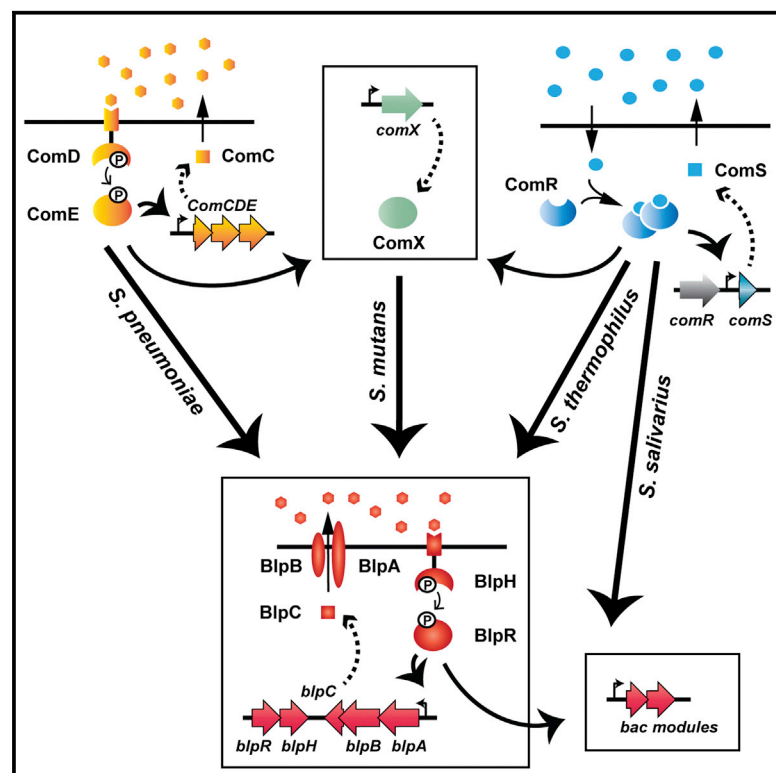


Cell Reports

Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*

Graphical Abstract



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In Brief

Mignolet et al. performed RNA-seq, genetics, and physiological tests in *Streptococcus salivarius* to demonstrate an extensive circuitry reorganization in the bacteriocin (small antibacterial peptide) control. They show that the pheromone-activated ComR directly promotes the synthesis of ComX (master competence regulator) and potent bacteriocins active against a wide spectrum of bacteria.

Highlights

- Bacteriocin control (predation) is rewired in *Streptococcus salivarius*
- ComR directly activates bacteriocin production
- ComR couples competence to predation
- *S. salivarius* secretes potent bacteriocins against a broad range of Gram⁺ bacteria

Data and Software Availability

GSE100416

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Circuitry Rewiring Directly Couples Competence to Predation in the Gut Dweller *Streptococcus salivarius*

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SUMMARY

Small distortions in transcriptional networks might lead to drastic phenotypical changes, especially in cellular developmental programs such as competence for natural transformation. Here, we report a pervasive circuitry rewiring for competence and predation interplay in commensal streptococci. Canonically, in streptococci paradigms such as *Streptococcus pneumoniae* and *Streptococcus mutans*, the pheromone-based two-component system BlpRH is a central node that orchestrates the production of antimicrobial compounds (bacteriocins) and incorporates signal from the competence activation cascade. However, the human commensal *Streptococcus salivarius* does not contain a functional BlpRH pair, while the competence signaling system ComRS directly couples bacteriocin production and competence commitment. This network shortcut might underlie an optimal adaptation against microbial competitors and explain the high prevalence of *S. salivarius* in the human digestive tract. Moreover, the broad spectrum of bacteriocin activity against pathogenic bacteria showcases the commensal and genetically tractable *S. salivarius* species as a user-friendly model for competence and bacterial predation.

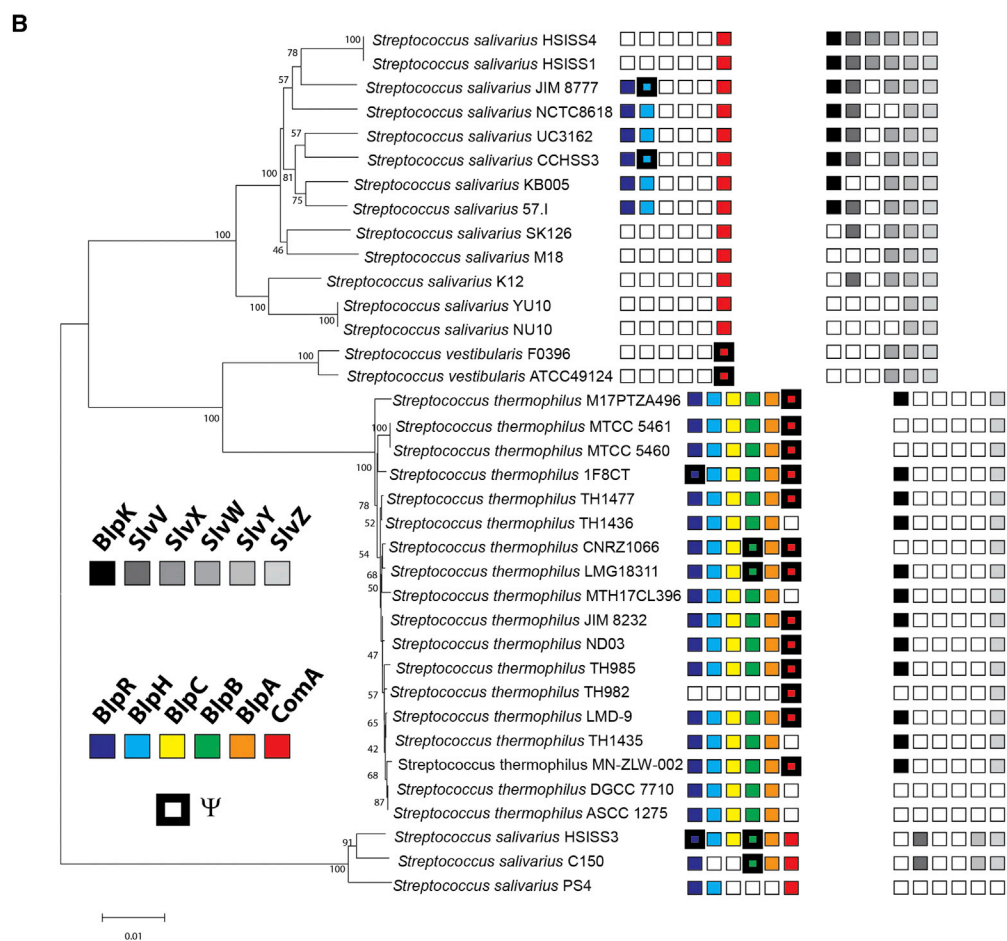
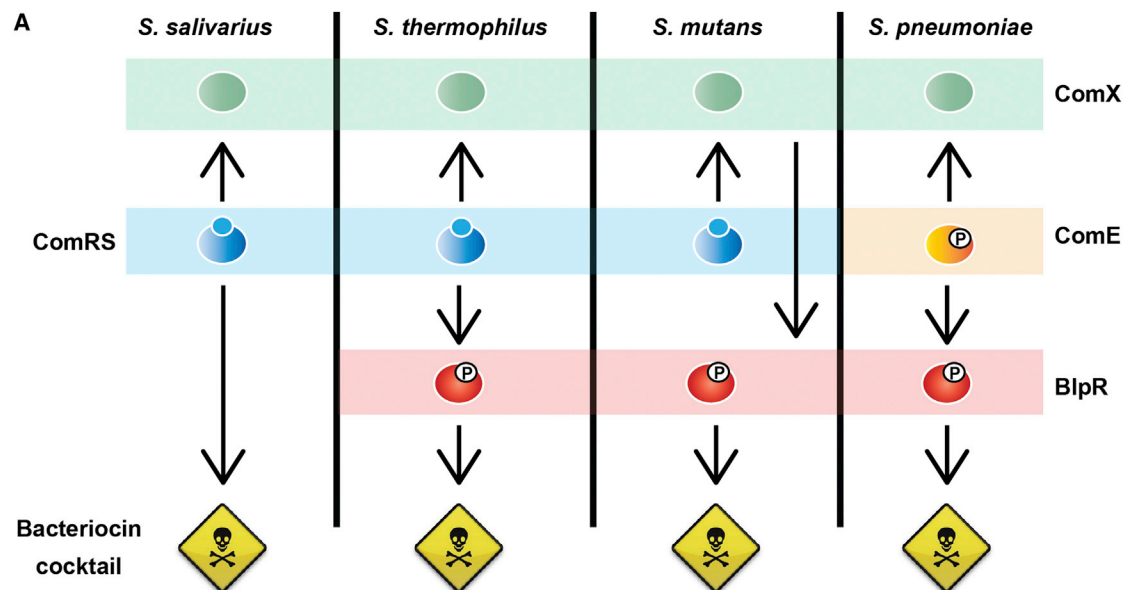
INTRODUCTION

Genome plasticity and reshuffling of transcriptional networks are keystones for the evolution of eukaryotes and prokaryotes (Reece-Hoyes et al., 2013; Sieber et al., 2017). Beside autonomous modifications (i.e., spontaneous mutation, gene duplication/deletion, intra-chromosomal remodeling), bacteria have developed intricate active mechanisms, such as conjugation

and natural DNA transformation, to acquire new genetic traits from phylogenetically close and distant species. These active processes of horizontal gene transfer are widespread and frequent in both Gram-positive and Gram-negative bacteria (Götgarten et al., 2002) and are responsible for the emergence of multidrug-resistant pathogenic bacteria (Ochman et al., 2000).

While the molecular mechanisms of natural DNA transformation differ in distant bacterial clades, a general scheme can be drawn up (Chen and Dubnau, 2004; Johnsborg et al., 2007; Johnston et al., 2014). Canonically, bacteria have to enter a transient state known as competence, during which a master regulator induces *de novo* synthesis of the transformation machinery, encoded by the so-called late competence (*com*) genes (Chen and Dubnau, 2004; Johnston et al., 2014). This machinery is in charge of capturing extracellular naked DNA (eDNA), translocating it through the bacterial envelope, and conveying internalized eDNA to the final steps of incorporation into the genome (Johnston et al., 2014). Competence is a burden for cell fitness, because it might interfere with essential bacterial processes (e.g., DNA replication, transcription, metabolism, energy/nutrient consumption) (Haijema et al., 2001; Nester and Stocker, 1963; Zaccaria et al., 2016). Therefore, bacteria minimize the time window during which they activate competence and concomitantly produce proteins that ensure a rapid competence shutoff (Turgay et al., 1998; Weng et al., 2013). To optimize transformation, bacteria simultaneously synthesize accessory antimicrobial polypeptides (for instance, bacteriocins) that they secrete to kill surrounding competitors (Claverys and Håvarstein, 2007; Veening and Blokesch, 2017). This predation behavior has two major benefits. First, it replenishes the medium with fresh nutrients released from dead cells. Second, DNA fragments are released and can be used in the natural transformation process and in biofilm matrix assembly. In this predation context, the bacteriocins, which usually target components of the cell envelope (Hécharad and Sahl, 2002), are of particular interest as therapeutics or food preservatives (Yang et al., 2014). They are short peptides with a broad variety of sequences, typically encoded in tandem with at least one immunity gene that protects the producer of





(legend on next page)

bacteriocins from their toxic effects. As ribosomally encoded antimicrobials, they are amenable for gene-based engineering (Tiwari et al., 2015).

In the genus *Streptococcus*, the central regulator of competence is the alternative sigma factor ComX (σ^X or SigX) (Fontaine et al., 2015). It binds a specific sequence known as the cin-box (TACGAATA in *Streptococcus pneumoniae*) and transiently associates with the RNA polymerase to activate promoters of the late *com* genes (Fontaine et al., 2015; Luo and Morrison, 2003). Two types of pheromone-responsive (cell-cell communication) systems guarantee the proximal transcriptional control of *comX*. They are engaged in a positive-feedback loop to robustly trigger competence when the signaling molecule reaches a threshold concentration (Figures 1A and S1A). In *S. pneumoniae*, when the inner membrane histidine kinase ComD binds the small extracellular signaling peptide CSP (competence-stimulating peptide; mature form of ComC), it autophosphorylates and transmits the phosphate moiety to the response regulator ComE. On the one hand, phosphorylated ComE binds to the *comCDE* promoter to accelerate CSP production (Martin et al., 2013; Pestova et al., 1996). On the other hand, it activates *comX* transcription (Figures 1A and S1A). In *Streptococcus mutans* and *Streptococcus thermophilus*, the loop does not rely on a membrane-tethered two-component system but is based on ComR, a cytoplasmic transcriptional regulator of the RNPP (Rap/NprR/PlcR/PrgX) family (Fontaine et al., 2010, 2013; Mashburn-Warren et al., 2010; Talagas et al., 2016). ComR is composed of two domains: a DNA-binding motif and a TPR (tetratricopeptide repeat) domain that specifically interacts with the cognate short signaling peptide XIP (*comX*/*sigX*-inducing peptide, mature form of ComS) (Talagas et al., 2016). The precursor of the pheromone XIP is processed, exported extracellularly, and then reimported into the cytoplasm via an oligopeptide ABC transporter (Opp/Ami system) (Gardan et al., 2009) and subsequently establishes a contact interaction with ComR (Talagas et al., 2016). This ComR•XIP complex (henceforth termed ComRS) then activates the positive-feedback loop and the competence state by activating the promoters of *comS* and *comX*, respectively (Fontaine et al., 2010, 2013; Haustenne et al., 2015) (Figures 1A and S1A).

In all model species of streptococci, most bacteriocin-encoding genes are under the direct control of the Blp system, a paralog of the ComCDE system (ambiguously named ComCDE in *S. mutans*). It is composed of the pheromone BlpC, the membrane-spanning histidine kinase BlpH, and the response regulator BlpR that binds promoters of bacteriocin genes (Figures 1A and S1A) (Shanker and Federle, 2017). How these bacteria connect competence commitment and BlpCHR activation is variable. In *S. pneumoniae*, production of BlpC and its exporter BlpAB is under the direct control of ComE (Kjos et al., 2016;

Wholey et al., 2016), while ComX activates the *blpRH* promoter in *S. mutans* (Figures 1A and S1A) (Reck et al., 2015). Interestingly, the *S. pneumoniae* ComX is additionally able to positively regulate the two-peptide bacteriocin CibAB and the lytic hydrolases LytA and CbpD (Guiral et al., 2005; Peterson et al., 2004), diversifying the spectrum, the mode of regulation, and the release timing of toxins. In *S. thermophilus*, ComX and ComR directly or indirectly modulate the expression level of many bacteriocin-related genes, but it is still unclear whether and how they affect BlpRH activity (Fontaine et al., 2007, 2013).

In the present study, we unearthed a new circuitry that directly couples competence to predation in *Streptococcus salivarius* HSISS4, a commensal strain of the human digestive tract lacking the canonical bacteriocin regulator BlpR (Figure 1B). Notably, we highlight that the ComRS complex serves as the connector that directly regulates both *comX* and bacteriocin genes, in contrast with other streptococci in which ComR or ComD indirectly control the production of main bacteriocins via the BlpCHR system. These results underline the diversity of transcriptional networks that drive homologous functions in streptococci.

RESULTS

The ComRS Module Mediates Competence Activation in *S. salivarius*

S. salivarius HSISS4 is a persistent strain isolated from the human gut (Mignolet et al., 2016; Van den Bogert et al., 2013). Even though strain HSISS4 was not reported as naturally competent, its chromosome contains all the genes required for competence, including the *comX* gene and several operons encompassing the late *com* genes involved in DNA capture, transport, and integration. In addition, its genome codes for proteins that share 94% (282/299 amino acids [aa]) and 83% (20/24 aa) identity with *S. thermophilus* ComR and ComS, respectively, suggesting that competence can be initiated in *S. salivarius* HSISS4 with a similar activation cascade. Under standard laboratory conditions (complex and defined media), competence was constitutively OFF, and we were unable to select transformants when providing a selective linear DNA-borne antibiotic marker (Table 1). However, addition of a synthetic form of ComS (LPYFAGCL, henceforth termed sComS) to the medium mimicked activating conditions by increasing the extracellular XIP concentration and induced natural transformation (Table 1), as shown previously with two other *S. salivarius* strains (Fontaine et al., 2010). We engineered strains with a deletion in the *comR* ($\Delta comR$) or *comX* ($\Delta comX$) genes and showed that these strains were irresponsive to sComS regarding the transformation rate (Table 1). Conversely, strong and mild overexpression of *comR* under the control of xylose-inducible promoters (P_{xy1} and P_{xy12} , respectively; Figure S2) mirrored the exogenous addition of

Figure 1. Bacteriocin Regulation in Streptococci and Degeneration of *blpABCRH* Locus in *S. salivarius*

(A) Transcriptional dependencies (arrows) between the competence activation module (ComRS and ComE), ComX, BlpR, and bacteriocins (skull cartoon) in streptococci model species. P indicates the phosphate moiety that covalently binds the ComE and BlpR response regulators. (B) Conservation of bacteriocins and the bacteriocin regulatory system across the salivarius streptococci group. BlpR (dark blue); BlpH (light blue); BlpC (yellow); BlpB (green); BlpA (orange); ComA (red); and the bacteriocins BlpK, SlvV, SlvX, SlvW, SlvY, and SlvZ (gray scale). The phylogenetic tree (100 bootstrap replicates) was adapted from Yu et al. 2015. An empty box means that no ortholog was found in the species genome. Boxes with thickened borders highlight genes that are potentially inactive (frameshift or truncation, ψ). Scale bar, 0.01 substitution per site. See also Figure S1.

Table 1. Competence Development (Transformation Frequency^a) in *S. salivarius* HSISS4 Derivatives

Strains	No Xylose		Xylose 1%	
	–	sComS	–	sComS
Wild-type	ND	2.6 (±1.5) E–05	NA	NA
$\Delta comR$	ND	ND	NA	NA
$\Delta comX$	ND	ND	NA	NA
$P_{xy11}-comR$	4.2 (±2.9) E–07	1.3 (±0.7) E–04	2.2 (±0.6) E–04	2.5 (±0.6) E–03
$P_{xy12}-comR$	ND	3.7 (±0.7) E–05	3.7 (±0.7) E–05	7.2 (±6.4) E–04

^aCalculated as the ratio of transformants (chloramphenicol-resistant colony-forming units [CFU]) to the total CFU count per 0.1 μ g of linear DNA. Transformation frequencies are expressed as the arithmetic mean of three independent experiments. Geometric means $\pm$ SD (expressed in log₁₀ between parentheses) are provided. ND, not detected (<1.0 E–08); NA, not applicable; –, no peptide. See also Figure S2.

sComS on wild-type (WT) strain and enhanced the transformation rate in combination with sComS (Table 1). *In silico* analyses of *comS* and *comX* promoters (P_{comS} and P_{comX} , respectively) disclosed a DNA motif in each promoter that matches the consensus sequence of dyad symmetry (TAGTGACAT-N₂-ATGTCATA) reported to be occupied by the sComS-bound ComR in *S. thermophilus* (Fontaine et al., 2013). Therefore, we designed a promoter-probe assay to quantify the maximal activity of P_{comS} and P_{comX} . For this purpose, these promoters were fused to the promoterless-*luxAB* genes at an ectopic locus. Consistently, the addition of sComS in the extracellular medium activated both P_{comS} (about 3,000-fold) and P_{comX} (about 40-fold) in a dose- and ComR-dependent manner (Figures 2A–2C). Altogether, our results indicate that the cell-cell communication RNPP regulator ComR, complexed to its cognate short peptide, binds and activates P_{comS} and P_{comX} to robustly unleash the competence state in *S. salivarius*. Furthermore, intracellular ComR and extracellular XIP concentration are two key factors in the dynamics of competence activation, confirming our previous mathematical modeling of induction of the *S. thermophilus* ComRS system (Haustenne et al., 2015).

ComRS Causes a *comX*-Independent Growth Defect

Interestingly, providing sComS at the beginning of the lag phase drastically postponed the moment at which cells reached mid-log phase (Figures 3A and S4A) but had no major impact on the growth rate. Inactivation of *comR* neutralized this sComS-induced phenotype, whereas *comR* overexpression promoted the growth curve shift and lowered the maximum optical density reached during the stationary phase (Figure 3B and S4B). This highlights the bacteriostatic or bactericidal effect of ComR activation and, presumably, competence development. Nevertheless, the growth curves of a *comX* mutant upon sComS addition fitted halfway between WT and $\Delta comR$ strains (Figures 3A and S4A), meaning that part of the phenotype caused by ComR activation is not related to ComX and downstream effects due to competence development.

Partial Overlap in ComR and ComX Regulons

We subsequently decided to carry out transcriptional profiling in order to elucidate specificities in ComR and ComX target genes. For this purpose, we extracted total mRNA of WT, $\Delta comR$, and $\Delta comX$ strains incubated with sComC for 30 min (optical density 600 [OD₆₀₀] = ~0.2) and performed deep-sequencing analyses. Comparison with the WT strain showed that the set of differentially expressed genes (cutoff: 5-fold change) is more extended in the $\Delta comX$ mutant (145 genes) than in the $\Delta comR$ mutant (55 genes) (Figure 4A; Table S1). Of these, 26 genes are downregulated in both mutants. They include genes required for pseudo-pilus biogenesis (i.e., *comGA*-GG, *comEA*/EC, *comFA*/FC, and *pilD*), DNA processing (e.g., *dprA*, *radC*, and *ssbA*) (Chen and Dubnau, 2004; Claverys et al., 2009), or other functions (e.g., *yoeB*, *yefM*, and *ackA*). We hypothesized that they form the direct regulon of ComX and represent the essential core of late *com* genes in *S. salivarius*. By comparing the promoter regions of this gene set, MEME analysis (<http://meme-suite.org/>) predicted a non-palindromic consensus motif, TTNCGAATA (Figure 4B), which markedly tallies with the cin-box sequence identified in *S. pneumoniae* (Campbell et al., 1998; Luo and Morrison, 2003), despite the low sequence conservation between ComX of both *Streptococcus* species (38% identity; 64% similarity). The 113 other genes of the ComX regulon probably resulted from indirect effects/regulation, as their differential expression level is weaker compared to genes with a promoter encompassing a cin-box (Table S1). These genes include those predicted to be involved in cellular physiological processes, such as protein degradation (*clpL*), membrane transport (20 genes), transcriptional control (6 genes), metabolism (19 genes), cell envelope homeostasis (9 genes), and DNA repair (*radA*).

Interestingly, half of the genes activated by ComR do not overlap with the ComX regulon. Besides *recX* (recombination), *gltA* and *citB* (citrate metabolism), *comEB* (nucleotide metabolism), and *pepP* (peptidase), which might have a direct role in competence because they are faintly downregulated (1.5 < fold change < 5) in the absence of *comX*, 21 genes show the opposite trend and are (often weakly) repressed by ComX (Figure 4A; Table S1). They are all members of transcriptional units that consist of predicted bacteriocin-related genes (Figure S1B), i.e., genes potentially involved in bacteriocin toxic effect, cognate immunity, or bacteriocin transport. Furthermore, the promoter of each operon encompasses a genuine ComR-box, which is indicative of a direct regulation (Figures 4A, 4C, and 4D). Surprisingly, there was no major read enrichment at the *comS* locus even though *comA*, the downstream gene supposed to be in operon with *comS*, is a top target of ComR. We can reconcile this discrepancy, considering that mRNAs smaller than 200 nt were discarded from the library generated for the RNA sequencing (RNA-seq) experiment. The unique ComR-box in the *comS*-*comA* upstream region suggests that the *comS*-*comA* single transcript might be cleaved in the intergenic region by an unknown mechanism, yielding two independent transcriptional units. In line with this hypothesis, RNA-seq performed on small RNAs (upper cutoff: 400 nt) revealed that the *comS* gene is, indeed, upregulated in the WT strain when sComS is provided in the medium 30 min before RNA extraction (Figure S5; Table S2).

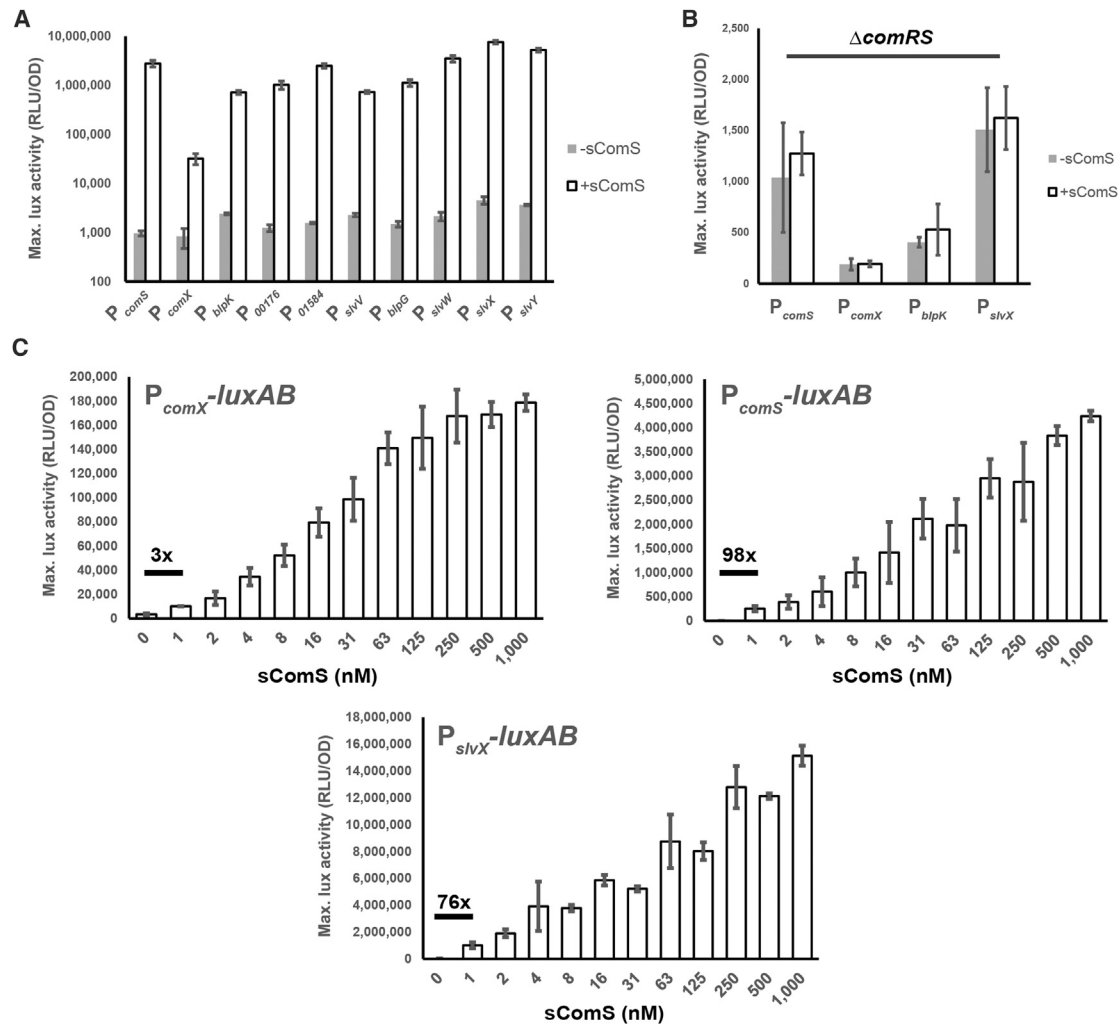


Figure 2. ComRS-Dependent Activation of Competence and Bacteriocin-Related Gene Promoters

Maximum luciferase activity/OD₆₀₀ ratio (relative light unit[RLU]/OD) of various *luxAB*-fused promoters in WT or $\Delta comRS$ mutant. Media were supplemented with sComS as indicated.

(A) Promoter activation of genes involved in competence or bacteriocin production (logarithmic scale) upon sComS addition (white bars) versus mock condition (gray bars).

(B) Activity of *comX*, *comS*, *blpK*, and *slvX* promoters in $\Delta comRS$ cells upon sComS addition (white bars) versus mock condition (gray bars).

(C) Dose-response charts of sComS concentration on *comX*, *comS*, and *slvX* promoter activation. Numbers in the bar charts describe the fold increase in promoter activation between control and the addition of 1 nM sComS.

Experimental values represent the averages (with SEM) of at least three independent biological replicates. See also Figure S3.

ComRS Directly Couples Competence and Bacteriocin Production

To validate our RNA-seq and *in silico* data, we performed promoter-probe assays to monitor the activity of operon promoters containing a ComR-box (Figure 2A). Consistently, promoters of *blpK* (P_{blpK}), *HSISS4_00176* (P_{00176}), *HSISS4_01584* (P_{01584}), *slvV* (P_{slvV}), *blpG* (P_{blpG}), *slvW* (P_{slvW}), *slvX* (P_{slvX}), and *slvY* (P_{slvY}) were all induced upon the addition of sComS, with a fold increase between 300 and 3,000 (Figure 2A). Compared to the 40-fold increase we observed for P_{comX} activation (Figure 2A), these values neatly correlate with our RNA-seq data that showed P_{comX} to be less sComS responsive than the promoters of bacte-

riocin-related genes (Table S1). In line with this, dose-response bar charts show that low concentrations of sComS are sufficient to markedly turn on P_{slvX} (and P_{comS}), while P_{comX} is nearly off (Figure 2C). Superimposed, P_{comX} is likely to activate with a slight delay, compared to P_{slvX} , at low concentrations of sComS, suggesting a weaker reactivity (Figure S3).

The HSISS4 genome encodes at least 6 putative bacteriocins; namely, BlpK (homolog of *S. thermophilus* BlpU), SlvV (salivaricine V; renamed from HSISS4_01594; role detailed later), SlvW (HSISS4_01653), SlvX (HSISS4_01665), SlvY (HSISS4_01742), and SlvZ (HSISS4_01743). They all feature an N-terminal double-glycine motif, which is a typical target

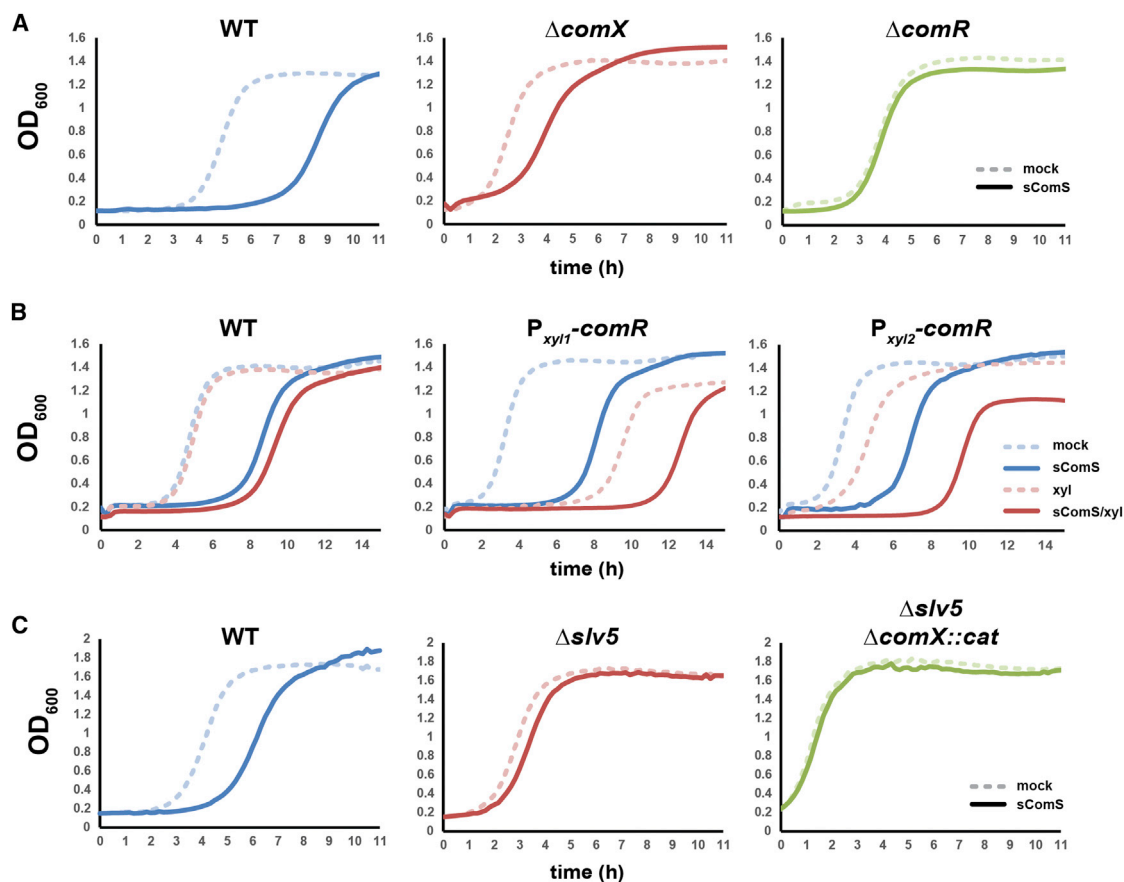


Figure 3. Impact of ComR, ComX, and Bacteriocin Production on Growth

(A) Growth in liquid chemically defined medium (CDM) of WT (blue), $\Delta comX$ (red), or $\Delta comR$ (green) cells upon sComS addition (continuous lines) versus mock condition (dashed lines). See also Figure S4A.

(B) Growth in liquid CDM of WT and two $comR$ -overexpressing strains (P_{xy11} or P_{xy12}) upon sComS addition (continuous lines) versus pheromone-free CDM (dashed lines). $comR$ overproduction was induced via xylose addition (red) and compared to CDM glucose (blue). See also Figure S4B.

(C) Growth in liquid CDM medium of WT (blue), $\Delta slv5$ (red), or double-mutant $\Delta slv5$ - $\Delta comX::cat$ (green) cells upon sComS addition (continuous lines) versus mock condition (dashed lines). See also Figure S4C.

In (A)–(C), each curve is a mean of three independent replicates.

See also Figure S4.

for processing via the ComA/BlpA family of transporters characterized by a C39 peptidase domain in class II peptides (Figure S1B) (Håvarstein et al., 1995). Interestingly, the corresponding genes are scattered throughout the chromosome in 5 different operons, including the incomplete *blp* locus encompassing *blpK* (Figure 4D). As no bacteriocin-mediated killing effect was reported in the literature for *S. salivarius* HISS4, we wondered how active this set of bacteriocins is and whether their production is determined by ComRS activation. Therefore, we evaluated the WT, $\Delta comR$, and $\Delta comX$ strains for bacteriocin production in the presence or absence of sComS. For this purpose, we qualitatively estimated the inhibitory halos due to *S. salivarius* derivatives grown on a soft-agar-embedded indicator strain (*S. thermophilus* LMD-9 $\Delta blpRH$ or *Lactococcus lactis* IL1403). In agreement with our luciferase tests, the addition of sComS to the agar feeding layer increases the inhibition zone surrounding the WT strain (Figure 5A). A xylose-driven overexpression of *comR* showed a similar effect, poisoning in

extreme cases (i.e., combined sComS addition and xylose induction) the producer strain itself (Figure 5B). Whereas $\Delta comR$ did not hinder growth of the indicator strains in any conditions, the halo bordering $\Delta comX$ cells was similar or even larger compared to the one surrounding WT cells, indicating that the toxic effect of bacteriocins is under the specific control of the ComRS system and does not require competence commitment. We demonstrated that this phenotype is polygenic, as single deletions of each bacteriocin locus ($\Delta slvX$ -01664, $\Delta blpK$ -I, $\Delta slvV$, $\Delta slvW$ -*blpG*, or $\Delta slvY$ -01744; Figure 4D) still sustain the bacterial prey inhibition. This shows the redundant toxicity caused by a cluster of bacteriocins (Figure 5C, left panels). Although double ($\Delta slv2$), triple ($\Delta slv3$), and quadruple ($\Delta slv4$) mutations in different bacteriocin loci mitigated the bacteriocidal effect of HISS4 to various extents, complete lack of killing of both indicator strains was only observed in a quintuple mutant ($\Delta slv5$), in which the 5 full bacteriocin loci (bacteriocin, immunity, and transporter genes) were deleted

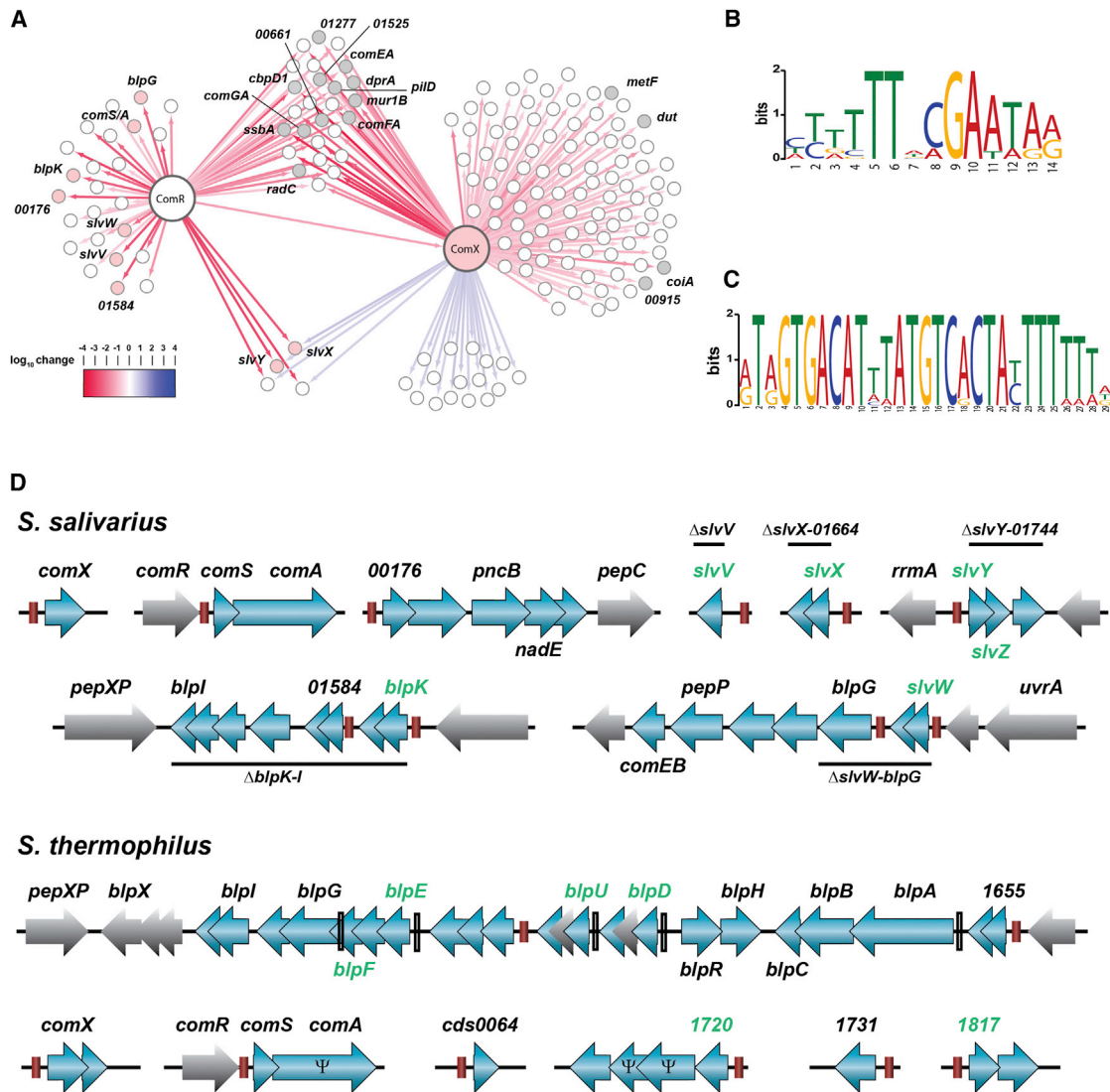


Figure 4. ComR and ComX Regulons

(A) Transcriptional network map of ComR and ComX regulons. Nodes depict genes differentially expressed in $\Delta comR$ or $\Delta comX$ mutant versus WT cells upon sComS addition. Arrows connecting ComR or ComX nodes to their target genes are color coded according to the fold change in gene expression (see also Figure S5 for the expression profile of the *comSA* locus). Annotated and stained nodes highlight genes that feature a ComR-box (light red) or a ComX-box (gray) in their promoter region.

(B and C) Weighted consensus sequence of nucleotide boxes recognized by ComR (B) or ComX (C) regulators. Information content is plotted as a function of nucleotide position. Sequence logo image was created using the MEME suite (<http://meme-suite.org>).

(D) Schematic representation (drawn to scale) of genes belonging to the ComR regulon in *S. salivarius* and *S. thermophilus*. ComR-controlled genes are colored in blue. In promoter regions, red bars and black double bars indicate typical ComR-boxes and BlpR-boxes, respectively. Gene names in green encoded the typical double-glycine bacteriocin-like peptides that are processed by the transporters ComA/BlpA. In *S. thermophilus*, *comA* is predicted to be a pseudogene (Ψ) because of several frameshifts inside the coding sequence. Part of the *S. salivarius* *blpK* locus was re-annotated with regard to the current NCBI genome annotation. Lines above and below *S. salivarius* loci highlight deleted regions in corresponding mutants.

See also Figure S5.

(Figure 5C, right panels). Interestingly, deletion of all 5 bacteriocin loci did not fully alleviate the growth shift caused by the addition of sComS to liquid culture (Figures 3C and S4C). Actually, we observed that deletion of *comX* in a $\Delta slv5$ background is sufficient to replicate the absence of growth shift observed for $\Delta comR$ cells, showing that both competence state entry and bacteriocin production provoke the ComR toxic effects (Figures

3C and S4C). In a time-course experiment, the cell viability was not drastically affected upon the addition of sComS in these mutants, as well as the WT strain (Figure S4D). Instead, the cell load appeared to stay equal at least during 30 min to 2 hr post-sComS addition. Therefore, we concluded that the ComRS effects are likely to be bacteriostatic rather than bactericidal.

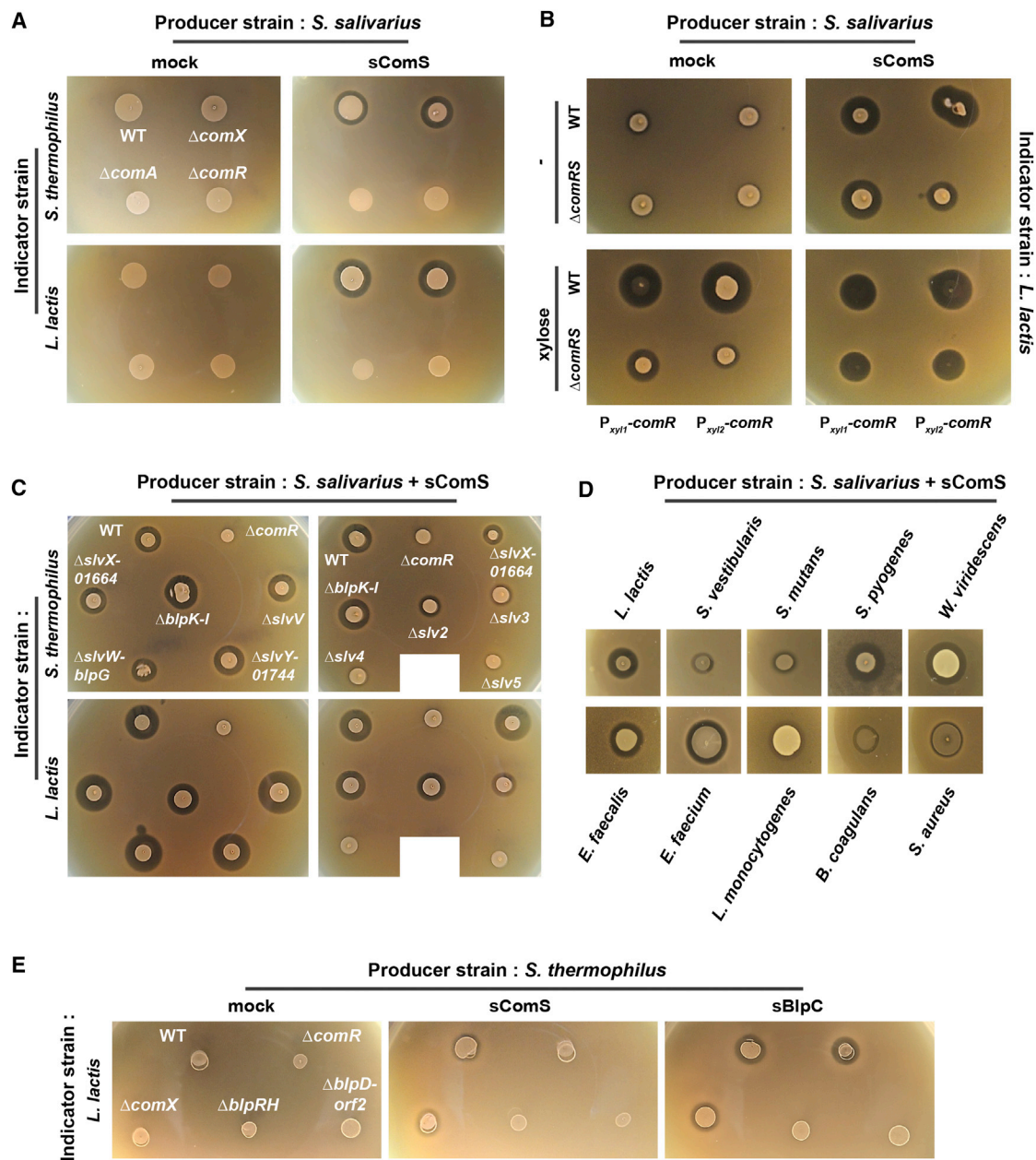


Figure 5. Species-Specific Requirement for Bacteriocin Production

(A–C) Bacteriocin inhibition assay of *S. salivarius* WT and mutant derivatives. Indicator strains (*L. lactis* or *S. thermophilus* $\Delta blpRH$) were embedded in the top soft agar layer, while sComS and/or xylose were supplemented into the bottom agar layer. Producer strains, including competence (A), overexpression (B), or bacteriocin (C) mutants, were spotted on top of the two agar layers.

(D) Bacteriocin inhibition assay of *S. salivarius* WT against various Gram-positive bacteria. Indicator strains are several well-known pathogenic or non-pathogenic bacteria. Plates were supplemented with sComS.

(E) Bacteriocin inhibition assay of *S. thermophilus* WT and mutant derivatives against *L. lactis*. Bottom agar layers were supplemented with the pheromones sBlpC or sComS as indicated.

See also Figures S6A and S6B.

***S. salivarius* Has a Broad Spectrum of Bacterial Prey**

The susceptibility of a non-streptococcal species to *S. salivarius* bacteriocins prompted us to assess the *S. salivarius* inhibition effect on various bacterial species. Strikingly, its antimicrobial activity targets closely related (*S. vestibularis*) to phylogenetically

more distant streptococci (*S. mutans* and *S. pyogenes*) (Figure 5D). Besides these species, *S. salivarius* is endowed with bacteriocins that affect several Gram-positive bacteria, including some serious (opportunistic) pathogens such as *Enterococcus faecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus*

(Figure 5D). We tried to dissect the contribution of each bacteriocin by testing our suite of single and multiple bacteriocin mutants against the set of the aforementioned sensitive strains. Although BlpK has an overwhelming cytotoxic activity in most cases (Figures S6A and S6B), the other minor bacteriocins, such as SlvX, might confer an extra benefit against specific species (Figures 5C and S6B). As expected, $\Delta slv5$ mutant cells grown in the presence of sComS never induced growth inhibition of the indicator strains, showing that *S. salivarius* bacteriocins are the sole determinants of antibacterial activity (Figures S6A and S6B). This broad spectrum of activity underlines the hypothesis that *S. salivarius* HSISS4 keeps a set of toxins at hand that can help it to compete and survive in a complex microbial environment.

ComA Substitutes for BlpA in *S. salivarius* and Contributes to Bacteriocin Translocation

In *S. salivarius*, the bacteriocin *blp* locus is incomplete (Figures 1B and 4D), with all strains missing the orthologous genes coding for BlpAB, the bacteriocin transporter system in *S. thermophilus* and some *S. pneumoniae* strains (Fontaine et al., 2007; Lux et al., 2007). Therefore, we questioned what protein(s) could perform the BlpAB function in *S. salivarius*. The ComRS-regulated ComA (Figures 4A and 4D; Table S1), which is homologous to BlpA and encoded downstream of *comS*, was identified as a promising candidate for bacteriocin secretion. Indeed, we observed that a $\Delta comA$ strain phenocopies $\Delta slv5$ or $\Delta comR$ cells with regard to bacteriocin production (Figure 5A). As $\Delta comA$ cells still expressed bacteriocin genes (Figure S6C), but were able to carry out natural transformation in the presence of sComS (data not shown), we therefore conclude that ComA is responsible for bacteriocin secretion and maturation in *S. salivarius*.

Rewiring of Bacteriocin Activation Cascade in Closely Related Streptococci

With *S. vestibularis*, *S. thermophilus* is a member of the salivarius streptococci. However, in contrast to *S. salivarius* and *S. vestibularis*, most *S. thermophilus* strains encode a functional BlpRH specifically dedicated to bacteriocin production (Fontaine et al., 2007; Fontaine and Hols, 2008). We thus investigate how competence and bacteriocin production are connected in this species. Interestingly, a microarray-based comparative survey of the transcriptome of *S. thermophilus* LMD-9 $\Delta comR$ and $\Delta comX$ null mutants revealed that all *blp* genes are positively controlled by ComR in competence-permissive conditions (Table S3). Those genes encode the determinants of thermophilin 9 production and activity: BlpRH; BlpAB (in *S. thermophilus*, a frameshift inactivates the *comA* gene function); the pheromone precursor BlpC; the four double-glycine bacteriocins BlpD, BlpU (homolog of *S. salivarius* BlpK), BlpE, and BlpF; and the disulfide bond maker BlpG, as well as predicted immunity peptides (Fontaine et al., 2007; Fontaine and Hols, 2008).

Bacteriocin detection assays showed that both sComS and sBlpC (synthetic form of BIP: SGWMDYINGFLKGGGQR TLPTKDYNIPQA) (Fontaine et al., 2007) induces bacteriocin production (Figure 5E). Akin to *S. salivarius*, this phenotype is not dependent on ComX (Figure 5E; Table S3), indicating that

bacteriocin production is part of the early steps of competence development in both species. However, *blpRH* or *blpD-orf2* deletion abrogated XIP- and BIP-mediated antimicrobial activity (Figure 5E). Since a canonical BlpR-box, but no ComR-box, is present in the *blpD* promoter (Figure 4D) (Fontaine et al., 2007), the ComR-mediated control of bacteriocin production is likely to be indirect through BlpRH activation. As expected, the addition of sBlpC still sustained bacteriocin production in $\Delta comR$ cells, while sComS did not (Figure 5E), confirming that BlpR can activate bacteriocin production independently of ComR. How *S. thermophilus* ComR influences BlpRH activity is still elusive. Nevertheless, as the *blpRH* and *blpABC* operons are part of the ComR regulon (Table S3), we suspect that ComR is able to activate *blpABC* expression via a transcriptional read-through of an upstream gene promoter (*ster_1655*) containing a ComR-box (Figure 4D) (Fontaine et al., 2013). Altogether, our results indicate that the BlpRH pair acts as a relay in the ComR activation cascade of bacteriocin production in *S. thermophilus*, in contrast to the direct ComR control that we demonstrated for *S. salivarius*.

DISCUSSION

In most streptococci, the competence-engaging system (either ComCDE or ComRS) modulates the activity of the two-component system BlpRH that, in turn, directly triggers bacteriocin production (Figures 1A and S1A) (Shanker and Federle, 2017). In contrast, we report here that, in a strain of *S. salivarius* isolated from the human digestive tract, one level of regulation is bypassed, and competence commitment is directly connected to bacteriocin production. *In silico* analyses revealed that the BlpRH/BlpC signaling system is missing or fragmentary in all sequenced *S. salivarius* strains (Figure 1B). These observations strongly suggest that the one level cascade that circumvents the BlpRH system is broadly conserved in the *salivarius* clade and, possibly, in the close relative *S. vestibularis*. The reason for such a shortcut in transcriptional circuitry is not known, although it may allow *S. salivarius* to respond more efficiently to intra- and/or inter-species competition, as it would secrete antimicrobial compounds more promptly, i.e., as soon as the intracellular ComS concentration exceeds a certain threshold. Given that bacteriocin promoters are much more (re)active than P_{comX} , with a lower activation threshold and a prompt induction (Figures 2C and S3), this would also suggest that bacteriocin secretion in the environment *de facto* precludes the entry into competence state. In this light, the bacteriocin-killing effect should provide extracellular DNA released from dead bacteria before the bacteriocin producers are physiologically competent. Finally, this differential promoter responsiveness strongly suggests that, in a certain range of ComS concentrations, bacteria would produce bacteriocins but would not initiate competence.

With the HSISS4 genome, we queried the Islandviewer server (<http://www.pathogenomics.sfu.ca/islandviewer/>) that predicted about 5.6% of foreign DNA. Besides a 5-kb fragment that has homology with DNA from various Gram-positive and Gram-negative bacteria, most of these regions are found in close streptococci and, to a lesser extent, in lactococci or lactobacilli. These data suggest that the *S. salivarius* HSISS4 genome is

mainly interspersed with foreign DNA coming from closely related bacteria (species inside the *Lactobacillales* order), even if bacteriocins can target more phylogenetically distant firmicutes. This might be explained by the constraint on the nature (homology) of the donor DNA fragment or, alternatively, by the biotope of the gastro-intestinal tract. Indeed, the challenged prey might have a limited dwelling time in the *S. salivarius* ecological niche, reducing the probability for *S. salivarius* to acquire part of their genome.

The wide variety of branching in regulatory circuits (Figures 1A and S1A) and the convergence of analogous proteins such as ComA and BlpAB, even in closely related streptococci, are intriguing and might reflect the adaptation to a different ecological niche and the diversity of interactions they set up with their neighbor microbes. A precedent of interchangeable function was already reported for the export of BlpC, and possibly pneumocins, in *S. pneumoniae* (Kjos et al., 2016; Wholey et al., 2016). Indeed, only 25% of *S. pneumoniae* strains encode a fully functional BlpAB transport system that can sustain BlpC secretion (Kjos et al., 2016). It was first thought that the remaining strains were “cheaters” that do not secrete endogenous BlpC but are still able to sense the exogenous pheromone (Son et al., 2011). However, it emerged that, in the absence of a functional BlpAB, the ComC transporter ComAB translocates BlpC in the extracellular medium (Kjos et al., 2016; Wholey et al., 2016). We observed a similar case in *S. salivarius* that lacks the BlpAB homolog and requisitions ComA (52% identity with *S. thermophilus* BlpA) to carry out bacteriocin export. Strikingly, the absence or loss of *blpAB* is shared by all *S. salivarius* strains (Figure 1B), whatever their origin (e.g., blood, milk, human skin, mouth, or gut), and suggests that ComA also secretes bacteriocins in these strains. Considering that the HSISS4 strain does not encode any homolog of the accessory BlpB (not essential for bacteriocin translocation in *S. thermophilus*; Fontaine et al., 2007) or ComB proteins, we suspect that ComA is, alone, responsible for the export of bacteriocins.

Our RNA-seq and microarray analyses unveiled an interesting property about competence cascade activation. It is noteworthy that the top downregulated targets in $\Delta comR$ (ComRS core regulon) are upregulated in $\Delta comX$, presumably driving the hyper-killing phenotype we observed against the indicator strain *S. thermophilus* $\Delta blpRH$ (Figure 5A). We already know from *S. thermophilus* that the anti-sigma factor MecA facilitates the Clp-dependent proteolysis of ComX to impose low ComX steady-state levels in non-permissive conditions and prevent inappropriate competence entry (Boutry et al., 2012; Wahl et al., 2014). It turned out that ComX or the ComX regulon are likely to be committed in a negative-feedback loop that targets the ComRS activity. This negative-feedback loop on ComRS activity was also observed in *S. thermophilus* and shown to be essential for the kinetics of competence shutoff (Haustenne et al., 2015). This cascade topology contrasts with the *S. mutans* model in which ComX binds an upstream region of the *comR* gene and activates its transcription (Khan et al., 2017). This provides a second safety latch to escape the competence state and narrow the time window during which bacteria are exposed to toxic side effects.

Interestingly, the ComR-dependent growth shift caused by ComS can be attributed to two factors. The first factor is the ComX activation (Figure 3A). The net ComX accumulation drastically reshapes at least 7% of the cell transcript profile, including numerous genes involved in proteolysis, DNA and RNA processing, the toxin-antitoxin system, metabolism, envelope homeostasis, and transcriptional regulation, which might reduce cell fitness or, in extreme cases, provoke a growth arrest. These results are in line with a previous report in *Streptococcus suis* highlighting that ComX remodels half of the transcriptome and, subsequently, the general metabolism (Zaccaria et al., 2016). The second factor is the production of a cocktail of previously undescribed bacteriocins (*S. salivarius* is well known for lantibiotic production; Hyink et al., 2007) (Figure 3C). It might impinge on the growth of producer cells because of a feeble protection by immunity proteins. Nevertheless, an alternative hypothesis would be that only a small part of the whole clonal population is responsive to XIP, secretes antimicrobial peptides, and activates *ad hoc* immunity, leaving the mass of “non-reactive” cells sensitive to bacteriocins. If confirmed, this would suggest that *S. salivarius* developed a bimodal strategy that provides the subpopulation of producer cells a competitive advantage toward non-producer cells that compensates for the deleterious side effects that occurred during competence activation.

Finally, considering its broad spectrum of bacteriocin targets, the strain HSISS4 is likely to be useful as a probiotic. Genetically tractable, this inhabitant of the oral cavity and small intestine might be engineered to encode several other bacteriocin/immunity modules in order to inhibit or modulate populations of digestive-tract pathogens without affecting the equilibrium of the whole microflora.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Oligonucleotides, PCR Fragments, and Growth Conditions

Growth conditions are described in the Supplemental Information. Bacterial strains, plasmids, oligonucleotides, and PCR fragments used in this study are listed in Tables S4, S5, S6, and S7 of the Supplemental Information, respectively.

Bacteriocin Detection Assay

The spot-on lawn (multilayer) detection method was performed as followed: 10 μ L of overnight cultures of producer strains were diluted in fresh M17G medium and grew to reach mid-log phase ($OD_{600} \approx 0.5$). In parallel, we cast plates with a bottom feeding layer (M17G 1.5% agar) supplemented with sComS where required. Next, we mixed 200 μ L of an overnight culture of an indicator strain in pre-warmed soft M17G medium (0.4% agar) and cast it as a top layer. Finally, we spotted 3 μ L of the producer strains on the top layer. Plates were incubated overnight before analysis of the inhibition zones surrounding the producer colonies.

Measurements of Growth and Luciferase Activity

Overnight precultures were diluted at a final OD_{600} of 0.05. A volume of 300 μ L of culture samples was incubated in the wells of a sterile covered white microplate with a transparent bottom (Greiner Bio-One, Alphen a/d Rijn, the Netherlands) for 75 min at 37°C and then supplemented with sComS (1 μ M, except if otherwise stated) or DMSO. Growth (OD_{600}) and luciferase (Lux) activity (expressed in relative light units) were monitored at 10-min intervals during 24 hr in a Varioskan Flash multi-mode reader (ThermoFisher Scientific,

Zellic, Belgium) as previously described (Fontaine et al., 2013). Experimental values represent the averages (with SEM) of at least three independent biological replicates. The time necessary to reach the mid-log phase (μ max) was calculated by fitting a standard logistic curve to the experimentally obtained growth curve data using the R package Growthcurver (Sprouffske and Wagner, 2016).

Deep Sequencing: RNA-Seq and smRNA-Seq

S. salivarius WT, $\Delta comR$, or $\Delta comX$ strains were pre-cultured overnight in CDMG at 37°C. They were resuspended in 50 mL of fresh pre-warmed CDMG to a final OD₆₀₀ of 0.05 and grown for approximately 2.5 hr (OD₆₀₀ = 0.2) at 37°C. We then supplemented the medium with either DMSO (negative control) or 1 μ M sComS and incubated for 30 min at 37°C. Cells were harvested by centrifugation (10 min; 4,050 $\times$ g), the supernatant was discarded, and the cell pellets were frozen with liquid nitrogen. Finally, RNA was extracted using the RiboPure Bacteria Kit (Ambion-Life Technologies) with an adapted protocol and subsequently sequenced (see Supplemental Information). Data analyses are summarized in Tables S1 and S2.

Microarray Data Analysis

Probes from triplicates were filtered by t test for significance at a threshold of $p < 0.05$. Significantly regulated probes were then defined based on a fold change higher than 2.0. Significantly regulated genes were defined as genes for which at least 50% of the probes are significantly regulated, with a mean absolute fold change (FC) of total probes of at least 2.0. Significantly induced probes adjacent to an induced coding DNA sequence (CDS) were also retained if the FC of the total probes of the adjacent CDS was at least 1.5. Data analyses were summarized in Table S3.

DATA AND SOFTWARE AVAILABILITY

The accession number for RNA-seq data reported in this paper is GEO: GSE100416. The accession number for microarray data reported in this paper is GEO: GSE35849.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and seven tables and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.01.055>.

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AUTHOR CONTRIBUTIONS

Conception and design, J. Mignolet, L.F., A.S., C.N., J. Mahillon, T.C., and P.H.; Acquisition of data, J. Mignolet, L.F., C.N., and A.S.; Analysis and interpretation of data, J. Mignolet, L.F., and P.H.; Drafting or revising the article, J. Mignolet, L.F., A.S., J. Mahillon, T.C., and P.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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