistance cassette fused to lox66 and lox71 sequences (<i>lox66-erm-lox71</i>)	pGIUD0855erm	ML45	ML46
	Up HR of <i>GOR</i> locus	HSISS4	AK458	AK452
	<i>erm</i> resistance cassette with lox66 and lox71 sequences with overlap flanking sequences	<i>lox66-erm-lox71</i>	AK455	AK462
	P ₃ constitutive promoter fused to a guide targeting the <i>luc</i> gene together with a <i>dcas9</i> -handle and terminator	pPEPX-P3-sgRNA _{luc}	AK463	AK464
	Dw HR of <i>GOR</i> locus	HSISS4	AK453	AK459
AK0037	Up HR of <i>tnpII</i> locus fused to a <i>spc</i> resistance cassette and the <i>luxAB</i> genes	AK0030	AK262	AK364
	P ₃₂ promoter	pJIMcat	AK361	AK362
	Dw HR of <i>tnpII</i> locus	HSISS4	AK264	AK265
AK0037	Up HR of <i>GOR</i> locus fused to the <i>erm</i> resistance, a terminator and the <i>dcas9</i> handle	AK0036	AK458	AK472
	<i>Dcas9</i> handle together with a guide targeting the P ₃₂ promoter	AK474	AK475	AK476
	P ₃ promoter together with Dw HR of <i>GOR</i> locus	AK0036	AK473	AK459
AK0038 AK0039 AK0040	Up HR of <i>GOR</i> locus fused to the <i>erm</i> resistance, a terminator and the <i>dcas9</i> handle	AK0036	AK458	AK472
	<i>Dcas9</i> handle together with a guide targeting a modified version of the P _{covRS} promoter (P _{covRS+})	AK534	AK475	AK476
	P ₃ promoter together with Dw HR of <i>GOR</i> locus	AK0036	AK473	AK459
AK0038 AK0039 AK0040	Up HR of <i>covRS</i> locus with 1 mutation in P _{covRS}	HSISS4	AK537	AK538
	<i>covRS</i> genes together with 1 mutation in P _{covRS}	HSISS4	AK539	AK187
	<i>cat</i> resistance cassette together with Dw HR of <i>covRS</i> locus	AK0011	Up.fw.lox66	AK544

AK0042	Up HR of LMD-9 <i>SUC</i> locus	LMD-9	AK565	AK566
	$P_{comX^{-}opt-gfp^{+}}$, <i>comX</i> promoter with 5'UTR optimized sequence fused to codon-optimized <i>gfp</i> ⁺	AK0014	AK569	AK570
	Dw HR of LMD-9 <i>SUC</i> locus	LMD-9	AK567	AK568
	Up HR of LMD-9 <i>tRNA_{ser}</i> locus	LMD-9	AR901	PB11
	<i>xyIR-P_{xyI2}</i> xylose inducible promoter	JM1125	AK147	AK576
AK0042	<i>covR</i> with D98 residue from LMG18311	LMG18311	AK575	AK612
	<i>cat</i> resistance cassette	pJIMcat	Up.fw.lox66	AK148
	Dw HR of LMD-9 <i>tRNA_{ser}</i> locus	LMD-9	PB12	JB546
pBAD-covR-ST _{N-ter}	pBAD plasmid backbone	pBAD-comR-ST	AK287	AK291
	<i>covR_{WT}</i>	HSISS4	AK288	AK290
pBAD-covR _{D53A} -ST _{N-ter}	pBAD plasmid backbone	pBAD-comR-ST	AK287	AK291
	<i>covR_{D53A}</i>	AK0011	AK288	AK290
pBAD-covR _{D53E} -ST _{N-ter}	pBAD plasmid backbone	pBAD-comR-ST	AK287	AK291
	<i>covR_{D53E}</i>	AK0012	AK288	AK290
FV0002-FV0015	<i>cat</i> , chloramphenicol resistance cassette	JM1020	Up.fw.lox66	Dn.rv.lox71
FV0002	Up HR of <i>covS</i> (HSISS4_00247)	HSISS4	Up_F_covS	Up_R_covS_lox66
	Dw HR of <i>covS</i> (HSISS4_00247)	HSISS4	DN_F_covS_lox71	DN_R_covS

FV0003	Up HR of <i>ciaRH</i> (HSISS4_01096-01097)	HSISS4	Up_F_ciaRH	Up_R_ciaRH_lox66
	Dw HR of <i>ciaRH</i> (HSISS4_01096-01097)	HSISS4	DN_F_ciaRH_lox71	DN_R_ciaRH
FV0004	Up HR of <i>spaRK</i> (HSISS4_01036-01035)	HSISS4	Up_F_spaRK	Up_R_spaRK_lox66
	Dw HR of <i>spaRK</i> (HSISS4_01036-01035)	HSISS4	DN_F_spaRK_lox71	DN_R_spaRK
FV0005	Up HR of <i>vicK</i> (HSISS4_00837)	HSISS4	Up_F_vicK	Up_R_vicK_lox66
	Dw HR of <i>vicK</i> (HSISS4_00837)	HSISS4	DN_F_vicK_lox71	DN_R_vicK
FV0006	Up HR of <i>tcs06</i> (HSISS4_01178-01179)	HSISS4	Up_F_TCS06	Up_R_TC06_lox6
	Dw HR of <i>tcs06</i> (HSISS4_01178-01179)	HSISS4	DN_F_TCS06_lox7	DN_R_TC06
FV0007	Up HR of <i>bceRS</i> (HSISS4_01193-01192)	HSISS4	Up_F_bceRS	Up_R_bceRS_lox6
	Dw HR of <i>bceRS</i> (HSISS4_01193-01192)	HSISS4	DN_F_bceRS_lox7	DN_R_bceRS
FV0008	Up HR of <i>liaSR</i> (HSISS4_01345-01344)	HSISS4	Up_F_liaSR	Up_R_liaSR_lox66
	Dw HR of <i>liaSR</i> (HSISS4_01345-01344)	HSISS4	DN_F_liaSR_lox71	DN_R_liaSR
FV0009	Up HR of <i>tcs09</i> (HSISS4_00378-00379)	HSISS4	Up_F_TCS09	Up_R_TC09_lox6
	Dw HR of <i>tcs09</i> (HSISS4_00378-00379)	HSISS4	DN_F_TCS09_lox7	DN_R_TC09
FV0010	Up HR of <i>tcs10</i> (HSISS4_00352-00353)	HSISS4	Up_F_TCS10	Up_R_TC10_lox6
	Dw HR of <i>tcs10</i> (HSISS4_00352-00353)	HSISS4	DN_F_TCS10_lox7	DN_F_TC10_lox7
FV0011	Up HR of <i>vncRS</i> (HSISS4_01248-01249)	HSISS4	Up_F_vncRS	Up_R_vncRS_lox66
	Dw HR of <i>vncRS</i> (HSISS4_01248-01249)	HSISS4	DN_F_vncRS_lox71	DN_R_vncRS

FV0012	Up HR of <i>fasB</i> (HSISS4_01716)	HSISS4	Up_F_fasB	Up_R_fasB_lox66
	Dw HR of <i>fasB</i> (HSISS4_01716)	HSISS4	DN_F_fasB_lox71	DN_R_fasB
FV0013	Up HR of <i>tcs13</i> (HSISS4_01231-01230)	HSISS4	Up_F_TCS13	Up_R_TC S13_lox6
	Dw HR of <i>tcs13</i> (HSISS4_01231-01230)	HSISS4	DN_F_TC S13_lox7	DN_R_TC S13
FV0014	Up HR of <i>tcs14</i> (HSISS4_01447-1446)	HSISS4	Up_F_TCS14	Up_R_TC S14_lox6
	Dw HR of <i>tcs14</i> (HSISS4_01447-01446)	HSISS4	DN_F_TC S14_lox7	DN_R_TC S14
LF150	Up HR of LMG18311 <i>covRS</i> (stu0317-0318)	LMG18311	DD_UpDe lcoRS-1	DD_UpDe lcoRS-2
LF152	<i>cat</i> , chloramphenicol cassette resistance	pJIMcat	Up.fw.lox66	Dn.rv.lox71
LF154	Dw HR of LMG18311 <i>covRS</i> (stu0317-0318)	LMG18311	DD_DNDe lcoRS-1	DD_DNDe lcoRS-2
LF151	Up HR of LMG18311 <i>covRS</i> locus	LMG18311	SeqCovRS-F5	Up.insert CovRSkan
	<i>kan</i> resistance cassette	TOPO <i>kan^{Rb}</i>	Kan OVL1	Kan OVL2
	Dw HR of LMG18311 <i>covRS</i> locus	LMG18311	Dn-insertcov RSKAN-F	DN-covRS-2
LF156	Up HR of LMD-9 <i>covRS</i> locus	LMD-9	SeqCovRS-F5	Up.insert CovRSkan-R
	<i>kan</i> resistance cassette	TOPO <i>kan^{Rb}</i>	Kan OVL1	Kan OVL2
	Dw HR of LMD-9 <i>covRS</i> locus	LMD-9	Dn-insertcov RSKAN-F	DN-covRS-2
LF159	LMD-9 <i>covRS</i> locus	LMD-9	DD_UpDe lcoRS-1	DD_DNDe lcoRS-2
LF160	LMG18311 <i>covRS</i> locus	LMG18311	DD_UpDe lcoRS-1	DD_DNDe lcoRS-2

LF161	LMG18311 upstream <i>covRS</i> locus with D98E mutation introduction	LF151	Updelster <i>covRS</i> -A	LF-Up LMGCovR_ _D98E-
	LMG18311 upstream <i>covRS</i> locus with D98E mutation introduction and <i>kan</i> resistance cassette	LF151	LF- DN_LMG CovRD98 E_3'A	DNcovRS- 2
LF162	LMD-9 upstream <i>covRS</i> locus with E98D mutation introduction	LF156	Updelster <i>covRS</i> -A	LF-Up D9CovR_ E98D-5'B
	LMD-9 upstream <i>covRS</i> locus with E98D mutation introduction and <i>kan</i> resistance cassette	LF156	LF- DN_D9Co vR_E98D_	DNcovRS- 2
pMG36E T-covRS	<i>covRS</i> operon of LMG18311, to be restricted to perform ligation with restricted pMG36ET	LMG1831 1	LF- pMGcovR D9XbaI-F	LF- pMGcovS LMGPstI- R
EMSA probes		Templat	Primer	Primer
P_{comR} -HSISS4 (<i>comR</i> promoter of HSISS4, non-fluorescent probe)		HSISS4	AK351	AK305
P_{comR} -HSISS4-Cy3 (<i>comR</i> promoter of HSISS4 with Cy3 dye at the 5' end)		HSISS4	AK304	AK305
P_{comR} -HSISS4-Cy3-Cy5 (<i>comR</i> promoter of HSISS4 with Cy3 and Cy5 dyes at the 5' and 3')		HSISS4	AK304	AK326
P_{dnaE} -HSISS4 (<i>dnaE</i> promoter of HSISS4, non-fluorescent probe)		HSISS4	AK349	AK301
P_{dnaE} -HSISS4-Cy3 (<i>comR</i> promoter of HSISS4, with Cy3 dye at the 5' end)		HSISS4	AK300	AK301
P_{covR} -HSISS4-Cy3 (<i>covR</i> promoter of HSISS4, with Cy3 dye at the 5' end)		HSISS4	AK298	AK299
CDS _{<i>dnaE</i>} -HSISS4 (150 pb fragment of the <i>dnaE</i> coding sequence of HSISS4, non-fluorescent probe)		HSISS4	AK350	AK303
CDS _{<i>dnaE</i>} -HSISS4-Cy3 (150 pb fragment of the <i>dnaE</i> coding sequence of HSISS4, with Cy3 dye at the 5' end)		HSISS4	AK302	AK303

P_{dnaE} -HSISS4 _{synth} (<i>dnaE</i> promoter of <i>S. salivarius</i> , with Cy3 dye at the 5'end)	P_{dnaE} (DNA _s)	AK354	AK355
P_{comR} -HSISS4 _{synth} (<i>comR</i> promoter of <i>S. salivarius</i> , with Cy3 dye at the 5'end)	P_{comR} HSISS4 (DNA _s)	AK354	AK355
P_{comR} -LMD-9 _{synth} (<i>comR</i> promoter of <i>S. thermophilus</i> , with Cy3 dye at the 5'end)	P_{comR} LMD-9 (DNA _s)	AK354	AK355
P_{comR} -NTC12167 _{synth} (<i>comR</i> promoter of <i>S. vestibularis</i> , with Cy3 dye at the 5'end)	P_{comR} NTC1216 7 (DNA _s)	AK354	AK355

^agBlocks™ DNA are synthetic dsDNA provided by Integrated DNA Technologies (IDT)

^bTOPO® plasmid from Thermo-Fischer

REFERENCES

- [1] Gogarten, J.P., Doolittle, W.F., Lawrence, J.G., 2002. Prokaryotic Evolution in Light of Gene Transfer. *Molecular Biology and Evolution* 19(12): 2226–38, Doi: 10.1093/oxfordjournals.molbev.a004046.
- [2] Soucy, S.M., Huang, J., Gogarten, J.P., 2015. Horizontal gene transfer: Building the web of life. *Nature Reviews Genetics*: 472–82, Doi: 10.1038/nrg3962.
- [3] Fontaine, L., Wahl, A., Fléchar, M., Mignolet, J., Hols, P., 2015. Regulation of competence for natural transformation in streptococci. *Infection, Genetics and Evolution* 33: 343–60, Doi: 10.1016/j.meegid.2014.09.010.
- [4] Zaccaria, E., Wells, J.M., Baarlen, P. Van., 2016. Metabolic Context of the Competence-Induced Checkpoint for Cell Replication in *Streptococcus suis*. *PLOS One* 11(5): 1–13, Doi: 10.1371/journal.pone.0153571.
- [5] Moradigaravand, D., Engelstädter, J., 2013. The evolution of natural competence: Disentangling costs and benefits of sex in bacteria. *American Naturalist* 182(4), Doi: 10.1086/671909.
- [6] Eldar, A., Elowitz, M.B., 2010. Functional roles for noise in genetic circuits. *Nature*: 167–73, Doi: 10.1038/nature09326.
- [7] Süel, G.M., Garcia-Ojalvo, J., Liberman, L.M., Elowitz, M.B., 2006. An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440(7083): 545–50, Doi: 10.1038/nature04588.
- [8] Leisner, M., Stingl, K., Frey, E., Maier, B., 2008. Stochastic switching to competence. *Current Opinion in Microbiology*: 553–9, Doi: 10.1016/j.mib.2008.09.020.
- [9] Leisner, M., Kuhr, J.T., Rädler, J.O., Frey, E., Maier, B., 2009. Kinetics of genetic switching into the state of bacterial competence. *Biophysical Journal* 96(3): 1178–88, Doi: 10.1016/j.bpj.2008.10.034.
- [10] Lemme, A., Gröbe, L., Reck, M., Tomasch, J., Wagner-Döbler, I., 2011. Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced *Streptococcus mutans*. *Journal of Bacteriology* 193(8): 1863–77, Doi: 10.1128/JB.01363-10.
- [11] Son, M., Ahn, S.-J., Guo, Q., Burne, R.A., Hagen, S.J., 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of comX. *Molecular Microbiology* 86(2): 258–72, Doi: 10.1111/j.1365-2958.2012.08187.x.
- [12] Hagen, S.J., Son, M., 2017. Origins of heterogeneity in *Streptococcus mutans* competence: interpreting an environment-sensitive signaling pathway. *Physical Biology* 14(1): 015001,

Doi: 10.1088/1478-3975/aa546c.

- [13] Son, M., Shields, R.C., Ahn, S.-J., Burne, R.A., Hagen, S.J., 2015. Bidirectional signaling in the competence regulatory pathway of *Streptococcus mutans*. *FEMS Microbiology Letters* 362, Doi: 10.1093/femsle/fnv159.
- [14] Reck, M., Tomasch, J., Wagner-Döbler, I., 2015. The Alternative Sigma Factor SigX Controls Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in *Streptococcus mutans*. *PLoS Genetics* 11(7), Doi: 10.1371/journal.pgen.1005353.
- [15] Moreno-Gómez, S., Sorg, R.A., Domenech, A., Kjos, M., Weissing, F.J., van Doorn, G.S., et al., 2017. Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. *Nature Communications* 8(1): 854, Doi: 10.1038/s41467-017-00903-y.
- [16] Fontaine, L., Boutry, C., Henry De Frahan, M., Delplace, B., Fremaux, C., Horvath, P., et al., 2010. A Novel Pheromone Quorum-Sensing System Controls the Development of Natural Competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. *JOURNAL OF BACTERIOLOGY* 192(5): 1444–54, Doi: 10.1128/JB.01251-09.
- [17] Gardan, R., Besset, C., Gitton, C., Guillot, A., Fontaine, L., Hols, P., et al., 2013. Extracellular life cycle of ComS, the competence-stimulating peptide of *Streptococcus thermophilus*. *Journal of Bacteriology* 195(8): 1845–55, Doi: 10.1128/JB.02196-12.
- [18] Fontaine, L., Goffin, P., Dubout, H., Delplace, B., Baulard, A., Lecat-Guillet, N., et al., 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. *Molecular Microbiology* 87(6): 1113–32, Doi: 10.1111/mmi.12157.
- [19] Talagas, A., Fontaine, L., Ledesma-García, L., Mignolet, J., Li de la Sierra-Gallay, I., Lazar, N., et al., 2017. Structural Insights into Streptococcal Competence Regulation by the Cell-to-Cell Communication System ComRS. *Annual Reviews of Genetics* 12(12): e1005980, Doi: 10.1371/journal.ppat.1005980.
- [20] Lingeswaran, A., Metton, C., Henry, C., Monnet, V., Juillard, V., Gardan, R., 2020. Export of Rgg Quorum Sensing Peptides is Mediated by the PptAB ABC Transporter in *Streptococcus Thermophilus* Strain LMD-9. *Genes* 11(9): 1096, Doi: 10.3390/genes11091096.
- [21] Ledesma-Garcia, L., Thuillier, J., Guzman-Espinola, A., Ensink, I., de la Sierra-Gallay, I.L., Lazar, N., et al., 2020. Molecular dissection of pheromone selectivity in the competence signaling system ComRS of streptococci. *Proceedings of the National Academy of Sciences of the United States of America* 117(14): 7745–54, Doi: 10.1073/pnas.1916085117.
- [22] Mignolet, J., Fontaine, L., Sass, A., Nannan, C., Mahillon, J., Coenye, T., et al., 2018. Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*. *Cell Reports* 22(7): 1627–38, Doi: 10.1016/j.celrep.2018.01.055.
- [23] Haustenne, L., Bastin, G., Hols, P., Fontaine, L., 2015. Modeling of the ComRS signaling pathway reveals the limiting factors controlling competence in *Streptococcus thermophilus*. *Frontiers in Microbiology* 6(DEC), Doi: 10.3389/fmicb.2015.01413.

-
- [24] Ozbudak, E.M., Thattai, M., Lim, H.H., Shraiman, B.I., Van Oudenaarden, A., 2004. Multistability in the lactose utilization network of *Escherichia coli*. *Nature* 427(6976): 737–40, Doi: 10.1038/nature02298.
- [25] Maamar, H., Dubnau, D., 2005. Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. *Molecular Microbiology* 56(3): 615–24, Doi: 10.1111/j.1365-2958.2005.04592.x.
- [26] Shanker, E., Federle, M.J., 2017. Quorum sensing regulation of competence and bacteriocins in *Streptococcus pneumoniae* and mutants. *Genes*, Doi: 10.3390/genes8010015.
- [27] Senadheera, M.D., Guggenheim, B., Spatafora, G.A., Huang, Y.C.C., Choi, J., Hung, D.C.I., et al., 2005. A VicRK signal transduction system in *Streptococcus mutans* affects gtfBCD, gbpB, and ftf expression, biofilm formation, and genetic competence development. *Journal of Bacteriology* 187(12): 4064–76, Doi: 10.1128/JB.187.12.4064-4076.2005.
- [28] Mattos-Graner, R.O., Duncan, M.J., 2017. Two-component signal transduction systems in oral bacteria. *Journal of Oral Microbiology* 9(1): 1400858, Doi: 10.1080/20002297.2017.1400858.
- [29] Finn, M.B., Ramsey, K.M., Dove, S.L., Wessels, M.R., 2021. Identification of Group A *Streptococcus* Genes Directly Regulated by CsrRS and Novel Intermediate Regulators. *MBio*, Doi: 10.1128/mbio.01642-21.
- [30] Mazzuoli, M.V., Daunesse, M., Varet, H., Rosinski-Chupin, I., Legendre, R., Sismeiro, O., et al., 2021. The CovR regulatory network drives the evolution of Group B *Streptococcus* virulence. *PLoS Genetics* 17(9): e1009761, Doi: 10.1371/journal.pgen.1009761.
- [31] Gryllos, I., Levin, J.C., Wessels, M.R., 2003. The CsrR/CsrS two-component system of group A *Streptococcus* responds to environmental Mg²⁺. *Proceedings of the National Academy of Sciences of the United States of America* 100(7): 4227–32, Doi: 10.1073/pnas.0636231100.
- [32] Gryllos, I., Tran-Winkler, H.J., Cheng, M.F., Chung, H., Bolcome, R., Lu, W., et al., 2008. Induction of group A *Streptococcus* virulence by a human antimicrobial peptide. *Proceedings of the National Academy of Sciences of the United States of America* 105(43): 16755–60, Doi: 10.1073/pnas.0803815105.
- [33] Dalton, T.L., Scott, J.R., 2004. CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. *Journal of Bacteriology* 186(12): 3928–37, Doi: 10.1128/JB.186.12.3928-3937.2004.
- [34] Horstmann, N., Sahasrabhojane, P., Saldaña, M., Ajami, N.J., Flores, A.R., Sumby, P., et al., 2015. Characterization of the Effect of the Histidine Kinase CovS on Response Regulator Phosphorylation in Group A *Streptococcus*. *Infection and Immunity* 83(3): 1068, Doi: 10.1128/IAI.02659-14.

- [35] Gusa, A.A., Gao, J., Stringer, V., Churchward, G., Scott, J.R., 2006. Phosphorylation of the group A Streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. *Journal of Bacteriology* 188(13): 4620–6, Doi: 10.1128/JB.00198-06.
- [36] Federle, M.J., Scott, J.R., 2002. Identification of binding sites for the group A streptococcal global regulator CovR. *Molecular Microbiology* 43(5): 1161–72.
- [37] Gusa, A.A., Gao, J., Stringer, V., Churchward, G., Scott, J.R., 2006. Phosphorylation of the group A streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. *Journal of Bacteriology* 188(13): 4620–6, Doi: 10.1128/JB.00198-06.
- [38] Gusa, A.A., Scott, J.R., 2005. The CovR response regulator of group A streptococcus (GAS) acts directly to repress its own promoter. *Molecular Microbiology* 56(5): 1195–207, Doi: 10.1111/j.1365-2958.2005.04623.x.
- [39] Lembo, A., Gurney, M.A., Burnside, K., Banerjee, A., De Los Reyes, M., Connelly, J.E., et al., 2010. Regulation of CovR expression in Group B Streptococcus impacts blood-brain barrier penetration. *Molecular Microbiology* 77(2): 431–43, Doi: 10.1111/j.1365-2958.2010.07215.x.
- [40] Zotta, T., Asterinou, K., Rossano, R., Ricciardi, A., Varcamonti, M., Parente, E., 2009. Effect of inactivation of stress response regulators on the growth and survival of *Streptococcus thermophilus* Sfi39. *International Journal of Food Microbiology* 129(3): 211–20, Doi: 10.1016/j.ijfoodmicro.2008.11.024.
- [41] Fontaine, L., Dandoy, D., Boutry, C., Delplace, B., de Frahan, M.H., Fremaux, C., et al., 2010. Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in *Streptococcus thermophilus*. *Applied and Environmental Microbiology* 76(23): 7870–7, Doi: 10.1128/AEM.01671-10.
- [42] Liu, X., Gallay, C., Kjos, M., Domenech, A., Slager, J., van Kessel, S.P., et al., 2017. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Molecular Systems Biology* 13(5): 931, Doi: 10.15252/msb.20167449.
- [43] Chang, J.C., Federle, M.J., 2016. PptAB exports Rgg quorum-sensing peptides in *Streptococcus*. *PLoS ONE* 11(12), Doi: 10.1371/journal.pone.0168461.
- [44] Underhill, S.A.M., Shields, R.C., Kaspar, J.R., Haider, M., Burne, R.A., Hagen, S.J., 2018. Intracellular Signaling by the comRS System in *Streptococcus mutans* Genetic Competence. *MSphere* 3(5): e00444-18, Doi: 10.1128/mSphere.00444-18.
- [45] Underhill, S.A.M., Shields, R.C., Burne, R.A., Hagen, S.J., 2019. Carbohydrate and PepO control bimodality in competence development by *Streptococcus mutans*. *Molecular Microbiology* 112(5): 1388–402, Doi: 10.1111/mmi.14367.
- [46] Wilkening, R. V., Chang, J.C., Federle, M.J., 2016. PepO, a CovRS-controlled endopeptidase, disrupts *Streptococcus pyogenes* quorum sensing. *Molecular Microbiology* 99(1): 71–87, Doi: 10.1111/mmi.13216.

-
- [47] Dmitriev, A., Mohapatra, S.S., Chong, P., Neely, M., Biswas, S., Biswas, I., 2011. CovR-controlled global regulation of gene expression in streptococcus mutans. *PLoS ONE* 6(5), Doi: 10.1371/journal.pone.0020127.
- [48] Graham, M.R., Smoot, L.M., Migliaccio, C.A.L., Virtaneva, K., Sturdevant, D.E., Porcella, S.F., et al., 2002. Virulence control in group A *Streptococcus* by a two-component gene regulatory system: Global expression profiling and in vivo infection modeling. *Proceedings of the National Academy of Sciences of the United States of America* 99(21): 13855–60, Doi: 10.1073/pnas.202353699.
- [49] Horstmann, N., Saldaña, M., Sahasrabhojane, P., Yao, H., Su, X., Thompson, E., et al., 2014. Dual-Site Phosphorylation of the Control of Virulence Regulator Impacts Group A Streptococcal Global Gene Expression and Pathogenesis. *PLoS Pathogens* 10(5), Doi: 10.1371/journal.ppat.1004088.
- [50] Jiang, S.M., Cieslewicz, M.J., Kasper, D.L., Wessels, M.R., 2005. Regulation of virulence by a two-component system in group B *Streptococcus*. *Journal of Bacteriology* 187(3): 1105–13, Doi: 10.1128/JB.187.3.1105-1113.2005.
- [51] Horstmann, N., Tran, C.N., Brumlow, C., DebRoy, S., Yao, H., Nogueras Gonzalez, G., et al., 2018. Phosphatase activity of the control of virulence sensor kinase CovS is critical for the pathogenesis of group A streptococcus. *PLoS Pathogens* 14(10), Doi: 10.1371/journal.ppat.1007354.
- [52] Jakobs, M., Meinhardt, F., 2014. What renders Bacilli genetically competent? A gaze beyond the model organism. *Applied Microbiology and Biotechnology* 99(4): 1557–70, Doi: 10.1007/s00253-014-6316-0.
- [53] Horstmann, N., Sahasrabhojane, P., Yao, H., Su, X., Shelburne, S.A., 2017. Use of a Phosphorylation Site Mutant To Identify Distinct Modes of Gene Repression by the Control of Virulence Regulator (CovR) in *Streptococcus pyogenes*. *Journal of Bacteriology* 199(18), Doi: 10.1128/JB.00835-16.
- [54] Churchward, G., 2007. The two faces of Janus: virulence gene regulation by CovR/S in group A streptococci. *Molecular Microbiology* 64(1): 34–41, Doi: 10.1111/j.1365-2958.2007.05649.x.
- [55] Horstmann, N., Tran, C.N., Brumlow, C., DebRoy, S., Yao, H., Nogueras Gonzalez, G., et al., 2018. Phosphatase activity of the control of virulence sensor kinase CovS is critical for the pathogenesis of group A streptococcus. *PLoS Pathogens* 14(10): e1007354, Doi: 10.1371/journal.ppat.1007354.
- [56] Chiang-Ni, C., Chiou, H.-J., Tseng, H.-C., Hsu, C.-Y., Chiu, C.-H., 2020. RocA Regulates Phosphatase Activity of Virulence Sensor CovS of Group A *Streptococcus* in Growth Phase- and pH-Dependent Manners. *MSphere* 5(3), Doi: 10.1128/msphere.00361-20.
- [57] Biswas, I., Scott, J.R., 2003. Identification of rocA, a positive regulator of covR expression in the group A streptococcus. *Journal of Bacteriology* 185(10): 3081–90, Doi: 10.1128/JB.185.10.3081-3090.2003.

- [58] Steiner, K., Malke, H., 2001. *relA*-independent amino acid starvation response network of *Streptococcus pyogenes*. *Journal of Bacteriology*, vol. 183. J Bacteriol p. 7354–64.
- [59] Dalton, T.L., Collins, J.T., Barnett, T.C., Scott, J.R., 2006. RscA, a member of the MDR1 family of transporters, is repressed by CovR and required for growth of *Streptococcus pyogenes* under heat stress. *Journal of Bacteriology* 188(1): 77–85, Doi: 10.1128/JB.188.1.77-85.2006.
- [60] Gryllos, I., Tran-Winkler, H.J., Cheng, M.-F., Chung, H., Bolcome, R., Lu, W., et al., 2008. Induction of group A *Streptococcus* virulence by a human antimicrobial peptide. *Proceedings of the National Academy of Sciences of the United States of America* 105(43): 16755–60, Doi: 10.1073/pnas.0803815105.
- [61] Froehlich, B.J., Bates, C., Scott, J.R., 2009. *Streptococcus pyogenes* CovRS mediates growth in iron starvation and in the presence of the human cationic antimicrobial peptide LL-37. *Journal of Bacteriology* 191(2): 673–7, Doi: 10.1128/JB.01256-08.
- [62] Park, S.E., Jiang, S., Wessels, M.R., 2012. CsrRS and environmental pH regulate group B *Streptococcus* adherence to human epithelial cells and extracellular matrix. *Infection and Immunity* 80(11): 3975–84, Doi: 10.1128/IAI.00699-12.
- [63] Di Palo, B., Rippa, V., Santi, I., Brettoni, C., Muzzi, A., Metruccio, M.M.E., et al., 2013. Adaptive Response of Group B *Streptococcus* to High Glucose Conditions: New Insights on the CovRS Regulation Network. *PLoS ONE* 8(4), Doi: 10.1371/journal.pone.0061294.
- [64] Mignolet, J., Fontaine, L., Kleerebezem, M., Hols, P., 2016. Complete genome sequence of *Streptococcus salivarius* HSISS4, a human commensal bacterium highly prevalent in the digestive tract. *Genome Announcements* 4(1), Doi: 10.1128/genomeA.01637-15.
- [65] Letort, C., Juillard, V., 2001. Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. *Journal of Applied Microbiology* 91(6): 1023–9, Doi: 10.1046/j.1365-2672.2001.01469.x.
- [66] Dower, W.J., Miller, J.F., Ragsdale, C.W., 1988. High efficiency transformation of *E.coli* by high voltage electroporation. *Nucleic Acids Research* 16(13): 6127–45, Doi: 10.1093/nar/16.13.6127.
- [67] Ducret, A., Quardokus, E.M., Brun, Y. V., 2016. MicrobeJ, a tool for high throughput bacterial cell detection and quantitative analysis. *Nature Microbiology* 1(7), Doi: 10.1038/nmicrobiol.2016.77.
- [68] Thevenard, B., Rasoava, N., Fourcassié, P., Monnet, V., Boyaval, P., Rul, F., 2011. Characterization of *Streptococcus thermophilus* two-component systems: In silico analysis, functional analysis and expression of response regulator genes in pure or mixed culture with its yogurt partner, *Lactobacillus delbrueckii* subsp. *bulgaricus*. *International Journal of Food Microbiology* 151(2): 171–81, Doi: 10.1016/j.ijfoodmicro.2011.08.019.

-
- [69] Throup, J.P., Koretke, K.K., Bryant, A.P., Ingraham, K.A., Chalker, A.F., Yigong, G., et al., 2000. A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. *Molecular Microbiology* 35(3): 566–76, Doi: 10.1046/j.1365-2958.2000.01725.x.
- [70] Buckley, S.J., Timms, P., Davies, M.R., McMillan, D.J., 2018. In silico characterisation of the two-component system regulators of *Streptococcus pyogenes*. *PLoS ONE* 13(6), Doi: 10.1371/journal.pone.0199163.
- [71] Lévesque, C.M., Mair, R.W., Perry, J.A., Lau, P.C.Y., Li, Y.H., Cvitkovitch, D.G., 2007. Systemic inactivation and phenotypic characterization of two-component systems in expression of *Streptococcus mutans* virulence properties. *Letters in Applied Microbiology* 45(4): 398–404, Doi: 10.1111/j.1472-765X.2007.02203.x.
- [72] Van Den Bogert, B., Boekhorst, J., Herrmann, R., Smid, E.J., Zoetendal, E.G., Kleerebezem, M., 2013. Comparative genomics analysis of *Streptococcus* isolates from the human small intestine reveals their adaptation to a highly dynamic ecosystem. *PLoS ONE* 8(12): 83418, Doi: 10.1371/journal.pone.0083418.
- [73] Mignolet, J., Cerckel, G., Damoczi, J., Ledesma-Garcia, L., Sass, A., Coenye, T., et al., 2019. Subtle selectivity in a pheromone sensor triumvirate desynchronizes competence and predation in a human gut commensal. *eLife* 8, Doi: 10.7554/eLife.47139.
- [74] Fontaine, L., Hols, P., 2008. The inhibitory spectrum of thermophilin 9 from *Streptococcus thermophilus* LMD-9 depends on the production of multiple peptides and the activity of BlpGSt, a thiol-disulfide oxidase. *Applied and Environmental Microbiology* 74(4): 1102–10, Doi: 10.1128/AEM.02030-07.

CHAPTER II

Competence shut-off by intracellular pheromone degradation in *Streptococcus* *salivarius*

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AK and PH conceived and designed the study. The laboratory work was carried out by AK (Fig. 2.1-2.7, S2.1-2.10), AW (Fig. 2.4A), ML (pre-data), BD (Fig. 2.2BC, 2.4), HD (Fig 2.4B), and LLG (Fig S2.7). AK designed and interpreted the mathematical model simulations. AK, LLG, FD and PH analyzed and interpreted the data. AK, LLG, PM, FD and PH wrote or revised the manuscript.

After deciphering the molecular mechanisms governing the activation of competence in *Streptococcus salivarius*, we sought to investigate the deactivation of the ComRS system. This process, known as competence shut-off, is crucial to restrict competence to a limited window of time. Indeed, since competence is a particular state that switches dramatically cell physiology, its extended activation is toxic.

Previous work in *Streptococcus thermophilus* had suggested the presence of an unknown shut-off player under the control of ComX. This assumption was confirmed experimentally by showing that *comX* deletion resulted in longer and higher competence activation. Nevertheless, this hypothesis was never tested in *S. salivarius* before this thesis.

To get insights into the ComRS regulatory pathway, we started by building a mathematical model based on previous work done in *S. thermophilus*. While calibrating the model, we realized that low ComS activity was necessary to fit the data generated during the bimodality investigations. Combining this suggestion with the previous data of *S. thermophilus*, we assumed the existence of a ComS antagonist player under the control of ComX. Since several peptidases controlling Quorum-Sensing pheromones have been described lately, we hypothesized the presence of a peptidase controlled by ComX that was responsible for the shut-off.

We identified *in silico* four different peptidases in the ComX regulon that was previously determined. Among those, none of them showed an increase in competence activation and one of them, PepF, seemed to be essential since we were unable to knock it out. To overcome this issue, we used a conditional mutant system based on the CRISPR-interference technology. We were then able to show that *pepF* inhibition results in longer and more intense competence activation.

We next performed a biochemical characterization of PepF activity on the pheromone XIP and showed that the 4th and 5th residues of the 8-aa peptide are crucial for PepF-mediated degradation. Finally, we screened and combined key residue mutations in the XIP sequence allowing shut-off escape and resulting in longer competence activation.

Finally, this study helps us to understand a critical regulatory feature of competence in the *Salivarius* group and showcases the importance of peptidases in bacterial QS systems.

ABSTRACT

Competence is a major bacterial adaptive and survival strategy. Yet successful, this strategy is energy-consuming and shifts the metabolism to such an extent that regular cell cycle is impaired. In streptococci, complex regulatory pathways control competence to narrow its activation to a sharp window of time, a process known as competence shut-off. Although characterized in competent streptococci activated by the ComCDE signaling pathway, it remains unclear for those controlled by the ComRS system. In this work, we investigate competence shut-off in the major human gut commensal *Streptococcus salivarius*. Using a deterministic mathematical model of the ComRS system, we predicted a negative player under the control of the central regulator ComX as involved in ComS/XIP pheromone degradation through a negative feedback loop. Systematic inactivation of peptidases belonging to the ComX regulon identified PepF as an essential oligoendopeptidase in *S. salivarius*. Combining conditional mutants, transcriptional analyses, and biochemical characterization of pheromone cleavage sites, we validated the reciprocal role of PepF and XIP in ComRS shut-off. Notably, engineering cleavage site residues generated ultra-resistant peptides producing high and long-lasting competence activation. Altogether, this study reveals and describes a proteolytic shut-off mechanism of competence in the salivarius group, and suggests that this mechanism could be shared by other ComRS streptococcal species.

The human microbiota that includes *Streptococcus salivarius* is one of the most competitive and challenging microbial ecosystems. Limited resources, physico-chemical inconstancy and bacterial high density have fostered the emergence of powerful survival strategies [1–3]. Sophisticated regulation networks ensure a tight control over those tactics, converting miscellaneous environmental inputs to population-wide synchronized outcome [4–6]. Orchestrating coordinated bacteriocin production and natural transformation, competence in streptococci is a glaring illustration of such ploy. While the production of antimicrobial compounds eliminates competitors, rival cell lysis guarantees genomic material availability for subsequent DNA uptake [7–9].

In streptococci, competence activation relies on two distinct pheromone-dependent mechanisms. In both cases, initiation occurs through the production of a genome-encoded small peptide, acting as a pheromone after secretion and maturation [10]. In two streptococcal groups (*i.e.* mitis and anginosus), the signaling peptide ComC sparks a phosphorelay after binding to its cognate histidine kinase ComD, ultimately stimulating dimerization of the transcriptional factor ComE and its transcriptional activity [11,12]. On the other hand, all other streptococci groups (*i.e.* mutans, bovis, pyogenes and salivarius) operate with a differing pattern [10]. After secretion through the PptAB ABC transporter [13], the pheromone precursor ComS is matured to form XIP (ComX-Inducing-Peptide) and internalized by the generic oligopeptide transporter Opp [14,15]. Subsequent binding to the ComR intracellular receptor triggers dimerization of the complex (ComR-XIP), leading to the establishment of a positive feedback loop via *comS* transcriptional activation [16]. Concomitantly, ComR-XIP will trigger the production of the alternative sigma factor ComX which will guide RNA polymerase to promoters of late competence genes responsible for the transformosome assembly [10].

The last two decades, genomic massive data accumulation together with multi-species investigations reshaped our perception of competence as a major driving force in adaptation and evolution of streptococci [17–21]. Even though competence long-term benefits for bacteria are undeniable, the process is costly and reshuffle the metabolism to such an extent that regular cell cycle is transitory impaired [22,23]. To curtail this dramatic impact on the survival chance of the population, streptococci exploited complex regulatory networks to restrict competence activation to a subpopulation. This tactic known as bimodality (bistability) allows only a subset of the community to play the dangerous but yet promising game of competence [4,24]. An auxiliary strategy aims to narrow the activation time window of this peculiar physiological state to restore routine cell cycle once DNA has been

internalized and integrated. This mechanism termed competence shut-off, has been thoroughly described in *Streptococcus pneumoniae*, where at least two distinct mechanisms were highlighted. The first one involves the late competence protein DprA, the RecA loader involved in single stranded DNA recombination during transformation [25]. Strikingly, this protein was shown to have a supplemental function in competence shut-off by interfering with ComE~P, thereby interrupting the positive feedback loop acting on the *comCDE* operon [26,27]. Moreover, this mechanism was shown to require ComX to spatially coordinate the interaction between DprA and ComE~P at the cell poles [28]. Another mechanism was suggested by Martin and co-workers, showing that ComE accumulation due to the activation of the *comCDE* operon would compete with ComE~P for P_{comCDE} binding, ultimately leading to competence shut-off [29].

In ComRS-streptococci, a number of competence negative players have been depicted. Among them, the endopeptidase PepO was shown in *Streptococcus mutans* to control XIP abundance. However, since *pepO* transcription is not stimulated through competence, the endopeptidase would rather stand as a locking device than a proper shut-off player [30]. Two other antagonist mechanisms controlled by competence and thereby acting through a negative feedback loop were described in streptococci. The small protein paratox (Prx) from a prophage of *Streptococcus pyogenes* was shown to be controlled by ComX and to prevent ComR-XIP association [31,32]. An analogous model in *S. mutans* involves a peptide termed XrpA that interferes with ComR dimerization [33]. Surprisingly, this peptide is encoded within the *comX* coding sequence and therefore is upregulated along with competence activation [34]. Although those two ComRS antagonists are rooted in a negative feedback loop, their inactivation was not shown to extend the competence time window [31,35], suggesting they do not stand as true shut-off effectors.

How competence shutdown takes place in the salivarius group is still a pending question. While previous work had ruled out shut-off implication of DprA in *Streptococcus thermophilus* [36], no homologs of Prx and no gene embedded in *comX* could be identified, covering tracks for a putative streptococcal shared inhibitor. In the present study, we questioned ComRS topology by developing a deterministic mathematical model describing competence in *S. salivarius*. Confronting model predictions with experimental data suggested the presence of a pheromone inhibitor under the control of ComX, which was experimentally identified as PepF, a widely-distributed oligoendopeptidase in streptococci. We confirmed its role in competence shut-off through XIP pheromone degradation and pinpointed key peptide residues involved in its specific processing. Those results outline

the benefits of combining computational and experimental methodologies to decipher the first proper shut-off effector in ComRS streptococci.

RESULTS

Mathematical model suggests pheromone degradation

Previous investigations in *S. salivarius* HSISS4 [37] have highlighted the central role of ComR abundance in competence activation [7,36]. Strikingly, while pheromone genetic overexpression could not trigger *comRS* transcriptional activation, synthetic pheromone (sXIP) addition generated a strong and unimodal response in the population [24]. To puzzle out this intriguing observation, we investigated ComRS topology by designing a deterministic model based on previous *in silico* modeling of *S. thermophilus* and *S. mutans* [36,38]. While calibrating the model based on single-cell and luciferase data (see Appendix 1 for details), we realized that low pheromone activity was essential for the model. Since high XIP-ComR affinity and high *comS* expression have been experimentally established [7,16,39], we hypothesized the presence of a negative player controlling ComS/XIP abundance to explain low pheromone activity. We introduced this unknown inhibitor in our model (named *deg*), adjusted the free parameters and compared the evolution of the cellular concentration of ComR, ComX and ComS with or without the *deg* variable (Fig. 2.1A and B). In both cases, we simulated the overexpression of ComR to high levels to trigger competence (up to 7000 molecules per cells, according to a western-blot semi quantification previously performed [24]). As expected, ComR intracellular concentration increases until a sharp drop corresponding to its entry into the ComR-XIP complex. Concomitantly, ComX concentration starts to increase in both cases at 200 minutes to reach about 150 molecules per cells, which corresponds to the predicted number of ComX box in the HSISS4 genome [7]. On the other hand, free ComS/XIP reaches high concentration (~6000 molecules/cells) when simulated with the *deg* player, while only rising to 5 molecules/cells in its absence. This counter-intuitive result is explained by the nature of the *deg* player simulated. Indeed, we modeled a ComS/XIP degradation machinery quickly saturated (Fig. 2.1A and Fig. 2.1B 4th panel) which avoids spontaneous competence activation but allows high ComS production once overwhelmed. This resulted in a theoretical maximum free XIP concentration of 6 nM produced during competence (Fig. 2.1C), which exceeds the previous experimentally determined sXIP threshold concentration (800 pM) required for activation [24].

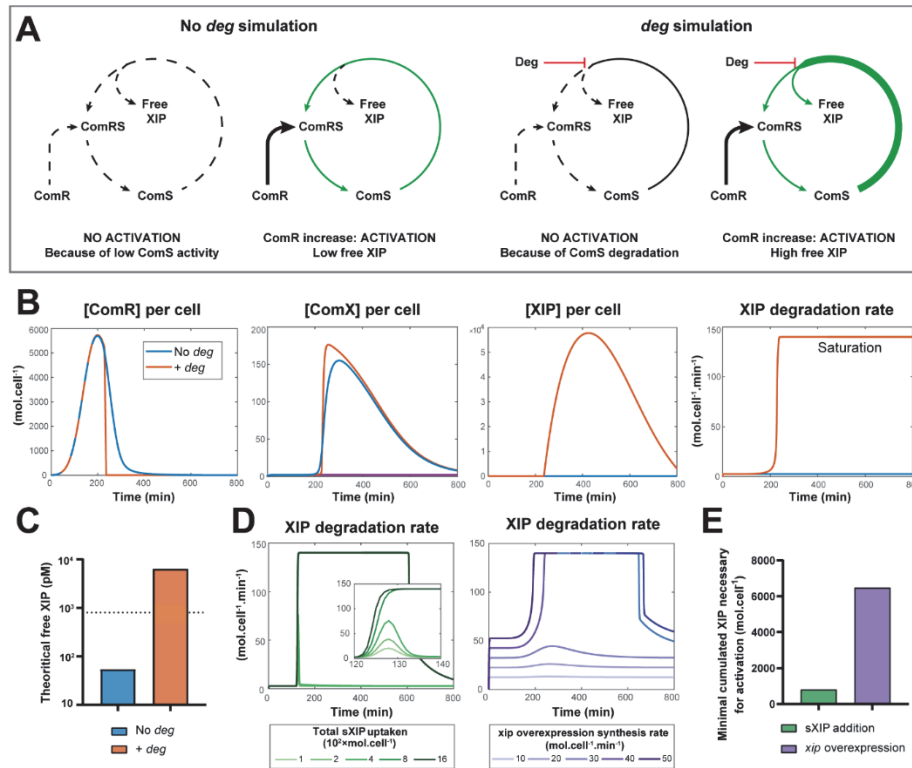


Figure 2.1. ComRS modelling suggests a peptidase activity. **A.** Cartoons depicting the two types of simulations involving or not the *deg* player. **B.** Simulated ComR, ComX and ComS intracellular concentrations together with ComS degradation rate over time. Two different simulations output are depicted, one considering a degradation machinery (*deg*, blue) of ComS being fastly saturated and one without the *deg* player (No *deg*, orange). In both cases, competence was induced via increasing by a 8.5-fold ComR concentration, mimicking its genetic overexpression. **C.** Theoretical maximum free ComS (not included in the ComR-XIP complex) are computed for both simulations shown in A. Dotted line denotes the minimum sXIP extracellular concentration experimentally determined to trigger competence in *S. salivarius* [24]. **D.** ComS degradation rate over time computed for the model including the *deg* variable in two types of competence inducing processes. First panel (green) simulates the extracellular addition of various sXIP concentration and second panel (purple) simulates the genetic overexpression of *xip* at various levels. **E.** Minimal quantity of XIP molecules to reach the threshold to activate competence in both scenarios modeled in D.

Moreover, the introduction of the *deg* player generated a system only reactive when higher ComR levels are provided and was able to fit previous ComR-overexpression single-cell data [24] (Fig. S2.1).

We next investigated the initial question regarding the differential sensitivity of the system to synthetic supplemented sXIP vs the genetically-encoded pheromone overexpression. To this aim, we simulated two different activations both including the *deg* player. In the first scenario, we simulated

at $t = 120$ min a high concentration of sXIP internalized within 15 minutes by the cells. In a second scenario, we mimicked the *xip* overexpression by a constant production rate of XIP. We next ran those two simulations multiple times with a gradual increase of both XIP production parameters. For both induction systems, competence is only activated once the *deg* player is saturated (Fig. 2.1D). Notably, the sXIP addition simulation requires less XIP to trigger the system than the *xip* overexpression simulation (Fig. 2.1E), presumably because a sharp increase in XIP concentration saturates the peptidase faster than a progressive production by the genetically-encoded peptide.

Altogether, those *in silico* results suggest the existence of a quickly saturated negative player limiting the pheromone abundance.

PepF negatively impacts competence activation

Since our model suggested the presence of a negative effector lowering pheromone activity, we aimed to search for such player. Because recent studies showed post-translational pheromone control through peptidase activity in other streptococci [30,40] and a previous model advocated for the presence of a shut-off player under ComX control [36], we searched within the ComX regulon for putative peptidases. Using previous transcriptional data on ComX regulon [7] together with the MEROPS database [41], we found 4 ComX-regulated peptidases : *pepO*, *pepP*, *pepQ*, and *pepF*. We succeeded to knock-out the first 3 peptidases that showed no impact on competence activation (Fig. S2.2), but were unsuccessful to mutate *pepF* (*HSISS4_00369*), encoding a Zinc oligoendopeptidase from family M3 (MEROPS identifier M003.007). Testing the involvement of *pepF* in competence shut-off the other way around, we overexpressed the peptidase gene with a strong constitutive promoter ($P_{32-pepF}$) and monitored P_{comX} activation thanks to luciferase activity ($P_{comX-luxAB}$). Competence was triggered either with a xylose-inducible promoter fused to *comR* ($P_{xyl2-comR}$) or with sXIP addition (Fig. 2.2A). For both competence-inducing conditions, we observed a decrease of P_{comX} activation in the *pepF* overexpressing strain, supporting previous theoretical assumptions.

As *pepF* overexpression inactivates competence, we expected *PepF* inhibition to result in higher competence activation. Since we were unsuccessful to knock out the gene, we designed a CRISPR-interference (CRISPRi) guide targeting the upstream region of *pepF*. Using the same reporter ($P_{comX-luxAB}$) and competence-inducer ($P_{xyl2-comR}$) systems as reported above, we monitored P_{comX} activation over time at different xylose concentrations (Fig. 2.2B, C, and D).

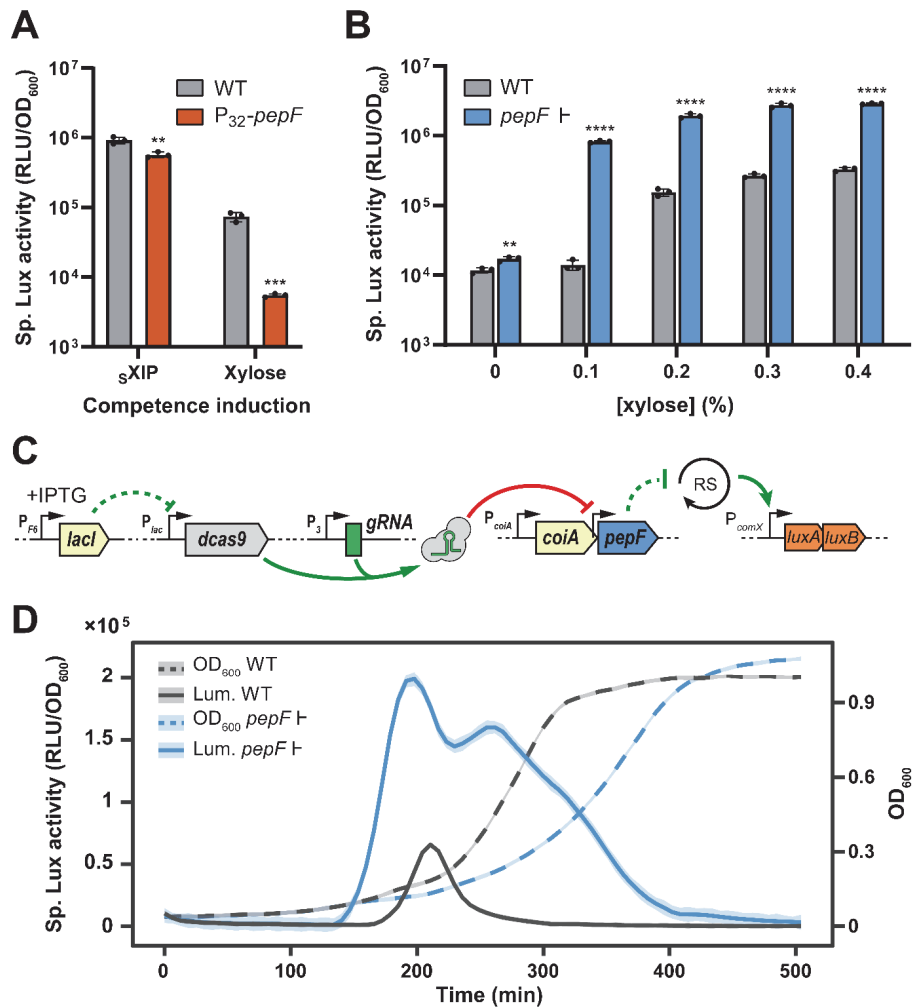


Figure 2.2. PepF down-regulates competence and constrains its activation to a limited time window. **A.** Effect of *pepF* overexpression on P_{comX} activity. Data show specific luciferase activity (RLU/OD₆₀₀) measured for WT (with P_{comX}-*luxAB* as proxy) compared to a strain carrying a constitutive overexpression of *pepF* (P₃₂-*pepF*). Competence was activated thanks to synthetic XIP addition (sXIP, 50 nM) or *comR* overexpression via a xylose-inducible promoter fused to *comR* (P_{xyI2}-*comR*, induced at 0.25% xylose). **B.** Effect of *pepF* inhibition on P_{comX} activity. Data show specific luciferase activity measured for WT (with P_{comX}-*luxAB* as proxy) compared to a strain expressing a guide targeting the promoter of *pepF* (P₃-*gRNA*₉). Both strains carry the IPTG-inducible dCas9 system (P_{F6}-*lacI* P_{lac}-*dcas9*) together with a xylose-inducible promoter fused to *comR* (P_{xyI2}-*comR*). Competence was activated at different xylose concentrations (0, 0.1, 0.2, 0.3 and 0.4%) and the dCas9 repression system was activated at 100 μM of IPTG. Dots show biological triplicates (*n* = 3), the bar shows the mean and error bar denotes standard deviation. Statistical *t*-test was performed for each condition in comparison to the related control (WT) (**, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001) **C.** Cartoon depicting the interference strategy used to repress *pepF* in B and D (RS: ComRS activation). **D** Growth

(OD₆₀₀) and kinetics of P_{comX} specific luciferase activity (Lum.) monitored over time with (*pepF* |-) or without *pepF* inhibition (WT). Data shown are the xylose 0.4% induction of panel B. Full lines and dashed lines are representative of the mean of biological triplicates, shaded lines represent standard deviation.

Conversely to *PepF* overproduction, we observed higher competence induction in the *PepF*-depleted condition for each tested xylose concentration (Fig. 2.2B). Those observations were confirmed by fine-tuning the level of *pepF* inhibition in the same strain with an IPTG gradient (P_{lac-dcas9} under IPTG control), which displayed a gradual increase in intensity and timing (Fig. S2.3). Notably, luciferase kinetics showed longer and earlier competence activation in the *pepF*-interfered condition, suggesting a dual role of the peptidase as a shut-off player and a as locking device preventing activation (Fig. 2.2D). This latter feature was confirmed by spontaneous natural transformation upon *PepF* inhibition (transformation rate $\sim 10^{-5}$). Altogether, these results underline the antagonist activity of *PepF* to limit both competence initiation and the timing of activation.

PepF transcription is competence-dependent

Since a true shut-off implies its inception when the system turns on, we examined *pepF* competence dependency. A glimpse on its genomic context showed that *pepF* is located downstream of *coiA* (Fig. 2.3A), a widely conserved late competence gene yet with unclear function [42]. Previous transcriptional data indicated upregulation of this bicistronic operon upon competence activation [7]. We confirmed those results using a P_{coiA}-*luxAB* luciferase reporter system inserted at an ectopic locus (Fig. 2.3B and C), showing P_{coiA} activation upon sXIP addition. To monitor more accurately *pepF* expression, we designed a new construct where we fused the whole *coiA* gene to the luciferase reporter genes (P_{coiA}-*coiA*-P_{pepF}-*luxAB*). As expected, we recorded an up-regulation upon competence activation via sXIP addition, presumably through P_{coiA} activation (Fig. 2.3B and C). However, in contrast to the direct P_{coiA} fusion, this construct displayed a higher basal luciferase activity. Suspecting an additional *pepF* promoter within the *coiA* gene, we built a direct reporter fusion with the 3' end of the *coiA* coding sequence (P_{pepF}-*luxAB*). Luciferase monitoring revealed a high basal transcriptional activity of this region, slightly influenced by competence activation (Fig. 2.3B and C). Together, these results suggest that *pepF* has a constitutive level of expression that is increased when competence is activated through the specific activation of P_{coiA}.

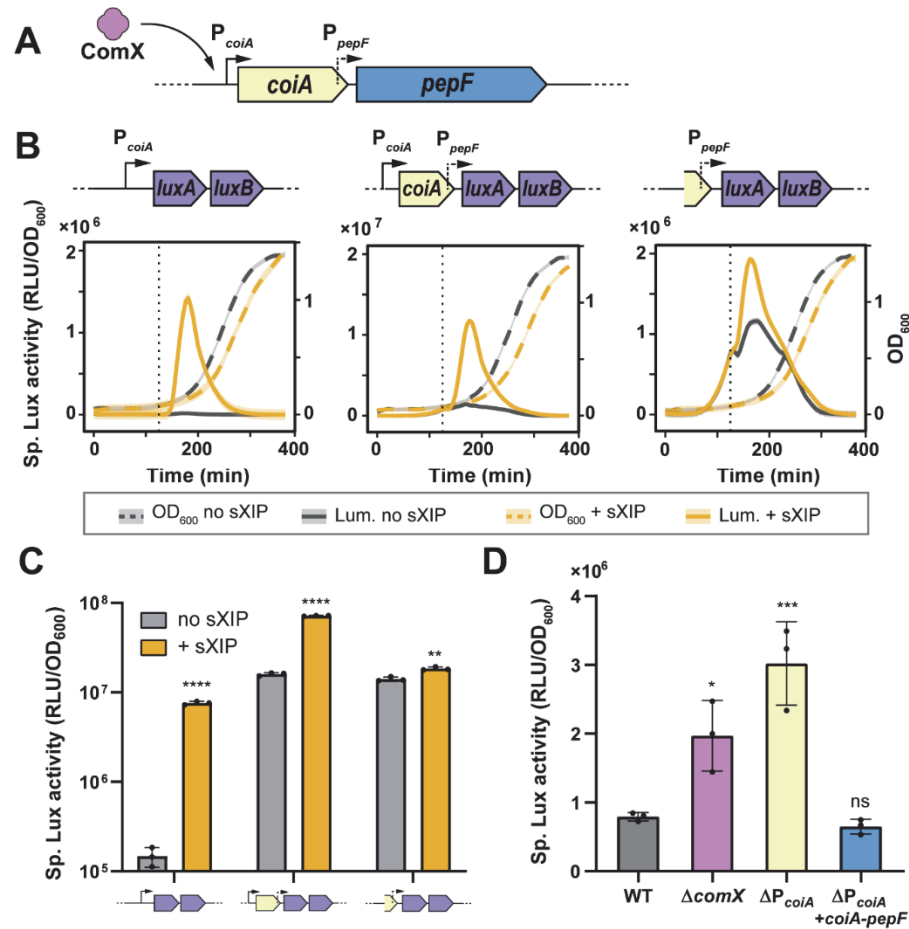


Figure 2.3. Transcriptional analysis of *pepF* and its ComX control. **A.** Cartoon depicting the organization of the *coiA-pepF* operon. ComX controls P_{coiA} and a constitutive promoter of *pepF* was found in the coding sequence of *coiA*. **B** and **C.** Kinetics (growth, OD₆₀₀ and Lux activity, Lum.) (**B**) and mean specific luciferase activity (RLU/OD₆₀₀) (**C**) reporting the transcription of the *coiA-pepF* operon upon competence activation. Lux activity was monitored with P_{coiA} -*luxAB*, P_{coiA} -*coiA*- P_{pepF} -*luxAB*, and P_{pepF} -*luxAB* luciferase reporter systems. The three constructs were incubated with (+ sXIP) or without (no sXIP) sXIP at a final concentration of 500 nM. **D.** Impact on competence activation of the ComX-control unplug on the expression of the *coiA-pepF* operon. Data show specific luciferase activity measured with a P_{comX} -*luxAB* reporter strain (WT) and derivatives with *comX* deletion ($\Delta comX$), P_{coiA} deletion (ΔP_{coiA}), and P_{coiA} deletion complemented with an ectopic copy of a P_{coiA} -*coiA-pepF* operon (ΔP_{coiA} + *coiA-pepF*). Competence was activated thanks to a xylose-inducible promoter fused to *comR* (P_{xyl2} -*comR*, induced at 1% xylose). In panel B, full lines and dashed lines are representative of the mean of biological triplicates, shaded lines depict standard deviation. In panels C and D, dots show biological triplicates (n=3), the bar shows the mean and error bar the standard deviation. Statistical *t*-tests (**C.**) or one-way ANOVA with Dunett's test (**D.**) were performed for each condition in comparison to the related mock to generate *P* values (*, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001; ****, *P* < 0.0001; ns, non-significative).

To confirm the role of PepF as a shut-off effector under ComX control, we unplugged *pepF* from competence activation through the inactivation of *comX* ($\Delta comX$) or the deletion of the promoter of *coiA* (ΔP_{coiA}). Triggering competence thanks to ComR overexpression and monitoring activation with a P_{comX} -*luxAB* fusion, we observed higher signals for the two ComX-unwired constructs (Fig. 2.3D). Supporting those results, a ΔP_{coiA} complementation with an ectopic insertion of the *coiA-pepF* operon ($\Delta P_{coiA} + coiA-pepF$) restored the signal observed in the wild-type background (Fig. 2.3D).

Altogether, those results showcase that PepF has a dual impact on competence regulation. While its control through ComX triggers competence shut-off, its basal production through the constitutive promoter in *coiA* coding sequence could prevent competence initiation, acting as a locking device.

PepF degrades XIP in vitro

To get insights into the molecular mechanism of the PepF-mediated competence inhibition, we purified PepF (Fig. 2.4A) and incubated it with its most plausible target of the ComRS system, namely XIP. We next analyzed the peptide integrity thanks to mass spectrometry. While incubation of the peptide without the peptidase generated a major peak corresponding to the intact sXIP peptide (m/z value of 882.42, LPYFAGCL), incubation with PepF resulted in the loss of the full-length peptide and the appearance of two main peaks (m/z values of 539.28 and 610.32), corresponding to LPYF and LPYFA fragments, respectively (Fig. 2.4B).

We then sought to evaluate the affinity of the peptidase for the pheromone. We used an indirect pheromone quantification method [40] by monitoring luminescence from a P_{comS} -*luxAB* reporter strain defective for the genome-encoded XIP ($\Delta comS$). By incubating the sXIP peptide at a constant concentration (500 nM) with a gradient of PepF concentration, we measured an enzyme concentration affording half luminescence of ~ 0.2 nM, corresponding to a ~ 2500 -fold pheromone-peptidase ratio or a turnover of about 13 seconds (Fig. 2.4C). This measurement falls within the range reported for other streptococcal oligoendopeptidases (0.4 to 600 seconds) although degradation protocols are different and therefore comparisons should be done with caution [30,40]. Together, those data suggest that PepF competence inhibition acts through XIP degradation.

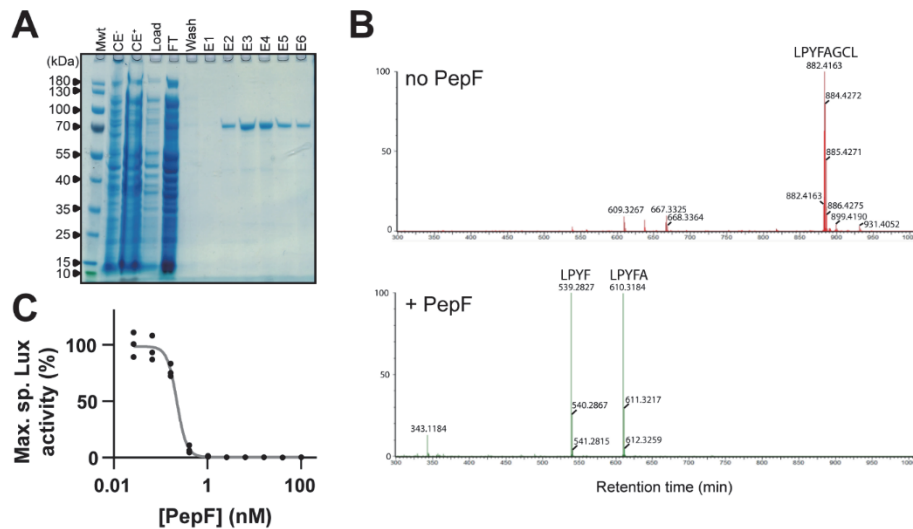


Figure 2.4. PepF degrades XIP *in vitro*. **A.** SDS-PAGE of PepF purification (Mwt: Molecular weight, CE: Crude extract with (+) or without arabinose induction (-), FT: Flow-through, E1-6: elution fractions 1 to 6). **B.** LC-MS chromatogram of sXIP incubated without (no PepF, red) or with PepF (+ PepF, green). sXIP was incubated at 37°C for 4 h at a concentration of 500 nM together with PepF at a concentration of 100 nM. The m/z value of 883.42 corresponds to the unprocessed peptide LPYFAGCL. The m/z values of 539.28 and 610.31 correspond to fragments LPYF and LPYFA, respectively. **C.** Maximum luciferase activity of a competence reporter strain defective for the genetically-encoded pheromone XIP (P_{comS} -*luxAB* $\Delta comS$). sXIP was incubated at 37°C for 4 hours at a concentration of 500 nM together with PepF at concentrations of 0, 0.025, 0.065, 0.16, 1, 2.56, 6.4, 16, 40 and 100 nM. The reaction mixture was 10-fold diluted by addition to an exponential growing culture (CDM) of the reporter strain. Maximum specific luciferase activity (RLU/OD₆₀₀) is displayed as the percentage of signal in comparison to a control culture with an addition of sXIP without PepF digestion. Dots represent technical replicates and the curve a non-linear fit of inhibition. Enzyme concentration affording half-luminescence = 0.22 ± 0.01 nM (standard error).

XIP positions 4 and 5 are crucial for PepF activity in vitro

We next investigated which residues of the pheromone were crucial for peptidase cleavage and screened 20 peptides variants with single mutations. We incubated those peptides with PepF at a mid-range concentration previously identified for the native XIP ([PepF] of 7 nM and [XIP] of 500 nM). Next, we added the peptides incubated or not with PepF to the P_{comS} -*luxAB* $\Delta comS$ reporter strain and analyzed the percentage of signal loss upon incubation with the peptidase. Comparing the signal loss of the peptide variants at the maximum luciferase activity, we identified 4 mutations (F4Y, F4W, A5I, A5V) suggesting a higher peptidase resistance (Fig. S2.4). Notably, those mutations were located at XIP positions 4 and 5, both at position -1 (P1) regarding the two cleavage sites reported above. To quantify more

accurately the PepF resistance of variants at those two positions, we used the same degradation assay as used for native sXIP and measured the enzyme concentration affording half-luminescence for each variant (Fig. 2.5A and Fig. S2.5). Since F4W, F4Y and A5I variants were the most resistant, we combined mutations at positions 4 and 5 in a single peptide to increase its robustness. The F4W-A5I and F4Y-A5I variants exhibited high resistance *in vitro* (Fig. 2.5A) and the F4W-A5I variant showed a reduced degradation kinetics compared to the WT pheromone (Fig. 2.5B). Because our degradation assay is based on an indirect method of quantification through the use of a reporter strain, bias could be introduced by the effect of the mutations on the ComR-pheromone affinity. To rule out this possibility, we measured the ComR-binding properties of the peptides by electrophoretic mobility shift assays (Fig. S2.6). Since no correlation between the peptide affinity for ComR and the degradation assay could be drawn, we concluded that the luciferase degradation assay reflects the peptide susceptibility to PepF.

Together, those results show that the residues at XIP positions 4 and 5 play a key role for PepF degradation *in vitro*.

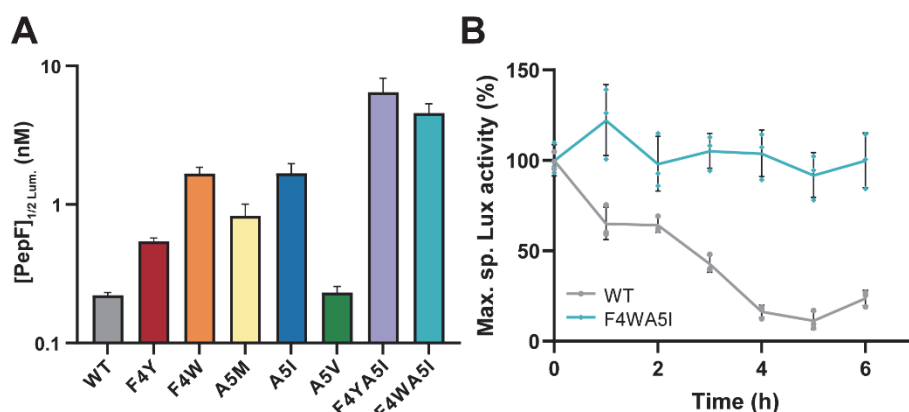


Figure 2.5. Degradation of PepF-resistant sXIP peptides. **A.** PepF degradation efficiency for XIP (LPY[F/Y/W][A/M/I/V]GCL) variants. PepF was incubated 4 hours at 37°C at various concentration with 500 nM of XIP variants and enzyme concentration affording half luminescence ($[PepF]_{1/2 Lum.}$) was determined (Fig. S2.5). Bars denote standard deviation on the IC₅₀ computation. **B.** XIP and XIP_{F4WA5I} kinetics of degradation by PepF. 8 nM of PepF and 500 nM of the peptides were incubated 6 hours at 37°C and sampled every hour for analysis. Reaction was stopped by adding 1 mM EDTA and further added to the *P_{comS}-luxAB ΔcomS* reporter strain as previously described. Error bars denote standard errors, dots technical triplicates.

PepF-resistant XIP variants result in higher and longer competence activation

To confirm *in vivo* the relevance of the *in vitro* data, we first measured the activation of a competence reporter strain defective for the pheromone (P_{comS} -*luxAB* $\Delta comS$) with the addition of synthetic peptides. To avoid a saturation of PepF with synthetic peptides that would mask the effect of the mutations, we supplemented the culture of the reporter strain with very low concentrations (5 nM) of pheromone variants (Fig. 2.6A and S2.7). For most of the peptides tested, we observed a higher and longer activation. Strikingly, the most resistant peptides *in vitro* did not result in the best activators, presumably because of the observed change in ComR-affinity (Fig. S2.6) or because of a sudden sXIP increase that overflows the effect of PepF.

To overcome this issue and to evaluate the effect of those mutations in a more relevant physiological context, we next transferred the most resistant peptides variants to the native *comS* locus of *S. salivarius*. We introduced the variants as full-length precursors (24 aa) under the P_{comS} control together with the upstream deletion of *comR* to avoid any toxic effect due to spontaneous competence activation [7]. The strain also carried a P_{comX} -*luxAB* reporter fusion, a xylose-inducible *comR* system as competence activator, and a CRISPRi repression system targeting *pepF*. In a first set of experiments, we overexpressed *comR* and monitored competence activation with the different XIP variants (WT, F4Y, F4W, A5I, F4Y-A5I, F4W-A5I, Fig. 2.6B). Simple mutation at position 4 or 5 resulted in longer and higher competence activation but double mutations F4W-A5I and F4Y-A5I showcased dramatic effects on both physiology and competence activation, resulting in a 7-hours growth delay and a ~10 and ~15-fold increase in competence activation, respectively (Fig. 2.6B and C).

We next induced the CRISPRi system to repress PepF-mediated shut-off together with *comR* overexpression to activate competence. We assumed that for a PepF-resistant peptide, inhibiting *pepF* would not increase competence activation. We observed a lower increase of competence activation for F4W-A5I and F4Y-A5I peptides upon *pepF* inhibition (Fig. 2.6C and D), suggesting that ultra-resistant pheromones can bypass the PepF-mediated shut-off.

Together, those results show that PepF-resistant XIP variants escape the shut-off mechanism, which drastically alter the kinetics and the strength of competence activation in *S. salivarius*.

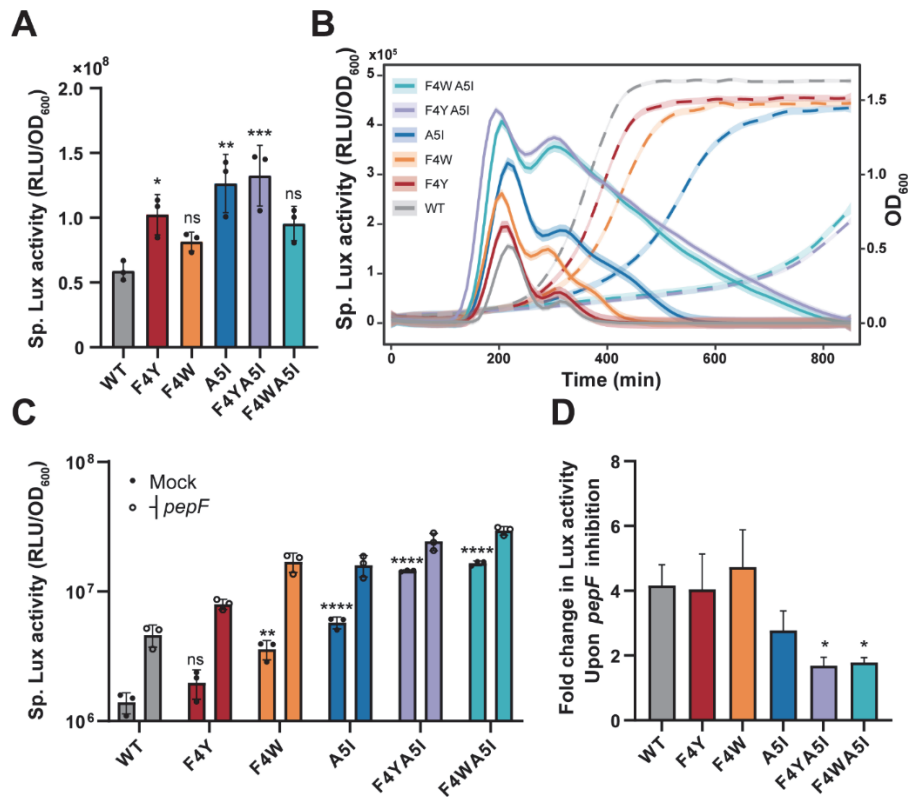


Figure 2.6. PepF-resistant peptides escape shut-off *in vivo*. **A.** Impact on competence activation of the addition of the synthetic peptide variants. Data show specific luciferase activity (RLU/OD₆₀₀) measured with a *P_{comS}-luxAB* reporter strain defective for the pheromone ($\Delta comS$) supplemented with the synthetic peptide variants at 5 nM. **B** and **C.** Kinetics (OD₆₀₀, dashed line; Sp. Lux activity, solid line) (B.) and mean specific luciferase (C.) of competence activation by genetically-encoded peptide variants (ComS, 24 aa). The *comS* native gene was mutated to produce the XIP variants into a xylose-inducible *comR* (*P_{xyI2}-comR*) *P_{comX}-luxAB* reporter background together with the *pepF* dCas9 inhibition module. The *comR* native gene was deleted to avoid any toxic spontaneous competence activation. Competence was induced by adding 0.5% of xylose. The bar represents the mean of biological triplicates (plain dots for competence activation and empty dots for competence activation together with *pepF* inhibition). **D.** Fold change in specific luciferase activity for competence activation upon *pepF* inhibition for each peptide variant based on results presented in C. Bars denote standard deviations on specific luciferase activity or fold change computation. Dots denote biological replicates. In panel B, solid lines and dashed lines are representative of the mean of biological triplicates of specific luciferase activity and growth, respectively. Shaded lines depict standard deviation. One-way ANOVA with Dunett's test was performed for each condition in comparison to WT (WT without *pepF* inhibition for panel C; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

DISCUSSION

Although competence activation has been extensively studied in streptococci, authentic exit mechanisms from ComRS initiation remained challenging to identify. Investigations presented in this work show that PepF, an essential oligoendopeptidase in *S. salivarius*, is responsible for the shut-off of the ComRS pathway through ComX activation. This finding bridges the positive feedback loop on the ComS pheromone production with a negative feedback loop acting on ComS/XIP degradation and set up a new model (Fig. 2.7) explaining the excitability of the system and the limited time window of competence activation in *S. salivarius*.

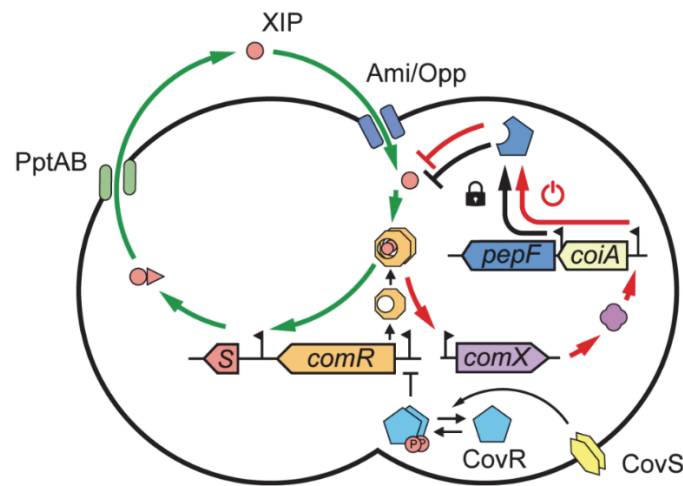


Figure 2.7. Model of competence regulation integrating the PepF-mediated shut-off in *S. salivarius*. Upon CovRS repression release, ComR reaches a threshold concentration allowing the activation of a positive feedback loop (green arrows). The positive loop is triggered by XIP binding to ComR, producing the ComR·XIP complex which activate *comS* transcription. ComS is then exported by the transporter PptAB and matured. The mature XIP pheromone can then enter the cell by the oligopeptide generic transporter Ami/Opp and bind ComR to enhance the loop. This activation can take place if XIP levels are sufficient to saturate the activity of PepF at native expression (locker icon). In parallel, the ComR·XIP complex will trigger the transcription of *comX*, encoding the central regulator of competence. This will activate all the late genes necessary for natural transformation including the *coiA-pepF* operon. PepF accumulation will result in XIP degradation, generating a negative feedback-loop (red arrows, power-off icon) on the ComRS system, ultimately leading to competence exit.

One striking feature of this new ComRS antagonist player is its essentiality in *S. salivarius*. Although PepF inactivation in *Lactococcus lactis* results in a small growth defect only in chemically-defined medium [43] and has no major impact in *Bacillus subtilis* [44], we were unable to produce a knock-out of the peptidase (Fig. S2.2) and observed a strong growth defect upon *pepF*

transcriptional inhibition (Fig. 2.2D). Remarkably, we could uncouple *pepF* from the negative feedback loop by knocking out the promoter of *coiA* controlled by ComX (Figure 2.3D), which suggests that essentiality is associated to its constitutive production rather than its role in competence shut-off. Since *pepF* inhibition remains toxic in a competent-deficient strain (Fig. S2.8), *pepF* essentiality in *S. salivarius* originates most probably from another function than competence regulation. In *Lactococcus lactis*, PepF was shown to play a major role in signal peptide recycling [45], a function also described for the homolog of PepF (OpdA) in *Salmonella enterica* [46]. In addition, OpdA of *Escherichia coli* was shown to be linked to cell cycle regulation [47]. Those results suggest that PepF may have multiple functions in *S. salivarius* in addition to competence regulation that could explain its essentiality.

As previously mentioned, PepF is a metallo-endopeptidase from family M3 containing a classical Zinc-binding motif HEXXH [48], which is involved in the catalysis of the peptide cleavage [49]. Regarding the structure of the M3 family, crystallization of two M3 peptidases revealed two types of structures. While the bacterial Pz-peptidase A from *Geobacillus collagenovorans* is globular and displays an inner closed channel where the peptide docks, the eukaryotic thimet oligopeptidase resembles an open bivalve with two distinct domains [49]. Unfortunately, no structural data are available to know which typical M3 structure PepF displays when bound to its substrate, which could be of main importance to understand its specificity. Yet, the analysis of peptide substrates of PepF homologs from *L. lactis* and *Streptococcus agalactiae* provides interesting data on PepF specificity and cleavage site. Indeed, the peptidase shows a wide specificity with size-limitation from 7 to 17 amino-acids and with a high prevalence of hydrophobic residues at the P1 site (position -1 regarding the cleavage site). Phenylalanine was reported in most cases, but no P1 alanine was observed [48,50]. Those results can be compared to our data showing two fragments, one after the fourth residue (phenylalanine) and another after the fifth residue (alanine). Interestingly, among the 20 XIP variants maintaining competence activation properties that were screened for PepF resistance, the most resistant pheromones exhibited mutations at P1 regarding the two cleavage sites. For single substitutions, *in vitro* degradation data (Fig. 2.5A) showed that F4W and A5I variants generated the most resistant peptides. Since tryptophan and isoleucine are the bulkiest residues tested at this position, it is likely that the higher steric effect of those residues at P1 interferes with the peptide access to the catalytic site or its positioning regarding catalytic residues. Considering PepF efficiency for pheromone proteolysis, XIP degradation measurement showed a turnover of about 13 seconds. This measurement can be compared

to other Gram-positive pheromone-peptidase tandems such as the *S. pyogenes* PepO-SHP2/3 (~0.4/4 seconds) and the *S. mutans* PepO-XIP (~600 seconds), the latter exhibiting very low activity *in vitro* although active *in vivo* [30,40]. Yet, the comparison with the PepO-SHP2/3 should be cautiously interpreted, since the degradation assays were performed at 30°C unlike our assay at 37°C.

In this work, we have uncovered a true shut-off mechanism in *S. salivarius* involving the degradation of XIP through a negative feedback loop restricting competence to a limited time window. One could question if this mechanism could be expanded to other species. In the *Salivarius* group, the XIP C-terminal signal sequence is common for *S. salivarius* HSISS4 and *S. thermophilus* LMD-9/LMG18311 (LPYFAGCL), suggesting that PepF could also limit the pheromone availability in those two strains. Yet, the third member of the *Salivarius* group, *S. vestibularis*, displays another XIP C-terminal signal sequence (XIP_{Sve}, VPFFMIYY). Interestingly, PepF from HSISS4 sharing high identity (98,5%) with the *S. vestibularis* PepF_{Sve} (Fig. S2.9A) is also able to degrade XIP_{Sve} (Fig. S2.9B). Notably, the PepF-degradation is less efficient for XIP_{Sve} ([PepF]_{1/2lum} = 3.06 ± 0.74) in comparison to XIP_{Ssa/Sth} ([PepF]_{1/2lum} = 0.22 ± 0.01). Since XIP_{Sve} displays lower affinity for its cognate ComR [39], it is tempting to speculate that the pheromone sequence has evolved differently in those species to maintain a balance between peptidase susceptibility and ComR affinity. PepF has a broad spectrum for peptide recognition [48,50], and we can ask therefore whether that peptidase plays a role in other ComRS species in competence shut-off and initiation. Although streptococci present high XIP pheromone diversity (for review, see [10]), PepF in other ComRS-containing streptococci could have different specificities, more adapted to recognize their native XIP peptides. Moreover, the highly conserved synteny between *coiA* and *pepF* in Firmicutes (Fig. S2.10) suggests a key-maintained role of PepF in competence control, as previously shown in *B. subtilis* where PepF controls the peptide signalization of competence and sporulation [44].

This study sheds light on the first true competence shut-off mechanism in ComRS-containing streptococci, which is mediated by the activation of a peptidase embedded in a negative feedback loop. Since quorum-sensing based on peptide pheromones is a widely spread system in Gram-positive bacteria, it is likely that this proteolytic mechanism can be broadened to many other communication devices.

MATERIAL & METHODS

Bacterial strains, plasmids, and oligonucleotides

Bacterial strains, plasmids, and oligonucleotides used in this study are listed and described in supplementary information (Tables S2.1-2.4).

Mathematical modeling

A deterministic model was built using ODEs (Ordinary Differential Equations) with the MATLAB software and its ode23 solver. The mathematical formulation of the ComRS system was mainly built on two pre-existing models [36,38]. In short, we used luciferase data (P_{comR} -luxAB, P_{comX} -luxAB and P_{comS} -luxAB) to calibrate the model and find parameters values as previously described [36]. We then validated the model using single-cell microscopic data together with semi-quantitative immunoblotting of ComR (P_{comX} -gfp⁺ P_{xylZ} -comR, see Appendix 1 and [24]). Details on the modeling methodology and the MATLAB code can be found in supplemental Appendix 1.

Growth conditions

S. salivarius HSISS4 [51] and derivatives were grown at 37°C without shaking in M17 (Difco Laboratories, Detroit, MI) or in chemically-defined medium (CDM) [52] supplemented with 1% (w/v) glucose (M17G, CDMG, respectively). *E. coli* TOP10 (Invitrogen) was cultivated with shaking at 37°C in LB (Lysogeny Broth). Chromosomal genetic constructions were introduced in *S. salivarius* via natural transformation [53]. Electrotransformation of *E. coli* was performed as previously described [54]. We added D-xylose (0.1 to 1 %; w/v), IPTG (1mM), ampicillin (250 µg/ml), spectinomycin (200 µg/ml), chloramphenicol (5 µg/ml) or erythromycin (10 µg/ml), as required. The synthetic peptides (purity of 95%), were supplied by Peptide 2.0 Inc. (Chantilly, VA) and resuspended first in dimethyl-formamide (DMF) and diluted in water to reach low DMF concentration (final of 0.02%). Solid plates inoculated with streptococci cells were incubated anaerobically (BBL GasPak systems, Becton Dickinson, Franklin lakes, NJ) at 37°C.

Competence induction and construction of mutants

To induce competence, overnight CDMG precultures were diluted at a final OD₆₀₀ of 0.05 in 500 µl of fresh CDMG and incubated 100 min at 37°C. Then, the pheromone sXIP and DNA (Gibson assembled PCR products or plasmids) were added and cells were incubated for 3 h at 37°C before plating on M17G agar supplemented with antibiotics when required. Null mutants were

constructed by exchanging (double homologous recombination) the coding sequences (CDS) of target genes (sequence between start and stop codons) for chloramphenicol, erythromycin or spectinomycin resistance cassette. If stated, mutants were cleaned for the *lox* site-flanked resistance cassette as previously described [53]. Integration of the antibiotic resistance cassette at the right location was subsequently checked by PCR and sequencing.

Genetic *comR* or *pepF* overexpression were designed by ATG fusion to a mild xylose-inducible promoter (P_{xyl2} , associated to the *xylR* regulator gene) or to a strong 5'-UTR optimized constitutive promoter (P_{32-opt}), respectively. The constructs were associated to a chloramphenicol resistance cassette and integrated at the permissive tRNA serine locus (*HSISS4_r00062*) for $P_{xyl2-comR}$ or at a second permissive locus (*tnpII*, downstream of *HSISS4_01854*) for $P_{32-opt-pepF}$. Luciferase reporter systems were constructed by fusing promoters to the *luxAB* reporter genes and inserted with a spectinomycin resistance cassette at the permissive tRNA threonine locus (*HSISS4_r00061*) or *tnpII* locus (downstream of *HSISS4_01854*).

For transcriptional inhibition of *pepF* by CRISPR-interference, the previously constructed strain harboring a codon-optimized version of *laci* together with a Cas9 catalytic mutant (dCas9) under the control of a P_{lac} promoter with a $P_{comX-luxAB}$ reporter system was used [24,55]. We introduced in this strain a xylose-inducible *comR* module ($P_{xyl2-comR}$) at the *suc* locus (upstream of *HSISS4_01641*). After excising the newly introduced resistance cassette by the Cre-*lox* system, a guide targeting the upstream region of *pepF* was introduced at the *gor* locus (downstream of *HSISS4_00325*) as previously described [24,55].

For genetically-encoded *comS* mutants, a scareless OroP system was used [56]. Briefly, a $P_{32-cat-oroP}$ cassette was introduced at the *comRS* locus and cassette insertion was selected on chloramphenicol. A second transformation was performed on the strain, introducing a *comS* modified construct with *comR* deletion. The transformants were selected on plates with 1 mg/ml 5-Fluoroorotic acid (5-FOA) and sequenced to validate the sequence insertion. The same strategy was used to generate the *comS*-defective mutant used as a reporter for sXIP-PepF degradation assays.

For ΔP_{coiA} complementation, the complete *PcoiA-coiA-pepF* operon was amplified from WT and introduced at an ectopic neutral locus (*fba*, upstream of *HSISS4_01793*). Finally, to construct the $P_{comS-opt-gfp}^+$ strain used for model validation, a 5'-UTR optimized version of P_{comS} was order as a Gblock (IDT) and introduces at the *tRNA_{thr}* locus with a spectinomycin resistance cassette.

Luciferase activity

Overnight precultures were diluted at a final OD₆₀₀ of 0.05. A volume of 300 µl of culture samples was transferred in the wells of a sterile covered white microplate with a transparent bottom (Greiner, Alphen a/d Rijn, The Netherlands). These culture samples were supplemented with D-xylose or IPTG if stated or further incubated for 100 min at 37°C before supplementation with sXIP or other peptides variants. Growth (OD₆₀₀) and luciferase (Lux) activity (expressed in relative light units, RLU) were monitored at 10 min intervals during 6 to 24 h in a Hidex Sense microplate reader (Hidex, Lemminkäisenkatu, Finland). Specific or maximum specific Lux activity were obtained by dividing Lux activity by the OD₆₀₀ for each measure and summing all the data obtained over time or selecting the maximum value, respectively. Biological or technical triplicates were then averaged and compared with *t*-test (unilateral distribution, heteroscedastic) with standard deviations and *P* values.

PepF purification

First, the PCR-amplified *pepF* gene from strains HSISS4 was cloned into the pBAD-covR-ST_{N-ter} vector [24]. Because the catalytic region of PepF is predicted at the C-terminus, the StrepTag was placed at the N-terminus by Gibson assembly, electroporated into *E. coli* Top10 and the final construct was verified by DNA sequencing. The StrepTag-PepF recombinant protein was overproduced in *E. coli* and purified without EDTA in standard native conditions on Strep-Tactin agarose beads (IBA, Germany) as previously described [57].

Mass spectrometry analysis

sXIP was incubated with or without rPepF at 37°C for 4 hours in reaction buffer (20mM Tris HCl pH7) at a final concentration of 100nM and 500nM, respectively. sXIP alone was diluted to an equal volume of TFA (Trifluoroacetic acid) 0.1% and the peptidase-sXIP mix was 10-fold diluted in TFA 1%. Both samples were then purified and concentrated using a ZipTip C18 reverse phased column (Merck-Millipore), using 20µl of acetonitrile 50% and formic acid 0.1% as elution. 5µl of the eluate were then injected on a LC-MS device.

Peptide degradation experiments

sXIP and derivatives were incubated at 500 nM with PepF at different concentrations ranging from 100 to 0.025 nM during 4 h at 37°C as described in the previous section. 30 µl of the mix was then added to 270 µl of

exponential phase culture of a P_{comS} reporter strain defective for the pheromone (P_{comS} -*luxAB* $\Delta comS$) and maximum luciferase activity was computed as reported above. Maximum values obtained were normalized by the signal recorded for the peptide incubated without the peptidase and enzyme concentration affording half-luminescence was extrapolated using Graphpad Prism and the non-linear extrapolation equation: $Signal = b + \frac{(t-b)}{(1 + \frac{IC_{50}}{[PepF]^{HS}})}$ where t stands for top, b for bottom, and HS for Hill-Slope.

Electrophoretic Mobility Shift Assays (EMSAs)

A fixed concentration of purified ComR protein was mixed with a 30-bp dsDNA fragment (20 ng) carrying the ComR box of P_{comX} coupled to the Cy3 fluorophore in absence or presence of increasing amounts of synthetic XIP peptide variants as previously described [57]. Purified StrepTagged ComR (3 μ M) was mixed with twofold serial dilutions of XIP variants at an initial concentration of 6 μ M. The samples (20 μ L) prepared in binding buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol [v/v], 1 mg·mL⁻¹ BSA) were incubated at 37 °C for 10 min prior to run at 100 V for approximately 2 h on a native 4-20% gradient gel (iD PAGE gel; Eurogentec) in Tris-MOPS buffer (50 mM Tris-base, 50 mM 3-(*N*-morpholino) propanesulfonic acid, 1 mM EDTA, pH 7.7). DNA complexes were detected by fluorescence on the Amersham Typhoon Scanner with bandpass excitation and emission filters of 540/25 and 595/25 nm, respectively (GE Healthcare).

SUPPLEMENTARY INFORMATION

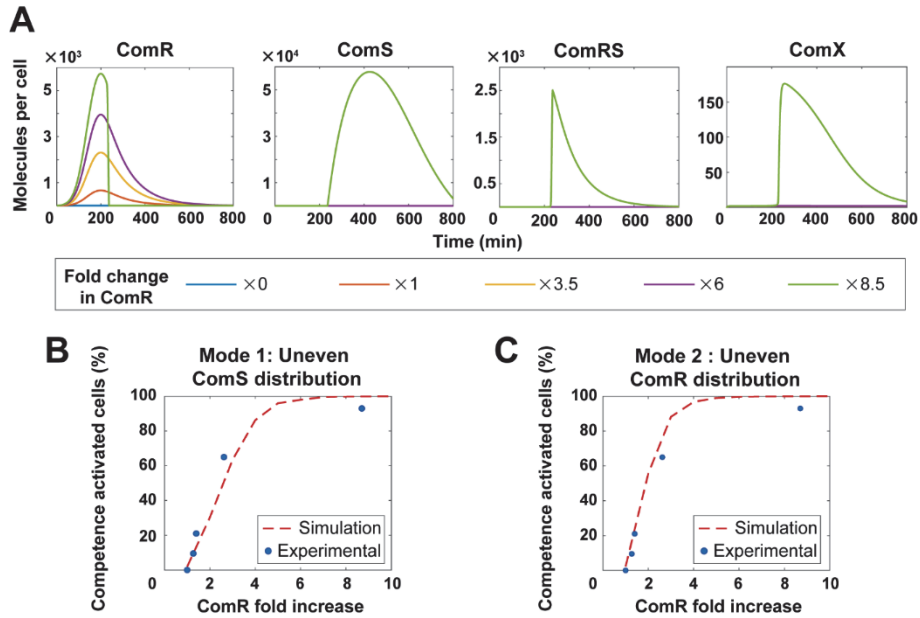


Figure S2.1. Model validation. **A.** ComR, ComS, ComRS and ComX intracellular concentrations computed over time regarding different fold changes in ComR abundance. Fold changes and kinetics of ComR abundance were computed based on experimental data (*i.e.* immunoblotting semi-quantification and luciferase activity of a P_{comR} -*luxAB* reporter strain [24]). ComS, ComRS and ComX abundance (basal and upon activation) were inferred from a previous model of *S. thermophilus* [36], luciferase activity (P_{comS} -*luxAB* and P_{comX} -*luxAB* reporter strains) and biochemical characterization [16] (see supplemental Appendix 1 for complete methodology). **B.** and **C.** Validation of the model using single cell microscopy data displaying the percentage of P_{comX} -activated cells in the population related to fold change in ComR abundance computed with immunoblotting semi-quantification. The model uses as a source of heterogeneity either uneven number of ComS (**B.**) or ComR (**C.**). The distribution of those players in the population was computed thanks to single cell distribution of P_{comS} -*gfp*⁺ or P_{comR} -*gfp*⁺ activation, respectively.

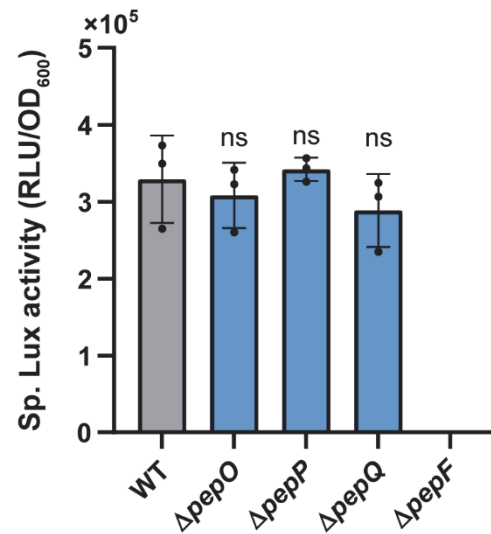


Figure S2.2. Effect of ComX-regulated peptidases deletions on competence activation. Specific luciferase activity measured for WT (with P_{comX} - $luxAB$ as proxy) compared to strains where $pepO$, $pepP$ or $pepQ$ are deleted. Competence was activated with $comR$ overexpression thanks to a xylose-inducible promoter fused to $comR$ (P_{xyl2} - $comR$, induced at 0.5% xylose). One-way ANOVA with Dunett's test were performed for each condition in comparison to the WT to generate p-values (ns, non-significative)

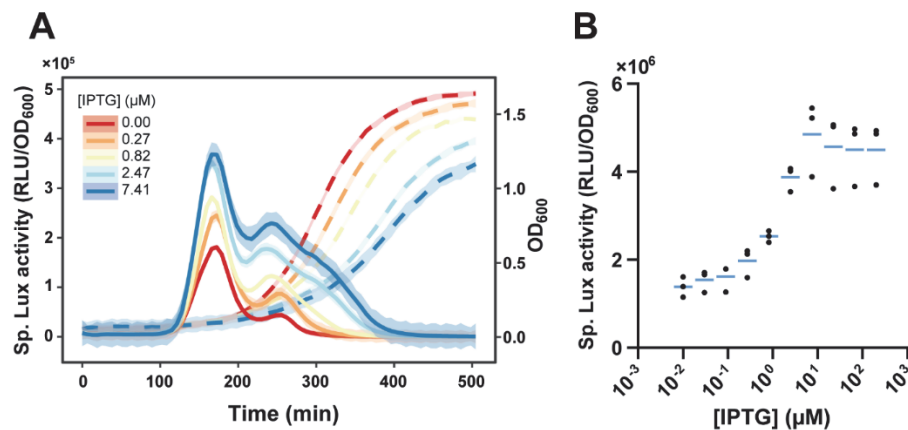
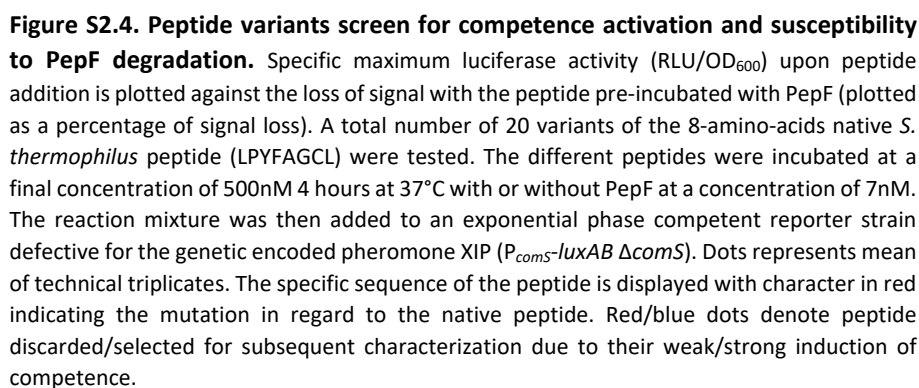


Figure S2.3. Progressive inhibition of $pepF$. A strain harboring a P_{comX} - $luxAB$ reporter system with a $dcas9$ module targeting $pepF$ (P_{F6} - $laci$ P_{lac} - $dcas9$ P_3 - $gRNA_9$) and a $comR$ xylose-inducible module (P_{xyl2} - $comR$) was incubated at various IPTG concentration (0, 0.01, 0.03, 0.09, 0.27, 0.82, 2.5, 7.4, 22, 66 and 200 μM) at 0.25% xylose. **A.** Kinetics of activation showing the mean specific luciferase activity (solid lines) and mean growth (OD₆₀₀, dashed lines) for biological triplicates at 5 chosen concentrations of IPTG (0, 0.27, 0.82, 2.47, 7.41 μM). Shaded lines denote standard deviation. **B.** Total specific luciferase activity for the different concentrations in IPTG. The bars denote the mean and the dots biological triplicates.



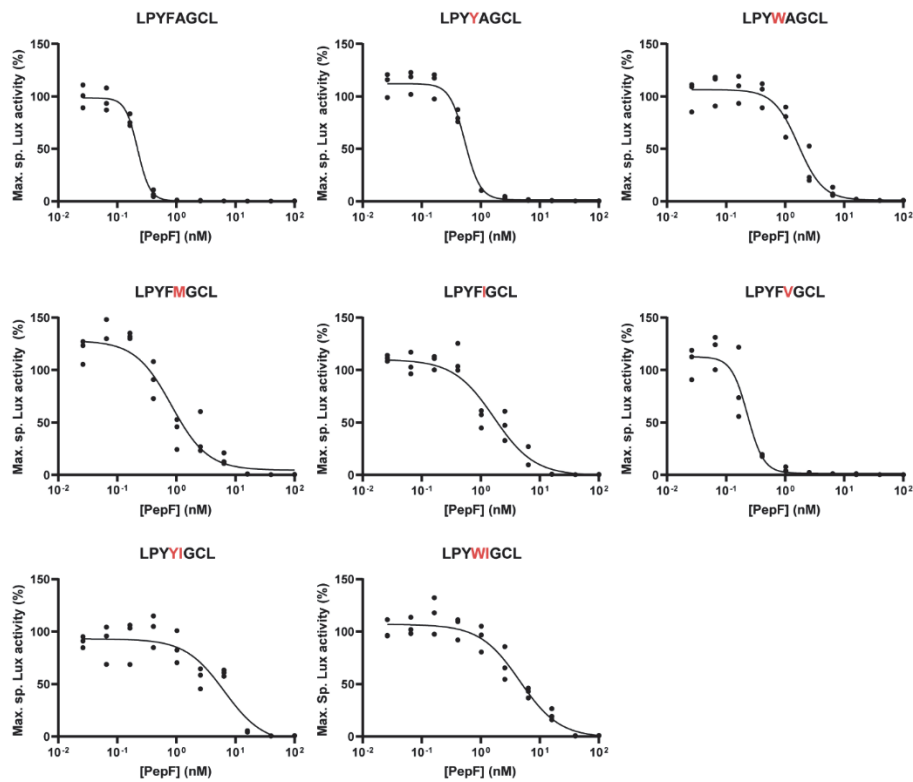


Figure S2.5. Peptide variant susceptibility to PepF degradation, complete set of data. Maximum luciferase activities of a competent reporter strain defective for the genetic-encoded pheromone XIP (*P_{comS}-luxAB ΔcomS*). XIP was incubated at 37°C for 4 hours at a concentration of 500nM together with PepF at concentrations of 100, 40, 16, 6.4, 2.56, 1, 0.4, 0.16, 0.065, 0.025 and 0nM and the reaction mixture was diluted ten times in a CDM exponential growing culture of the reporter strain. Maximum specific luciferase activity (RLU/OD₆₀₀) is displayed as the percentage of signal in comparison to an addition of XIP without PepF digestion. Dots represent technical replicates. The curve is a non-linear fit of inhibition.

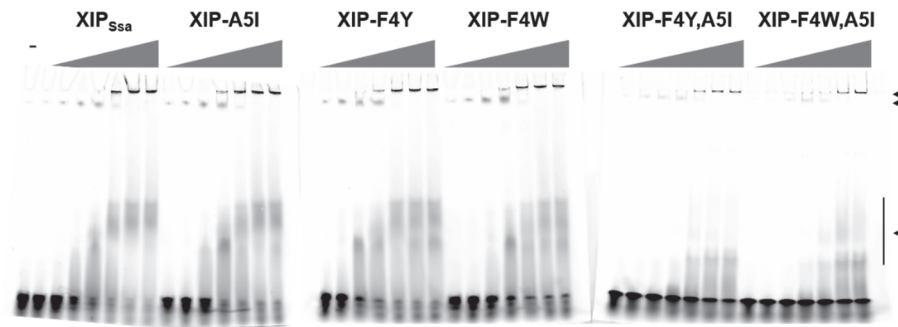


Figure S2.6. Electrophoretic mobility shift assay measurement of the sXIP pheromone variants affinity for ComR. Labeled P_{comX} 30-bp DNA fragments (20 ng) were incubated with a fixed concentration of ComR_{Ssa} WT (3 μ M) in absence of peptide (-) or in presence of increasing concentrations of peptide variants (0, 0.9, 0.19, 0.38, 0.75, 1.5, 3, and 6 μ M). The ComR-XIP-DNA complex and multimeric complexes are indicated by arrowheads.

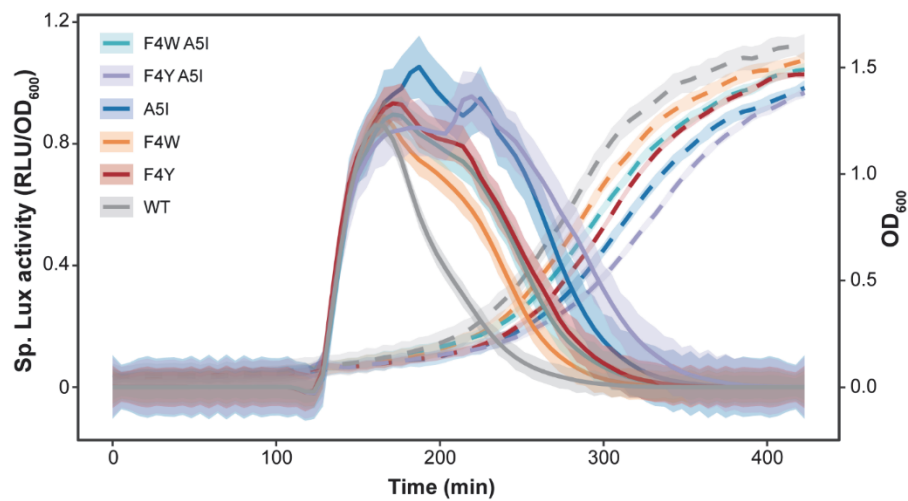


Figure S2.7. Kinetics of competence activation with synthetic peptides. Impact on competence activation of the addition of the synthetic peptide variants. Data show specific luciferase activity (RLU/OD₆₀₀, solid lines) and growth (OD₆₀₀, dashed lines) measured with a P_{comS} -*luxAB* reporter strain defective for the pheromone ($\Delta comS$) supplemented with 5nM of the synthetic peptide variants at t=120min. Shaded lines represent standard deviations.

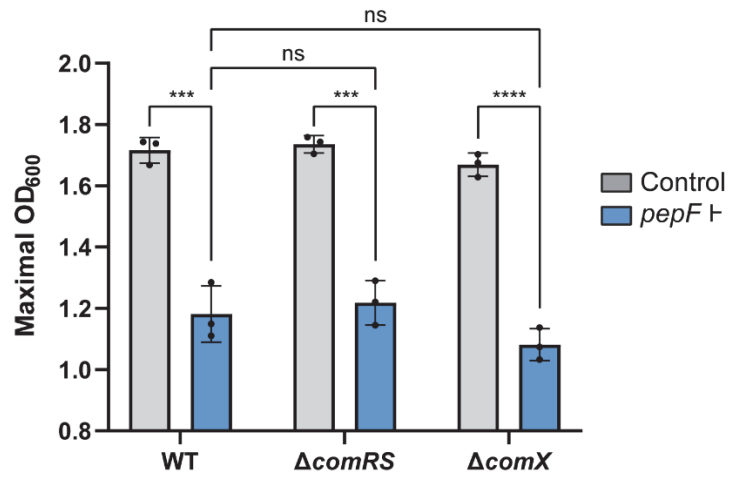
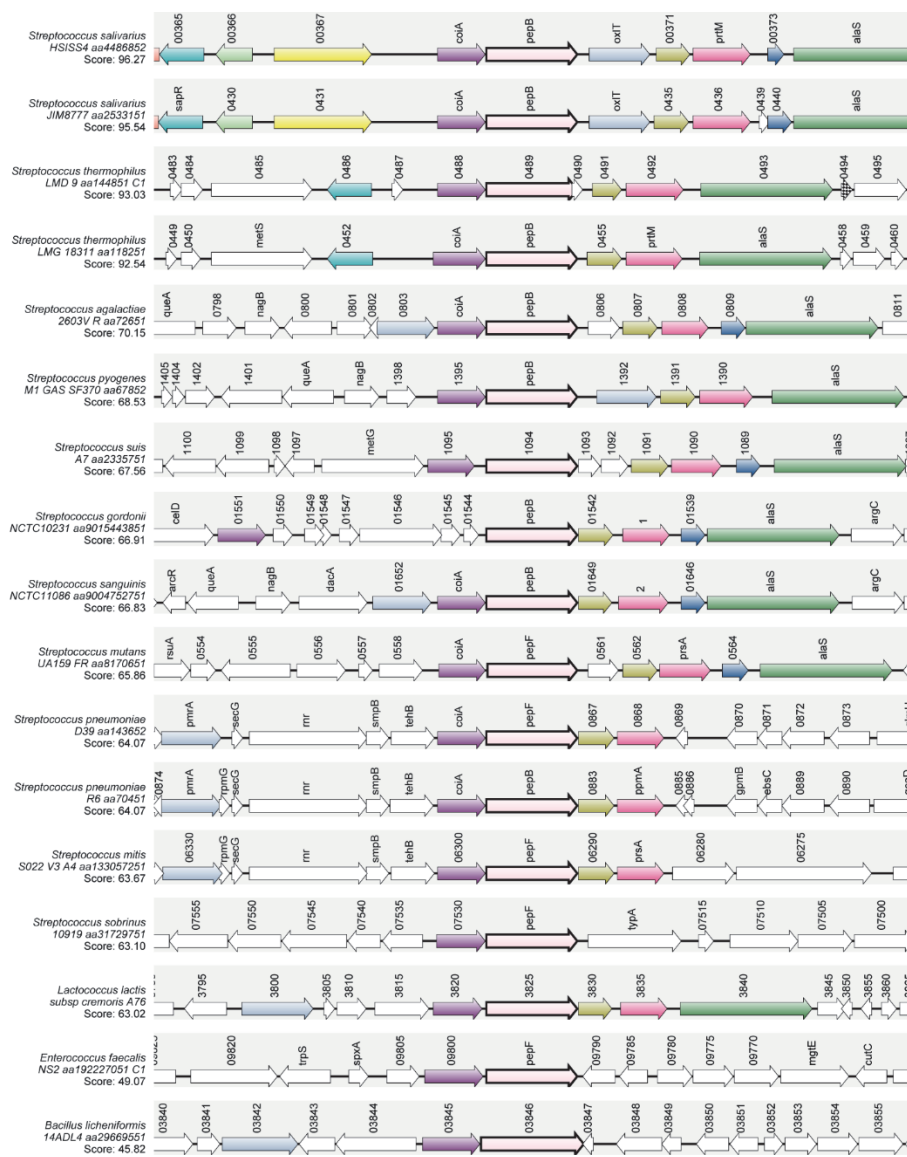


Figure S2.8. PepF essentiality is not competence-related. Maximum OD₆₀₀ measured after growth with (PepF⁻) or without (Control) *pepF* inhibition in strains with the full set of competence gene (WT) or deficient for early (Δ*comRS*) or late (Δ*comX*) competence genes. In the three strains, *pepF* was inhibited thanks to a guide targeting the promoter of *pepF* (P_{3-gRNA_9}) together with the IPTG-inducible dCas9 system (P_{F6-lacI} P_{lac-dcas9}) induced at 1 mM IPTG. Statistical *t*-test was performed for each strain in comparison to the related control and one-way ANOVA with Dunett's test were performed to compare the strains with *pepF* inhibition in comparison to the WT to generate p-values (***, *P* < 0.001; ****, *P* < 0.0001; ns, non-significative).



← **Figure S2.9. Conservation of PepF in salivarius streptococci and *S. vestibularis* pheromone susceptibility to PepF degradation.** **A.** Alignment of CovR performed with the aid of the CLC Main Workbench multiple alignment tool. Four representative strains were used for both alignments: *S. salivarius* HSIS4, *S. thermophilus* LMD-9, *S. thermophilus* LMG18311 and *S. vestibularis* NTC12167. Red denote perfect consensus, orange 75% conservation and yellow 50% conservation. **B.** Maximum luciferase activities of a *S. thermophilus* LMD-9 competent reporter strain defective for the genetic-encoded pheromone XIP and harboring the ComR of *S. vestibularis* (P_{comS} -luxAB $\Delta comS$ comR_{Sve}). XIP_{Sve} was incubated at 37°C for 4 hours at a concentration of 500 nM together with PepF at concentrations of 100, 40, 16, 6.4, 2.56, 1, 0.4, 0.16, 0.065, 0.025 and 0 nM and the reaction mixture was diluted ten times in a CDM exponential growing culture of the reporter strain. Maximum specific luciferase activity (RLU/OD₆₀₀) is displayed as the percentage of signal in comparison to an addition of XIP without PepF digestion. Dots represent technical triplicates. The curve is a non-linear fit of inhibition. The enzyme concentration affording half luminescence computed is 3.06 ± 0.74 .



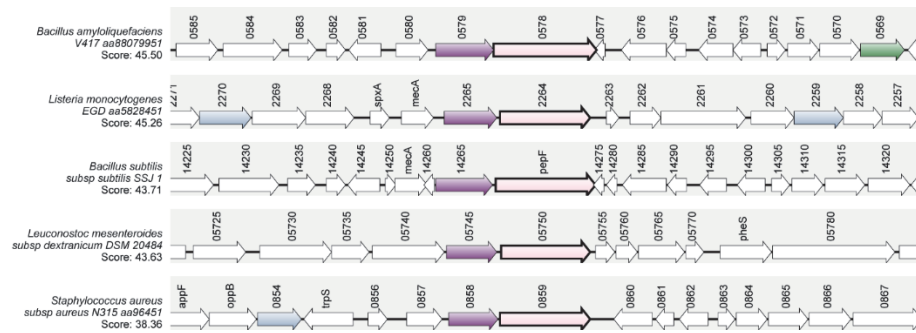


Figure S2.10. SyntTax synteny analysis of *pepF* in firmicutes. The HSISS4 sequence of PepF was used as a proxy for genomic context analysis of *pepF* in firmicutes thanks to the SyntTax server [58]. Species were picked from various genera across firmicutes. Central bold arrow: *pepF* homologs, left light blue: *sapR* homologs (transcriptional regulator from LysR family), light green: hypothetical, yellow: hypothetical, purple: *coiA* homolog, right light blue: *oxlT* homologs, right green: O-methyltransferase family protein C1 homologs, pink: *prtM* homologs, blue: hypothetical protein, green: *alaS* homologs.

Table S2.1. List of bacterial strains used in this study

Strain	Characteristics	Reference/source
<i>Escherichia coli</i>		
TOP10	<i>mcrA</i> , $\Delta(mrr-hsdRMS-mcrBC)$, Φ hi80 <i>lacZ</i> (<i>del</i>)M15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (<i>SmR</i>), <i>endA1</i> , <i>nupG</i>	Invitrogen, CA
<i>Streptococcus salivarius</i>		
HSISS4	Wild-type gastro-intestinal tract isolate	[37]
AK0004	<i>HSISS4 tRNA^{thr}::P_{comX}-luxAB-spc</i> <i>tRNA^{ser}::P_{xyl2}-comR-cat</i>	[24]
AK0010	<i>HSISS4 tRNA^{thr}::P_{comR}-luxAB-spc</i>	[24]
AK0022	<i>HSISS4 tRNA^{thr}::P_{comR}-opt-gfp-spc</i>	[24]
AK0043	<i>HSISS4 tRNA^{thr}::P_{comS}-opt-gfp-spc</i>	This work
AK0044	<i>HSISS4 tRNA^{thr}::P_{comS}-luxAB-spc</i> <i>tRNA^{ser}::P_{xyl2}-comR-cat</i>	This work
AK0045	<i>HSISS4 tRNA^{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA^{ser}::P_{xyl2}-comR-lox72</i> <i>tnpII::P₃₂-opt-pepF-cat</i>	This work
AK0046	<i>HSISS4 tRNA^{thr}::P_{F6}-lacI-lox72</i> <i>tRNA^{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i>	This work
AK0047	<i>HSISS4 tRNA^{thr}::P_{F6}-lacI-lox72</i> <i>tRNA^{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g₉(P_{pepF})-erm</i>	This work
AK0048	<i>HSISS4 tRNA^{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA^{ser}::P_{xyl2}-comR-lox72</i> Δ <i>pepO::cat</i>	This work

AK0049	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔpepP::cat</i>	This work
AK0050	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔpepQ::cat</i>	This work
AK0051	HSISS4 <i>tRNA_{thr}::P_{coiA}-luxAB-spc</i>	This work
AK0052	HSISS4 <i>tRNA_{thr}::P_{pepF}-luxAB-spc</i>	This work
AK0053	HSISS4 <i>tRNA_{thr}::P_{coiA-coiA-PpepF}-luxAB-spc</i>	This work
AK0054	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔcomX::erm</i>	This work
AK0055	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔPcoiA::spc</i>	This work
AK0056	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-lox72</i> <i>tRNA_{ser}::P_{xyl2}-comR-lox72</i> <i>ΔPcoiA::spc</i> <i>FBA::P_{coiA}-coiA-P_{pepF}-pepF</i>	This work
AK0057	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g₉(PpepF)-erm</i> <i>ΔcomR-comS_{WT}</i>	This work
AK0058	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g₉(PpepF)-erm</i> <i>ΔcomR-comS_{F4Y}</i>	This work

AK0059	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g_9(PpepF)-erm</i> $\Delta comR-comS_{F4W}$	This work
AK0060	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g_9(PpepF)-erm</i> $\Delta comR-comS_{A5I}$	This work
AK0061	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g_9(PpepF)-erm</i> $\Delta comR-comS_{F4YA5I}$	This work
AK0062	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g_9(PpepF)-erm</i> $\Delta comR-comS_{F4WA5I}$	This work
AK0063	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyl2}-comR-lox72</i> <i>GOR::P3-g_9(PpepF)-erm</i> $\Delta comRS::cat$	This work

AK0064	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P3-g₉(PpepF)-erm</i> <i>ΔcomX ::cat</i>	This work
LL0052	<i>HSISS4 tRNA_{thr}::PcomS-luxAB-spc</i>	This work
LL053	<i>HSISS4 tRNA_{thr}::PcomS-luxAB-spc</i> <i>ΔcomS</i>	This work
<i>Streptococcus thermophilus</i>		
LL01	<i>LMD-9 (blpD-blpX)::PcomS-luxAB</i> <i>comR::comR_{Sve}</i> <i>ΔcomS::P₃₂-cat</i>	[39]

Table S2.2. List of plasmids used in this study

Plasmid	Characteristics	Reference/source
<i>pBad-covR-ST_{N-ter}</i>	pBAD plasmid encoding CovR _{HSISS4} fused to a N-terminal StreptagII	[24]
<i>pBad-pepF-ST_{N-ter}</i>	pBAD- <i>covR-ST_{N-ter}</i> derivative encoding <i>pepF</i> _{HSISS4} fused to a N-terminal StreptagII	This work
<i>pGhostcre</i>	Thermosensitive replication origin vector in <i>S. salivarius</i> , encoding the Cre recombinase; <i>erm</i>	[53]
pGIUD0855erm	pUC18 derivative containing the <i>erm</i> gene	[53]
pJUDspecmut1- <i>gfp</i> +ter	Terminator associated- <i>gfp</i> + ORF cloned in pJUDspec	[7]
pJIMcat	pJIM4900 derivative with a <i>cat</i> cassette	[7]

Table S2.3. List of oligonucleotides used in this study

NAME	SEQUENCE (5' TO 3')
1022SA	TTATAAAATTTAATGAATATTATTCCGGTC
1110SA	TAAGGAAGATAAATCCCATAAGG
AK72	GCAAAATGATAGAGTTACGTG
AK73	TAATGATGAAATCTGTCCAAAAATG
AK74	CATTTTGGACAGATTTTCATTATAAGGAAGATAAATCCCATAAGG
AK75	GGGGAATTTTGTATAATGAAAGCTTCACGTTACTAAAGGGAATGTA
AK76	GCTTTCATTATAACAAAATTCCC
AK77	CAAATCATTCAAGCCAAATC
AK78	GGCTCAAATGTTTTATAGAATC
AK79	GATTTGGAGGAATACTTTATGC
AK80	GGCATAAAGTATTCCTCCAAATCTAAGGAAGATAAATCCCATAAGG
AK81	CCTAAAAGACCAAAGGAGTAATTTATATTCACGTTACTAAAGGGAATGT A
AK82	TATAAATTACTCCTTTGGTCTTTTAG
AK83	GACTATTTTGGTTTTAGAGTTAG
AK88	CTTATGGGATTTATCTTCCTTATAAATAATAAAAAAGCCGGATTAATAAT
AK91	CCTTATGGGATTTATCTTCCTTACTATTAGGTATATTCCATGTG
AK106	CTTTTGAAAAATTGAGAGGTATAAATCAACTGCAGAAAATTACAATAAG
AK211	CATACAGTCTTTGGTCAATTG
AK212	CCTTATGGGATTTATCTTCCTTAGATGTCTAATTAATAAACATGGG
AK213	TACATTCCCTTTAGTAACGTGAATATTTCTTACACTCATTCTGTC
AK214	TGATACCGTCTACTTGTGTTG
AK262	CATTTTGACTGCTATCTTTG
AK264	TTAAGTAGGAGTGCTTAATTC
AK265	AAATGAGTTATCCTTTCTCC
AK266	TTAAAATATAGATAAATATCATTGATTAGTATTTGTTACGTTACTAAA GGGAATGTA
AK316	CTTTTGAAAAATTGAGAGGTATAAATCAACTATTTTAAATGCTTATGTGG
AK317	GTAAGCAAAAAGTTTCCAAATTCATAACTCTCTCCTTCGTGCAC
AK325	CTATTGTTCTGTAAAAATGCATTAAC
AK330	CCTTATGGGATTTATCTTCCTTAGTATAGTAACTTCTTTTAAAGAAAG
AK331	GTTTATACTCGTTCATATAATCAAATTGGAATGTCAGACAATCGTACTC
AK338	TTTTACCTCCTATTTTTTTTCTAG
AK353	GTTTGAATTAAGCACTCCTACTTAACTTTTGAAAAATTGAGAGG
AK356	CTTTTGAAAAATTGAGAGGTATAAATCAAGAAAAGAGATCAAATGGAC
AK357	GTATGTAAGCAAAAAGTTTCCAAATTCATAATATTCTCCTTAATTGTGT C

AK384	CTTCTAAATGAGTACGATTGTCTGAACCAGCAGCACCTTTCTCG
AK385	GAAAAAGGTGTTACCTTTCTTAAGAATTCTAGAAGCTTGGC
AK386	CGAGAAAGGTGCTGCTGGTTCAGACAATCGTACTCATTTAGAAG
AK387	GCCAAGCTTCTAGAATTCTTAAGAAAGGTGAACACCTTTTTC
AK458	GTAGCAACACTCTTGTTTAAAG
AK459	GTGGCTGAATTATCAAAATAAATC
AK472	AAGTTTAAATAAGGCTAGTCCGTTATCAACTTG
AK473	TATAGTTATTATACCAGGGGGAC
AK475	CGGACTAGCCTTATTTAAAC
AK476	GTCCCCCTGGTATAATAACTATA
AK487	CTATTAAATTCCTTTATAGCTTTACC
AK488	TTAAGACGATAGGCACTACTG
AK489	GTTTTAAATAAAGTTTGGATAGAGG
AK490	TGACTATTTTCTTACTTGTCTG
AK495	GACTAATCCTCTATCCAACTTTATTTAAACTTCACGTTACTAAAGGGA ATGTA
AK496	CCTTTTTTTAGACAATAAATGCG
AK497	GAAAAGTATGAAGCAGAGTAG
AK498	CTTTTCTTTATGAGCCATTTAAC
AK499	ATCGTAAAGAAAAGCTAAACG
AK516	GTACAGACTACTCTGCTTCATACTTTTCACTTATAGGGGTAACACTTAAA AAAG
AK517	GCTTTTTTAGTTAAATGGCTCATAAAGAAAAGTTCACGTTACTAAAGGG AATGTA
AK521	AACGTTGACTGTAACATACAAC
AK522	TACATTCCCTTTAGTAACGTGAATTCTTCAAGGAAAAATCCCC
AK523	CCTTATGGGATTTATCTTCCTTAATGTTAATTGCAAGAAATCAGAAAAG
AK524	CAATCCAACGTTCCGTAAAG
AK531	CTTTTGAAAAATTGAGAGGTATAAATCAAGAAAAATCCAAGTGGATG
AK532	GTAAGCAAAAAGTTTCCAAATTTTATAATATTCTCCTTAATTGTGTC
AK563	AGTACAGTCGGCATTATCTCATATTATAAAAATTTAATGAATATTATTCC
AK564	TATGAGATAATGCCGACTGTACT
AK577	CTACCAGTAGTGCCTATCGTCTTAAGAAAAGAGATCAAATGGACTG
AK580	CCTTATGGGATTTATCTTCCTTATAACTTCTTTTAAAGAAAGGTGAAC
AK657	CCTTATGGGATTTATCTTCCTTAAAAATAAGGCAAGATAGTGATAAG
AK658	GACCGGAATAATATTCATTAAATTTTATAAATGGGTTGTCTTTAATAAG
AK677	GATATTTTGACATGGTGACTCCTTATTACAAAATAAACTCCTTTAACT G
AK678	CATAACTAACAGTTAAAAGGAGTTTTATTTGTAATAAGGAGTCACCAT
AK687	CATTTATGTCACCACCATTTCTACAAGAGACACTCCTTTATTTTCG

AK688	CGAAATAAAGGAGTGTCTCTTGTAGAAATGGTGGTGACATAAATG
AK689	AGACAACCTATAAAATAAGGCAAGATAGTGATAAG
AK690	CCTTATTTTATAGGTTGTCTTTAATAAGGAGTC
AK691	CAACCTGCATAATAAGGCAAGATAGTGATAAGTAG
AK692	TTGCCTTATTATGCAGGTTGTCTTTAATAAG
AK693	ACAACCTGCCAATAAGGCAAGATAGTGATAAGTAG
AK694	CCTTATTGGGCAGGTTGTCTTTAATAAGGAGTCACCATG
AK695	AAGACAACCTATATAATAAGGCAAGATAGTGATAAGTAG
AK696	CTTATTATATAGGTTGTCTTTAATAAGGAGTC
AK697	AAAGACAACCTATCCAATAAGGCAAGATAGTGATAAGTAG
AK698	TTATTGGATAGGTTGTCTTTAATAAGGAGTC
Dn.comX 578	TACTGGCTTCTTAGTATCCT
Dn.Rv.lox	TTCACGTTACTAAAGGGAATGTA
DR.THR	AAGGAGAAAATTATGTACAC
DR_SER	TTGGATAAGGTCTTGACTTC
DR_THR	AAGGAGAAAATTATGTACAC
LL61	CATGGGAGTTCTCATCCTATAAAATCAACCTCTTTGAACATA
luxAB.AT	ATGAAATTTGGAACTTTTTGC
ML42	CCTTATGGGATTATCTTCCTTATCAACCTCCTATTAATAGATATAATTTT TG
ML43	TACATTCCCTTTAGTAACGTGAATCCTTAATATAGAACCTTGACTG
ML45	TAAGGAAGATAAATCCCATAAGGTAAACATAAACATGCAAGGAG
ML46	TTCACGTTACTAAAGGGAATGTAGAAAAGGCAATTGCGTTACC
PH497	TTGCTTACAGTTGCTATGGT
PH499	TCATCACAATGGTCACATCT
R_spec	ATAGGATGAGAACTCCCATG
UF.THR	TGTCAAAGGATTAGGAAAAC
UF_SER	CAAGATTAACCATGACCTTC
UF_THR	TGTCAAAGGATTAGGAAAAC
Up.comX 557	CAAGATGAGATTTCCCATGA
Up.fw.lox	TAAGGAAGATAAATCCCATAAGG
UR.THR	TTGATTATACCTCTCAATTT

Table S2.4. List of PCR fragments, synthetic DNA constructs, and EMSA probes

Strain/ Plasmid	PCR fragment	Template DNA	Primer 1	Primer 2
AK0043	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF_THR	UR_THR
	<i>P_{comS}-opt-gfp⁺</i> , <i>comS</i> promoter with 5'UTR optimized sequence fused to codon-optimized <i>gfp⁺</i>	<i>P_{comS}-gfp⁺</i> (gBlocks DNA ^a)	AK106	AK88
	<i>Spc</i> fused-Dw HR of <i>tRNA_{thr}</i>	Strain AK0001 ^b	Up.fw.lo x66	DR_THR
AK0044	<i>P_{xyl2}-comR</i> overexpression system fused to a chloramphenicol resistance cassette at the <i>tRNA_{ser}</i> locus	Strain AK0004 ^b	UF_SER	DR_SER
AK0045	Up HR of <i>tnpII</i>	HSISS4	AK262	AK266
	<i>cat</i> resistance cassette	pJIMcat	Up.fw.lo x66	Dn.rv.lo x71
	<i>pepB</i>	HSISS4	AK330	AK331
	<i>P₃₂-opt</i> , constitutive 5'UTR optimized <i>P₃₂</i> promoter	<i>P₃₂-opt-gfp⁺</i> (gBlocks DNA ^a)	AK338	AK353
	Dw HR of <i>tnpII</i>	HSISS4	AK264	AK265
AK0046	Up HR of <i>SUC</i>	HSISS4	AK496	AK497
	<i>P_{xyl2}</i> xylose-inducible promoter fused to <i>comR</i> and to a chloramphenicol cassette resistance <i>cat</i>	Strain AK0004 ^b	AK516	AK517
	Dw HR of <i>SUC</i>	HSISS4	AK498	AK499

AK0047	Up HR of <i>GOR</i> locus fused to the <i>erm</i> resistance, a terminator and the <i>dcas9</i> handle	Strain AK0036 ^b	AK458	AK472
	<i>Dcas9</i> handle together with a guide targeting the promoter of <i>pepF</i> (P_{pepF})	AK482	AK475	AK476
	P_3 promoter together with Dw HR of <i>GOR</i> locus	Strain AK0036 ^b	AK473	AK459
AK0048	Up HR of <i>pepO</i>	HSISS4	AK72	AK73
	<i>cat</i> resistance cassette	pJIMcat	AK74	AK75
	Dw HR of <i>pepO</i>	HSISS4	AK76	AK77
AK0049	Up HR of <i>pepP</i>	HSISS4	AK78	AK79
	<i>cat</i> resistance cassette	pJIMcat	AK80	AK81
	Dw HR of <i>pepP</i>	HSISS4	AK82	AK83
AK0050	Up HR of <i>pepQ</i>	HSISS4	AK211	AK212
	<i>cat</i> resistance cassette	pJIMcat	Up.fw.lo x66	Dn.rv.lo x71
	Dw HR of <i>pepQ</i>	HSISS4	AK213	AK214
AK0051	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF.THR	UR.THR
	Promoter of <i>coiA</i> P_{coiA}	HSISS4	AK316	AK317
	<i>luxAB</i> genes together with <i>spc</i> resistance cassette and Dw HR of <i>tRNA_{thr}</i>	Strain AK0001 ^b	luxAB.AT G	DR.THR
AK0052	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF.THR	UR.THR
	Promoter of <i>pepF</i> (P_{pepF})	HSISS4	AK531	AK532
	<i>luxAB</i> genes together with <i>spc</i> resistance cassette and Dw HR of <i>tRNA_{thr}</i>	Strain AK0001 ^b	luxAB.AT G	DR.THR

	Up HR of <i>tRNA_{thr}</i>	HSISS4	UF.THR	UR.THR
AK0053	Promoter of <i>coiA</i> P_{coiA} fused to <i>coiA</i> and the <i>pepF</i> promoter (P_{coiA} - <i>coiA</i> - P_{pepF})	HSISS4	AK356	AK357
	<i>luxAB</i> genes together with <i>spc</i> resistance cassette and Dw HR of <i>tRNA_{thr}</i>	Strain AK0001 ^b	luxAB.ATG	DR.THR
AK0054	Up HR of <i>comX</i>	HSISS4	AK563	ML42
	<i>Erm</i> resistance cassette	pGIUD0855ery	ML45	ML46
	Dw HR of <i>comX</i>	HSISS4	ML43	AK564
AK0055	Up HR of P_{coiA}	HSISS4	AK521	AK522
	<i>spc</i> resistance cassette	pJUDspecmut1-gfp+ter	Dn.Rv.lo x71	Up.Fw.Iox66
	Dw HR of P_{coiA}	HSISS4	AK523	AK524
AK0056	Up HR of <i>FBA</i> locus	HSISS4	AK487	AK488
	P_{coiA} - <i>coiA</i> - P_{pepF} - <i>pepF</i> operon	HSISS4	AK577	AK580
	<i>Cat</i> resistance cassette	pJIMcat	Up.Fw.Iox66	AK495
	Dw HR of <i>FBA</i> locus		AK489	AK490
AK0057	Up HR of <i>comRS</i> locus	HSISS4	AK325	AK657
AK0058				
AK0059	<i>cat-oroP</i> cassette	<i>cat-oroP</i> cassette ^d	1110SA	1022SA
AK0060				
AK0061	Dw HR of <i>comRS</i> locus	HSISS4	AK658	PH499
AK0057	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
	Dw HR of <i>comS</i>	HSISS4	AK688	PH499
AK0058	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
	P_{comS} - <i>comS</i> , introducing F4Y mutation	HSISS4	AK688	AK691
	Dw HR of <i>comS</i> , introducing F4Y mutation	HSISS4	AK692	PH499

	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
AK0059	<i>P_{comS}-comS</i> introducing F4W mutation	HSISS4	AK688	AK693
	Dw HR of <i>comS</i> , introducing F4W mutation	HSISS4	AK694	PH499
	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
AK0060	<i>P_{comS}-comS</i> introducing A5I mutation	HSISS4	AK688	AK689
	Dw HR of <i>comS</i> , introducing A5I mutation	HSISS4	AK690	PH499
	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
AK0061	<i>P_{comS}-comS</i> introducing F4YA5I mutation	HSISS4	AK688	AK695
	Dw HR of <i>comS</i> , introducing F4YA5I mutation	HSISS4	AK696	PH499
	Up HR of <i>comRS</i> locus, without <i>comR</i>	HSISS4	PH497	AK687
AK0062	<i>P_{comS}-comS</i> introducing F4YA5I mutation	HSISS4	AK688	AK697
	Dw HR of <i>comS</i> , introducing F4YA5I mutation	HSISS4	AK698	PH499
AK0063	Up and Dw HR of <i>comRS</i> locus fused to <i>cat</i>	Strain JM1002 ^c	PH497	PH499
AK0064	Up and Dw HR of <i>comX</i> locus fused to <i>cat</i>	Strain JM1013 ^c	Up.comX 557	Dn.comX 578
	Up HR of <i>tRNA^{thr}</i> locus fused to <i>P_{comS}-luxAB</i>	Strain JM1019 ^c	UF_THR	AK91
LL0052	<i>spc</i> , spectinomycin resistance cassette	pJUDspecmu t1-gfp+ter	Up.fw.lo x66	R_spec
	Dw HR of <i>tRNA^{thr}</i> locus	HSISS4	LL61	DR_THR
LL0053	Up HR of <i>comS</i>	HSISS4	PH497	AK677
	Dw HR of <i>comS</i>	HSISS4	AK678	PH499
pBad- <i>pepF</i> - <i>ST_{N-ter}</i>	pBAD plasmid backbone	pBAD-covR-ST _{Nter}	AK384	AK385
	<i>pepF_{HSISS4}</i>	HSISS4	AK386	AK387

EMSA probes	Template	Primer 1	Primer 2
HSISS4 promoter of <i>comX</i> fused to Cy3	HSISS4	Cy3_F_ PcomX	R_Pco mX

^agBlocks™ DNA are synthetic dsDNA provided by Integrated DNA Technologies (IDT)

^b[24]

^c[7]

^d[56]

APPENDIX 1: Mathematic model of ComRS Bimodality in *Streptococcus salivarius*

The ComRS signalization pathway has been extensively studied in *salivarius* streptococci but remains partially understood. In the previous chapter, we determined experimentally the key processes involved in competence bimodality *i. e.* the heterogeneous activation of competence restricted to a subpopulation. We showed that ComR abundance is central to bimodality and highlighted the essentiality of a functional positive feedback loop for bimodal activation. Unexpectedly, the system did not activate upon the pheromone *comS* overexpression at ComR native abundance while activating at low synthetic XIP addition (sXIP). To understand such counter-intuitive mechanisms, we aimed to build a deterministic model describing the ComRS system in *salivarius*. Of note, because we characterized thoroughly the ComRS system in *S. salivarius* at the single cell level in the previous chapter, most of the experimental data used to parametrize and validate the model will come from this source.

1. Materials and experimental data

Strains, primers, and methods used for generating experimental data used for the parametrization and validation of the model are described in the Materials and Methods section of the main text. The experimental data used for the modeling are numerically available at the supplementary file at the end of this chapter. The Matlab code and the data used to run the model are available numerically in the supplementary file section at the end of this chapter. Those two files can be run together using Matlab and take approximately 45 min to complete.

2. Key processes in ComRS signalization

The ComRS activation has been shown to mostly rely on ComR abundance [7,24,36]. Also, experimental data shown in chapter 1 have underlined the necessity of a functional positive feedback loop for bimodal activation. In addition, those results showed a low sensitivity of the system regarding ComS (peptide pheromone signal), PptAB (peptide exporter), and Opp (peptide importer) abundance. While those three players are crucial to maintain the functionality of the positive loop, it seems they don't stand as bottlenecks for activation. For this reason, we chose to build a model neglecting the export-processing-reimportation of the peptide by assuming the peptide maturation as a single process. In consequence, we will consider that the mature form of ComS (XIP) is equal to ComS and the ComS term will only be used, except if else stated. The characterization of the formation of

the ComRS complex in *Streptococcus thermophilus* showed a high affinity between ComR and ComS [16,39]. We therefore simulated in the model that the binding between the two players is irreversible, even though the complex can be further degraded after binding.

Finally, because competence induces a cell-division arrest [23], we chose to neglect the dilution of the different players through cellular growth. Since we model the system during, before, and after competence induction, we will have an effect of this dilution. However, we decided to include it in the degradation rate of the different players.

In summary, the different assumptions are:

1. Exportation, maturation, and reimportation of the peptide is neglected (XIP=ComS).
2. ComR and ComS binding is irreversible.
3. Dilution of the players through growth is included in the degradation rate.

3. Mathematical formulation

The model was built using the four main players of the system: ComS (the pheromone peptide), ComR (intracellular receptor), the ComRS complex (formed by two ComS and two ComR) and ComX (alternative sigma factor activating the late phase of competence). Since we had few quantitative data on the ComR control through CovRS, we decided to model the basal abundance of ComR fluctuating over time according to luciferase expression data, independently of any other player. We based mainly our model on two other models described for *S. thermophilus* [36] and *Streptococcus mutans* [38]. Ordinary differential equations (ODEs) of the model were written as follow:

$$\frac{dComR}{dt} = b_R(t) - d_R \cdot ComR(t) - k_{RS}(ComR(t) \cdot ComS(t))^n$$

$$\frac{dComS}{dt} = max_S \left(\left(\frac{ComRS(t)}{ComRS(t) + K_S} \right) + b_S \right) - d_S \cdot ComS(t) - k_{RS}(ComR(t) \cdot ComS(t))^n$$

$$\frac{dComRS}{dt} = \frac{1}{2} k_{RS}(ComR(t) \cdot ComS(t))^n - d_{RS} \cdot ComRS(t)$$

$$\frac{dComX}{dt} = max_X \left(\left(\frac{ComRS(t)}{ComRS(t) + K_X} \right) + b_X \right) - d_X \cdot ComX(t)$$

We modeled the fluctuation in ComR abundance as the result of basal rate production over time, subtracted by its degradation rate and its inclusion into the ComRS complex (through a non-linear mass action law modeled by a Hill equation). ComS amount was modeled as the result of a basal constant production plus the transcriptional activation by ComRS (through a classical Michaelis-Menten equation involving saturation). Similarly to ComR, the negative terms were associated to degradation and inclusion into the ComRS complex. Regarding ComRS, we described its production as the half quantity (since dimeric) of the non-linear association between ComS and ComR minus its degradation. Finally, ComX was computed as the result of a basal rate of production, plus the transcriptional activation through ComRS (Michaelis-Menten kinetics) and minus its degradation. The different parameters, their meaning, corresponding values, and origin are described in the next section.

3.1. Model calibration

With the mathematic model described above, we ended up with a total of 12 parameters. Fortunately, a previous modeling of the system in *S. thermophilus* [36] and experimental data obtained during this work helped us to determine the range of values for each parameter. Table S2.5 at the end of this appendix provides an overview of all the parameters of the model.

3.1.1. Literature-based parametrization

Previous deterministic modeling of the close relative *S. thermophilus* species helped us to determine mostly the degradation parameters: ComR, ComRS and ComX degradation rate (d_R , d_{RS} , d_X). Regarding the degradation rate of ComS (d_S), we chose not to use the previously reported parameter from the *S. thermophilus* model because of contradictory experimental results obtained in *S. salivarius*.

K_S and K_X represent the affinity of the ComRS complex for P_{comS} and P_{comX} , respectively. Because ComR-binding box at the DNA level and its interaction with the ComRS complex are highly conserved between *S. thermophilus* and *S. salivarius* [7,39], we chose to keep an upscaled version of the parameters used in our previous *S. thermophilus* model [36]. Since the amount of ComRS molecules produced in our model in comparison to the *S. thermophilus* model is 322 times higher, we upscaled K_S and K_X by this factor.

We used a Hill coefficient for ComR binding to ComS of two due to the dimeric nature of the binding, which involves two molecules of ComS and two molecules of ComR [16].

3.1.2. Experimental parametrization

Of note, all the experimental luciferase and growth data used in this section ($P_{comS}/P_{comR}/P_{comX}$ -luxAB) and available online were shifted (delayed or brought forward) in time in order to have similar growth curves, allowing us to compute and compare different reporter strains together.

ComR basal rate ($b_R(t)$)

Since competence is a transient state that is mainly depending on ComR abundance, we suspected that *comR* expression is also transient. Transcriptional fusion between the *comR* promoter and a luciferase gene (P_{comR} -luxAB) described in the previous chapter allowed us to monitor the activation of the promoter over time. Indeed, we observed a strong activation at the early stage of exponential phase, with a sharp decrease upon entry into stationary phase (Fig. S2.8A).

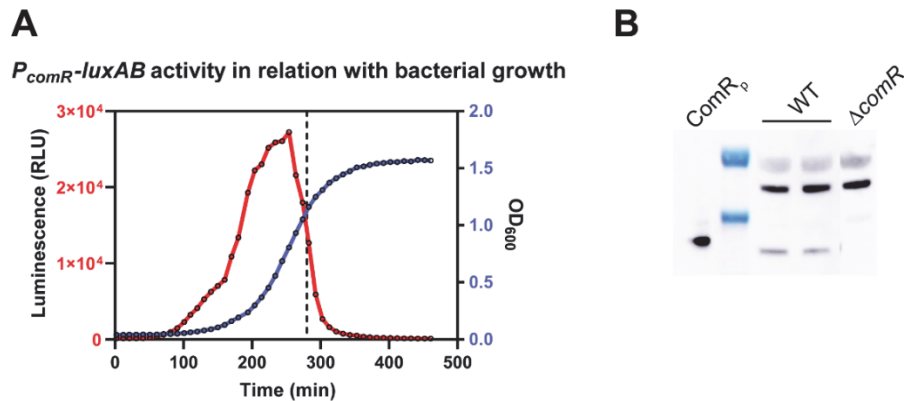


Figure S2.8. **A.** P_{comR} -luxAB activity in comparison to growth. Red and blue lines denote experimental mean values (biological triplicates) of luminescence measurement and optical density at 600nm, respectively. The vertical black dashed line shows the time of sampling for Immunoblotting. **B.** ComR semi-quantification through western blot. The ComR amount in WT was quantified thanks to the recombinant purified CovR_p used as a standard. Number of ComR molecules on the blot were then divided by the number of cells present in the original extract, assuming a complete lysis of the cells.

In parallel, semi-quantification through western blotting experiments on the WT strain gave us an estimate of the amount of ComR (Fig. S2.8B) at a given time (280 min, dashed line on Fig. S2.8A). Combining those two data, we were able to generate an approximation of ComR production over time. We used the same method as previously described [36] for processing luminescence measurement into protein amount production per cell. The

only change that was brought to this method was the choice of the luminescence to protein amount factor based on the western blot semi-quantification (Fig. S2.8B). Of note, this quantification relies on the assumption of a complete lysis of the cell population before blotting. Because this hypothesis is certainly not accurate, this quantification should be taken as a model parametrization and certainly not as an absolute real value.

Maximum synthesis rate of comS (max_s)

We used the same approach for modeling the maximum synthesis of ComS than for the basal synthesis of ComR. In short, we used absorbance data describing growth and luciferase expression of a P_{comS} -*luxAB* strain to model the growth curve and growth rate together with the activity of the P_{comS} promoter upon competence activation. For activating the competence, we overexpressed *comR* thanks to a xylose-inducible promoter fusion (P_{xyI2} -*comR*) present in a neutral locus of the strain by adding xylose at a final concentration of 0.5%.

Since we had no quantification of ComS or XIP available, we used the same RLU_{mol} factor obtained for P_{comR} -*luxAB*. Because we know that transcription efficiency can vary from one fusion to another and that translation properties of a mRNA will influence translation efficiency, we compared the total amount of ComS produced during our modeling with existing data in *S. mutans* [59] for validation (see below). Finally, we calculated the maximum production rate and used it for max_s .

Basal rate of comS (b_s)

As previously described, we first computed the growth curve and growth rate for a non-activating competence population (0% xylose added) and then modeled the ComS synthesis rate thanks to the P_{comR} RLU_{mol} factor previously calculated. We then compared the maximum synthesis rate obtained for the non-activating vs activating competence population to obtain the fold increase in P_{comS} expression upon competence activation. We then used this fold increase (384 fold) to compute the basal rate of ComS synthesis (b_s) such as:

$$\frac{1 + b_s}{b_s} = \text{fold increase}$$

Where 1 represents the maximum P_{comS} activation through the ComRS complex and $(1+b_s)$ is thus the maximum rate synthesis (basal rate and ComRS induction).

Basal rate of *comX* (b_x)

We used exactly the same procedure for b_x as we used for b_s . The max_x parameter used as well as the RLU_{mol} factor were chosen as described in the next free parameter calibration section. We used the same equations as described for b_s computation, and calculated a P_{comX} fold-change between competence activated cells and non-competent activated cells of 95.

3.1.3. Free parameters calibration

To calibrate our model with the three *comS*-related free parameters left describing ComS activity (k_{rs} , max_s , d_s), we used microscopic data showing that *Ssa* cells do not activate *comX* at basal ComR amount ($401 \pm 32,5$ molecules/cell) while they nearly all (95%) activate *comX* at higher ComR amount (3507 ± 713 molecules/cell representing a 8.5-fold increase). We used those two data (0% of cells activated vs ~100%) in a deterministic frame, considering the bacterial population as one entity being either ON (~100%) or OFF (0%). Because the three parameters (k_{rs} , max_s , d_s) influence ComS activity, we calibrated them together, computing the maximum number of ComX produced per simulation, using either low or high ComR values (Fig. S2.9). As we expected the system to be in an ON state (production of ComX) for high ComR values and in an OFF state (no production of ComX) for low ones, we used as output the factor $ComX_{ON/OFF}$:

$$ComX_{ON/OFF} = \text{Max}(ComX_{High\ ComR}(t)) - \text{Max}(ComX_{Low\ ComR}(t))$$

and draw the output as surface values for different parameter values as shown in Figure S2.9. High values of $ComX_{ON/OFF}$ are associated with permissive parameters for bimodality (*i.e.* ComX not activated at basal ComR amount and ComX activated at 8.5-fold basal ComR amount). Of note, higher k_{rs} values can generate a region where $ComX_{ON/OFF}$ has intermediate values meaning that the basal or 8.5-fold basal ComR system is not completely OFF or ON within the range of parameters under consideration. For this reason, we will consider those parameter ranges as non-permissive for bimodality.

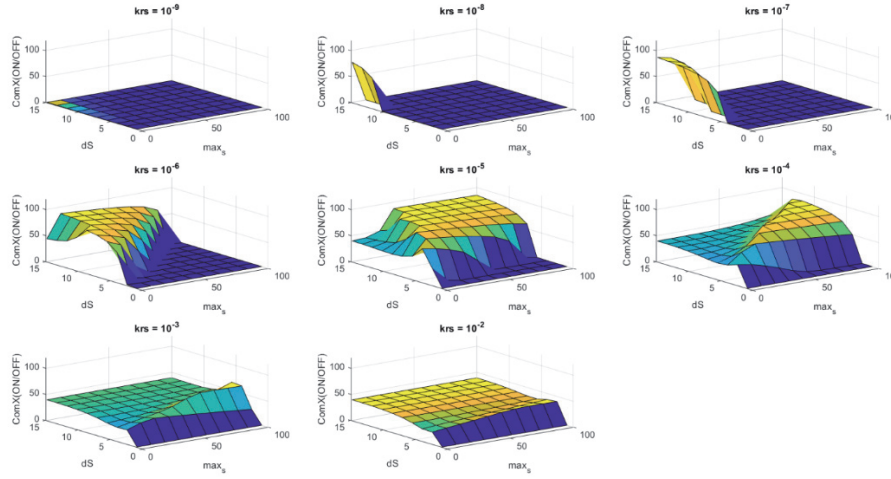


Figure S2.9. Surface plots showing the value of $\text{ComX}_{\text{ON/OFF}}$ for different parameter values of k_{RS} , d_S and max_S . Results show that a bimodal state (meaning high $\text{ComX}_{\text{ON/OFF}}$ values) would only be possible for parameters values ranging from 10^{-8} to 10^{-4} for k_{RS} , and either high d_S rate for low k_{RS} or high max_S for higher k_{RS} .

Using parameter values permissive for bimodal activation (k_{RS} values of 10^{-8} to 10^{-4} , d_S of 5 to 15 min^{-1} and max_S production of 1 to $100 \text{ mol.cell}^{-1}.\text{min}^{-1}$), we were able to generate a non-linear solution resulting in ComRS activation only at 8.5 (or higher) ComR fold-change (Fig. S2.10). Dynamics of the system displayed on the figure S2.10 showed that our model responds in a non-linear fashion and could produce bimodality. Indeed, we had a system turning ON for small increase in ComR basal expression, suggesting that small heterogeneity in the ComR distribution could produce different phenotypes. We used those results to tune the amount of ComX produced when competence was activated based on the amount of ComX boxes found in the genome of *S. salivarius* (~ 150 , [7]). For this purpose, we set the max_X parameter at $40 \text{ mol.cell}^{-1}.\text{min}^{-1}$, resulting in a maximum concentration of $150 \text{ mol.cell}^{-1}$.

While our system showed bimodal properties, the free pheromone ComS produced in the cell was extremely low (max. $\sim 5 \text{ mol/cell}$, Fig. S2.10 panel 3) and not in agreement with previous reports [15,59]. Because the parametrization of max_S , k_{RS} and d_S had shown that a low activity of ComS was necessary for bimodal activation (Fig. S2.9) and that experimental data suggested high k_{RS} and max_S values, we tried to conciliate those observations by re-examining the degradation rate.

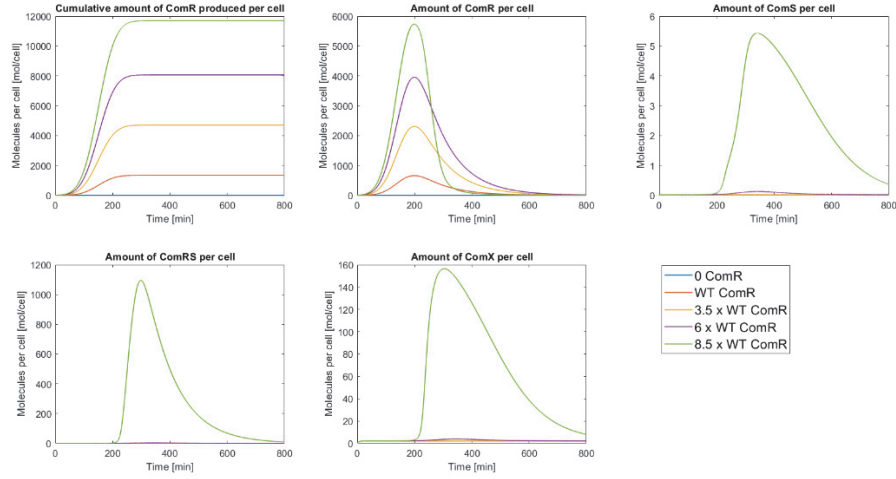


Figure S2.10 Simulation of the four variables of the system (ComR, ComS, ComRS and ComX) over time depending on the ComR concentration. First chart depicts cumulative amount of ComR for the 5 simulations, neglecting ComR degradation and incorporation into the ComRS complex. Second, third, fourth and fifth chart show the evolution over time of the different variables (ComR, ComS, ComRS, and ComX, respectively) regarding the amount of basal ComR computed in the system.

Various types of negative regulation on the activity of the communication pheromone have been described in streptococci: peptide interference [33], transcriptional inactivation [60,61], peptidase degradation [30,40]. Other mechanisms such as specific RNase activity have also been hypothesized [7], suggesting a high diversity of competence control mechanisms through peptide negative regulation. Therefore, we hypothesized the presence of an additional negative player of the system, which is degrading ComS. We aimed to model the presence of a constitutively expressed player (named *deg*) that would reduce the basal amount of ComS. In parallel, this new player had to be quickly saturated by ComS upon ComRS activation in order to allow high ComS concentration in competence activated cells. We modeled this *deg* saturating activity through a new Michaelis-Menten equation term in the ComS equation:

$$-k_{cat} \times deg \times \frac{ComS}{ComS + K_{deg}}$$

Where k_{cat} is the apparent constant rate of degradation of ComS through the negative regulator, *deg* is the constant *deg* concentration due to constitutive

expression, and K_{deg} is the ComS concentration required for half max degradation of ComS.

We implemented this new term in our model and searched for permissive values for bimodal activation with parameters $d_s = 0.01 \text{ min}^{-1}$, $max_s = 1000 \text{ mol.cell}^{-1}.\text{min}^{-1}$ and $k_{rs} = 0.01$ (d_s and k_{rs} values were chosen based on the previous simulation [36]. The max_s value was chosen to approach literature data [59], suggesting ComS production upon competence activation in the nanomolar to micromolar range. This value was also chosen in accordance with recent data published on the rate quantification of bacterial translation synthesis [62].

We used the same approach described before to generate surface plot showing bimodal landscape (data not shown) but using instead $ComS_{ON/OFF}$ in function of two parameters values: $k_{cat} \times deg$ and K_{deg} . $ComS_{ON/OFF}$ was used in order to have an idea of the maximum ComS values for activating cells. Because computation time were far too long for low K_{deg} values permissive for bimodal activation, we decided to keep low k_{rs} values ($10^{-6} \text{ cell}^3.\text{mol}^{-3}.\text{min}^{-1}$). We realized that those two parameters (K_{deg} and k_{rs}) were responsible for the same effect on the model: increasing the ComS production necessary for activating the system. While low K_{deg} has much more biological significance than k_{rs} regarding our *in vitro* ComRS binding data, low K_{deg} was too difficult to compute in our model.

The parameter landscape regarding $ComS_{ON/OFF}$ (thus bimodality) showed that only some particular values were permissive for bimodality. Because low K_{deg} was necessary for bimodality and low $k_{cat} \times deg$ could provide a fair number of ComS molecules produced when competence is activated, we hypothesized that the properties of the negative player would directly influence the bimodal behavior. While high ComS affinity for the degradation regulator could provide a bimodal activation through efficient low ComS degradation, fast saturation can account for the high ComS production in competence activating cells.

Then, we used this new set of equations to model competence over time in relation to ComR abundance. Regarding previous parametrization, we decided to use $k_{cat} \times deg = 140 \text{ mol.cell}^{-1}.\text{min}^{-1}$ and $K_{deg} = 1 \text{ mol.cell}^{-1}$ for computations (Fig. S2.11). As expected, we could not see any competence activation for lower values than $8.5 \times \text{ComR}$. However, unlike our previous model (showing low level of unbound ComS when competence was activated), we could reach about 60 000 molecules per cell of ComS in the

activating state. Because this amount would correspond to a nanomolar range of concentration in the growth medium, we concluded that our degradation model described more accurately the features of the ComRS system.

Of note, the ComS amount at the late stage of growth in our model is still important (Fig. S2.11 panel 3), probably because we did not include a specific shut-off mechanism of the system. We designed our model with this typology because we were mainly interested in using the simplest model as possible describing bimodal activation and were lacking experimental data regarding shut-off mechanisms at this point of the research.

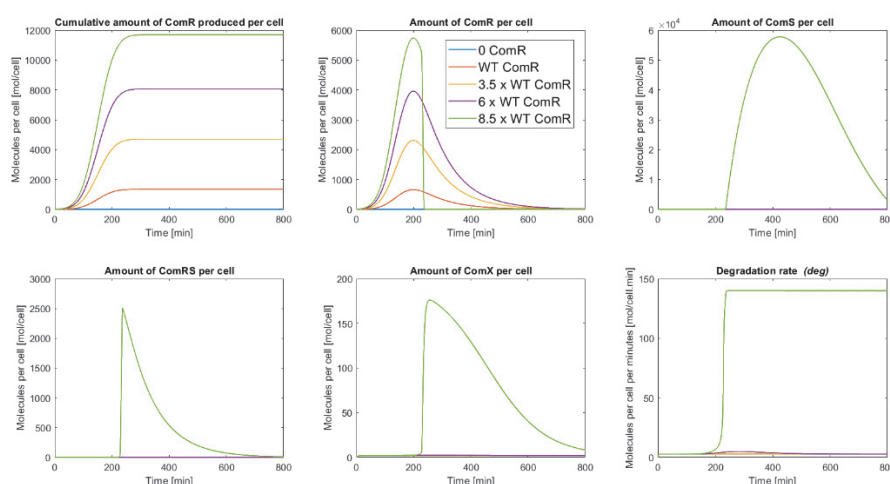


Figure S2.11. Modeling of the four variables of the system (ComR, ComS, ComRS and ComX) and the degradation rate of ComS over time depending on ComR concentration. First chart depicts cumulative amount of ComR for the 5 simulations, neglecting ComR degradation and incorporation into the ComRS complex. Second, third, fourth and fifth charts show the evolution over time of the different variables (ComR, ComS, ComRS, ComX and the degradation rate, respectively) regarding the amount of basal ComR computed in the system. Only the 8.5-times ComR increase triggers ComS, ComRS and ComX production and saturates fastly the degradation mechanism (panel 6).

3.2. Validation

We then sought to validate our model with a set of experimental data. To do this, we determined if our model could predict the percentage of cells activating competence in ComR overexpressing strains (see previous chapter).

To this aim, we first determined the hypothetical maximum and minimum basal ComS production. For the minimum value, we used the fact that an 8.5-fold increase in WT ComR abundance is the threshold for the activation of the whole population. It means that all the cells in this situation, even the ones with the lowest basal ComS values, are able to trigger the system. We therefore determined that the minimum basal ComS production for the population was $b_s = 0.0026$, the value used in our simulation until now.

To compute the maximum basal ComS value, we used our microscopy data together with ComR semi-quantitative immunoblotting showing that a 1.27-fold ComR increase results in the first cells to activate competence.

We computed the basal ComS value necessary to obtain activation at a threshold of 1.27-fold ComR increase. We found a value of 0.03 for activating the system. We therefore hypothesized that the ComS basal distribution within the population should range from 0.0026 to 0.03, which corresponds to a basal rate synthesis of ComS of 2.6 to 31 mol.cell⁻¹.min⁻¹. Of note, because experimental data obtained in the previous chapter showed that ComR is homogeneously distributed in the population, we neglected the effect of small variations in ComR abundance from cell to cell. This approximation seems relevant, as weakly expressed genes such as *comS* show higher noise in expression [63]. Nevertheless, this assumption was not proven experimentally in *S. salivarius*, and still needs to be validated.

Recent theoretical and single molecule studies have shown that the random distribution of proteins can be modeled as a simple gamma function, knowing the mean and the dispersion of the protein amount in the population [38,64]. We considered the rough assumption that b_s is directly linked to the basal ComS concentration and we modeled this basal rate as a gamma function using single-cell data to have an idea of the dispersion and the mean of b_s . For this purpose, we analyzed the fluorescence of a $P_{comS}\text{-}gfp^+$ strain for competence non-activated cells (Fig. S2.12). Those cells were displaying only non-activated *comS* promoters, which correspond to basal ComS production. We first computed the Fano factor (F) where μ stands for the mean and σ^2 for the variance.

$$F = \frac{\sigma^2}{\mu}$$

Then, we used those values to generate a gamma distribution of the fluorescent intensity of the $P_{comS-luxAB}$ population thanks to the equation [64]:

$$p(x) = \frac{x^{a-1} \cdot e^{-\frac{x}{b}}}{\Gamma(a) \cdot b^a}$$

Where Γ is a gamma function, a is the inverse of the noise, and b is the Fano factor of the fluorescent distribution. With this equation, we generated the probability density function and the cumulative density function of the fluorescence intensity, as shown on the Figure S2.12.

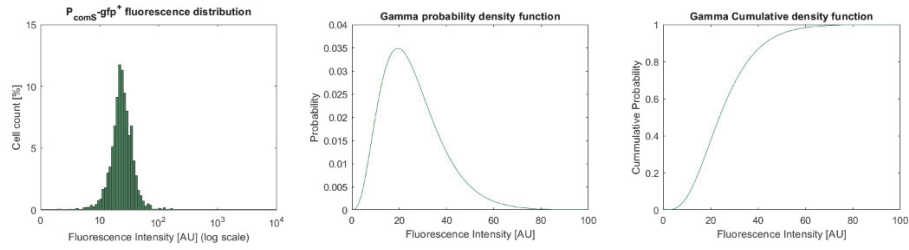


Figure S2.12. ComS distribution within the population. Left panel shows the experimental values obtained from a $P_{comS-gfp^+}$ strain for a competence non-activated population. Central and left panels show the associated gamma distribution.

Knowing that extreme values of fluorescence intensity would be associated to extreme values of predicted basal rates, we used the lower and upper quantile of the cumulative gamma function ($\alpha=0.05$) and computed their corresponding intensities (8.8 and 50, respectively). Using the maximum and minimum basal rate of ComS ($b_{Smin}=0.0026$ and $b_{Smax}=0.03$), we were able to scale the fluorescence intensity to the basal rate thanks to the equation:

$$I = b_S \times \frac{(I_{max}-I_{min})}{(b_{Smax}-b_{Smin})} [AU]$$

We then computed the critical b_{Scrit} values for competence activation for several ComR concentrations and computed the associated I_{crit} factor thanks to the scaling equation (Fig. S2.13). Finally, we reported the I_{crit} value computed on the gamma cumulative density function (Fig. S2.12 panel 3) for every ComR fold increase to determine the percentage of cells with higher intensities than the I_{crit} value. This percentage represents cells with sufficient ComS basal activity to trigger the system. We were then able to generate a chart depicting the percentage of activated cells in the populations as a

function of ComR abundance and compared it to experimental data obtained (Fig. S2.13 panel 2).

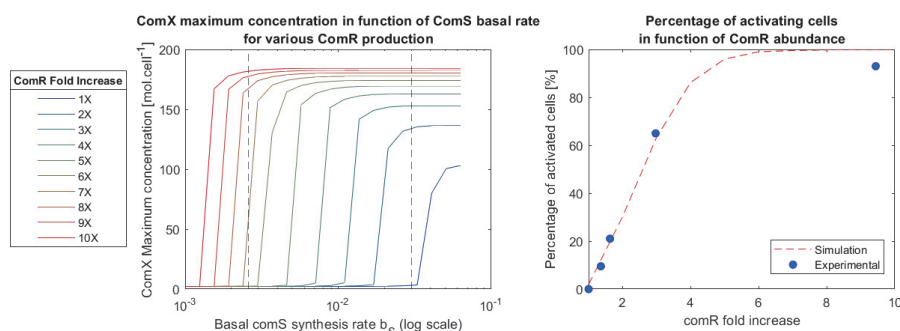


Figure S2.13. Model validation using percentage of competence-activated cells for various ComR concentrations. **A.** ComX maximum concentration computed in function of ComS basal rate for various ComR-fold increase. **B.** Subsequent percentage of cells activating competence determined based on the inferred gamma distribution of b_S (Fig. S2.12 panel 3). Dashed lines in panel A depict threshold values for b_S (0.0026 and 0.03).

The comparison between experimental values and simulation showed that our model was able to describe quantitatively the bimodal competence activation through the ComRS system.

3.2.1. Competence bimodality and *comR* heterogeneity

Because our system has shown to be extremely ComR sensitive, we thought that although its distribution in the population is quite homogeneous (see experimental data on $P_{comR-gfp}^+$, $P_{comR-comR::gfp}^+$ and XIP population-wide dose in the previous chapter) it was possible that small uneven ComR distribution in the population could account for bimodality. To validate this hypothesis, we challenged our model with a similar approach as performed with the *comS* distribution. First, we generated a gamma distribution fitted to the $P_{comR-gfp}^+$ data as previously described for *comS* (Fig. S2.14).

We first generated a 1.27-fold gamma distribution representing the ComR distribution in an overexpressing strain harboring a xylose-inducible promoter fused to *comR* and induced with 0.1% of xylose. To do this, we multiplied the mean and the standard deviation of our experimental data in the WT by 1.27 and generated a new fano factor and a noise parameter that we used to compute the gamma distribution of the 1.27-fold ComR amount strain. This strategy implies that the new distribution generated has the same mean over standard deviation ratio. We could reasonably make this

assumption since $P_{xyl2}\text{-}gfp^+$ data obtained suggested homogeneous P_{xyl2} activation (Fig. S1.2). and would therefore only increase ComR amount without changing its distribution among the population

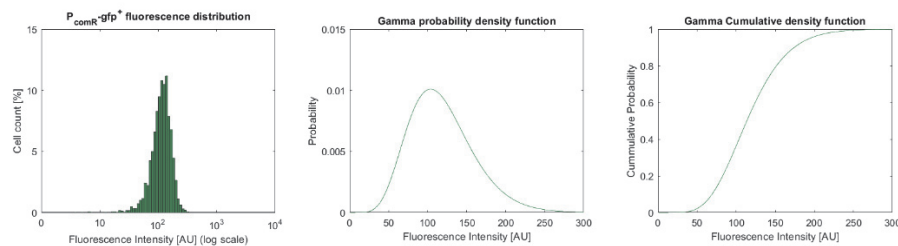


Figure S2.14. ComR distribution within *S. salivarius* population. Left panel shows the experimental values obtained from a $P_{comR}\text{-}gfp^+$ strain for a competence non-activated population. Central and left panel show the associated gamma distribution.

We next used the 0.90 quantile value (corresponding to the 10% of cells activated measured experimentally) of the 1.27-fold distribution generated and defined it as the critical value for activation. This value corresponds to a 1.9-fold ComR change.

We next generated several ComR gamma distribution for different fold changes and computed the number of cells activated with the threshold (1.9-fold) defined. We then compared the computed percentages obtained with experimental values (Figure S2.15).

Figure S2.15 shows clearly that ComR heterogeneity can be sufficient to explain bimodal activation of competence. Nevertheless, the parametrization used to obtain the fit presented in this figure must be considered for interpreting the validation. Indeed, the threshold determined based on experimental values is quite low (1.9-fold ComR increase). For triggering the system at this ComR concentration, high basal rate of ComS is needed (about $40 \text{ mol}\cdot\text{cell}^{-1}\cdot\text{min}^{-1}$) which is not in accordance with experimental data, suggesting low *comS* expression. Since the model suggests that both ComS and ComR heterogeneity could explain the bimodal activation of competence, it is most likely that heterogeneity on both partners is at the core of the stochastic process giving rise to bimodality.

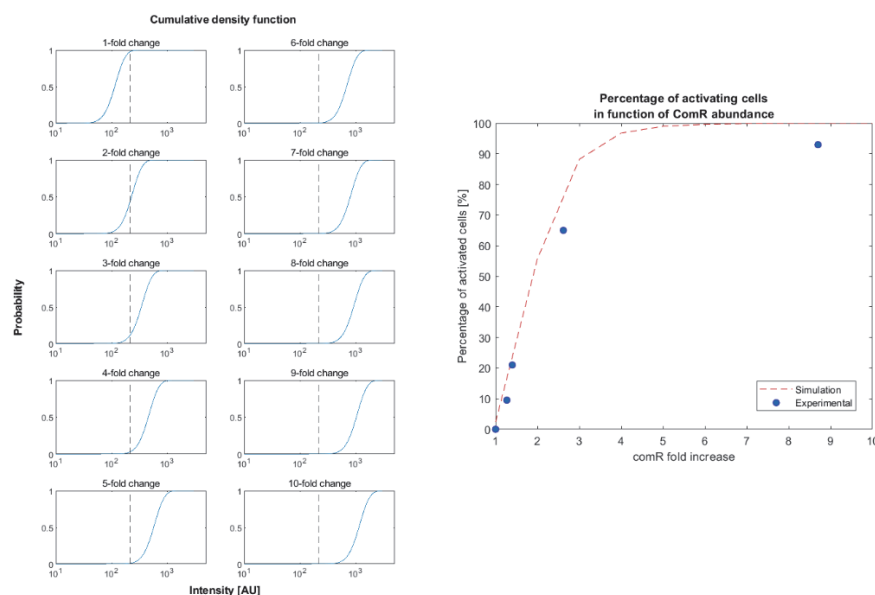


Figure S2.15. Validation of the model based on a ComR noisy expression as a source for bimodal competence. Left panel shows the computed gamma cumulative distribution based on experimental $P_{\text{comR-gfp}^+}$ data. The distribution is shifted to higher intensities values to simulate ComR overexpression. Dashed line denotes the ComR threshold value for competence activation previously computed. Right panel shows the simulated (red dashed line) vs the experimental percentage of activated cells (blue dots) for different values of ComR fold increase (regarding WT).

3.3. XIP₅ addition vs *xip* overexpression prediction

Now that we had performed parametrization and validation, we could explore the model to ask biological relevant questions. We first investigated puzzling experimental results obtained regarding pheromone-based competence activation in *S. salivarius*. Particularly, the addition of nanomolar concentration of synthetic sXIP (sXIP) is able to trigger the system while the overexpression of the 12-amino acid matured form of ComS (*xip*) fails to activate competence. In order to evaluate if our model could explain such unexpected behavior, we modeled the addition of sXIP by a sharp and sudden increase in ComS concentration and modeled the *xip* overexpression by a constant ComS production over time. Of note, we will still consider in this section that XIP and ComS are equivalent.

Since recent investigations have shown the high processivity of the Opp transporter in *B. subtilis* [65], we assumed that addition of XIP in the nanomolar concentration range would result in a fast uptake by the cells

within a few minutes. Because our luciferase experimental data show an activation of the ComRS complex after 5 min upon sXIP addition, we modeled the total uptake taking place within 15 minutes and reaching a maximum rate after 5 minutes. In parallel, experimental sXIP titration with $P_{comX}\text{-}gfp^+$ strains showed a threshold activation at a ~ 1 nM concentration (see Fig. 1.2). Since we do not know the processivity of the Opp transporter and the resulting intracellular concentration in sXIP, we performed a simulation for sXIP uptake gradient, starting with a maximum value and considering that every molecule added to the medium is imported by the cells. To model this, we assumed that at $t = 120$ min, XIP concentration in the medium reach suddenly 10^{-9} M. Direct uptake through the Opp transporter results in an intracellular concentration given by the equation:

$$[XIP]_{cell} = \frac{[XIP]_{out}}{X(t=120)} [\text{mol.cell}^{-1}]$$

Where $[XIP]_{cell}$ is the intracellular concentration of XIP, $[XIP]_{out}$ is the extracellular concentration of sXIP added and $X(t=120)$ is the bacterial concentration in the medium at the time $t = 120$ min. Because a complete uptake of every single sXIP molecule present in the medium by the cells is rather unrealistic, we chose a range of total amount of XIP_s uptaken by the cell weaker than the one calculated for a complete uptake with a sXIP concentration in the medium of 10^{-9} M. We then generated Figure 9, showing the effect of sXIP addition on the different variables of the system.

We found an intracellular concentration of $1.5 \mu\text{M}$ (500 molecules per cell) sufficient to overcome the degradation rate through the degradation machinery (*deg*) and trigger competence. Regarding the experimental data showing an effect starting at 1 nM, it would suggest that the Opp system is quite efficient.

To investigate and compare the effect of an intracellular overexpression of *xip*, we modeled a constant production of ComS over time and checked how the overexpression was able to activate the system (Fig. S2.17).

We then computed the total amount of ComS produced until competence was activated ($t = 225$ min for the highest *xip* overexpression) through *xip* overexpression and compare it to the sXIP quantity necessary for competence activation. We found a much higher ComS value produced through overexpression ($6469 \text{ mol.cell}^{-1}$) than through sXIP addition ($500 \text{ mol.cell}^{-1}$). This difference is explained by the discrepancy between the two modes of ComS production. In the first one, the overexpression is gradual

and rather slow, giving the time for the negative player *deg* to degrade it. By contrast, sXIP addition results in a sharp increase of ComS, directly overcoming the *deg* capacity. Of note, those speculations are based on the suggested high processivity of the Opp transporter [65]. Nevertheless, regarding the experimental reactivity of the system to sXIP addition, this can reasonably be hypothesized.

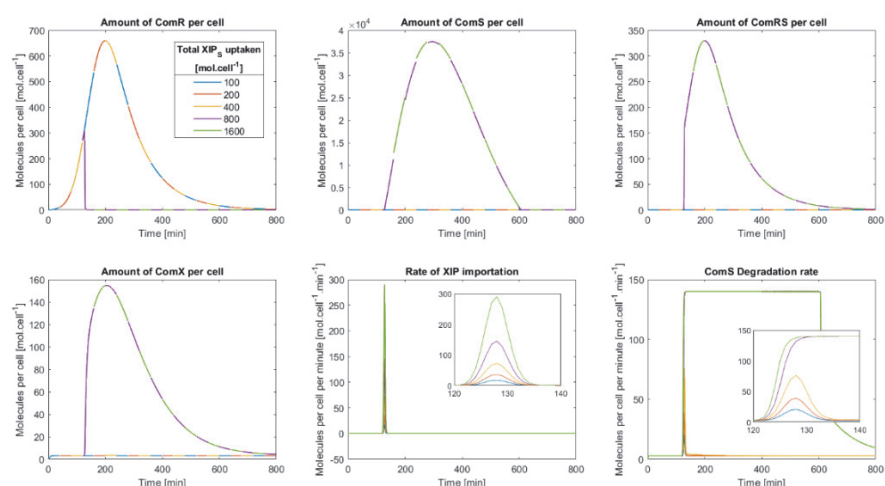


Figure S2.16. Modeling of sXIP importation efficiency and its effect on competence activation. ComR, ComS, ComRS, ComX abundance, XIP importation rate, and ComS degradation rate were computed over time depending on sXIP uptake. Total amount of sXIP molecules imported through the Opp transporter are depicted by color (blue=100, orange=200 ComR, yellow=400, purple =800 and green=1600 mol.cell⁻¹). Those values correspond to an intracellular XIP concentration of 300, 600, 1200, 2400 and 4800 nM.

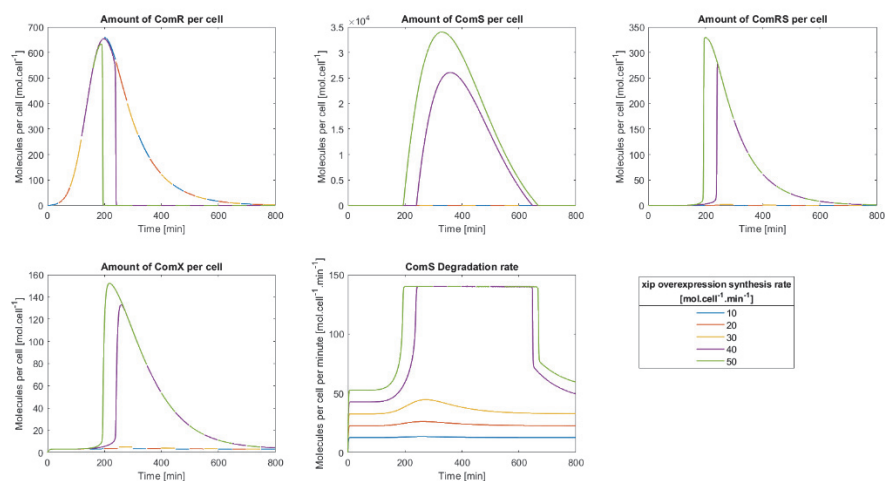


Figure S2.17. Modeling of xip overexpression and its effect on competence activation. ComR, ComS, ComRS, ComX abundance, and ComS degradation rate, were computed over time depending on the xip overexpression level. Computed XIP synthesis rates are depicted by color (blue=15, orange=20 ComR, yellow=25, purple =30 and green=35 mol.cell⁻¹). Those values correspond to a cumulative intracellular ComS concentration of 2250, 3656, 5063, 6469 and 7875 mol.cell⁻¹, respectively.

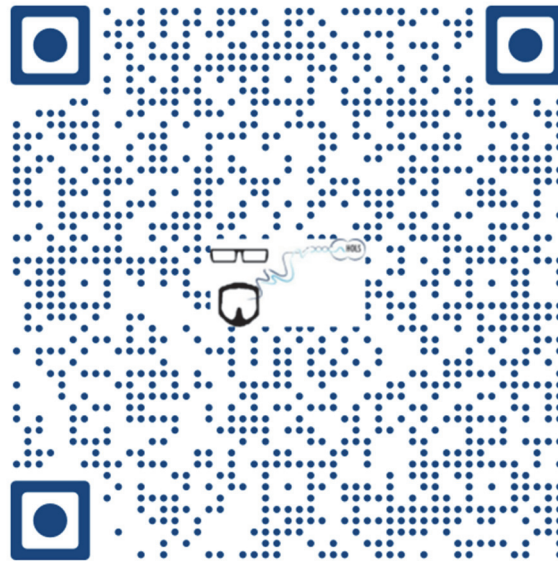
Table S2.5. Final parameters values used in the model

Parameter	Description	Value	Unit	Origin
$b_R(t)$	Basal synthesis rate of ComR (fluctuates over time)	0-12	[mol.cell ⁻¹ .min ⁻¹]	Experimental
d_R	ComR degradation rate constant	0.01	[min ⁻¹]	[36]
k_{RS}	Complex formation rate constant	10 ⁻⁶	[cell ³ .mol ⁻³ .min ⁻¹]	Free parameter
n	Hill coefficient	2	[]	[16]
max_S	Maximum synthesis rate of ComS upon ComRS activation	1000	[mol.cell ⁻¹ .min ⁻¹]	Free parameter
K_S	Required concentration of ComRS for half maximum synthesis rate of ComS	166	[mol.cell ⁻¹]	[36]
b_S	Basal synthesis rate of ComS constant	0.0026-0.031	[]	Experimental & Free parameter
d_S	ComS degradation rate constant	0.01	[min ⁻¹]	Free parameter
d_{RS}	ComRS degradation rate constant	0.01	[min ⁻¹]	[36]
max_X	Maximum synthesis rate of ComX upon ComRS activation	60	[mol.cell ⁻¹ .min ⁻¹]	Free parameter
K_X	Required concentration of ComRS for half maximum synthesis rate of ComX	322	[mol.cell ⁻¹]	[36]
b_X	Basal synthesis rate of ComX constant	0.0107	[]	Experimental
d_X	ComX degradation rate constant	0.2	[min ⁻¹]	[36]
$k_{cat} \times deg$	Basal number of XIP-peptidase molecules times specific degradation rate	140	[mol.cell ⁻¹ .min ⁻¹]	Free parameter
K_{deg}	Required concentration of ComS for half maximum degradation rate	1	[mol.cell ⁻¹]	Free parameter

SUPPLEMENTARY FILES

Supplementary numeric files necessary to run the model are available following the QR code or the web link. Those files include:

- The experimental data used to run the model (Dataset1.xlsx)
- The Matlab code (ssa_model.m)



(<https://drive.google.com/drive/folders/11PXYfBxdZUB6AhsQejifqHqGWx1Ve9n2?usp=sharing>)

REFERENCES

- [1] Huang, R., Li, M., Gregory, R.L., 2011. Bacterial interactions in dental biofilm. *Virulence*: 435–44, Doi: 10.4161/viru.2.5.16140.
- [2] Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., et al., 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486(7402): 207–14, Doi: 10.1038/nature11234.
- [3] Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., et al., 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526(7575): 719–22, Doi: 10.1038/nature15524.
- [4] Shanker, E., Federle, M.J., 2017. Quorum sensing regulation of competence and bacteriocins in *Streptococcus pneumoniae* and mutants. *Genes*, Doi: 10.3390/genes8010015.
- [5] Son, M., Ghoreishi, D., Ahn, S.-J., Burne, R.A., Hagen, S.J., 2015. Sharply Tuned pH Response of Genetic Competence Regulation in *Streptococcus* mutants: a Microfluidic Study of the Environmental Sensitivity of comX. *Applied and Environmental Microbiology* 81(16): 5622–31, Doi: 10.1128/AEM.01421-15.
- [6] Reck, M., Tomasch, J., Wagner-Döbler, I., 2015. The Alternative Sigma Factor SigX Controls Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in *Streptococcus* mutants. *PLoS Genetics* 11(7), Doi: 10.1371/journal.pgen.1005353.
- [7] Mignolet, J., Fontaine, L., Sass, A., Nannan, C., Mahillon, J., Coenye, T., et al., 2018. Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*. *Cell Reports* 22(7): 1627–38, Doi: 10.1016/j.celrep.2018.01.055.
- [8] Wang, C.Y., Dawid, S., 2018. Mobilization of Bacteriocins during Competence in *Streptococci*. vol. 26, pp. 389–91.
- [9] Veening, J.W., Blokesch, M., 2017. Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria. *Nature Reviews Microbiology*: 621–9, Doi: 10.1038/nrmicro.2017.66.
- [10] Fontaine, L., Wahl, A., Fléchar, M., Mignolet, J., Hols, P., 2015. Regulation of competence for natural transformation in streptococci. *Infection, Genetics and Evolution* 33: 343–60, Doi: 10.1016/j.meegid.2014.09.010.
- [11] Håvarstein, L.S., Coomaraswamy, G., Morrison, D.A., 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* 92(24): 11140–4, Doi: 10.1073/pnas.92.24.11140.
- [12] Lee, M.S., Morrison, D.A., 1999. Identification of a new regulator in *Streptococcus pneumoniae* linking quorum sensing to competence for genetic transformation. *Journal of Bacteriology* 181(16): 5004–16.

- [13] Lingeswaran, A., Metton, C., Henry, C., Monnet, V., Juillard, V., Gardan, R., 2020. Export of Rgg Quorum Sensing Peptides is Mediated by the PptAB ABC Transporter in *Streptococcus Thermophilus* Strain LMD-9. *Genes* 11(9): 1096, Doi: 10.3390/genes11091096.
- [14] Fontaine, L., Boutry, C., Henry De Frahan, M., Delplace, B., Fremaux, C., Horvath, P., et al., 2010. A Novel Pheromone Quorum-Sensing System Controls the Development of Natural Competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. *JOURNAL OF BACTERIOLOGY* 192(5): 1444–54, Doi: 10.1128/JB.01251-09.
- [15] Gardan, R., Besset, C., Gitton, C., Guillot, A., Fontaine, L., Hols, P., et al., 2013. Extracellular life cycle of ComS, the competence-stimulating peptide of *Streptococcus thermophilus*. *Journal of Bacteriology* 195(8): 1845–55, Doi: 10.1128/JB.02196-12.
- [16] Talagas, A., Fontaine, L., Ledesma-García, L., Mignolet, J., Li de la Sierra-Gallay, I., Lazar, N., et al., 2017. Structural Insights into Streptococcal Competence Regulation by the Cell-to-Cell Communication System ComRS. *Annual Reviews of Genetics* 12(12): e1005980, Doi: 10.1371/journal.ppat.1005980.
- [17] Blokesch, M., 2017. In and out—contribution of natural transformation to the shuffling of large genomic regions. *Current Opinion in Microbiology*: 22–9, Doi: 10.1016/j.mib.2017.04.001.
- [18] Dowson, C.G., Hutchison, A., Woodford, N., Johnson, A.P., George, R.C., Spratt, B.G., 1990. Penicillin-resistant viridans streptococci have obtained altered penicillin-binding protein genes from penicillin-resistant strains of *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* 87(15): 5858–62, Doi: 10.1073/pnas.87.15.5858.
- [19] Dowson, C.G., Coffey, T.J., Kell, C., Whiley, R.A., 1993. Evolution of penicillin resistance in *Streptococcus pneumoniae*; the role of *Streptococcus mitis* in the formation of a low affinity PBP2B in *S. pneumoniae*. *Molecular Microbiology* 9(3): 635–43, Doi: 10.1111/j.1365-2958.1993.tb01723.x.
- [20] Pletz, M.W.R., McGee, L., Van Beneden, C.A., Petit, S., Bardsley, M., Barlow, M., et al., 2006. Fluoroquinolone resistance in invasive *Streptococcus pyogenes* isolates due to spontaneous mutation and horizontal gene transfer. *Antimicrobial Agents and Chemotherapy* 50(3): 943–8, Doi: 10.1128/AAC.50.3.943-948.2006.
- [21] Balsalobre, L., Ferrándiz, M.J., Liñares, J., Tubau, F., De la Campa, A.G., 2003. Viridans group streptococci are donors in horizontal transfer of topoisomerase IV genes to *Streptococcus pneumoniae*. *Antimicrobial Agents and Chemotherapy* 47(7): 2072–81, Doi: 10.1128/AAC.47.7.2072-2081.2003.
- [22] Bergé, M.J., Mercy, C., Mortier-Barrière, I., Vannieuwenhze, M.S., Brun, Y. V., Grangeasse, C., et al., 2017. A programmed cell division delay preserves genome integrity during natural genetic transformation in *Streptococcus pneumoniae*. *Nature Communications* 8(1), Doi: 10.1038/s41467-017-01716-9.
- [23] Zaccaria, E., Wells, J.M., Baarlen, P. Van., 2016. Metabolic Context of the Competence- Induced Checkpoint for Cell Replication in *Streptococcus suis*. *PLOS One* 11(5): 1–13, Doi: 10.1371/journal.pone.0153571.

- [24] Knoops, A., Vande Cappelle, F., Fontaine, L., Verhaeghen, M., Mignolet, J., Goffin, P., et al., 2022. The CovRS environmental sensor directly controls the ComRS signaling system to orchestrate competence bimodality in *Salivarius streptococci*. MBio (in press).
- [25] Lisboa, J., Andreani, J., Sanchez, D., Boudes, M., Collinet, B., Liger, D., et al., 2014. Molecular determinants of the DprA-RecA interaction for nucleation on ssDNA. Nucleic Acids Research 42(11): 7395–408, Doi: 10.1093/nar/gku349.
- [26] Mirouze, N., Bergé, M.A., Soulet, A.-L., Mortier-Barrière, I., Quentin, Y., Fichant, G., et al., 2013. Direct involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of pneumococcal competence. Proceedings of the National Academy of Sciences of the United States of America 110(11): E1035-44, Doi: 10.1073/pnas.1219868110.
- [27] Weng, L., Piotrowski, A., Morrison, D.A., 2013. Exit from competence for genetic transformation in *Streptococcus pneumoniae* is regulated at multiple levels. PloS One 8(5): e64197, Doi: 10.1371/journal.pone.0064197.
- [28] Johnston, C.H.G., Soulet, A.L., Bergé, M., Prudhomme, M., Lemos, D. De., Polard, P., 2020. The alternative sigma factor σ^X mediates competence shut-off at the cell pole in *streptococcus pneumoniae*. ELife 9: 1–27, Doi: 10.7554/eLife.62907.
- [29] Martin, B., Soulet, A.L., Mirouze, N., Prudhomme, M., Mortier-Barrière, I., Granadel, C., et al., 2013. ComE/ComE~P interplay dictates activation or extinction status of pneumococcal X-state (competence). Molecular Microbiology 87(2): 394–411, Doi: 10.1111/mmi.12104.
- [30] Underhill, S.A.M., Shields, R.C., Burne, R.A., Hagen, S.J., 2019. Carbohydrate and PepO control bimodality in competence development by *Streptococcus mutans*. Molecular Microbiology 112(5): 1388–402, Doi: 10.1111/mmi.14367.
- [31] Mashburn-Warren, L., Goodman, S.D., Federle, M.J., Prehna, G., 2018. The conserved mosaic prophage protein paratox inhibits the natural competence regulator ComR in *Streptococcus*. Scientific Reports 8(1): 16535, Doi: 10.1038/s41598-018-34816-7.
- [32] Rutbeek, N.R., Rezasoltani, H., Patel, T.R., Khajepour, M., Prehna, G., 2021. Molecular mechanism of quorum sensing inhibition in *Streptococcus* by the phage protein paratox. Journal of Biological Chemistry 297(3): 100992, Doi: 10.1016/j.jbc.2021.100992.
- [33] Kaspar, J., Shields, R.C., Burne, R.A., 2018. Competence inhibition by the XrpA peptide encoded within the *comX* gene of *Streptococcus mutans*. Molecular Microbiology 109(3): 345–64, Doi: 10.1111/mmi.13989.
- [34] Kaspar, J., Ahn, S.J., Palmer, S.R., Choi, S.C., Stanhope, M.J., Burne, R.A., 2015. A unique open reading frame within the *comX* gene of *Streptococcus mutans* regulates genetic competence and oxidative stress tolerance. Molecular Microbiology 96(3): 463–82, Doi: 10.1111/mmi.12948.
- [35] Son, M., Kaspar, J., Ahn, S.J., Burne, R.A., Hagen, S.J., 2018. Threshold regulation and stochasticity from the MecA/ClpCP proteolytic system in *Streptococcus mutans* competence. Molecular Microbiology 110(6): 914–30, Doi: 10.1111/mmi.13992.

- [36] Haustenne, L., Bastin, G., Hols, P., Fontaine, L., 2015. Modeling of the ComRS signaling pathway reveals the limiting factors controlling competence in *Streptococcus thermophilus*. *Frontiers in Microbiology* 6(DEC), Doi: 10.3389/fmicb.2015.01413.
- [37] Van Den Bogert, B., Boekhorst, J., Herrmann, R., Smid, E.J., Zoetendal, E.G., Kleerebezem, M., 2013. Comparative genomics analysis of *Streptococcus* isolates from the human small intestine reveals their adaptation to a highly dynamic ecosystem. *PLoS ONE* 8(12): 83418, Doi: 10.1371/journal.pone.0083418.
- [38] Son, M., Ahn, S.-J., Guo, Q., Burne, R.A., Hagen, S.J., 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of comX. *Molecular Microbiology* 86(2): 258–72, Doi: 10.1111/j.1365-2958.2012.08187.x.
- [39] Ledesma-Garcia, L., Thuillier, J., Guzman-Espinola, A., Ensink, I., de la Sierra-Gallay, I.L., Lazar, N., et al., 2020. Molecular dissection of pheromone selectivity in the competence signaling system ComRS of streptococci. *Proceedings of the National Academy of Sciences of the United States of America* 117(14): 7745–54, Doi: 10.1073/pnas.1916085117.
- [40] Wilkening, R. V., Chang, J.C., Federle, M.J., 2016. PepO, a CovRS-controlled endopeptidase, disrupts *Streptococcus pyogenes* quorum sensing. *Molecular Microbiology* 99(1): 71–87, Doi: 10.1111/mmi.13216.
- [41] Rawlings, N.D., Barrett, A.J., Thomas, P.D., Huang, X., Bateman, A., Finn, R.D., 2018. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Research* 46(D1): D624–32, Doi: 10.1093/nar/gkx1134.
- [42] Desai, B. V., Morrison, D.A., 2006. An unstable competence-induced protein, CoiA, promotes processing of donor DNA after uptake during genetic transformation in *Streptococcus pneumoniae*. *Journal of Bacteriology* 188(14): 5177–86, Doi: 10.1128/JB.00103-06.
- [43] Nardi, M., Renault, P., Monnet, V., 1997. Duplication of the pepF gene and shuffling of DNA fragments on the lactose plasmid of *Lactococcus lactis*. *Journal of Bacteriology* 179(13): 4164–71, Doi: 10.1128/jb.179.13.4164-4171.1997.
- [44] Kanamaru, K., Stephenson, S., Perego, M., 2002. Overexpression of the PepF oligopeptidase inhibits sporulation initiation in *Bacillus subtilis*. *Journal of Bacteriology* 184(1): 43–50, Doi: 10.1128/JB.184.1.43-50.2002.
- [45] Kleine, L.L., Monnet, V., Pechoux, C., Trubuil, A., 2008. Role of bacterial peptidase f inferred by statistical analysis and further experimental validation. *HFSP Journal* 2(1): 29–41, Doi: 10.2976/1.2820377.
- [46] Vimr, E.R., Green, L., Miller, C.G., 1983. Oligopeptidase-deficient mutants of *Salmonella typhimurium*. *Journal of Bacteriology* 153(3): 1259–65, Doi: 10.1128/jb.153.3.1259-1265.1983.
- [47] Jiang, X., Zhang, M., Ding, Y., Yao, J., Chen, H., Zhu, D., et al., 1998. *Escherichia coli* prlC Gene Encodes a Trypsin-Like Proteinase Regulating the Cell Cycle. *Journal of Biochemistry* 124(5): 980–5, Doi: 10.1093/oxfordjournals.jbchem.a022216.

- [48] Monnet, V., Nardi, M., Chopin, A., Chopin, M.C., Gripon, J.C., 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. *Journal of Biological Chemistry* 269(51): 32070–6, Doi: 10.1016/s0021-9258(18)31602-8.
- [49] Kawasaki, A., Nakano, H., Hosokawa, A., Nakatsu, T., Kato, H., Watanabe, K., 2010. The exquisite structure and reaction mechanism of bacterial Pz-peptidase a toward collagenous peptides: X-ray crystallographic structure analysis of Pz-peptidase a reveals differences from mammalian thimet oligopeptidase. *Journal of Biological Chemistry* 285(45): 34972–80, Doi: 10.1074/jbc.M110.141838.
- [50] Lin, B., Averett, W.F., Novák, J., Winn Chatham, W., Hollingshead, S.K., Coligan, J.E., et al., 1996. Characterization of PepB, a group B streptococcal oligopeptidase. *Infection and Immunity* 64(8): 3401–6, Doi: 10.1128/iai.64.8.3401-3406.1996.
- [51] Mignolet, J., Fontaine, L., Kleerebezem, M., Hols, P., 2016. Complete genome sequence of *Streptococcus salivarius* HSISS4, a human commensal bacterium highly prevalent in the digestive tract. *Genome Announcements* 4(1), Doi: 10.1128/genomeA.01637-15.
- [52] Letort, C., Juillard, V., 2001. Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. *Journal of Applied Microbiology* 91(6): 1023–9, Doi: 10.1046/j.1365-2672.2001.01469.x.
- [53] Fontaine, L., Dandoy, D., Boutry, C., Delplace, B., de Frahan, M.H., Fremaux, C., et al., 2010. Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in *Streptococcus thermophilus*. *Applied and Environmental Microbiology* 76(23): 7870–7, Doi: 10.1128/AEM.01671-10.
- [54] Dower, W.J., Miller, J.F., Ragsdale, C.W., 1988. High efficiency transformation of *E.coli* by high voltage electroporation. *Nucleic Acids Research* 16(13): 6127–45, Doi: 10.1093/nar/16.13.6127.
- [55] Liu, X., Gallay, C., Kjos, M., Domenech, A., Slager, J., van Kessel, S.P., et al., 2017. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Molecular Systems Biology* 13(5): 931, Doi: 10.15252/msb.20167449.
- [56] Dorrazehi, G.M., Worms, S., Chirakadavil, J.B., Mignolet, J., Hols, P., Soumillion, P., 2020. Building Scarless Gene Libraries in the Chromosome of Bacteria. In: Iranzo, OlgaRoque, A.C.A., editor. *Peptide and Protein engineering*, Springer US p. 189–211.
- [57] Fontaine, L., Goffin, P., Dubout, H., Delplace, B., Baulard, A., Lecat-Guillet, N., et al., 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. *Molecular Microbiology* 87(6): 1113–32, Doi: 10.1111/mmi.12157.
- [58] Oberto, J., 2013. SyntTax: A web server linking synteny to prokaryotic taxonomy. *BMC Bioinformatics* 14(1), Doi: 10.1186/1471-2105-14-4.
- [59] Ricomini Filho, A.P., Khan, R., Åmdal, H.A., Petersen, F.C., 2019. Conserved Pheromone Production, Response and Degradation by *Streptococcus mutans*. *Frontiers in Microbiology* 10: 2140, Doi: 10.3389/fmicb.2019.02140.
- [60] Schnorpfel, A., Kranz, M., Kovács, M., Kirsch, C., Gartmann, J., Brunner, I., et al., 2013. Target evaluation of the non-coding csRNAs reveals a link of the two-component regulatory system CiaRH to competence control in *Streptococcus pneumoniae* R6. *Molecular Microbiology* 89(2): 334–49, Doi: 10.1111/mmi.12277.

- [61] Laux, A., Sexauer, A., Sivaselvarajah, D., Kaysen, A., Brückner, R., 2015. Control of competence by related non-coding csRNAs in *Streptococcus pneumoniae* R6. *Frontiers in Genetics* 6(JUL): 246, Doi: 10.3389/fgene.2015.00246.
- [62] Li, G.-W., Burkhardt, D., Gross, C., Weissman, J.S., 2014. Quantifying absolute protein synthesis rates reveals principles underlying allocation of cellular resources. *Cell* 157(3): 624–35, Doi: 10.1016/j.cell.2014.02.033.
- [63] Elowitz, M.B., Levine, A.J., Siggia, E.D., Swain, P.S., 2002. Stochastic Gene Expression in a Single Cell. *Science* 297(5584): 1183–6, Doi: 10.1126/SCIENCE.1070919.
- [64] Taniguchi, Y., Choi, P.J., Li, G.W., Chen, H., Babu, M., Hearn, J., et al., 2010. Quantifying *E. coli* proteome and transcriptome with single-molecule sensitivity in single cells. *Science* 329(5991): 533–8, Doi: 10.1126/science.1188308.
- [65] Babel, H., Naranjo-Meneses, P., Trauth, S., Schulmeister, S., Malengo, G., Sourjik, V., et al., 2020. Ratiometric population sensing by a pump-probe signaling system in *Bacillus subtilis*. *Nature Communications* 11(1), Doi: 10.1038/s41467-020-14840-w.

CHAPTER III

A CRISPRi screen reveals a StkP-mediated connection between cell-wall integrity and competence in *Streptococcus salivarius*

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AK and PH conceived and designed the study. The laboratory work was carried out by AK (Fig. 3.1-3.6, S3.1-3.3, Table 3.1-3.3), AW (pre-data), ML (pre-data) and BD (pre-data). AK and PH analyzed and interpreted the data. AK and PH wrote and revised the manuscript.

The previous chapter was focused on the study of a specific shut-off mechanism in *Streptococcus salivarius* involving a negative feedback loop based on the peptidase PepF. Nevertheless, the knock-down of the peptidase was not sufficient to completely abolish competence exit, suggesting additional shut-off mechanisms.

To screen for other shut-off players, we decided to design a method allowing the selection or screening of essential genes, unlike the classical Tn-seq method. Indeed, *pepF* was a glaring example of an essential gene involved in the shut-off and we did not want to miss such other players. To this aim, we decided to develop a genome-wide CRISPR-interference screen.

We first introduced a library of high-density guides into three different reporter strains allowing three selection/screening methods. The first screen is based on spontaneous natural transformation upon competence-antagonist gene inhibition. The second screen is taking advantage of the toxicity of competence overactivation in the case of an interference on a shut-off gene. This property was used to monitor by Next-Generation Sequencing (NGS) the depletion of guides associated to a toxic phenotype when competence is induced. The third old-fashioned but still efficient screen is based on the activity of a β -galactosidase reporter under the control of the promoter of *comX*. The library was screened on X-gal plates and dark blue phenotypes were associated to shut-off impairment and longer competence activation.

The efficacy of the three different screens were all confirmed by the identification of known inhibitors/activators of the ComRS system such as the previously identified shut-off gene *pepF*. Strikingly, the three methods identified several key genes involved in cell envelope biogenesis, including peptidoglycan, extracellular polysaccharides, wall teichoic acids and lipoteichoic acids synthesis pathways. Furthermore, several key TCS involved in the sensing of cell-wall damages and the serine threonine kinase StkP were identified.

All those data suggested that an environmental sensor could sense cell-wall impairment and transmit this information to activate competence. Since we found several hits of the serine threonine kinase StkP in the screens, we investigated more deeply its role in competence regulation. Using CRISPR-interference, we were able to show that StkP inhibits *comX* transcription while having no effect on *comR* transcription. We also demonstrate that StkP can sense specific cell-wall debris, suggesting a shut-off mediated by the cell-wall processing.

Although this study was initially designed to screen only shut-off players, we uncovered an unknown link between cell envelope defects and competence activation. This connection sheds light on the importance of competence as a survival strategy triggered in stress conditions. Furthermore, the three different screens have revealed several other key genes that would be involved in competence regulation. Future functional investigations of those genes will probably deepen our understanding of competence regulation in streptococci.

ABSTRACT

Combining production of antibacterial compounds and integration of DNA released by dead cells, competence is one of the most efficient bacterial survival strategies. In most streptococci, this tactic is orchestrated by the ComRS system, a pheromone communication device providing subpopulation competence initiation and a limited time window of activation. Understanding how this apparatus integrates multiple inputs to fine-tune the adequate response is a long-standing question. Particularly, essential genes involved in the regulation of ComRS have been challenging to study. In this work, we built a conditional mutant library using the CRISPR-interference technology and developed three distinct screens to investigate competence genetic regulation in *Streptococcus salivarius*, a major human commensal species. All screens highlighted a competence increase upon cell-wall impairment, suggesting a connection between envelope stress and competence activation. Besides, we report that the serine-threonine kinase StkP which regulates cell-wall assembly, controls competence by a mechanism involving muropeptides sensing. Together, those data suggest a key muropeptide sensing mechanism triggering competence as a stress response to cell-wall impairment.

In the human digestive tract, bacteria face highly competitive pressure and physico-chemical inconstancy. Surviving in this environment requires powerful and efficient strategies which must be tightly controlled and collectively coordinated [1–3]. Quorum sensing (QS) devices are particularly suited to control concerted survival tactics since they perform bacterial density sensing. Initially thought to be restricted to this role, recent evidences suggest that QS systems can operate as autocrine modules and process multiple inputs [4]. On the one hand, QS autocrine signaling allows heterogeneity amplification by positive feedback loop, a key feature for sub-populations activation [5–7]. On the other hand, environmental stimuli can fine-tune the sensitivity of the pheromone-based apparatus [8,9]. This latter property is switching the QS opinion from a cell-density to a multi-input device, integrating diverse stimuli to calibrate population-wide strategies [4].

One of the best characterized QS system in Gram-positive is probably competence regulation [10]. Orchestrating predation through bacteriocin production together with natural transformation, competence is regulated by two types of signaling systems in streptococci [11]. The ComCDE system found in the mitis and anginosus groups relies on the sensing of the extracellular pheromone CSP (Competence stimulating peptide) that induces a phosphorelay leading to transcriptional activation of several genes comprising *comX*, the master competence regulator [12]. The alternative predominant system in streptococci is based on the production of the pheromone XIP (ComX-inducer peptide), which can be internalized by the Opp transporter and binds the intracellular receptor ComR [13,14]. Subsequently, the dimeric ComR·XIP complex activates several genes including *comX* [15,16].

Uncovering the environmental triggers allowing permissive conditions for competence QS has remained challenging in streptococci [17]. Since two-components systems (TCS) and serine-threonine kinases (STK) are dedicated to sense the outside world, they constitute attractive targets to couple environmental stimuli to QS reactivity. In *Streptococcus pneumoniae*, several of those sensors (StkP, CiaRH, VicRK) have been highlighted to control the ComCDE activity upon pH, O₂, cell density or antibiotic stresses [8,18–22]. In the cariogenic *Streptococcus mutans* species, other distal regulators have been highlighted such as ScnRK, HdrM, BrsRM, CiaRH or StkP and a link between competence activation and various conditions (pH, carbohydrate source, oxygen, cell density) have been established [23–30]. Besides, we uncovered in the first chapter of this work a direct link between the CovRS environmental sensor and the ComRS signaling system in salivarius streptococci [7]. As exemplified by those three cases, despite environmental

triggers can be shared, environmental sensors bridging detection to competence can be highly divergent in between species.

To investigate key sensors generating permissive conditions for competence activation, genome-wide screens are the fastest and most-suited approaches. While Tn-seq strategies have already revealed several regulators in *S. mutans* and *S. pneumoniae* [31,32], classical knock-out characterization of the identified genes is often impaired by their essentiality. Recently, a CRISPR-interference (CRISPRi) genome wide screen method was shown to overcome this drawback [33]. This technique combines the use of a guide RNA library targeting the whole genome together with a catalytically dead mutant of Cas9 (dCas9), producing transcription interference upon DNA binding. Plugging the dCas9 under the control of an inducible promoter allows the construction of a conditional mutant library which can be used for genetic screens and further knock-down characterization of essential genes [33,34].

In this work, we used this technique in combination with three distinct screens to investigate positive and negative unknown competence regulators. Cross-validation of the hits obtained for the three screens converged to a connection between cell-wall biogenesis impairment and competence activation. Besides, several sensors of the bacterial envelope integrity were identified among which StkP, suggesting a putative signaling pathway bridging cell-wall stresses to competence activation.

RESULTS

Genome-wide CRISPRi inhibition and screening for spontaneous transformation

To identify unknown negative players of competence in *S. salivarius* HSISS4 [35], we designed a genome-wide CRISPRi screen. We fostered the use of this technique over classical Tn-seq for its genetic conditional inactivation feature allowing the study of essential genes. To design guides on the whole genome of HSISS4, we subset all the 20 nt sequences followed by a protospacer adjacent motif (PAM, NGG sequence) and retained only the guides targeting the coding strand of CDSs and both strands in intergenic regions [33]. We ended up with a total of 83.103 guides (Table S3.1 in supplementary files) that were introduced under the control of a P₃ constitutive promoter in a chromosomal neutral locus. The random chromosomal distribution of the guides in the library was validated by the direct sequencing of 40 individual clones (Fig. S3.1A).

The transfer of the library was initially performed in a strain carrying a lactose-inducible dCas9 ($P_{F6-lacI}$; $P_{lac-dcas9}$) (Fig. 3.1).

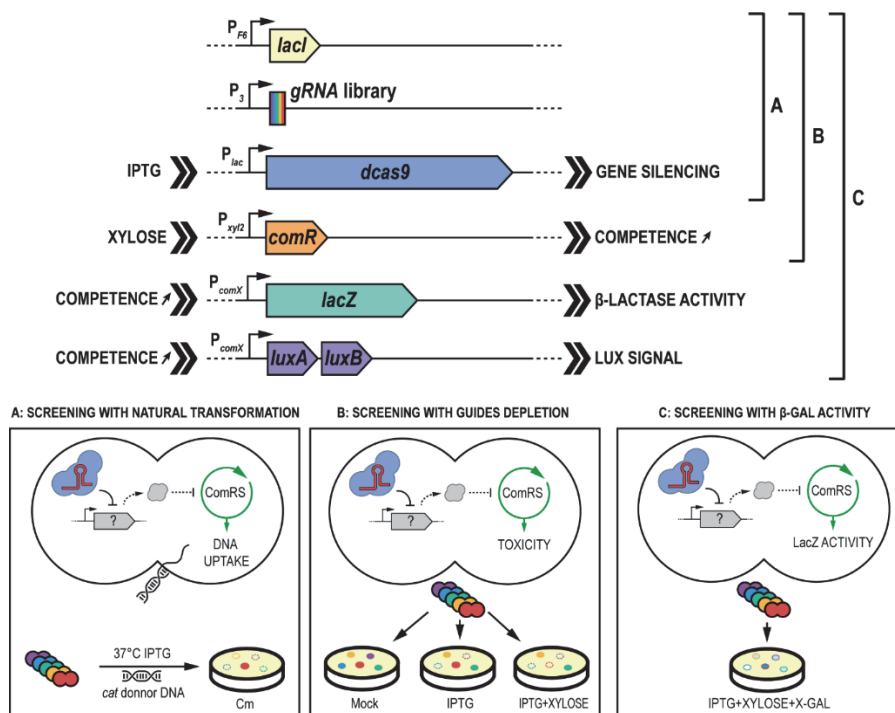


Figure 3.1. CRISPRi screening strategy for identification of shut-off actors of competence in *S. salivarius*. A library of gRNA was designed and introduced (P_3 -gRNA) in an engineered strain of *S. salivarius* harboring an IPTG-inducible system for *dcas9* ($P_{F6-lacI}$; $P_{lac-dcas9}$). A first library was screened for spontaneous competence activation upon *dcas9*-inhibition, by growing cells in chemically defined medium in presence of IPTG and *cat* donor DNA. The selection on chloramphenicol plate was associated with competence negative actors inhibition (A). A second library was generated by introducing the gRNA library into the same background with a supplemental construct consisting of a xylose-incucible promoter fused to *comR* ($P_{xyl2-comR}$). The library was spread on a control (mock), a gRNA library-induced (IPTG) or a gRNA library and competence induced (IPTG+xylose) plates. NGS analysis of guides depletion in the three conditions was performed to search for genes essentiality only associated to competence (B). A third library was built by adding *lacZ* under the control of P_{comX} ($P_{comX-lacZ}$) together with a competence luciferase reporter system ($P_{comX-luxAB}$) to the previous strain and transferring the gRNAs library into this background. The generated library was screened on plates containing IPTG, xylose and X-gal. Guides targeting potential shut-off or competence necessary genes were associated to dark blue or white phenotypes, respectively (C).

This first strain was next screened for the activation of spontaneous natural transformation. We hypothesized that dCas9-mediated repression of genes involved in competence inhibition would result in spontaneous natural

transformation and donor DNA integration. We activated the interference library by adding IPTG (dCas9 activation) to a liquid culture supplemented with donor DNA containing a chloramphenicol resistance cassette and after 8 hours of incubation, we plated the induced library onto chloramphenicol plates. Integration of the donor DNA in transformant candidates was confirmed by PCR and the guides were sequenced to identify their targets. After 3 independent rounds of selection, we were able to isolate 16 candidates, all harboring a different guide (Table 3.1). Finally, to confirm the phenotype generated by these guides, we back-transformed them individually into the original strain harboring the dCas9 module and assessed their transformability. Spontaneous transformation was confirmed for 10 candidates (Table 3.1). Importantly, the screen succeeded to identify two previously described negative effectors of competence acting on ComX or XIP stability (*clpC* and *pepF*, respectively) [36,37]. Besides, other players inhibition such as rRNAs, a bactoprenol glucosyltransferase, and 3 hypothetical proteins leading to spontaneous transformation were identified with no previous reported link with competence regulation. Specifically, interference on the putative bactoprenol glucosyltransferase (HSISS4_01391) resulted in a high transformation rate (3.4×10^{-2}) suggesting an important role of this player for natural transformation.

Screening based on guide's depletion

This strategy is based on the assumption that since competence overactivation is toxic in HSISS4 [38], repression of genes involved in competence inhibition would produce a fitness cost that will result in guides depletion targeting the corresponding genes. To set up this strategy, a second screening strain was generated by introducing a supplemental construct consisting of a xylose-inducible *comR*, allowing competence activation upon xylose addition [7] (Fig. 3.1).

After introducing the guide's library into this strain, we spread it on three different solid culture conditions. The first condition (#1) without any inducer was used as control (mock). The second condition (#2) was induced with IPTG alone to activate the CRISPRi library and the third condition (#3) was supplemented with IPTG and xylose to concomitantly activate the CRISPRi library and competence. We hypothesized that we could identify "competence essential genes" by comparing the guide's depletion between conditions #2 and #3.

Table 3.1. gRNA identification in spontaneous transformants

<i>Guide ID</i>	<i>Genomic position</i>	<i>Interference target</i>	<i>Gene name</i>	<i>Locus tag</i>	<i>Comment/function</i>	<i>Transformation rate</i>
<i>g_30</i>	1823312	Gene		HSISS4_01622	Hypothetical protein	2.00E-06
<i>g_27</i>	1589000	Gene		HSISS4_01391	Bactoprenol glucosyltransferase	3.40E-02
<i>g_31</i>	714120	Gene		HSISS4_00663	Extracellular nuclease 2	N.D ^a
<i>g_32</i>	875880	Gene		HSISS4_00805	Hypothetical protein	5.80E-04
<i>g_35</i>	1442100	Gene		HSISS4_01302	Hypothetical protein	4.00E-06
<i>g_26</i>	112760	Gene	<i>gpmB</i>	HSISS4_00092	Phosphoglycerate mutase	N.D
<i>g_37</i>	83910	Gene	<i>clpC</i>	HSISS4_00061	ComX degradation machinery ClpC	2.00E-06
<i>g_38</i>	85320	Gene	<i>clpC</i>	HSISS4_00061	ComX degradation machinery ClpC	6.00E-06
<i>g_39</i>	412742	Gene	<i>pepF</i>	HSISS4_00369	Oligoendopeptidase F	4.00E-06
<i>g_40</i>	1270227	Gene	<i>scuR</i>	HSISS4_01166	Intracellular receptor bacteriocin-related communication system	N.D
	1272924	Gene	<i>sarF</i>	HSISS4_01169	Intracellular receptor bacteriocin-related communication system	N.D
<i>g_41</i>	1775841	Gene	<i>pepXP</i>	HSISS4_01580	Dipeptidyl - peptidase	N.D
<i>g_42</i>	499523	Gene	<i>carB</i>	HSISS4_00444	Carbamoyl synthase	N.D
<i>g_43</i>	664598	Intergenic				N.D
<i>g_33</i>	multiple sites	Ribosomal RNA			16S ribosomal RNA	4.00E-06
<i>g_34</i>	multiple sites	Ribosomal RNA			16S ribosomal RNA	2.00E-06
<i>g_36</i>	multiple sites	Ribosomal RNA			16S ribosomal RNA	2.00E-06

^a N.D: Not detected

To this aim, we performed deep sequencing to quantify each guide abundance per condition. We first validated the randomness and homogeneity of the guides distribution without any selection pressure (condition #1) by visualizing the mapping of the guides on the genome of HSISS4 (Fig. S3.1B), which validated our previous Sanger-sequencing data (Fig. S3.1A). We next used the MAGeCK algorithm [39] to compare depletion of guides within genes from one condition to another. As expected, guides depletion analysis of condition #2 in comparison to the control condition #1

uncovered well-known essential genes in streptococci (Table S3.2 in supplementary files and Fig. S3.2A), as well as competence-related genes (e.g. *covR*, *pepF*) whose inactivation was shown to be lethal in strain HSISS4 [7,37]. We next performed the same analysis for the comparison of condition #3 with the mock (Table S3.3 in supplementary files and Fig. S3.2B) and plotted against each other the two scores obtained from the two comparisons (i.e. #2 vs #1 and #3 vs #1) (Fig. S3.3). As expected, depletion scores in the two comparisons displayed high correlation showing that genes essentiality was conserved with or without competence activation (linear regression of $R^2=0.9653$) but several outliers were present. Because the outliers represent essential genes in one condition but not in the other, we analyzed the standardized residuals of the linear regression (Fig. 3.2A and Table S3.4 in supplementary files). We set up an arbitrary cut-off at +2.5 and -2.5 to identify outliers and highlighted several genes with a putative downregulation effect on competence (Std residuals < -2.5, Table 3.2) such as the ComX degradation machinery adaptor *mecA*. Unexpectedly, several crucial genes for competence activation (*comR*, *amiACDEF*) or competence-based bacteriocin production (*slvX*) were also selected. In HSISS4, competence, bacteriocins and bacteriocin-immunity genes are concomitantly activated [38]. Therefore, those genes might have been selected because a lack of competence goes along with a lack of bacteriocin immunity, ultimately leading to a high fitness cost due to fratricide.

Screening based on β -galactosidase activity

The last screening strategy was based on β -galactosidase activity that allows the evaluation of competence activation level in individual clones on plates. For this purpose, a strain was generated by plugging the promoter of *comX* in front of the native *lacZ* gene together with a P_{comX} luciferase reporter system (P_{comX} -*luxAB*) into the previous screening strain (Fig. 3.1). We transformed the guide's library into this genetic background and spread it onto M17GL plates supplemented with IPTG, xylose, and X-gal for detection of β -galactosidase activity. We examined ~94 000 isolated colonies, searching for white and dark blue phenotypes. While white phenotype is associated to guides targeting genes disrupting competence activation, dark blue phenotype is related to gene inhibition producing higher competence activation. We next re-isolated the selected colonies to confirm their phenotypes and ended up with 141 dark blue and 68 white phenotype clones. We sequenced their guides to identify the interference target and quantified their guide inhibition effect on P_{comX} activation thanks to the P_{comX} -*luxAB* module present in the strain. To this aim, we slightly overexpressed

comR with xylose thanks to the P_{xyI2} -*comR* module and induced the guide-related inhibition system by adding IPTG. We compared the specific luciferase activity of all the selected clones to the initial strain harboring no guide RNA. The sequence of the guides, their identified target and their fold change in P_{comX} activation are displayed in the Table S3.5 (in supplementary files).

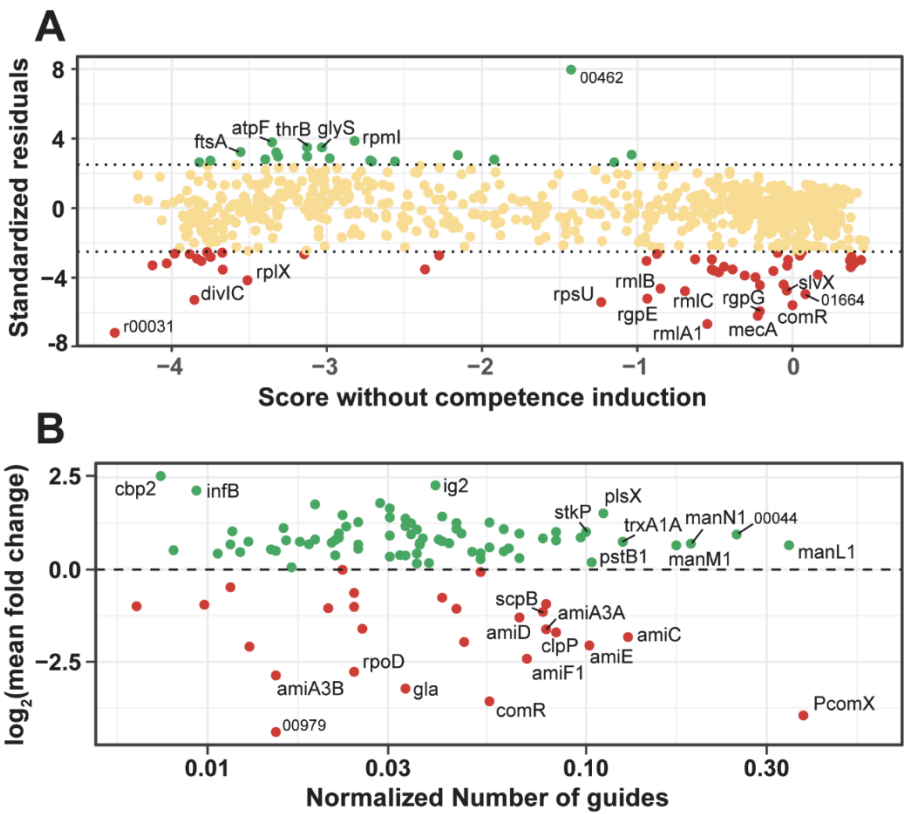


Figure 3.2. CRISPRi screen guides selection. **A. Guides depletion screen.** The guide's library was spread on M17G with no induction (i) and *dcas9* library inhibition induction without (ii) or with competence induction (iii). Bacteria were grown on plate for 16 hours at 37°C, yielding an average of 12 generations. After genomic extraction, the locus containing the guides was amplified by PCR and sequenced by Illumina. Four technical replicates were analyzed per conditions, yielding a total of 40M reads mapped thanks to the MAGeCK-VISPR algorithm [39]. Using the same bioinformatic tool, we compared both (ii) and (iii) to the (i) condition to identify lethal guides depletion linked to (ii) library induction and (iii) library induction plus competence induction. We then compared the guides depletion scores for each gene in both induction systems and performed a linear regression. Standardized residuals of the regression were then computed and plotted in function of the score of each gene in the condition without competence induction (ii). Positive (green) and negative (red) standardized residuals denote guides enriched or depleted, respectively, when competence is activated.

We used an arbitrary limit of 2.5 and -2.5 for standardized residual to define outlier genes. Genes within this range are considered as non-significative (yellow). **B. β -gal screen.** After library production, $\sim 10^5$ colonies were screened for dark blue phenotypes and white phenotypes on M17GL plates supplemented with IPTG, xylose, and X-gal thanks to the reporter system P_{comX} -*lacZ*. A total of 158 dark blue phenotypes and 155 white phenotypes were re-isolated for phenotype confirmation and luciferase test (P_{comX} -*luxAB*) was performed in comparison with the parental strain harboring no gRNA. Guides with the most dissimilar luciferase phenotypes were sequenced for guide identification. The plot's y-axis displays the mean fold change log value of luciferase activity computed on all the guides targeting the same gene. The x-axis displays the number of guides targeting the same gene normalized by the potential total number of guides present in the library for this gene. Green dots and red dots denote gene inhibition resulting in competence overexpression or repression, respectively.

Since both frequency of selected guides targeting the same gene and fold change in P_{comX} activation were relevant features to identify new competence regulators, we combined those two parameters in the same analysis. On one hand, we computed the mean fold change in P_{comX} activation for all guides targeting the same gene. On the other hand, we counted the number of selected guides targeting the same gene. In addition, we normalized the count by the total hypothetical number of guides of the library targeting each defined gene to avoid any gene-size bias (the probability to encounter a guide targeting a high-size gene is higher) (Table S3.6 in supplementary files). We plotted those two values (activation fold-change vs normalized guide frequency) (Fig. 3.2B) and validated the screen by finding most of the proximal effectors of the ComRS system: *comR*, *amiACDEF*, *clpC* and P_{comX} .

We next applied an arbitrary cut-off (normalized count > 0.02 and $\log_2(\text{FC}) > 0.5$) to find the most significative genes with antagonist activity towards competence (Table 3.3). We thereby selected several players whose competence inhibition was suggested also with the guides depletion screen, such as the phosphate transporter (*pstB*) and the serine-threonine kinase (*stkP*). Strikingly, the mannose/glucosamine PTS transporter (*manLMN* operon) was particularly overrepresented, even though absent from the previous screens. This result might be an artefact consequence of a carbon metabolism shift enhancing xylose uptake ultimately resulting in higher *comR* induction, but could also be due to a link between mannose and competence, as reported in *S. mutans* [30].

Table 3.2. Identification of competence guides depleted genes

Gene name	Locus tag	Comment/function	Fitness-cost score without competence induction ^a	Std residuals ^b
	HSISS4_r00031	tRNA Met	-4.3663	-7.1637
<i>rmlA1</i>	HSISS4_00723	Rhamnose synthesis protein	-0.5488	-6.6618
<i>mecA</i>	HSISS4_00128	ComX degradation machinery adaptative protein	-0.2235	-6.1869
<i>rgpG</i>	HSISS4_00129	Rhamnan extracellular synthesis protein	-0.2101	-5.9241
<i>comR</i>	HSISS4_00217	Competence intracellular receptor	0.0004	-5.5756
<i>rpsU</i>	HSISS4_01396	Small subunit rRNA	-1.2325	-5.4007
<i>divlC</i>	HSISS4_00008	Cell division protein	-3.8533	-5.2690
<i>rgpE</i>	HSISS4_01379	Rhamnan extracellular synthesis protein	-0.9354	-5.2037
	HSISS4_01664	Hypothetical protein	0.0831	-4.9400
<i>rmlC</i>	HSISS4_00724	Rhamnose synthesis protein	-0.6924	-4.7668
<i>slvX</i>	HSISS4_01665	Bacteriocin	-0.0369	-4.7340
<i>rmlB</i>	HSISS4_00725	Rhamnose synthesis protein	-0.8507	-4.6220
<i>rgpF</i>	HSISS4_01378	Rhamnan extracellular synthesis protein	-0.2094	-4.4280
<i>prfB</i>	HSISS4_00848	Peptide chain release factor	-0.0572	-4.3906
<i>rplX</i>	HSISS4_01812	Large subunit rRNA	-3.5117	-4.1461
	HSISS4_00889	Membrane protein involved in the export of O-antigen, teichoic acids, lipoteichoic acids	-0.2386	-3.9719
<i>dltB</i>	HSISS4_01110	D-alanyl transfer protein	-0.3073	-3.8741
	HSISS4_00523	Hypothetical protein	0.1635	-3.8180
<i>dltC</i>	HSISS4_01109	D-Alanine phosphoribitol ligase subunit 2	-0.4759	-3.6841
<i>amiE</i>	HSISS4_01362	Oligopeptide ABC transporter, ATP-binding subunit E	-0.4741	-3.6834
<i>pgmA</i>	HSISS4_01102	Phosphoglucomutase	-0.1227	-3.6094
<i>amiC</i>	HSISS4_01364	Oligopeptide ABC transporter, permease subunit C	-0.5067	-3.5877
<i>rplM</i>	HSISS4_00076	Large subunit rRNA	-3.6706	-3.5315
<i>amiD</i>	HSISS4_01363	Oligopeptide ABC transporter, permease subunit D	-0.5198	-3.5283
<i>dltA</i>	HSISS4_01111	D-Alanine phosphoribitol ligase subunit 1	-0.3858	-3.5209
<i>rgpA2</i>	HSISS4_01383	Rhamnan extracellular synthesis protein	-2.3673	-3.5178
<i>pstC2</i>	HSISS4_00937	Phosphate transport, permease protein	0.3755	-3.3905
<i>amiA3A</i>	HSISS4_01365	Oligopeptide ABC transporter, oligopeptide binding subunit A	-0.4413	-3.3654
	HSISS4_00888	Hypothetical protein	-0.0337	-3.3046
<i>ftsL</i>	HSISS4_01598	Cell division protein	-4.1237	-3.2877
<i>pstC1</i>	HSISS4_00938	Phosphate transport, permease protein	0.3849	-3.2684
	HSISS4_r00059	tRNA Glu	-4.0320	-3.1711
<i>pstS</i>	HSISS4_00939	Phosphate transport, phosphate binding protein	0.3996	-3.1412
<i>rpsF</i>	HSISS4_01661	Small subunit rRNA	-3.8082	-3.0378

<i>pyrH</i>	HSISS4_00354	Uridine monophosphate kinase	-0.9401	-3.0328
<i>pstB1</i>	HSISS4_00936	Phosphate transport, ATP binding protein	0.3704	-2.9993
<i>stkP</i>	HSISS4_01348	Serine-Threonine kinase	0.4418	-2.9834
<i>dltD</i>	HSISS4_01108	Poly(glycerolphosphate chain) D-alanine transfer protein	-0.0282	-2.9760
<i>amiF1</i>	HSISS4_01361	Oligopeptide ABC transporter, ATP-binding subunit F	-0.5192	-2.9446
	HSISS4_01112	Hypothetical protein	-0.6275	-2.9349
<i>rpsS</i>	HSISS4_01819	Small subunit rRNA	-3.8370	-2.8990
	HSISS4_00271	Ribosomal protein	-3.7487	-2.8048
<i>spxA1</i>	HSISS4_00943	Transcriptional regulator	0.3812	-2.8044
	HSISS4_00898	Permease	0.0470	-2.7254
<i>dgk</i>	HSISS4_00536	Lipid carrier recycler	-2.2765	-2.7134
<i>atpE</i>	HSISS4_00399	ATP synthase	-3.1457	-2.6477
<i>rpsE</i>	HSISS4_01806	Small subunit rRNA	-3.8839	-2.6386
<i>sipA</i>	HSISS4_01673	Secretory signal peptidase	-0.8736	-2.6321
<i>murG</i>	HSISS4_00684	Peptidoglycan Lipid II precursor synthesis	-3.9806	-2.6157
<i>ctsR</i>	HSISS4_00060	Stress transcriptional regulator	-0.0949	-2.5720
	HSISS4_r00070	tRNA Arg	-3.6741	-2.5622
<i>acpP1</i>	HSISS4_00021	Acyl carrier protein	0.0610	-2.5388
<i>proS</i>	HSISS4_00152	Prolyl tRNA synthetase	-3.7720	-2.5262

^a The Fitness-cost scores were computed by the Mageck algorithm [39] by comparing the total guides depletion per gene in the mock condition (#1) in comparison with the library-induced condition (#2)

^b The standard residuals were computed as the deviation from the linear regression performed with the fitness-cost scores for conditions with library induction (#2) and with library induction together with competence induction (#3).

Table 3.3. Identification of antagonist competence genes from β -gal screen

Gene name	Locus tag	Comment/function	Normalized count ^a	Mean log ₂ (FC) Lux ^b
	HSISS4_00044	hypothetical transmembrane protein	0.2500	0.9521
<i>manL1</i>	HSISS4_00257	PTS system, mannose-specific IIA component/PTS system, mannose-specific IIB component	0.3438	0.6586
<i>plsX</i>	HSISS4_00020	Phosphate:acyl-ACP acyltransferase	0.1111	1.5243
<i>manN1</i>	HSISS4_00255	PTS system, mannose-specific IID component	0.1892	0.7021
<i>manM1</i>	HSISS4_00256	PTS system, mannose-specific IIC component	0.1731	0.6565
<i>stkP</i>	HSISS4_01348	Serine-threonine protein kinase	0.1000	1.0199
<i>trxA1A</i>	HSISS4_00080	Thioredoxin	0.1250	0.7578
	HSISS4_01010	Phenazine biosynthesis protein PhfZ-like	0.1250	0.7490
	HSISS4_01867	Transcriptional regulator, PadR family	0.0833	1.0171
<i>scrK</i>	HSISS4_01640	Fructokinase	0.0968	0.8673
<i>murJ</i>	HSISS4_00717	Lipid II flippase	0.0556	1.2767
<i>pepP</i>	HSISS4_01648	Aminopeptidase YpdF (MP-, MA-, MS-, AP-, NP- specific)	0.0606	1.0951
	HSISS4_01531	RNA binding protein	0.0833	0.7911

<i>tpiA</i>	HSISS4_00409	Triosephosphate isomerase	0.0769	0.8452
	HSISS4_01426	Acetyltransferase	0.0667	0.9742
<i>rgpX3</i>	HSISS4_01386	Heteropolysaccharide repeat unit export protein	0.0417	1.4369
<i>sdaA</i>	HSISS4_01162	L-serine dehydratase, alpha subunit	0.0455	1.2622
	HSISS4_01230	putative histidine kinase "hk13"	0.0286	1.8028
<i>nusA</i>	HSISS4_00269	Transcription termination protein NusA	0.0303	1.6579
	HSISS4_00833	Glutamate transport permease protein	0.0455	1.1025
<i>ig2</i>	IG2	Intergenic region	0.0400	1.1775
<i>livJ</i>	HSISS4_00287	High-affinity leucine-specific transport system	0.0333	1.3815
<i>pgmA</i>	HSISS4_01102	Phosphoglucomutase	0.0469	0.9758
	HSISS4_00832	Glutamate transport membrane-spanning protein	0.0345	1.2552
<i>murZ</i>	HSISS4_01465	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	0.0303	1.4123
<i>LiaF</i>	HSISS4_01346	Transporter associated with LiaSR	0.0357	1.0845
<i>ig3</i>	IG3	Intergenic region	0.0667	0.5455
	HSISS4_01089	Nucleoside-binding protein	0.0625	0.5749
<i>pcsB2</i>	HSISS4_00358	GBS surface immunogenic protein	0.0227	1.4773
<i>livG2</i>	HSISS4_00477	ABC-type multidrug transport transport system, ATPase component	0.0556	0.6012
<i>murC</i>	HSISS4_00190	UDP-N-acetylmuramate--alanine ligase	0.0408	0.8137
	HSISS4_01307	hypothetical protein	0.0345	0.9545
<i>pstC1</i>	HSISS4_00938	Phosphate transport system permease protein PstC	0.0250	1.2858
<i>glnP</i>	HSISS4_01405	Glutamine ABC transporter/ glutamine-binding permease GlnP	0.0417	0.7673
	HSISS4_00330	Lipopolysaccharide biosynthesis protein	0.0370	0.8456
<i>dnaA</i>	HSISS4_00001	Chromosomal replication initiator	0.0435	0.7136
<i>asp3</i>	HSISS4_01316	Accessory secretory protein	0.0435	0.7105
	HSISS4_00614	hypothetical protein	0.0303	0.9211
<i>dltB</i>	HSISS4_01110	D-alanyl transfer protein DltB	0.0233	1.1658
	HSISS4_01826	Acyltransferase family	0.0364	0.7157
<i>dltA</i>	HSISS4_01111	D-alanine--poly(phosphoribitol) ligase subunit 1	0.0364	0.6731
<i>argJ</i>	HSISS4_00385	Glutamate N-acetyltransferase/N-acetylglutamate synthase	0.0222	0.9805
<i>gcaD</i>	HSISS4_00481	N-acetylglucosamine-1-phosphate uridyltransferase/Glucosamine-1-phosphate N-acetyltransferase	0.0222	0.8576
<i>glmS</i>	HSISS4_01060	Glucosamine--fructose-6-phosphate aminotransferase isomerizing	0.0217	0.8009
<i>galR</i>	HSISS4_01243	Galactose operon repressor	0.0250	0.6792
<i>ig6</i>	IG6	Intergenic region	0.0333	0.5055
<i>pepS</i>	HSISS4_00051	Aminopeptidase S	0.0213	0.7266
<i>cshA</i>	HSISS4_01831	Chromosome segregation helicase	0.0250	0.5635

^a The normalized counts were computed by dividing the number of guides found during the screen targeting the same gene by the number of putative total guides targeting the same gene present in the library.

^b The mean of the log₂ fold-change is an average of all the fold-changes in specific Lux activity for the different guides targeting the same genes tested.

Cell-wall integrity is competence-related

Because of the important number of genes selected by the three screening approaches, we hypothesized that specific pathways involving multiple enzymes, transporters and signalization were targeted. To investigate this assumption, we used the Clusters of Orthologous Groups of proteins (COG) database [40] to classify the different genes identified thanks to the prediction of their general function. For this analysis, we selected only the genes whose inhibition resulted in competence increase, *i.e.* all the guides from the first screen, genes with standardized residuals < -2.5 for the second screen, and genes with normalized count > 0.02 and $\log_2(\text{FC}) > 0.5$ for the third screen. We next assessed the importance of the different pathways for competence activation. For this purpose, we counted the number of genes per screen involved in one COG function and normalized it by the total number of genes within this COG function in the HSISS4 genome (Fig. 3.3A). This analysis indicated that the most represented function was the cell-wall/membrane/envelope biogenesis (23% of all the genes highlighted). Furthermore, we observed a limited number of overlapping genes between the guide depletion and the β -galactosidase screens but all of them except the phosphoglucomutase *pgmA* were directly or indirectly involved in cell-envelope assembly. Indeed, the *dltA* and *dltB* genes involved in teichoic acid D-alanylation, the cell-wall-related serine threonine kinase *stkP* and the phosphate transporter *pstC1* with an important role for polyphosphate teichoic acids synthesis were identified in both screens (Figure 3.3B).

We next tried to draw a more precise view of the different cell-wall pathways targeted by gene interference that lead to competence activation. We found that synthesis of peptidoglycan, teichoic acids and extracellular polysaccharides were all down-regulated (Fig. 3.4). In parallel, key sensors (*StkP*, *LiaFSR*) or mediators (*SpxA1*) known to be triggered by cell-wall alterations were also found in the screens [29,41–45], suggesting a possible link between cell-wall integrity and ComRS activation.

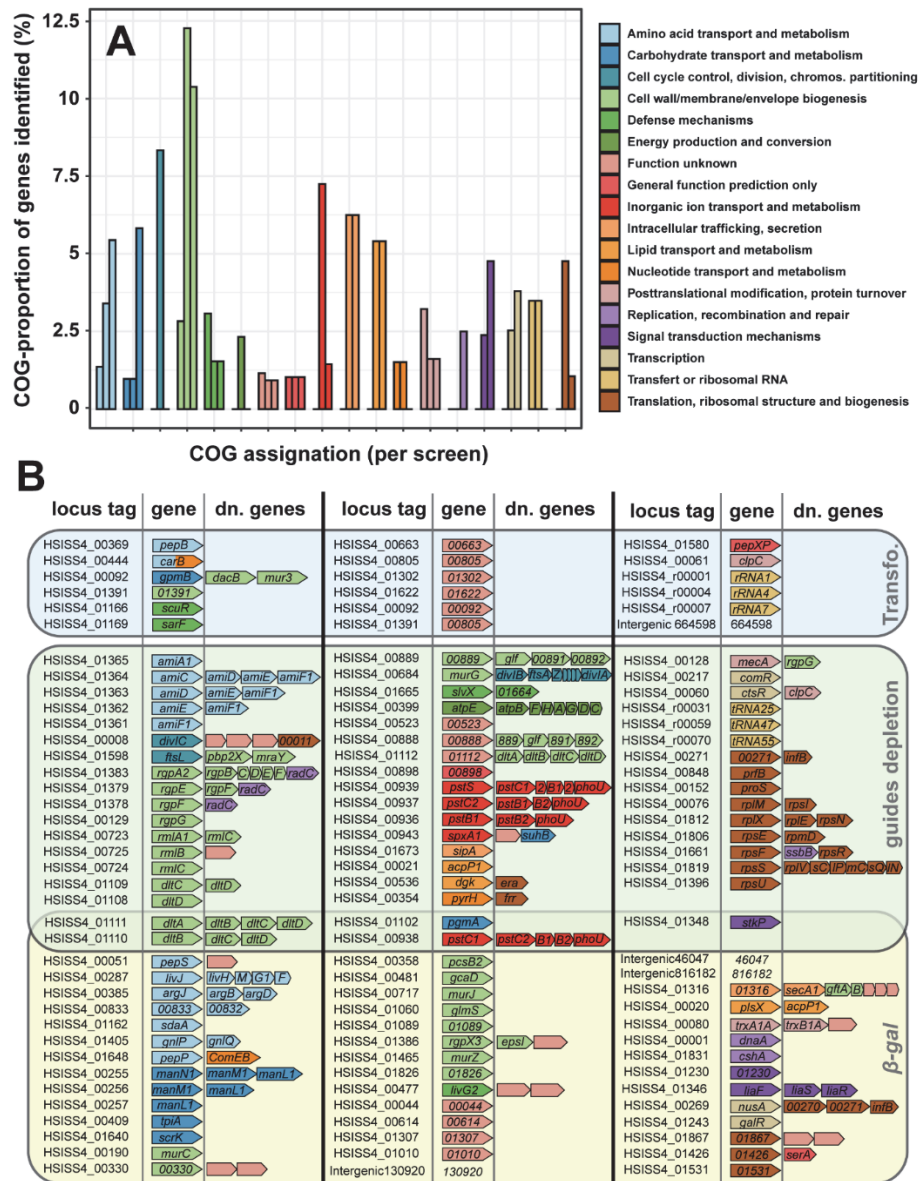


Figure 3.3. Genes screen-selected functions. A. COG proportion of gene identified per screen. A COG assignment was associated to every gene from the HSISS4 genome. For each COG type, the proportion of the genes selected with the screen was computed against all the genes with this COG assignment of the genome (%). This proportion is displayed per screen (1st bar: Transformation screen, 2nd bar: guides depletion screen, 3rd bar: β -gal screen). **B.** Details of all the genes selected displayed per screen. Operons are shown since Crispr-interference silences also downstream genes. Genes are colored depending on their COG assignment.

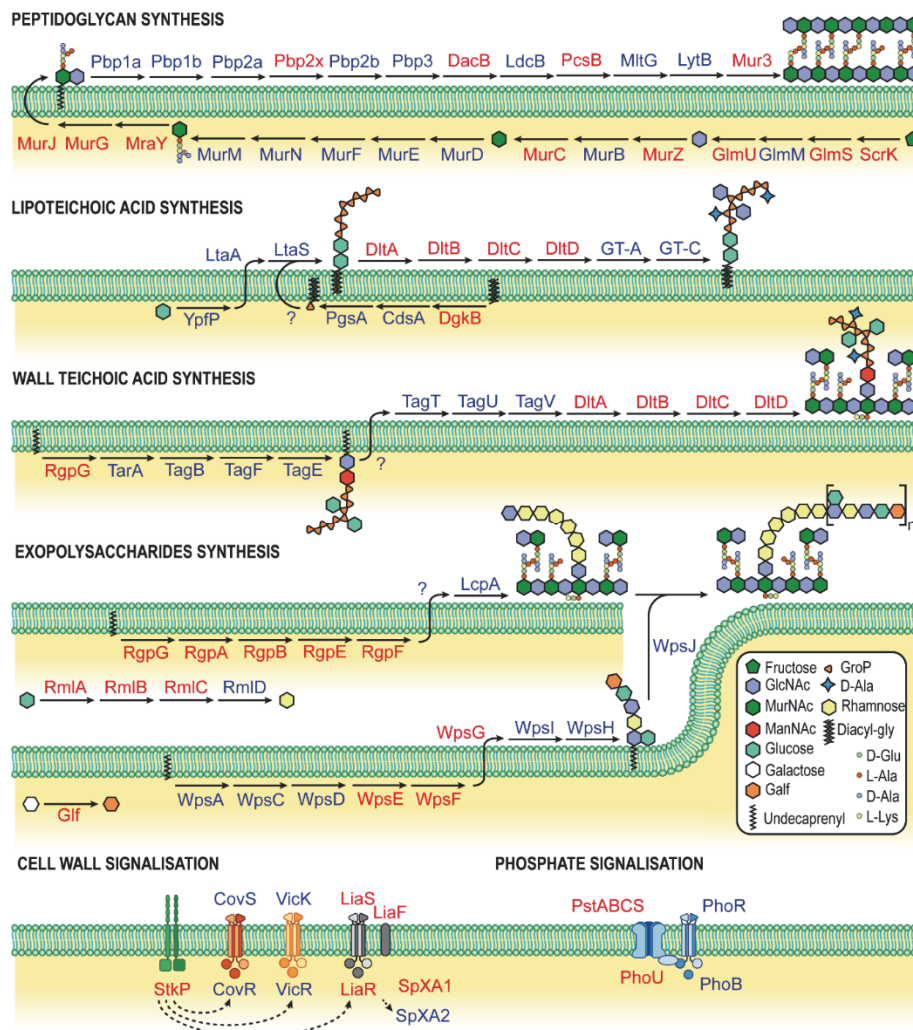


Figure 3.4. Cell wall pathways and genes selected from screens. The cell-wall main synthesis and signalization pathways are depicted. Proteins selected by any of the screens are showed in red. In the absence of literature for complete reconstructed pathways, lipoteichoic acid synthesis is based on literature from *S. aureus*, wall teichoic acid from *B. subtilis* 168 and polysaccharide synthesis from *L. lactis*. HSISS4_00889, 00890, 00891 and 00892 were renamed with *L. lactis* homologs *wpsG*, *glf*, *wpsE* and *wpsF*, respectively. GlcNAc: N-acetyl glucosamine, MurNAc: N-acetyl muramic acid, ManNAc: N-acetylmannosamine, Galf: Galactofuranose, Diacyl-gly: Diacyl-glycerol, GroP: Glycerol-phosphate.

StkP controls comX expression through muropeptide binding

Since StkP was highlighted by two of our screens and is cell-wall associated, we decided to investigate further its link with competence activation. Serine-threonine kinases are pleiotropic regulators that control key cellular processes such as dormancy, virulence, cell division and cell wall synthesis through protein phosphorylation [46,47]. In *S. salivarius*, only one serine-threonine kinase homolog is present and displays PASTA motifs shown in *B. subtilis* to bind muropeptides [48]. Besides, StkP of *S. pneumoniae* has been shown to coordinate cell-wall synthesis and septation [29,49–51] and yet unclear, a link with competence has been suggested in *S. mutans* and *S. pneumoniae* [20,21,29].

In a first set of experiment, we transferred a guide targeting *stkP* in a strain harboring the dCas9 module ($P_{F6-lacI}$ $P_{lac-dcas9}$) together with a luciferase reporter of the transcriptional activity of *comR* ($P_{comR-luxAB}$) or *comX* with the xylose-inducible module allowing competence activation ($P_{comX-luxAB}$ $P_{xyl2-comR}$). Monitoring activation of those two promoters with or without *stkP* inhibition suggested that StkP level influences *comX* activity but has no influence on *comR* transcription (Fig. 3.5A). We next used the same *comX* reporter strain with increasing xylose concentrations for *comR* induction and measured P_{comX} activation with or without *stkP* inhibition (Fig. 3.5B). Higher impacts of *stkP* inhibition were recorded for lower ComR levels, suggesting that ComR overproduction interferes with the StkP-mediated regulation of *comX*.

Since StkP was shown to bind specific muropeptides via its PASTA domains [48], we investigated if the addition of peptidoglycan extracts was able to modulate competence. To this aim, we purified peptidoglycan from *S. salivarius* (L-Lys pentapeptide) or *Bacillus subtilis* (Meso-DAP pentapeptide) and measured the activation of the $P_{comX-luxAB}$ reporter strain with (-| *stkP*) or without (mock) dCas9 interference on *stkP* expression (Fig. 3.5C and D). While *S. salivarius* peptidoglycan could decrease P_{comX} activation, *B. subtilis* extracts did not result in any significative reduction. Moreover, peptidoglycan from *S. salivarius* lost its ability to reduce P_{comX} activation when *stkP* was inhibited, suggesting that StkP mediates the signalization (Fig. 3.5D).

Altogether, these results suggest that StkP interferes with the transcriptional activity of the ComR-XIP complex by an unknown mechanism, which is modulated by the binding of specific muropeptides.

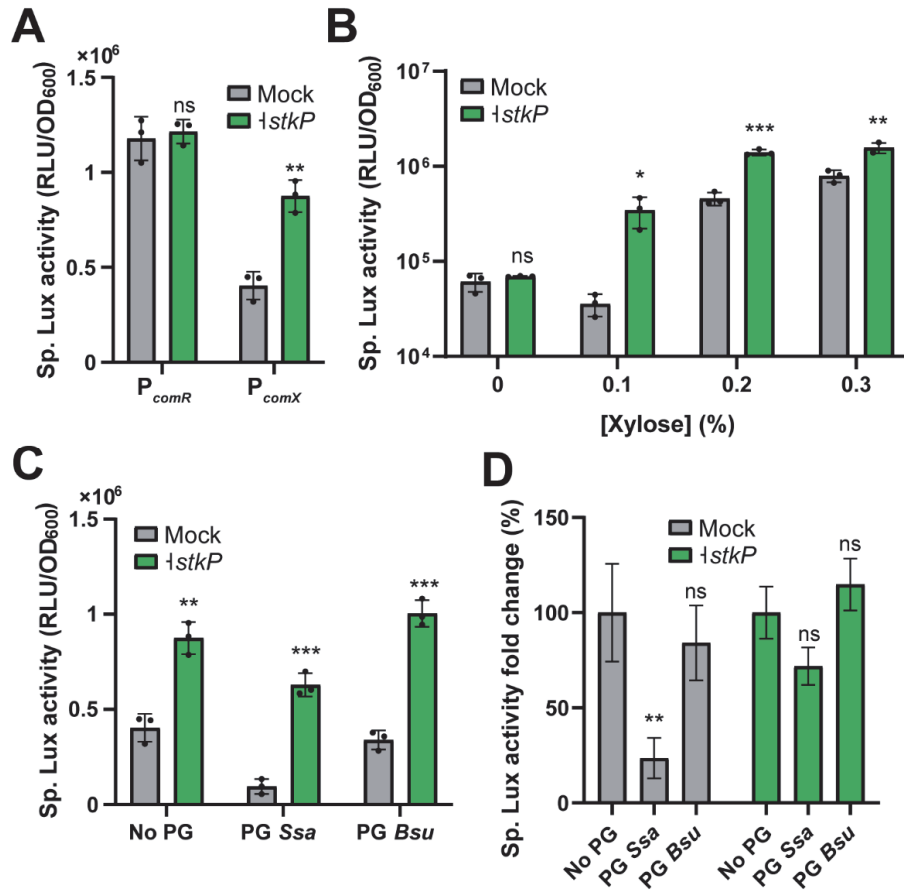


Figure 3.5. StkP controls *comX* expression via cell-wall debris sensing. **A.** Effect of the inhibition of *stkP* on *comR* and *comX* expression. A *dcas9* module (*P_{F6}-lacI*; *P_{lac}-dcas9*) together with a guide targeting *stkP* (*P_{3-g_23}*) were used to inhibit *stkP* transcription with a *P_{comR}-luxAB* or a *P_{comX}-luxAB* reporter system together with a xylose-inducible *comR* module (*P_{xyI2}-comR*). Mock denotes the same strain without *dcas9* interference. **B.** *stkP* activation of *comX* and its dependency on the ComR levels. The *P_{comX}-luxAB P_{xyI2}-comR* strain described in A was incubated at various xylose concentration (0, 0.1, 0.2, 0.3%) with or without *stkP* inhibition. **C.** Cell-wall debris effect on StkP-mediated activation of *comX*. Cell-wall debris of *S. salivarius* (PG *Ssa*) or *B. subtilis* (PG *Bsu*) were added at a final concentration of 0.3mg/ml in a culture of the *P_{comX}-luxAB* strain described in A. **D.** Specific Lux activity fold-change computed between the culture with no cell-wall extracts added (No PG) and the related condition. Ratio were calculated with the data presented in C. For *P_{comX}-luxAB* activation, xylose 0.25% was used except if else stated. For CRISPRi *stkP* inhibition, IPTG 1mM was used. Dots denote technical triplicates values for WT and biological triplicates values for mutants, $\pm$ standard deviation. Statistical *t*-test was performed for each condition in comparison to related control (Ctrl: mock, ABD.) or one-way ANOVA with Dunett's test for multiple comparison (Ctrl: No PG, E.) (ns, $P > 0.05$; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$)

DISCUSSION

How QS modules integrate multiple inputs to fine-tune their sensitivity and optimize collective behavior is a challenging topic. In this work, we performed a genome-wide screen coupled to three different readouts to uncover key triggers of the ComRS competence communication apparatus. Using a conditional mutant library, we were able to highlight a link between cell-wall biogenesis deficiency and competence activation. Moreover, we uncover a link between muropeptide sensing via the serine threonine StkP and competence activation. Those two evidences suggest a key role of cell-wall stress on competence response (Fig. 3.6).

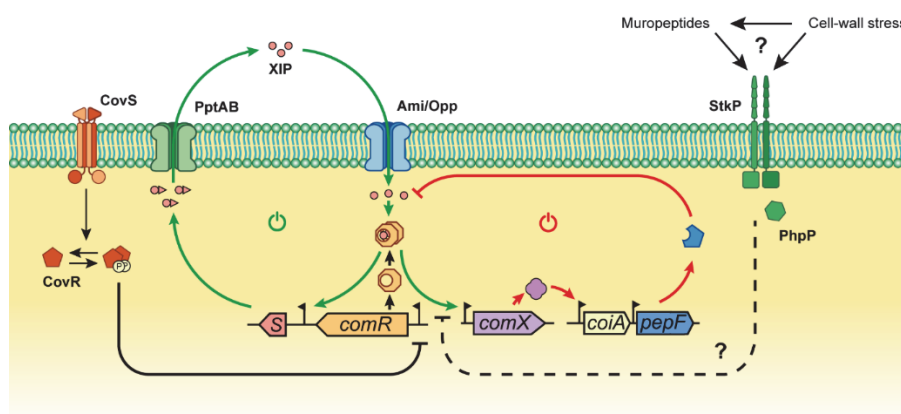


Figure 3.6. Model of competence regulation integrating the StkP-mediated cell-wall regulation in *S. salivarius*. Upon CovRS repression release, ComR reaches a threshold concentration allowing the activation of a positive feedback loop (green arrows, power-on icon). The positive loop is triggered by XIP binding to ComR, producing the ComR·XIP complex which activates *comS* transcription. ComS is then exported by the transporter PptAB and matured. The mature XIP pheromone can then enter the cell by the oligopeptide generic transporter Ami/Opp and bind ComR to enhance the loop. In parallel, the ComR·XIP complex will trigger the transcription of *comX*, encoding the central regulator of competence. This will activate all the late genes necessary for natural transformation including the *coiA-pepF* operon. PepF accumulation will result in XIP degradation, generating a negative feedback-loop (red arrows, power-off icon) on the ComRS system, ultimately leading to competence exit. In parallel, cell-wall stress and/or free muropeptides can be sensed by the serine-threonine kinase StkP to modulate the transcriptional activation of *comX* most probably via the ComR·XIP complex.

To discover new players involved in competence regulation, we built a CRISPRi library and performed three distinct screens. The interference technology offers several advantages over the classical Tn-seq method [52] but its key advantage is the production of conditional mutants allowing the study of essential genes. This property was of main importance for the first

transformation screen where the library was transiently induced without generating a toxic acquired phenotype due to constitutive activation of natural transformation, providing a direct screening method for DNA integration. This strategy allowed us to select guides targeting essential genes among which *pepF*, a key competence shut-off gene in *S. salivarius* [37]. In addition, we also selected two different guides targeting *clpC*, a gene encoding a component of the MecA-ClpCP machinery responsible of ComX degradation [36,53]. Those results confirm the key roles of PepF and ClpC to prevent spontaneous competence activation at the early and late stage of competence, respectively [36,37,53]. Moreover, the transformation screen highlighted a putative bactoprenol glucosyltransferase (*HSISS4_01391*) which shows important transformation rate upon inhibition. As many genes involved in polysaccharides and teichoic-acids synthesis were selected by the different screens, this suggests that this protein could be involved in the first step of an extracellular polysaccharide synthesis of main importance for competence regulation.

Although the transformation screen displays the best features to select and study essential genes connected to competence development, it is limited by the guide-mediated transformation rate. As exemplified by some guides displaying transformation rates as low as 10^{-6} (Table 3.1), our high-density guides library would require a massive number of cells to ensure a complete coverage. This is probably the reason why we were unsuccessful to select a guide targeting the CovRS two-components system, which we previously showed to repress *comR* transcription and natural transformation [37]. This issue is not present in the guide's depletion screen, where NGS high throughput is exploited to map and quantify all the guides, generating a complete coverage. Nevertheless, the identification of the genes is based on the competence-related toxic phenotype. This feature biases the detection of essential genes whose inhibition has a high fitness cost, as illustrated by the absence of *pepF* or *covRS* identification. Similarly, the β -galactosidase screen is not adequate to identify essential genes involved in competence regulation. As the library is spread on plate with competence induction, gene inhibition leading to high toxicity will not produce viable colonies. Yet, unlike the depletion screen, selection is based on P_{comX} activity and therefore avoid any fitness bias. Of note, the fitness bias present in the guide depletion screen could explain some intriguing results. NGS data showed a depletion of the guides associated to genes involved in the downregulation of competence such as *mecA*. Less expected is the selection of guides targeting crucial genes for competence activation such as *comR* or the *ami/opp* operon. To conciliate those two results, one must remember that competence triggers bacteriocin production. The libraries were spread on

plates at relatively high colony density, and it is most likely that a strain lacking a functional competence is impaired for the activation of the bacteriocin-related immunity genes. Since competence is activated in the surrounding colonies, sensitivity to bacteriocin could explain the recorded guide depletion. This hypothesis is strengthened by the high competence-fitness cost observed for several bacteriocin and immunity encoding genes such as *slvX* (-4.73), its associated immunity gene (*HSISS4_01664*, -4.94) and the immunity gene of *blpK* (*HSISS4_01585*, -2.19).

Besides those specificities, the three screens converge to select guides involved in key envelope biogenesis processes and its control by cell-wall sensors (Fig. 3.4). The connection between cell-wall and competence has only been reported in a similar experiment with Tn-seq in *S. mutans* [54]. Strikingly, the authors report that inactivation by transposon insertion of the cell-wall related genes *pknB* (homolog of *stkP*), *rgpL*, *dltA* and *liaS* result in a lower activation of competence, contradicting the results obtained in this study. Opposite effects of competence regulators in *S. salivarius* and *S. mutans* have already been reported for the CovRS system [7,55] and showcase that species have evolved controls suited to their own lifestyles.

Aside from the cell-wall synthesis, several other pathways were highlighted. One of them is the translation, with several important players targeted (rRNAs, tRNAs, *prfB* (peptide chain release factor), ribosomal proteins, and tRNA synthetases). This correlation is interesting in the light of the work of Stevens and coworkers, who showed that translation fidelity impairment promotes competence activation via the extracellular serine protease HtrA in *S. pneumoniae* [56]. The screens also highlighted enzymes or transporters involved in amino acids synthesis and uptake: *carB* and *argJ* (arginine biosynthesis), *HSISS4_00833* and *00832* (glutamate transporters), *livJ* (leucine transporter), and *gnlP* (glutamine transporter). Amino acids starvation is known to trigger the stringent response via RelA and the production of (p)ppGpp alarmones [57]. Since it was shown in *S. mutans* that (p)ppGpp influences competence regulation [58], inhibition of genes involved in amino acids synthesis and uptake may trigger competence in *S. salivarius*. Interestingly, important genes involved in division were also underlined by the screens: *divIC*, *ftsL* and *dnaA* (Table 3.2 and 3.3). In *S. pneumoniae*, replication stress has been shown to induce competence, but the exact mechanism is controversial. While increasing gene dosage due to *comCDE* proximity with *oriC* has been suggested by Slager and colleagues, the Polard's team showed that unrepaired replication fork is most likely the trigger, independently from gene dosage increase [59,60]. In *S. salivarius*, gene dosage is unlikely because *comRS* is not located near *oriC* and conversely to *S. pneumoniae*, the inhibition of *dnaA* results in competence

activation. Therefore, it is most likely that another mechanism is present to explain the link between division and competence activation in *S. salivarius*.

In this work, we specifically investigate StkP as a key sensor of cell-wall integrity (a role recently demonstrated in *S. pneumoniae* [44]) that was highlighted in two screens and targeted by many different guides. We showed that *stkP* depletion promotes a higher *comX* activation without affecting *comR* expression. This suggests a mechanism acting directly on ComR, by increasing its transcriptional properties. This hypothesis is strengthened by the fact that ComR overexpression curtails the effect of StkP on *comX* (Fig. 3.5B). The exact process remains to be discovered, but may suggest a direct phosphorylation of ComR by StkP.

In other streptococci, StkP was shown to phosphorylate classical response regulators at key threonine residues. In *Streptococcus agalactiae* and *Streptococcus pyogenes*, StkP phosphorylates CovR at a position mutually exclusive with the classical aspartate phosphorylation, ultimately leading to virulence activation [61,62]. StkP also phosphorylates a threonine residue of ComE in *S. pneumoniae*, triggering a distinct regulon from the aspartate phosphorelay initiated by ComD [22]. Finally, crosstalk between the cell-wall sensor VicRK and StkP has been highlighted in *S. mutans*, *S. pneumoniae* and *B. subtilis* [29,44,63]. Besides its crosstalk with response regulators, StkP is also involved in septation and division and phosphorylates several key cell-wall proteins [50,64–66]. The pleiotropic effects of StkP and its involvement in major cellular processes is probably the reason why its impact on competence has been reported but remains controversial in *S. pneumoniae* [20–22]. Also, the dual functions of StkP as a cell-wall sensor and orchestrator questions its function in competence activation. Indeed, the kinase inhibition could trigger competence by a lack of muropeptide signaling or by a disfunction of the cell-wall synthesis, which would be detected by another sensor such as VicRK or LiaSRF [41,67]. While the experiments with peptidoglycan extracts presented here (Fig. 3.5D and E) advocate for the first possibility, both mechanisms could be at work.

In this study, we screened a conditional mutant library and uncovered a connection between cell-wall impairment and competence activation. This link not only shows that competence activation is influenced by multiple inputs, but also highlights its importance for survival in stress conditions.

MATERIAL & METHODS

Bacterial strains, plasmids, sgRNA and oligonucleotides

Bacterial strains, plasmids, sgRNA and oligonucleotides used in this study are listed and described in supplementary information (Tables S3.7-S3.10).

Growth conditions and competence induction

S. salivarius HSISS4 [68] and derivatives were grown at 37°C without shaking in M17 (Difco Laboratories, Detroit, MI) or in chemically-defined medium (CDM) [69] supplemented with 1% (w/v) glucose (M17G, CDMG, respectively). Chromosomal genetic constructions were introduced in *S. salivarius* via natural transformation [70]. We added D-xylose (0.1 to 1 %; w/v), IPTG (1mM), spectinomycin (200 µg/ml), chloramphenicol (5 µg/ml) or erythromycin (10 µg/ml), as required. The synthetic peptides (purity of 95%), were supplied by Peptide 2.0 Inc. (Chantilly, VA) and resuspended first in dimethyl-formamide (DMF) and diluted in water to reach low DMF concentration (final of 0.02%). Solid plates inoculated with streptococci cells were incubated anaerobically (BBL GasPak systems, Becton Dickinson, Franklin lakes, NJ) at 37°C. To induce competence, overnight CDMG precultures were diluted at a final OD₆₀₀ of 0.05 in 500 µl of fresh CDMG and incubated 100 min at 37°C. Then, the pheromone sXIP and DNA (Gibson assembled PCR products or plasmids) were added and cells were incubated for 3 h at 37°C before plating on M17G agar supplemented with antibiotics when required.

Library design and construction

The guides library was designed by selecting all the 20nt followed by a PAM sequence within the genome of HSISS4. Since it was shown that efficient interference in open reading frame occurs only with guides targeting the coding strand [33], we filtered the library to keep only guides targeting the coding strand in open reading frame and targeting both strands in intergenic regions. We ended up with a high-density library of 83 104 guides resulting in a theoretical base pairing every 25pb on the HSISS4 genome. Of note, we chose to use a high-density library to target unknown small genetic elements such as siRNA or small peptides. We ordered the guides as single strand DNA (Twist BioSciences) and amplified the oligopool thanks to common upstream and downstream region using primers AK475 and AK476. To reduce any amplification sequence-bias we used 10 cycles of amplification.

The PCR products were then purified (Monarch kit, NEB) and gibson-joined to the pre-amplified upstream homologous region of the neutral locus *gor*

(downstream of *HSISS4_00325*) containing an erythromycin resistance and to the downstream homologous region of the same locus fused to a P_3 constitutive promoter. We performed 20 independent Gibson assembly, which we latter transformed into 20 independent HSISS4 culture by natural transformation into HSISS4 strains containing a *dcas9* lactose-inducible module ($P_{F6-lacI}$; $P_{lac-dcas9}$) and a competence luciferase reporter system ($P_{comX-luxAB}$) as previously described [7]. Supplemental specific genetic constructions were presents in those strains depending on the screen to achieve. For every library produced, we computed the transformants number to ensure a minimum 15-fold transformants over diversity rate, ensuring theoretically that 99,9% of the diversity would be present in the library [71]. We finally pooled all the transformants in Phosphate Saline Buffer (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 , 1.8mM KH_2PO_4) and measured OD₆₀₀ prior to -80°C storing.

Dcas9 transformation screen

For the spontaneous transformation screen, we first introduced the guides library in the lactose-inducible strain harboring a P_{comX} luciferase reporter system ($P_{F6-lacI}$; $P_{lac-dcas9}$; $P_{comX-luxAB}$). We next diluted the cells in 15ml of fresh CDMG supplemented with IPTG 1mM at an OD of 0.01. We grew this culture at 37°C for 8 hours and added every 30minutes a PCR-amplified product consisting of a chloramphenicol cassette with 2000pb up and down homologous recombination arms at a final concentration of 0.25nM. We centrifugated this culture, plated it on chloramphenicol plates and grew it overnight. Colonies were picked and donor DNA integration was confirmed by PCR. We next amplified the locus containing the guides before Sanger sequencing.

Dcas9 lacZ activity screen

We first produced a new genetic background by introducing into the strain described in the previous section an ectopic copy of *comR* under the control of a xylose inducible promoter ($P_{xyl2-comR}$) together with a chloramphenicol cassette at the neutral locus *suc* (upstream of *HSISS4_01641*). We next fused the promoter of *comX* to the native *lacZ* gene of HSISS4 together with a spectinomycin resistance ($P_{comX-lacZ}$) and introduced the guides library into this strain. The resulting library was spread on M17 0.5% glucose, 0.5% lactose, IPTG 100μM, xylose 1% and X-gal 100mg/ml for screening for dark-blue (highly competent) and white (competence loss) colonies. A total of ~94 000 colonies were examined. Dark blue or white phenotypes were confirmed by re-isolation of the selected colonies and guides were amplified by PCR for Sanger-sequencing.

Dcas9 guides depletion screen

For the guides depletion analysis, we used the same strain as described in the previous section lacking the P_{comX} -*lacZ* module. After introducing the guides in this background, we spread the resulting library onto three different solid media (M17G; M17G IPTG 100 μ M; M17G 100 μ M xylose 1%) resulting in an average of 9.6×10^6 CFU per large plates. Technical quadruplates were incubated 16 hours at 37°C to yield an estimated mean of 12.34 generations. Cells were then collected, pooled in PBS buffer and homogenized for each replicate. After genomic extraction (GenElute SigmaAldrich) with a minimum of 1.5×10^9 cells per replicates, we PCR-amplified the locus containing the guides prior to sequence it. We used an optimized PCR protocol with high primers concentration (5 μ M), low template genomic DNA (2ng/ μ l) and low number of cycles (15 cycles) to avoid any chimeric products due to the highly randomized guides sequences. The 219pb amplicons were next gel-purified (Monarch DNA Gel Extraction kit, NEB) and sent with a minimum amount of 4 pmol for Illumina sequencing (GeneWiz). High-Seq Illumina sequencing was performed with 30% Phix and generated an average of 30M reads per replicate.

NGS analysis

We used the MAGeCK algorithm to map the reads on the guides library [39]. Approximately 30% of the total reads were mapped, producing about 10M reads per replicates. Following the MAGeCK guidelines, we next pooled the reads from the quadruplates, ultimately generating a total of 40M reads per condition. Of note, out of the 83104 guides, 160 could not be found in any conditions. In a first analysis, we compared the guides depletion in the IPTG-induced condition with the mock to determine all the essential genes from HSISS4. For the sake of clarity, we only compared guides targeting ORF, since targets of intergenic guides are much more complicated to determine. We next compared the guides depletion for each gene in the IPTG- and IPTG-xylose-induced conditions in comparison to the mock condition thanks to the MAGeCK algorithm. The depletion scores generated per genes for the two induced conditions were then plotted against each other and a linear regression was fitted to the plot (*lm* function, R) and outliers were identified by standardizing the residuals.

COG analysis

The conserved domain database of NCBI was used to infer functions of the genes from the genome of HSISS4 [40,72] and only the highest scored function for each gene were retained. The number of genes of the whole genome involved in each function prediction was then computed, generating

a function prediction frequency matrix. This matrix was then used to weight the number of genes with a specific predicted function highlighted in the different screens.

Luciferase assay

Overnight precultures were diluted at a final OD₆₀₀ of 0.05. A volume of 300 µl of culture was transferred in the wells of a sterile covered white microplate with a transparent bottom (Greiner, Alphen a/d Rijn, The Netherlands). These culture samples were supplemented with D-xylose, IPTG or cell-wall extracts if stated. Growth (OD₆₀₀) and luciferase (Lux) activity (expressed in relative light units, RLU) were monitored at 10 min intervals during 8 to 24 h in a Hidex Sense microplate reader (Hidex, Lemminkäisenkatu, Finland). Specific Lux activity were obtained by dividing Lux activity by the OD₆₀₀ over time and summing all the data obtained over time. When stated, biological or technical triplicates were averaged and compared with *t*-test (unilateral distribution, heteroscedastic) or one-way ANOVA with Dunnett's test with standard deviations and p-values.

Transformation test

The CDMG preculture of HSISS4 and derivatives was rediluted in 500µl of CDMG supplemented with 1mM IPTG at an OD₆₀₀ of 0.005. The culture was grown at 37°C for 8 hours and we added every 30minutes a PCR-amplified product consisting of a chloramphenicol cassette with 2000pb up and down homologous recombination arms at a final concentration of 0.25nM. We next performed serial dilution of the culture and plated the various dilutions on M17G plates supplemented with or without chloramphenicol 5µg/ml. We next computed the transformation rate based on the CFU of the two plates.

Cell-wall extraction

Cell wall debris were extracted according to [48]. One hundred milliliter of *S. salivarius* HSISS4 or *Bacillus subtilis* 168 were grown to an OD₆₀₀ of ~1.2 in M17 or LB, respectively. Cells were collected by centrifugation, washed with 0.8% NaCl, resuspended in hot 4% SDS, boiled for 30 min, and incubated at room temperature (RT) overnight. The suspension was then boiled for 10 min and the SDS-insoluble cell wall material was collected by centrifugation at 15k for 15 min at RT. The pellet-containing cell wall peptidoglycan was washed four times with water and finally resuspended in 1 ml sterile water. The resuspended PG was next digested with mutanolysin (10 µg/ml) overnight at 37°C prior to inactivation of mutanolysin at 80°C for 20 min.

SUPPLEMENTARY INFORMATION

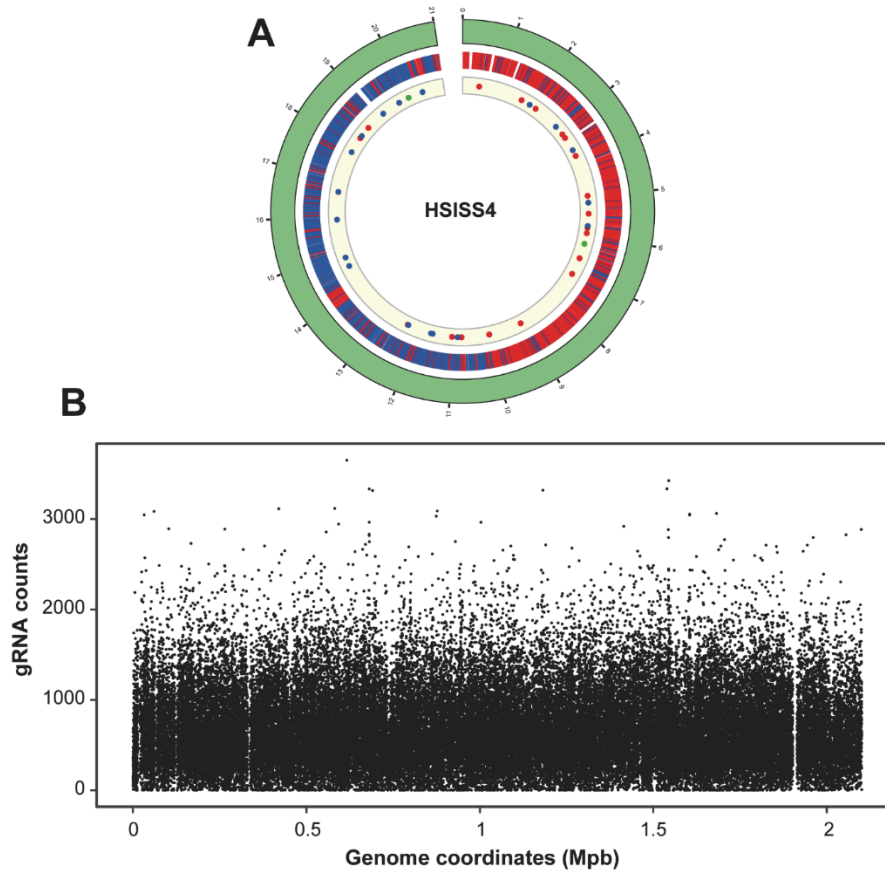


Figure S3.1. Random chromosomal distribution of the guides library. **A.** 40 randomly picked colonies from the guides library were PCR amplified, Sanger-sequenced and mapped on the *Streptococcus salivarius* HSISS4 genome. From outside to center: numbers denote the genomic position (x10⁵ pair of base), red and blue regions depict coding strand being on (+) or (-) strand, respectively. Large color-empty regions correspond to clusters of tRNAs or rRNAs. Red and blue dots show the mapping of guides targeting the (+) or (-) strands, respectively. Green dots show guides targeting intergenic regions. **B.** NGS mapping of the reads from gRNA in the mock library with no library induction. The number of reads per guides are shown. Guides with common sequences were discarded since their mapping at multiple sites bias the analysis. Low guides mapping density on the graph are associated to highly similar sequences such as rRNA, tRNA or multiple transposons insertion. Removal of the guides with the same sequences from the analysis particularly influences the mapping in those regions.

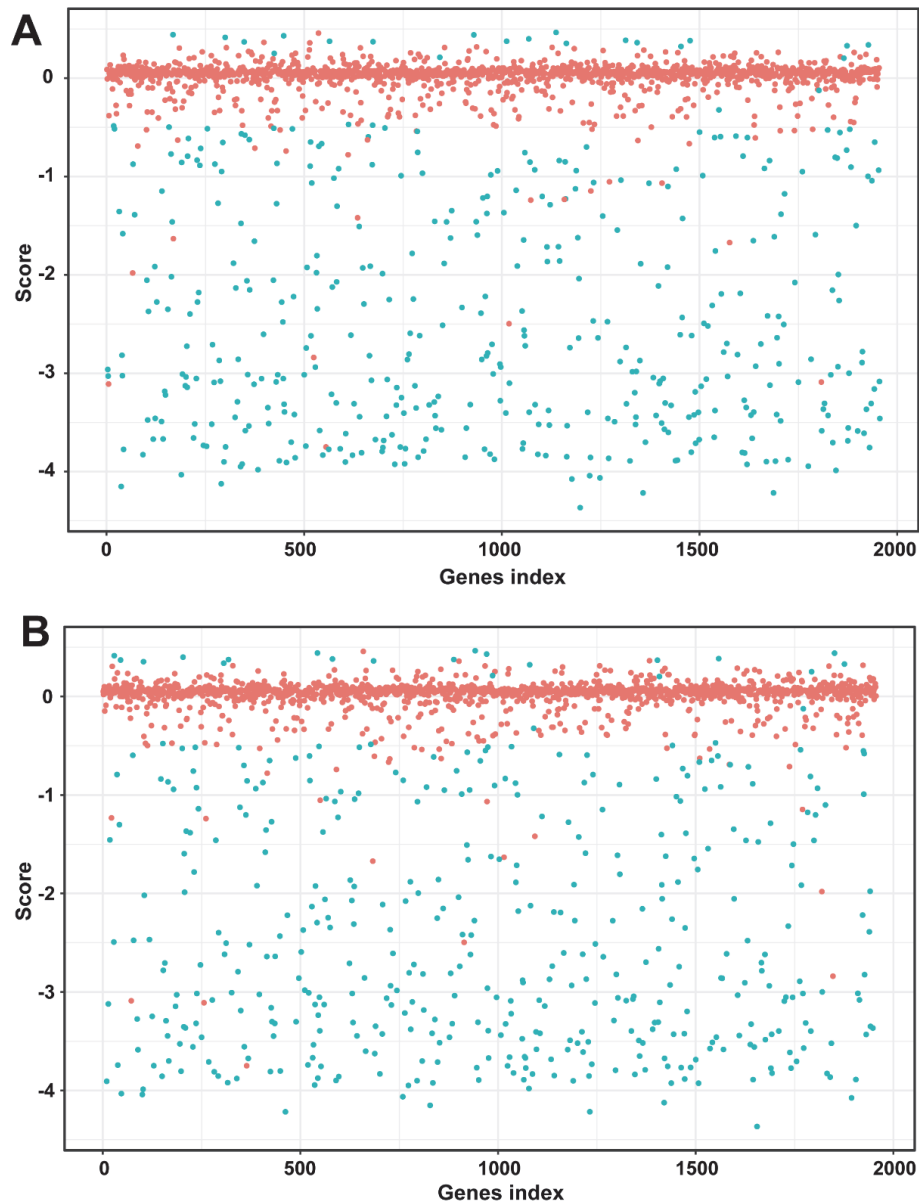


Figure S3.2. Gene's guides depletion scores. Guides reads computed with the library induction (A) and with the library induction plus competence activation (B) were both compared to the reads computed in the mock condition thanks to the MAGeCK algorithm [39]. The algorithm generated a score translating the total guide's depletion for one gene and a False Discovery Rate value (FDR), as a significative marker of the score. The plots show the score computed for each genes (red: FDR>0.05, blue:FDR<0.05). Each gene was associated to a random number (index) for the sake of clarity. Values for each gene can be found in Table S1 and S2 (in supplementary files).

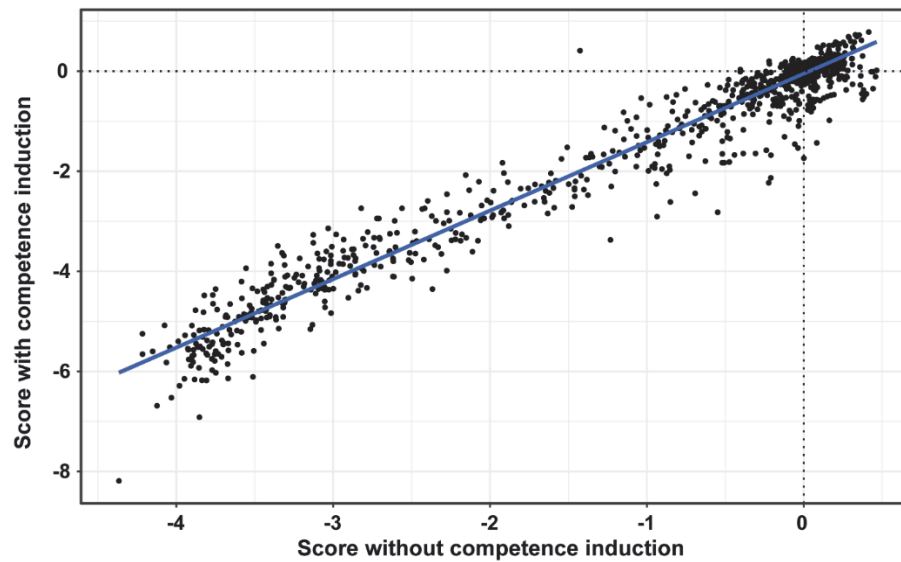


Figure S3.3 Linear regression of gene's guides depletion scores. Guides depletion for each gene in comparison to the mock condition (Fig. S3.2) are plotted against each other. The linear regression was computed thanks to the *lm* function of R using QR method ($R^2=0.9653$). Dashed lines denote a score of zero associated to a neutral fitness effect of the gene inhibition.

Table S3.7. List of bacterial strains used in this study

Strain	Characteristics	Reference/source
<i>Streptococcus salivarius</i>		
HSISS4	Wild-type gastro-intestinal tract isolate	[35]
AK0004	HSISS4 <i>tRNA_{thr}::P_{comX}-luxAB-spec</i> <i>tRNA_{ser}::P_{xyI2}-comR-cat</i>	[7]
AK0033	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i>	[7]
AK0034	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-lox72</i>	[7]
AK0046	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i>	[37]
AK0065	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-cat</i> <i>P_{comX}-lacZ-spc</i>	This work
AK0066	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_30}(HSISS4_01622)-erm</i>	This work
AK0067	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_27}(HSISS4_01391)-erm</i>	This work

AK0068	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_31}(HSISS4_00663)-erm</i>	This work
AK0069	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_32}(HSISS4_00805)-erm</i>	This work
AK0070	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_35}(HSISS4_01302)-erm</i>	This work
AK0071	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_26}(gpmB-dacB-mur3)-erm</i>	This work
AK0072	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_37}(clpC)-erm</i>	This work
AK0073	<i>HSISS4 tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_38}(clpC)-erm</i>	This work

AK0074	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_39}(pepF)-erm</i>	This work
AK0075	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_40}(scuR/sarF)-erm</i>	This work
AK0076	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_41}(pepXP)-erm</i>	This work
AK0077	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_42}(carB)-erm</i>	This work
AK0078	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_43}(IG664598)-erm</i>	This work
AK0079	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comR}-luxAB-lox72</i> <i>GOR::P_{3-g_23}(stkP)-erm</i>	This work

AK0080	HSISS4 <i>tRNA_{thr}::P_{F6}-lacI-lox72</i> <i>tRNA_{ser}::P_{lac}-dcas9-lox72</i> <i>tnpII::P_{comX}-luxAB-lox72</i> <i>SUC::P_{xyI2}-comR-lox72</i> <i>GOR::P_{3-g_23}(stkP)-erm</i>	This work
<i>Bacillus subtilis</i>		
168	Wild-type	J. Mahillon, Laboratory collection

Table S8. List of plasmids used in this study

Plasmid	Characteristics	Reference/ source
<i>pGhostcre</i>	Thermosensitive replication origin vector in <i>S. salivarius</i> , encoding the Cre recombinase; <i>ery^R</i>	[70]
pGIUD0855ery	pUC18 derivative containing the <i>erm</i> gene	[70]
pJUDspecmut1- <i>gfp</i> +ter	Terminator associated- <i>gfp</i> +ORF cloned in	[38]
pJIMcat	pJIM4900 derivative with a <i>cat</i> cassette	[38]

Table S9. List of oligonucleotides used in this study

Name	Sequence (5' to 3')
AK396	TCAACCTCCTATTAATAGATATAATTTTG
AK458	GTAGCAACACTCTTGTTTAAAG
AK459	GTGGCTGAATTATCAAAATAAATC
AK465	TATAGATTTTCATTGCTGGAC
AK484	GCCTTAGCCAAATCGTAATC
AK485	AACAGGAGGTTTTTCGTAATGG
AK518	GTATTCAAAAAACAATAACAGG
AK519	CCAACGTCCAGCAATGAAATCTATATAAGGAAGATAAATCCCATAAGG
AK520	GTTATTACCTTCAAAAAAGGAGAATAATCTTTCACGTTACTAAAGGGAATGTA
Dn.Rv.lox71	TTCACGTTACTAAAGGGAATGTA
ML7	GGAGGCAAAGTCCAATAATACTTAAGGAAGATAAATCCCATAAG
ML8	GTTATTACCTTCAAAAAAGGAGAATAATCTTTCACGTTACTAAAGGGAATG
ML9	AGATTATTCTCCTTTTTGAAG
ML11	GTTTGAATTTTTTCAGTCGTGTTTCATTCAACCTCCTATTAATAGATATAATTTTG
ML13	TTAGTTGAGTGGTTCAATCATG
Up.Fw.lox6	TAAGGAAGATAAATCCCATAAGG

Table S10. List of PCR fragments

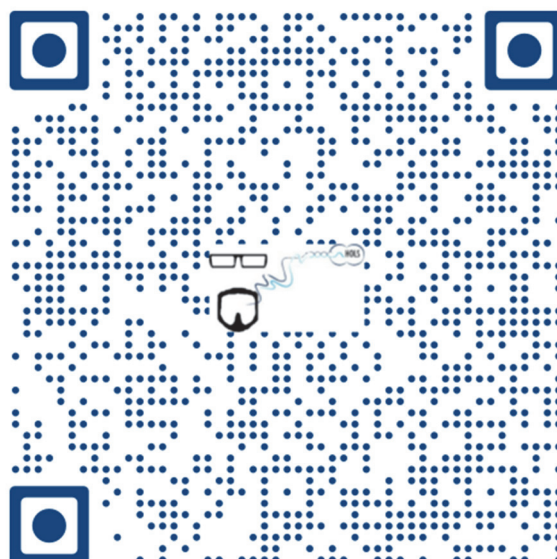
Strain/ DNA	PCR fragment	Template DNA	Primer 1	Primer 2
AK0065	Up HR of <i>lacZ</i> locus	HSISS4	ML13	ML11
	Promoter of <i>comX</i> , <i>P_{comX}</i>	HSISS4	AK396	AK465
	Spectinomycine cassette resistance, <i>spc</i>	pJUD <i>specmut1-gfp</i> +ter	AK519	AK520
	Dw HR of <i>lacZ</i> locus	HSISS4	ML9	AK518
AK0066	P _{3-g_30} (HSISS4_01622) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #2	AK458	AK459
AK0067	P _{3-g_27} (HSISS4_01391) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #5	AK458	AK459
AK0068	P _{3-g_31} (HSISS4_00663) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #6	AK458	AK459
AK0069	P _{3-g_32} (HSISS4_00805) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #9	AK458	AK459
AK0070	P _{3-g_35} (HSISS4_01302) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #13	AK458	AK459
AK0071	P _{3-g_26} (<i>gpmB-dacB-mur3</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #16	AK458	AK459
AK0072	P _{3-g_37} (<i>clpC</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #17	AK458	AK459
AK0073	P _{3-g_38} (<i>clpC</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #18	AK458	AK459
AK0074	P _{3-g_39} (<i>pepF</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #21	AK458	AK459

AK0075	P _{3-g_40} (<i>scuR/sarF</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #22	AK458	AK459
AK0076	P _{3-g_41} (<i>pepXP</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #23	AK458	AK459
AK0077	P _{3-g_42} (<i>carB</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #25	AK458	AK459
AK0078	P _{3-g_43} (IG664598) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from transformation screen #28	AK458	AK459
AK0079, AK0080	P _{3-g_27} (<i>stkP</i>) fused to the two HR of the GOR locus together with <i>erm</i>	Selected colony from the <i>lacZ</i> screen	AK458	AK459
Δ <i>lacZ</i> :: <i>Cat</i>	Up HR of <i>lacZ</i> locus (2000bp)	HSISS4	AK484	ML7
	Chloramphenicol cassette resistance <i>cat</i>	pJIM <i>cat</i>	Up.Fw.lox66	Dn.Rv.lox71
	Dw HR of <i>lacZ</i> locus (2000bp)	HSISS4	ML8	AK485

SUPPLEMENTARY FILES

Supplementary numeric files are available following the QR code or the web link. Those files include:

- Table S3.1
- Table S3.2
- Table S3.3
- Table S3.4
- Table S3.5
- Table S3.6



(<https://drive.google.com/drive/folders/11PXYfBxdZUB6AhsQejfqHqGWx1Ve9n2?usp=sharing>)

REFERENCES

- [1] Huang, R., Li, M., Gregory, R.L., 2011. Bacterial interactions in dental biofilm. *Virulence*: 435–44, Doi: 10.4161/viru.2.5.16140.
- [2] Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., et al., 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486(7402): 207–14, Doi: 10.1038/nature11234.
- [3] Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., et al., 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526(7575): 719–22, Doi: 10.1038/nature15524.
- [4] Bettenworth, V., Steinfeld, B., Duin, H., Petersen, K., Streit, W.R., Bischofs, I., et al., 2019. Phenotypic Heterogeneity in Bacterial Quorum Sensing Systems. *Journal of Molecular Biology*: 4530–46, Doi: 10.1016/j.jmb.2019.04.036.
- [5] Domenech, A., Slager, J., Veening, J.W., 2018. Antibiotic-Induced Cell Chaining Triggers Pneumococcal Competence by Reshaping Quorum Sensing to Autocrine-Like Signaling. *Cell Reports* 25(9): 2390–2400.e3, Doi: 10.1016/j.celrep.2018.11.007.
- [6] Doğaner, B.A., Yan, L.K.Q., Youk, H., 2016. Autocrine Signaling and Quorum Sensing: Extreme Ends of a Common Spectrum. *Trends in Cell Biology*: 262–71, Doi: 10.1016/j.tcb.2015.11.002.
- [7] Knoop, A., Vande Cappelle, F., Fontaine, L., Verhaeghen, M., Mignolet, J., Goffin, P., et al., 2022. The CovRS environmental sensor directly controls the ComRS signaling system to orchestrate competence bimodality in *Salivarius streptococci*. *MBio* (in press).
- [8] Moreno-Gómez, S., Sorg, R.A., Domenech, A., Kjos, M., Weissing, F.J., van Doorn, G.S., et al., 2017. Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. *Nature Communications* 8(1): 854, Doi: 10.1038/s41467-017-00903-y.
- [9] Chang, J.C., Jimenez, J.C., Federle, M.J., 2015. Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme. *Molecular Microbiology* 97(6): 1097–113, Doi: 10.1111/mmi.13088.
- [10] Neiditch, M.B., Capodagli, G.C., Prehna, G., Federle, M.J., 2017. Genetic and Structural Analyses of RRNPP Intercellular Peptide Signaling of Gram-Positive Bacteria. *Annual Review of Genetics*: 311–33, Doi: 10.1146/annurev-genet-120116-023507.
- [11] Fontaine, L., Wahl, A., Fléhard, M., Mignolet, J., Hols, P., 2015. Regulation of competence for natural transformation in streptococci. *Infection, Genetics and Evolution* 33: 343–60, Doi: 10.1016/j.meegid.2014.09.010.
- [12] Shanker, E., Federle, M.J., 2017. Quorum sensing regulation of competence and bacteriocins in *Streptococcus pneumoniae* and mutants. *Genes*, Doi: 10.3390/genes8010015.

- [13] Fontaine, L., Boutry, C., Henry De Frahan, M., Delplace, B., Fremaux, C., Horvath, P., et al., 2010. A Novel Pheromone Quorum-Sensing System Controls the Development of Natural Competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. *JOURNAL OF BACTERIOLOGY* 192(5): 1444–54, Doi: 10.1128/JB.01251-09.
- [14] Gardan, R., Besset, C., Gitton, C., Guillot, A., Fontaine, L., Hols, P., et al., 2013. Extracellular life cycle of ComS, the competence-stimulating peptide of *Streptococcus thermophilus*. *Journal of Bacteriology* 195(8): 1845–55, Doi: 10.1128/JB.02196-12.
- [15] Fontaine, L., Goffin, P., Dubout, H., Delplace, B., Baulard, A., Lecat-Guillet, N., et al., 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. *Molecular Microbiology* 87(6): 1113–32, Doi: 10.1111/mmi.12157.
- [16] Talagas, A., Fontaine, L., Ledesma-García, L., Mignolet, J., Li de la Sierra-Gallay, I., Lazar, N., et al., 2017. Structural Insights into Streptococcal Competence Regulation by the Cell-to-Cell Communication System ComRS. *Annual Reviews of Genetics* 12(12): e1005980, Doi: 10.1371/journal.ppat.1005980.
- [17] Hagen, S.J., Son, M., 2017. Origins of heterogeneity in *Streptococcus mutans* competence: interpreting an environment-sensitive signaling pathway. *Physical Biology* 14(1): 015001, Doi: 10.1088/1478-3975/aa546c.
- [18] Echenique, J.R., Chapuy-Regaud, S., Trombe, M.C., 2000. Competence regulation by oxygen in *Streptococcus pneumoniae*: Involvement of ciaRH and comCDE. *Molecular Microbiology* 36(3): 688–96, Doi: 10.1046/j.1365-2958.2000.01891.x.
- [19] Echenique, J.R., Trombe, M.C., 2001. Competence repression under oxygen limitation through the two-component MicAB signal-transducing system in *Streptococcus pneumoniae* and involvement of the PAS domain of MicB. *Journal of Bacteriology* 183(15): 4599–608, Doi: 10.1128/JB.183.15.4599-4608.2001.
- [20] Echenique, J., Kadioglu, A., Romao, S., Andrew, P.W., Trombe, M.C., 2004. Protein Serine/Threonine Kinase StkP Positively Controls Virulence and Competence in *Streptococcus pneumoniae*. *Infection and Immunity* 72(4): 2434–7, Doi: 10.1128/IAI.72.4.2434-2437.2004.
- [21] Sasková, L., Nováková, L., Basler, M., Branny, P., 2007. Eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus pneumoniae*. *Journal of Bacteriology* 189(11): 4168–79, Doi: 10.1128/JB.01616-06.
- [22] Piñas, G.E., Reinoso-Vizcaino, N.M., Yandar Barahona, N.Y., Cortes, P.R., Duran, R., Badapanda, C., et al., 2018. Crosstalk between the serine/threonine kinase StkP and the response regulator ComE controls the stress response and intracellular survival of *Streptococcus pneumoniae*, Doi: 10.1371/journal.ppat.1007118.
- [23] Lévesque, C.M., Mair, R.W., Perry, J.A., Lau, P.C.Y., Li, Y.H., Cvitkovitch, D.G., 2007. Systemic inactivation and phenotypic characterization of two-component systems in expression of *Streptococcus mutans* virulence properties. *Letters in Applied Microbiology* 45(4): 398–404, Doi: 10.1111/j.1472-765X.2007.02203.x.

- [24] Qi, F., Merritt, J., Lux, R., Shi, W., 2004. Inactivation of the *ciaH* gene in *Streptococcus mutans* diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance. *Infection and Immunity* 72(8): 4895–9, Doi: 10.1128/IAI.72.8.4895-4899.2004.
- [25] Kim, J.N., Stanhope, M.J., Burne, R.A., 2013. Core-gene-encoded peptide regulating virulence-associated traits in *Streptococcus mutans*. *Journal of Bacteriology* 195(12): 2912–20, Doi: 10.1128/JB.00189-13.
- [26] Okinaga, T., Xie, Z., Niu, G., Qi, F., Merritt, J., 2010. Examination of the *hdrRM* regulon yields insight into the competence system of *Streptococcus mutans*. *Molecular Oral Microbiology* 25(3): 165–77, Doi: 10.1111/j.2041-1014.2010.00574.x.
- [27] Xie, Z., Okinaga, T., Niu, G., Qi, F., Merritt, J., 2010. Identification of a novel bacteriocin regulatory system in *Streptococcus mutans*. *Molecular Microbiology* 78(6): 1431–47, Doi: 10.1111/j.1365-2958.2010.07417.x.
- [28] Dufour, D., Cordova, M., Cvitkovitch, D.G., Lévesque, C.M., 2011. Regulation of the competence pathway as a novel role associated with a *Streptococcal* bacteriocin. *Journal of Bacteriology* 193(23): 6552–9, Doi: 10.1128/JB.05968-11.
- [29] Banu, L.D., Conrads, G., Rehrauer, H., Hussain, H., Allan, E., Van Der Ploeg, J.R., 2010. The *Streptococcus mutans* serine/threonine kinase, PknB, regulates competence development, bacteriocin production, and cell wall metabolism. *Infection and Immunity* 78(5): 2209–20, Doi: 10.1128/IAI.01167-09.
- [30] Underhill, S.A.M., Shields, R.C., Burne, R.A., Hagen, S.J., 2019. Carbohydrate and PepO control bimodality in competence development by *Streptococcus mutans*. *Molecular Microbiology* 112(5): 1388–402, Doi: 10.1111/mmi.14367.
- [31] Chastanet, A., Prudhomme, M., Claverys, J.P., Msadek, T., 2001. Regulation of *Streptococcus pneumoniae* *clp* genes and their role in competence development and stress survival. *Journal of Bacteriology*, vol. 183. p. 7295–307.
- [32] Shields, R.C., Zeng, L., Culp, D.J., Burne, R.A., 2018. Genomewide Identification of Essential Genes and Fitness Determinants of *Streptococcus mutans* UA159. *MSphere* 3(1), Doi: 10.1128/mSphere.00031-18.
- [33] Cui, L., Vigouroux, A., Rousset, F., Varet, H., Khanna, V., Bikard, D., 2018. A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nature Communications* 9(1): 1912, Doi: 10.1038/s41467-018-04209-5.
- [34] Liu, X., Gallay, C., Kjos, M., Domenech, A., Slager, J., van Kessel, S.P., et al., 2017. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Molecular Systems Biology* 13(5): 931, Doi: 10.15252/msb.20167449.
- [35] Van Den Bogert, B., Boekhorst, J., Herrmann, R., Smid, E.J., Zoetendal, E.G., Kleerebezem, M., 2013. Comparative genomics analysis of *Streptococcus* isolates from the human small intestine reveals their adaptation to a highly dynamic ecosystem. *PLoS ONE* 8(12): 83418, Doi: 10.1371/journal.pone.0083418.

- [36] Boutry, C., Wahl, A., Delplace, B., Clippe, A., Fontaine, L., Hols, P., 2012. Adaptor protein MecA is a negative regulator of the expression of late competence genes in *Streptococcus thermophilus*. *Journal of Bacteriology* 194(7): 1777–88, Doi: 10.1128/JB.06800-11.
- [37] Knoop, A., Ledesma-García, L., Alexandra, W., Lamontagne, M., Decat, B., Degand, H., et al., 2021. Competence shut-off by intracellular pheromone degradation in *Streptococcus salivarius*. In Preparation.
- [38] Mignolet, J., Fontaine, L., Sass, A., Nannan, C., Mahillon, J., Coenye, T., et al., 2018. Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*. *Cell Reports* 22(7): 1627–38, Doi: 10.1016/j.celrep.2018.01.055.
- [39] Li, W., Köster, J., Xu, H., Chen, C.H., Xiao, T., Liu, J.S., et al., 2015. Quality control, modeling, and visualization of CRISPR screens with MAGeCK-VISPR. *Genome Biology* 16(1): 281, Doi: 10.1186/s13059-015-0843-6.
- [40] Tatusov, R.L., Galperin, M.Y., Natale, D.A., Koonin, E. V., 2000. The COG database: A tool for genome-scale analysis of protein functions and evolution. *Nucleic Acids Research*: 33–6, Doi: 10.1093/nar/28.1.33.
- [41] Eldholm, V., Gutt, B., Johnsborg, O., Brückner, R., Maurer, P., Hakenbeck, R., et al., 2010. The pneumococcal cell envelope stress-sensing system LiaFSR is activated by murein hydrolases and lipid II-interacting antibiotics. *Journal of Bacteriology* 192(7): 1761–73, Doi: 10.1128/JB.01489-09.
- [42] Rojas-Tapias, D.F., Helmann, J.D., 2018. Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. *PLOS Genetics* 14(7): e1007531, Doi: 10.1371/journal.pgen.1007531.
- [43] Suntharalingam, P., Senadheera, M.D., Mair, R.W., Levesque, C.M., Cvitkovitch, D.G., 2009. The LiaFSR system regulates the cell envelope stress response in *Streptococcus mutans*. *Journal of Bacteriology* 191(9): 2973–84, Doi: 10.1128/JB.01563-08.
- [44] Ulrych, A., Fabrik, I., Kupčík, R., Vajrychová, M., Doubravová, L., Branny, P., 2021. Cell wall stress stimulates the activity of the protein kinase StkP of *Streptococcus pneumoniae*, leading to multiple phosphorylation. *Journal of Molecular Biology*: 167319, Doi: 10.1016/j.jmb.2021.167319.
- [45] Ganguly, T., Kajfasz, J.K., Abranches, J., Lemos, J.A., 2020. Regulatory circuits controlling Spx levels in *Streptococcus mutans*. *Molecular Microbiology* 114(1): 109–26, Doi: 10.1111/mmi.14499.
- [46] Pereira, S.F.F., Goss, L., Dworkin, J., 2011. Eukaryote-Like Serine/Threonine Kinases and Phosphatases in Bacteria. *Microbiology and Molecular Biology Reviews* 75(1): 192–212, Doi: 10.1128/mmbr.00042-10.
- [47] Henry, C., Haller, L., Blein-Nicolas, M., Zivy, M., Canette, A., Verbrugghe, M., et al., 2019. Identification of Hanks-type kinase PknB-specific targets in the *Streptococcus thermophilus* phosphoproteome. *Frontiers in Microbiology* 10(JUN), Doi: 10.3389/fmicb.2019.01329.

- [48] Shah, I.M., Laaberki, M.H., Popham, D.L., Dworkin, J., 2008. A Eukaryotic-like Ser/Thr Kinase Signals Bacteria to Exit Dormancy in Response to Peptidoglycan Fragments. *Cell* 135(3): 486–96, Doi: 10.1016/j.cell.2008.08.039.
- [49] Dias, R., Félix, D., Caniça, M., Trombe, M.C., 2009. The highly conserved serine threonine kinase StkP of *Streptococcus pneumoniae* contributes to penicillin susceptibility independently from genes encoding penicillin-binding proteins. *BMC Microbiology* 9: 121, Doi: 10.1186/1471-2180-9-121.
- [50] Beilharz, K., Nováková, L., Fadda, D., Branny, P., Massidda, O., Veening, J.W., 2012. Control of cell division in *Streptococcus pneumoniae* by the conserved Ser/Thr protein kinase StkP. *Proceedings of the National Academy of Sciences of the United States of America* 109(15): E905–13, Doi: 10.1073/pnas.1119172109.
- [51] Fleurie, A., Cluzel, C., Guiral, S., Freton, C., Galisson, F., Zanella-Cleon, I., et al., 2012. Mutational dissection of the S/T-kinase StkP reveals crucial roles in cell division of *Streptococcus pneumoniae*. *Molecular Microbiology* 83(4): 746–58, Doi: 10.1111/j.1365-2958.2011.07962.x.
- [52] Yan, A., Xiong, Z.-Q., Gu, Y., Shao, S., Zhang, R., Xu, W., et al., 2021. Gene Silencing Through CRISPR Interference in Bacteria: Current Advances and Future Prospects. *Front. Microbiol* 12: 635227, Doi: 10.3389/fmicb.2021.635227.
- [53] Tian, X.L., Dong, G., Liu, T., Gomez, Z.A., Wahl, A., Hols, P., et al., 2013. MecA protein acts as a negative regulator of genetic competence in *Streptococcus mutans*. *Journal of Bacteriology* 195(22): 5196–206, Doi: 10.1128/JB.00821-13.
- [54] Shields, R.C., O'Brien, G., Maricic, N., Kesterson, A., Grace, M., Hagen, S.J., et al., 2018. Genome-wide screens reveal new gene products that influence genetic competence in *Streptococcus mutans*. *Journal of Bacteriology* 200(2), Doi: 10.1128/JB.00508-17.
- [55] Dmitriev, A., Mohapatra, S.S., Chong, P., Neely, M., Biswas, S., Biswas, I., 2011. CovR-controlled global regulation of gene expression in *streptococcus mutans*. *PLoS ONE* 6(5), Doi: 10.1371/journal.pone.0020127.
- [56] Stevens, K.E., Chang, D., Zwack, E.E., Seibert, M.E., 2011. Competence in *streptococcus pneumoniae* is regulated by the rate of ribosomal decoding errors. *MBio* 2(5), Doi: 10.1128/mBio.00071-11.
- [57] Winther, K.S., Roghanian, M., Gerdes, K., 2018. Activation of the Stringent Response by Loading of RelA-tRNA Complexes at the Ribosomal A-Site. *Molecular Cell* 70(1): 95-105.e4, Doi: 10.1016/j.molcel.2018.02.033.
- [58] Kaspar, J., Kim, J.N., Ahn, S.-J., Burne, R.A., 2016. An Essential Role for (p)ppGpp in the Integration of Stress Tolerance, Peptide Signaling, and Competence Development in *Streptococcus mutans*. *Frontiers in Microbiology* 7(JUL): 1162, Doi: 10.3389/fmicb.2016.01162.
- [59] Slager, J., Kjos, M., Attaiech, L., Veening, J.W., 2014. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. *Cell* 157(2): 395–406, Doi: 10.1016/j.cell.2014.01.068.

- [60] Khemici, V., Prudhomme, M., Polard, P., 2021. Tight interplay between replication stress and competence induction in *Streptococcus pneumoniae*. *Cells* 10(8), Doi: 10.3390/cells10081938.
- [61] Lin, W.-J.J., Walthers, D., Connelly, J.E., Burnside, K., Jewell, K.A., Kenney, L.J., et al., 2009. Threonine phosphorylation prevents promoter DNA binding of the Group B *Streptococcus* response regulator CovR. *Molecular Microbiology* 71(6): 1477–95, Doi: 10.1111/j.1365-2958.2009.06616.x.
- [62] Horstmann, N., Saldaña, M., Sahasrabhojane, P., Yao, H., Su, X., Thompson, E., et al., 2014. Dual-Site Phosphorylation of the Control of Virulence Regulator Impacts Group A *Streptococcal* Global Gene Expression and Pathogenesis. *PLoS Pathogens* 10(5), Doi: 10.1371/journal.ppat.1004088.
- [63] Libby, E.A., Goss, L.A., Dworkin, J., 2015. The Eukaryotic-Like Ser/Thr Kinase PrkC Regulates the Essential WalRK Two-Component System in *Bacillus subtilis*. *PLoS Genetics* 11(6), Doi: 10.1371/journal.pgen.1005275.
- [64] Zucchini, L., Mercy, C., Garcia, P.S., Cluzel, C., Gueguen-Chaignon, V., Galisson, F., et al., 2018. PASTA repeats of the protein kinase StkP interconnect cell constriction and separation of *Streptococcus pneumoniae*. *Nature Microbiology* 3(2): 197–209, Doi: 10.1038/s41564-017-0069-3.
- [65] Fleurie, A., Manuse, S., Zhao, C., Campo, N., Cluzel, C., Lavergne, J.-P., et al., 2014. Interplay of the Serine/Threonine-Kinase StkP and the Paralogs DivIVA and GpsB in *Pneumococcal* Cell Elongation and Division. *PLoS Genetics* 10(4): e1004275, Doi: 10.1371/journal.pgen.1004275.
- [66] Hirschfeld, C., Gómez-Mejía, A., Bartel, J., Hentschker, C., Rohde, M., Maaß, S., et al., 2020. Proteomic Investigation Uncovers Potential Targets and Target Sites of *Pneumococcal* Serine-Threonine Kinase StkP and Phosphatase PhpP. *Frontiers in Microbiology* 10: 3101, Doi: 10.3389/fmicb.2019.03101.
- [67] Dubrac, S., Bisicchia, P., Devine, K.M., Msadek, T., 2008. A matter of life and death: Cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Molecular Microbiology*: 1307–22, Doi: 10.1111/j.1365-2958.2008.06483.x.
- [68] Mignolet, J., Fontaine, L., Kleerebezem, M., Hols, P., 2016. Complete genome sequence of *Streptococcus salivarius* HSISS4, a human commensal bacterium highly prevalent in the digestive tract. *Genome Announcements* 4(1), Doi: 10.1128/genomeA.01637-15.
- [69] Letort, C., Juillard, V., 2001. Development of a minimal chemically-defined medium for the exponential growth of *Streptococcus thermophilus*. *Journal of Applied Microbiology* 91(6): 1023–9, Doi: 10.1046/j.1365-2672.2001.01469.x.
- [70] Fontaine, L., Dandoy, D., Boutry, C., Delplace, B., de Frahan, M.H., Fremaux, C., et al., 2010. Development of a versatile procedure based on natural transformation for marker-free targeted genetic modification in *Streptococcus thermophilus*. *Applied and Environmental Microbiology* 76(23): 7870–7, Doi: 10.1128/AEM.01671-10.

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- [71] Dorrazehi, G.M., Worms, S., Chirakadavil, J.B., Mignolet, J., Hols, P., Soumilion, P., 2020. Building Scarless Gene Libraries in the Chromosome of Bacteria. In: Iranzo, OlgaRoque, A.C.A., editor. *Peptide and Protein engineering*, Springer US p. 189–211.
- [72] Lu, S., Wang, J., Chitsaz, F., Derbyshire, M.K., Geer, R.C., Gonzales, N.R., et al., 2020. CDD/SPARCLE: The conserved domain database in 2020. *Nucleic Acids Research* 48(D1): D265–8, Doi: 10.1093/nar/gkz991.

GENERAL DISCUSSION

In this thesis, we investigated the molecular mechanisms governing dynamic of activation and deactivation of competence via the ComRS system in the commensal species *Streptococcus salivarius*. Beyond the comprehension of a specific biochemical pathway, this study helps us to address fundamental biological questions regarding bacterial communication, collective behavior and stress adaptation.

Specifically, the main objective of this thesis was to dissect the ComRS system in *S. salivarius* in order to:

1. Understand the molecular basis explaining heterogeneous activation of competence.
2. Identify and characterize the exit from competence.
3. Uncover novel genetic antagonists of competence with a genome-wide screen.

The results obtained, their significance, the biological questions they raise and their perspectives will be discussed in this section.

1 Bimodality

In the first part of this work, we questioned the unclear processes generating heterogeneous activation of competence in the salivarius group. This investigation led us to characterize the molecular mechanisms of ComRS necessary to generate heterogeneity and to identify CovRS as a key distal controller of this phenomenon (Fig. 17).

1.1 ComRS topology and heterogeneous activation

Phenotypic heterogeneity and more specifically bet-hedging, is a key collective behavior to share responsibility at the community level to activate risky strategies such as competence. The first report of competent subpopulation activation in ComRS streptococci has been described more than a decade ago [90]. Even though extensive researches on the origin of bimodality in *S. mutants* have been conducted since then [88,89,91,146], the existence of a supplemental pheromone system controlling competence has substantially slow down and complexified its study.

In this thesis, we used the *S. salivarius* model to demonstrate that the concentration of the intracellular receptor ComR is a pivotal feature to determine the proportion of activated cells in a population. We also provide evidences that competence heterogeneous activation requires a functional positive feedback loop on the pheromone ComS. Since the ComRS system was

shown to be crucial to generate bimodality in *S. mutans* [88,89], we suggest that the central role of ComR and the essentiality of the loop for bimodality are most likely common to ComRS streptococci.

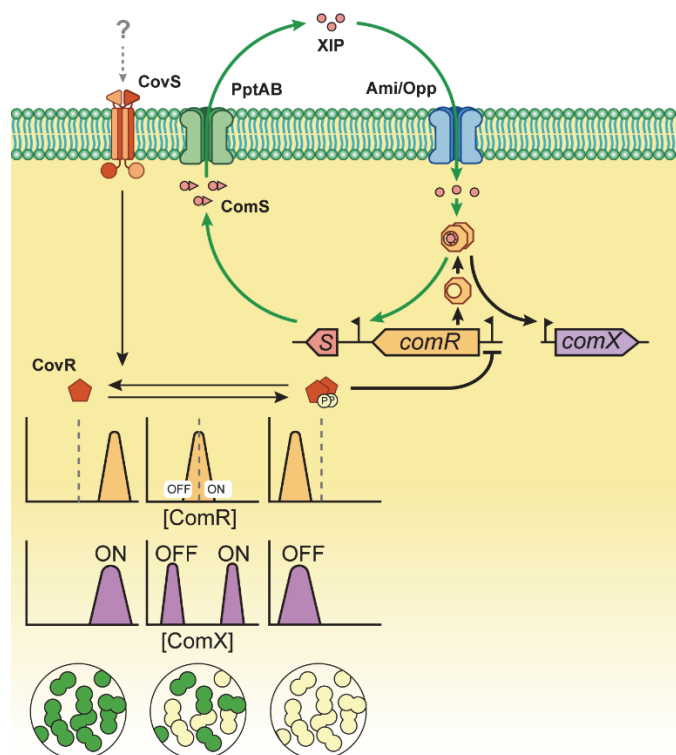


Figure 17. The bimodal model in *S. salivarius*. The environmental sensor CovS interacts with CovR to tune the phosphorylation state of CovR. Phosphorylated CovR will dimerize and bind P_{comR} to repress *comR* transcription. Low levels of phosphorylated CovR will trigger an increase of ComR concentration to a level sufficient to launch the positive feedback loop driven by *comS* (S). The loop will be activated in part of the population generating bimodality.

Of note, the positive feedback loop mechanism described here is in accordance with *B. subtilis* data, where the emergence of a competence subpopulation also requires a loop acting on the central regulator ComK [16,140]. This suggests that this topology is a broad bacterial strategy used to amplify noise and generate differential response within a community.

Although this study provides experimental evidences to generate a consistent ComRS model of bimodality, the observation of a progressive and unimodal activation upon increasing sXIP addition (Fig. 1.2D) is challenging to integrate in the frame of this new model. Indeed, since the system is

predicted to respond in a non-linear fashion thanks to the positive feedback loop, even a small amount of the pheromone should trigger the loop and result in a maximum activation. To explain such a discrepancy between the model and experimental data, the most plausible hypothesis is that the loop is not functional at the timing of sXIP addition. The addition of sXIP would then titrate the ComR molecules present at the time of addition, without generating a fully activated system. Since Opp and ComR are necessary for sXIP activation, this implies that another key player of the loop (ComS or PptAB) must be unfunctional at early stage of growth when sXIP is provided. This assumption can fit with our experimental data, since observation of bimodality in *S. salivarius* occurs later during the growth phase, presumably due to the timing of ComR overproduction by the xylose-inducible promoter.

1.1 CovRS control

Investigations conducted during this thesis also led us to identify the environmental sensor CovRS as a major regulator of competence in the *salivarius* group. Particularly, we showed that CovR performs direct transcriptional repression on *comR* to fine-tune the fraction of competent cells.

To our knowledge, this is the first report of a direct transcriptional control on the widely-distributed pheromone receptors of the RRNPP family in Gram-positive bacteria. Those Quorum-Sensing systems are increasingly seen as communication devices integrating multiple inputs to fine tune their activation [112], and the CovRS control described here strengthen this view. Particularly, this regulation showcases how an environmental sensor can transmit a signal to a complex communication device to generate a differential response in a bacterial population.

One could question if the CovRS control of competence is a shared mechanism among streptococci. While suggested by transcriptomics analyses in *S. mutans* [293], this environmental sensor seems only involved in virulence in the pathogenic Group A and B streptococci (GAS and GBS). Indeed, extensive transcriptomics data generated in those species did not report any link between CovRS and competence [264,289,290,362], suggesting that the commensal *salivarius* species has diverted this sensor from a virulence to a competence regulator.

Although the *comR* transcriptional regulation by CovR has been well characterized in this study, less clear is the CovR-CovS signalization. In particular, none of the reported triggers for CovS streptococcal homologs (Mg^{2+} , LL-37, iron starvation and high glucose concentration) [264–270] except pH

modulation generated a significative activation, underlining the existence of alternative stimuli. Besides, the absence of the expected triggers could be related to a different signalization process involving cross-talk with other histidine kinase(s) or unknown modulators as reported in GAS and GBS [276,277,363]. Further study of the CovR-CovS interactions together with the identification of putative partners will help decipher the key determinants for CovRS activation. This is of main importance, since it will help us to understand the driving forces that modulate the proportion of competent cells in *S. salivarius* communities, and more broadly, provides ecological evidences to explain collective behavior.

2 PepF-mediated shut-off

2.1 PepF as a shut-off player

During this work, we uncovered the intracellular pheromone degradation as a ComRS antagonist system by combining mathematical simulations and previous transcriptomics data [4]. We show that the oligoendopeptidase PepF is embedded in a negative feedback loop through its activation by ComX, thereby triggering competence exit by XIP degradation. Moreover, we show that PepF acts also as a locking device, preventing spontaneous competence activation (Fig. 18).

Although at least two competence shut-off mechanisms have been described in the ComCDE streptococci [14,15,105], the PepF mechanism described here is the first report of a true shut-off in ComRS streptococci. Indeed, several antagonistic mechanisms have been described in *S. mutans* and *S. pyogenes* [98,104,106,108,109,364], but none of them stands as a true shut-off *i. e.* being controlled by competence activation and generating shorter activation time window.

In this work, we also showed that PepF displays a degradation efficiency depending on the sequence of its peptide substrate. Yet, PepF from *S. salivarius* was able to degrade XIP from *S. vestibularis* (Fig. S2.9), confirming previous reports showing low specificity [365,366]. Regarding the fact that ComRS streptococci display high XIP variability [19], PepF homologs in other streptococci could display alternative residues specificity or cleavage at different positions. This assumption is strengthened by the high synteny conservation between the late competence gene *coiA* and *pepF* in Firmicutes, suggesting a key role of *pepF* in competence control. Moreover, PepF was shown in *B. subtilis* to control other communication pheromones [367], strengthening the prevalent role of PepF as a pheromone antagonist. More broadly, the

pheromone control of XIP by PepF describes here is a supplemental illustration of the importance of oligoendopeptidases to fine-tune QS devices in streptococci [103,104] and highlights their importance for bacterial communication.

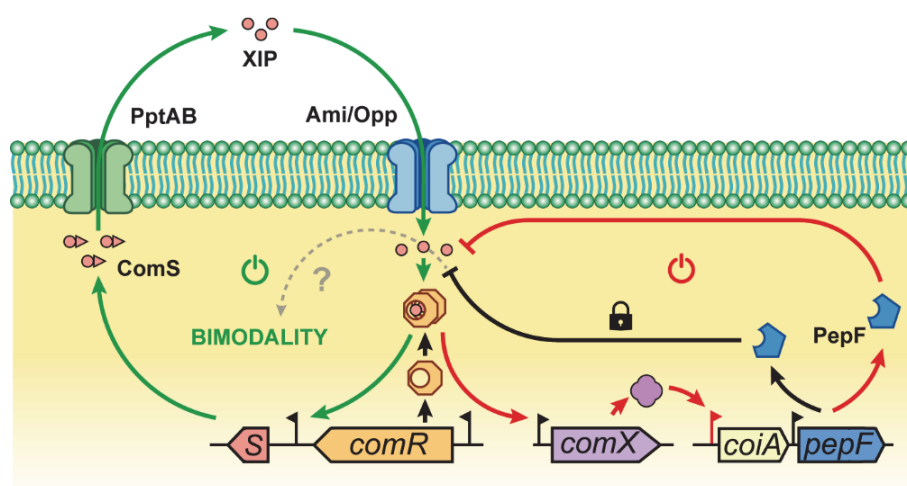


Figure 18. PepF shut-off and lockdown effects on ComRS. ComRS activation can take place if XIP levels are sufficient to saturate the activity of PepF at native expression (locker icon). Concomitantly, the ComR-XIP complex trigger the transcription of *comX*, encoding the central regulator of competence. This will activate all the late genes necessary for natural transformation including the *coiA-pepF* operon. PepF accumulation will result in XIP degradation, generating a negative feedback-loop (red arrows, power-off icon) on the ComRS system, ultimately leading to competence exit. In parallel, PepF could play a key role in bimodality by limiting XIP availability.

2.2 PepF and bimodality

In this thesis, we show that PepF has two main roles, one as a shut-off player and another as a locking device preventing competence initiation through pheromone limitation. In parallel, investigations on bimodality highlighted the unresponsiveness of the ComRS system to the overproduction of the pheromone while synthetic pheromone addition triggered unimodal activation. Our mathematical model conciliates those two observations. It suggests that PepF is quickly saturated upon synthetic addition while displaying progressive degradation along with *xip* gene overexpression.

This hypothesis implies that PepF has a major role in the control of the pheromone basal level. Since the origin of noise to trigger bimodality is still unclear (heterogeneous ComR or ComS/XIP concentrations), PepF could play a major role for bimodality in limiting the abundance of XIP (Fig. 18). Such

mechanism has been described in *S. mutans*, where the endopeptidase PepO is crucial to limit the pheromone concentration [104]. In this species, the deletion of the endopeptidase *pepO* shifts the bimodal activation of competence to a unimodal activation, suggesting that PepO plays a pivotal role to produce heterogeneity [104]. Investigations on the role of PepF in bimodality have not been addressed during this thesis, particularly because PepF inhibition results in a toxic phenotype. In those conditions, single-cell activation analyses can be difficult to conduct, as exemplified by the study of the essential gene *covR* in the context of bimodality.

3 CRISPRi screen and StkP

In the last set of investigations, we designed and performed three CRISPR-interference screens to uncover new (essential) genetic antagonists of competence. The method was validated by highlighting key regulators previously described and sheds light on a connection between cell-wall alteration and competence activation. Moreover, we show that the threonine kinase StkP interferes with *comX* activation during competence by an unknown mechanism involving muropeptide sensing (Fig. 19).

3.1 CRISPRi as a new major physiological study tool

In this work, we adapted the CRISPRi conditional mutant designed for *S. pneumoniae* [368] to *S. salivarius*, and show its functionality allowing inhibition of transcription of essential genes. Moreover, we demonstrate the efficiency of its genome-wide screen application to uncover essential genes involved in competence activation or limitation.

The key feature of this method is its ability to produce conditional mutants. Particularly, this technique allowed us to study the role of two essential genes (*pepF* and *covR*) of main importance for competence regulation. We also investigated the role of the serine/threonine kinase on competence regulation and showcase that inhibition and deletion (data not shown) can have profoundly different outcomes. This difference arises most probably because the generation of deletions implies transformation protocols with several rounds of growth, which provides more time for compensatory mutations to take place [369,370]. Interestingly, such compensatory mutations have been recently described in *S. pneumoniae* mutants defective for *stkP* and its phosphatase partner *phpP* [66]. Since CRISPR-interference allows the study of essential genes and avoid compensatory mutations, this technique will most probably become a major tool for geneticists in the coming years.

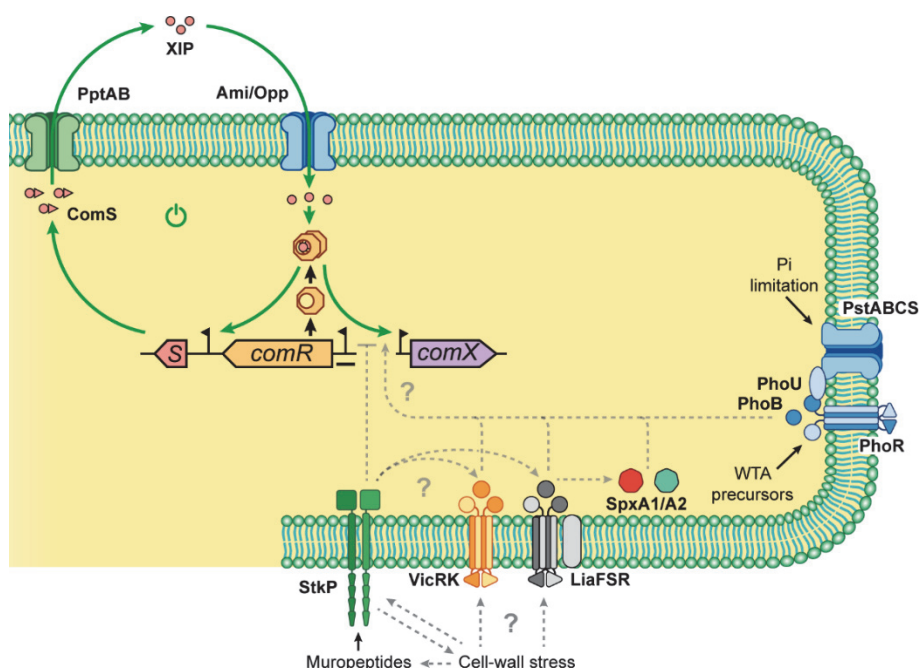


Figure 19. Cell-wall and StkP regulation of ComRS. ComRS activation loop is influenced by cell-wall impairment and many putative envelope sensors were selected or suggested by the CRISPRi screens. (1) StkP influences *comX* transcription by an unknown mechanism involving cell-wall and/or muropeptides sensing. (2) TCS envelope sensors such as VicRK or LiaFSR could be activated by StkP or sense cell-wall damage and transmit the signal directly to the ComRS system or to (3) SpxA1/A2 that could activate competence as reported in *S. pneumoniae*. (4) The PstABCS transporter could transmit a phosphate limitation signal to PhoR or (5) WTA precursors accumulation could activate PhoR which in both cases would lead to PhoB activation.

In parallel, the genome-wide screen application of the method has lately gained major interests because of its key advantages over the classical Tn-Seq (discussed in the introduction) [355]. Combining the genetic screen and the study of the genes selected by interference inhibition is thus a powerful and efficient strategy to explore genetic systems involving essential genes. Its design and development during this thesis in *S. salivarius* open multiple possibility in this species to investigate physiological pathways such as bacteriocin production and other pheromone-based communication systems.

3.2 StkP, a supplemental layer of shut-off?

In *S. pneumoniae*, at least two shut-off mechanisms contribute to competence exit [14,15,105]. Since competence is a toxic physiological state in *S. salivarius* [4], multiplying the shut-off mechanisms seems a relevant strategy to ensure a tight temporal control. In this work, we report a shut-off

mechanism acting directly on the pheromone availability via PepF. Nevertheless, we were not successful to produce a never-ending competence activation by inhibiting this pathway. One could hypothesize that our interference module is not efficient enough to completely remove PepF from the system, which would produce slower but not interrupted shut-down. Since we were unsuccessful to knock-out *pepF* (which was later shown to be essential in our CRISPRi screen), this assumption is hard to challenge.

An alternative to explain the lack of a complete shut-off impairment is to consider a supplemental shut-off pathway acting directly on ComR or ComR-XIP, and several evidences in this thesis advocate for such hypothesis. Indeed, the CRISPRi screen suggested new inhibitors of the ComRS system including the serine-threonine kinase StkP. We confirmed its antagonism towards ComRS and showed that StkP limits the activation of *comX* without visible effect on *comR* transcription. This indicates that StkP decrease *comX* transcription by an unknown mechanism that could take place at various levels. The most direct and simple hypothesis is the direct phosphorylation of ComR for its inactivation. This assumption should be tested in the future by co-overproducing StkP and ComR and analyze the phosphorylation status of ComR by mass spectrometry. In addition, purification of both proteins followed by a phosphorylation assay *in vitro* could be performed.

Although some peptidoglycan recycling pathways exist, muropeptides reach important concentrations during growth and peptidoglycan remodeling, particularly in Gram-positive bacteria [232]. In this work, we show that muropeptides mediate *comX* inhibition via StkP. Since it was shown in *B. subtilis* that StkP acts as a growth phase sensor by muropeptide sensing [236], it is tempting to speculate that StkP limits competence activation in *S. salivarius* by an unknown mechanism once a critical growth stage has been reached. Interestingly, this picture would fit perfectly into the frame of the work of Dufour and colleagues, who investigated the shut-off in *S. mutans* [371]. Their work showed that upon a threshold cellular density, competence completely shuts-down through the down-regulation of *comX*. Particularly, they produced extensive single mutant data on the 13 TCSs and the known QS regulators of *S. mutans* but were unsuccessful to inactivate the cellular-density exit from competence. They also demonstrated that this shut-off is not only triggered by *S. mutans* density but also from other species, suggesting a non-specific sensing mechanism. As they did not attempt to inactivate StkP, this peptidoglycan sensor stands as the perfect suspect. If existing, this type of extinction would not stand as a true shut-off, since it would be

independent from competence activation. Yet, this conclusion must be temperate by the fact that StkP deletion was shown to downregulate competence in *S. mutans* [63], even though constitutive mutant data should be cautiously considered for such a pleiotropic regulator.

3.3 Cell-wall and competence connection

The genome-wide screen performed in this work highlights a correlation between the impairment of cell-wall genes and competence activation. This connection suggests that competence is somehow used by *S. salivarius* to resist in case of envelope damages and underlines the importance of competence for survival in stress conditions.

This unexpected link has also been reported for a genome-wide screen in *S. mutans* [149], although the correlation between cell-wall impairment and competence is negative. Unfortunately, no further work to decipher how cell-wall damages and competence activation are associated is available. Since this connection is present in two different species presenting distinct lifestyles, we can raise the question whether cell-wall competence association is a common feature in streptococci. As competence produces bacteriocins to defend against competitors and provides a way to gain new genetic traits, triggering competence in case of cell-wall attack could make sense.

Exploring how cell-wall triggers competence in *S. salivarius* seems crucial to get insights into this stress response. Since extracellular sensing is mainly performed by two-components systems and the serine threonine kinase, they provide the first track for further investigations. Particularly, StkP was strongly highlighted in our screen, possesses key PASTA motifs recognizing muropeptides and was recently described in *S. pneumoniae* as a cell-wall stress sensor [66]. For those reasons, this kinase stands as a putative cell wall sensor. Yet, the most documented view of StkP defines it as a cell-wall and division orchestrator, using the repeated PASTA domains to localize at the septum where it phosphorylates key cell-wall and division proteins [153,326,330–332].

Having those two roles in mind, we can question whether StkP has a role in competence activation in *S. salivarius* because of its sensor feature or because its inhibition results in cell-wall impairment, which is later perceived by another sensor controlling competence. Although non-exclusives, those two distinct mechanisms would result in the same effect on competence upon *stkP* CRISPR-inhibition while capturing profoundly different stimuli. Yet, the CRISPRi screen selected many other genes involved in cell-wall

biogenesis. StkP-based or not, this indicates the existence of a cell-wall sensor mechanism, which activates competence upon envelope stress.

Among the putative two-components cell-wall sensors, LiaRSF is the most likely. Indeed, we found a hit corresponding to the *liaRSF* operon in our CRISPRi β -gal screen and the deletion of the *liaSR* tandem results in a 3-fold decrease in *comR* expression (Fig. 1.4A, Florence Vande Capelle data). Moreover, several homologs of this TCS have been shown to sense envelope stresses produced by cell-wall targeting antibiotics [372]. Particularly, crosstalk between StkP and LiaR has been reported in *S. aureus* [348], suggesting that those two sensors could also work together in *S. salivarius*.

Another putative sensor is the VicRK TCS also called WalRK. VicRK localizes at the septum of the cells and has a major role in cell growth and regulation of cell-wall metabolism [373]. Although the trigger of this sensor is not completely clear, heat stress and lipid II precursor have been suggested [343,374]. Notably, homologs of StkP have been shown to crosstalk with the response regulator VicR in *B. subtilis*, *S. pneumoniae* and *S. mutans*, triggering the activation of a similar regulon than the classical VicK activation [61,63,66]. In *S. salivarius*, VicR deletion seems lethal and we reported a slight decrease in *comR* expression in the *vicK* mutant (Fig. 1.4A, FVDC data).

The PstABCS together with its PhoU regulator were also highlighted by two of the screens. In *B. subtilis*, it was shown that upon phosphate limitation, this phosphate transporter can change its conformation and transmit the information to the PhoR histidine kinase through PhoU [349]. This ultimately leads to phosphorylation of the PhoB/PhoP response regulator, which activates optimized phosphate pathways such as the synthesis of teichuronic acids in place of the highly phosphate-consuming teichoic acid production. Besides, PhoR HK activity was shown to be sensitive to the accumulation of WTA precursors in case of phosphate shortages or antibiotic activity [350]. Regarding the fact that several LTA, WTA, and extracellular polysaccharides key enzymes were targeted in our screen, PhoRB stands as another putative sensor bridging cell-wall impairment and competence activation in *S. salivarius*. Unfortunately, there is no clear PhoRB homolog in *S. salivarius*, and further TCS investigations will help understand how the phosphate uptake pathway is connected to extracellular matrix and competence.

Finally, the Spx pathway stands also as a putative bridge between cell-wall impairment and competence activation. Indeed, it was shown in *B. subtilis* that cell wall stress can induce Spx activation by two independent pathways

[345,375], resulting in a cell-wall rescue response. Since SpxA1 was highlighted by our screen and because this protein was shown to activate competence in *S. pneumoniae* [376], one could hypothesize that the cell-wall stress is transmitted through this protein for competence activation. Furthermore, recent work in *S. pneumoniae* has shown that SpxA1 was activated upon cell-wall stress [66] although this regulator was previously shown in *S. mutans* to be the oxidative sensor of the SpxA1/A2 couple [347].

3.4 Lightning up stationary phase

To identify the key sensor(s) involved in the cell-wall-competence regulation, the simplest strategy consists of inhibiting a key cell-wall component to trigger the competence response in strains impaired for the different putative sensors. The disruption of the signalization in one of the mutants would then be associated to the sensor of the cell-wall-competence connection. Nevertheless, the cell-wall activation of competence seems to take place at late stage of growth. Since the transcriptional reporter LuxAB mostly used in our lab uses as a cofactor FMNH₂, which is scarce in stationary phase, we must find another reporter to monitor competence activation. Other luciferases with improved signal and using different cofactors have been developed such as the NanoLuc [377] or the *Renilla* luciferase [378] and their use might improve the signal in stationary phase.

The other key reporter we can think of is GFP. Since it only requires oxygen, it is suited for stationary phase experiments. Nevertheless, GFP signal is very low in *S. salivarius*, most probably because of its thick peptidoglycan layer, acidic intracellular concentration and low oxygen availability [379,380]. Notably, the optimization of GFP and other fluorescent tools has been the first task conducted during this thesis (data not shown) and turned out to be complicated and time-consuming. The optimized GFP generated by this work showed an increase in signal, but insufficient to be observed in high throughput multiplate readers. A solution to bypass the intracellular non-permissive conditions for fluorescence in *S. salivarius* would be to design a GFP-secretion system. The hijacking of a secretion machinery such as the Tat exporter [381] would allow intracellular folding and subsequent export of the reporter proteins, which would benefit from extracellular conditions to shine.

4 Conclusion

Competence is a physiological state of main survival relevance, involving predation via bacteriocin production, and exogenous DNA integration resulting in new traits acquisition or DNA repair. In this work, we focused on the dynamics of competence in the highly prevalent human commensal species *S. salivarius*.

More specifically, we show that the pheromone-based communication system ComRS integrates modulation from the environmental sensor CovRS to fine-tune collective behavior at the population scale. Importantly, those results showcase how environmental conditions can be perceived by bacteria and transmitted to complex regulatory pathways to generate heterogeneity.

Using a mathematical model together with classical biomolecular and biochemical tools, we also highlight the importance of the oligoendopeptidase PepF to limit competence activation to a sharp window of time. This control is the first true shut-off reported in ComRS streptococci and illustrates the importance of endopeptidase for Gram-positive communication devices. Also, this investigation underlines the relevance of simulation-guided experiments.

Finally, we set up a conditional mutant method associated to a genome-wide screen in *S. salivarius* which sheds light on a connection between cell-wall and competence activation. As the method developed has important perspectives to study essential genes, the cell-wall-competence connection reveals a key role for competence in stress conditions.

More broadly, those investigations show that competence is regulated at multiple levels to ensure an environmental and stress-dependent response, with population-wide outcomes. The new model for competence activation in *S. salivarius* built from those results is presented in an integrative view at Figure 20.

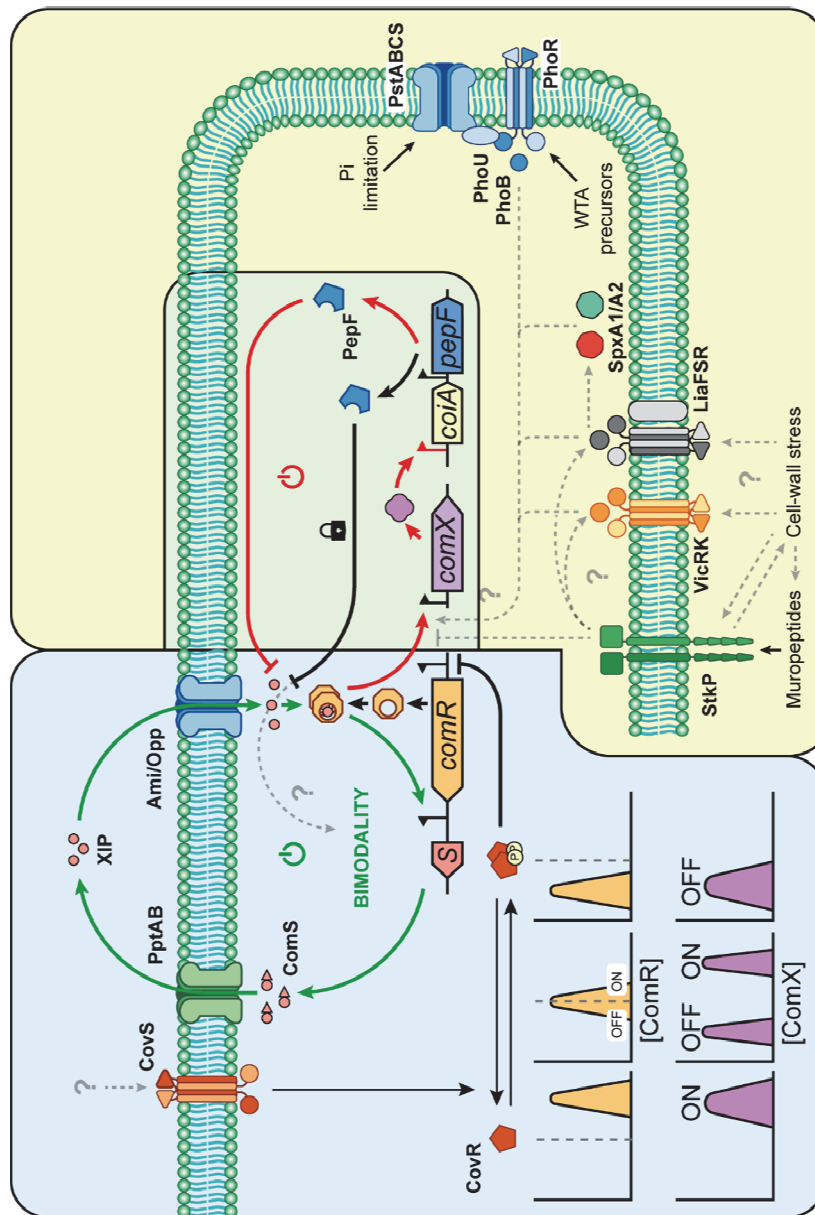


Figure 20. Integrative model presenting the major outcomes and perspectives from the thesis. Blue, green and yellow panels depict results presented in the first, second or third chapter of this thesis, respectively.

REFERENCES

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- [1] Facklam, R., 2002. What happened to the streptococci: Overview of taxonomic and nomenclature changes. *Clinical Microbiology Reviews*: 613–30, Doi: 10.1128/CMR.15.4.613-630.2002.
- [2] Delorme, C., Abraham, A.L., Renault, P., Guédon, E., 2015. Genomics of *Streptococcus salivarius*, a major human commensal. *Infection, Genetics and Evolution* 33: 381–92, Doi: 10.1016/j.meegid.2014.10.001.
- [3] Cosseau, C., Devine, D.A., Dullaghan, E., Gardy, J.L., Chikatarla, A., Gellatly, S., et al., 2008. The commensal *Streptococcus salivarius* K12 downregulates the innate immune responses of human epithelial cells and promotes host-microbe homeostasis. *Infection and Immunity* 76(9): 4163–75, Doi: 10.1128/IAI.00188-08.
- [4] Mignolet, J., Fontaine, L., Sass, A., Nannan, C., Mahillon, J., Coenye, T., et al., 2018. Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*. *Cell Reports* 22(7): 1627–38, Doi: 10.1016/j.celrep.2018.01.055.
- [5] Huang, R., Li, M., Gregory, R.L., 2011. Bacterial interactions in dental biofilm. *Virulence*: 435–44, Doi: 10.4161/viru.2.5.16140.
- [6] Huttenhower, C., Gevers, D., Knight, R., Abubucker, S., Badger, J.H., Chinwalla, A.T., et al., 2012. Structure, function and diversity of the healthy human microbiome. *Nature* 486(7402): 207–14, Doi: 10.1038/nature11234.
- [7] Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., et al., 2015. Bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. *Nature* 526(7575): 719–22, Doi: 10.1038/nature15524.
- [8] Bowen, W.H., Burne, R.A., Wu, H., Koo, H., 2018. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. *Trends in Microbiology*: 229–42, Doi: 10.1016/j.tim.2017.09.008.
- [9] Coyte, K.Z., Rakoff-Nahoum, S., 2019. Understanding Competition and Cooperation within the Mammalian Gut Microbiome. *Current Biology*: R538–44, Doi: 10.1016/j.cub.2019.04.017.
- [10] Veening, J.W., Blokesch, M., 2017. Interbacterial predation as a strategy for DNA acquisition in naturally competent bacteria. *Nature Reviews Microbiology*: 621–9, Doi: 10.1038/nrmicro.2017.66.
- [11] Wang, C.Y., Dawid, S., 2018. Mobilization of Bacteriocins during Competence in *Streptococci*. vol. 26, pp. 389–91.
- [12] Bergé, M.J., Mercy, C., Mortier-Barrière, I., Vannieuwenhze, M.S., Brun, Y. V., Grangeasse, C., et al., 2017. A programmed cell division delay preserves genome integrity during natural genetic transformation in *Streptococcus pneumoniae*. *Nature Communications* 8(1), Doi: 10.1038/s41467-017-01716-9.

-
- [13] Zaccaria, E., Wells, J.M., Baarlen, P. Van., 2016. Metabolic Context of the Competence- Induced Checkpoint for Cell Replication in *Streptococcus suis*. *PLOS One* 11(5): 1–13, Doi: 10.1371/journal.pone.0153571.
- [14] Mirouze, N., Bergé, M.A., Soulet, A.-L., Mortier-Barrière, I., Quentin, Y., Fichant, G., et al., 2013. Direct involvement of DprA, the transformation-dedicated RecA loader, in the shut-off of pneumococcal competence. *Proceedings of the National Academy of Sciences of the United States of America* 110(11): E1035–44, Doi: 10.1073/pnas.1219868110.
- [15] Weng, L., Piotrowski, A., Morrison, D.A., 2013. Exit from competence for genetic transformation in *Streptococcus pneumoniae* is regulated at multiple levels. *PloS One* 8(5): e64197, Doi: 10.1371/journal.pone.0064197.
- [16] Süel, G.M., Garcia-Ojalvo, J., Liberman, L.M., Elowitz, M.B., 2006. An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440(7083): 545–50, Doi: 10.1038/nature04588.
- [17] Leisner, M., Stingl, K., Frey, E., Maier, B., 2008. Stochastic switching to competence. *Current Opinion in Microbiology*: 553–9, Doi: 10.1016/j.mib.2008.09.020.
- [18] Gnugge, R., Dharmarajan, L., Lang, M., Stelling, J., 2016. An Orthogonal Permease-Inducer-Repressor Feedback Loop Shows Bistability. *ACS Synthetic Biology* 5(10): 1098–107, Doi: 10.1021/acssynbio.6b00013.
- [19] Fontaine, L., Wahl, A., Fléchar, M., Mignolet, J., Hols, P., 2015. Regulation of competence for natural transformation in streptococci. *Infection, Genetics and Evolution* 33: 343–60, Doi: 10.1016/j.meegid.2014.09.010.
- [20] Martin, B., Quentin, Y., Fichant, G., Claverys, J.P., 2006. Independent evolution of competence regulatory cascades in streptococci? *Trends in Microbiology*: 339–45, Doi: 10.1016/j.tim.2006.06.007.
- [21] Straume, D., Stamsås, G.A., Håvarstein, L.S., 2015. Natural transformation and genome evolution in *Streptococcus pneumoniae*. *Infection, Genetics and Evolution*: 371–80, Doi: 10.1016/j.meegid.2014.10.020.
- [22] Peterson, S.N., Sung, C.K., Cline, R., Desai, B. V., Snesrud, E.C., Luo, P., et al., 2004. Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Molecular Microbiology* 51(4): 1051–70, Doi: 10.1046/j.1365-2958.2003.03907.x.
- [23] Burton, B., Dubnau, D., 2010. Membrane-associated DNA transport machines. *Cold Spring Harbor Perspectives in Biology*, Doi: 10.1101/cshperspect.a000406.
- [24] Claverys, J.P., Martin, B., Polard, P., 2009. The genetic transformation machinery: Composition, localization, and mechanism. *FEMS Microbiology Reviews*, vol. 33. p. 643–56.

- [25] Dubnau, D., Blokesch, M., 2019. Mechanisms of DNA Uptake by Naturally Competent Bacteria. *Annual Review of Genetics*: 217–37, Doi: 10.1146/annurev-genet-112618-043641.
- [26] Chung, Y.S., Dubnau, D., 1998. All seven comG open reading frames are required for DNA binding during transformation of competent *Bacillus subtilis*. *Journal of Bacteriology* 180(1): 41–5, Doi: 10.1128/jb.180.1.41-45.1998.
- [27] Muschiol, S., Erlendsson, S., Aschtgen, M.S., Oliveira, V., Schmieder, P., De Lichtenberg, C., et al., 2017. Structure of the competence pilus major pilin ComGC in *Streptococcus pneumoniae*. *Journal of Biological Chemistry* 292(34): 14134–46, Doi: 10.1074/jbc.M117.787671.
- [28] Chung, Y.S., Dubnau, D., 1995. ComC is required for the processing and translocation of ComGC, a pilin-like competence protein of *Bacillus subtilis*. *Molecular Microbiology* 15(3): 543–51, Doi: 10.1111/j.1365-2958.1995.tb02267.x.
- [29] Sheppard, D., Berry, J.-L., Denise, R., Rocha, E.P.C., Matthews, S., Pelicic, V., 2020. The major subunit of widespread competence pili exhibits a novel and conserved type IV pilin fold, Doi: 10.1074/jbc.RA120.013316.
- [30] Laurenceau, R., Péhau-Arnaudet, G., Baconnais, S., Gault, J., Malosse, C., Dujeancourt, A., et al., 2013. A Type IV Pilus Mediates DNA Binding during Natural Transformation in *Streptococcus pneumoniae*. *PLoS Pathogens* 9(6): e1003473, Doi: 10.1371/journal.ppat.1003473.
- [31] Lam, T., Ellison, C.K., Eddington, D.T., Brun, Y. V., Dalia, A.B., Morrison, D.A., 2021. Competence pili in *Streptococcus pneumoniae* are highly dynamic structures that retract to promote DNA uptake. *Molecular Microbiology* 116(2): 381–96, Doi: 10.1111/mmi.14718.
- [32] Attaiech, L., Olivier, A., Mortier-Barrière, I., Soulet, A.-L., Granadel, C., Martin, B., et al., 2011. Role of the Single-Stranded DNA-Binding Protein SsbB in Pneumococcal Transformation: Maintenance of a Reservoir for Genetic Plasticity. *PLoS Genetics* 7(6): e1002156, Doi: 10.1371/journal.pgen.1002156.
- [33] Chen, Z., Yang, H., Pavletich, N.P., 2008. Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature* 453(7194): 489–94, Doi: 10.1038/nature06971.
- [34] Bell, C.E., 2005. Structure and mechanism of *Escherichia coli* RecA ATPase. *Molecular Microbiology*: 358–66, Doi: 10.1111/j.1365-2958.2005.04876.x.
- [35] Lisboa, J., Andreani, J., Sanchez, D., Boudes, M., Collinet, B., Liger, D., et al., 2014. Molecular determinants of the DprA-RecA interaction for nucleation on ssDNA. *Nucleic Acids Research* 42(11): 7395–408, Doi: 10.1093/nar/gku349.
- [36] Marie, L., Rapisarda, C., Morales, V., Bergé, M., Perry, T., Soulet, A.L., et al., 2017. Bacterial RadA is a DnaB-Type helicase interacting with RecA to promote bidirectional D-loop extension. *Nature Communications* 8, Doi: 10.1038/ncomms15638.

- [37] Desai, B. V., Morrison, D.A., 2006. An unstable competence-induced protein, *CoiA*, promotes processing of donor DNA after uptake during genetic transformation in *Streptococcus pneumoniae*. *Journal of Bacteriology* 188(14): 5177–86, Doi: 10.1128/JB.00103-06.
- [38] Matthey, N., Blokesch, M., 2016. The DNA-Uptake Process of Naturally Competent *Vibrio cholerae*. *Trends in Microbiology*: 98–110, Doi: 10.1016/j.tim.2015.10.008.
- [39] Solanki, V., Kapoor, S., Thakur, K.G., 2018. Structural insights into the mechanism of Type IVa pilus extension and retraction ATPase motors. *The FEBS Journal* 285(18): 3402–21, Doi: 10.1111/febs.14619.
- [40] Chlebek, J.L., Hughes, H.Q., Ratkiewicz, A.S., Rayyan, R., Wang, J.C.Y., Herrin, B.E., et al., 2019. PilT and PilU are homohexameric ATPases that coordinate to retract type IVa pili. *PLoS Genetics* 15(10), Doi: 10.1371/journal.pgen.1008448.
- [41] Jakobs, M., Meinhardt, F., 2014. What renders Bacilli genetically competent? A gaze beyond the model organism. *Applied Microbiology and Biotechnology* 99(4): 1557–70, Doi: 10.1007/s00253-014-6316-0.
- [42] Metzger, L.C., Blokesch, M., 2016. Regulation of competence-mediated horizontal gene transfer in the natural habitat of *Vibrio cholerae*. *Current Opinion in Microbiology*: 1–7, Doi: 10.1016/j.mib.2015.10.007.
- [43] Johnston, C., Martin, B., Fichant, G., Polard, P., Claverys, J.P., 2014. Bacterial transformation: Distribution, shared mechanisms and divergent control. *Nature Reviews Microbiology*: 181–96, Doi: 10.1038/nrmicro3199.
- [44] Shanker, E., Federle, M.J., 2017. Quorum sensing regulation of competence and bacteriocins in *Streptococcus pneumoniae* and mutants. *Genes*, Doi: 10.3390/genes8010015.
- [45] Håvarstein, L.S., Coomaraswamy, G., Morrison, D.A., 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America* 92(24): 11140–4, Doi: 10.1073/pnas.92.24.11140.
- [46] Piotrowski, A., Luo, P., Morrison, D.A., 2009. Competence for genetic transformation in *Streptococcus pneumoniae*: Termination of activity of the alternative sigma factor ComX is independent of proteolysis of ComX and ComW. *Journal of Bacteriology* 191(10): 3359–66, Doi: 10.1128/JB.01750-08.
- [47] Inniss, N.L., Prehna, G., Morrison, D.A., 2019. The pneumococcal σ activator, ComW, is a DNA-binding protein critical for natural transformation. *Journal of Biological Chemistry* 294(29): 11101–18, Doi: 10.1074/jbc.RA119.007571.
- [48] Chang, K.S., Morrison, D.A., 2005. Two distinct functions of ComW in stabilization and activation of the alternative sigma factor ComX in *Streptococcus pneumoniae*. *Journal of Bacteriology* 187(9): 3052–61, Doi: 10.1128/JB.187.9.3052-3061.2005.

- [49] Luo, P., Li, H., Morrison, D.A., 2004. Identification of ComW as a new component in the regulation of genetic transformation in *Streptococcus pneumoniae*. *Molecular Microbiology* 54(1): 172–83, Doi: 10.1111/j.1365-2958.2004.04254.x.
- [50] Echenique, J.R., Chapuy-Regaud, S., Trombe, M.C., 2000. Competence regulation by oxygen in *Streptococcus pneumoniae*: Involvement of *ciaRH* and *comCDE*. *Molecular Microbiology* 36(3): 688–96, Doi: 10.1046/j.1365-2958.2000.01891.x.
- [51] Martin, B., Prudhomme, M., Alloing, G., Granadel, C., Claverys, J.P., 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. *Molecular Microbiology* 38(4): 867–78, Doi: 10.1046/j.1365-2958.2000.02187.x.
- [52] Zähler, D., Grebe, T., Guenzi, E., Krau, J., Van Linden, M. Der., Terhune, K., et al., 1996. Resistance determinants for β -lactam antibiotics in laboratory mutants of *Streptococcus pneumoniae* that are involved in genetic competence. *Microbial Drug Resistance* 2(2): 187–91, Doi: 10.1089/mdr.1996.2.187.
- [53] Gómez-Mejía, A., Gámez, G., Hammerschmidt, S., 2018. *Streptococcus pneumoniae* two-component regulatory systems: The interplay of the pneumococcus with its environment. *International Journal of Medical Microbiology*: 722–37, Doi: 10.1016/j.ijmm.2017.11.012.
- [54] Mascher, T., Zähler, D., Merai, M., Balmelle, N., De Saizieu, A.B., Hakenbeck, R., 2003. The *Streptococcus pneumoniae* *cia* regulon: *CiaR* target sites and transcription profile analysis. *Journal of Bacteriology* 185(1): 60–70, Doi: 10.1128/JB.185.1.60-70.2003.
- [55] Cassone, M., Gagne, A.L., Spruce, L.A., Seeholzer, S.H., Seibert, M.E., 2012. The HtrA protease from *Streptococcus pneumoniae* digests both denatured proteins and the competence-stimulating peptide. *Journal of Biological Chemistry* 287(46): 38449–59, Doi: 10.1074/jbc.M112.391482.
- [56] Laux, A., Sexauer, A., Sivaselvarajah, D., Kaysen, A., Brückner, R., 2015. Control of competence by related non-coding csRNAs in *Streptococcus pneumoniae* R6. *Frontiers in Genetics* 6(JUL): 246, Doi: 10.3389/fgene.2015.00246.
- [57] Eldholm, V., Gutt, B., Johnsborg, O., Brückner, R., Maurer, P., Hakenbeck, R., et al., 2010. The pneumococcal cell envelope stress-sensing system LiaFSR is activated by murein hydrolases and lipid II-interacting antibiotics. *Journal of Bacteriology* 192(7): 1761–73, Doi: 10.1128/JB.01489-09.
- [58] Mohedano, M.L., Overweg, K., De La Fuente, A., Reuter, M., Altabe, S., Mulholland, F., et al., 2005. Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. *Journal of Bacteriology* 187(7): 2357–67, Doi: 10.1128/JB.187.7.2357-2367.2005.

- [59] Echenique, J.R., Trombe, M.C., 2001. Competence repression under oxygen limitation through the two-component MicAB signal-transducing system in *Streptococcus pneumoniae* and involvement of the PAS domain of MicB. *Journal of Bacteriology* 183(15): 4599–608, Doi: 10.1128/JB.183.15.4599-4608.2001.
- [60] Ng, W.L., Tsui, H.C.T., Winkler, M.E., 2005. Regulation of the *pspA* virulence factor and essential *pcsB* murein biosynthetic genes by the phosphorylated VicR (YycF) response regulator in *Streptococcus pneumoniae*. *Journal of Bacteriology* 187(21): 7444–59, Doi: 10.1128/JB.187.21.7444-7459.2005.
- [61] Libby, E.A., Goss, L.A., Dworkin, J., 2015. The Eukaryotic-Like Ser/Thr Kinase PrkC Regulates the Essential WalRK Two-Component System in *Bacillus subtilis*. *PLoS Genetics* 11(6), Doi: 10.1371/journal.pgen.1005275.
- [62] Reck, M., Rutz, K., Kunze, B., Tomasch, J., Surapaneni, S.K., Schulz, S., et al., 2011. The Biofilm Inhibitor Carolacton Disturbs Membrane Integrity and Cell Division of *Streptococcus mutans* through the Serine/Threonine Protein Kinase PknB †. *Journal of Bacteriology* 193(20): 5692–706, Doi: 10.1128/JB.05424-11.
- [63] Banu, L.D., Conrads, G., Rehrauer, H., Hussain, H., Allan, E., Van Der Ploeg, J.R., 2010. The *Streptococcus mutans* serine/threonine kinase, PknB, regulates competence development, bacteriocin production, and cell wall metabolism. *Infection and Immunity* 78(5): 2209–20, Doi: 10.1128/IAI.01167-09.
- [64] Echenique, J., Kadioglu, A., Romao, S., Andrew, P.W., Trombe, M.C., 2004. Protein Serine/Threonine Kinase StkP Positively Controls Virulence and Competence in *Streptococcus pneumoniae*. *Infection and Immunity* 72(4): 2434–7, Doi: 10.1128/IAI.72.4.2434-2437.2004.
- [65] Sasková, L., Nováková, L., Basler, M., Branny, P., 2007. Eukaryotic-type serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus pneumoniae*. *Journal of Bacteriology* 189(11): 4168–79, Doi: 10.1128/JB.01616-06.
- [66] Ulrych, A., Fabrik, I., Kupčík, R., Vajrychová, M., Doubravová, L., Branny, P., 2021. Cell wall stress stimulates the activity of the protein kinase StkP of *Streptococcus pneumoniae*, leading to multiple phosphorylation. *Journal of Molecular Biology*: 167319, Doi: 10.1016/j.jmb.2021.167319.
- [67] Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., Claverys, J.P., 2006. Antibiotic stress induces genetic transformability in the human pathogen *streptococcus pneumoniae*. *Science* 313(5783): 89–92, Doi: 10.1126/science.1127912.
- [68] Slager, J., Kjos, M., Attaiech, L., Veening, J.W., 2014. Antibiotic-induced replication stress triggers bacterial competence by increasing gene dosage near the origin. *Cell* 157(2): 395–406, Doi: 10.1016/j.cell.2014.01.068.

- [69] Mortier-Barrière, I., Velten, M., Dupaigne, P., Mirouze, N., Piétrement, O., McGovern, S., et al., 2007. A Key Presynaptic Role in Transformation for a Widespread Bacterial Protein: DprA Conveys Incoming ssDNA to RecA. *Cell* 130(5): 824–36, Doi: 10.1016/j.cell.2007.07.038.
- [70] Johnston, C.H.G., Soulet, A.L., Bergé, M., Prudhomme, M., Lemos, D. De., Polard, P., 2020. The alternative sigma factor σ_x mediates competence shut-off at the cell pole in *Streptococcus pneumoniae*. *ELife* 9: 1–27, Doi: 10.7554/eLife.62907.
- [71] Fontaine, L., Boutry, C., Henry De Frahan, M., Delplace, B., Fremaux, C., Horvath, P., et al., 2010. A Novel Pheromone Quorum-Sensing System Controls the Development of Natural Competence in *Streptococcus thermophilus* and *Streptococcus salivarius*. *JOURNAL OF BACTERIOLOGY* 192(5): 1444–54, Doi: 10.1128/JB.01251-09.
- [72] Gardan, R., Besset, C., Gitton, C., Guillot, A., Fontaine, L., Hols, P., et al., 2013. Extracellular life cycle of ComS, the competence-stimulating peptide of *Streptococcus thermophilus*. *Journal of Bacteriology* 195(8): 1845–55, Doi: 10.1128/JB.02196-12.
- [73] Lingeswaran, A., Metton, C., Henry, C., Monnet, V., Juillard, V., Gardan, R., 2020. Export of Rgg Quorum Sensing Peptides is Mediated by the PptAB ABC Transporter in *Streptococcus Thermophilus* Strain LMD-9. *Genes* 11(9): 1096, Doi: 10.3390/genes11091096.
- [74] Chang, J.C., Federle, M.J., 2016. PptAB exports Rgg quorum-sensing peptides in *Streptococcus*. *PLoS ONE* 11(12), Doi: 10.1371/journal.pone.0168461.
- [75] Fontaine, L., Goffin, P., Dubout, H., Delplace, B., Baulard, A., Lecat-Guillet, N., et al., 2013. Mechanism of competence activation by the ComRS signalling system in streptococci. *Molecular Microbiology* 87(6): 1113–32, Doi: 10.1111/mmi.12157.
- [76] Ledesma-Garcia, L., Thuillier, J., Guzman-Espinola, A., Ensink, I., de la Sierra-Gallay, I.L., Lazar, N., et al., 2020. Molecular dissection of pheromone selectivity in the competence signaling system ComRS of streptococci. *Proceedings of the National Academy of Sciences of the United States of America* 117(14): 7745–54, Doi: 10.1073/pnas.1916085117.
- [77] Kaspar, J., Underhill, S.A.M., Shields, R.C., Reyes, A., Rosenzweig, S., Hagen, S.J., et al., 2017. Intercellular communication via the comX-Inducing Peptide (XIP) of *Streptococcus mutans*. *Journal of Bacteriology* 199(21), Doi: 10.1128/JB.00404-17.
- [78] Moreno-Gámez, S., Sorg, R.A., Domenech, A., Kjos, M., Weissing, F.J., van Doorn, G.S., et al., 2017. Quorum sensing integrates environmental cues, cell density and cell history to control bacterial competence. *Nature Communications* 8(1): 854, Doi: 10.1038/s41467-017-00903-y.
- [79] Prudhomme, M., Berge, M., Martin, B., Polard, P., 2016. Pneumococcal Competence Coordination Relies on a Cell-Contact Sensing Mechanism. *PLoS Genetics* 12(6): e1006113, Doi: 10.1371/journal.pgen.1006113.

- [80] Neiditch, M.B., Capodagli, G.C., Prehna, G., Federle, M.J., 2017. Genetic and Structural Analyses of RRNPP Intercellular Peptide Signaling of Gram-Positive Bacteria. *Annual Review of Genetics*: 311–33, Doi: 10.1146/annurev-genet-120116-023507.
- [81] Talagas, A., Fontaine, L., Ledesma-García, L., Mignolet, J., Li de la Sierra-Gallay, I., Lazar, N., et al., 2017. Structural Insights into Streptococcal Competence Regulation by the Cell-to-Cell Communication System ComRS. *Annual Reviews of Genetics* 12(12): e1005980, Doi: 10.1371/journal.ppat.1005980.
- [82] Boutry, C., Wahl, A., Delplace, B., Clippe, A., Fontaine, L., Hols, P., 2012. Adaptor protein MecA is a negative regulator of the expression of late competence genes in *Streptococcus thermophilus*. *Journal of Bacteriology* 194(7): 1777–88, Doi: 10.1128/JB.06800-11.
- [83] Tian, X.L., Dong, G., Liu, T., Gomez, Z.A., Wahl, A., Hols, P., et al., 2013. MecA protein acts as a negative regulator of genetic competence in *Streptococcus mutans*. *Journal of Bacteriology* 195(22): 5196–206, Doi: 10.1128/JB.00821-13.
- [84] Kim, J.N., Stanhope, M.J., Burne, R.A., 2013. Core-gene-encoded peptide regulating virulence-associated traits in *Streptococcus mutans*. *Journal of Bacteriology* 195(12): 2912–20, Doi: 10.1128/JB.00189-13.
- [85] Li, Y.H., Tang, N., Aspiras, M.B., Lau, P.C.Y., Lee, J.H., Ellen, R.P., et al., 2002. A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation. *Journal of Bacteriology* 184(10): 2699–708, Doi: 10.1128/JB.184.10.2699-2708.2002.
- [86] Khan, R., Rukke, H. V., Filho, A.P.R., Fimland, G., Arntzen, M., Thiede, B., et al., 2012. Extracellular identification of a processed type II ComR/ComS pheromone of *Streptococcus mutans*. *Journal of Bacteriology* 194(15): 3781–8, Doi: 10.1128/JB.00624-12.
- [87] Desai, K., Mashburn-Warren, L., Federle, M.J., Morrison, D.A., 2012. Development of competence for genetic transformation of *Streptococcus mutans* in a chemically defined medium. *Journal of Bacteriology* 194(15): 3774–80, Doi: 10.1128/JB.00337-12.
- [88] Son, M., Ahn, S.-J., Guo, Q., Burne, R.A., Hagen, S.J., 2012. Microfluidic study of competence regulation in *Streptococcus mutans*: environmental inputs modulate bimodal and unimodal expression of comX. *Molecular Microbiology* 86(2): 258–72, Doi: 10.1111/j.1365-2958.2012.08187.x.
- [89] Reck, M., Tomasch, J., Wagner-Döbler, I., 2015. The Alternative Sigma Factor SigX Controls Bacteriocin Synthesis and Competence, the Two Quorum Sensing Regulated Traits in *Streptococcus mutans*. *PLoS Genetics* 11(7), Doi: 10.1371/journal.pgen.1005353.

- [90] Lemme, A., Gröbe, L., Reck, M., Tomasch, J., Wagner-Döbler, I., 2011. Subpopulation-specific transcriptome analysis of competence-stimulating-peptide-induced *Streptococcus mutans*. *Journal of Bacteriology* 193(8): 1863–77, Doi: 10.1128/JB.01363-10.
- [91] Hagen, S.J., Son, M., 2017. Origins of heterogeneity in *Streptococcus mutans* competence: interpreting an environment-sensitive signaling pathway. *Physical Biology* 14(1): 015001, Doi: 10.1088/1478-3975/aa546c.
- [92] Perry, J.A., Jones, M.B., Peterson, S.N., Cvitkovitch, D.G., Lévesque, C.M., 2009. Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. *Molecular Microbiology* 72(4): 905–17, Doi: 10.1111/j.1365-2958.2009.06693.x.
- [93] Hale, J.D.F., Ting, Y.T., Jack, R.W., Tagg, J.R., Heng, N.C.K., 2005. Bacteriocin (mutacin) production by *Streptococcus mutans* genome sequence reference strain UA159: Elucidation of the antimicrobial repertoire by genetic dissection. *Applied and Environmental Microbiology* 71(11): 7613–7, Doi: 10.1128/AEM.71.11.7613-7617.2005.
- [94] Qi, F., Merritt, J., Lux, R., Shi, W., 2004. Inactivation of the *ciaH* gene in *Streptococcus mutans* diminishes mutacin production and competence development, alters sucrose-dependent biofilm formation, and reduces stress tolerance. *Infection and Immunity* 72(8): 4895–9, Doi: 10.1128/IAI.72.8.4895-4899.2004.
- [95] Okinaga, T., Xie, Z., Niu, G., Qi, F., Merritt, J., 2010. Examination of the *hdrRM* regulon yields insight into the competence system of *Streptococcus mutans*. *Molecular Oral Microbiology* 25(3): 165–77, Doi: 10.1111/j.2041-1014.2010.00574.x.
- [96] Okinaga, T., Niu, G., Xie, Z., Qi, F., Merritt, J., 2010. The *hdrRM* operon of *Streptococcus mutans* encodes a novel regulatory system for coordinated competence development and bacteriocin production. *Journal of Bacteriology* 192(7): 1844–52, Doi: 10.1128/JB.01667-09.
- [97] Xie, Z., Okinaga, T., Niu, G., Qi, F., Merritt, J., 2010. Identification of a novel bacteriocin regulatory system in *Streptococcus mutans*. *Molecular Microbiology* 78(6): 1431–47, Doi: 10.1111/j.1365-2958.2010.07417.x.
- [98] Qin, H., Zou, Z., Anderson, D., Sang, Y., Higashi, D., Kreth, J., et al., 2021. The transcription regulator BrsR serves as a network hub of natural competence protein-protein interactions in *Streptococcus mutans*. *Proceedings of the National Academy of Sciences of the United States of America* 118(39), Doi: 10.1073/pnas.2106048118.
- [99] Dufour, D., Cordova, M., Cvitkovitch, D.G., Lévesque, C.M., 2011. Regulation of the competence pathway as a novel role associated with a Streptococcal bacteriocin. *Journal of Bacteriology* 193(23): 6552–9, Doi: 10.1128/JB.05968-11.
- [100] Underhill, S.A.M., Shields, R.C., Kaspar, J.R., Haider, M., Burne, R.A., Hagen, S.J., 2018. Intracellular Signaling by the *comRS* System in *Streptococcus mutans* Genetic Competence. *MSphere* 3(5): e00444-18, Doi: 10.1128/msphere.00444-18.

- [101] Chang, J.C., LaSarre, B., Jimenez, J.C., Aggarwal, C., Federle, M.J., 2011. Two Group A Streptococcal Peptide Pheromones Act through Opposing Rgg Regulators to Control Biofilm Development. *PLoS Pathogens* 7(8): e1002190, Doi: 10.1371/journal.ppat.1002190.
- [102] Chang, J.C., Jimenez, J.C., Federle, M.J., 2015. Induction of a quorum sensing pathway by environmental signals enhances group A streptococcal resistance to lysozyme. *Molecular Microbiology* 97(6): 1097–113, Doi: 10.1111/mmi.13088.
- [103] Wilkening, R. V., Chang, J.C., Federle, M.J., 2016. PepO, a CovRS-controlled endopeptidase, disrupts *Streptococcus pyogenes* quorum sensing. *Molecular Microbiology* 99(1): 71–87, Doi: 10.1111/mmi.13216.
- [104] Underhill, S.A.M., Shields, R.C., Burne, R.A., Hagen, S.J., 2019. Carbohydrate and PepO control bimodality in competence development by *Streptococcus mutans*. *Molecular Microbiology* 112(5): 1388–402, Doi: 10.1111/mmi.14367.
- [105] Martin, B., Soulet, A.L., Mirouze, N., Prudhomme, M., Mortier-Barrière, I., Granadel, C., et al., 2013. ComE/ComE~P interplay dictates activation or extinction status of pneumococcal X-state (competence). *Molecular Microbiology* 87(2): 394–411, Doi: 10.1111/mmi.12104.
- [106] Kaspar, J., Ahn, S.J., Palmer, S.R., Choi, S.C., Stanhope, M.J., Burne, R.A., 2015. A unique open reading frame within the comX gene of *Streptococcus mutans* regulates genetic competence and oxidative stress tolerance. *Molecular Microbiology* 96(3): 463–82, Doi: 10.1111/mmi.12948.
- [107] Kaspar, J., Shields, R.C., Burne, R.A., 2018. Competence inhibition by the XrpA peptide encoded within the comX gene of *Streptococcus mutans*. *Molecular Microbiology* 109(3): 345–64, Doi: 10.1111/mmi.13989.
- [108] Mashburn-Warren, L., Goodman, S.D., Federle, M.J., Prehna, G., 2018. The conserved mosaic prophage protein paratox inhibits the natural competence regulator ComR in *Streptococcus*. *Scientific Reports* 8(1): 16535, Doi: 10.1038/s41598-018-34816-7.
- [109] Rutbeek, N.R., Rezasoltani, H., Patel, T.R., Khajepour, M., Prehna, G., 2021. Molecular mechanism of quorum sensing inhibition in *Streptococcus* by the phage protein paratox. *Journal of Biological Chemistry* 297(3): 100992, Doi: 10.1016/j.jbc.2021.100992.
- [110] Haustenne, L., Bastin, G., Hols, P., Fontaine, L., 2015. Modeling of the ComRS signaling pathway reveals the limiting factors controlling competence in *Streptococcus thermophilus*. *Frontiers in Microbiology* 6(DEC), Doi: 10.3389/fmicb.2015.01413.
- [111] Veening, J.-W., Smits, W.K., Kuipers, O.P., 2008. Bistability, Epigenetics, and Bet-Hedging in Bacteria. *Annu. Rev. Microbiol* 62: 193–210, Doi: 10.1146/annurev.micro.62.081307.163002.

-
- [112] Bettenworth, V., Steinfeld, B., Duin, H., Petersen, K., Streit, W.R., Bischofs, I., et al., 2019. Phenotypic Heterogeneity in Bacterial Quorum Sensing Systems. *Journal of Molecular Biology*: 4530–46, Doi: 10.1016/j.jmb.2019.04.036.
- [113] López, D., Vlamakis, H., Losick, R., Kolter, R., 2009. Paracrine signaling in a bacterium. *Genes and Development* 23(14): 1631–8, Doi: 10.1101/gad.1813709.
- [114] van Gestel, J., Vlamakis, H., Kolter, R., 2015. From Cell Differentiation to Cell Collectives: *Bacillus subtilis* Uses Division of Labor to Migrate. *PLoS Biology* 13(4): e1002141, Doi: 10.1371/journal.pbio.1002141.
- [115] Elowitz, M.B., Levine, A.J., Siggia, E.D., Swain, P.S., 2002. Stochastic Gene Expression in a Single Cell. *Science* 297(5584): 1183–6, Doi: 10.1126/SCIENCE.1070919.
- [116] Pedraza, J.H., Van Oudenaarden, A., 2005. Noise propagations in gene networks. *Science* 307(5717): 1965–9, Doi: 10.1126/science.1109090.
- [117] Thattai, M., Van Oudenaarden, A., 2001. Intrinsic noise in gene regulatory networks. *Proceedings of the National Academy of Sciences of the United States of America* 98(15): 8614–9, Doi: 10.1073/pnas.151588598.
- [118] Kim, S., Jacobs-Wagner, C., 2018. Effects of mRNA Degradation and Site-Specific Transcriptional Pausing on Protein Expression Noise. *Biophysical Journal* 114(7): 1718–29, Doi: 10.1016/j.bpj.2018.02.010.
- [119] Bury-Moné, S., Sclavi, B., 2017. Stochasticity of gene expression as a motor of epigenetics in bacteria: from individual to collective behaviors. *Research in Microbiology* 168(6): 503–14, Doi: 10.1016/j.resmic.2017.03.009.
- [120] Smits, W.K., Kuipers, O.P., Veening, J.W., 2006. Phenotypic variation in bacteria: The role of feedback regulation. *Nature Reviews Microbiology*: 259–71, Doi: 10.1038/nrmicro1381.
- [121] Gunawardena, J., 2014. Models in biology: “Accurate descriptions of our pathetic thinking.” *BMC Biology*: 29, Doi: 10.1186/1741-7007-12-29.
- [122] Uphoff, S., Lord, N.D., Okumus, B., Potvin-Trottier, L., Sherratt, D.J., Paulsson, J., 2016. Stochastic activation of a DNA damage response causes cell-to-cell mutation rate variation. *Science* 351(6277): 1094–7, Doi: 10.1126/science.aac9786.
- [123] Gardner, T.S., Cantor, C.R., Collins, J.J., 2000. Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403(6767): 339–42, Doi: 10.1038/35002131.
- [124] Yildirim, N., 2012. Mathematical modeling of the low and high affinity arabinose transport systems in *Escherichia coli*. *Molecular BioSystems* 8(4): 1319, Doi: 10.1039/c2mb05352g.
- [125] Bhalla, U.S., Ram, P.T., Iyengar, R., 2002. MAP kinase phosphatase as a locus of flexibility in a mitogen-activated protein kinase signaling network. *Science* 297(5583): 1018–23, Doi: 10.1126/science.1068873.

-
- [126] Hahl, S.K., Kremling, A., 2016. A Comparison of Deterministic and Stochastic Modeling Approaches for Biochemical Reaction Systems: On Fixed Points, Means, and Modes. *Frontiers in Genetics* 7(AUG): 157, Doi: 10.3389/fgene.2016.00157.
- [127] Sauro, H.M., 2014. *Systems Biology: Introduction to Pathway Modeling*. Ambrosius Publishing.
- [128] Gillespie, D.T., 2001. Approximate accelerated stochastic simulation of chemically reacting systems. *Journal of Chemical Physics* 115(4): 1716–33, Doi: 10.1063/1.1378322.
- [129] Chatterjee, A., Vlachos, D.G., Katsoulakis, M.A., 2005. Binomial distribution based τ -leap accelerated stochastic simulation. *Journal of Chemical Physics* 122(2): 024112, Doi: 10.1063/1.1833357.
- [130] Camacho, D.M., Collins, K.M., Powers, R.K., Costello, J.C., Collins, J.J., 2018. Next-Generation Machine Learning for Biological Networks. *Cell*: 1581–92, Doi: 10.1016/j.cell.2018.05.015.
- [131] Son, M., Ghoreishi, D., Ahn, S.-J., Burne, R.A., Hagen, S.J., 2015. Sharply Tuned pH Response of Genetic Competence Regulation in *Streptococcus mutans*: a Microfluidic Study of the Environmental Sensitivity of comX. *Applied and Environmental Microbiology* 81(16): 5622–31, Doi: 10.1128/AEM.01421-15.
- [132] Cahn, F.H., Fox, M.S., 1968. Fractionation of transformable bacteria from ocompetent cultures of *Bacillus subtilis* on renografin gradients. *Journal of Bacteriology* 95(3): 867–75, Doi: 10.1128/jb.95.3.867-875.1968.
- [133] Hadden, C., Nester, E.W., 1968. Purification of competent cells in the *Bacillus subtilis* transformation system. *Journal of Bacteriology* 95(3): 876–85, Doi: 10.1128/jb.95.3.876-885.1968.
- [134] Nester, E.W., Stocker, B.A.D., 1963. Biosynthetic latency in early stages of deoxyribonucleic acid transformation in *Bacillus subtilis*. *Journal of Bacteriology* 86(4): 785–96, Doi: 10.1128/JB.86.4.785-796.1963.
- [135] Haijema, B.-J., Hahn, J., Haynes, J., Dubnau, D., 2001. A ComGA-dependent checkpoint limits growth during the escape from competence. *Molecular Microbiology* 40(1): 52–64, Doi: 10.1046/j.1365-2958.2001.02363.x.
- [136] Hamoen, L.W., Venema, G., Kuipers, O.P., 2003. Controlling competence in *Bacillus subtilis*: Shared use of regulators. *Microbiology*: 9–17, Doi: 10.1099/mic.0.26003-0.
- [137] Eldar, A., Elowitz, M.B., 2010. Functional roles for noise in genetic circuits. *Nature*: 167–73, Doi: 10.1038/nature09326.
- [138] Leisner, M., Kuhr, J.T., Rädler, J.O., Frey, E., Maier, B., 2009. Kinetics of genetic switching into the state of bacterial competence. *Biophysical Journal* 96(3): 1178–88, Doi: 10.1016/j.bpj.2008.10.034.

- [139] Alon, U., 2007. Network motifs: Theory and experimental approaches. *Nature Reviews Genetics*: 450–61, Doi: 10.1038/nrg2102.
- [140] Maamar, H., Dubnau, D., 2005. Bistability in the *Bacillus subtilis* K-state (competence) system requires a positive feedback loop. *Molecular Microbiology* 56(3): 615–24, Doi: 10.1111/j.1365-2958.2005.04592.x.
- [141] Turgay, K., Hamoen, L.W., Venema, G., Dubnau, D., 1997. Biochemical characterization of a molecular switch involving the heat shock protein ClpC, which controls the activity of ComK, the competence transcription factor of *Bacillus subtilis*. *Genes and Development* 11(1): 119–28, Doi: 10.1101/gad.11.1.119.
- [142] K, T., J, H., J, B., D, D., 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *The EMBO Journal* 17(22): 6730–8, Doi: 10.1093/emboj/17.22.6730.
- [143] Ogura, M., Liu, L., LaCelle, M., Nakano, M.M., Zuber, P., 1999. Mutational analysis of ComS: Evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis*. *Molecular Microbiology* 32(4): 799–812, Doi: 10.1046/j.1365-2958.1999.01399.x.
- [144] Süel, G.M., Kulkarni, R.P., Dworkin, J., Garcia-Ojalvo, J., Elowitz, M.B., 2007. Tunability and noise dependence in differentiation dynamics. *Science* 315(5819): 1716–9, Doi: 10.1126/science.1137455.
- [145] Li, Y.H., Lau, P.C.Y., Lee, J.H., Ellen, R.P., Cvitkovitch, D.G., 2001. Natural genetic transformation of *Streptococcus mutans* growing in biofilms. *Journal of Bacteriology* 183(3): 897–908, Doi: 10.1128/JB.183.3.897-908.2001.
- [146] Son, M., Shields, R.C., Ahn, S.-J., Burne, R.A., Hagen, S.J., 2015. Bidirectional signaling in the competence regulatory pathway of *Streptococcus mutans*. *FEMS Microbiology Letters* 362, Doi: 10.1093/femsle/fnv159.
- [147] De Furio, M., Ahn, S.J., Burne, R.A., Hagen, S.J., 2017. Oxidative stressors modify the response of *Streptococcus mutans* to its competence signal peptides. *Applied and Environmental Microbiology* 83(22), Doi: 10.1128/AEM.01345-17.
- [148] Moye, Z.D., Son, M., Rosa-Alberty, A.E., Zeng, L., Ahn, S.-J.J., Hagen, S.J., et al., 2016. Effects of Carbohydrate Source on Genetic Competence in *Streptococcus mutans*. *Applied and Environmental Microbiology* 82(15): 4821–34, Doi: 10.1128/aem.01205-16.
- [149] Shields, R.C., O'Brien, G., Maricic, N., Kesterson, A., Grace, M., Hagen, S.J., et al., 2018. Genome-wide screens reveal new gene products that influence genetic competence in *Streptococcus mutans*. *Journal of Bacteriology* 200(2), Doi: 10.1128/JB.00508-17.
- [150] Mirouze, N., Ferret, C., Cornilleau, C., Carballido-López, R., 2018. Antibiotic sensitivity reveals that wall teichoic acids mediate DNA binding during competence in *Bacillus subtilis*. *Nature Communications* 9(1): 1–11, Doi: 10.1038/s41467-018-07553-8.

- [151] Silhavy, T.J., Kahne, D., Walker, S., 2010. The bacterial cell envelope. Cold Spring Harbor Perspectives in Biology, Doi: 10.1101/cshperspect.a000414.
- [152] Egan, A.J.F., Errington, J., Vollmer, W., 2020. Regulation of peptidoglycan synthesis and remodelling. Nature Reviews Microbiology: 446–60, Doi: 10.1038/s41579-020-0366-3.
- [153] Vollmer, W., Massidda, O., Tomasz, A., 2019. The Cell Wall of *Streptococcus pneumoniae*. Microbiology Spectrum 7(3), Doi: 10.1128/microbiolspec.gpp3-0018-2018.
- [154] Barreteau, H., Kovač, A., Boniface, A., Sova, M., Gobec, S., Blanot, D., 2008. Cytoplasmic steps of peptidoglycan biosynthesis. FEMS Microbiology Reviews: 168–207, Doi: 10.1111/j.1574-6976.2008.00104.x.
- [155] Vollmer, W., Blanot, D., De Pedro, M.A., 2008. Peptidoglycan structure and architecture. FEMS Microbiology Reviews 32(2): 149–67, Doi: 10.1111/j.1574-6976.2007.00094.x.
- [156] Schleifer, K.H., Kandler, O., 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews: 407–77, Doi: 10.1128/mmbr.36.4.407-477.1972.
- [157] N.d. KEGG PATHWAY: Amino sugar and nucleotide sugar metabolism - *Streptococcus salivarius* JIM8777. <https://www.kegg.jp/pathway/stj00520>. [accessed July 15, 2021].
- [158] Filipe, S.R., Pinho, M.G., Tomasz, A., 2000. Characterization of the murMN operon involved in the synthesis of branched peptidoglycan peptides in *Streptococcus pneumoniae*. Journal of Biological Chemistry 275(36): 27768–74, Doi: 10.1074/jbc.M004675200.
- [159] Fiser, A., Filipe, S.R., Tomasz, A., 2003. Cell wall branches, penicillin resistance and the secrets of the MurM protein. Trends in Microbiology 11(12): 547–53, Doi: 10.1016/j.tim.2003.10.003.
- [160] Shepherd, J., Ibba, M., 2013. Direction of aminoacylated transfer RNAs into antibiotic synthesis and peptidoglycan-mediated antibiotic resistance. FEBS Letters: 2895–904, Doi: 10.1016/j.febslet.2013.07.036.
- [161] Mohammadi, T., Van Dam, V., Sijbrandi, R., Vernet, T., Zapun, A., Bouhss, A., et al., 2011. Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. EMBO Journal 30(8): 1425–32, Doi: 10.1038/emboj.2011.61.
- [162] Sham, L.T., Butler, E.K., Lebar, M.D., Kahne, D., Bernhardt, T.G., Ruiz, N., 2014. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. Science 345(6193): 220–2, Doi: 10.1126/science.1254522.

- [163] Ruiz, N., 2008. Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 105(40): 15553–7, Doi: 10.1073/pnas.0808352105.
- [164] Taguchi, A., Welsh, M.A., Marmont, L.S., Lee, W., Sjodt, M., Kruse, A.C., et al., 2019. FtsW is a peptidoglycan polymerase that is functional only in complex with its cognate penicillin-binding protein. *Nature Microbiology* 4(4): 587–94, Doi: 10.1038/s41564-018-0345-x.
- [165] Emami, K., Guyet, A., Kawai, Y., Devi, J., Wu, L.J., Allenby, N., et al., 2017. RodA as the missing glycosyltransferase in *Bacillus subtilis* and antibiotic discovery for the peptidoglycan polymerase pathway. *Nature Microbiology* 2, Doi: 10.1038/nmicrobiol.2016.253.
- [166] Sjodt, M., Brock, K., Dobihal, G., Rohs, P.D.A., Green, A.G., Hopf, T.A., et al., 2018. Structure of the peptidoglycan polymerase RodA resolved by evolutionary coupling analysis. *Nature* 556(7699): 118–21, Doi: 10.1038/nature25985.
- [167] Meeske, A.J., Sham, L.T., Kimsey, H., Koo, B.M., Gross, C.A., Bernhardt, T.G., et al., 2015. MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences of the United States of America* 112(20): 6437–42, Doi: 10.1073/pnas.1504967112.
- [168] Straume, D., Piechowiak, K.W., Kjos, M., Håvarstein, L.S., 2021. Class A PBPs: It is time to rethink traditional paradigms. *Molecular Microbiology*: 41–52, Doi: 10.1111/mmi.14714.
- [169] Meeske, A.J., Riley, E.P., Robins, W.P., Uehara, T., Mekalanos, J.J., Kahne, D., et al., 2016. SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* 537(7622): 634–8, Doi: 10.1038/nature19331.
- [170] Straume, D., Piechowiak, K.W., Olsen, S., Stamsås, G.A., Berg, K.H., Kjos, M., et al., 2020. Class A PBPs have a distinct and unique role in the construction of the pneumococcal cell wall. *Proceedings of the National Academy of Sciences of the United States of America* 117(11): 6129–38, Doi: 10.1073/pnas.1917820117.
- [171] Irazoki, O., Hernandez, S.B., Cava, F., 2019. Peptidoglycan muropeptides: Release, perception, and functions as signaling molecules. *Frontiers in Microbiology*, Doi: 10.3389/fmicb.2019.00500.
- [172] De Las Rivas, B., García, J.L., López, R., García, P., 2002. Purification and polar localization of pneumococcal LytB, a putative endo- β -N-acetylglucosaminidase: The chain-dispersing murein hydrolase. *Journal of Bacteriology* 184(18): 4988–5000, Doi: 10.1128/JB.184.18.4988-5000.2002.
- [173] Duchêne, M.C., Rolain, T., Knoop, A., Courtin, P., Chapot-Chartier, M.P., Dufrêne, Y.F., et al., 2019. Distinct and specific role of NlpC/P60 endopeptidases LytA and LytB in cell elongation and division of *Lactobacillus plantarum*. *Frontiers in Microbiology* 10(APR), Doi: 10.3389/fmicb.2019.00713.

- [174] Bai, X.H., Chen, H.J., Jiang, Y.L., Wen, Z., Huang, Y., Cheng, W., et al., 2014. Structure of pneumococcal peptidoglycan hydrolase LytB reveals insights into the bacterial cell wall remodeling and pathogenesis. *Journal of Biological Chemistry* 289(34): 23403–16, Doi: 10.1074/jbc.M114.579714.
- [175] López, R., P. González, M., García, E., L. García, J., García, P., 2000. Biological roles of two new murein hydrolases of *Streptococcus pneumoniae* representing examples of module shuffling. *Research in Microbiology*, vol. 151. Elsevier Masson SAS p. 437–43.
- [176] Garcia, P., Gonzalez, M.P., Garcia, E., Garcia, J.L., Lopez, R., 1999. The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. *Molecular Microbiology* 33(1): 128–38, Doi: 10.1046/j.1365-2958.1999.01455.x.
- [177] Tsui, H.-C.T., Zheng, J.J., Magallon, A.N., Ryan, J.D., Yunck, R., Rued, B.E., et al., 2016. Suppression of a deletion mutation in the gene encoding essential PBP2b reveals a new lytic transglycosylase involved in peripheral peptidoglycan synthesis in *Streptococcus pneumoniae* D39. *Molecular Microbiology* 100(6): 1039–65, Doi: 10.1111/mmi.13366.
- [178] Sham, L.T., Barendt, S.M., Kopecky, K.E., Winkler, M.E., 2011. Essential PcsB putative peptidoglycan hydrolase interacts with the essential FtsX Spn cell division protein in *Streptococcus pneumoniae* D39. *Proceedings of the National Academy of Sciences of the United States of America* 108(45): E1061–9, Doi: 10.1073/pnas.1108323108.
- [179] Morlot, C., Pernot, L., Le Gouellec, A., Di Guilmi, A.M., Vernet, T., Dideberg, O., et al., 2005. Crystal structure of a peptidoglycan synthesis regulatory factor (PBP3) from *Streptococcus pneumoniae*. *Journal of Biological Chemistry* 280(16): 15984–91, Doi: 10.1074/jbc.M408446200.
- [180] Barendt, S.M., Sham, L.T., Winkler, M.E., 2011. Characterization of mutants deficient in the L,D-carboxypeptidase (DacB) and WalRK (VicRK) Regulon, involved in peptidoglycan maturation of *Streptococcus pneumoniae* serotype 2 strain D39. *Journal of Bacteriology* 193(9): 2290–300, Doi: 10.1128/JB.01555-10.
- [181] Hoyland, C.N., Aldridge, C., Cleverley, R.M., Duchêne, M.C., Minasov, G., Onopriyenko, O., et al., 2014. Structure of the LdcB LD-carboxypeptidase reveals the molecular basis of peptidoglycan recognition. *Structure* 22(7): 949–60, Doi: 10.1016/j.str.2014.04.015.
- [182] Chapot-Chartier, M.P., Kulakauskas, S., 2014. Cell wall structure and function in lactic acid bacteria. *Microbial Cell Factories* 13(Suppl 1): S9, Doi: 10.1186/1475-2859-13-S1-S9.
- [183] Brown, S., Santa, J.P., Jr, M., Walker, S., n.d. Wall Teichoic Acids of Gram-Positive Bacteria, Doi: 10.1146/annurev-micro-092412-155620.
- [184] Percy, M.G., Gründling, A., 2014. Lipoteichoic Acid Synthesis and Function in Gram-Positive Bacteria. *Annual Review of Microbiology* 68(1): 81–100, Doi: 10.1146/annurev-micro-091213-112949.

- [185] Swoboda, J.G., Campbell, J., Meredith, T.C., Walker, S., 2010. Wall teichoic acid function, biosynthesis, and inhibition. *ChemBioChem*: 35–45, Doi: 10.1002/cbic.200900557.
- [186] Neuhaus, F.C., Baddiley, J., 2003. A Continuum of Anionic Charge: Structures and Functions of d -Alanyl-Teichoic Acids in Gram-Positive Bacteria . *Microbiology and Molecular Biology Reviews* 67(4): 686–723, Doi: 10.1128/mubr.67.4.686-723.2003.
- [187] Anderson, A.J., Green, R.S., Sturman, A.J., Archibald, A.R., 1978. Cell wall assembly in *Bacillus subtilis*: Location of wall material incorporated during pulsed release of phosphate limitation, its accessibility to bacteriophages and concanavalin A, and its susceptibility to turnover. *Journal of Bacteriology* 136(3): 886–99, Doi: 10.1128/jb.136.3.886-899.1978.
- [188] Soldo, B., Lazarevic, V., Karamata, D., 2002. tagO is involved in the synthesis of all anionic cell-wall polymers in *Bacillus subtilis* 168. *Microbiology* 148(7): 2079–87, Doi: 10.1099/00221287-148-7-2079.
- [189] D’Elia, M.A., Henderson, J.A., Beveridge, T.J., Heinrichs, D.E., Brown, E.D., 2009. The N-acetylmannosamine transferase catalyzes the first committed step of teichoic acid assembly in *Bacillus subtilis* and *Staphylococcus aureus*. *Journal of Bacteriology* 191(12): 4030–4, Doi: 10.1128/JB.00611-08.
- [190] Soldo, B., Lazarevic, V., Pooley, H.M., Karamata, D., 2002. Characterization of a *Bacillus subtilis* thermosensitive teichoic acid-deficient mutant: Gene *mnaA* (*yvyH*) encodes the UDP-N-Acetylglucosamine 2-epimerase. *Journal of Bacteriology* 184(15): 4316–20, Doi: 10.1128/JB.184.15.4316-4320.2002.
- [191] Bhavsar, A.P., Truant, R., Brown, E.D., 2005. The TagB protein in *Bacillus subtilis* 168 is an intracellular peripheral membrane protein that can incorporate glycerol phosphate onto a membrane-bound acceptor in vitro. *Journal of Biological Chemistry* 280(44): 36691–700, Doi: 10.1074/jbc.M507154200.
- [192] Lovering, A.L., Lin, L.Y.C., Sewell, E.W., Spreter, T., Brown, E.D., Strynadka, N.C.J., 2010. Structure of the bacterial teichoic acid polymerase TagF provides insights into membrane association and catalysis. *Nature Structural and Molecular Biology* 17(5): 582–9, Doi: 10.1038/nsmb.1819.
- [193] Pereira, M.P., Schertzer, J.W., D’Elia, M.A., Koteva, K.P., Hughes, D.W., Wright, G.D., et al., 2008. The wall teichoic acid polymerase TagF efficiently synthesizes poly(glycerol phosphate) on the TagB product lipid III. *ChemBioChem* 9(9): 1385–90, Doi: 10.1002/cbic.200800026.
- [194] Young Seo Park., Sweitzer, T.D., Dixon, J.E., Kent, C., 1993. Expression, purification, and characterization of CTP:glycerol-3-phosphate cytidyltransferase from *Bacillus subtilis*. *Journal of Biological Chemistry* 268(22): 16648–54, Doi: 10.1016/s0021-9258(19)85467-4.

- [195] Allison, S.E., D'Elia, M.A., Arar, S., Monteiro, M.A., Brown, E.D., 2011. Studies of the genetics, function, and kinetic mechanism of TagE, the wall teichoic acid glycosyltransferase in *Bacillus subtilis* 168. *Journal of Biological Chemistry* 286(27): 23708–16, Doi: 10.1074/jbc.M111.241265.
- [196] Bernal, P., Zloh, M., Taylor, P.W., 2009. Disruption of D-alanyl esterification of *Staphylococcus aureus* cell wall teichoic acid by the β -lactam resistance modifier (-)-epicatechin gallate. *Journal of Antimicrobial Chemotherapy* 63(6): 1156–62, Doi: 10.1093/jac/dkp094.
- [197] Brown, S., Xia, G., Luhachack, L.G., Campbell, J., Meredith, T.C., Chen, C., et al., 2012. Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *Proceedings of the National Academy of Sciences of the United States of America* 109(46): 18909–14, Doi: 10.1073/pnas.1209126109.
- [198] Xia, G., Kohler, T., Peschel, A., 2010. The wall teichoic acid and lipoteichoic acid polymers of *Staphylococcus aureus*. *International Journal of Medical Microbiology*: 148–54, Doi: 10.1016/j.ijmm.2009.10.001.
- [199] Kawai, Y., Marles-Wright, J., Cleverley, R.M., Emmins, R., Ishikawa, S., Kuwano, M., et al., 2011. A widespread family of bacterial cell wall assembly proteins. *EMBO Journal* 30(24): 4931–41, Doi: 10.1038/emboj.2011.358.
- [200] Lazarevic, V., Karamata, D., 1995. The tagGH operon of *Bacillus subtilis* 168 encodes a two-component ABC transporter involved in the metabolism of two wall teichoic acids. *Molecular Microbiology* 16(2): 345–55, Doi: 10.1111/j.1365-2958.1995.tb02306.x.
- [201] Schirner, K., Stone, L.K., Walker, S., 2011. ABC transporters required for export of wall teichoic acids do not discriminate between different main chain polymers. *ACS Chemical Biology*, vol. 6. ACS Chem Biol p. 407–12.
- [202] Perego, M., Glaser, P., Minutello, A., Strauch, M.A., Leopold, K., Fischer, W., 1995. Incorporation of D-alanine into lipoteichoic acid and wall teichoic acid in *Bacillus subtilis*: Identification of genes and regulation. *Journal of Biological Chemistry* 270(26): 15598–606, Doi: 10.1074/jbc.270.26.15598.
- [203] May, J.J., Finking, R., Wiegeshoff, F., Weber, T.T., Bandur, N., Koert, U., et al., 2005. Inhibition of the D-alanine:D-alanyl carrier protein ligase from *Bacillus subtilis* increases the bacterium's susceptibility to antibiotics that target the cell wall. *FEBS Journal* 272(12): 2993–3003, Doi: 10.1111/j.1742-4658.2005.04700.x.
- [204] Kovács, M., Halfmann, A., Fedtke, I., Heintz, M., Peschel, A., Vollmer, W., et al., 2006. A functional dlt operon, encoding proteins required for incorporation of D-alanine in teichoic acids in gram-positive bacteria, confers resistance to cationic antimicrobial peptides in *Streptococcus pneumoniae*. *Journal of Bacteriology* 188(16): 5797–805, Doi: 10.1128/JB.00336-06.

- [205] Gründling, A., Schneewind, O., 2007. Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. *Journal of Bacteriology* 189(6): 2521–30, Doi: 10.1128/JB.01683-06.
- [206] Jorasch, P., Warnecke, D.C., Lindner, B., Zähringer, U., Heinz, E., 2000. Novel processive and nonprocessive glycosyltransferases from *Staphylococcus aureus* and *Arabidopsis thaliana* synthesize glycoglycerolipids, glycopospholipids, glycosphingolipids and glycosylsterols. *European Journal of Biochemistry* 267(12): 3770–83, Doi: 10.1046/j.1432-1327.2000.01414.x.
- [207] Jorasch, P., Wolter, F.P., Zähringer, U., Heinz, E., 1998. A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: Expression of ypfP in *Escherichia coli* and structural analysis of its reaction products. *Molecular Microbiology* 29(2): 419–30, Doi: 10.1046/j.1365-2958.1998.00930.x.
- [208] Kiriukhin, M.Y., Debabov, D. V., Shinabarger, D.L., Neuhaus, F.C., 2001. Biosynthesis of the glycolipid anchor in lipoteichoic acid of *Staphylococcus aureus* RN4220: Role of YpfP, the diglucosyldiacylglycerol synthase. *Journal of Bacteriology* 183(11): 3506–14, Doi: 10.1128/JB.183.11.3506-3514.2001.
- [209] Zhang, B., Liu, X., Lambert, E., Mas, G., Hiller, S., Veening, J.W., et al., 2020. Structure of a proton-dependent lipid transporter involved in lipoteichoic acids biosynthesis. *Nature Structural and Molecular Biology* 27(6): 561–9, Doi: 10.1038/s41594-020-0425-5.
- [210] Gründling, A., Schneewind, O., 2007. Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proceedings of the National Academy of Sciences of the United States of America* 104(20): 8478–83, Doi: 10.1073/pnas.0701821104.
- [211] Koch, H.U., Haas, R., Fischer, W., 1984. The role of lipoteichoic acid biosynthesis in membrane lipid metabolism of growing *Staphylococcus aureus*. *European Journal of Biochemistry* 138(2): 357–63, Doi: 10.1111/j.1432-1033.1984.tb07923.x.
- [212] Taron, D.J., Childs, W.C., Neuhaus, F.C., 1983. Biosynthesis of D-alanyl-lipoteichoic acid: Role of diglyceride kinase in the synthesis of phosphatidylglycerol for chain elongation. *Journal of Bacteriology* 154(3): 1110–6, Doi: 10.1128/jb.154.3.1110-1116.1983.
- [213] Arakawa, H., Shimada, A., Ishimoto, N., Ito, E., 1981. Occurrence of ribitol-containing lipoteichoic acid in *Staphylococcus aureus* h and its glycosylation. *Journal of Biochemistry* 89(5): 1555–63, Doi: 10.1093/oxfordjournals.jbchem.a133349.
- [214] Heaton, M.P., Neuhaus, F.C., 1992. Biosynthesis of D-alanyl-lipoteichoic acid: Cloning, nucleotide sequence, and expression of the *Lactobacillus casei* gene for the D-alanine-activating enzyme. *Journal of Bacteriology* 174(14): 4707–17, Doi: 10.1128/jb.174.14.4707-4717.1992.

- [215] Iwasaki, H., Shimada, A., Yokoyama, K., Ito, E., 1989. Structure and glycosylation of lipoteichoic acids in *Bacillus* strains. *Journal of Bacteriology* 171(1): 424–9, Doi: 10.1128/jb.171.1.424-429.1989.
- [216] Iwasaki, H., Shimada, A., Ito, E., 1986. Comparative studies of lipoteichoic acids from several *Bacillus* strains. *Journal of Bacteriology* 167(2): 508–16, Doi: 10.1128/jb.167.2.508-516.1986.
- [217] Miller, D.J., Jerga, A., Rock, C.O., White, S.W., 2008. Analysis of the *Staphylococcus aureus* DgkB Structure Reveals a Common Catalytic Mechanism for the Soluble Diacylglycerol Kinases. *Structure* 16(7): 1036–46, Doi: 10.1016/j.str.2008.03.019.
- [218] Jerga, A., Miller, D.J., White, S.W., Rock, C.O., 2009. Molecular determinants for interfacial binding and conformational change in a soluble diacylglycerol kinase. *Journal of Biological Chemistry* 284(11): 7246–54, Doi: 10.1074/jbc.M805962200.
- [219] Jerga, A., Lu, Y.J., Schuijman, G.E., De Mendoza, D., Rock, C.O., 2007. Identification of a soluble diacylglycerol kinase required for lipoteichoic acid production in *Bacillus subtilis*. *Journal of Biological Chemistry* 282(30): 21738–45, Doi: 10.1074/jbc.M703536200.
- [220] Mistou, M.Y., Sutcliffe, I.C., Van Sorge, N.M., 2016. Bacterial glycobiology: Rhamnose-containing cell wall polysaccharides in gram-positive bacteria. *FEMS Microbiology Reviews*: 464–79, Doi: 10.1093/femsre/fuw006.
- [221] Yother, J., 2011. Capsules of *Streptococcus pneumoniae* and other bacteria: Paradigms for polysaccharide biosynthesis and regulation. *Annual Review of Microbiology*: 563–81, Doi: 10.1146/annurev.micro.62.081307.162944.
- [222] Soliman, C., Pier, G.B., Ramsland, P.A., 2020. Antibody recognition of bacterial surfaces and extracellular polysaccharides. *Current Opinion in Structural Biology*: 48–55, Doi: 10.1016/j.sbi.2019.12.001.
- [223] Lancefield, R.C., 1933. A serological differentiation of human and other groups of hemolytic streptococci. *Journal of Experimental Medicine* 57(4): 571–94, Doi: 10.1084/jem.57.4.571.
- [224] Van Sorge, N.M., Cole, J.N., Kuipers, K., Henningham, A., Aziz, R.K., Kasirer-Friede, A., et al., 2014. The classical lancefield antigen of group A *Streptococcus* is a virulence determinant with implications for vaccine design. *Cell Host and Microbe* 15(6): 729–40, Doi: 10.1016/j.chom.2014.05.009.
- [225] Caliot, É., Dramsi, S., Chapot-Chartier, M.P., Courtin, P., Kulakauskas, S., Péchoux, C., et al., 2012. Role of the group B antigen of *Streptococcus agalactiae*: A peptidoglycan-anchored polysaccharide involved in cell wall biogenesis. *PLoS Pathogens* 8(6), Doi: 10.1371/journal.ppat.1002756.
- [226] Sadovskaya, I., Vinogradov, E., Courtin, P., Armalyte, J., Meyrand, M., Giaouris, E., et al., 2017. Another brick in the wall: A rhamnan polysaccharide trapped inside peptidoglycan of *Lactococcus lactis*. *MBio* 8(5), Doi: 10.1128/mBio.01303-17.

- [227] Theodorou, I., Courtin, P., Palussière, S., Kulakauskas, S., Bidnenko, E., Péchoux, C., et al., 2019. A dual-chain assembly pathway generates the high structural diversity of cell-wall polysaccharides in *Lactococcus lactis*. *Journal of Biological Chemistry* 294(46): 17612–25, Doi: 10.1074/jbc.RA119.009957.
- [228] Köhler, W., 2007. The present state of species within the genera *Streptococcus* and *Enterococcus*. *International Journal of Medical Microbiology*: 133–50, Doi: 10.1016/j.ijmm.2006.11.008.
- [229] Giraud, M.F., Naismith, J.H., 2000. The rhamnose pathway. *Current Opinion in Structural Biology*: 687–96, Doi: 10.1016/S0959-440X(00)00145-7.
- [230] Doyle, R.J., Chaloupka, J., Vinter, V., 1988. Turnover of cell walls in microorganisms. *Microbiological Reviews*: 554–67, Doi: 10.1128/mmr.52.4.554-567.1988.
- [231] Mauck, J., Glaser, L., 1970. Turnover of the cell wall of *Bacillus subtilis* W-23 during logarithmic growth. *Biochemical and Biophysical Research Communications* 39(4): 699–706, Doi: 10.1016/0006-291X(70)90261-5.
- [232] Reith, J., Mayer, C., 2011. Peptidoglycan turnover and recycling in Gram-Positive bacteria. *Applied Microbiology and Biotechnology*: 1–11, Doi: 10.1007/s00253-011-3486-x.
- [233] Borisova, M., Gaupp, R., Duckworth, A., Schneider, A., Dalügge, D., Mühleck, M., et al., 2016. Peptidoglycan recycling in gram-positive bacteria is crucial for survival in stationary phase. *MBio* 7(5), Doi: 10.1128/mBio.00923-16.
- [234] Kluj, R.M., Ebner, P., Adamek, M., Ziemert, N., Mayer, C., Borisova, M., 2018. Recovery of the peptidoglycan turnover product released by the Autolysin Atl in *Staphylococcus aureus* involves the phosphotransferase system transporter MurP and the Novel 6-phospho-N-acetylmuramidase MupG. *Frontiers in Microbiology* 9(NOV), Doi: 10.3389/fmicb.2018.02725.
- [235] Mir, M., Asong, J., Li, X., Cardot, J., Boons, G.J., Husson, R.N., 2011. The extracytoplasmic domain of the mycobacterium tuberculosis ser/thr kinase PknB binds specific muropeptides and is required for PknB localization. *PLoS Pathogens* 7(7), Doi: 10.1371/journal.ppat.1002182.
- [236] Shah, I.M., Laaberki, M.H., Popham, D.L., Dworkin, J., 2008. A Eukaryotic-like Ser/Thr Kinase Signals Bacteria to Exit Dormancy in Response to Peptidoglycan Fragments. *Cell* 135(3): 486–96, Doi: 10.1016/j.cell.2008.08.039.
- [237] Righino, B., Galisson, F., Pirolli, D., Vitale, S., Réty, S., Gouet, P., et al., 2018. Structural model of the full-length Ser/Thr protein kinase StkP from *S. pneumoniae* and its recognition of peptidoglycan fragments. *Journal of Biomolecular Structure and Dynamics* 36(14): 3666–79, Doi: 10.1080/07391102.2017.1395767.

- [238] Wang, Q., Marchetti, R., Prsic, S., Ishii, K., Arai, Y., Ohta, I., et al., 2017. A Comprehensive Study of the Interaction between Peptidoglycan Fragments and the Extracellular Domain of Mycobacterium tuberculosis Ser/Thr Kinase PknB. *ChemBioChem* 18(21): 2094–8, Doi: 10.1002/cbic.201700385.
- [239] Buschiazio, A., Trajtenberg, F., 2019. Two-component sensing and regulation: How do histidine kinases talk with response regulators at the molecular level? *Annual Review of Microbiology*: 507–28, Doi: 10.1146/annurev-micro-091018-054627.
- [240] Jacob-Dubuisson, F., Mechaly, A., Betton, J.M., Antoine, R., 2018. Structural insights into the signalling mechanisms of two-component systems. *Nature Reviews Microbiology*: 585–93, Doi: 10.1038/s41579-018-0055-7.
- [241] Cousin, C., Derouiche, A., Shi, L., Pagot, Y., Poncet, S., Mijakovic, I., 2013. Protein-serine/threonine/tyrosine kinases in bacterial signaling and regulation. *FEMS Microbiology Letters* 346(1): 11–9, Doi: 10.1111/1574-6968.12189.
- [242] Choi, H.S., Kim, J.R., Lee, S.W., Cho, K.H., 2008. Why have serine/threonine/tyrosine kinases been evolutionarily selected in eukaryotic signaling cascades? *Computational Biology and Chemistry* 32(3): 218–21, Doi: 10.1016/j.compbiolchem.2008.02.005.
- [243] Ulrich, L.E., Zhulin, I.B., 2009. The MiST2 database: A comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Research* 38(SUPPL.1), Doi: 10.1093/nar/gkp940.
- [244] Zschiedrich, C.P., Keidel, V., Szurmant, H., 2016. Molecular Mechanisms of Two-Component Signal Transduction. *Journal of Molecular Biology*: 3752–75, Doi: 10.1016/j.jmb.2016.08.003.
- [245] Casino, P., Rubio, V., Marina, A., 2010. The mechanism of signal transduction by two-component systems. *Current Opinion in Structural Biology*: 763–71, Doi: 10.1016/j.sbi.2010.09.010.
- [246] Gushchin, I., Gordeliy, V., 2018. Transmembrane Signal Transduction in Two-Component Systems: Piston, Scissoring, or Helical Rotation? *BioEssays* 40(2): 1700197, Doi: 10.1002/bies.201700197.
- [247] Neiditch, M.B., Federle, M.J., Pompeani, A.J., Kelly, R.C., Swem, D.L., Jeffrey, P.D., et al., 2006. Ligand-Induced Asymmetry in Histidine Sensor Kinase Complex Regulates Quorum Sensing. *Cell* 126(6): 1095–108, Doi: 10.1016/j.cell.2006.07.032.
- [248] Wang, B., Zhao, A., Novick, R.P., Muir, T.W., 2014. Activation and inhibition of the receptor histidine kinase AgrC occurs through opposite helical transduction motions. *Molecular Cell* 53(6): 929–40, Doi: 10.1016/j.molcel.2014.02.029.
- [249] Szurmant, H., Bunn, M.W., Cho, S.H., Ordal, G.W., 2004. Ligand-induced conformational changes in the Bacillus subtilis chemoreceptor McpB determined by disulfide crosslinking in vivo. *Journal of Molecular Biology* 344(4): 919–28, Doi: 10.1016/j.jmb.2004.09.093.

- [250] Moukhametzianov, R., Klare, J.P., Efremov, R., Baeken, C., Göppner, A., Labahn, J., et al., 2006. Development of the signal in sensory rhodopsin and its transfer to the cognate transducer. *Nature* 440(7080): 115–9, Doi: 10.1038/nature04520.
- [251] Molnar, K.S., Bonomi, M., Pellarin, R., Clinthorne, G.D., Gonzalez, G., Goldberg, S.D., et al., 2014. Cys-Scanning disulfide crosslinking and bayesian modeling probe the transmembrane signaling mechanism of the histidine kinase, PhoQ. *Structure* 22(9): 1239–51, Doi: 10.1016/j.str.2014.04.019.
- [252] Gushchin, I., Melnikov, I., Polovinkin, V., Ishchenko, A., Yuzhakova, A., Buslaev, P., et al., 2017. Mechanism of transmembrane signaling by sensor histidine kinases. *Science* 356(6342): eaah6345, Doi: 10.1126/science.aah6345.
- [253] Casino, P., Rubio, V., Marina, A., 2009. Structural Insight into Partner Specificity and Phosphoryl Transfer in Two-Component Signal Transduction. *Cell* 139(2): 325–36, Doi: 10.1016/j.cell.2009.08.032.
- [254] Yang, Y., Inouye, M., 1991. Intermolecular complementation between two defective mutant signal- transducing receptors of *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* 88(24): 11057–61, Doi: 10.1073/pnas.88.24.11057.
- [255] Ninfa, E.G., Atkinson, M.R., Kamberov, E.S., Ninfa, A.J., 1993. Mechanism of autophosphorylation of *Escherichia coli* nitrogen regulator II (NR(II) or NtrB): trans-Phosphorylation between subunits. *Journal of Bacteriology* 175(21): 7024–32, Doi: 10.1128/jb.175.21.7024-7032.1993.
- [256] Bhate, M.A.P., Molnar, K.A.S., Goulian, M., Degrado, W.F., 2015. Signal Transduction in Histidine Kinases: Insights from New Structures. *Structure*: 981–94, Doi: 10.1016/j.str.2015.04.002.
- [257] Casino, P., Miguel-Romero, L., Marina, A., 2014. Visualizing autophosphorylation in histidine kinases. *Nature Communications* 5(1): 1–12, Doi: 10.1038/ncomms4258.
- [258] Stock, A.M., Robinson, V.L., Goudreau, P.N., 2000. Two-component signal transduction. *Annual Review of Biochemistry*: 183–215, Doi: 10.1146/annurev.biochem.69.1.183.
- [259] Nguyen, M.P., Yoon, J.M., Cho, M.H., Lee, S.W., 2015. Prokaryotic 2-component systems and the ompr/phob superfamily. *Canadian Journal of Microbiology*: 799–810, Doi: 10.1139/cjm-2015-0345.
- [260] Lee, S.Y., Cho, H.S., Pelton, J.G., Yan, D., Henderson, R.K., King, D.S., et al., 2001. Crystal structure of an activated response regulator bound to its target. *Nature Structural Biology* 8(1): 52–6, Doi: 10.1038/83053.
- [261] Kleerebezem, M., Quadri, L.E.N., Kuipers, O.P., De Vos, W.M., 1997. Quorum sensing by peptide pheromones and two-component signal- transduction systems in Gram-positive bacteria. *Molecular Microbiology* 24(5): 895–904, Doi: 10.1046/j.1365-2958.1997.4251782.x.

- [262] Levin, J.C., Wessels, M.R., 1998. Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A streptococcus. *Molecular Microbiology* 30(1): 209–19, Doi: 10.1046/j.1365-2958.1998.01057.x.
- [263] Jiang, S.M., Cieslewicz, M.J., Kasper, D.L., Wessels, M.R., 2005. Regulation of virulence by a two-component system in group B *Streptococcus*. *Journal of Bacteriology* 187(3): 1105–13, Doi: 10.1128/JB.187.3.1105-1113.2005.
- [264] Gryllos, I., Levin, J.C., Wessels, M.R., 2003. The *CsrR/CsrS* two-component system of group A *Streptococcus* responds to environmental Mg^{2+} . *Proceedings of the National Academy of Sciences of the United States of America* 100(7): 4227–32, Doi: 10.1073/pnas.0636231100.
- [265] Gryllos, I., Grifantini, R., Colaprico, A., Jiang, S., DeForce, E., Hakansson, A., et al., 2007. Mg^{2+} signalling defines the group a streptococcal *CsrRS* (*CovRS*) regulon. *Molecular Microbiology* 65(3): 671–83, Doi: 10.1111/j.1365-2958.2007.05818.x.
- [266] Gryllos, I., Tran-Winkler, H.J., Cheng, M.-F., Chung, H., Bolcome, R., Lu, W., et al., 2008. Induction of group A *Streptococcus* virulence by a human antimicrobial peptide. *Proceedings of the National Academy of Sciences of the United States of America* 105(43): 16755–60, Doi: 10.1073/pnas.0803815105.
- [267] Froehlich, B.J., Bates, C., Scott, J.R., 2009. *Streptococcus pyogenes* *CovRS* mediates growth in iron starvation and in the presence of the human cationic antimicrobial peptide LL-37. *Journal of Bacteriology* 191(2): 673–7, Doi: 10.1128/JB.01256-08.
- [268] Park, S.E., Jiang, S., Wessels, M.R., 2012. *CsrRS* and environmental pH regulate group B *Streptococcus* adherence to human epithelial cells and extracellular matrix. *Infection and Immunity* 80(11): 3975–84, Doi: 10.1128/IAI.00699-12.
- [269] Di Palo, B., Rippa, V., Santi, I., Brettoni, C., Muzzi, A., Metruccio, M.M.E., et al., 2013. Adaptive Response of Group B *Streptococcus* to High Glucose Conditions: New Insights on the *CovRS* Regulation Network. *PLoS ONE* 8(4), Doi: 10.1371/journal.pone.0061294.
- [270] Velarde, J.J., Ashbaugh, M., Wessels, M.R., 2014. The human antimicrobial peptide LL-37 binds directly to *CsrS*, a sensor histidine kinase of group A *Streptococcus*, to activate expression of virulence factors. *The Journal of Biological Chemistry* 289(52): 36315–24, Doi: 10.1074/jbc.M114.605394.
- [271] Horstmann, N., Tran, C.N., Brumlow, C., DebRoy, S., Yao, H., Noguera Gonzalez, G., et al., 2018. Phosphatase activity of the control of virulence sensor kinase *CovS* is critical for the pathogenesis of group A streptococcus. *PLoS Pathogens* 14(10), Doi: 10.1371/journal.ppat.1007354.
- [272] Horstmann, N., Sahasrabhojane, P., Saldaña, M., Ajami, N.J., Flores, A.R., Sumby, P., et al., 2015. Characterization of the Effect of the Histidine Kinase *CovS* on Response Regulator Phosphorylation in Group A *Streptococcus*. *Infection and Immunity* 83(3): 1068, Doi: 10.1128/IAI.02659-14.

- [273] Gusa, A.A., Gao, J., Stringer, V., Churchward, G., Scott, J.R., 2006. Phosphorylation of the group A Streptococcal CovR response regulator causes dimerization and promoter-specific recruitment by RNA polymerase. *Journal of Bacteriology* 188(13): 4620–6, Doi: 10.1128/JB.00198-06.
- [274] Horstmann, N., Saldaña, M., Sahasrabhojane, P., Yao, H., Su, X., Thompson, E., et al., 2014. Dual-Site Phosphorylation of the Control of Virulence Regulator Impacts Group A Streptococcal Global Gene Expression and Pathogenesis. *PLoS Pathogens* 10(5), Doi: 10.1371/journal.ppat.1004088.
- [275] Dalton, T.L., Scott, J.R., 2004. CovS inactivates CovR and is required for growth under conditions of general stress in *Streptococcus pyogenes*. *Journal of Bacteriology* 186(12): 3928–37, Doi: 10.1128/JB.186.12.3928-3937.2004.
- [276] Biswas, I., Scott, J.R., 2003. Identification of rocA, a positive regulator of covR expression in the group A streptococcus. *Journal of Bacteriology* 185(10): 3081–90, Doi: 10.1128/JB.185.10.3081-3090.2003.
- [277] Chiang-Ni, C., Chiou, H.-J., Tseng, H.-C., Hsu, C.-Y., Chiu, C.-H., 2020. RocA Regulates Phosphatase Activity of Virulence Sensor CovS of Group A Streptococcus in Growth Phase- and pH-Dependent Manners . *MSphere* 5(3), Doi: 10.1128/msphere.00361-20.
- [278] Jain, I., Miller, E.W., Danger, J.L., Pflughoeft, K.J., Sumby, P., 2017. RocA Is an Accessory Protein to the Virulence-Regulating CovRS Two-Component System in Group A Streptococcus. *Infection and Immunity* 85(11), Doi: 10.1128/IAI.00274-17.
- [279] Lynskey, N.N., Velarde, J.J., Finn, M.B., Dove, S.L., Wessels, M.R., 2019. RocA binds csrs to modulate CsrRS-mediated generegulation in group a streptococcus. *MBio* 10(4), Doi: 10.1128/mBio.01495-19.
- [280] Feng, W., Minor, D., Liu, M., Li, J., Ishaq, S.L., Yeoman, C., et al., 2017. Null mutations of group a streptococcus orphan kinase RocA: Selection in mouse infection and comparison with covS mutations in alteration of in vitro and in vivo protease SpeB expression and virulence. *Infection and Immunity* 85(1), Doi: 10.1128/IAI.00790-16.
- [281] Lin, W.-J.J., Walthers, D., Connelly, J.E., Burnside, K., Jewell, K.A., Kenney, L.J., et al., 2009. Threonine phosphorylation prevents promoter DNA binding of the Group B Streptococcus response regulator CovR. *Molecular Microbiology* 71(6): 1477–95, Doi: 10.1111/j.1365-2958.2009.06616.x.
- [282] Agarwal, S., Agarwal, S., Pancholi, P., Pancholi, V., 2011. Role of serine/threonine phosphatase (SP-STP) in *Streptococcus pyogenes* physiology and virulence. *Journal of Biological Chemistry* 286(48): 41368–80, Doi: 10.1074/jbc.M111.286690.
- [283] Bernish, B., Van De Rijn, I., 1999. Characterization of a two-component system in *Streptococcus pyogenes* which is involved in regulation of hyaluronic acid production. *Journal of Biological Chemistry* 274(8): 4786–93, Doi: 10.1074/jbc.274.8.4786.

- [284] Federle, M.J., Scott, J.R., 2002. Identification of binding sites for the group A streptococcal global regulator CovR. *Molecular Microbiology* 43(5): 1161–72, Doi: 10.1046/j.1365-2958.2002.02810.x.
- [285] Gusa, A.A., Scott, J.R., 2005. The CovR response regulator of group A streptococcus (GAS) acts directly to repress its own promoter. *Molecular Microbiology* 56(5): 1195–207, Doi: 10.1111/j.1365-2958.2005.04623.x.
- [286] Gao, J., Gusa, A.A., Scott, J.R., Churchward, G., 2005. Binding of the global response regulator protein CovR to the sag promoter of *Streptococcus pyogenes* reveals a new mode of CovR-DNA interaction. *Journal of Biological Chemistry* 280(47): 38948–56, Doi: 10.1074/jbc.M506121200.
- [287] Churchward, G., 2007. The two faces of Janus: virulence gene regulation by CovR/S in group A streptococci. *Molecular Microbiology* 64(1): 34–41, Doi: 10.1111/j.1365-2958.2007.05649.x.
- [288] Churchward, G., Bates, C., Gusa, A.A., Stringert, V., Scott, J.R., 2009. Regulation of streptokinase expression by CovR/S in *Streptococcus pyogenes*: CovR acts through a single high-affinity binding site. *Microbiology* 155(2): 566–75, Doi: 10.1099/mic.0.024620-0.
- [289] Graham, M.R., Smoot, L.M., Migliaccio, C.A.L., Virtaneva, K., Sturdevant, D.E., Porcella, S.F., et al., 2002. Virulence control in group A *Streptococcus* by a two-component gene regulatory system: Global expression profiling and in vivo infection modeling. *Proceedings of the National Academy of Sciences of the United States of America* 99(21): 13855–60, Doi: 10.1073/pnas.202353699.
- [290] Jiang, S.M., Ishmael, N., Hotopp, J.D., Puliti, M., Tissi, L., Kumar, N., et al., 2008. Variation in the group B *Streptococcus* CsrRS regulon and effects on pathogenicity. *Journal of Bacteriology* 190(6): 1956–65, Doi: 10.1128/JB.01677-07.
- [291] Sumby, P., Whitney, A.R., Graviss, E.A., DeLeo, F.R., Musser, J.M., 2006. Genome-wide analysis of group A streptococci reveals a mutation that modulates global phenotype and disease specificity. *PLoS Pathogens* 2(1): 0041–9, Doi: 10.1371/journal.ppat.0020005.
- [292] Lembo, A., Gurney, M.A., Burnside, K., Banerjee, A., De Los Reyes, M., Connelly, J.E., et al., 2010. Regulation of CovR expression in Group B *Streptococcus* impacts blood-brain barrier penetration. *Molecular Microbiology* 77(2): 431–43, Doi: 10.1111/j.1365-2958.2010.07215.x.
- [293] Dmitriev, A., Mohapatra, S.S., Chong, P., Neely, M., Biswas, S., Biswas, I., 2011. CovR-controlled global regulation of gene expression in streptococcus mutans. *PLoS ONE* 6(5), Doi: 10.1371/journal.pone.0020127.
- [294] Hanks, S.K., Hunter, T., 1995. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification 1. *The FASEB Journal* 9(8): 576–96, Doi: 10.1096/fasebj.9.8.7768349.

- [295] Pereira, S.F.F., Goss, L., Dworkin, J., 2011. Eukaryote-Like Serine/Threonine Kinases and Phosphatases in Bacteria. *Microbiology and Molecular Biology Reviews* 75(1): 192–212, Doi: 10.1128/mmbr.00042-10.
- [296] Bakal, C.J., Davies, J.E., 2000. No longer an exclusive club: Eukaryotic signalling domains in bacteria. *Trends in Cell Biology*: 32–8, Doi: 10.1016/S0962-8924(99)01681-5.
- [297] Stancik, I.A., Šestak, M.S., Ji, B., Axelson-Fisk, M., Franjevic, D., Jers, C., et al., 2018. Serine/Threonine Protein Kinases from Bacteria, Archaea and Eukarya Share a Common Evolutionary Origin Deeply Rooted in the Tree of Life. *Journal of Molecular Biology* 430(1): 27–32, Doi: 10.1016/j.jmb.2017.11.004.
- [298] Kornev, A.P., Taylor, S.S., 2010. Defining the conserved internal architecture of a protein kinase. *Biochimica et Biophysica Acta - Proteins and Proteomics*: 440–4, Doi: 10.1016/j.bbapap.2009.10.017.
- [299] Nolen, B., Taylor, S., Ghosh, G., 2004. Regulation of protein kinases: Controlling activity through activation segment conformation. *Molecular Cell*: 661–75, Doi: 10.1016/j.molcel.2004.08.024.
- [300] Ortiz-Lombardía, M., Pompeo, F., Boitel, B., Alzari, P.M., 2003. Crystal structure of the catalytic domain of the PknB serine/threonine kinase from *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* 278(15): 13094–100, Doi: 10.1074/jbc.M300660200.
- [301] Young, T.A., Delagoutte, B., Endrizzi, J.A., Falick, A.M., Alber, T., 2003. Structure of *Mycobacterium tuberculosis* PknB supports a universal activation mechanism for Ser/Thr protein kinases. *Nature Structural Biology* 10(3): 168–74, Doi: 10.1038/nsb897.
- [302] Cox, S., Radzio-Andzelm, E., Taylor, S.S., 1994. Domain movements in protein kinases. *Current Opinion in Structural Biology* 4(6): 893–901, Doi: 10.1016/0959-440X(94)90272-0.
- [303] Greenstein, A.E., Echols, N., Lombana, T.N., King, D.S., Alber, T., 2007. Allosteric activation by dimerization of the PknD receptor Ser/Thr protein kinase from *Mycobacterium tuberculosis*. *Journal of Biological Chemistry* 282(15): 11427–35, Doi: 10.1074/jbc.M610193200.
- [304] Lombana, T.N., Echols, N., Good, M.C., Thomsen, N.D., Ng, H.L., Greenstein, A.E., et al., 2010. Allosteric activation mechanism of the *mycobacterium tuberculosis* receptor Ser/Thr protein Kinase, PknB. *Structure* 18(12): 1667–77, Doi: 10.1016/j.str.2010.09.019.
- [305] Good, M.C., Greenstein, A.E., Young, T.A., Ng, H.L., Alber, T., 2004. Sensor domain of the *Mycobacterium tuberculosis* receptor Ser/Thr protein kinase, PknD, forms a highly symmetric β propeller. *Journal of Molecular Biology* 339(2): 459–69, Doi: 10.1016/j.jmb.2004.03.063.

- [306] Cavazos, A., Prigozhin, D.M., Alber, T., 2012. Structure of the sensor domain of mycobacterium tuberculosis PknH receptor kinase reveals a conserved binding cleft. *Journal of Molecular Biology* 422(4): 488–94, Doi: 10.1016/j.jmb.2012.06.011.
- [307] Yan, Q., Jiang, D., Qian, L., Zhang, Q., Zhang, W., Zhou, W., et al., 2017. Structural Insight into the Activation of PknI Kinase from *M. tuberculosis* via Dimerization of the Extracellular Sensor Domain. *Structure* 25(8): 1286–1294.e4, Doi: 10.1016/j.str.2017.06.010.
- [308] Barthe, P., Mukamolova, G. V., Roumestand, C., Cohen-Gonsaud, M., 2010. The structure of PknB extracellular PASTA domain from mycobacterium tuberculosis suggests a ligand-dependent kinase activation. *Structure* 18(5): 606–15, Doi: 10.1016/j.str.2010.02.013.
- [309] Prigozhin, D.M., Papavinasasundaram, K.G., Baer, C.E., Murphy, K.C., Moskaleva, A., Chen, T.Y., et al., 2016. Structural and genetic analyses of the mycobacterium tuberculosis protein kinase B sensor domain identify a potential ligand-binding site. *Journal of Biological Chemistry* 291(44): 22961–9, Doi: 10.1074/jbc.M116.731760.
- [310] Hardt, P., Engels, I., Rausch, M., Gajdiss, M., Ulm, H., Sass, P., et al., 2017. The cell wall precursor lipid II acts as a molecular signal for the Ser/Thr kinase PknB of *Staphylococcus aureus*. *International Journal of Medical Microbiology* 307(1): 1–10, Doi: 10.1016/j.ijmm.2016.12.001.
- [311] Kaur, P., Rausch, M., Malakar, B., Watson, U., Damle, N.P., Chawla, Y., et al., 2019. LipidII interaction with specific residues of Mycobacterium tuberculosis PknB extracytoplasmic domain governs its optimal activation. *Nature Communications* 10(1), Doi: 10.1038/s41467-019-09223-9.
- [312] Zucchini, L., Mercy, C., Garcia, P.S., Cluzel, C., Gueguen-Chaignon, V., Galisson, F., et al., 2018. PASTA repeats of the protein kinase StkP interconnect cell constriction and separation of *Streptococcus pneumoniae*. *Nature Microbiology* 3(2): 197–209, Doi: 10.1038/s41564-017-0069-3.
- [313] Bellinzoni, M., Wehenkel, A.M., Durán, R., Alzari, P.M., 2019. Novel mechanistic insights into physiological signaling pathways mediated by mycobacterial Ser/Thr protein kinases. *Genes and Immunity*: 383–93, Doi: 10.1038/s41435-019-0069-9.
- [314] Osaki, M., Arcondéguy, T., Bastide, A., Touriol, C., Prats, H., Trombe, M.C., 2009. The StkP/PhpP signaling couple in *Streptococcus pneumoniae*: Cellular organization and physiological characterization. *Journal of Bacteriology* 191(15): 4943–50, Doi: 10.1128/JB.00196-09.
- [315] Jin, H., Pancholi, V., 2006. Identification and biochemical characterization of a eukaryotic-type serine/threonine kinase and its cognate phosphatase in *streptococcus pyogenes*: Their biological functions and substrate identification. *Journal of Molecular Biology* 357(5): 1351–72, Doi: 10.1016/j.jmb.2006.01.020.

- [316] Boitel, B., Ortiz-Lombardía, M., Durán, R., Pompeo, F., Cole, S.T., Cerveñansky, C., et al., 2003. PknB kinase activity is regulated by phosphorylation in two Thr residues and dephosphorylation by PstP, the cognate phospho-Ser/Thr phosphatase, in *Mycobacterium tuberculosis*. *Molecular Microbiology* 49(6): 1493–508, Doi: 10.1046/j.1365-2958.2003.03657.x.
- [317] Chopra, P., Singh, B., Singh, R., Vohra, R., Koul, A., Meena, L.S., et al., 2003. Phosphoprotein phosphatase of *Mycobacterium tuberculosis* dephosphorylates serine-threonine kinases PknA and PknB. *Biochemical and Biophysical Research Communications* 311(1): 112–20, Doi: 10.1016/j.bbrc.2003.09.173.
- [318] Nováková, L., Sasková, L., Pallová, P., Janeček, J., Novotná, J., Ulrych, A., et al., 2005. Characterization of a eukaryotic type serine/threonine protein kinase and protein phosphatase of *Streptococcus pneumoniae* and identification of kinase substrates. *FEBS Journal* 272(5): 1243–54, Doi: 10.1111/j.1742-4658.2005.04560.x.
- [319] Obuchowski, M., Madec, E., Delattre, D., Boel, G., Iwanicki, A., Foulger, D., et al., 2000. Characterization of PrpC from *Bacillus subtilis*, a member of the PPM phosphatase family. *Journal of Bacteriology* 182(19): 5634–8, Doi: 10.1128/JB.182.19.5634-5638.2000.
- [320] Pietack, N., Becher, D., Schmidl, S.R., Saier, M.H., Hecker, M., Commichau, F.M., et al., 2010. In vitro phosphorylation of key metabolic enzymes from *Bacillus subtilis*: PrkC phosphorylates enzymes from different branches of basic metabolism. *Journal of Molecular Microbiology and Biotechnology* 18(3): 129–40, Doi: 10.1159/000308512.
- [321] Zhang, C., Sun, W., Tan, M., Dong, M., Liu, W., Gao, T., et al., 2017. The eukaryote-like serine/threonine kinase stk regulates the growth and metabolism of zoonotic *Streptococcus suis*. *Frontiers in Cellular and Infection Microbiology* 7(MAR), Doi: 10.3389/fcimb.2017.00066.
- [322] Ohlsen, K., Donat, S., 2010. The impact of serine/threonine phosphorylation in *Staphylococcus aureus*. *International Journal of Medical Microbiology*: 137–41, Doi: 10.1016/j.ijmm.2009.08.016.
- [323] Débarbouillé, M., Dramsi, S., Dussurget, O., Nahori, M.A., Vaganay, E., Jouvion, G., et al., 2009. Characterization of a serine/threonine kinase involved in virulence of *Staphylococcus aureus*. *Journal of Bacteriology* 191(13): 4070–81, Doi: 10.1128/JB.01813-08.
- [324] Didier, J.-P., Cozzone, A.J., Duclos, B., 2010. Phosphorylation of the virulence regulator SarA modulates its ability to bind DNA in *Staphylococcus aureus*. *FEMS Microbiology Letters* 306(1): 30–6, Doi: 10.1111/j.1574-6968.2010.01930.x.
- [325] Depardieu, F., Didier, J.-P., Bernheim, A., Sherlock, A., Molina, H., Duclos, B., et al., 2016. A Eukaryotic-like Serine/Threonine Kinase Protects *Staphylococci* against Phages. *Cell Host and Microbe* 20: 471–81, Doi: 10.1016/j.chom.2016.08.010.

- [326] Kang, C.M., Abbott, D.W., Sang, T.P., Dascher, C.C., Cantley, L.C., Husson, R.N., 2005. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: Substrate identification and regulation of cell shape. *Genes and Development* 19(14): 1692–704, Doi: 10.1101/gad.1311105.
- [327] Fiuza, M., Canova, M.J., Zanella-Cléon, I., Becchi, M., Cozzone, A.J., Mateos, L.M., et al., 2008. From the characterization of the four serine/threonine protein kinases (PknA/B/G/L) of *Corynebacterium glutamicum* toward the role of PknA and PknB in cell division. *Journal of Biological Chemistry* 283(26): 18099–112, Doi: 10.1074/jbc.M802615200.
- [328] Beltramini, A.M., Mukhopadhyay, C.D., Pancholi, V., 2009. Modulation of cell wall structure and antimicrobial susceptibility by a *Staphylococcus aureus* eukaryote-like serine/threonine kinase and phosphatase. *Infection and Immunity* 77(4): 1406–16, Doi: 10.1128/IAI.01499-08.
- [329] Dias, R., Félix, D., Caniça, M., Trombe, M.C., 2009. The highly conserved serine threonine kinase StkP of *Streptococcus pneumoniae* contributes to penicillin susceptibility independently from genes encoding penicillin-binding proteins. *BMC Microbiology* 9: 121, Doi: 10.1186/1471-2180-9-121.
- [330] Rajagopal, L., Clancy, A., Rubens, C.E., 2003. A eukaryotic type serine/threonine kinase and phosphatase in *Streptococcus agalactiae* reversibly phosphorylate an inorganic pyrophosphatase and affect growth, cell segregation, and virulence. *Journal of Biological Chemistry* 278(16): 14429–41, Doi: 10.1074/jbc.M212747200.
- [331] Beilharz, K., Nováková, L., Fadda, D., Branny, P., Massidda, O., Veening, J.W., 2012. Control of cell division in *Streptococcus pneumoniae* by the conserved Ser/Thr protein kinase StkP. *Proceedings of the National Academy of Sciences of the United States of America* 109(15): E905–13, Doi: 10.1073/pnas.1119172109.
- [332] Manuse, S., Fleurie, A., Zucchini, L., Lesterlin, C., Grangeasse, C., 2015. Role of eukaryotic-like serine/threonine kinases in bacterial cell division and morphogenesis. *FEMS Microbiology Reviews*: 41–56, Doi: 10.1093/femsre/fuv041.
- [333] Fleurie, A., Cluzel, C., Guiral, S., Freton, C., Galisson, F., Zanella-Cleón, I., et al., 2012. Mutational dissection of the S/T-kinase StkP reveals crucial roles in cell division of *Streptococcus pneumoniae*. *Molecular Microbiology* 83(4): 746–58, Doi: 10.1111/j.1365-2958.2011.07962.x.
- [334] Dasgupta, A., Datta, P., Kundu, M., Basu, J., 2006. The serine/threonine kinase PknB of *Mycobacterium tuberculosis* phosphorylates PBPA, a penicillin-binding protein required for cell division. *Microbiology* 152(2): 493–504, Doi: 10.1099/mic.0.28630-0.
- [335] Holečková, N., Doubravová, L., Massidda, O., Molle, V., Buriánková, K., Benada, O., et al., 2014. LocZ is a new cell division protein involved in proper septum placement in *Streptococcus pneumoniae*. *MBio* 6(1), Doi: 10.1128/mBio.01700-14.

- [336] Fleurie, A., Manuse, S., Zhao, C., Campo, N., Cluzel, C., Lavergne, J.-P., et al., 2014. Interplay of the Serine/Threonine-Kinase StkP and the Paralogs DivIVA and GpsB in Pneumococcal Cell Elongation and Division. *PLoS Genetics* 10(4): e1004275, Doi: 10.1371/journal.pgen.1004275.
- [337] Gieffing, C., Jelencsics, K.E., Gelbmann, D., Senn, B.M., Nagy, E., 2010. The pneumococcal eukaryotic-type serine/threonine protein kinase StkP co-localizes with the cell division apparatus and interacts with FtsZ in vitro. *Microbiology* 156(6): 1697–707, Doi: 10.1099/mic.0.036335-0.
- [338] Jolly, L., Ferrari, P., Blanot, D., Van Heijenoort, J., Fassy, F., Mengin-Lecreulx, D., 1999. Reaction mechanism of phosphoglucosamine mutase from *Escherichia coli*. *European Journal of Biochemistry* 262(1): 202–10, Doi: 10.1046/j.1432-1327.1999.00373.x.
- [339] Hirschfeld, C., Gómez-Mejia, A., Bartel, J., Hentschker, C., Rohde, M., Maaß, S., et al., 2020. Proteomic Investigation Uncovers Potential Targets and Target Sites of Pneumococcal Serine-Threonine Kinase StkP and Phosphatase PhpP. *Frontiers in Microbiology* 10: 3101, Doi: 10.3389/fmicb.2019.03101.
- [340] Piñas, G.E., Reinoso-Vizcaino, N.M., Yandar Barahona, N.Y., Cortes, P.R., Duran, R., Badapanda, C., et al., 2018. Crosstalk between the serine/threonine kinase StkP and the response regulator ComE controls the stress response and intracellular survival of *Streptococcus pneumoniae*, Doi: 10.1371/journal.ppat.1007118.
- [341] Evans, J.J., Bolz, D.D., 2019. Regulation of virulence and antibiotic resistance in Gram-positive microbes in response to cell wall-active antibiotics. *Current Opinion in Infectious Diseases*: 217–22, Doi: 10.1097/QCO.0000000000000542.
- [342] Kristich, C.J., Wells, C.L., Dunny, G.M., 2007. A eukaryotic-type Ser/Thr kinase in *Enterococcus faecalis* mediates antimicrobial resistance and intestinal persistence. *Proceedings of the National Academy of Sciences of the United States of America* 104(9): 3508–13, Doi: 10.1073/pnas.0608742104.
- [343] Dubrac, S., Bisicchia, P., Devine, K.M., Msadek, T., 2008. A matter of life and death: Cell wall homeostasis and the WalKR (YycGF) essential signal transduction pathway. *Molecular Microbiology*: 1307–22, Doi: 10.1111/j.1365-2958.2008.06483.x.
- [344] Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., Hiramatsu, K., 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Molecular Microbiology* 49(3): 807–21, Doi: 10.1046/j.1365-2958.2003.03599.x.
- [345] Rojas-Tapias, D.F., Helmann, J.D., 2018. Stabilization of *Bacillus subtilis* Spx under cell wall stress requires the anti-adaptor protein YirB. *PLOS Genetics* 14(7): e1007531, Doi: 10.1371/journal.pgen.1007531.
- [346] Rojas-Tapias, D.F., Helmann, J.D., 2018. Induction of the Spx regulon by cell wall stress reveals novel regulatory mechanisms in *Bacillus subtilis*. *Molecular Microbiology* 107(5): 659–74, Doi: 10.1111/mmi.13906.

- [347] Ganguly, T., Kajfasz, J.K., Abranches, J., Lemos, J.A., 2020. Regulatory circuits controlling Spx levels in *Streptococcus mutans*. *Molecular Microbiology* 114(1): 109–26, Doi: 10.1111/mmi.14499.
- [348] Canova, M.J., Baronian, G., Brelle, S., Cohen-Gonsaud, M., Bischoff, M., Molle, V., 2014. A novel mode of regulation of the *Staphylococcus aureus* Vancomycin-resistance-associated response regulator VraR mediated by Stk1 protein phosphorylation. *Biochemical and Biophysical Research Communications* 447(1): 165–71, Doi: 10.1016/j.bbrc.2014.03.128.
- [349] Devine, K.M., 2018. Activation of the PhoPR-Mediated Response to Phosphate Limitation Is Regulated by Wall Teichoic Acid Metabolism in *Bacillus subtilis*. *Frontiers in Microbiology* 9(NOV): 2678, Doi: 10.3389/fmicb.2018.02678.
- [350] Prunty, M.P., Noone, D., Devine, K.M., 2018. The distinct PhoPR mediated responses to phosphate limitation in *Bacillus subtilis* subspecies *subtilis* and *spizizenii* stem from differences in wall teichoic acid composition and metabolism. *Molecular Microbiology* 109(1): 23–40, Doi: 10.1111/mmi.13965.
- [351] van Opijnen, T., Bodi, K.L., Camilli, A., 2009. Tn-seq: high-throughput parallel sequencing for fitness and genetic interaction studies in microorganisms. *Nature Methods* 6(10): 767–72, Doi: 10.1038/nmeth.1377.
- [352] Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315(5819): 1709–12, Doi: 10.1126/science.1138140.
- [353] Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E., 2012. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (New York, N.Y.)* 337(6096): 816–21, Doi: 10.1126/science.1225829.
- [354] Qi, L.S., Larson, M.H., Gilbert, L.A., Doudna, J.A., Weissman, J.S., Arkin, A.P., et al., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152(5): 1173–83, Doi: 10.1016/j.cell.2013.02.022.
- [355] Yan, A., Xiong, Z.-Q., Gu, Y., Shao, S., Zhang, R., Xu, W., et al., 2021. Gene Silencing Through CRISPR Interference in Bacteria: Current Advances and Future Prospects. *Front. Microbiol* 12: 635227, Doi: 10.3389/fmicb.2021.635227.
- [356] Vigouroux, A., Oldewurtel, E., Cui, L., Bikard, D., Teeffelen, S., 2018. Tuning dCas9's ability to block transcription enables robust, noiseless knockdown of bacterial genes. *Molecular Systems Biology* 14(3), Doi: 10.15252/msb.20177899.
- [357] Li, X.T., Jun, Y., Erickstad, M.J., Brown, S.D., Parks, A., Court, D.L., et al., 2016. TCRISPRi: Tunable and reversible, one-step control of gene expression. *Scientific Reports* 6(1): 1–12, Doi: 10.1038/srep39076.
- [358] Cui, L., Vigouroux, A., Rousset, F., Varet, H., Khanna, V., Bikard, D., 2018. A CRISPRi screen in *E. coli* reveals sequence-specific toxicity of dCas9. *Nature Communications* 9(1): 1912, Doi: 10.1038/s41467-018-04209-5.

- [359] Lee, H.H., Ostrov, N., Wong, B.G., Gold, M.A., Khalil, A.S., Church, G.M., 2019. Functional genomics of the rapidly replicating bacterium *Vibrio natriegens* by CRISPRi. *Nature Microbiology*: 1105–13, Doi: 10.1038/s41564-019-0423-8.
- [360] Cho, S., Choe, D., Lee, E., Kim, S.C., Palsson, B., Cho, B.K., 2018. High-Level dCas9 Expression Induces Abnormal Cell Morphology in *Escherichia coli*. *ACS Synthetic Biology* 7(4): 1085–94, Doi: 10.1021/acssynbio.7b00462.
- [361] Beisel, C.L., Storz, G., 2010. Base pairing small RNAs and their roles in global regulatory networks. *FEMS Microbiology Reviews*: 866–82, Doi: 10.1111/j.1574-6976.2010.00241.x.
- [362] Mazzuoli, M.V., Daunesse, M., Varet, H., Rosinski-Chupin, I., Legendre, R., Sismeiro, O., et al., 2021. The CovR regulatory network drives the evolution of Group B *Streptococcus* virulence. *PLoS Genetics* 17(9): e1009761, Doi: 10.1371/journal.pgen.1009761.
- [363] Jain, I., Miller, E.W., Danger, J.L., Pflughoeft, K.J., Sumby, P., 2017. RocA is an accessory protein to the virulence-regulating CovRS twocomponent system in group A *Streptococcus*. *Infection and Immunity* 85(11), Doi: 10.1128/IAI.00274-17.
- [364] Kaspar, J., Shields, R.C., Burne, R.A., 2018. Competence inhibition by the XrpA peptide encoded within the *comX* gene of *Streptococcus mutans*. *Molecular Microbiology* 109(3): 345–64, Doi: 10.1111/mmi.13989.
- [365] Lin, B., Averett, W.F., Novák, J., Winn Chatham, W., Hollingshead, S.K., Coligan, J.E., et al., 1996. Characterization of PepB, a group B streptococcal oligopeptidase. *Infection and Immunity* 64(8): 3401–6, Doi: 10.1128/iai.64.8.3401-3406.1996.
- [366] Monnet, V., Nardi, M., Chopin, A., Chopin, M.C., Gripon, J.C., 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. *Journal of Biological Chemistry* 269(51): 32070–6, Doi: 10.1016/s0021-9258(18)31602-8.
- [367] Kanamaru, K., Stephenson, S., Perego, M., 2002. Overexpression of the PepF oligopeptidase inhibits sporulation initiation in *Bacillus subtilis*. *Journal of Bacteriology* 184(1): 43–50, Doi: 10.1128/JB.184.1.43-50.2002.
- [368] Liu, X., Galloway, C., Kjos, M., Domenech, A., Slager, J., van Kessel, S.P., et al., 2017. High-throughput CRISPRi phenotyping identifies new essential genes in *Streptococcus pneumoniae*. *Molecular Systems Biology* 13(5): 931, Doi: 10.15252/msb.20167449.
- [369] Maisnier-Patin, S., Berg, O.G., Liljas, L., Andersson, D.I., 2002. Compensatory adaptation to the deleterious effect of antibiotic resistance in *Salmonella typhimurium*. *Molecular Microbiology* 46(2): 355–66, Doi: 10.1046/j.1365-2958.2002.03173.x.
- [370] Poon, A., Chao, L., 2005. The rate of compensatory mutation in the DNA bacteriophage ϕ X174. *Genetics* 170(3): 989–99, Doi: 10.1534/genetics.104.039438.

- [371] Dufour, D., Villemin, C., Perry, J.A., Lévesque, C.M., 2016. Escape from the competence state in *Streptococcus mutans* is governed by the bacterial population density. *Molecular Oral Microbiology* 31(6): 501–14, Doi: 10.1111/omi.12145.
- [372] Wolf, D., Kalamorz, F., Wecke, T., Juszczak, A., Mäder, U., Homuth, G., et al., 2010. In-depth profiling of the LiaR response of *Bacillus subtilis*. *Journal of Bacteriology* 192(18): 4680–93, Doi: 10.1128/JB.00543-10.
- [373] Takada, H., Yoshikawa, H., 2018. Essentiality and function of WalK/WalR two-component system: The past, present, and future of research. *Bioscience, Biotechnology and Biochemistry*: 741–51, Doi: 10.1080/09168451.2018.1444466.
- [374] Takada, H., Shiwa, Y., Takino, Y., Osaka, N., Ueda, S., Watanabe, S., et al., 2018. Essentiality of walRK for growth in *Bacillus subtilis* and its role during heat stress. *Microbiology (United Kingdom)* 164(4): 670–84, Doi: 10.1099/mic.0.000625.
- [375] Helmann, J.D., 2016. *Bacillus subtilis* extracytoplasmic function (ECF) sigma factors and defense of the cell envelope. *Current Opinion in Microbiology*: 122–32, Doi: 10.1016/j.mib.2016.02.002.
- [376] Turlan, C., Prudhomme, M., Fichant, G., Martin, B., Gutierrez, C., 2009. SpxA1, a novel transcriptional regulator involved in X-state (competence) development in *Streptococcus pneumoniae*. *Molecular Microbiology* 73(3): 492–506, Doi: 10.1111/j.1365-2958.2009.06789.x.
- [377] England, C.G., Ehlerding, E.B., Cai, W., 2016. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. *Bioconjugate Chemistry*: 1175–87, Doi: 10.1021/acs.bioconjchem.6b00112.
- [378] Wiles, S., Ferguson, K., Stefanidou, M., Young, D.B., Robertson, B.D., 2005. Alternative luciferase for monitoring bacterial cells under adverse conditions. *Applied and Environmental Microbiology* 71(7): 3427–32, Doi: 10.1128/AEM.71.7.3427-3432.2005.
- [379] Kneen, M., Farinas, J., Li, Y., Verkman, A.S., 1998. Green fluorescent protein as a noninvasive intracellular pH indicator. *Biophysical Journal* 74(3): 1591–9, Doi: 10.1016/S0006-3495(98)77870-1.
- [380] Hansen, M.C., Palmer, R.J., Udsen, C., White, D.C., Molin, S., 2001. Assessment of GFP fluorescence in cells of *Streptococcus gordonii* under conditions of low pH and low oxygen concentration. *Microbiology* 147(5): 1383–91, Doi: 10.1099/00221287-147-5-1383.
- [381] Goosens, V.J., Monteferrante, C.G., Van Dijk, J.M., 2014. The Tat system of Gram-positive bacteria. *Biochimica et Biophysica Acta - Molecular Cell Research*: 1698–706, Doi: 10.1016/j.bbamcr.2013.10.008.