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***Staphylococcus epidermidis* biofilms :
new insights in their formation and
development of control strategies**

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Abstract

Bacterial biofilms are the root cause of most chronic infections associated with implanted materials. For the opportunistic pathogen *Staphylococcus epidermidis*, the development of biofilms is considered as a major virulence factor, responsible for its resilience in hospitals and in patients. There is a clear need to improve our understanding of the influences of the environment on *S. epidermidis* biofilm formation to propose more adapted clinical treatments to biofilms. This study aimed to gather more insights on the mechanisms of bacterial adhesion and aggregation, two essential phases of biofilm development and to develop methods to detect and control *S. epidermidis* in biofilms.

First, the adhesion of *S. epidermidis* to fibrinogen-coated surfaces was investigated. In particular, the contribution of SdrG, a cell surface adhesin, in the modulation of the adhesion of *S. epidermidis* to adsorbed fibrinogen was assessed. Secondly, a method to detect adhering bacteria by means of a impedimetric biosensor was developed. An original combination of optical and electrical measurements allowed to confirm the detection selectivity towards *Staphylococci*.

Then, a novel type of aggregation was reported. Several isolates of *Staphylococcus aureus* and *S. epidermidis* were found to rapidly form large cell clumps in a reversible fashion. The implications of this phenotype on biofilm formation was also demonstrated.

Finally, enzymes were used to challenge mature *S. epidermidis* biofilms. Lysostaphin, lysins of phage K and Twort and an analog of dispersin B were identified as potent candidates with anti-biofilm activity. Lysostaphin and lysin K could also cause significant cell lysis. These enzymes represent alternatives to the failure of antibiotics and disinfectants to eradicate biofilms.

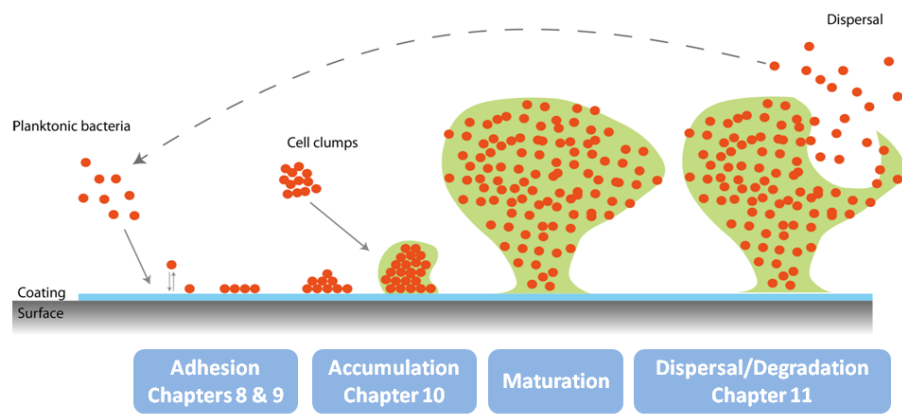
Keywords: adhesion, aggregation, biofilms, detection, fibrinogen, ionic strength, lysins, nosocomial infections, *Staphylococcus epidermidis*.

Preamble

This PhD thesis was supported by a grant of the F.R.S - FNRS. The work was carried out in the Earth and Life Institute (ELI), in the Applied Microbiology cluster (ELIM) and more precisely, in the Food and Environmental Microbiology laboratory (MIAE).

For *S. epidermidis*, one of the leading cause of healthcare-associated infections related to implanted medical devices, biofilm formation ability is a key asset to invade the human host and increase its persistence. Three main leads were developed in this thesis. The first was to improve the understanding of the mechanisms underlying the formation of biofilms by *S. epidermidis*. In particular, the phases of adhesion to surfaces coated by the plasma protein Fibrinogen and of accumulation to form large cell clumps were investigated. In a second step, a method involving sensors able to detect bacteria adhering on a surface was adapted to highlight the presence of *Staphylococci* in a biological fluid. This has been developed in close collaboration with N. Couniot (ICTM/ICTEAM), PhD student in electronics. Finally, a strategy to challenge mature, well-established biofilms was explored. It relies on bacteriolytic and matrix-degrading enzymes to achieve significant cell lysis and biofilm removal.

This thesis comprises five manuscripts (two published, one accepted and one other submitted for publication and one in preparation). These works were performed in close collaboration with several researchers in the laboratory, in other laboratories at within and outside UCL. The results are shown in Chapters 8, 9, 10 and 11 and notes on the author's contribution are available. Some parts of the work that did not reach a sufficient level of maturity for publication are presented in the Appendices. A list of scientific communications is also reported at the end of this thesis.



Included manuscripts

Published papers

- **Forces driving the attachment of *Staphylococcus epidermidis* to fibrinogen-coated surfaces.** P. Herman, S. El-Kirat-Chatel, A. Beaussart, J. A. Geoghegan, T. Vanzieleghem, T. J. Foster, P. Hols, J. Mahillon, and Y. F. Dufrêne. *Langmuir*, vol. 29, pages 13018-13022, 2013
- **Lytic enzymes as selectivity means for label-free, microfluidic and impedimetric detection of whole-cell bacteria using ALD- Al_2O_3 passivated microelectrodes.** N. Courniot, T. Vanzieleghem, J. Rasson, N. Van Overstraete-Schlögel, O. Poncelet, J. Mahillon, L. A. Francis, D. Flandre. *Biosensors and Bioelectronics*, In press, available from May 2015.

Submitted manuscripts

- ***Staphylococcus epidermidis* affinity for Fibrinogen-coated surfaces correlates with the abundance of the SdrG adhesin on the cell surface.** T. Vanzieleghem, P. Herman, Y. F. Dufrêne, J. Mahillon. Accepted for publication in *Langmuir* on March 30, 2015.
- **Come together: ionic strength modulates cell aggregation and biofilm formation in *Staphylococci*.** T. Vanzieleghem, N. Courniot, P. Herman, D. Flandre, Y. F. Dufrêne, J. Mahillon. Submitted April 2015.

In preparation

- **Enzymatic degradation of *Staphylococcus epidermidis* biofilms.** T. Vanzieleghem, F. Vande Cappelle, M. Schmelcher, M. Loessner, J. Mahillon, in preparation.

List of Abbreviations

Aae	An autolysin
AB	A cid B ase interactions
ACME	A rginine C atabolic M obile E lement
AFM	A tomical F orce M icroscopy
AI	A uto I nducer
AtlE	A uto lysin E
CCD	C harge C oupled D evice
CDC	C enter for D isease C ontrol
CFSTR	C ontinuous F low S tirred T ank R eactor
Clp	A serine peptidase
DLVO	D erjaguin L andau V erwey O verbeek
Ebp	E lastin binding protein
eDNA	extracellular DNA
EL	E lectrostatic interactions
Embp	E xtracellular m atrix- b inding protein
EPS	E xtracellular P olymeric S ubstances
Fg	F ibrinogen
Fn	F ibronectin
FTIR	F ourier T ransform I nfra <i>Red</i>
GehD	A lipase
HGT	H orizontal G ene T ransfer
HRT	H ydraulic R etention T ime
icaABCD	i nter c ellular aggregation ABCD
IR	I nverted R epeats
IS	I nsertion S equences
LST	L ypo S taphin
LTA	L ipo T eichoic A cid
LW	L ifshitz V an der Waals

LysK	L ysin of phage K
MDT	M ulti- D rug T olerance
MLST	M ulti L ocus S equences T yping
MRD	M odified R obbins D evice
MRI	M agnetic R esonance I maging
MRSA	M ethicillin R esistant <i>Staphylococcus aureus</i>
MSCRAMM	M icrobial S urface C omponents R ecognizing A dhesive M atrix M olecules
MTP	M icro T iter P lates
NP	N ano P articles
OPA	O psono P hagocytosis A ssay
OS	O smotic interactions
PAI	P athogenicity I slands
PEG	P oly E thylene G lycol
PEO	P oly E thylene O xide
PET	P oly- E thylene T erephthalate
PFGE	P ulse F ield G el E lectrophoresis
PFR	P lug F low R eactor
PG	P eptido G lycan
PGA	P oly G lutamic A cid
PLA	P oly L actic A cid
PMMA	P oly M ethyl M eth A crylate
PNAG	P oly N - A cetyl G lucosamine
PoC	P oint of C are
PPO	P oly P ropylene O xide
PTFE	P oly- T etra F luoro E thylene
<i>pur</i>	p urine synthesis genes
QQ	Q uorum Q uenching
QS	Q uorum S ensing
QSI	Q uorum S ensing I nhibitors
ROS	R eactive O xygen S pecies
<i>rsb</i>	regulator of sigma B
RSM	R esponse S urface M ethodology
<i>sar</i>	staphylococcal accessory regulator
SAT	S alt A ggregation T est

SCV	Small Colony Variants
SERAM	Secreted E xpanded R epertoire of A dhesive M olecules
σ_B	A major transcription regulator
Spx	A transcription regulator
TA	Toxin A ntitoxin system
TCA	TriCarboxylic A cid cycle
TSB	Tryptic Soy B roth
Tw	Lysin of phage T wort
VISA	Vancomycin Intermediate <i>Staphylococcus aureus</i>
Vn	Vibronectin
VNC	Viable Non Culturable
WGS	Whole G enome S equencing

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Part I

Introduction and Objectives

The context - Bacterial biofilms

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1.1 Historic context

Biofilms result from the natural organisation of micro-organisms as a community [Costerton 1995] [Westall 2006]. Bacteria and archaea have colonized almost all available natural habitats, and in about 90% of the cases, they live in the form of a biofilm [Costerton 1987]. The first observation of biofilm was made in the late 17th century by Antonie Van Leeuwenhoek (1632-1723), a dutch tradesman passionate about science. He conceived rudimentary microscopes and could observe what he called "animalculi", a word he used to describe micro-organisms isolated from his dental plaque. He said "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom.". The scurf he mentioned is, in fact, what we now call a biofilm. Unfortunately, his reports were welcomed with much scepticism amongst scientist of that time. The leads he proposed to pursue were left aside and his publications did not receive the echo they deserved in the scientific community.

On the impulsion of Robert Koch (1841-1910), the paradigm of microbiology was centered on a gold standard : the pure culture of planktonic bacteria. Indeed, he put pure culture at the core of its strategy to establish his four postulates to link an etiological agent to a diseases. He stated that: the etiological agent of a disease should be present in high numbers in the patients, should be cultivated *in vitro*, should provoke the disease when reinoculated in a healthy individual and should be isolated again from the contaminated body [Loeffler 1884]. Still profoundly anchored in mentalities, this pure culture paradigm occulted, almost totally, important questions regarding the ecology of bacteria in their natural environments. In 1930's and 1940's though, some voices arose to reinstate the essence of Van Leeuwenhoek observations : "Surfaces enable bacteria to develop in substrates otherwise too dilute for growth. Development takes place either as bacterial slime or colonial growth attached to surfaces." [Heukelekian 1940]. Their statements were supported by experimental evidences that in sea water metabolic activity and growth increased close to surfaces upon which

bacteria develop [Henrici 1933] [Zobell 1943] [Allen 1949].

From then to the 1970's, the number and the relevance of such observations on "surface-bound" life of bacteria were numerous: at air-water, air-soil, water-soil, water-rocks interfaces [Geesey 1977] [Characklis 1983] [Costerton 1995]. More recently, some forms of biofilms such as granules were shown to grow without the need for an external interface, thriving on their own extracellular polymeric substance (EPS) matrix [Barr 2010]. Based on the pioneering works of Bill Costerton and Bill Characklis, the first center for the study of biofilms was created in Montana in 1990. Ever since, biofilm research has grown immensely popular, as indicated by the exponentially increasing number of publications related to the topic.

1.2 Definition of a biofilm

Nowadays, a commonly admitted definition for a biofilm can be put as follows: a community of aggregating microorganisms, dwelling on biotic or abiotic interfaces, engulfed in a self produced matrix of EPS [Costerton 1995]. This matrix of polymers, a feature specific to biofilms, is generally composed of polysaccharides, proteins, eDNA (extracellular DNA) and minor components such as minerals and lipids [Flemming 2010]. Due to its intrinsic properties, the EPS matrix plays various roles in the growth and maturation of a biofilm (as explained in details later). In nature, several species are commonly present in these sessile populations called multi-species biofilms. They thrive together as an organized consortium. In some rarer cases, monospecies biofilms are encountered.

1.3 A paradigm for the development of bacterial biofilms

The following section is dedicated to detailing the biofilm life cycle as it is commonly accepted today.

1.3.1 Four steps to the biofilm life cycle

The development of a biofilm involves four main steps [Monds 2009]. First, free-floating or planktonic bacteria come in contact with a surface (either biotic or abiotic). Upon contact, adhesion may occur and bacterial cells anchor themselves, reversibly. Over time, two scenarios are then possible. In the first case, the cell-surface interaction weakens and the bacterium is set free from the surface. In the second case, reversible adhesion is fastened, turning into irreversible adhesion [Marshall 1971] [Hermansson 1999] [Hinsa 2003] [Boks 2008b].

Second, the adhered bacterial cells, in the presence of nutrients, can grow and divide to form microcolonies. This step is called accumulation. It is mainly supported by the production of the EPS matrix, a biological glue that keeps cells together thanks to electrostatic, Van der Waals and acid-base interactions [Flemming 2010]. Depending on the species, alterations of the EPS with, for example, acetyl residues can modulate the aggregative properties of the matrix [Tielen 2005]. In many species, specific cell surface proteins also play a role in cell-cell interactions, as it is the case by *Lactobacilli* with the Cfp protein [Schachtsiek 2004] or for *Enterococci* with the Asc10 and Asa1 proteins [Paganelli 2012].

Third, comes a maturation process while micro-colonies undergo dramatic changes in their structure and, if their density is high enough, they can merge. At this stage, quorum sensing (QS), a mechanism of bacterial communication based on the recognition of self-emitted small molecules [Waters 2005], plays an important role in the organisation of bacteria in the micro- and macro-colonies. The impact of

quorum sensing on the development of staphylococcal biofilms will be thoroughly explained in Section 3.5.2. The build up of micro-colonies into macro-colonies usually involve the development of towers or mushrooms shapes, but not always [Claessen 2014]. On the contrary, some micro-colonies partly disaggregate to allow water channels to form into the biofilm core [Monds 2009]. It results in a complex heterogeneous three-dimensional architecture comprising a variety of micro-environments [Stewart 2008] [Periasamy 2012]. As a consequence of its heterogeneity, a biofilm is composed of cells in different metabolic and physiological states, the most famous of which are persister cells [Keren 2004a] [Lewis 2010].

Finally, mature biofilms come to release planktonic cells during a stage commonly called dispersal. The mechanisms for detachment are extrinsic as well as intrinsic. Extrinsic factors are, for example, shear forces from the liquid moving on the surface of the biofilm [Purevdorj 2002], tearing off bits of the bacterial community, nutrient starvation [Hunt 2004], oxygen depletion [Thormann 2005], or temperature fluctuations [Kaplan 2002]. Intrinsic factors are, among others, the release of EPS degrading enzymes by biofilm bacteria, like nuclease [Chaignon 2007], polysaccharide degrading enzymes [Allison 1998] and genetic down regulation of EPS production. The latter is notably influenced by environmental stimuli. Gradients of nutrients, gases, i.e. nitric oxide (NO), and metabolic wastes occurring in biofilms can be sensed by biofilm bacteria and interpreted as a signal to initiate the dispersal phase [McDougald 2012].

The life cycle of a biofilm is represented in Fig. 1.1.

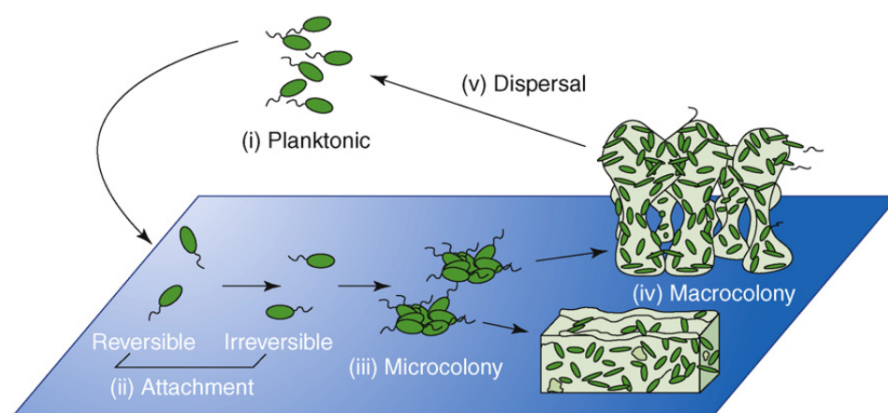


Figure 1.1: The 4 steps of biofilm development. Planktonic (or free-floating) cells (i) come in contact with a substratum (in blue). Attachment (ii) occurs first in a reversible, then in an irreversible fashion through bond strengthening. Bacteria anchored on the substratum divide and accumulate to form microcolonies (iii). During maturation, microcolonies turn into macrocolonies (iv) which shapes and architecture may be diverse. Finally, macrocolonies release planktonic cells in the surrounding environment, a phenomenon called dispersal (v). Source : [Monds 2009].

1.4 The biofilm - the genuine lifestyle of bacteria

The ubiquity of biofilms in nature has led scientists to question the former paradigm of microbiology based on the assumption that the majority of bacteria were living in free-floating forms. Jefferson argued that biofilms could well be the "default" lifestyle of bacteria [Jefferson 2004]. Based on the increased fitness of biofilms compared to planktonic cells, the latter can be regarded as "seeds" of biofilms being carried along to a new substrate to colonize. In the next sections, several arguments demonstrating the increased fitness provided by the biofilm to bacteria will be presented.

1.4.1 The biofilms : a defense against environmental threats

Firstly, a biofilm is a stronghold, an efficient mechanism of defense. Indeed, biofilms provide increased protection against all types of envi-

ronmental threats such as pH variation, dessication, oxidative stress, UV radiations or predation [Flemming 1993] [Davey 2000] [Tamaru 2005] [Flemming 2010]. Faced with such adverse conditions, a planktonic population will systematically suffer a much larger decrease in viability. In most species, biofilm formation is even genetically triggered as a response to stresses. In *Escherichia coli* for instance, the starvation stress induces the expression of the YcfR protein that decreases cell hydrophobicity and regulates biofilm buildup [Zhang 2007]. Similarly, in *Bacillus subtilis*, the stress-response pathway YwcC-SlrA strongly influences biofilm formation [Chai 2009] [Vlamakis 2013] and, in *Vibrio cholerae*, the biofilm production increases under the influence of stringent response (bacterial reaction to low nutrient availability) mediators [He 2012].

The key biofilm component providing this increased protection is the EPS matrix. Its stickiness holds the biofilm together under external loading. The reticulated web of EPS slows down mass transfer considerably inside the biofilm. EPS are highly hydrated polymers, also allowing a very good hygroscopic retention of water [Flemming 2010], a feature often crucial in nature. When water availability is changing, biofilms are able to delay dessication substantially whereas planktonic populations will not be able to avoid water evaporation. Biofilms thus contribute to bacterial resilience [Jefferson 2004].

1.4.2 Colonization of surfaces

Besides protection, the second reason for the success of the biofilm life form is its ability to colonize. The word "colonization" can be interpreted in two different ways.

The first is the capacity to stay at a certain place in order to secure an implantation. Biofilms do that quite well, notably thanks to specific adhesins, cell surface proteins, that fasten the community to the surface on which they thrives. For example, *Streptococcus pyogenes* possesses two surface proteins, encoded by the *prtF* and *emm* genes, binding

to fibronectin and fibrinogen, respectively [Patti 1994b]. In the same vein, SspA and SspB of *Streptococcus gordonii*, two binding proteins of saliva agglutinins, facilitates the anchorage of cells on the teeth surface [Burne 1998]. Also, the flagella, pili and fimbriae have been shown to contribute to bacterial adherence to surfaces for *Salmonella enterica*, *Pseudomonas aeruginosa* and *V. cholerae* [Lejeune 2003].

The second interpretation of colonization relates to the ability to migrate and occupy new ecological niches. As mentioned above, a well established surface dwelling community continuously releases planktonic cells, sent as emissaries to look for greener grass elsewhere. Some of these bacterial cells will encounter surfaces on which they can grow and develop a new biofilm which will, in turn, release cells as it comes to maturity. This strategy can be quite efficient in environments such as rivers, oceans, industrial plants [Abee 2011] where mass transport around the biofilm is abundant.

1.4.3 Biofilms : a cooperative hub for bacteria

A third advantage for bacteria to live in biofilms is the community. This is where bacteria resemble multicellular organisms the most: they cooperate, divide the metabolic burden and even perform horizontal gene transfer more efficiently [Molin 2003]. These activities are particularly relevant in mixed species biofilms. Indeed, as the pool of available genes is larger, the community is capable to perform tasks that mono species cannot. The group of Pr. Springael at KULeuven showed that degradation of the pesticide linuron by a consortium of three soil bacteria, each able to perform one step of the three-step degradation pathway, was only effective when the three species were entangled in the macrocolonies of a biofilm. Oppositely, biofilms developed with citrate as a carbon source, mainly composed of mono species macrocolonies, were not able to significantly degrade the pesticide [Breugelmans 2010] [Horemans 2013]. Another example is the anammox reaction in bacterial granules for wastewater treatment. Anammox requires a specific set of conditions for several functional

groups of bacteria to transform NH_4^+ and NO_2^- into N_2 . These conditions are only met in granules (dense aggregates of bacteria) but not in activated sludge, the most used technology to date [VanDongen 2001].

The hypothesis that biofilm is the default mode of growth of the overwhelming majority of bacteria in nature tends to be widely accepted. Though the shift in paradigm from "pure culture" to "biofilm as default growth mode" took time to operate, partly due to a lack of appropriate technology to grow biofilms in the laboratories. However, progress has now been made and biofilms are routinely grown *in vitro* in the laboratory with reliable methods [Goeres 2005] [Hu 2008] [Brenner 2012].

1.5 Impact of biofilm on humans

The span of biofilms impact on human in general is broad and can be either beneficial or detrimental. An overview of the significance of biofilms is proposed in the following paragraphs.

1.5.1 Beneficial biofilms

Even before humans made their own use of biofilms, they revealed themselves useful in nature. Indeed, an American Society for Microbiology (ASM) report called "Global environmental changes: Microbial contributions and Microbial solutions" states that biofilms are key players in the biogeochemical cycles happening in soils, rivers and oceans acting as major driving forces in the recycling of organic matter [King 2014]. Biofilms also largely contribute to the nutrient uptake of plants through symbiotic relationships with plants [Bogino 2013]. Besides this involvement in natural processes, humans have put biofilms to work in more than one fashion. Biofilms are used for the conversion of biomass into gases (mostly methane and hydrogen) subsequently burnt to produce heat and energy [Parawira 2008]. In the form of activated sludge or granules, biofilms are the core the our wastewater treatment technology [Nicoletta 2000]. Biofilms have also been

grown in bioreactors to produce high value added compounds such as enzymes [Sarkar 2011] or to perform defined chemical reactions, acting as catalysts [Halan 2012]. Other promising technologies are being currently developed with biofilms : microbial fuel cells designed to produce electricity by degradation of organic matter (i.e. lactate, acetate) [Liu 2004] or bioreactors to remove micropollutants (i.e. pesticides and heavy metals) from treated waste water [Horemans 2013].

1.5.2 Detrimental biofilms

The detrimental effects of biofilms on humans are numerous, the most notable are presented here. First, the involvement of biofilms in various pathologies makes them of high clinical importance. In fact, they are the causative agents of, among others, nosocomial infections associated with implanted devices [Costerton 1999] [Huebner 1999] [Donlan 2001] [Hoiby 2011], otitis [Bakaletz 2007], tooth decay, gingivitis [Jenkinson 2005], complications in wounds [Wolcott 2008]. Hence, bacteria in biofilms tend to tolerate antibiotic concentration up to 50 times greater than the minimal inhibitory concentrations of planktonic bacteria (MIC) [García-Castillo 2007]. Second, in industrial settings, biofilms contaminate food production lines [Kumar 1998], heat exchangers, water pipes [Mattila-Sandholm 1992] and actively corrode metal alloys and concrete [Zuo 2007]. These problems are often tackled with difficulty and at high costs. Finally, biofouling, including bacterial biofilms, algae and macrofouling by marine organisms, on ship hulls provokes increased corrosion and increased fuel consumption, due to higher shear forces. As an example, the annual supplementary cost in fuel for a mid-size destroyer of the US Marine is 50 million dollars, imputable to drag force caused by biofilms [Schultz 2011].

1.6 Biofilms in the laboratory

1.6.1 Model species

Four species have been used as models to investigate biofilm formation : *P. aeruginosa*, *E. coli*, *B. subtilis* and *Staphylococcus aureus* [López 2010].

P. aeruginosa is ubiquitous, Gram-negative, versatile pathogen often involved in complication in cystic fibrosis patients [Moreau-Marquis 2008]. This species forms dense biofilms with abundant EPS production, a feature that can be recognized as a virulence factor [Mann 2012]. In industrial settings, contamination due to *Pseudomonas* spp. is rather frequent causing, for example, major losses in the food industry and also reduces the yield of heat exchanger [Costerton 1987]. The problems associated with *P. aeruginosa* led the scientific community to use it as a reference species for biofilm formation. Klausen *et al.* grew biofilms of *P. aeruginosa* and noticed the appearance of mushroom like structure upon changing carbon source [Klausen 2003]. Others found that twitching mobility by flagella was important for biofilm morphology [Heydorn 2002].

E. coli is an enterobacterium also well-known as an entero- and uropathogen. In *E. coli*, the role played by surface appendages such as fimbriae, pili and flagella in biofilm formation was extensively studied [Pratt 1998] [Wright 2007]. It was also shown that *E. coli* produced curli proteins that were in fact amyloid fibers, drivers of the biofilm stability and architecture [Chapman 2002] [Blanco 2012].

B. subtilis is a soil bacterium that readily forms biofilm at air-liquid interfaces. It dwells on plant roots and relies on biofilm formation to increase its colonization of the rhizosphere [López 2010]. Essential features of biofilms have been highlighted in *B. subtilis* thanks to mutant generation [Vlamakis 2013]. Famous examples are, among others, the composition of the EPS matrix (poly- γ -glutamate and amyloid fibers)

and the cellular components that trigger and regulate biofilm development and dispersal such as, the Spo0A pathway involved in sporulation [Fujita 2005] and SlrR-SinR switch that govern cell chaining [Chai 2010].

Finally *S. aureus* and more recently *Staphylococcus epidermidis* have become reference species to study biofilm formation since their ability to form biofilm was recognized as a major virulence factors in nosocomial infections [Otto 2012]. Staphylococci have evolved to bind a variety of host proteins, cells and tissues as well as biomaterials, and accumulate on these surfaces as dense biofilms [Otto 2009]. These are mainly composed of polysaccharides that confer protection against aggressions from the immune system (anti-microbial peptides, neutrophils and antibodies) [Vuong 2004a] [Kristian 2008].

1.6.2 *In vitro* Culture techniques

Concerning biofilm cultivation in the laboratory, two main categories are currently available: static or flow displacement systems.

Static conditions

The most frequently used microtiter plates (MTP) technique has been extensively implemented for almost 30 years according to a protocol developed by Christensen *et al.* in 1985. Biofilms in MTP are often grown directly on the polystyrene well surface, but coupons have also been used in 6, 12 or 24 wells MTP. Alternatively, the so-called Calgary device allows to grow biofilms on pegs (plastic protrusions, see Fig. 1.2 B.) dipped into the wells on the MTP. The pegs can be removed from the plate containing the spent medium and dipped into another plate, which makes it convenient to assess Minimal Biofilm Eradication Concentration (MBEC) [Ceri 1999]. Finally, static biofilms can also be developed on the surface of agar or at the liquid-air interface in a bottle of growth medium. Fig. 1.2 illustrates the different techniques used to grow biofilms in static conditions.

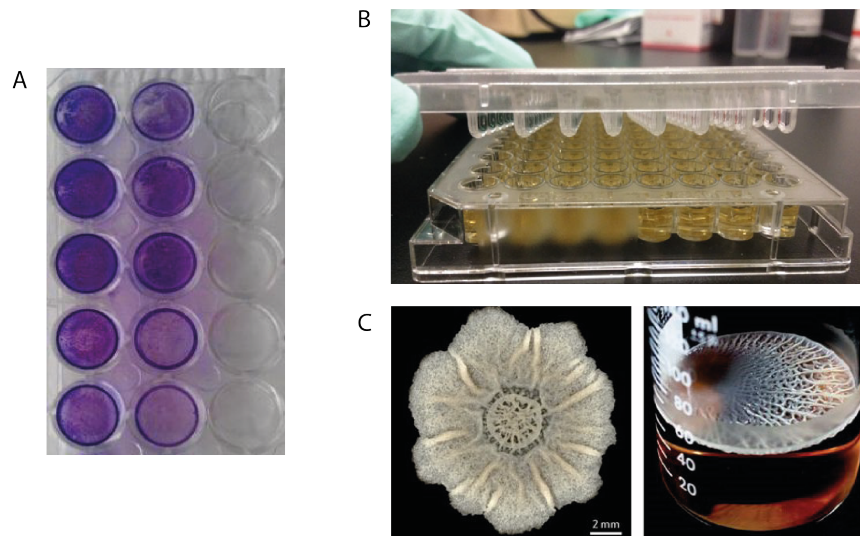


Figure 1.2: Static biofilm growth in laboratories. A - Conventional biofilm growth in MTP, followed by coloration with crystal violet to reveal biofilm formation. Biofilm develops on the bottom and walls of the wells. B - Calgary device, composed of two parts : the standard MTP containing the growth medium and the upper plate with the pegs dipped into the wells. Biofilms grow on the pegs of the upper plate which can be transferred from one plate to another, for example to challenge biofilms with antibiotics. C - To the left hand side, a *B. subtilis* biofilm on on nutrient agar surface, to the right hand side, a biofilm of the same species at the air-liquid interface in a medium bottle (Source : [Vlamakis 2013]).

In MTP systems, a fixed quantity of medium is added to the well containing a bacterial inoculum. There is no replacement of the medium during the experiment unless the experimentator does so. Uncontrolled shear stress can be applied by shaking the MTP. The key for the success of this method is its high throughput rate and low reagent consumption [Coenye 2010].

Flow displacement systems

In contrast to the closed systems, a variety of "open" systems have been developed. They are continuously fed with fresh medium and can be classified in two main categories: the Continuous Flow Stirred Tank Reactor (CFSTR) and the Plug Flow Reactors (PFR) [Heersink 2003].

Schematic representation and characteristics of MTP, CFSTR and PFR are shown in Fig.1.3.

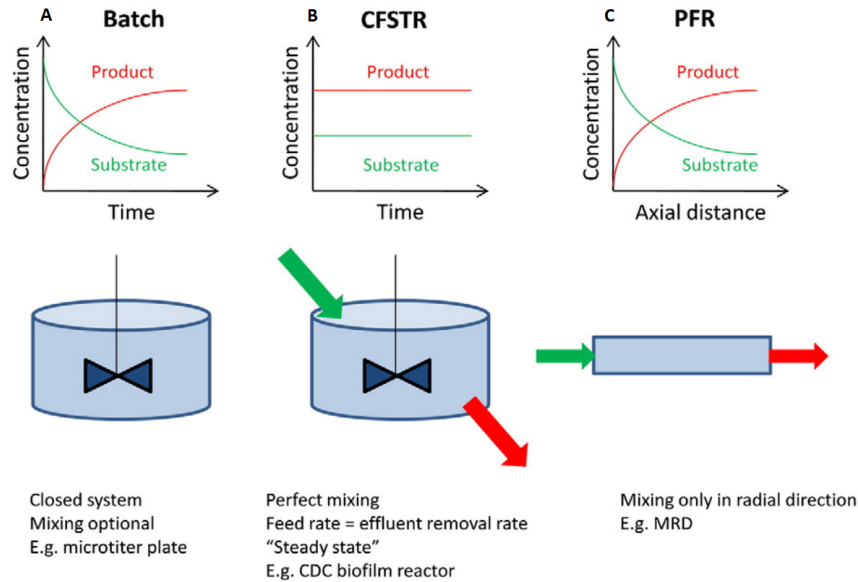


Figure 1.3: The 3 main modes of *in vitro* biofilm growth. A - Batch mode: a closed system with or without mixing. No liquid goes in or out the reactor. The substrate availability decreases as the biofilm grows and starvation inevitably occurs at a certain point. The most common example is MTP. B - Continuous flow stirred tank reactor (CFSTR), mixing happens in all directions and substrate availability is monitored and kept constant, the reactor achieves a steady-state. C - Reactors fed by plug flow imply that spent medium is pushed out of the chamber by injection of the fresh medium according to the direction of the flow. Mixing only occurs radially, the substrate is thus consumed by the biofilm as it flows along the length of the PFR chamber. The residence time of the medium in the chamber is governed by flow rate. As a consequence, the substrate/product diagram is drawn in function of the axial distance and not the time as for CFSTR and MTP. Source: [Coenye 2010]

CFSTR systems work in steady-state: the flow rate of fresh medium in the tank is the same as the outflow of waste products. The hydraulic retention time ($HRT [s] = \text{Volume of the tank [m}^3] / \text{Flow rate [m}^3/\text{s}]$) is chosen to be shorter than the doubling time of the cultivated species. In that way, planktonic bacteria are regularly washed away, only the sessile cells remain. CFSTR are operated with one or several coupons

of a chosen material upon which biofilms may grow. A mixing arm stirs the reactor in order to ensure a good homogeneity in the solution [Coenye 2010]. The major drawback of the method is the bench space and equipment needed to run the system. A good example of such devices is the Center for Disease Control (CDC) biofilm reactor [Goeres 2005] shown in Fig. 1.4 a. This reactor was found to be an appropriate tool to grow *Streptococcus pneumoniae* [Donlan 2004] and *P. aeruginosa* [Coenye 2010] biofilms.

In PFR systems, a flux of fresh medium is fed in the reactor from the input and the incoming medium pushes the spent medium towards the output [Heersink 2003]. The concentration of nutrient tends to diminish over the length of the PFR whereas the waste products concentration builds up. PFRs are usually much smaller in size than CFSTR and can easily be paralleled. The mixing in such devices is only occurring radially and waste products are totally removed from the reactor. As a consequence, PFRs do not reach a steady state. Two examples of PFRs are : the modified Robbins Device (MRD) [McCoy 1981], flow cells such as STOVALL (also called microfluidic devices if the channel dimensions are below the millimeter). In some cases, the surface available for biofilm growth cannot be recovered after the experiment Both devices are depicted in Fig. 1.4 b and c.

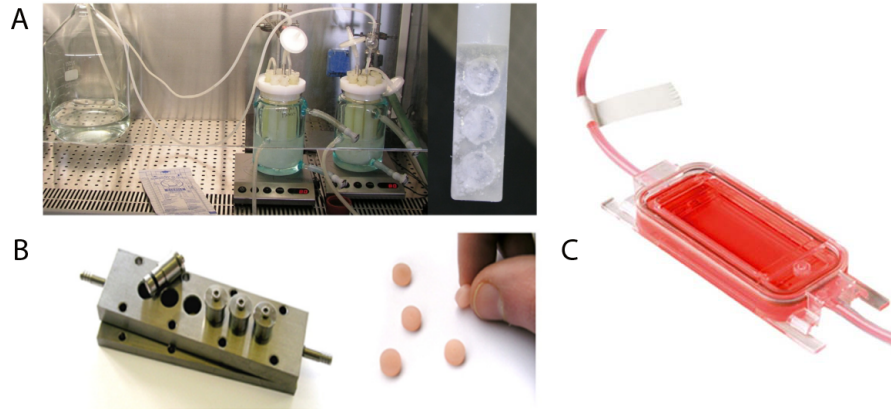


Figure 1.4: A - The CDC biofilm reactor, belonging to the class of CFSTR, two vessels are run in parallel, fed with a stock of fresh medium. B - Modified Robbins Device (MRD), a PFR, composed of a main channel in which coupons can be inserted orthogonally, flow runs tangentially to the coupon. Up to 6 coupons can be run at the same time. C - A PFR flow cell device (STOVALL), the chamber accepts a plastic slide for biofilm growth. Source : Adapted from [Coenye 2010] for A and B, from Stovall Life Science, Inc. for C.

The growth method chosen to cultivate *in vitro* biofilms influences the characteristics of sessile bacterial communities. It is important to keep in mind that there is no panacea yet, each method presents its pros and cons. One must therefore carefully choose the device according to the questions being addressed [Coenye 2010].

1.6.3 *In vivo* biofilm development

Although growing biofilms *in vivo* is more relevant, it is also more time consuming. A variety of organisms have already been used for *in vivo* biofilm studies: non-mammalian such as the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, and the zebrafish *Danio rerio* and mammalian such as murine models and rabbits [Lebeaux 2013].

Non mammalian models offer interesting perspectives in terms of throughput, ethical considerations, cost and experimental possibilities.

C. elegans, for instance, has a generation time of 4 to 7 days and presents analogies to mammals in its immune response. It has been found to be well suited for the study of gut colonization [Marsh 2012]. This model has been employed for the study of biofilm formation by species of *Shigella*, *Mycobacterium* and *Listeria* [Lebeaux 2013]. However, it is more distantly related to mammalian pathology.

Murine models, on the other hand, are particularly relevant for clinical research but suffer from low throughput rates, ethics and labour-intensive logistics [Coenye 2010] [Lebeaux 2013]. They have notably proved valuable to understand the development of biofilm-related pathologies on implantable medical devices such as vascular catheters [Rupp 2001] [Kokai-Kun 2009]. In particular, a recent work has highlighted the importance of biofilm formation as a way for bacteria to avoid macrophage phagocytosis and to significantly reduce inflammation, i.e. exposition to the immune system [Thurlow 2011].

1.6.4 Biofilm analysis and characterization

Quantification of biofilms can be performed by several techniques. First, the number of Colony Forming Units (CFU) is usually determined by standard plate count method after recovering the biofilm biomass. This technique has some limitations, notably due to the presence of Viable but Non Culturable cells (VNC) [Paulson 2009]. Biomass quantification can also be performed by staining with non-specific dyes (e.g. crystal violet or safranin) and measuring absorbance. Morphological analysis of biofilm *in situ* is best performed with confocal microscopy (i.e. fluorescence). The z resolution of this microscope offers precious information on the three-dimensional structure of the biofilm [Neu 2010].

More recently, combined electron microscopy with fluorescent hybridization and confocal microscopy have been successfully implemented to visualise biofilms with much details [Schaudinn 2009]. In order to extract quantitative morphological parameters from microscopy

images, sophisticated algorithms for image treatment have also been developed, e.g. COMSTAT [Heydorn 2000]. These algorithms are able to compute, among others, biofilm roughness, substrate coverage and total biomass volume based on stacks of confocal microscopy images.

The mechanical properties of biofilms can be approached by rheology [Stoodley 1999]. Because EPS display visco-elastic properties, rheometry allows to draw stress-strain curves, indicating how a biofilm yields under a define mechanical load. This parameter is of importance to apprehend the resilience of biofilms in environments with high shear stresses (e.g. in industrial pipes, on river rocks or on ship hulls).

Molecular biology techniques can also prove useful to analyse biofilms. For example, quantitative PCR coupled with propidium monoazide has been used to determine the levels of live *versus* dead cells in biofilm samples [Alvarez 2013] or to assess the level of expression of specific genes in biofilms [França 2012].

In addition, sensors can be employed to quantify the amount of biofilm forming on a surface or to follow specific parameters such as the concentration in oxygen or the pH [Wang 2013]. These sensors can be optical [Kwak 2014], electrical [Paredes 2013] or even mechanical such as microcantilevers to measure cohesion in biofilms [Aggarwal 2010].

Spectroscopy has recently gained much popularity in the analysis of biofilms due to its non-invasiveness. In particular, Raman spectroscopy has been employed to assess the distribution of bacterial species within a biofilm without the need for labelling [Pätzold 2006] and Fourier Transform Infra-Red spectroscopy (FTIR) allows to follow the apparition of chemical bonds, for example by the production of biopolymers (such as EPS), with a spatial resolution of a few micrometers [Holman 2009]. Magnetic Resonance Imaging (MRI) has been used to get access to biofilm composition and to map mass transfer from and to a biofilm, mainly due to the difference in relaxation time of excited nuclei in the

biofilm compared to the bulk phase [Neu 2010].

Observation of biofilms *in vivo*, for example in murine models, has been implemented using bioluminescent bacteria. Briefly, a highly sensitive CCD camera is fixed in a dark room where the anesthetized animals are placed. The camera collects photons emitted by bioluminescent bacteria present in the animal. Generated images display the zones colonized by the biofilm inside the infected mouse. This technique is non invasive and is particularly useful to follow and localize infections sites on live individuals through time [Kadurugamuwa 2008].

Why focusing on *S. epidermidis*?

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This work was dedicated to studying *S. epidermidis*. In this Chapter, the key characteristics of this species are presented. Particular emphasis will be put on the pathogenesis of *S. epidermidis* and notably the role of biofilm formation.

2.1 General characteristics and classification

Staphylococci were first observed in the late nineteenth century, isolated from pus. Due to the lack of discriminant criteria other than Gram-positive, non-motile, non-sporulant, spheres of $0.5\ \mu\text{m}$ to $1.5\ \mu\text{m}$, their classification has been changed numerous times. In 1975, a simple but efficient classification scheme based on metabolic features, notably the capacity to produce a coagulase and the utilization of various carbons sources, was proposed [Kloos 1975]. Though it is only

in 1990 that Staphylococci were classified in a family (i.e. *Staphylococcaceae*) of their own, therefore separated from the *Micrococaceae* family to which they belonged. The main reasons for that was their facultative anaerobic metabolism and a higher G+C content [LeLoir 2010].

To date, ca. 50 species belonging to the genus have been described. The ability to produce a coagulase is widely used to classify the species in two groups. The "coagulase-positive" group comprise 7 species including the famous pathogen *S. aureus* whereas the "coagulase-negative" group is composed of more than 40 species among which *S. epidermidis* [LeLoir 2010].

The genomes of *Staphylococci* usually comprise between 2.5 and 3 Mb (million base pairs), corresponding to ca. 2,500 coding sequences and have a G-C content of about 33 % [Zhang 2003] [Baba 2008]. The occurrence of plasmids, often of small size (below 10 kb), and transposons is quite high [Gill 2005]. The impact of the latter on the genetic evolution of Staphylococci will be discussed in Section 2.3.

2.2 Ecology and habitat

S. epidermidis and *S. aureus* are both colonizers of the skin and mucosa of mammals. *S. epidermidis* is the most frequently encountered species on human skin whereas *S. aureus* is present in smaller numbers and, according to a recent study, only in about 10 to 25 % of the human population [den Heijer 2013]. Several mechanisms confer to *S. epidermidis* an ecological advantage to reside on the skin. Firstly, it secretes a cell surface protein Aap (accumulation associated protein) that is able to bind to corneocytes, fastening cells on the skin [Macintosh 2009]. Secondly, the capsule produced by most of the strains, made of poly- γ -glutamic acid (PGA), has an osmoprotective role against the relatively high salinity of the skin. PGA also inhibits the action of antimicrobial peptides secreted by the host [Kocianova 2005]. *S. epidermidis* is also able to sense these antimicrobial peptides and activate specific resistance mechanisms [Li 2007]. Finally, the species possesses several

Na⁺ and H⁺ exchangers to face saline bursts [Hiramatsu 1998].

For healthy individuals, the presence of *S. epidermidis* on the skin is beneficial as it limits the colonization of other species, like *S. aureus* or other pathogenic Gram-positive bacteria [Lina 2003] [Cogen 2010]. However, immunocompromised people are unfortunately subject to infections by opportunistic pathogens, a class in which *S. epidermidis* has now acquired a solid reputation [Otto 2009]. The pathogenicity of *S. epidermidis* and *S. aureus* will be discussed in Section 2.4.

2.3 Genetic variability

Molecular techniques such as Pulsed Field Gel Electrophoresis (PFGE), Multi Locus Sequence Typing (MLST), DNA-DNA hybridization and Whole Genome Sequencing (WGS) coupled with *in silico* analysis have highlighted the diversity amongst *S. epidermidis* [Lina 1992] [Jamaluddin 2008] [Schoenfelder 2010] and *S. aureus* genomes [Fitzgerald 2001] [Lindsay 2004] [Maiden 2006] [LeLoir 2010]. Several mechanisms account for the reported intra-species diversity.

The first type of mechanism supporting the constant evolution of Staphylococci is horizontal gene transfer (HGT). Three main types of HGT exist : transformation, conjugation and transduction.

Transformation consists in the uptake, by so-called competent bacterial cells, of DNA from the environment. It has long been a question whether *S. aureus* is naturally competent [LeLoir 2010], but a recent study demonstrates chromosomal DNA (i.e. methicillin resistance genes) uptake in *S. aureus* [Morikawa 2012]. However, it also showed that this phenomenon probably occurs at a low frequency as only a minor fraction displayed natural competence, upon the expression of a cryptic sigma B factor, SigH.

On the contrary, conjugation amongst Staphylococci is rather frequent. Conjugative plasmids self-transfer from one strain to another

thanks to a specific transfer machinery. Conjugation facilitates the mobility of DNA inside the species, the genus and even further, depending on the host range of plasmids. Mobile plasmids frequently carry pathogenicity islands and antibiotic resistance genes [Grohmann 2003] [McCarthy 2012]. It is worthy to note that conjugation has been found to be highly dependent on the conditions inside biofilms [Hausner 1999] [Ehlers 1999] [Madsen 2012]. It was also recently shown for plasmid transfer amongst Staphylococci in biofilms [Savage 2013].

The last type of HGT is transduction, it happens upon phage infection as the viral particle contains, in its genome, bits of genomic DNA from another host. These carried fragments can be integrated into the genome of the infected cells. Pathogenicity Islands (PAI) are amongst the sequences usually transferred through this pathway. Some of which are former prophages having lost their ability to complete a lytic cycle [Novick 2003]. Transduction is thought to be the major mode of HGT in Staphylococci [LeLoir 2010].

Secondly, events of mutation, deletion, insertion of single base pairs participate to the genetic plasticity of *S. epidermidis* and *S. aureus* genomes. Such events normally happen at low frequency ($\sim 10^{-9}$ per base pair), though in some isolates mutations were shown to occur at a higher frequency, from 10 to 1000-fold [Hall 2006]. Spontaneous mutation or recombination is a strong driving force of bacterial evolution in adverse conditions, it has been shown that resistance to several antibiotics originates from it. For example, point mutations in Penicillin Binding Protein 2 (PBP2) conferred resistance to methicillin in *S. aureus* isolates [Hackbarth 1995] [Ba 2014]. Mutations in gyrase A and B (*gyrA* and *gyrB*) genes suppressed susceptibility to quinolones [Ito 1994]. Finally, resistance to daptomycin can be mediated by mutations in several genes with various functions, from protease to nucleotide synthesis (*walK*, *agrA*, *clpP* and *prs*) [Song 2013].

Thirdly, an important part of genomic variability can be attributed to Insertion Sequences (IS). These elements, composed of two Inverted

Repeats (IR) sequences, and a transposase/recombinase are "jumping" across the genome [Mahillon 1998]. Transposition can inactivate some genes or disturb their expression. Therefore, IS are potent agents of genetic remodeling. Several IS have been spotted in genomes of Staphylococci, some of which, i.e. IS256 and IS257, are involved in the regulation of biofilm formation and antibiotic resistance by *S. epidermidis* [Kozitskaya 2004] [Gill 2005] [Schoenfelder 2010].

Finally, recombination of small chromosomal fragments, often brought by HGT, or even replacement of large portions of chromosomes (up to 500 kb) which are taken up by bacterial cells in a yet unclear fashion, have been reported in Staphylococci [Robinson 2004] [Everitt 2014]. This type of genetic rearrangements create a diversity in staphylococcal genomes. Though happening at low frequencies, it could be one of the driving forces of bacterial evolution in environments with high selective pressure.

2.4 Pathogenicity - *S. epidermidis* the bad and *S. aureus* the ugly ? or vice-versa

S. epidermidis and *S. aureus* share some common features but they also differ as it comes to pathogenicity.

2.4.1 Statistics

S. aureus is recognized as a major threat to public health. It causes acute bloodstream infections, purulent wounds, abscesses, furuncles, osteomyelitis and implanted device infections [Archer 1998] [Gordon 2008]. The most dangerous infections are caused by Methicillin-Resistant *S. aureus* (MRSA) and Vancomycin Intermediate *S. aureus* (VISA) since they are insensitive to several clinically used antibiotics. According to CDC, in 2014, the overall incidence of MRSA infections in the US was estimated to a rate (community and hospital acquired confounded) of 0.03 %, i.e. one person out of 3,000 on the global population in the US. Two categories of people were pointed out as

being clearly more at risk (up to 100 fold): dialysis patients and +65 people [Dantes 2013]. Mortality of MRSA and VISA infections was estimated at 27 % on 700 patients with bacteriemia between 2002 and 2007 [Pastagia 2012]. Comparable mortality rate of 21.5 % was found in another study considering deaths associated with all types of MRSA infections, not only bloodstream [Datta 2008].

S. aureus is also responsible for food intoxications due to the production of several toxins in food products [Argudín 2010]. Such episodes are very rarely lethal and represent a minority of reported food associated diseases [Mead 1999] [LeLoir 2003].

S. epidermidis has long been considered as a benign skin colonizer before its ability to develop implanted device-related infections was apprehended [McCann 2008] [Otto 2009]. The number of device-related infections caused by coagulase negative Staphylococci is estimated at 250,000 cases each year in the US, or about 1.5 % of the 150 million devices implanted each year in this country [Raad 2007]. Because of its ubiquity on human skin and mucosa [Grice 2011], *S. epidermidis* is the most frequent causative agent of these infections [Otto 2012]. Among the consequences, patients stay longer in the hospital (7 days on average) and the treatment represents heavy financial burden for the healthcare system [O’Grady 2002] [Raad 2007]. Hence, the associated mortality rate of *S. epidermidis* infections ranges from 1 to 25 % depending on situations [Otto 2012].

Being less virulent than *S. aureus*, *S. epidermidis* particularly affects immunodepressed people and neonates [Vuong 2002].

2.4.1.1 Staphylococcal virulence factors

S. aureus is a highly virulent pathogen, carrying an impressive arsenal of toxins : Panton-Valentine leukocidin, Toxic-shock syndrome toxin, pore-forming α -toxin, enterotoxins that possess superantigenic properties, haemolytic toxins [Bergdoll 1981] [Johnson 1991] [Lina 1999]

[Gill 2005]. The list presented here is not exhaustive but demonstrate the extent of *S. aureus* resources. Therefore, the infections it causes are usually rapidly deteriorating patients state, under the assault of several cytotoxic toxins. The number of antibiotic resistance genes *S. aureus* isolates display associated to the quick evolution of the disease, make *S. aureus* infections difficult to handle for clinicians [Klevens 2007].

On the other hand, *S. epidermidis* has virtually no virulence factors *sensu stricto*, except phenol soluble modulins (PSM), toxins with a weak cytolytic activity. It proceeds in a different manner to invade the host. Indeed, the strategy developed by *S. epidermidis* is based on its ability to grow in the form of biofilms, the main cause of persistent infections.

2.4.2 The role of biofilms in staphylococcal pathogenesis

A common scenario for biofilm-related infection is as follows. After the initial contamination of the implanted device, a long period of latent infection is taking place during which *S. epidermidis* develops slowly and fastens its anchorage in the body. Once a mature biofilm is formed, inflammation symptoms occur, causing discomfort to the patients [del Pozo 2007]. This is the consequence of dispersal, the fourth phase of biofilm development, that provokes a release of planktonic cells in surrounding tissues and blood [Boles 2011]. On top of the redoubtable ability of *S. epidermidis* to resist antimicrobial therapies, bacteria in biofilms profit from an increased tolerance to these antibacterial compound [Fux 2005a] [Hoiby 2011]. Ultimately, due to the failure of antibiotics to eradicate *S. epidermidis* biofilms, the removal of the infected device appears as a necessary step to eliminate them [Shapiro 2011]. In some cases though, the pathogen dispersed in blood by the biofilm formed at a primary site of infection, i.e. medical device, has colonized host tissues somewhere in the body. These affected tissues are called secondary sites of infection and complicate the treatment even further [Kiedrowski 2011].

While biofilms are the cornerstone of *S. epidermidis* pathogenicity, it is a less preponderant factor in *S. aureus* infective strategy. It is rather a way for *S. aureus* to increase its persistence in the host [Fux 2005a].

Biofilm formation by *S. epidermidis* - The specifics

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In the first chapter, the four steps of biofilm development have been described in a manner that remained general for most bacterial genera. Evolution has led each bacterial species to acquire a variety of specific factors to initiate, develop and regulate its life in biofilms. This section presents the formation of biofilms by *S. epidermidis* in details.

3.1 First step - Adhesion to surfaces

Adhesion to surfaces is the first step to the formation of biofilms. The extent of colonization by the biofilm crucially depends on the establishment of adhesive bonds between bacterial cells and the surface. In fact, adhesion remains critical throughout the life of the biofilm, to ensure that the community is not loosening its grip on its substratum. *S. epidermidis* is able to bind to surfaces in two ways.

The first is non-specific adhesion, happening through physical interactions. Alternatively, specific *S. epidermidis* adhesion to host proteins can occur thanks to receptor-ligand interactions, mediated by specific binding cell surface proteins. Specific adhesion is an important mechanism in the process of colonization of the host by *S. epidermidis*.

3.1.1 Non-specific adhesion of *S. epidermidis*

3.1.1.1 Theory of bacterial adhesion

S. epidermidis can adhere non-specifically on a substratum. Adhesion occurs through the interplay of the ELectrostatic (EL), Lifshitz Van-derWaals forces (LW), Acid-Base (AB) and OSmotic (OS) interactions proposed by Van Oss as an extension of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, in 1989. Thanks to this theory, Gibbs free energy can be calculated to assess the thermodynamic feasibility of the event. Bacteria would adhere if ΔG_{tot} is negative [Van Oss 1989].

Various factors affect the calculation of the free energy, including pH, ionic strength, temperature or the presence of biopolymers in suspension [Hermansson 1999]. One other critical factor on which the free energy components AB, LW, OS and EL all depend on is the cell-substratum distance. As a result, ΔG_{tot} , computed as a sum of the EL, LW, AB and OS interactions, is usually plotted as a function of the cell-substratum distance, assuming all other forecited parameters are kept constant. In Fig. 3.1, an example of plot showing the free energy as a function of

distance of a negatively charged bacterium approaching a negatively charged substratum is represented. In this Fig., the electrostatic (EL) and acid-base (AB) repulsions are balanced by attractive VanderWaals interactions.

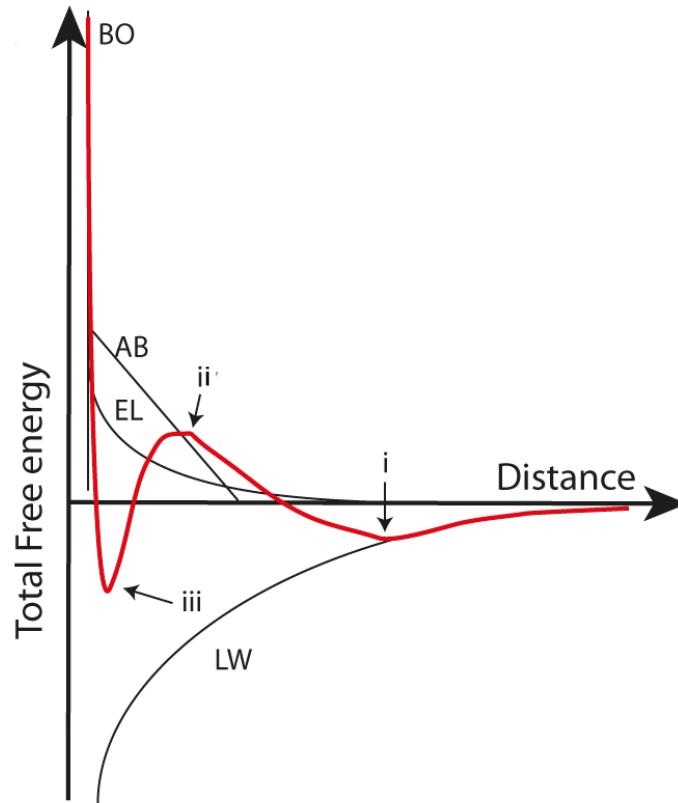


Figure 3.1: Schematic representation of free energy components as a negatively charged bacterium comes in contact with a negatively charged substratum, according to DLVO theory. EL, LW and AB interactions all decay with the distance. Although it may not be properly represented on the graphs, AB and EL decay exponentially and LW decays proportionally to $1/d^2$. BO is the born repulsion caused by the fact that atoms and molecules cannot approach one another at a distance smaller than 0.14 nm. The resulting free energy is plotted in red. **i** - The secondary minimum at which the total energy of interaction is favorable to attraction, LW are overcoming the EL and AB repulsions. **ii** - A local maximum, or energy barrier between the two minima. The total energy of interaction is positive, adhesion is not thermodynamically favored. **iii** - At a short distance, a primary minimum exist. Adapted from [Van Oss 1989].

The presence of one or several minima and maxima in ΔG_{tot} indicate a distance at which bacterial cells will respectively be retained to or repelled from the surface with a force proportional to the depth of the minimum/maximum. A bacterium caught in a secondary minimum adheres in a reversible fashion. External forces such as shear stress, if sufficient to outbalance the one that anchors the cell on the substratum, can induce detachment. In an intermediate state, a bacterium can still be weakly attached to the substrate while being carried away by the flow, this is called mobile adhesion [Boks 2009]. Reversible adhesion can, on the one hand, turn into irreversible when bacteria "jump" the energy barrier (see Fig. 3.1 B) to get, closer to the substrate, to the primary minimum. At this point, the cell is anchored on the substrate in a favorable situation with regard to free energy. This step is called bond strengthening [Boks 2008a].

There are important assumptions in this theory coming from the science of colloids. First, a bacterium should present a smooth cell surface and ideally show no deformation under loading. Second, the surface chemistry of the colloid, i.e. the bacterial cell, is not supposed to evolve over time. Finally, the substratum should be flat or with a minimal surface roughness [Van Oss 1989]. It should be noticed that these conditions are hardly ever met in real situations [Hermansson 1999]. Since roughness can induce tangential forces not apprehended by the extended DLVO theory [Hermansson 1999], adaptations were proposed to take surface roughness into account via a numerically calculated surface element integration that allow better prediction of energy profiles locally [Hoek 2006].

A recent work reports the calculation of a predictive free energy ΔG_{tot} as a function of the distance with measured contact angles of bacteria with water, formamide, α - bromonaphthalene and methylene iodide as well as with the zeta potential. In the absence of external forces such as hydrodynamic shear stress, their model was able to predict the adhesion of *S. epidermidis* cells to glass and hydrophobic glass at large distance but not close to the substratum [Boks 2008b]. The authors

recognize that precautions should be taken while interpreting DLVO curves because it does not take into account the surface appendages harbored by bacteria and the relative softness of the bacterial cell. Any protrusion that differs from the perfectly smooth substratum or cell surface could cause bridging effects and facilitate the transition towards irreversible adhesion [Bos 1999] [Hermansson 1999].

Another study, focussing on the measurement of bond strengthening by Atomic force microscopy (AFM) on both hydrophobic and hydrophilic glass surfaces with *S. epidermidis* has come to conclusions that were not in accordance with the theory. Interestingly, the authors observed a larger increase in bond strength over time on hydrophilic glass compared to hydrophobic coated glass whereas the theory predicted a larger minimum in free energy for the hydrophobic substratum. This phenomenon was attributed to the conformational changes operated by proteins of the cell surface to increase their interaction with the substrate [Boks 2008a]. In this case, the adaptability of *S. epidermidis* could not be integrated in the model calculations [Bos 1994].

The extended DLVO theory has its limits but can be carefully employed to explain non-specific adhesion of bacterial cells to surfaces.

3.1.1.2 Impact of surface properties on *S. epidermidis* adhesion

Physico-chemical properties of substrata have a significant influence on the adhesion of *S. epidermidis*. On hydrophobic materials, a general tendency is that *S. epidermidis* adheres in high numbers. However, when the surface has previously been coated with host proteins, adhesion largely decreases (up to 2 log). This was demonstrated *in vitro* on PolyTetraFluoroEthylene (PTFE) coated with Fibronectin (Fn) [Abusalim 2013], on Elasthane coated with bovine serum [Patel 2006], and on four types of surfaces including silicone and PolyEthyleneTerephthalate (PET) coated with bovine serum albumin (BSA) or with Fn [Linnes 2012]. On hydrophilic materials, the effect of adsorbed proteins

on *S. epidermidis* adhesion to surfaces is similar as for hydrophobic ones but to a lesser extent [MacKintosh 2006] [Linnes 2012].

These tendencies can be explained by the repelling properties of a proteic film on surface towards bacteria. At physiological pH, most serum proteins are negatively charged, as is the surface of *S. epidermidis* cells. In view of the DLVO theory, a significant increase in EL interactions occurs when bacteria come close to the protein layer coating the surface. Hence, AB interactions between the biopolymers of the cell surface and adsorbed proteins are also repulsive [Nune 2012].

To overcome such barriers hampering colonization of coated surfaces, biofilm formation and survival in the host, *S. epidermidis* expresses a panoply of cell surface receptors to specifically bind to host proteins. The next section presents the different classes of these factors called adhesins.

3.1.2 Specific adhesion of *S. epidermidis* to substrates

S. epidermidis adhesins are macromolecules that specifically recognize host proteins in a receptor-ligand fashion [Patti 1994a] [Klemm 2000]. Two main groups are distinguished: the Microbial Surface Components Recognizing Extracellular Matrix Molecules (MSCRAMMs) which are covalently bound to the cell surface and the Secretable Expanded Repertoire of Adhesive Molecules (SERAMs) that are excreted in the environment, without covalent binding to the cell surface [Heilmann 2011].

3.1.2.1 MSCRAMMs

S. epidermidis genomes comprise up to 12 genes encoding MSCRAMMs. These proteins are characterized by a LPXTG motif that allows a sortase, i.e. a transpeptidase, to anchor them covalently to an amine group of the peptidoglycan [Schneewind 1993].

Amongst the MSCRAMMs of *S. epidermidis*, there is a family of protein containing several serine-aspartate repeats (Sdr). Two of

its members are well characterized : SdrG that binds Fibrinogen (Fg) with high affinity according to a "dock, lock and latch" model [Hartford 2001] [Ponnuraj 2003], and SdrF, a protein that recognizes collagen [Arrecubieta 2007]. Another family of surface proteins, without repeats, called *S. epidermidis* surface proteins (Ses), has been discovered in 2005 and shown to be expressed in the course of an infection as revealed by the presence of anti-Ses antibodies in patients blood [Bowden 2005]. Their role in *S. epidermidis* adhesion however, remains unclear [Fey 2010].

3.1.2.2 SERAMs

Contrary to the MSCRAMMs, SERAMs are bound to cell surface by yet unknown mechanisms. Several proteins fall into this category. Notably, the Extracellular matrix-binding protein (Embp), a giant extracellular protein enables binding to Fn [Williams 2002], *GehD*, a lipase with an affinity for collagen [Bowden 2002] and Ebp an analog to *S. aureus* protein EbpS that binds to elastin [Park 1996]. Also, autolysins (Aae and Atle), on top of their essential role in cell wall remodelling, were shown to bind Fn, Fg and Vitronectin (Vn) [Hirschhausen 2010].

3.1.3 The transition from reversible to irreversible adhesion

The role of adhesins in the process of *S. epidermidis* attachment to substrata is to favor the transition from reversible to irreversible adhesion (see Section 3.1.1.1). By adding up specific interactions to a situation of reversible adhesion, a loosely bound bacterium is able to fasten itself on its substratum by bridging the energy gap shown in Fig. 3.1. It is clear that adhesins confer to the bacteria an ecological advantage to colonize certain types of surfaces. They secure the adhesion of bacterial cells in order to allow the initiation of the second phase of biofilm formation : the accumulation and the development of microcolonies (see Fig. 1.1).

3.2 Second step - Accumulation

In this phase of the biofilm development, the surface bound cells divide and grow into microcolonies. These are multi-layered structures spanning in three dimensions. According to the DLVO theory, negatively charged staphylococcal cells agglutination does not occur spontaneously since repulsive EL interactions keep cells apart. Besides, AB interactions between two cell surfaces loaded with biopolymers should also be somewhat repulsive [Van Oss 1989]. In brief, the ΔG_{tot} of cell-cell aggregation should normally be greater than zero, and cell-cell clumping should not happen. This can be observed in pure cultures in which planktonic bacteria seldom aggregate.

S. epidermidis compensate this cell-cell repulsion through the production of several biopolymers that make cell-cell aggregation effective. In particular, two surface anchored proteins have the ability to bring cells together and the polymers of the EPS matrix act as a glue that engulfs the growing microcolonies.

3.2.1 Surface proteins mediating cell-cell aggregation

These two surface proteins are the 140 kDa accumulation associated protein (Aap) [Hussain 1997] and the 237 kDa biofilm-associated-like protein (Bhp), an homolog of Bap first described for *S. aureus* [Cucarella 2001].

Aap is a protein that requires proteolytic activation to form polymeric fibrils [Rohde 2005]. These fibrils self-assemble by dimerization of the so-called G5 domains in the presence of Zn^{2+} to yield a flexible, spring-like link [Conrady 2012]. Interestingly, it was found that only a subpopulation of *S. epidermidis* harbored fibrils while the rest did not. The mechanisms leading to this specialization are yet unknown [Otto 2012]. In strains deficient for PNAG production, Aap is a key factor in biofilm formation. The biofilms formed by this type of strains

are commonly referred to as proteinaceous biofilms.

Bhp is a large surface protein composed of several tandem repeats. The gene from which it is encoded can be located on pathogenicity islands [Tormo 2005a]. Its precise role is not yet fully elucidated. Bhp is thought not to be necessary but rather helpful in the formation of biofilms [Lasa 2006].

3.2.2 The role of matrix polymers

The polymeric matrix of EPS is, for strains that are able to produce it, of crucial importance in the development of mature biofilms [Flemming 2010]. As it was mentioned in Section 7.2, the EPS are composed, in variable proportions of polysaccharides, proteins, DNA, and in minor quantities, lipids and minerals. In the case of *S. epidermidis*, poly-N-acetylglucosamine (PNAG) also known as polysaccharide intercellular adhesin (PIA) is the major compound of the matrix [Otto 2009].

The genetic determinants encoding the synthesis of PNAG are located on the *ica* locus, composed of four genes *icaA-D* and a regulator, *icaR*. IcaA and IcaD are two subunits of an N-acetylglucosamine-transferase that assembles NAG monomers together by forming β 1-6 bonds. IcaC is responsible for the elongation and the excretion of the polymer chain. Finally, IcaB, located at the cell surface, is a N-acetylase that cleaves some acetyl residues from elongated PNAG chains, composed of 130 monomers on average [Heilmann 1996] [Gerke 1998] [Otto 2012]. Fig 3.2 summarizes the production and excretion of PNAG.

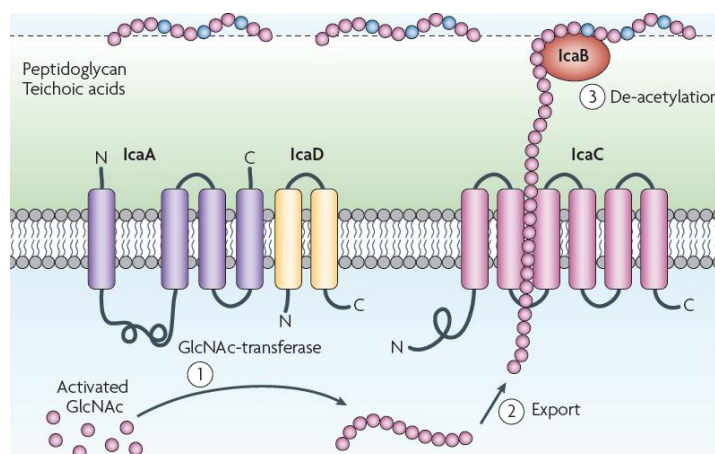


Figure 3.2: Assembly and excretion of PNAG. IcaA and IcaD polymerize the NAG monomers (purple balls). The chains in elongation are exported outside the cell by IcaC. IcaB finishes the process by deacetylating a portion of the polymerized monomers (blue balls). Source : [Otto 2009].

Partial deacetylation confers to PNAG a positive charge. This feature is critical to diminish the repulsion of the negatively charged cells and ensure cell-cell aggregation in microcolonies. Although not required for the formation of a biofilm as such, evidences suggest that proteinaceous biofilms developed by strains deficient in the *ica* locus are less resistant to mechanical stresses than their homologs producing PNAG [Rohde 2007]. Besides this role of intercellular adhesin, PNAG also has the ability to protect *S. epidermidis* from the action of antimicrobial peptides, immunoglobulins (Ig) and neutrophils [Vuong 2004a]. Eventually, its implication in virulence is presumed but a lack of evidence prevented authors of previous works to conclude on the subject [Otto 2012]. In the early moments of a biofilm development, PNAG thus plays an important role that goes beyond its properties of sticking cells together.

Another type of cell surface polymers play important role in biofilm development. Teichoic acids (TA), prominent cell surface polymers composed of phosphate, glycerol and polyols, were found to favor cell-cell interactions. Indeed, mutants in *tagO*, a gene responsible for the

biosynthesis of wall teichoic acids (WTA), showed an impaired adhesion and accumulation on hydrophobic polystyrene (PS), partly due to a decrease in *icaABCD* expression and PNAG synthesis [Holland 2011].

Finally, over time, the proportion of EPS other than PNAG increases, especially eDNA. Released upon autolysis (by autolysins AtlE) of cells within the biofilm [Qin 2007], eDNA plays a role in the stabilization of the biofilm (bacterial adhesion and aggregation) through favorable acid-base interactions [Das 2010].

3.3 Third step - Maturation towards a 3-D architecture

The growth of microcolonies, supported by PNAG and other matrix components, leads them to increase their thickness and to merge with adjacent microcolonies if they are close enough. At this point, due to the heterogeneity of the conditions inside the biofilm, bacteria undergo a dramatic switch in their physiological states [Rani 2007]. Either in response to environmental conditions, by stochastic gene activation/inactivation, distinct subpopulations appear [Stewart 2008]. Some lose the ability to produce PNAG in a reversible fashion [Ziebuhr 1999], others excrete small tensioactive peptides called phenol soluble modulins (PSMs) that locally decrease the cohesion of the biofilm [Wang 2011]. The result of this specialization of the subpopulations is the build up of a multi-layered 3-D architecture. In general, it is composed of mushroom (or tower) shaped macrocolonies in and around which water filled channels permit the circulation of the medium [Costerton 1995]. Owing to this intraspecies diversity [Schoenfelder 2010], various phenotypes can be observed in the biofilms formed by *S. epidermidis* strains [Schaudinn 2014]. Fig. 3.3 illustrates the transition from the adhering bacteria to the mature biofilm.

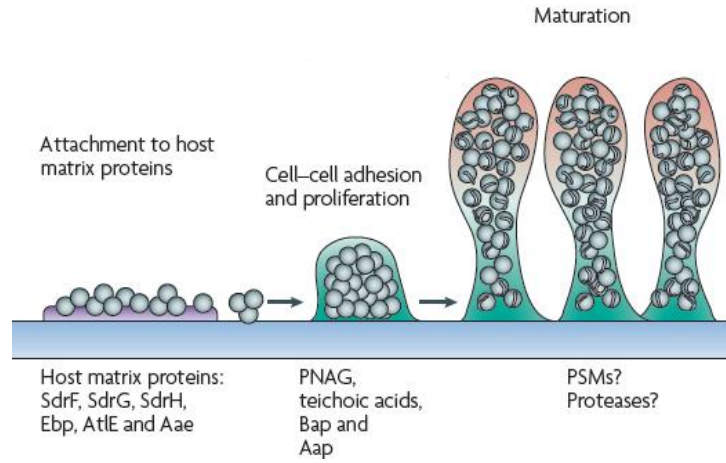


Figure 3.3: Development of surface bound *S. epidermidis* cells into a mature biofilm. From left to right, adhesion on host matrix proteins, accumulation in microcolonies, and maturation in a specific 3-D architecture with towers and channels. Color gradient in the towers indicates the heterogeneity of conditions inside the mature biofilm. Source : adapted from [Otto 2009].

In the maturation phase, the biofilm also reinforces mechanisms of protection towards the aggressions of its environment. First, the increased proportion of EPS ensures a mechanical stability against mechanical disruptive forces while delaying the penetration of host immune defenses as Ig and Anti-Microbial Peptides (AMPs) inside the biofilm [Vuong 2004b]. Secondly, exoenzymes such as proteases and lipases are produced by the cells in the biofilms to actively degrade AMPs [Lai 2007], and transform some toxic host compounds, such as specific fatty acids, into harmless degradation products [Chamberlain 1997].

3.4 Fourth step - Dispersal

The last step to complete the life cycle of a biofilm is dispersal. As it was underlined in Chapter 1, the release of individual cells or larger sections of the biofilm is a strategy to support the colonization of new spots. Although little is known about the dispersal mechanisms, PSMs and proteases have been shown to be involved in a similar fashion as in the maturation phase [Boles 2008] [Wang 2011]. Quorum Sensing (QS),

especially the *agr* system, was also pointed out as an important regulator of detachment, through the regulation of PSM and proteases expression [Vuong 2004b]. The specific regulatory aspects of biofilms are addressed in the next section.

3.5 Regulation of staphylococcal biofilm formation

The development of a mature biofilm requires a fine temporal control of the expression of factors mediating adhesion, accumulation, maturation and dispersal. Besides, in order to maintain its fitness in changing conditions, a biofilm should be able to respond to a series of environmental stimuli. In this section, regulatory mechanisms, i.e. transcriptional regulation, QS and phase variation, are developed. The response to environmental stimuli is presented as well.

3.5.1 Transcriptional regulation of PNAG

The expression of PNAG, a central element in biofilm regulation, is associated with the *ica* locus. The transcriptional regulator IcaR represses the transcription of the other genes of the *ica* locus, i.e. *icaABCD* [Conlon 2002] [Gotz 2002]. The transcriptional activity of the *icaR* promoter is controlled by σ_B which is itself influenced by RsbV, RsbW and RsbU, i.e. regulators of sigma factor B, according to a chain of phosphorylation (see Fig. 3.4) [Knobloch 2004]. Besides σ_B down-regulation of *icaR* transcription, SarX is able to hamper the binding of IcaR on *icaABCD* promoter. Levels of SarX are influenced by the transcriptional activator Rbf that up-regulates *sarX*. Rbf thus has a positive effect on biofilm formation [Cue 2013]. On the contrary, up-regulation of *icaR* transcription can occur via the regulatory protein Spx, leading to decreased *icaABCD* transcription. Normally, ClpP protease degrade Spx and counterbalance the repressive effect of Spx on PNAG production [Pamp 2006] [Wang 2010].

Three pathways for direct *icaABCD* regulation, i.e. IcaR independent, have been described. The first is dependent on staphylococcal accessory regulators *sarA* and *sarZ*. The promoter of *sarA* is activated by high levels of σ_B and high levels of SarA enhance expression of *icaABCD* promoter [Wang 2008]. Contrary to SarA, SarZ has a repressive effect on *icaABCD* transcription. Finally, the transcription of *icaABCD* is upregulated by the purine repression operon (*purR*) [Tormo 2005b].

Environmental cues have also been shown to impact biofilm formation by *S. epidermidis in vitro*. For example, ethanol favors the expression of *icaABCD* by repressing *icaR* transcription [Conlon 2002], glucose activates the synthetic pathways of PNAG [Dobinsky 2003] and NaCl favors *icaABCD* expression via RsbU and σ^B [Knobloch 2004]. It has also been shown that the activity of the tricarboxylic cycle (TCA) has a large influence over the production of PNAG. Indeed, a lower activity of TCA cycle induce a larger amount of PNAG produced, probably as a response to a metabolic stress. Similarly, in conditions of high TCA cycle activity, PNAG synthesis was found to be diminished [Vuong 2005] [Sadykov 2010]. The understanding of the exact mode of action of such regulations is still incomplete.

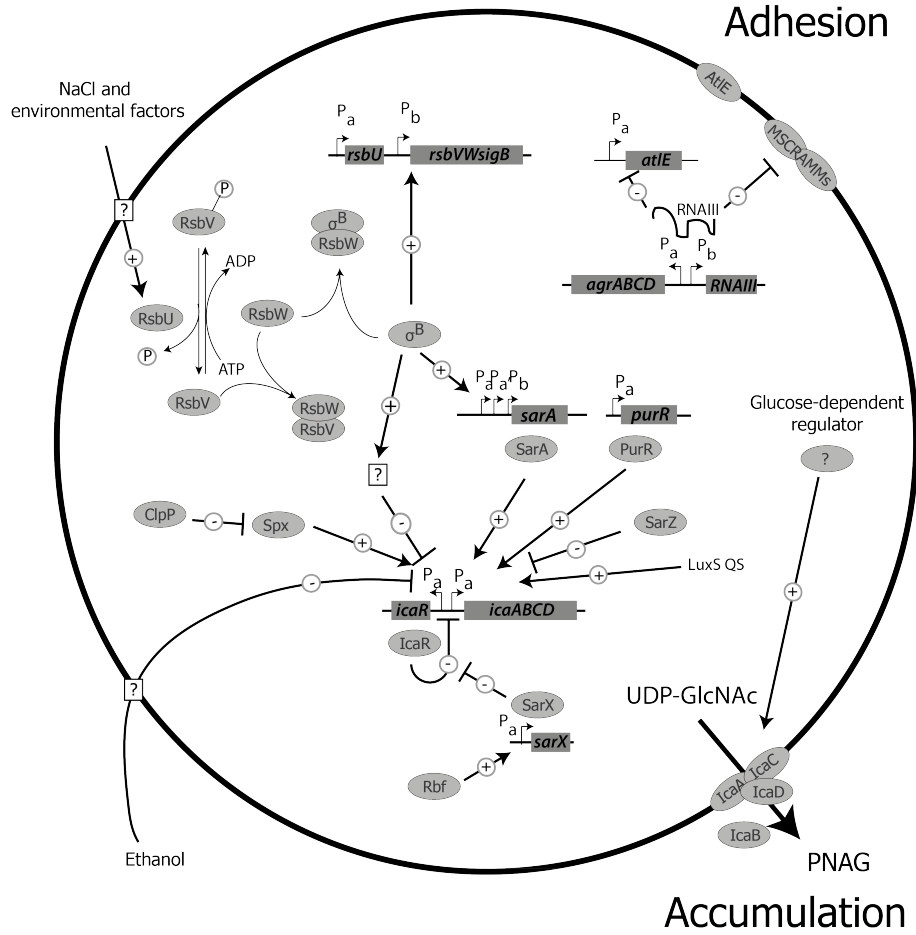


Figure 3.4: Summary of transcriptional regulation of the *icaABCD* operon. The large circle defines the cell membrane. Rectangles represent genes and ovals proteins. Arrows indicate a positive regulation whereas straight lines blocked by a perpendicular line indicate down-regulation. Promoters are indicated by little arrows upstream of genes. For details, see main text. Source : inspired from [Mack 2007].

3.5.2 QS regulation

QS is the cell-cell communication mechanism used by bacteria to adapt their behaviour in response to population density [Waters 2005]. In *S. epidermidis*, two systems are reported to date : Agr and LuxS. Their modes of action and impacts on biofilm formation are presented here.

3.5.2.1 Agr system

Agr QS is encoded by an operon composed of *agrABCD* and RNAIII. It is a relatively common system among Gram-positive bacteria [Cegelski 2008]. Its principle of action relies upon the secretion of an Auto-Inducing Peptide (AIP). AIPs are presynthesized by AgrD, processed and externalized by AgrB in the form of semi-cyclic molecules: a cycle is formed by a thioester bond between a conserved cysteine and another amino acid while a tail of 2 to 5 amino acid long is attached to this cycle [Lyon 2004]. Fig. 3.5 shows the different families of AIPs discovered in *S. aureus*. Extracellular AIP binds to AgrC, an histidine kinase sensor that will in turn phosphorylate AgrA, the transcription regulator for the *agr* operon [Yarwood 2003]. When the concentration in extracellular AIP reaches a threshold, sufficient levels of activated AgrA are produced and the system enters in a positive feed-back loop. Fig. 3.6 illustrates the principle of *agr* QS.

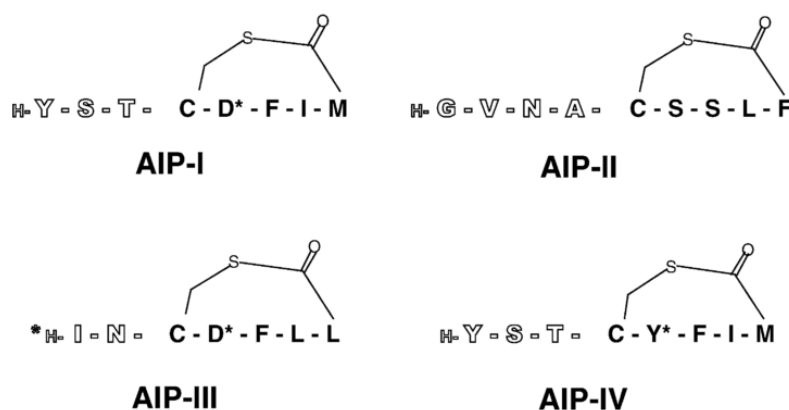


Figure 3.5: Four families of AIP found in *S. aureus*. The aminoacids are represent according to the standard one-letter code. Outlined letters represent the amino acids of the AIP tail. The thioester bond is closing the cycle between the cysteine (C) and the C-terminal amino acid. Residues crucial to the recognition of AIP by AgrC are indicated by an asterisk. Source : [Lyon 2004].

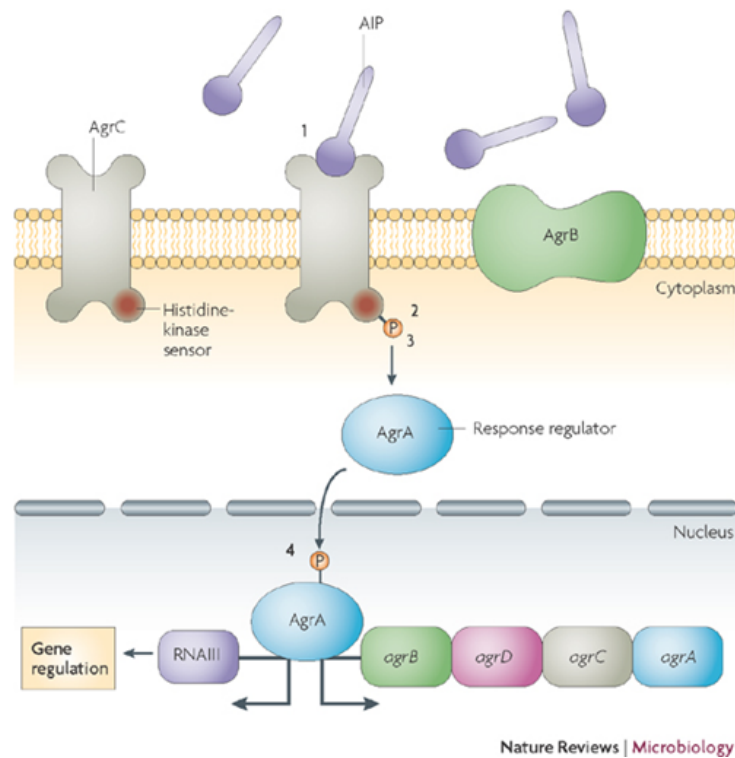


Figure 3.6: Mechanism of Agr QS in Gram-positive bacteria. The *agr* locus composed of *agrA-D* genes encode respectively : a transcriptional regulator of the operon (*agrA*), a membrane protein to export AIPs (*agrB*), an histidine kinase receptor (*agrC*), an enzyme that catalyses the production of AIPs (*agrD*). RNAIII expression is under the control of phosphorylated AgrA, that in turn depends on extracellular levels of AIP. RNAIII has pleiotropic effects on gene regulation, notably a repressive control over adhesive factors in *Staphylococci*. The sequence of action is as follows: 1. AIP is recognized by AgrC, 2. and 3. AgrC phosphorylates AgrA and 4. AgrA acts as a transcriptional regulator of the operon. Source : [Cegelski 2008].

The effector molecule of Agr QS is RNAIII. This molecule regulates the transcription rates of various target genes. Amongst these, RNAIII exert repressive control over MSCRAMMs synthesis, therefore inhibiting adhesion of *S. epidermidis* to host proteins. RNAIII also down-regulates the production of AtlE, an enzymes known to be essential to the release of eDNA [Qin 2007] (see Fig. 3.4). On the contrary, agr QS boosts the expression of virulence factors and triggers dispersal by *S. epidermidis*, notably via an increase in PSM, protease and lipase excre-

tion as confirmed by proteomic analysis [Vuong 2000] [Batzilla 2006] [Kretschmer 2012]. It seems that *agr* governs the transition from the colonizing to pathogenic behaviour of *S. epidermidis* in biofilms.

Besides these direct regulatory effects on biofilms, Agr QS has a pleiotropic effect on bacterial growth, indirectly affecting biofilm development [Mack 2007].

3.5.2.2 LuxS system

LuxS QS works through the excretion of the Auto-Inducing molecule 2 (AI2), an homoserine lactone, that is in turn internalized. The exact mechanism of AI2 intracellular signalling are not yet fully understood [Li 2008].

In *S. epidermidis*, $\Delta luxS$ mutant was found to increase the expression of the *icaABCD* up to four-fold, resulting in the formation of thicker biofilms *in vitro* [Xu 2006]. LuxS QS thus down regulates the production of PNAG, the main component of *S. epidermidis* biofilms. A more recent study has shown that LuxS affected *ica* expression in an IcaR dependent manner in *S. aureus* [Yu 2012]. In addition to this effect on *icaABCD*, LuxS influences virulence and dispersal in a way that remains to be elucidated.

As for Agr, LuxS has pleiotropic effects on the growth of Staphylococci, not limited to the formation of biofilms.

3.5.3 Phase variation

Phase variation is a mechanism by which bacteria regulate the expression of their genes in a "on/off" fashion [Seifert 1996] [Fux 2005b]. Pathogenic bacteria such as *Salmonella enterica*, *Neisseria gonorrhoeae* or some streptococcal species (*S. mutans*, *S. pneumoniae*) use this mechanism to silence the expression of antigenic molecules [Ziebuhr 1999].

In *S. epidermidis*, one of the major driving force of phase variation are insertion sequences (IS) or transposons. These mobile genetic elements, described in Section 2.3, can insert themselves in specific genes and inactivate them. IS256 has been found to cause reversible inactivation of the *ica* locus, as well as *rsbU* and *sarA* genes [Ziebuhr 1999] [Kozitskaya 2004] [Conlon 2004]. Fig. 3.7 illustrates how IS256 participates to the genetic plasticity of *S. epidermidis* genomes. Besides IS, phase variation can also be mediated by point mutations, DNA rearrangements, altered methylation or frame-shift mutations [Ziebuhr 1999] [Otto 2012]. Of note, the insertion of the transposon Tn917 in loci involved in regulation of *icaABCD* expression, or in *icaABCD* itself, has also been reported. This is a major mechanism to modulate biofilm regulation in *S. epidermidis* [Knobloch 2001] [Dobinsky 2002].

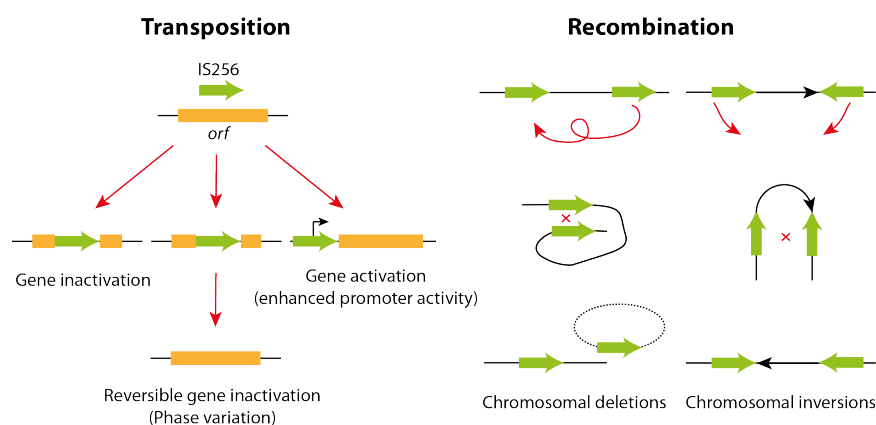


Figure 3.7: IS256 and its effects of genome flexibility of *S. epidermidis*. Two modes of action are shown : transposition and recombination. Source : [Schoenfelder 2010].

Phase variation is one of the mechanisms that generate a notable phenotypic diversity amongst subpopulations of *S. epidermidis* in biofilms. It is a feature essential to the development of the specific 3-D architecture of mature biofilms and to the coordination of the bacterial population metabolic activity [Conlon 2004] [McCann 2008].

3.5.4 pH homeostasis

During the development of a biofilm, the maintenance of stable pH throughout the biofilm is of importance. Generation of acidic metabolic waste, in particular in the anaerobic zones of a biofilm, tends to cause an acid stress [Stewart 2008]. To counterbalance this effect, a recent study demonstrates that *S. epidermidis* is able to produce ammonia by degradation of arginine thanks to a deiminase coded by the *arc* genes. Strains lacking the Arginine Catabolic Mobile Element (ACME), i.e. carrying the *arc* genes, formed significantly less viable biofilms due to a growth dependent pH drop [Lindgren 2014].

3.6 Persistence and persister cells

To survive in challenging environments, biofilms of *S. epidermidis* have put persistence at the core of their survival strategy. One of the main reasons for this increased persistence is the occurrence of dormant cells called persisters [Lewis 2005] [Schoenfelder 2010].

The existence of persister cells was highlighted in dose-response studies of antibiotics on bacterial populations [Bigger 1944]. They stochastically appear in planktonic cultures as well as in biofilms in various proportions depending on, among other things, growth stage, DNA damage and oxidative stress [Wu 2012]. Experiments have proven that the persister phenotype was reversible [Brooun 2000] [Keren 2004a]. It was also proposed that antibiotic tolerance exhibited by persisters was due to the combination of slow growth and expression of specific genes. These genes encode Multi-Drug Tolerance (MDT) proteins that interfere with the lethal action of bactericidal antibiotics after they bound their target [Keren 2004b]. MDT confers tolerance to multiple antibiotics since it inactivates a target often common to several bactericidal molecules.

In *E. coli*, toxin/anti-toxin (TA) systems were identified as responsible for the tolerance to multiple antibiotics [Lewis 2005]. A TA

system is composed of a toxin, and its antitoxin, usually less stable. When in excess, the toxin inhibits a cellular target (see Fig. 3.8 B (ii)). Examples of cellular target of TA systems are: DNA gyrase, mRNA, ribosome, ATP synthase. TA systems can be encoded on both plasmids and chromosomes. On plasmids, their primary function is to ensure partitioning of the plasmids in the daughter cells during host replication [Naito 1995] [Hayes 2003] [Cooper 2010]. Thanks to HGT, TA systems colonize an ever increasing number of bacterial species, notably via conjugation [Van Melderen 2009].

The occurrence of persister cells induced by TA systems can be attributed to a stochastic variation in toxin and antitoxin concentration in the cytosol [Cohen 2013]. Indeed, in persister cells, the concentration of the toxin compared to the antitoxin in their cytosol is higher (Fig. 3.8 C) [Gerdes 2012]. As a consequence, the toxin inactivates its cellular target, leading to slow growth. TA systems can induce tolerance to antibiotics if they compete for the inactivation of the same cellular target (see Fig. 3.8 B (iv)). In this case, persisters are protected from the action of cidal antibiotics, that cannot corrupt the cell function already inactivated by the TA toxin [Lewis 2010]. For example, aminoglycosides interrupt the translation of mRNA into proteins and generate misfolded peptides. These create pores in the cell membranes, leading to cell death. TA systems that inactivate the ribosomes prevent the action of aminoglycosides [Lewis 2005].

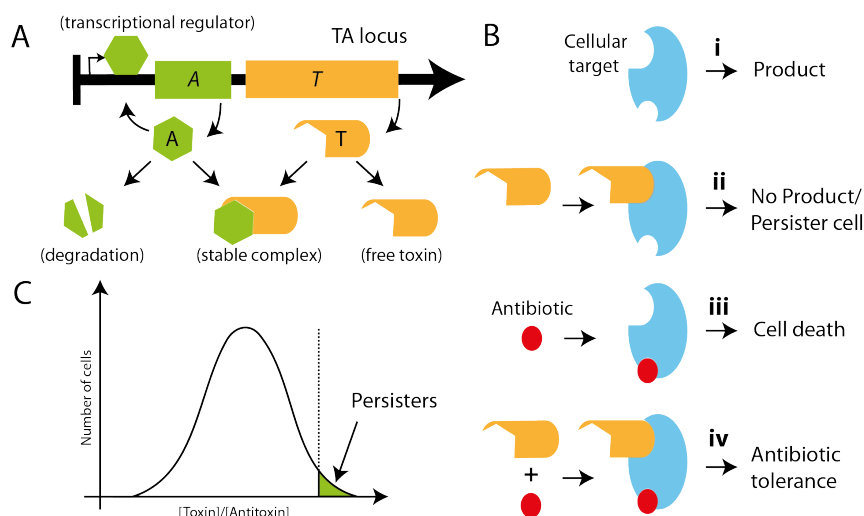


Figure 3.8: TA systems as a mechanism for persister cell formation. **A.** The TA locus, with the antitoxin gene (A) and the toxin gene (T). The antitoxin (hexagone) has three fates: be a transcriptional regulator the TA locus expression, bind the toxin to form an inactive complex or be degraded. The toxin can be either complexed by the antitoxin or left free. **B.** (i) The cellular target, i.e. ribosome, mRNA, DNA gyrase..., is working normally when there is no toxin bound to it. (ii) The toxin left free by a lack of antitoxin, binds the cellular target (blue oval) and induces persistence. (iii) The antibiotics bind a cellular target without previous modification by the toxin, the cell dies. (iv) The toxin is already bound to the cellular target, the antibiotic cannot corrupt its target, the cell is a persister. **C.** A schematic representation of the stochastic variability in the ratio of toxin and antitoxin concentration in a bacterial population. Persisters are characterized by higher values of this ratio, i.e. more toxin than antitoxin available, leading to the protection of specific cellular target from the corruptive action of antibiotics. Source : modified from [Lewis 2005].

In *S. epidermidis*, several Fst-like TA systems were identified [Kwong 2010]. Fst-like system belongs to a family of RNA-regulated toxins causing severe membrane stresses due to dramatic changes in the expression of transporters [Greenfield 2002]. The exact mechanism of action of Fst-like system is still largely unknown [Brinkman 2013]. Also, in *S. aureus*, the presence of RNA cleaving TA systems of the mazEF family has been spotted [Fu 2007]. The implications of TA systems in the presence of high levels of persisters in staphylococcal populations could not yet be demonstrated [Shapiro 2011]. Though,

based on evidences in *E. coli*, one can reasonably suppose that TA systems could induce the persister phenotype in Staphylococci.

Another type of mechanism related to MDT in *S. aureus*, is the generation of Small Colony Variants (SCV). SCV are so called because of the relatively small size of the colonies grown on non selective medium. They present phenotypic and pathogenic traits that differ from the parental strain [von Eiff 2008]. A majority of SCV carry mutations in *menD* and *hemB* genes, leaving them auxotrophic for menadione, hemin and thymidine biosynthesis [Bates 2003]. *S. epidermidis* is also able to produce SCVs, as confirmed by isolation from prosthetic joint infections [Maduka-Ezeh 2012]. Even though the antibiotic tolerance of SCV is usually higher than that of the parental strain, the mechanisms for drug tolerance by SCV are yet unknown [Lechner 2012].

There is still a controversy about the proportion of persisters in biofilms, especially whether this proportion is higher in biofilms than in planktonic cultures [Roberts 2005] [Lewis 2005]. Notwithstanding the quantity of persisters, the competitive advantage of persisters in biofilms lies in a supplementary protection provided by the EPS matrix. Indeed, as shown in Fig 3.9, where persisters of planktonic population that survived antibiotic treatment are mopped up by macrophages *in vivo*, those in biofilms avoid phagocytosis. When the bactericidal agent has faded away, the persisters in the biofilm can grow again. As a consequence, persister cells markedly improve the resilience of biofilms in the presence of antibacterial compounds, causing complications in the treatment of biofilm associated chronic infections [Otto 2008] [McCann 2008].

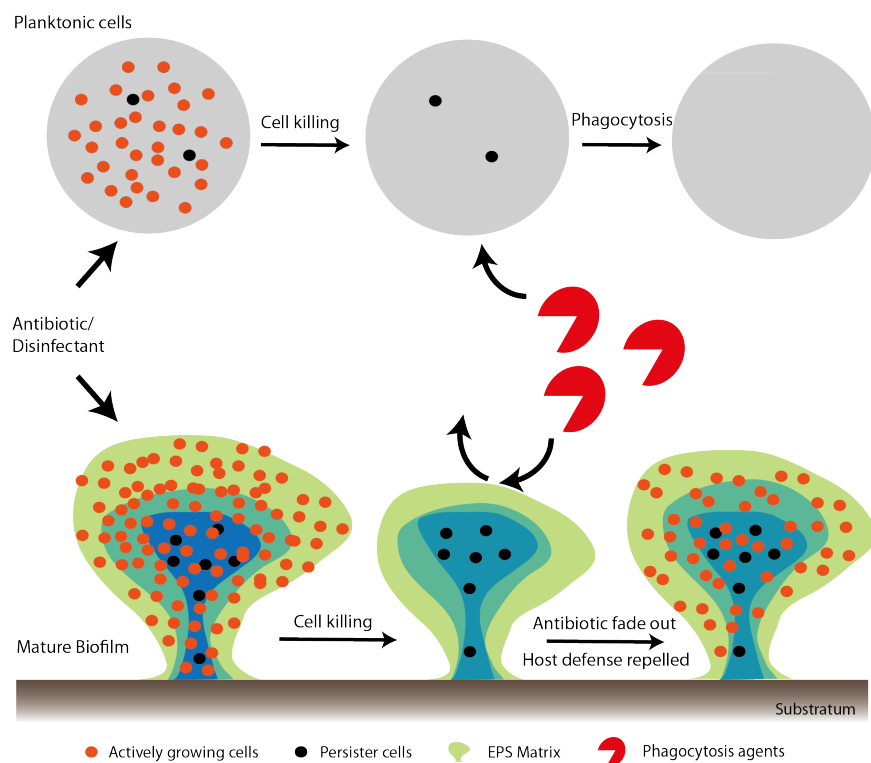


Figure 3.9: Role of persisters in biofilm resilience. The effect of bactericidal molecules on biofilms (bottom) and planktonic cells in liquid medium (gray circle at the top) are compared. Persisters (in black) are protected from phagocytosis by the EPS matrix of the biofilm where planktonic cells are vulnerable. Note : the layers (from greenish to blue) indicate the heterogeneity of conditions in the biofilm.

Strategies to control clinically relevant biofilms

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The prevalence of implanted device-related nosocomial infections increases every year. Nowadays, biofilms are involved in 65% of these infections [de la Fuente-Núñez 2013]. In most cases, the failure of antibiotics to cope with the infection leaves no choice but to proceed to the surgical removal of infected devices. This therapeutic dead-end has drawn the attention from the scientific community over the last two decades. In the quest for novel therapeutics strategies, two main directions are being pursued: the first is preventive by avoiding biofilm formation from the start, the second is curative by eradicating mature biofilms. Both approaches are complementary as to this day, there is no silver bullet to prevent or treat biofilm associated chronic infections. In this chapter, when relevant, the focus will be put on methods to prevent infections by *Staphylococci*.

4.1 Preventive strategies

So far, preventive approaches to tackle the biofilm issue have essentially focussed on two means. The first is to modify the physicochemical properties of the implantable materials in order to decrease adhesion and/or kill bacteria upon contact. The second is to make use of host natural defences, with appropriate vaccines, to target an infection in its early moments.

4.1.1 Modified surfaces

Modified biomaterials aim to reduce bacterial adhesion and/or kill bacteria as they come in contact with their surface. Of note, all the surface treatment applied to implantable materials should meet the requirements of biocompatibility, i.e. support host cell growth, and not be toxic to the host [Kohn 2004].

4.1.1.1 Anti-adhesive surfaces

The goal in designing anti-adhesive surface is to reduce the amount of attractive interactions between the surface itself and bacteria. The paragraphs here below present several examples of works that aimed to achieve bacteria-repellent coatings.

The simplest approach is to increase surface hydrophilicity. Some studies have used physical treatment such as plasma [Everaert 1998], others have used heparin to passivate the surface towards bacterial adhesion [Appelgren 1996]. The clinical success of this approach was, however, quite moderate, most probably due to the variety of mechanism that bacteria employ to achieve adhesion [Habash 1999]. Hence, polymer coating, if not covalently bound, can be displaced either by human biopolymers or excreted bacterial polymers. The properties of the modified surface can therefore be significantly altered [Rodrigues 2011]. Interestingly, in some cases, although adhesion decreased, biofilm formation *in vivo* was shown to be increased on modified biomaterial compared to be untreated control [Everaert 1998]

[MacKintosh 2006].

The main issue in the first approach was the adsorption of biopolymers, some of which facilitate bacterial adhesion. To prevent adsorption of biopolymers, another type of surfaces was proposed. They are coated with highly hydrated polymers such as PolyEthyleneGlycol (PEG) [Kingshott 2003], hydrophilic polyurethanes [Pavithra 2008] or polymer brushes, usually made of poloxamers (block copolymers) [Kaper 2003]. The effectiveness of such coatings may vary greatly according to the bacterial species involved [Hetrick 2006]. It was also noticed that this type of surface modifications only delay initial bacterial adhesion. Indeed, they tend to be gradually inactivated by a conditioning film of host proteins or dead bacteria over time [Bryers 2008].

In conclusion, these approaches to simply prevent bacterial adhesion with coatings proved to be effective *in vitro*, but they did not have the expected success in clinical trials [Habash 1999]. However, recent advances in coating technologies and surface modifications have helped to develop more reliable biomaterials, notably with respect to tissue integration [Richards 2012]. For example, a more recent trend relying on the control of surface topography at micro- and nano-scale in order to repel bacteria has been paid much attention to. The presence of surface features, their height, width and spacing were shown to be critical to prevent adhesion of bacteria and biofilm development [Graham 2014]. Notably, surfaces mimicking the topography of echinoderms and shark skin showed a pronounced inhibition of adhesion and biofilm formation by *P. aeruginosa*, *E. coli* and *S. aureus* [Epstein 2013].

4.1.1.2 Anti-microbial coatings

Since bacterial adhesion on implants *in vivo* could not be completely avoided, much efforts have been put in the design of biomaterials releasing or immobilizing anti-bacterial compounds. Several classes of anti-bacterial agents have been used to dope the surface of implants, including, among others : silver ions, antibiotics, anti-microbial pep-

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tides, quaternary ammonia, lactic acid and phages [Donlan 2009] [Rodrigues 2011]. Almost all the studies implementing this approach have also included a surface modification described in the previous section, in order to keep the anti-adhesive property.

Three possibilities have been explored to dope biomaterials with anti-bacterial agents. All three are presented in Fig. 4.1.

First, in most cases, these anti-bacterial molecules are released from the bulk of the biomaterial by simple diffusion towards the environment. It has been, for example, implemented for : PolyMethylMethAcrylate (PMMA) bone cement supplemented with antibiotic-filled beads [Webb 2007] and vascular grafts with an hydrogel coating soaked with ampicillin, gentamicin or cefazolin [Pritchard 2013], in which the hydrogel is essential to delay the release of antibiotics from minutes to days [Blanchemain 2005]. Nanoparticles have also been employed to increase surface/volume ratio and retention time of silver ions or to encapsulate antibiotics in implantable materials [Rai 2009] [Huh 2011]. The major drawback of the methods is that the passivation by a conditioning film of proteins inside the host tends to slow the diffusion process [Rodrigues 2011].

Second, biomaterials featuring a controlled release by progressive biodegradation of the implant polymer have been developed. This is notably the case with Poly Lactic Acid (PLA) coatings that are degraded by the host. Antibiotics concealed in the bulk of PLA are set free upon PLA dissolution. Such a strategy was proposed to coat Kirschner-wires, metallic pins used in bone surgery [Gollwitzer 2003].

A last possibility consists in the direct grafting of anti-bacterial compounds on the biomaterial. Grafted anti-bacterial agents perform as well as their non-grafted counterparts if the linker molecule that binds them to the substrate allows a sufficient mobility [Statz 2008]. Numerous studies have highlighted the *in vitro* efficacy of the technique.

For example, anti-bacterial properties coupled with enhanced tissue integration were observed with AMPs and arginine-glycine-aspartate (RGD) peptides covalently bound to Pluronic F127 (a commercially available poloxamer) grafted on biomaterials [Muszanska 2014]. Immobilization of ampetoids (synthetic polypeptides mimicking the effect of AMP but with increased resistance to proteases [Chongsiriwatana 2008]) on TiO_2 surface has proven efficient in preventing biofilm development while preserving good tissue integration properties [Statz 2008].

A special mention should be made to biosurfactants as they display both anti-adhesive and anti-microbial properties [Muthusamy 2008]. They can be glycolipids, peptides, phospholipids, fatty acids, lipoproteins and are synthesized by various bacterial species including: *B. subtilis*, *Lactobacillus* spp., *Acinetobacter calcoaceticus*, *P. aeruginosa* and *E. coli*. [Rodrigues 2011]. Their potential use in commercial implantable devices has been demonstrated for several applications. For example, urethral catheters pre-coated with surfactin, a biosurfactant produced by *B. subtilis*, were found to reduce biofilm formation of *S. enterica* and *E. coli* as model uropathogens [Mireles 2001]. In another study, a surfactant of *Lactobacillus paracasei* showed anti-adhesive and anti-microbial activity against *S. aureus* and *S. epidermidis* among others [Gudiña 2010]. Although the potential for biomedical applications of biosurfactants has been largely demonstrated, three factors explain the absence of commercial products on the market: the methods to bind them to surfaces are not yet optimal, cost of large scale production are still too high and toxicity towards mammalian cells requires further investigation [Rodrigues 2011].

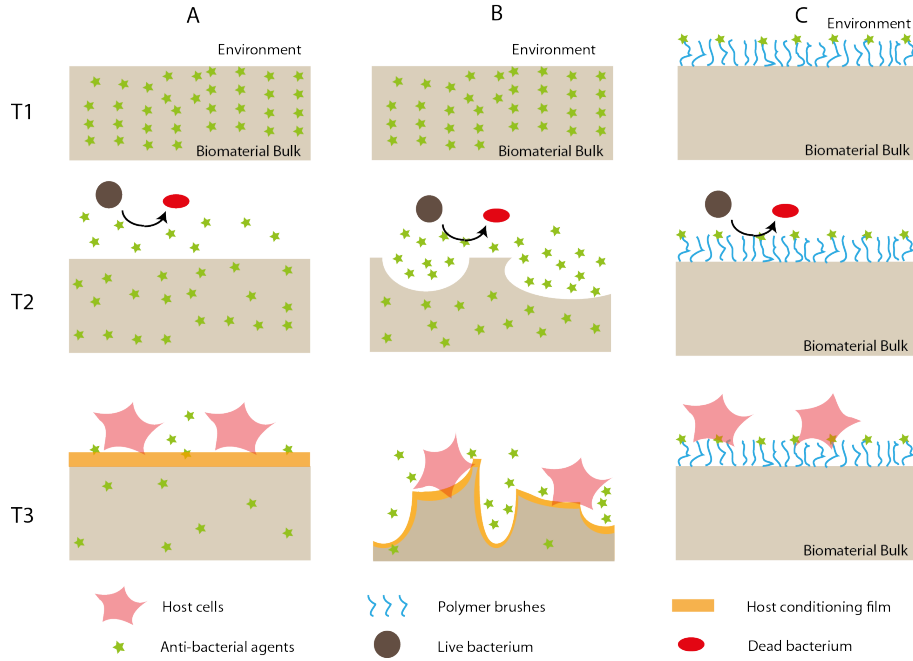


Figure 4.1: The three types of anti-bacterial agents releasing biomaterials. Time scales are: **T1** corresponds to the initial state of the biomaterial, before implantation, **T2** is shortly after biomaterial implantation and **T3** is hours to days after implant insertion in the body. **A.** Implant material is soaked in anti-bacterial agent, upon implantation, they are progressively released in the environment. When bulk concentration decreases an a conditioning film forms, diffusion is less pronounced. **B.** Implant is made of biodegradable biomaterial, i.e. PLA, filled with anti-bacterial agents. PLA is slowly degraded inside the host, releasing the trapped anti-bacterial molecules. **C.** Polymer brushes coat the biomaterial, some of them carry anti-bacterial agents at their non surface-bound end. Bacteria are repelled by polymer brushes and kill by contact with anti-bacterial molecules. These surfaces are still compatible with host tissue integration.

4.1.2 Immunotherapy

Make use of the strength of the immune system by means of immunotherapy to combat infections at an early stage is appealing. Although plenty of work has been done to identify potential epitopes expressed at the surface of bacteria in the course of infections, there is currently no immunotherapy approved to combat Staphylococcal infections [Kiedrowski 2011]. Yet, several therapies are still in clinical

and preclinical trials, some others have failed to pass the tests or proved to be inefficient [Jansen 2013].

Amongst the products admitted in clinical trials, two kinds are to be distinguished. First, the vaccines that trigger the production of antibody directed at a certain target, known as active immunization. Second, the therapies that stimulate the humoral response and bring a dose of non-host produced IgGs, also called passive immunization [Ohlsen 2010]. Both methods, though meant to be used for distinct classes of patients, target the same following compounds expressed at the staphylococcal surface *in vivo*: the PNAG, the capsular polymer Poly Glutamic Acid (PGA), MSCRAMMs, LipoTeichoic Acids (LTA), the iron surface determinant and toxins [Burlak 2007] [Ohlsen 2010]. Notably, an attempt to conceive a whole cell vaccine is at the phase I of clinical trials [Jansen 2013].

The major issues in creating effective vaccines to prevent staphylococcal infections are the following. Firstly, not all antibodies have the same capacity to induce the phagocytosis of bacterial cells by neutrophils (opsonosis). An accurate method exists to assess this feature *in vitro* (i.e. OpsonoPhagocytosis Assay (OPA), an assay to measure the rate of bacterial killing), but it is currently under used, probably due to its complexity [Nanra 2013]. Secondly, the expression of virulence factors by Staphylococci is highly dependent on the environment they colonize, hence the intraspecies variability in virulence can be great. Therefore, single antigens are often unable to provide effective host protection against invading bacteria [Ohlsen 2010]. Finally, the patients may react to vaccines in various ways, that clearly affect the quality of the protection. Typically, age, fitness and lifestyle of individuals may have a great impact. In addition, people more at risk for *S. epidermidis* infections usually are immunodeficient therefore limiting the options for active immunization. All these factors should be taken into account in the design of novel vaccine formulations [Jansen 2013].

4.2 Curative strategies

Despite the tremendous research efforts being made to develop preventive strategies, many hurdles are to be crossed before they reach the market and could be used on patients. As most probably none of them will turn out to be 100 % effective, there is a need for curative strategies that are able to tackle biofilm infections. This section reviews the currently used therapeutic strategies and the prospective leads.

4.2.1 Antibiotics

Staphylococci in biofilms are highly resistant and/or tolerant to various antibiotics. Owing to the presence of persisters, the relatively slow metabolism, the surface bound state [Muszanska 2012] and the frequent occurrence of resistance genes, MBEC of an antibiotic molecule can be up to 100 times higher than the Minimal Inhibitory Concentration (MIC) established for planktonic cells. That was shown for several clinically relevant antibiotics including among others: rifampicin, tigecyclin, vancomycin and ciprofloxacin [Molina-Manso 2012]. In addition to the high rates of survival, sub-inhibitory concentrations of certain antibiotics induce a cascade of genetic response that, in part, increases the production of PNAG [Rachid 2000] and therefore favors the transcription of *aap* and *atlE* [Reiter 2014]. These considerations have led to reconsider the paradigm of the single antibiotic therapy.

Although some antibiotics, such as daptomycin, seem to remain active against mature biofilms of Staphylococci [García 2010a] [Bauer 2013], the tendency is now to combine antibiotics with either other antibiotics, or adjuvants that sensitize bacteria in biofilms [Cushnie 2014] [Brooks 2014] [Beloin 2014]. These approaches aim to take advantage of synergistic effects of two compounds to increase bactericidal activity in biofilms [Gill 2015]. Also, coupling two or more antibiotics should limit the appearance of resistant mutants. Recent works have shown that combinatorial anti-biofilm treatment are promising. For example, the use of peptide 1018 in combination with ciprofloxacin has

proved efficient to enhance killing of *P. aeruginosa* in biofilms and decrease dispersal [Reffuveille 2014]. Other approaches, though not particularly focused on biofilm resistance, could prove useful to increase the sensitivity of biofilm bacteria towards antibiotics. These are notably the use of membrane permeabilizers or efflux pump inhibitors [Gill 2015].

4.2.2 Antimicrobial peptides

AMPs are 15 to 30 amino acids long peptides synthesized by numerous organisms, including crustaceans, insects, plants, bacteria, fungi and amphibians [Jorge 2012], to protect themselves from invasion of bacterial pathogens [Rossi 2008]. Their amphipathic properties allow them to selectively permeabilize bacterial membranes, inducing leakage, depolarization and death [Shai 2002] [Beckloff 2007]. Known resistance to AMPs is relatively rare, which makes them good candidates to be used as anti-infective therapeutic agents [Hancock 2006] [Sang 2008]. Recently, synthetic versions of AMPs called ampetoids, either mimetic or non mimetic, have been shown to be more resistant to proteolysis and as efficient at lysing bacterial cells as the natural ones [Chongsiriwatana 2008]. The still largely untapped potential for novel natural AMPs discovery and synthesis of effective peptoids is about to be explored thanks to bioinformatic tools [Jorge 2012].

An abundant literature covering the prophylactic use of AMPs as a pre-treatment to prevent biofilm formation is available. Yet, reports of the use of AMPs or ampetoids to control mature biofilms are relatively recent. This effect was observed on *S. epidermidis*, *S. aureus* and *P. aeruginosa* biofilms with both natural AMPs [Shi 2009] [Molhoek 2011] [Kharidia 2011] and synthetic ampetoids [Flemming 2009] [Kapoor 2011] [Liu 2013]. Two general mechanisms of action have been proposed. In the first place, AMPs and ampetoids disrupt the stability of the EPS matrix, therefore fragilizing the biofilm [Kapoor 2011]. Secondly, they are able to interfere with the expression of biofilm formation related genes by binding DNA [Beckloff 2007]

[Dean 2011].

Biofilms may however display tolerance and resistance to AMP and synthetic peptoids. As far as tolerance is concerned, the EPS matrix plays a key role. Indeed, it is able to significantly slow down the diffusion of such molecules inside the community, notably thanks to the addition of charged moieties to EPS [Folkesson 2008]. Active mechanisms such as AMP sensing and efflux pumps also participate to the protection of the bacterial cell against AMPs [Li 2007] [Otto 2009]. Finally, resistance may occur due to mutations in intracellular targets of AMPs [Herbert 2007].

Although the preliminary results of AMPs as anti-biofilm agents are promising, several difficulties lie in the path to their commercialization as approved therapies [Jorge 2012]. First, their production is expensive and fastidious. Then, post AMP treatment effects are still largely unknown, both regarding the response of host (e.g. immunity) and of the biofilm. Indeed, the response of the biofilm to AMP is not yet well understood [Jorge 2012].

4.2.3 Quorum sensing or quorum quenching

The influence of QS on the development of biofilms was developed in Section 3.5.2. Quorum Sensing Inhibitors (QSI), or Quorum Quenching (QQ), interfere with QS mechanism [Hoiby 2011] at various stages of the process: production of the AI molecules, sequestrations of AIs or binding to the QS receptors without triggering the expected response. Just as AMPs, QSI are synthesized by a wide variety of organisms [Kalia 2013]. The possible application of QSI to combat *P. aeruginosa* and *S. aureus* biofilms in chronic infections has been proposed [Bjarnsholt 2005] [Kiran 2008] [Njoroge 2009]. In general, inhibition of QS induce a physiological shift towards less virulence and impaired biofilm formation [Kalia 2013].

In *Staphylococci*, successful eradication of biofilms were performed by activating Agr QS *in vitro* [Lauderdale 2010] [Wang 2011]. Proof of concept for combinatorial therapies in which QSI act as potentiators of antibiotics have also been successfully performed. This was the case for three QQ molecules (biaryl hydroxyketones) that promoted the healing of wound infected with MRSA in biofilms on animal models. These molecules could also significantly sensitize MRSA to nafcillin and cephalothin [Kuo 2015]. However, *in vivo*, this strategy might have thus some undesirable consequences: the release of planktonic cells *in vivo* and the expression of virulence factors (for both *S. aureus* and *S. epidermidis*) [Yarwood 2003] [Boles 2008].

4.2.4 Nanoantibiotics

Advances in nanotechnologies have made possible to fabricate NanoParticles (NPs) with potent antimicrobial properties [Huh 2011]. Several types of NPs have been designed and can act according to different mechanisms including, among others, the local production of Reactive Oxygen Species (ROS), protein damage, DNA mutagenesis or membrane leakage [Hajipour 2012].

Treating biofilms with NPs can be made easier by modifying their surfaces in order to increase the on-site delivery of antibiotics. For example, vancomycin loaded cationic liposomes displaying high affinity for *S. epidermidis* biofilms, mainly through non-specific electrostatic interactions and hydrogen bonding, could deliver high doses of vancomycin to the infection site [Sanderson 1996]. Also, immunoliposomes, coated with molecules targeting biofilm components, proved efficient at selective delivery of chlorhexidine to *Streptococcus oralis* biofilms [Forier 2014]. Finally, nano-encapsulated antibiotics displayed increased activity against biofilms of *P. aeruginosa* in chronic infections in lungs compared to classical inhaling therapies [Hadinoto 2014]. The difference comes from the better penetration of antibiotic loaded NPs inside the mucus and the biofilm EPS [Forier 2013]. In addition, since NPs are not recognized as foreign bodies, they are not rapidly cleared, resulting

in an improved retention of antibiotics in close vicinity of the biofilm [Weers 2009].

Though very effective and promising for the treatment of biofilms formed by multi-drug resistant bacteria, the toxicity of antimicrobial NPs in humans should be carefully considered. Potential adverse side effects can largely outweigh the benefits of using such a technology to therapeutic ends [El-Ansary 2013]. In particular, some NPs can successfully cross the blood-brain barrier, a very rare and dangerous property with regard to neurotoxicity [Hu 2010].

4.2.5 Enzymes

The production of exoenzymes is one of the key mechanisms by which bacteria favor dispersal of planktonic cells from biofilms [Lister 2014]. The same strategy can be employed by using enzymes as anti-biofilm agents, enabling for the dissolution of the matrix of EPS [Beloin 2014].

Amongst these enzymes, DNases, polysaccharidases and proteases are good candidates to degrade biofilms. For example, pulmozyme®, a commercial product, contains recombinant human DNase I (rhDNase) as a main active principle and is able to reduce mucus viscosity due to *P. aeruginosa* biofilms in cystic fibrosis patients [Frederiksen 2006]. The same type of rhDNase is also able to inhibit biofilm formation by *S. aureus* [Kaplan 2012]. Dispersin B, a PNAG degrading enzyme isolated from *Actinobacillus actinomycescomitans*, could significantly degrade the matrix of staphylococcal biofilms, of which PNAG is a major compound [Kaplan 2004] [Boles 2011]. Also, dispersin B in combination with other antimicrobial compounds showed synergistic effect on the killing of *S. aureus* compared to the bactericidal molecule alone [Darouiche 2009]. Finally, proteases have been reported to remove biofilms, although, their activity was clearly dependent on the composition of the biofilm [Chaignon 2007]. Interestingly however, a recent work have highlighted that Esp, a serine protease secreted by *S. epidermidis* could inhibit and degrade mature *S. aureus* biofilms [Iwase 2010]. It

might be one of the strategies developed by *S. epidermidis* to hamper the colonization of ecological niches by *S. aureus*.

Another class of enzymes, lysins, also known as Peptidoglycan (PG) hydrolases, is of interest in the struggle to combat bacterial biofilms. Lysins comprise bacterial lysins (exolysins, autolysins, lysozymes) and endolysins from phages. Four mechanistic classes of PG bond cleavage are distinguished: glycosidases, endopeptidases, specific amidohydrolases, and lytic transglycosylases [Nelson 2012]. Gram-negative lysins show a globular structure and a molecular weight of around 20 kDa [Briers 2007] whereas Gram-positive lysins typically display a modular architecture with one to three domains and a higher molecular weight [Díaz 1990]. Functional domains of lysins can include a Cell-wall Binding Domain (CBD) recognizing various motifs at the cell surface, and one or more of the enzymatic activities mentioned above.

For Staphylococci, generic PG composition and hydrolase activities of characterized lysins are schematically represented in Fig. 4.2.

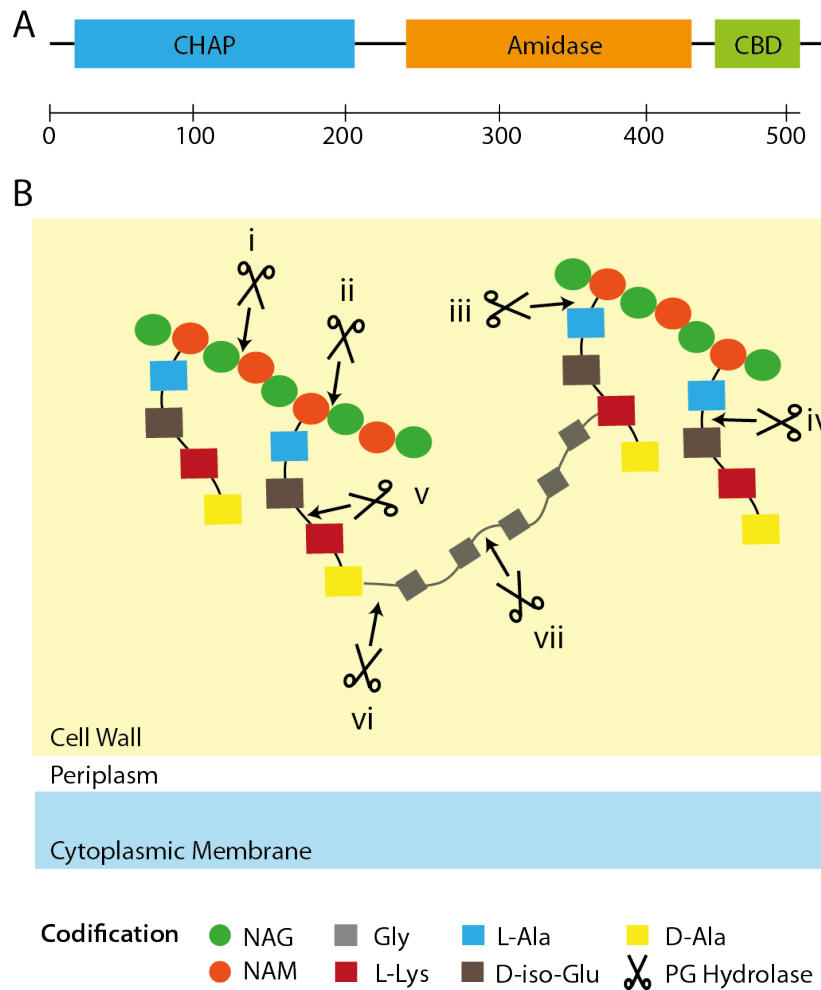


Figure 4.2: Structure of staphylococcal lysins and their PG cleavage sites. **A.** Typical modular structure of staphylococcal PG hydrolases. The scale indicates the length of the protein, in amino acids. The three domains are represented as an example are: a CHAP (standing for Cysteine Histidine dependent aminohydrolase/peptidase), an amidase and a CBD, usually from the SH3-5 family. It is notably the structure of LysK, and several other phage lysins. **B.** Cleavage sites of PG hydrolases in the staphylococcal cell wall. NAM stands for N-acetyl-muramic acid, and NAG for N-acetyl glucosamine. **i.** A N-acetyl glucosaminidase cleaves at the reducing end of NAG. **ii.** Cleavage between NAM and NAG at the reducing end of NAM. This activity is a N-acetyl muramidase typical of lysozymes. Of note, lytic transglycosidases cleave the same bond but follow a different mechanistic way [Nelson 2012]. **iii.** An N-acetylmuramoyl-L-alanine amidase cuts the bond between the glycan and peptide chains. **iv to vii.** Peptide bonds are cleaved by endopeptidase with defined specificities. Source : inspired from [Nelson 2012]

PG hydrolases have the property to lyse bacterial cells from the outside, as it was highlighted in 1940 by Delbruck [Delbrück 1940]. Indeed, when added exogeneously to the medium, lysins degrade PG of Gram-positive cells which lyse due to the large difference in osmotic pressure (up to 50 atm.) of their cytosol compared to the environment [Seltmann 2002] [Fischetti 2006]. For Gram-negative bacteria, lysis from outside is complicated by the presence of the outer membrane, which protects the PG from the lysins action. Lysis would occur when lysins act in synergy with surfactants or chelators to disrupt the outer membrane integrity [Vaara 1992] [Briers 2011].

The use of lysins as anti-microbials is appealing since they generally display a near-species specificity, rarely broader. Indeed, the fine chemical structure of PG presents variations from one bacterial species to another, affecting the susceptibility of the concerned species. Notably, the number and type of amino acids in the side chain can vary, the peptide bridge can connect peptide chains together at various sites and the composition of glycan chains is variable from one species to another [Schleifer 1972] [Vollmer 2008]. As a result, specific species can be targeted by using a lysin as bactericidal agent [Nakimbugwe 2006], avoiding the disadvantages of the usual broad range antibiotics [Nelson 2012].

Concerning the resistance of bacteria to lysis by PG hydrolases, phage endolysins should be distinguished from other lysins. Indeed, several mechanisms of resistance have been identified for bacterial lysins and lysozymes. For lysostaphin, a bacterial exolysin produced by *Staphylococcus simulans*, three resistance mechanisms have been discovered. The first involves the *lif* gene or immunity factor from *S. simulans* that protects the host from the lytic action of its lysin [DeHart 1995] [Thumm 1997]. Then mutations in *femA*, a factor essential to methicillin resistance in *S. aureus*, were shown to imply modifications in the mucopeptides leading to increased resistance towards lysostaphin [Sugai 1997] [Climo 2001]. Finally, *lyrA*, a lysostaphin resistance gene, was pointed out to mediated lysostaphin resistance, even though

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purified PG from resistant strains was still sensitive to lysostaphin. This suggests that *lyrA* acts by affecting the cell envelope without modifying the PG chemical structure [Gründling 2006]. Similarly to lysostaphin, resistance to lysozymes has been reported to happen through modifications in the glycan backbone of PG [Davis 2011]. On the contrary, there is so far no report of mutants resistant to phage endolysins [Nelson 2012]. Two studies have tried to submit bacterial populations to sublethal doses of endolysins but did not observe any change in susceptibility afterwards [Fischetti 2005]. It is hypothesized that the bacteria-phage co-evolution has driven the endolysins to target strongly conserved PG bonds [Nelson 2012].

During the last decade, a few studies have shown that both bacterial and phage lysins were able to eradicate staphylococcal biofilms. For example, phage endolysins $\Phi 11$ and LysK, composed of a CBD, an endopeptidase and an amidase, dissolved *S. aureus* biofilms *in vitro* in static models [O’Flaherty 2005] [Sass 2007]. Lysostaphin proved to be as effective on *S. epidermidis* and *S. aureus* biofilms *in vitro* and on catheters in infected mice *in vivo* [Walencka 2005] [Kokai-Kun 2009]. More recently, similar results were obtained with a chimeric lysin [Yang 2014]. These examples demonstrate the potential of lysins to play a role in the control of biofilms, mainly thanks to their ability to rapidly lyse slow growing cells [Donlan 2008].

The potency of lysostaphin and phage endolysins to combat staphylococcal infections has also been shown *in vivo*. For example, 50 μg of LysGH15, a lysin isolated from a staphylococcal phage named GH15, was able to protect mice from bacteremia following the intraperitoneal injection of a lethal dose of MRSA [Gu 2011]. Lysostaphin has been tested in *in vivo* models to cure burn wounds, ocular infections, nasal colonization [Nelson 2012]. Additionally, studies focusing on the treatment of aortic valve endocarditis in rabbits have obtained promising results [Climo 1998] [Patron 1999].

However, the clinical use of lysins, either topically or intravascularly, requires a more advanced knowledge of the potential adverse effects such as allergies and anaphylactic shock and more long term effects on the kidneys and liver [Climo 1998]. Antibodies inactivating the lysins can also be generated by the host. To test this hypothesis, studies were conducted *in vitro* to assess the activity of lysins in presence of IgGs. For Cpl-1, a pneumococcal endolysin, the activity of the enzyme was slowed but still present, indicating that inactivation by IgGs was not complete [Fischetti 2005]. The same enzyme was used *in vivo* for the treatment of mice with bacteremia [Loeffler 2003] or meningitis [Grandgirard 2008] and the animals showed no signs of adverse effects while bacterial titers were significantly reduced [Nelson 2012]. The same was noticed for lysostaphin in the treatment of bacteremia by intravenous injection [Schaffner 1967] [Climo 1998].

Altogether, these results indicate that an enzymatic treatment of recalcitrant staphylococcal infections should be possible in a not so far future. It is especially interesting since PG hydrolases have displayed synergies with other lysins and with some other anti-microbial agents. For example, lysostaphin and LysK (phage K lysin) lyse MRSA more efficiently in combination [Becker 2008], β -lactams display improved killing of MRSA when lysostaphin is present [Kiri 2002], and finally LysH5 a staphylococcal phage lysin acts in synergy with nisin to eradicate *S. aureus* in milk [García 2010b].

Objectives

Chronic foreign body infections associated with the development of biofilms represent a real threat to public health and a major burden for health care systems. *S. epidermidis*, formerly considered as a harmless commensal, now widely recognized as opportunistic pathogen is the main aetiological agent of these infections. The core of its infective strategy lies in the formation of a mature, persistent biofilm. Therefore, understanding how the environment is challenging *S. epidermidis* in its biofilm formation process, from adhesion to dispersal, is key to improve clinical management of such infections.

This study aims to get more insights on the impact of the environment on the biofilm formation process of *S. epidermidis* as well as to challenge mature biofilms with lytic and EPS-degrading enzymes.

The emphasis will be first set on understanding the complex dynamics of *S. epidermidis* biofilm formation. The particular roles of SdrG (Fg binding cell surface protein) in *S. epidermidis* adhesion to surfaces will be investigated. The dependence of bacterial aggregation on the ionic strength will also be addressed.

In line with the increasing public health concern about biofilm related infections, two strategies will be explored to prevent and challenge biofilms. First, an innovative method for the detection of surface bound bacteria by means of electrical sensing will be evaluated. Secondly, in the quest for novel therapeutic strategies to combat mature biofilms, *S. epidermidis* biofilms will be challenged *in vitro* with cocktails of lytic and matrix degrading enzymes.

Part II

Results

Scientific strategy

To achieve the goals of this study stated in the Objectives, several state-of-the-art techniques have been used. This Chapter presents an overview of these methods, notably AFM and impedance spectroscopy.

6.1 Atomic Force Microscopy (AFM) on living cells

AFM is a high spatial resolution technique that gives access to information such as: the topography or the elasticity of a sample. The working principle is shown in Fig. 6.1. Briefly, a tip (of about 20 nm wide) connected to a cantilever scans the surface of a sample. The deflection of the cantilever in z is measured through the reflection of a Lazer beam that is sensed by a photodiode. The piezoscanner on which the sample is mounted controls the x,y,z positions. Finally, a computer handles the feed-back loop between the deflection of the cantilever and the movement of the piezoscanner.

Scanning samples can be performed either in contact mode, in which the tip encounters the sample, that is the case for rheology assessment, or in tapping mode, in which there is very limited contact between tip and sample, mostly used for topographical or imaging studies [Alessandrini 2005].

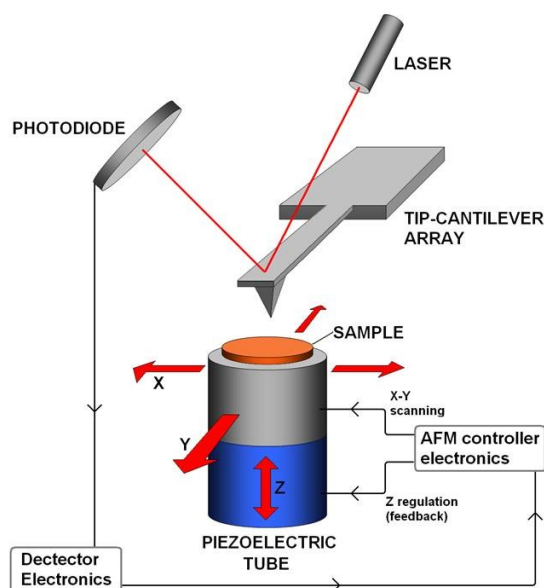


Figure 6.1: Schematic working principle of AFM. The deflection of the cantilever upon approach of the sample surface is sensed by the photodiode (the laser direction is changing). A feedback loop is operated between the piezoscanner that holds the sample and the photodiode signal. Source : <http://pharm.virginia.edu/facilities/atomic-force-microscope-afm/>

AFM has been applied to various fields such as food technology (study of polymer rheology, or interfacial phenomena) [Liu 2011] or materials science [Magonov 1997] but also importantly in biophysics [Alessandrini 2005]. The application of AFM to the imaging of live cells has yielded interesting findings on the organization of cell membranes and the structure of cell walls [Dufrene 1999] [Dufrêne 2008].

In this study, two applications of the AFM were used : the Single Molecule Force Spectroscopy (SMFS), also known as chemical force microscopy and the Single Cell Force Spectroscopy (SCFS). These techniques allow to measure the forces involved in the interactions of two or a few molecules. The magnitude of the forces usually range from dozens of picoNewton (pN) for weak forces to a couple of nanoNewton (nN) for

strong bonds. The next sections are dedicated to the presentation of SMFS and SCFS in further details.

6.1.1 Single Molecule Force Spectroscopy (SMFS)

In the SMFS setup, the AFM tip is chemically modified to harbor a defined chemistry or a single molecule. Sample scanning, in this case the surface of a bacterium, is usually performed in contact mode. The tip is brought in contact with the live bacterium and upon its retraction, the force that retains the tip to the cell surface is recorded. Fig. 6.2 displays an example of SMFS with a Fibrinogen modified tip, as used in this work (Chapter 8).

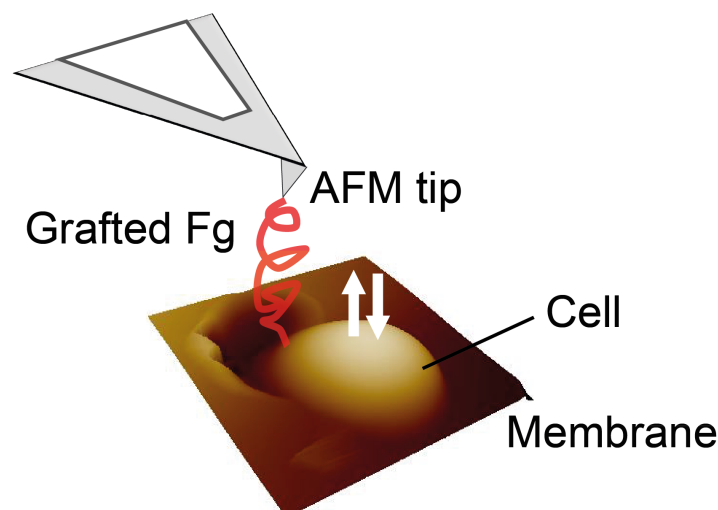


Figure 6.2: Example of SMFS in which a Fibrinogen modified AFM tip scans the surface of a live bacterium trapped in a membrane. Credit: P. Herman 2014.

This technique can be performed in wet conditions, which allow to collect information on chemical interaction of a living cell with a defined molecule in liquid. However, mastering the chemistry of AFM might reveal challenging. In this work, SMFS was appropriate to obtain a cartography of the presence of SdrG, a cell surface adhesin of *S. epidermidis*, on living bacteria.

6.1.2 Single Cell Force Spectroscopy (SCFS)

In this particular application of the AFM, a single cell is attached on a tip-less cantilever (Fig. 6.3). A colloidal bead is preferred to the tip in order to facilitate the binding of the bacterial cell. Attachment of the bacterium is performed with polydopamine, known as a biological glue, in order to make sure the cell remains bound to the colloid while scanning. Like SMFS, SCFS can be performed in liquid, a tremendous advantage to study bacterium-substrate interactions in relevant natural conditions. In this work (Chapter 8), SCFS was used to assess the binding of single cells to various silicone substrates, some of which were coated by proteins, notably Fibrinogen.

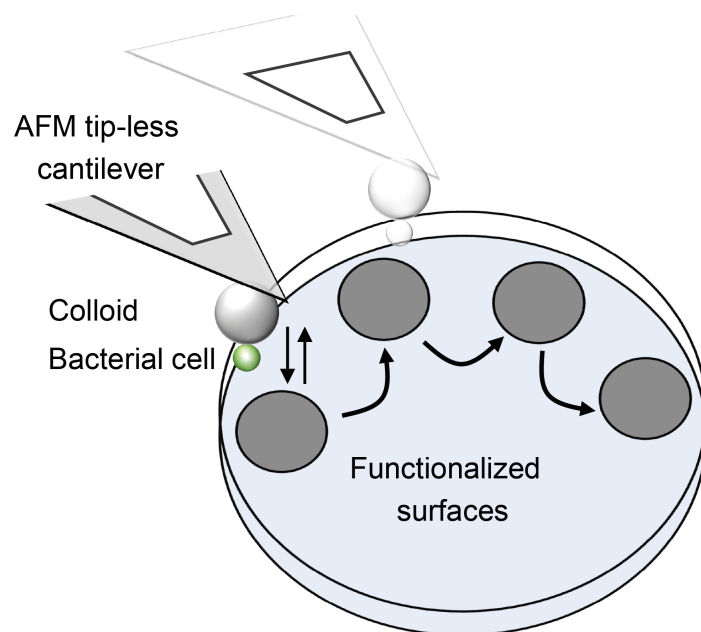


Figure 6.3: Representation of SCFS work flow. A single bacterial cell shown in green is attached to a colloid that bridges the gap to the AFM cantilever. The cantilever brings the bacterial cell in contact with the surfaces represented by gray disks then retracts the cell and the adhesion force is recorded. The procedure is repeated more than 100 times for each surface. Credit: P. Herman 2014.

6.2 Impedance spectroscopy to detect bacteria

In this work (Chapter 9), a method to selectively detect whole cell bacteria has been developed. It is based on impedance spectroscopy. The next paragraphs briefly describe the other biosensing methods available to date and why our choice was oriented towards impedimetric sensing of bacteria.

A wide variety of biosensors tailored for bacterial detection exist. The type of sensing can be: optical (using bioluminescence, fluorescence or colorimetry), acoustical, mechanical (e.g. affinity-based deflection of coated cantilevers), magnetic or electrical (potentiometry or charge sensing) [Lazcka 2007] [Su 2011] [Hasan 2014]. Amongst all these methods, impedimetric sensing was chosen for three main reasons. First and most importantly, impedimetric sensing presents the opportunity for advanced miniaturization and integration. Indeed, microelectrodes used in impedance spectroscopy can be made by using classical micro-fabrication techniques, also used in the production of microprocessors for computers [Varshney 2009]. Second, impedimetric sensors perform well in the detection of whole cell bacteria, especially when coupled to microfluidics, removing the need for extensive sample preparation [Yang 2008]. Finally, contrary to other sensing methods that use labels to increase the signal, impedance spectroscopy allows a direct, label free detection of bacteria [Tsouti 2011].

Amongst impedimetric biosensing techniques, two main classes can be distinguished. Some detect transient effect such as saline bursts originating from cell lysis (change in medium resistivity), whereas others detect a stable effect originating from the dielectric properties of bacterial cells. In this second category, the detection principle is based on the shift in impedance occurring at defined range of frequencies (of the electrical signal) when a bacterium happens to be in the interelectrode space. This shift is the bacterial impedance, Z_b (see Fig. 6.4 for the electrical circuit of label-free impedance spectroscopy). Z_b arises from the difference in permittivity of the bacterial cytosol compared to the

external medium and from the presence of a complex double layer of membrane and cell wall around the bacterial cell. That defines Z_b , the impedance of the bacterial cell. The measurement of Z_b is optimal at high frequencies (i.e. around 1 MHz or higher), where the signal of Z_b is much more pronounced compared to possible contamination such as dust or aggregates of polymers.

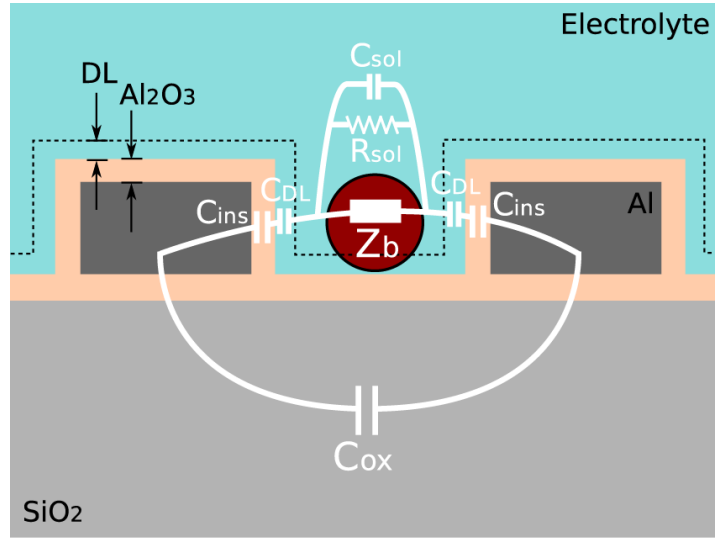


Figure 6.4: Schematic representation of the electrical circuit of microelectrodes, patterned on a SiO_2 wafer, for the sensing of whole cell bacteria. The red-filled circle is a bacterium, DL means Double-Layer, C accounts for Capacitance, R for resistance. Credit: N. Couniot 2014.

In this work, the method used is called capacitive sensing. Interdigitated electrodes have been employed because their design was shown to be adequate for bacterial detection [Tsouti 2011]. The measurements of impedance are performed in picoFarads (pF), relating to a change in the capacity or resistivity of the medium in between the interdigitated electrodes. The presence of bacterial cells in the interelectrode space increases the capacitance of the circuit (by addition of the Z_b contribution) and an increase in impedance (pF).

Diversity in *S. epidermidis*

Aim

In this Chapter, the characterization of a collection of *S. epidermidis* strains originating from the environment, the hospital and directly from patients suffering from an infection is presented. The goal was to highlight the genetic diversity related to the biofilm forming ability of *S. epidermidis*. A total of 16 genes or genetic determinants known to be involved in biofilm formation by *S. epidermidis* according to [Otto 2009] were selected. Their presence or absence in the strains of the collection was tested by PCR. Clustering methods were then used to analyse the dataset and to highlight the main trends in the results.

Material and methods

Strain collection

A total of 134 *S. epidermidis* strains were collected. Fifty were isolated from the environment, thirty six from clinical settings (i.e. various spots in hospital rooms : sinks, bed frame, tablets) and 46 are clinical isolates, originating from infected patient samples. Strains from patients admitted in Saint-Luc hospital (Woluwe, Belgium) were isolated from blood cultures (26 strains) and the 20 strains from Mont-Godinne university hospital (Godinne, Belgium) were collected by swabbing of infected wounds, catheters or samples drawn from patients. The two remaining were ATCC reference strains : ATCC 12228 and ATCC 35984. The strains were identified as *S. epidermidis* by 16S DNA sequencing or by MALDI-TOF IVD identification (Bruker).

Detection of biofilm related genes

The presence or absence of 16 genes or loci in the strains of the collection was assessed by PCR. Dilutions of DNA previously extracted using classical phenol-chloroform protocols was used as template. Table 7.1 displays the primer pairs used in this study. PCR were performed according to the protocols described in the referenced works. Table 7.2 summarizes the cycle that was used to perform the PCR with the primers designed in this study.

Clustering analysis

The data collected from the PCR were then used to build a matrix of 1 and 0, corresponding to presence and absence of the genes. A dendrogram was constructed with a hierarchical clustering method using the Ward method to compute distance between clusters in JMP (SAS).

Table 7.1: Primers used to detect the presence of biofilm related genes in the collection of *S. epidermidis* strains.

Gene	Primers (5' - 3')	Source
<i>aap</i>	F -TCACTAAACAACCTGTTGACGAA R -TTGATTTTATATCTGTTGAATGC	[Monk 2007]
<i>embp</i>	F -AGCGGTACAAATGTCAAT R -AGAAGTGCTCTAGCATCATCC	[Rohde 2004]
<i>atlE</i>	F -CAACTGCTCAACCGAGAACA R -TTTGTAGATGTTGTGCCCCA	[Rohde 2004]
<i>sdhG</i>	F -CTACAAAGTTCAGGTCAAGGACAAGG R -GCGTCGGCGTATATCCTTCAG	[Rohde 2004]
<i>IS256</i>	F -TGAAAAGCGAAGAGATTCAAAAGC R -ATGTAGGTCCATAAAGAACGGC	[Rohde 2004]
<i>IS257</i>	F -ACGTTCAATCAATCAACGGTC R -AGTGTTGCGCTTAACCTTGCTAG	[Rohde 2004]
<i>bhp</i>	F -ATGGTATTAGCAAGCTCTCAGCTGG R -AGGGTTTCCATCTGGATCCG	[Rohde 2004]
<i>aae</i>	F -CAGGGATCCGCAACAACGCATACAGTAAAAGTGG R -GTCCCTGCAGCTTAATGAATAAATTTGTAATTTCTCAC	[Heilmann 2003]
<i>psmB1</i>	F -TTAGCAGAAGCTATTGCAAATACA R -TTAGAAACCGAAGATTTTACCTAATACGCT	[Wang 2010]
<i>sepA</i>	F -AGAAAGCAGCTACTACAGGTACAGGTA R -TGGATTAGACATACTTCTTAAGGCATCTCCGC	[Wang 2010]
<i>icaADB</i>	F-TTATCAATGCCGCGAGTTGTC R -GTTTAAACGCGAGTGCGGTAT	[Frebourg 2000]

Table B.1 (*continued*): Primers used to detect the presence of biofilm related genes in the collection of *S. epidermidis* strains.

Gene	Primers (5' - 3')	Source
<i>sdrF</i>	F -CAATGGTTCTTCAACAGCACAGG R -CTCTAAACCTGTGAATTCATATT	This study
<i>capA</i>	F -GTGATACGAGAGACAAAGATAGC R -CAATCTCATTGACTACGACG	This study
<i>agrI</i>	F -GCTGCAACCAAGAAACAACC R -CGTGTATTCATAATATGCTTCGATT	[Li 2004]
<i>agrII</i>	F -TATGCAAGCCAAGCACTTG R -GTGCGAAAGCCGATAACAAT	[Li 2004]
<i>agrIII</i>	F -CCTTGGCTAGTACTACACCTTC R -GTGCTTGGCTTGCATAAACA	[Li 2004]

Table 7.2: PCR cycles used for primer pairs designed in this study.

Cycles	Temperature (°C)	Duration	Step
1 x	95	5 min	Denaturation
30 x	94	30 sec	Denaturation
	55	30 sec	Hybridization
	72	1 min	Elongation
1 x	72	7 min	Final elongation

Results and discussion

The result of the clustering is shown in Fig. 7.1. A decomposition in 7 clusters was justified by a breaking point in the distance graph shown at the bottom of Fig. 7.1. Clusters I, II and III are composed only of environmental isolates strains and are rather remote to the rest of the strains. Cluster IV regroups mainly isolates from surfaces in hospital rooms and also contains few environmental strains. In cluster V, a majority of environmental strains can be found along with 7 clinical isolates. Strikingly, clusters VI and VII solely contain strains originating from hospitals or directly from patients.

Of note, the *mecA* (methicillin resistance) gene strongly discriminates clinical strains from environmental isolates. Close to 90 % of strains isolated from patients samples carried *mecA*, while less than 10 % of environmental strains did. About 30 % of the strains originating from surfaces in hospital rooms carried at least a copy of the *mecA* gene. These data, in line with previous reports [Schoenfelder 2010] [den Heijer 2013], show that exposure to patients within healthcare facilities increases the likelihood for a bacterium to collect the *mecA* gene, either by conjugation with other bacteria, by transformation or by transduction with phages carrying the methicillin resistance gene.

The distribution of the strains in the clusters supports the hypothesis of the effect of a strong selective pressure in healthcare facilities. To a lesser extent, the strains isolated from infected patients appear to cluster apart from the majority of the strains collected from inert surfaces in hospitals, indicating that dwelling in patients body accentuates the need for bacteria to keep, maintain or acquire biofilm-related genes [Schoenfelder 2010].

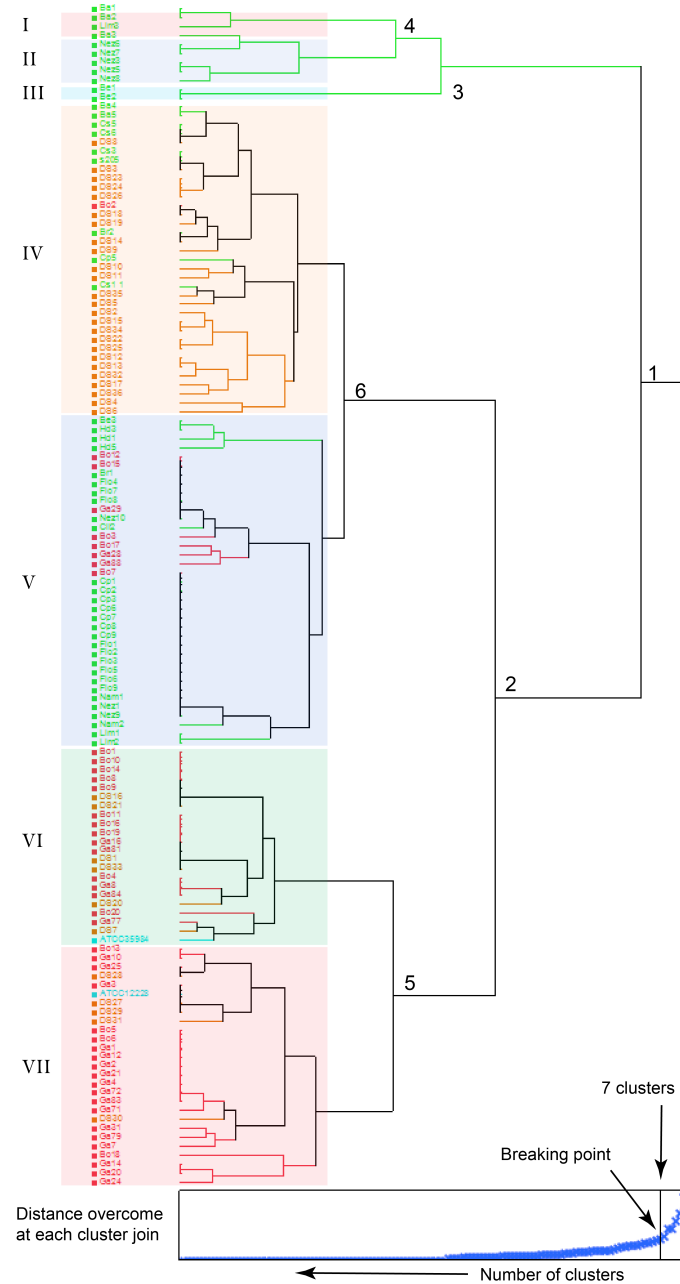


Figure 7.1: Dendrogram constructed from the PCR data of presence or absence of biofilm related genes in *S. epidermidis* strains. The colors indicate the origin of the strains: green is environmental, blue is reference (ATCC), orange is hospital surfaces and finally red is from patients. Numbers in capital indicate the number of the 7 clusters. Number in arabic show the branching events. The plot at the bottom of the figure represents the distance overcome at each cluster join. Clustering begins from the right and goes to left. A breaking point is clearly visible after 7 cluster joins, meaning that further clustering would not be relevant.

Adhesion of *S. epidermidis* to surfaces

Contents

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In this Chapter, two comprehensive studies of the process of *S. epidermidis* adhesion to Fg-coated surfaces are presented. The focus was set on SdrG, a cell surface adhesin, mainly responsible for the recognition and the attachment of *S. epidermidis* to the plasma protein Fg.

8.1 Role played by SdrG in *S. epidermidis* attachment to Fibrinogen coated surfaces

In this section, the force with which *S. epidermidis* strains bind fibrinogen (Fg) coated surfaces was assessed using an AFM approach called Single Cell Force Spectroscopy. To this end, two isogenic strains, HB and HB Δ SdrG have been used in order to verify that the observed forces could indeed be attributed to the SdrG-Fg interaction. It was shown that the so-called signature of the AFM retraction curves is typical of a multiple domain protein unfolding with, at last, a strong rupture force.

This work brings biophysical insights into the ligand-receptor binding of Fg and SdrG. Although the stabilisation of the ligand through a "dock, lock and latch" mechanism was discovered in 2003, a quantification of the force uniting SdrG and Fg was lacking. The fact that the extent of this force is unusually large supports the hypothesis that SdrG is a key asset for *S. epidermidis* to anchors on surfaces in its human host.

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Forces driving the attachment of *Staphylococcus epidermidis* to fibrinogen-coated surfaces

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My contribution to this work consisted of punctual help with experiments, biological interpretation of results and manuscript proof-reading.

Abstract

Cell surface proteins of bacteria play essential roles in mediating the attachment of pathogens to host tissues and, therefore, represent key targets for anti-adhesion therapy. In the opportunistic pathogen *S. epidermidis*, the adhesion protein SdrG mediates attachment of bacteria to the blood plasma protein fibrinogen (Fg) through a binding mechanism that is not yet fully understood. We report the direct measurement of the forces driving the adhesion of *S. epidermidis* to Fg-coated substrates using single cell force spectroscopy. We found that the *S. epidermidis*-Fg adhesion force is of around 150 pN magnitude and that the adhesion strength and adhesion probability strongly increase with the interaction time, suggesting that the adhesion process involves time-dependent conformational changes. Control experiments with mutant bacteria lacking SdrG and substrates coated with the Fg β 6-20 peptide, instead of the full Fg protein, demonstrate that these force signatures originate from the rupture of specific bonds between SdrG and its peptide ligand. Collectively, our results are consistent with a dynamic, multi-step ligand-binding mechanism called : dock, lock, and latch.

Introduction

S. epidermidis is a bacterial commensal of humans that colonizes the skin. It is a common cause of infections associated with indwelling medical devices [1] [2] [3]. Survival in the infected host is promoted by the ability of bacteria to attach to the surface of indwelling devices that have been conditioned with host plasma proteins, such as fibrinogen (Fg) and fibronectin. *S. epidermidis* expresses on its surface a protein of the microbial surface component recognizing adhesive matrix molecule (MSCRAMM) family called SdrG [4] [5], which binds to the β -chain of Fg by the "dock, lock, and latch" mechanism [6] [7]. The N-terminal ligand-binding A region is linked to the bacterial cell by a flexible stalk comprising a variable number of repeats of the dipeptide Ser-Asp. At the C-terminus, a sorting signal promotes covalent attachment of the SdrG protein to cell-wall peptidoglycan. SdrG binds to a short, unfolded peptide near the N-terminus of the Fg β -chain (residues 6-20) [7]. The peptide docks into a trench located between the separately folded subdomains N2 and N3 of region A. Once docked and stabilized

by hydrophobic interactions and hydrogen bonds, a C-terminal extension of subdomain N3 undergoes a conformational change and folds over the inserted Fg peptide to lock it in place and finally complements a β -sheet in subdomain N2, forming the latch. Despite a detailed molecular understanding of ligand binding by SdrG and other staphylococcal MSCRAMMs gained from *in vitro* experiments with purified proteins, little is known about the forces that govern attachment to and detachment from ligand-coated surfaces. While classical microbiological assays probe large ensembles of cells, single-cell microbiology uses advanced technologies to analyze single-cell heterogeneity and to reveal rare events and properties that were otherwise not accessible [8] [9] [10]. Among these new tools, atomic force microscopy (AFM) makes it possible to measure cell surface interactions at the single-molecule and single-cell levels [10] [11] [12] [13] [14] [15] [16]. Several protocols have been developed to attach bacterial cells onto AFM cantilevers to measure bacterial adhesion forces, including the use of chemical fixation [17], electrostatic interactions [18], and polydopamine adhesives [19]. Here, we combine the use of colloidal probes and polydopamine as a new AFM-based single-cell force spectroscopy (SCFS) approach, enabling us to quantify the specific forces driving the adhesion of *S. epidermidis* to Fg-coated substrates. The results show that the interaction between *S. epidermidis* and Fg involves essentially specific bonds between SdrG and its β 6-20 peptide ligand and that the interaction force strengthens with time, consistent with the "dock, lock, and latch" mechanism.

Materials and Methods

Bacterial cultures

We used *S. epidermidis* ATCC 12228 cells grown at 37 °C and 150 rpm in Tryptic Soy Broth (TSB), unless otherwise stated. Control experiments were performed with *S. epidermidis* HB strain, in which SdrG expression was impaired by gene disruption after pG+Host9'fbc plasmid integration in the *fbc* (SdrG) gene (SdrG(-)) [4]. This strain was cultivated in TSB broth supplemented with 10 $\mu\text{g mL}^{-1}$ erythromycin (Sigma, E5389). The cells were harvested in the stationary phase (16-18 h) by centrifugation for 10 min at 7500 x g and washed 3 times in phosphate-buffered saline (PBS) buffer. For cell probe preparation, 50 μL of a suspension of ca. 1×10^6 cells was transferred into a glass Petri dish.

Bacterial Cell Probes

Using a Nanoscope VIII Multimode atomic force microscope (Bruker Corporation, Santa Barbara, CA), triangular shaped tipless cantilevers (NP-O10, Microlevers, Veeco Metrology Group) were slowly immersed in a very thin layer of ultraviolet (UV)-curable glue (NOA 63, Norland Edmund Optics) spread on a glass slide and slowly brought into contact with a silica microsphere (6.1 μm diameter, Bangs Laboratories). After 3 min of contact, the colloidal probe was cured for 10 min under a UV lamp. The cantilever was then immersed for 1 h in a 10 mM Tris buffer + 150 mM NaCl solution (pH 8.5) containing 4 mg mL⁻¹ dopamine hydrochloride (99%, Sigma). The probe was then washed and dried under N₂. Proper attachment and positioning of bacteria on the colloidal probe were achieved using a BioScope Catalyst (Bruker Corporation, Santa Barbara, CA) equipped with a Zeiss Axio Observer Z1 and a Hamamatsu camera C10600. To check the viability of the bacteria, a LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen, Kit L7012) was used. Prior to attachment, 2 μL of a 1:1 Syto 9 (green-fluorescent nucleic acid stain)/propidium iodide (red-fluorescent nuclear and chromosome counterstain) mixture at 1.5 mM was added to a drop of 50 μL of bacteria suspension and mixed thoroughly. The suspension was deposited in the glass bottom Petri dish, where the protein model surfaces were previously attached, and the bacteria were left to incubate with the dyes for 15 min in the dark. Sedimented bacteria and Fg substrates were immersed by the addition of 4 mL of PBS buffer in the Petri dish. The colloidal probe was then mounted into the atomic force microscope and brought into contact with an isolated bacterium. After confirmation of proper attachment of the cell by fluorescence imaging, the cell probe was positioned over the Fg substrates without dewetting.

[

]Fg and β 6-20 Peptide Substrates To prepare Fg-coated substrates, clean glass coverslips coated with a thin layer of gold were immersed overnight in an ethanol solution containing 1 mM 10% 16-mercaptododecahexanoic acid/90% 1-mercapto-1-undecanol (Sigma), rinsed with ethanol, and dried with N₂. Substrates were then immersed for 30 min into a solution containing 10 mg mL⁻¹ Nhydroxysuccinimide (NHS) and 25 mg mL⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma), rinsed 5 times with Ultrapure water (ELGA LabWater), incubated with 0.2 mg mL⁻¹ of Fg for 1 h, rinsed further with PBS buffer, and then immedi-

ately used.

In a control experiment, β 6-20 peptide substrates were prepared using the same protocol, except that Fg was replaced by the β 6-20 peptide NEEGFFSARGHRPLD (Eurogentec).

Force Measurements

AFM measurements were performed at room temperature (20 °C) in PBS buffer at pH 7.4 using a BioScope Catalyst AFM (Bruker AXS Corporation, Santa Barbara, CA). Using the inverted optical microscope, the bacterial probe was engaged onto the substrates. Multiple force curves were recorded on three different spots using a maximum applied force of 250 pN, a contact time of 50 ms or 1 s, and constant approach and retraction speeds of 1000 nm s⁻¹. For each condition, at least three bacteria from independent cultures were probed. Optical imaging was used during the measurements to ensure that the cells remained properly positioned on the cantilever.

Results

SCFS Setup

To probe the SdrG-Fg interaction on a single-cell basis, we combined the use of colloidal probe cantilevers and a bioinspired polydopamine wet adhesive (Fig. 1a) [20]. The polydopamine-coated colloidal probes enabled us to attach single bacterial cells on AFM cantilevers without altering their viability (Fig. 1b). Using these cellular probes, we measured the forces between single *S. epidermidis* cells and Fg surfaces. Topographic imaging showed that the morphology of Fg-coated substrata was homogeneous and stable upon repeated scanning, indicating strong attachment of the macromolecules (Fig. 1c). Imaging a small area at a large force removed the Fg layer and enabled us to assess its thickness, approx. 4 nm (Fig. 1c; see square in the center of the image).

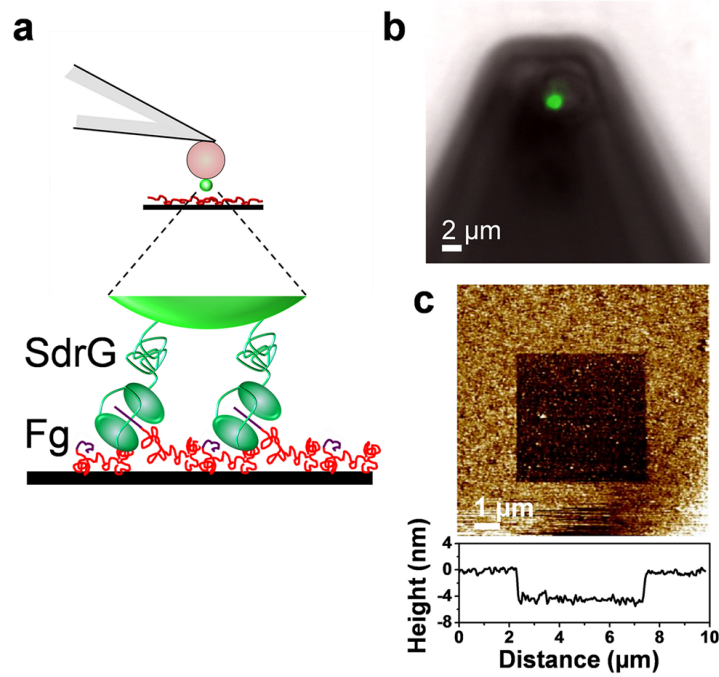


Figure 1: Single-bacterial cell force spectroscopy. (a) Living cells from the pathogen *S. epidermidis* are picked up with a polydopamine-coated colloidal probe, and the forces between individual bacteria and Fg-coated substrates are measured. The enlarged view (bottom) emphasizes specific bonds between the cell adhesion protein SdrG (green) and Fg (red). Two IgG-like subdomains of the SdrG molecule bind to a peptide sequence of 14 amino acids found in the N-terminal β chain of Fg (violet), through a putative dynamic multi-step ligand binding mechanism. (b) Use of an integrated AFM-inverted optical microscope shows that single *S. epidermidis* bacteria attached to the cantilever probes are properly located and alive (green color). (c) AFM height image (z scale = 10 nm; a vertical cross-section taken in the center of the image is shown beneath the image) recorded with a silicon nitride tip documenting the presence of a smooth, homogeneous layer of Fg molecules covalently attached to carboxylterminated surfaces. To determine the layer thickness (approx. 4 nm), a small square area was first scanned at large forces (>10 nN), followed by recording a larger image of the same area under smaller forces.

Measuring the Adhesion Forces between *S. epidermidis* and Fg

Multiple force-distance curves were recorded between single bacterial cells and Fg substrates. Fig. 2 shows the adhesion force and rupture length histograms, together with representative force curves, obtained at short contact time (50 ms) for three different cells from independent cultures. About 35% of the curves showed well-defined force peaks of 145 ± 27 pN magnitude and 25-250 nm rupture length (cells 1 and 2). The general features of the curves did not substantially change when recording consecutive force curves (up to several hundreds) on different spots, indicating that force measurements did not alter the interacting surfaces. While cells from independent cultures generally yielded adhesion properties that were in the same range (cells 1 and 2), some cells featured lower adhesion frequency (cell 3), an effect that we attribute to heterogeneity of the bacterial population.

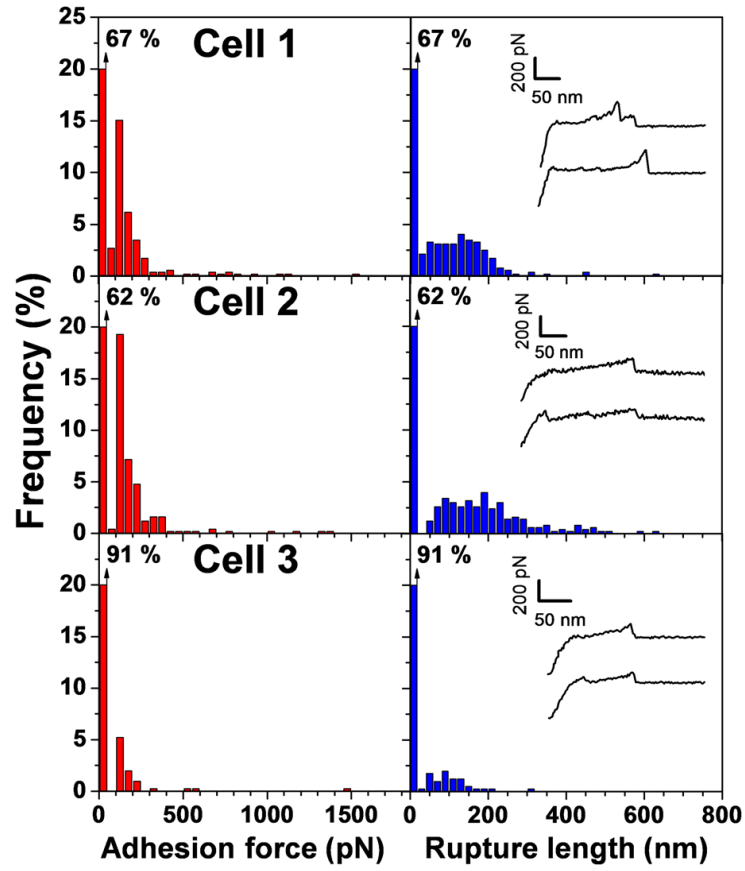


Figure 2: Measuring the adhesion forces between *S. epidermidis* and Fg. Adhesion force (red) and rupture length (blue) histograms, as well as representative retraction force curves (insets), obtained by recording multiple force curves in buffer at short contact time (50 ms) between single *S. epidermidis* cells and Fg substrates. Numbers listed in the top left corners of the graphs represent the percentages of zero adhesion forces. Results from three cells from independent cultures are shown ($n > 400$ force-distance curves for each cell).

In the light of several independent controls (see Fig. 3 and text below), we suggest that the measured force signatures reflect the rupture of single (or few) SdrG-Fg complexes. The approx. 150 pN binding force is larger than the value obtained at fairly comparable loading rates (around 60 000 pN s⁻¹) for other cell adhesion molecules, such as cadherins [21] and other bacterial adhesins [22] [23] including staphylococcal adhesins [24] which suggests that multiple receptor-ligand complexes are probed in parallel or that the SdrG-Fg bond is particularly strong. Adhesion force peaks could not be fitted with the worm-likechain(WLC) model, which usually describes the unfolding of protein secondary structures [25] [26]. This, together with the fact that multiple peaks were rarely observed, could mean that the adjacent subdomains containing IgG-like folds of SdrG are too stable to be unraveled one by one.

Notably, we found that increasing the contact time to 1 s (3) substantially changed the characteristics of the force profiles. First, a dramatic increase in adhesion frequency was noted (from 35 to 97%), suggesting that the probability to form SdrG-Fg bonds increases with the interaction time as observed for other receptor-ligand bonds [21] [22]. Second, the mean adhesion force generally increased from 145 to 250-1500 pN (cells 1 and 3). Some cells (cell 2) however did not show such a large increase in adhesion, suggesting again that the cell population was heterogeneous. Nevertheless, the observed time dependency may reflect the time necessary for conformational changes within the SdrG and Fg molecules to achieve optimal fitting. Specifically, the increased adhesion force is consistent with the "dock, lock, and latch" mechanism, which is thought to stabilize the SdrG-Fg bond. This model involves docking of the ligand in a pocket formed between two SdrG subdomains followed by the movement of a C-terminal extension of one subdomain to cover the ligand and to insert and complement a β -sheet in a neighboring subdomain [6]. Hence, our results suggest that the increased adhesion may originate from an increased number of SdrG-Fg bonds, as observed with animal cells [27] and/or from enhanced stability of the bonds.

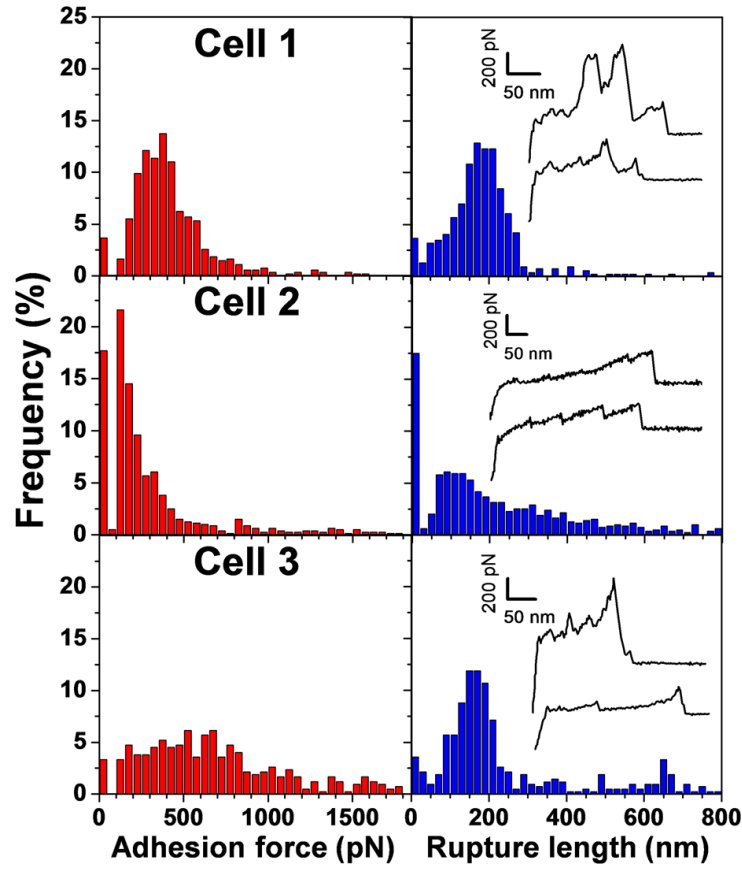


Figure 3: Adhesion forces depend upon contact time. Adhesion force (red) and rupture length (blue) histograms, as well as representative retraction force curves (insets), obtained by recording multiple force curves in buffer at prolonged contact time (1 s) between single *S. epidermidis* cells and Fg substrates. Results from three cells from independent cultures are shown ($n > 400$ force-distance curves for each cell). Cells 1-3 are the same as those probed at short contact time (Fig. 2).

Interaction Forces Originate from the Specific Adhesion between SdrG and Its Fg $\beta 6-20$ Peptide Ligand.

To determine the specificity of the measured adhesion forces and rule out the possibility of artifacts associated with the cell probe preparation, several control experiments were performed using a 1 s contact time (Fig. 4). First, use of polydopamine-coated probes instead of bacterial probes led to a major reduction of adhesion frequency (down to 60%; Fig. 4a), indicating that the adhesive events measured above were associated with

the *S. epidermidis* surface and not the polydopamine adhesive. Second, treatment of the cells with free Fg molecules led to a lower adhesion frequency (from 90 to 76%), suggesting that blocking of the SdrG molecules inhibits specific SdrG-Fg bonds. However, the observed inhibition was moderate, which could mean that Fg molecules on the cell surface may bind Fg on the substrate or that other cell surface molecules mediate adhesion (Fig. 4b). Third, use of a mutant *S. epidermidis* impaired in SdrG production (SdrG(-)) abolished most adhesion events (adhesion frequency of only 10%), providing a direct demonstration that the measured adhesion forces are indeed associated with specific SdrG-Fg bonds (Fig. 4c). Fourth, instead of the full Fg molecule, we tested a short synthetic peptide (β 6-20) corresponding to the SdrG binding site in Fg.6 Force curves recorded between WT *S. epidermidis* and a substrate covered with β 6-20 peptides featured force signatures that were similar to those obtained with the full proteins, thus confirming the specific nature of the probed interactions (Fig. 4d).

Accordingly, these observations demonstrate that the *S. epidermidis*-Fg interaction forces measured here originate from the specific adhesion between SdrG and its Fg β 6-20 peptide ligand.

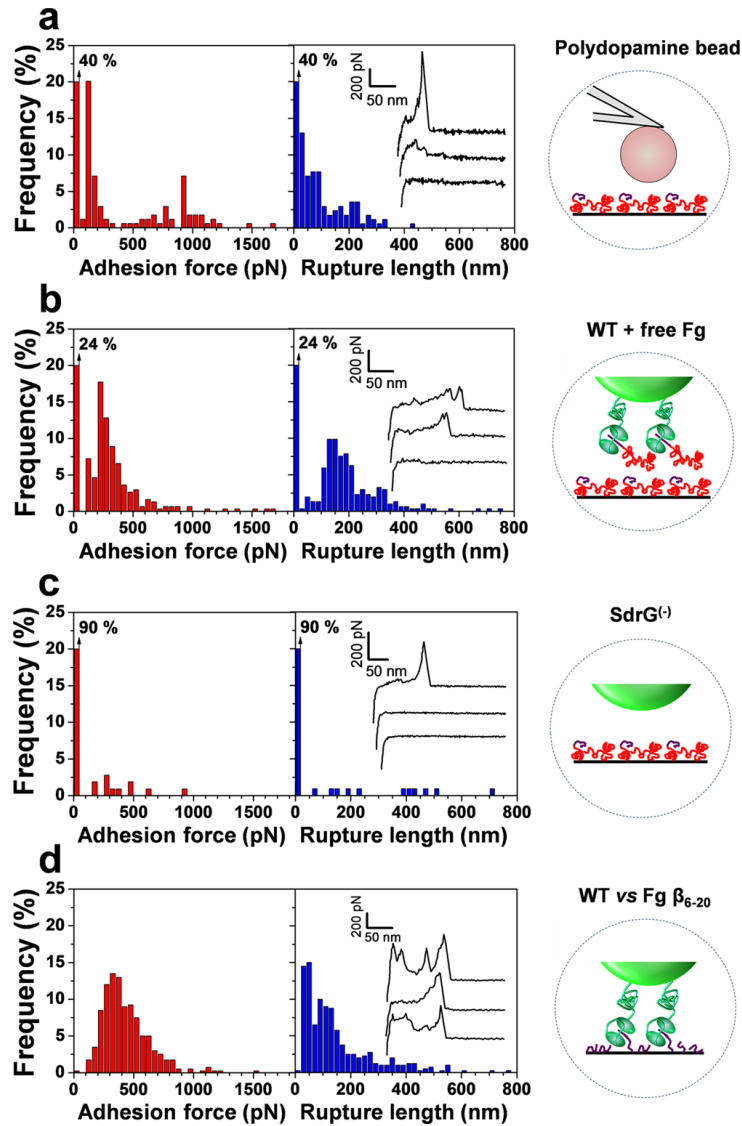


Figure 4: *S. epidermidis*-Fg adhesion forces involve specific bonds between SdrG and its peptide ligand. (a-d) Adhesion force and rupture length histograms, together with representative force curves, obtained by recording force curves at prolonged contact time (1 s) (a) between polydopamine-coated probes and Fg substrates, (b) between WT *S. epidermidis* cells and Fg substrates in the presence of free Fg (0.1 mg mL^{-1}) after 1 h of incubation, (c) between *S. epidermidis* mutant cells impaired in SdrG expression and Fg substrates, and (d) between WT *S. epidermidis* cells and substrates covered with the Fg β_{6-20} peptide. For each condition, similar data were obtained in duplicate experiments.

Conclusion

Knowledge of the fundamental forces driving the adhesion of Staphylococci to host cells and matrix components is critical to our understanding of the molecular bases of staphylococcal infections. In recent years, AFM techniques have provided quantitative information on the fundamental forces driving the adhesion of bacterial pathogens, including Staphylococci. In particular, the use of force spectroscopy with cell probe cantilevers has enabled researchers to measure the adhesion forces of *Pseudomonas aeruginosa* pili [15], to probe the interaction forces of *Candida parapsilosis* and *P. aeruginosa* to surfaces [12], to investigate the effect of cranberry on *Escherichia coli* adhesion [14], to study the influence of substrate properties on the adhesion of *S. epidermidis* [11], and to quantify the adhesion forces between *S. epidermidis* [13] or *S. aureus* [16] [28] and fibronectin. These results however are generally difficult to interpret at the molecular level for several reasons: cells were attached on the cantilever using protocols that lead to cell surface denaturation or cell death; multiple cells were attached and probed together; and cell positioning and cell-substrate contact area were poorly controlled.

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8.2 Cell surface abundance of SdrG modulates the affinity of *S. epidermidis* for Fibrinogen-coated surfaces

In this section, the role of the abundance of the SdrG adhesin on the cell surface of *S. epidermidis* was investigated. Previous works have described the mechanism by which SdrG is able to bind to Fg, alongside with the force involved in this binding event. These results established a correlation between the density of SdrG exhibited on the cell envelope and the ability of single cells as well as whole bacterial populations to adhere to Fg-coated substrates.

The results presented in this section establish that not only the presence but also the abundance of SdrG on the bacterial cell surface matter as it comes to adherence on a Fg coated substrate. This work highlights the need for future studies on the pathway of *fbe* expression to understand the factors influencing the density of active SdrG on the cell envelope.

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***Staphylococcus epidermidis* affinity for Fibrinogen-coated surfaces correlates with the abundance of the SdrG adhesin on the cell surface**

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Abstract

S. epidermidis is a world leading pathogen in health-care facilities, mainly causing medical device-associated infections. These nosocomial diseases often result in complications such as bacteremia, fibrosis or peritonitis. The virulence of *S. epidermidis* relies on its ability to colonize surfaces and develop thereupon in the form of biofilms. Bacterial adherence on biomaterials, usually covered with plasma proteins after implantation, is a critical step leading to biofilm infections. The cell surface protein SdrG mediates adhesion of *S. epidermidis* to Fibrinogen (Fg) through a specific "dock, lock and latch" mechanism, which results in greatly stabilized protein-ligand complexes. Here, we combine single-molecule, single-cell and whole population assays to investigate the extent to which the surface density of SdrG determines the ability of *S. epidermidis* clinical strains HB, ATCC 35984 and ATCC 12228 to bind to Fg-coated surfaces. Strains that showed enhanced adhesion on Fg-coated Poly-DiMethylSiloxane (PDMS) were characterized by increased amounts of SdrG proteins on the cell surface, as observed by single-molecule analysis. Consistent with previous reports showing increased expression of SdrG following *in vivo* exposure, this work provides direct evidence that abundance of SdrG on the cell surface of *S. epidermidis* strains dramatically improves their ability to bind to Fg-coated implanted medical devices.

Introduction

The opportunistic pathogen *S. epidermidis* is the main etiological agent of infections associated with implanted medical devices, especially vascular and urinary catheters [1]. In the US, infections involving *S. epidermidis* are estimated to reach 200.000 cases per year [2]. Most often, these diseases occur in a chronic form owing to the development of a biofilm on the implanted material [3] [4]. The treatment of chronic infections is complicated by the intrinsic resistance of bacteria or tolerance of biofilms to antibiotics, resulting in a significant burden for public health systems [5] [6].

Bacterial contamination of implanted devices in patients can happen during surgery or while the device is being used to inject drugs to or withdraw biological fluids from patients [7]. Adhesion of bacteria

on the surface of biomaterials will determine their ability to start an infection. In the human body, surfaces of medical devices tend to be readily coated by host factors upon contact with biological fluids. On its cell surface, *S. epidermidis* possesses a group of receptors, referred to as MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules), that specifically bind to human proteins such as Fg, Fibronectin (Fn) and Collagen (Cn) [8] [9] [10]. One of these MSCRAMMs is SdrG, a protein belonging to the Serine Aspartate Repeat family (Sdr), anchored to the cell wall by a LPXTG motif. SdrG mediates binding to Fg with a high affinity [11]. A sequence of 14 amino acids at the N-terminus of Fg is specifically recognized by SdrG according to a specific "dock, lock and latch" mechanism [12]. In this model, the target peptidic sequence is first trapped in between the two main domains of SdrG (N2 and N3), then, an arm of SdrG covers the peptidic sequence of Fg to secure the interaction. Recently, SdrG was found to bind to Fg with a force of 2 nN, which is remarkably strong [13]. It was also shown that contact time between the two protein influences the rate of efficient SdrG-Fg binding and consequently the fate of bacterial adhesion on Fg-coated surfaces [14].

While the molecular mechanism controlling the SdrG-Fg interaction is well established, little is known about the impact of SdrG surface density on the ability of various *S. epidermidis* strains to adhere on Fg-coated surfaces. By combining whole population, single-cell and single-molecule atomic force microscopy (AFM) [15] [16] approaches, we show that *S. epidermidis* strains that display a higher density of SdrG on their cell surface show enhanced adhesion to Fg-coated substrates. In light of these data, a model is proposed in which the density of cell surface sites displaying high Fg affinity (i.e. active SdrG molecules) correlates with the proportion of bacteria from a population that effectively bind to Fg-coated substrates.

Materials and Methods

Bacterial strains and cultures

Four *S. epidermidis* strains were used in this study: HB [17], HB SdrG(-) a mutant in which the *sdrG* gene is deleted, kindly provided by Prof. T. Foster [11], ATCC 12228 and ATCC 35984. Bacteria from -80°C frozen stocks were grown on Tryptic Soy Agar (TSA, Bio-Rad) for at least 16 h

at 37 °C. Single colonies were then used to inoculate Tryptic Soy Broth (TSB) and cultures were carried out under agitation at 120 RPM, 37 °C for 16 h.

Preparation of bacterial suspensions

Bacterial suspensions were prepared according to the following procedure. Stationary phase cultures were centrifuged, the supernatant was removed and the pellet was re-suspended in PBS at pH 7.4 (Tablets, Sigma). Centrifugation were performed either once or three times, at either 1,000 *g* or 6,000 *g* for 15 min.

Whole population adhesion assays in 96-well plates

The adhesion assays in static conditions were carried out in 96-well plates coated with PolyDiMethylSiloxane (PDMS). The coating of the wells was performed by adding, at the bottom of the wells, 10 μ L of 1:2 (v:v) solution of PDMS (Sylgard 184, Dow Corning) in hexane (VWR), previously filtered on 0.22 μ m. 96-well plates were then left to dry at 55 °C for at least 1 h.

To coat the PDMS wells for whole population adhesion assays, they were first filled either with PBS or a 0.22 μ m filtered solution of Human Fg (Sigma) 0.5 mg/ml in PBS or Bovine Serum Adult (BS, Sigma) diluted 1:10 in PBS, also previously filtered on 0.22 μ m and left to incubate at 37 °C for 1 h. Afterwards, 150 μ L were withdrawn, and 150 μ L of PBS were added as a rinse. This rinsing step was repeated five times. 50 μ L of liquid were left in the wells at each step to avoid un-wetting. In the last rinsing step, 100 μ L of liquid was withdrawn and 100 μ L of bacterial suspension was added. The content of each well was homogenized by pipetting up and down twice. Incubation was performed for 1 h at 37 °C. The same rinsing procedure was applied to the plates to remove unbound bacteria. Each strain was tested at least in four wells for each PDMS surface type (bare, Fg or BS coated) and repeated three times with independent cultures.

To quantify bacterial adhesion at the bottom of the wells, two bright field micrographs were taken in each well using a 40x long working distance objective (numerical aperture 0.6, Fluotar, Leica) mounted on an inverted microscope (Leica, DMI 6000) equipped with a sensitive B/W

cooled CCD camera (Leica, DFX 365). Surface bound bacteria were counted in ImageJ thanks to the particle analyzer function, after an appropriate thresholding was performed. Results (number of bacteria per image (covering $75.600 \mu\text{m}^2$)) were converted into bacterial surface densities expressed as the number of bacterial cells/ mm^2 .

Single molecule force spectroscopy

Single-molecule force spectroscopy (SMFS) measurements were performed at room temperature (20°C) in PBS buffer using a Nanoscope VIII Multimode AFM (Bruker corporation, Santa Barbara, CA) and oxide sharpened micro-fabricated Si_3N_4 cantilevers with a nominal spring constant of $\sim 0.01 \text{ N m}^{-1}$ (MSCT, Microlevers, Bruker Corporation). The spring constants of the cantilevers were measured using the thermal noise method (Picoforce, Bruker). Bacterial cells were immobilized by mechanical trapping into porous polycarbonate membranes (Millipore, Billerica, MA) with a pore size similar to the cell size [18] [19]. After filtering a cell suspension, the filter was gently rinsed with PBS, carefully cut ($1 \text{ cm} \times 1 \text{ cm}$), attached to a steel sample puck using a small piece of double face adhesive tape, and the mounted sample was transferred into the AFM liquid cell while avoiding de-wetting.

Fg-functionalized tips were obtained using PEG-benzaldehyde linkers [20]. Prior to functionalization, cantilevers were washed with chloroform and ethanol, placed in an UV-ozone-cleaner for 30 min, immersed overnight into an ethanolamine solution (3.3 g ethanolamine into 6 ml of DMSO), then washed 3 times with DMSO and twice with ethanol, and dried with N_2 . The ethanolamine-coated cantilevers were immersed for two hours in a solution prepared by mixing 1 mg Acetal-PEG-NHS dissolved in 0.5 ml of chloroform with $10 \mu\text{l}$ triethylamine, then washed with chloroform and dried with N_2 . Cantilevers were further immersed for 5 min in a 1 % citric acid solution, washed in Ultrapure water (ELGA LabWater), and covered with a $200 \mu\text{l}$ droplet of PBS solution containing human Fg ($2 \mu\text{M}$) to which $2 \mu\text{l}$ of a 1 M NaCNBH_3 solution were added. After 50 min, cantilevers were incubated with $5 \mu\text{l}$ of a 1 M ethanolamine solution in order to passivate unreacted aldehyde groups, and then washed three times with PBS and lastly with PBS supplemented with 0.01 % NaN_3 for storage. Bovine Serum Albumin (BSA) tips for control experiments were obtained according to the same proto-

col.

Bare tips were first used to localize and to image individual cells and then replaced by Fg-tips. Adhesion maps were obtained by recording 32×32 force-distance curves on areas of 500×500 nm, calculating the adhesion force for each force curve and displaying the adhesive events as grey pixels. Unless specified otherwise, all force curves were recorded at 100 ms contact time, with a maximum applied force of 250 pN and using a constant approach and retraction speed of $1,000 \text{ nm s}^{-1}$.

Single-cell force spectroscopy

PDMS-coated surfaces

Round glass cover slips were spin coated with PDMS. Briefly, a 1:5 solution of PDMS (Sylgard 184) in hexane (VWR) was prepared and 0.2 ml of this solution was dropped onto the cover slips before they were spun at 3,000 rpm for 1 min in the spin-coater (Technologies, model: WS-400B-6NPP/Lite). Surfaces were then cured at 95°C for 1 h to allow to PDMS to polymerize. PDMS coated cover slips were stored at room temperature in sealed Petri dishes until use.

Fibrinogen and bovine serum-coated substrates

To prepare Fg-coated substrates, as well as bovine serum substrates, for single-cell probe experiments, glass cover slips coated with a thin layer of gold were immersed overnight in an ethanol solution containing 1 mM of 10 % 16-mercapto-dodecahexanoic acid, 90 % 1-mercapto-1-undecanol (Sigma), rinsed with ethanol, and dried with N_2 . Substrates were then immersed for 30 min into a solution containing 10 mg ml⁻¹ N-hydroxysuccinimide (NHS) and 25 mg ml⁻¹ 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Sigma), and rinsed five times with Ultrapure water (ELGA LabWater). For Fg-coated surfaces, substrates were incubated with 0.5 mg ml⁻¹ of Human Fg (Sigma) for 1 h, rinsed with PBS buffer, and immediately used without de-wetting. For BS surfaces, substrates were incubated with bovine serum diluted 1:10 in PBS, rinsed with PBS buffer, and immediately used without de-wetting.

Single-cell force spectroscopy measurements

Bacterial cell probes were obtained as previously described [21] [22]. Briefly, colloidal probes were obtained by attaching single silica micro-

sphere (6.1 μm diameter, Bangs laboratories) with a thin layer of UV-curable glue (NOA 63, Norland Edmund Optics) on triangular-shaped tipless cantilevers (NP-O10, Microlevers, Veeco Metrology Group) and using a Nanoscope VIII Multimode AFM (Bruker corporation, Santa Barbara, CA). The cantilever was then immersed for 1 h in a 10 mM Tris Buffer, 150 mM NaCl solution (pH 8.5) containing 4 mg ml⁻¹ dopamine hydrochloride (99 %, Sigma). The probe was then rinsed in 10 mM Tris Buffer, 150 mM NaCl solution (pH 8.5) and used directly for cell probe preparation. The nominal spring constant of the colloidal probe cantilever was $\sim 0.06 \text{ N m}^{-1}$, as determined by the thermal noise method (Picoforce, Bruker).

For cell probe preparation, 50 μl of a suspension of ca. 1×10^6 CFU were transferred into a glass Petri dish in which the different substrates were attached. The cells were stained in the dark during 15 min using a BacLight viability kit (Invitrogen, kit L7012) following the manufacturer instructions to check the viability and positioning of the cell. After staining, 4 ml of PBS were added to immerse bacteria and the surfaces. The colloidal probe was brought into contact with an isolated bacterium. Single bacteria were attached on the centre of the colloidal probes using a Bioscope Catalyst AFM (Bruker Corporation, Santa Barbara, CA) equipped with a Zeiss Axio Observer Z1 and a Hamamatsu camera C10600. When proper attachment of the cell was confirmed by fluorescence imaging, the cell probe was positioned over the appropriate substrate without de-wetting. Single-cell interaction forces with surfaces were measured at room temperature (20 °C) by recording multiple forces curves on three different spots, using a maximum applied force of 250 pN, 1 s contact time, and constant approach and retraction speeds of 1,000 nm s⁻¹. For each condition, at least three bacterial cells from independent cultures were probed, 150 times for BS surfaces, and at least 350 times with PDMS and Fg-coated substrates.

Statistical analysis

Results of whole population assays were treated with JMP© (SAS) to calculate the means, standard deviations and confidence intervals at 95 % of bacterial surface densities on coated or bare PDMS surfaces.

Results

Fg binding activity of SdrG is sensitive to repeated frictional forces

The impact of centrifugation on the preparation of bacterial suspensions was assessed by means of whole population adhesion assays to Fg-coated PDMS. In brief, the bacterial suspensions were removed after 1 h of incubation in contact with the surface and the wells were thoroughly rinsed with PBS. Adherent bacterial cells were numbered on microscopic pictures to determine the surface density in terms of cells per mm². Fig. 1A presents the results for suspensions obtained by centrifuging the liquid cultures three times either at 1,000g or 6,000g for 10 min. Almost twice as many bacteria from the suspension centrifuged three times at 1,000g remained bound to the Fg-coated PDMS surface compared to the suspension centrifuged at 6,000g. These results strongly suggest a loss in SdrG activity in response to a higher centrifugation speed. The adherence to Fg-coated surfaces of bacterial cells that were centrifuged either once or three times at 6,000g was tested (Fig. 1B). Strikingly, increasing the number of centrifugation steps decreases the ability of a population of *S. epidermidis* to attach to surface bound Fg. Repeated exposures of the cell surface to mechanical stresses partially inactivate SdrG and may lead to underestimate its role in bacterial adhesion. In the following experiments, the preparation of bacterial suspensions was performed with only one centrifugation step to preserve the activity of SdrG on the bacterial cell surface.

Whole population adhesion assay of four *S. epidermidis* strains on bare and coated PDMS substrates

Fig. 2 presents the results of whole population adhesion assays of four *S. epidermidis* isolates: ATCC 12228, ATCC 35984, HB and HB SdrG(-), an isogenic mutant in which *fbe* has been knocked out, to PDMS surfaces, either bare or coated with BS or Fg in modified multi-well plates (Fig. 2A).

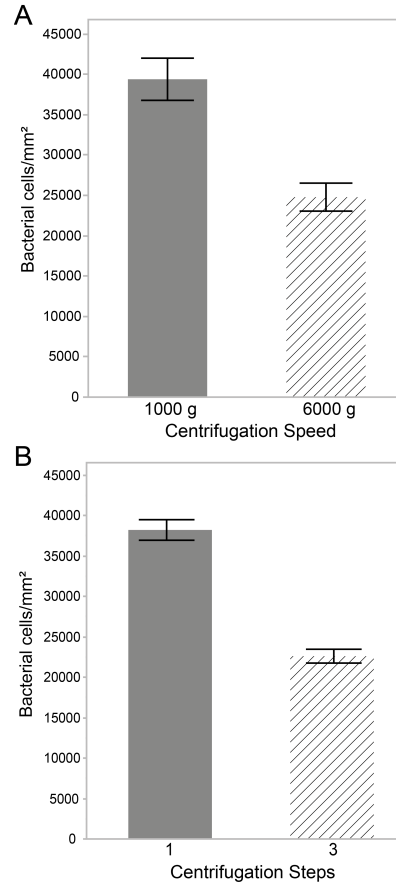


Figure 1: Influence of centrifugation on the adhesion of *S. epidermidis* strain HB to Fg-coated PDMS. Whole population adhesion assays performed with bacterial suspension of the HB strain centrifuged three times at 1,000 and 6,000g (**A**) and either once or three times at 6,000g (**B**). Plots represent the number of bacterial cells per mm² on the surface of the well. Error bars are confidence intervals at 95 %.

On bare PDMS, all strains tested in this study strongly adhered to cover the entire surface of the wells entirely as shown in Fig. 2B. This situation is achieved when around 150.000 cells can be found per square mm. On PDMS coated with BS, bacterial adhesion was generally weak (at least to orders of magnitude less than on bare PDMS). Bacterial surface densities of all tested strains could not be statistically discriminated and remained just above or close to the limit of detection of our method, i.e. 500 bacteria/mm².

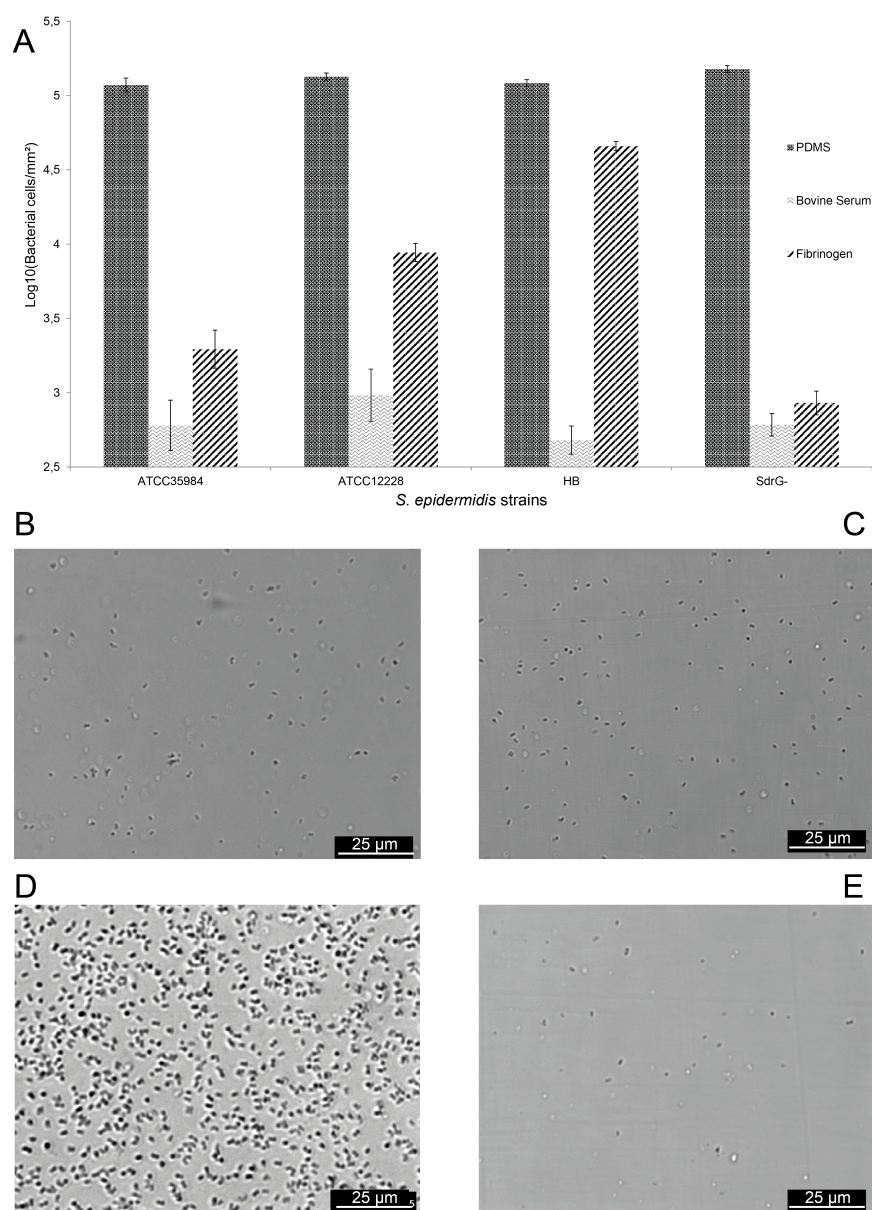


Figure 2: Whole population adhesion assays on bare and coated PDMS surfaces in 96-well plates. **(A)** Plot of the density of surface bound bacterial cells is expressed as the log10 of the number of bacterial cells per mm² on the surface of the well. Adhesion of *S. epidermidis* strains ATCC 12228, ATCC35984, HB and HB SdrG(-) to PDMS either bare or coated with Fg or BS is represented. Error bars are confidence intervals at 95 %. **(B-E)** Micrographs of the surface of a well of Fg-coated PDMS at the end of the adhesion assay performed with suspensions of ATCC 35984, ATCC 12228, HB and HB SdrG(-) respectively. The scale bar indicates 25 μ m.

On Fg-coated PDMS, SdrG(-) behaved as for the reference BS-coated PDMS, with about 1,000 surface-bound bacteria/mm² (see Fig. 2 E). This means that the mutant strain SdrG(-) interacts with the layer of adsorbed Fg in the same way as with a layer of other proteins, as expected given the absence of SdrG on its cell surface. For the other strains, WT HB strain (see Fig. 2 D) adhered significantly better than ATCC 12228 by 0.6 log (see Fig. 2 C), and the latter displayed a significantly higher affinity for Fg-coated surfaces than ATCC 35984, also marked by a 0.6 log difference in the bacterial surface density (see Fig. 2 B). The adhesion of ATCC 35984 on Fg could just be discriminated from its adhesion to the control BS-coated surface.

Single-cell adhesion forces

The binding forces between single *S. epidermidis* cells and PDMS, Fg-coated and BS-coated surfaces were then measured using SCFS [21] [22]. As illustrated in Fig. 3A, single-bacterial cells were picked up with colloidal probe cantilevers coated with polydopamine. Force-distance curves were then recorded between the cellular probes and the different substrates. Fig. 3B shows the adhesion force histograms, as well as representative force curves, obtained between three different cells (black, red and green histograms) of ATCC 12228, ATCC 35984, HB and HB SdrG(-) strains and bare, BS-coated and Fg-coated PDMS surfaces.

On bare PDMS, all strains displayed 100 % of adhesive events, indicating strong binding to hydrophobic surfaces. The binding forces varied amongst the strains, from ~ 250 pN to 1,500 pN. These forces most probably result from hydrophobic interactions between the cell surfaces and PDMS. The surface hydrophobicity of *S. epidermidis* has been reported to be strain-dependent [23] and be essentially mediated by surface proteins such as AtlE and Aae [8]. By contrast, on BS-coated surfaces, ATCC 35984, HB and SdrG(-) cells showed hardly any binding, while ATCC 12228 featured adhesion forces of 50-100 pN in 20 % of the curves.

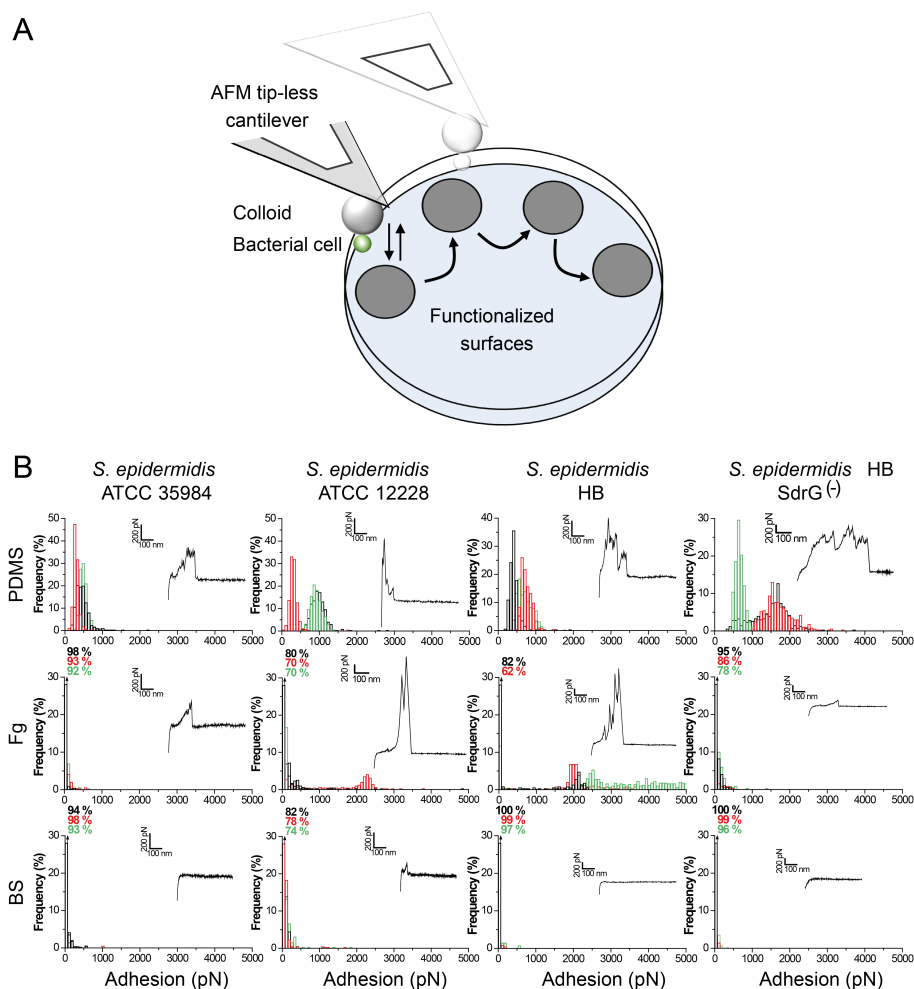


Figure 3: Single-cell force spectroscopy (SCFS) of the interaction between *S. epidermidis* strains and PDMS, Fg-, and BS-coated substrates. **(A)** Schematic representation of the SCFS protocol. The bacterial probe is composed of a bacterial cell, attached to a colloidal bead immobilized onto a tipless AFM cantilever. The same bacterial probe is brought into contact with PDMS, Fg, and BS-coated surfaces. **(B)** Adhesion force histograms, together with representative force curves (x axis represents the distance (in nm) while the y axis is the force (in pN), for the four *S. epidermidis* strains ATCC 12228, ATCC 35984, HB and HB SdrG(-) interacting with the three types of surfaces. The three colors (black, red and green) represent the results obtained with three independent bacterial cells.

On Fg-coated surfaces, the differences between the strains were more pronounced. While there was hardly any adhesion with ATCC 35984,

one out of the three ATCC 12228 cells showed strong interactions towards the Fg-coated surface. This *intra*-strain variation reflects heterogeneity of the cell population. The force value (~ 2.1 nN), as well as the force signatures, were similar to those previously observed and associated with the Fg-SdrG "dock, lock and latch" binding mechanism [13]. The adhesive ATCC 12228 cell presented a relatively low adhesion frequency of 30 % compared to what we found previously [13]. For strain HB, the adhesion frequency, the recorded force value and the force signatures were comparable to what was previously observed [13]. *S. epidermidis* HB however showed greater adhesion reflecting the possibility of probing multiple SdrG-Fg interactions in parallel. HB SdrG(-), in which the *fbe* gene has been disrupted, was then used to confirm the specificity of the SdrG-Fg interactions. As expected, HB SdrG(-) cells showed little to no adhesion.

Finally, BS-coated surfaces were tested to further control the specificity of binding on PDMS and Fg-coated substrates. ATCC 35984, HB and HB SdrG(-) cells showed hardly any adhesion reflecting the specific nature of the adhesion forces. ATCC 12228 however showed little adhesion characterized by weak adhesion forces (< 250 pN) with a low adhesion frequency (18 to 26 %).

In general, the trends observed in SCFS results are in line with those of whole population adhesion assays. Adhesion to Fg-coated surfaces occurring through the specific SdrG-Fg recognition is well documented in the SCFS data of the HB strain and still present but less marked in the strain ATCC 12228, whereas ATCC 35984 and SdrG(-) showed no sign of affinity for Fg. Hydrophobic PDMS is very reactive towards *S. epidermidis* cell surfaces and the protein layer covering the BS-coated PDMS almost totally inhibits the cell-surface interactions resulting in very low adhesiveness.

Mapping single SdrG proteins on the *S. epidermidis* cell surface

AFM-based single molecule force spectroscopy (SMFS) [24] [25] was used to map the distribution of single SdrG molecules on living bacteria. The cell surface of the ATCC 12228, ATCC 35984, HB and HB SdrG(-) strains was probed with a Fg-modified tip in order to detect the presence of SdrG (Fig. 4). Spatially-resolved SMFS of live cells immobilized

on porous membranes (Fig. 4A) allowed us to directly measure the localization and binding strength of single adhesins. Adhesive events were attributed to the specific detection of single SdrG molecules for two reasons: first, the procedure that we used was already validated in previous work [13], second, use of irrelevant tips, i.e. tips functionalized with BSA, hardly gave any adhesion forces on the four strains (Fig. 5, available in Supplementary information). The maps resulting from these experiments are shown in Fig. 4B; they display the spatial distribution of single SdrG molecules of a $0.25 \mu\text{m}^2$ area on the cell surface which represents about 1/10 of the total bacterial cell surface.

The highest density (between 19 and 29 %) of SdrG molecules was found on the HB strain. The strain ATCC 12228, ranked second as one of the tested cells, displayed 12 % of adhesive events, while the cell surfaces of the two others were almost non-adhesive. This behavior could reflect the natural heterogeneity of the bacterial population. Indeed, stochastic gene expression can generate diversity amongst the cells of a population [26] [27]. For the mutant strain SdrG(-), two cells displayed 10 % of adhesive events but these interactions are likely to be non-specific since no typical SdrG-Fg force signatures were observed (Fig. 4C). Finally, ATCC 35984 was essentially non-adhesive (99 % of non-adhesive events). This indicates that ATCC 35984 has the same reactivity towards Fg as the mutant strain in which the *fbe* gene coding for SdrG has been deleted. As this strain carries a copy of *fbe*, its lack of adhesion to Fg could be explained by a strain dependent genetic regulation, possibly cross-influenced by other regulatory/signaling pathways. The possibility to have a significant crowding by other cell surface polymers that would reduce the opportunity for SdrG to appropriately bind to Fg cannot be excluded.

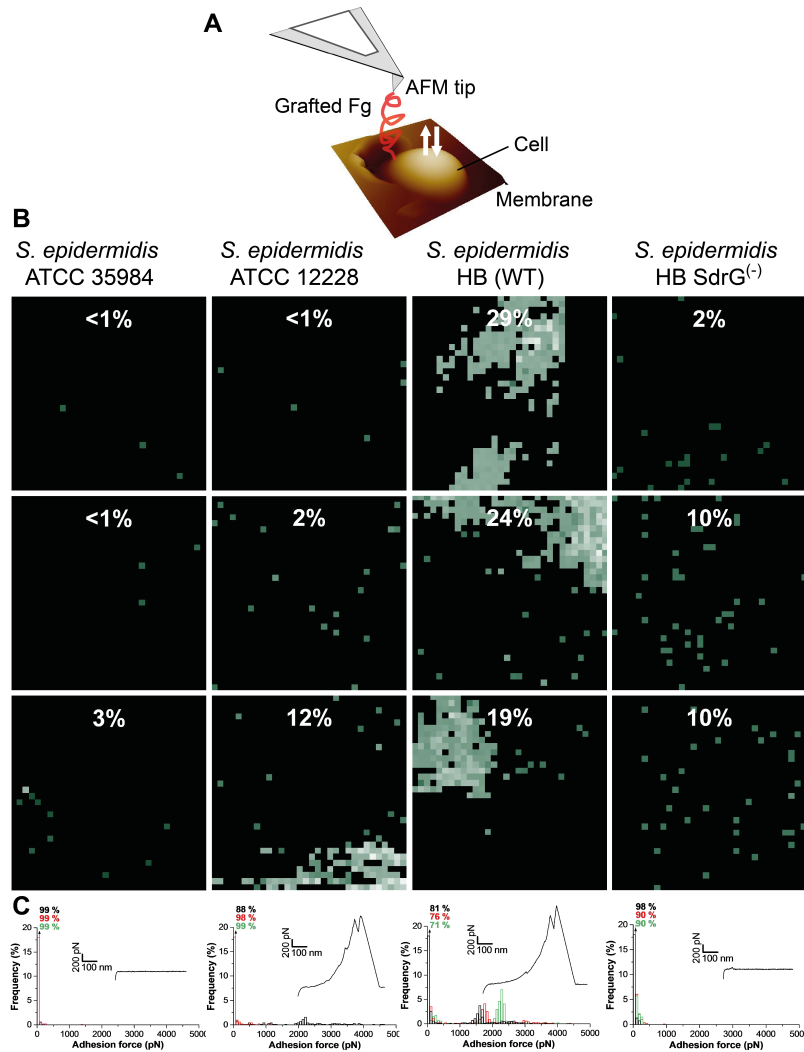


Figure 4: Imaging single SdrG proteins on the *S. epidermidis* cell surfaces using single-molecule force spectroscopy (SMFS) with Fg-modified AFM tips. (A) The surface of a bacterial cell trapped in a porous membrane is probed with a Fg-modified tip. Deflection image shows the emerging part of the trapped cell. (B) Adhesion forces maps (500 nm x 500 nm) recorded on the different strains using Fg-tips. Every bright pixel documents the detection of a single SdrG protein. Percentages indicated on top of the maps correspond to the detection frequency for every map. The data were obtained from three independent cells for each strain. (C) Adhesion force histograms (x -axis represents the magnitude of the binding forces while y -axis represents the percentage of events) together with representative force curves. For HB and ATCC 12228 strain, the large forces (~ 2 nN) are attributed to the binding of SdrG to Fg. The three colors (black, red and green) represent the results obtained with the three independent bacterial cells of each strain.

These SMFS results provide direct indication as to the abundance of SdrG on the cell surface of *S. epidermidis* strains. These strains can be classified in the following order: HB > ATCC 12228 > ATCC35984 $\simeq$ SdrG(-). This corresponds to the conclusions drawn from the whole population adhesion assays and the SCFS data on Fg-coated surfaces.

Discussion

Adhesion of bacteria on implanted biomaterials determines their fate in the host. Bacterial anchorage on surfaces paves the way to the development of biofilms and determines the ability to cause chronic infections [7]. In *S. epidermidis*, SdrG mediates strong and specific binding to Fg, a molecule most often present in the conditioning film of proteins that forms upon implantation of devices [28]. In this paper, we present pieces of evidence that underline the importance of the cell surface abundance of SdrG in the ability of single cells and whole populations to efficiently adhere to surface bound Fg.

First, it was observed that the Fg binding activity of SdrG on the cell surface is influenced by strong frictional forces. Adhesion assays performed with bacterial suspensions from the same culture prepared using distinct centrifugation parameters resulted in decrease bacterial surface density on Fg-coated PDMS for the suspension submitted to repeated centrifugation steps or to a higher centrifugation speed. Centrifugation has recently been reported to cause damages at the bacterial cell surface, notably causing a decrease in *S. aureus* adhesion to glass substrates [29]. It is likely that the quaternary structure of SdrG is altered by excessive frictional forces occurring between two cells surfaces, therefore impairing its ability to specifically recognize Fg. As a consequence, reducing manipulations that involve frictional forces as much as possible is highly recommended when studying the Fg-binding function of SdrG.

Using complementary population- and single-cell-based approaches, the adhesion of *S. epidermidis* to Fg-coated surfaces was found to be strongly strain dependent. Indeed, SCFS showed that the affinity of HB strain for surface bound Fg was high, ATCC 12228 affinity was less marked whereas ATCC 35984 and SdrG(-) did not display any specific recognition of Fg. In line with these conclusions, the adhesion of whole population assays resulted in high surface bacterial density of the HB

strain, moderate adhesion of ATCC 12228 and the two other strains displayed the same level of adhesion as on the control BS-coated PDMS, indicating the absence of specific SdrG-Fg recognition.

Based on these results, it was postulated that the differences between strains could originate from variations in the abundance of SdrG on the cell surface. To get more insights on the distribution of active SdrG molecules on the *S. epidermidis* surface, SMFS with a Fg-modified AFM tip was used to probe the surface of the strains. The proportion of sites that specifically recognized Fg were numerous for HB strain, with about 20 % of the surface of three independent HB cells that interacted with Fg according to the "dock, lock and latch" mechanism. For ATCC 12228, 12 % of the surface of one ATCC 12228 cell did recognize Fg, but no SdrG could be detected on the other two cells tested. On the last two strains ATCC 35984 and HB SdrG(-), the SdrG adhesin was not detected on the cell surface. Our interpretation is that the abundance of SdrG on the cell surface determines the ability of single cells and whole bacterial population to remain bound on a surface conditioned with Fg.

The results of bacterial adhesion on bare and BS-coated PDMS lead us to argue on the ecological role of SdrG in *S. epidermidis*. Indeed, since the attractive hydrophobic surfaces become repelling as they are conditioned with serum proteins [30] [31], SdrG provides bacteria with the ability to counterbalance the effect of the adhesion-inhibiting layer of proteins and mediate adhesion to the surface.

The expression of *fbe*, at both mRNA and protein levels, has already been shown to be rather low in *in vitro* culture and to significantly increase following exposure to *in vivo* environments, *i.e.* in a murine model [32]. In *S. aureus*, the expression of many virulence factors, including the fibrinogen binding protein, is significantly higher in human blood as confirmed by transcriptome analysis [33]. In the same vein, the expression of MSCRAMMs in *S. aureus* has recently been reported to strongly increase in the presence of human plasma *in vitro* [34].

Even though the regulatory pathway involved in *fbe* expression has not yet been elucidated, it is clear that *S. epidermidis* has developed a strategy to crowd its surface with SdrG when the bacteria are inside their host. Taken together with the results of this work, this suggests that *S. epidermidis* responds to environmental changes in order to improve its

adhesiveness to surfaces partially coated by Fg, such as indwelling medical devices and host tissues. It is generally admitted that increased adhesion to biomaterials is a key step for *S. epidermidis* to establish in the host in the form of a biofilm, therefore causing chronic infections.

In future work, it would be interesting to further investigate the impact of SdrG clustering on cell adhesion, including further investigation of the cell-to-cell variability. For instance, one could collect cells that adhere more strongly to fibrinogen and assess the surface density and clustering of SdrG to see whether those two factors are important for the adhesion phenotype.

In conclusion, this paper establishes a link between the abundance of SdrG on the cell surface of *S. epidermidis* and the adherence of this species to the surface of materials coated with Fg, *i.e.* vascular catheters and prostheses. Future works identifying the molecular regulation pathways that control *fbe* expression and surface density of SdrG and, hence, the adherence to Fg-coated surfaces would help to understand the critical factors that govern the onset of a chronic nosocomial infection involving biofilms of *S. epidermidis*.

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Supplementary information

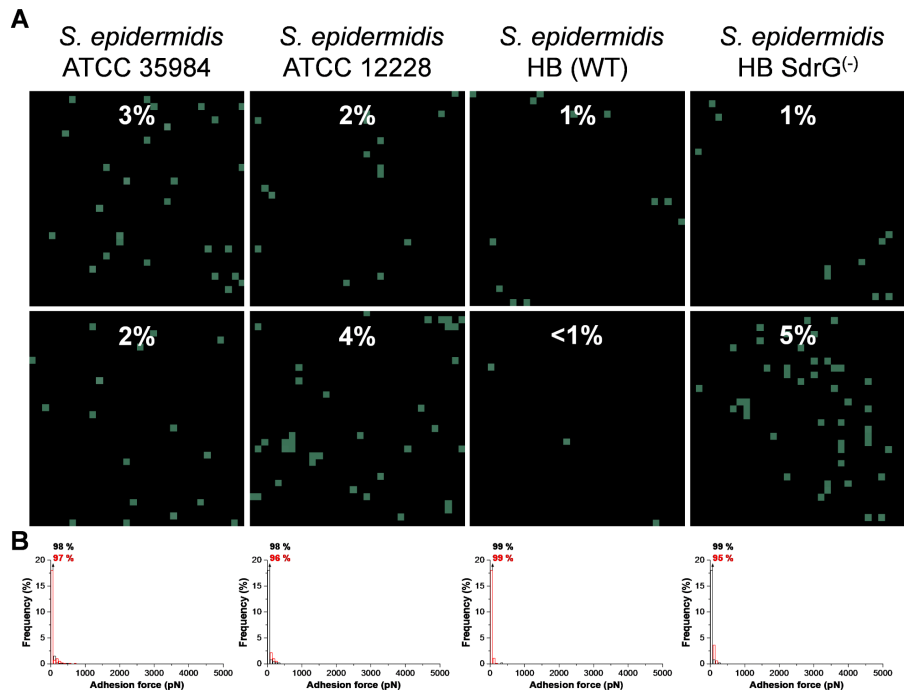


Figure 5: Probing the cell surface of *S. epidermidis* strains using single-molecule force spectroscopy (SMFS) with irrelevant BSA-modified AFM tips. (A) Adhesion forces maps (500 nm x 500 nm) recorded on the different strains using BSA-tips. Percentages indicated on top of the maps correspond to the detection frequency for every map. The data were obtained from two independent cells for each strain. (B) Adhesion force histograms (x-axis represents the magnitude of the binding forces while y-axis represents the percentage of events).

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Detection of surface-bound *Staphylococci*

This Chapter focuses on the selective detection of bacteria by means of an electrical biosensor. An experimental setup combines impedimetric detection of surface adhering bacteria on micro-electrodes and optical follow up by microscopy. The originality of this work resides in three essential points. First, the detection principle is solely based on intrinsic dielectric properties of bacterial cells whereas current methods involve electrochemical reactions or cell lysis. Second, the setup combines both electrical and optical sensing to accurately attribute electrical signals in the light of microscopic events. Finally, a strategy is proposed that selectively identifies the genera or the species of the attached bacteria with the help of lytic enzymes added exogenously.

The proof of concept presented here is based on the example of bacterial detection in urine but this technique is not limited to this particular application.

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Lytic enzymes as selectivity means for label-free, microfluidic and impedimetric detection of whole-cell bacteria using ALD-Al₂O₃ passivated microelectrodes

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In this work, the first author and myself were almost equally involved.

Abstract

Point-of-care (PoC) diagnostics for bacterial detection offer tremendous prospects for public healthcare improvement. However, such tools require the complex combination of the following performances: rapidity, selectivity, sensitivity, miniaturization and affordability. To meet these specifications, this paper presents a new selectivity method involving lysostaphin together with a CMOS-compatible impedance sensor for genus-specific bacterial detection. The method enables the sample matrix to be directly flown on the polydopamine-covered sensor surface without any pre-treatment, and considerably reduces the background noise. Experimental proof-of-concept, explored by simulations and confirmed through a setup combining simultaneous optical and electrical real-time monitoring, illustrates the selective and capacitive detection of *S. epidermidis* in synthetic urine also containing *Enterococcus faecium*. While providing capabilities for miniaturization and system integration thanks to CMOS compatibility, the sensors show a detection limit of ca. 10^8 (CFU/mL).min in a 1.5 μ L microfluidic chamber with an additional setup time of 50min. The potentials, advantages and limitations of the method are also discussed.

Introduction

Recently brought to huge medical interest, especially in the struggle against antibiotic resistant bacteria, lytic enzymes are molecules that specifically digest the cell wall of most Gram-positive bacteria. They can be produced by either bacteriophages or bacterial cells. In the first case, bacteriophage lytic enzymes, also called endolysins, break bonds in the thick cross-linked peptidoglycan to enable phages to inject their genetic material in their host to infect it [1]. In the second case, bacteria produce their own lytic enzymes, also called autolysins, which are tightly controlled and necessary for cell wall rearrangements during cell division. Finally, in some cases, evolution has driven bacteria to develop lytic enzymes to eliminate species competing for a specific environmental niche. This is notably the case for *S. simulans*, which produces lysostaphin, a peptidoglycan hydrolase active against almost all *Staphylococcus* species.

Besides their medical relevance, lytic enzymes and bacteriophages have also proven to be powerful tools to achieve high-selective impedimetric detection of bacteria [2], in a growth-dependent or independent way. In the first case, bacterial growth monitored through ion release is strongly affected in culture media containing phages, since target bacteria are continuously digested [3]. In the growth-independent method, phages are immobilized on the electrode surface and subsequently capture and digest bacteria in a selective way, releasing ions in the outer medium that are monitored by the impedimetric sensor [4]. Another electrochemical technique detects peptidoglycan fragments, products of the bacterial lysis, that have specifically bound on an antibody layer grafted on the sensor surface [5].

For Point-of-Care (PoC) diagnostics, growth-independent impedance detection provides a lower detection time. Despite essentials for Lab-on-Chip (LoC) systems, compactness, simplicity and autonomy remain challenging for the following reasons. First, most studies report gold microelectrodes hardly compatible with the Complementary Metal-Oxide-Semiconductor (CMOS) process. To benefit from low-cost, system integration and miniaturization capabilities of CMOS, microelectrodes can be patterned in the last CMOS metal layer and covered with an $\sim 0.1\ \mu\text{m}$ -etched [6] [7] or $\sim \mu\text{m}$ -intact [8] [9] passivation layer. However, this thick oxide layer causes a massive drop in sensitivity.

Secondly, grafting a biorecognition layer, *e.g.*, lytic enzymes, bacteriophages or antibodies, on the sensor surface typically faces problems such as reproducibility, uniformity and stability over time [10]. Although already complex, the functionalization protocol further needs to be adapted to every surface material and grafting molecule. In addition, the biorecognition layer is subject to nonspecific bindings in complex samples, requiring dedicated negative controls.

Thirdly, the largest sensitivity of bacterial impedance sensors is typically achieved in low-salt buffers of electrical conductivity close to $100\ \mu\text{S/m}$, such as 0.1% peptone water [11], 100 mM mannitol solution [12] [13], or even in deionized water [14]. Resuspension of bacteria in these buffers is then typically performed but requires at least three centrifugation steps to remove most ions contained in the initial matrix. This is time-consuming ($>1\ \text{h}$) and requires lab equipment incompatible with PoC and LoC applications. Furthermore, great care should be

taken in the result interpretation, since observed shifts can either be due to bacterial cells or the presence of remaining ions from the initial buffer.

In this paper, three notable innovations are brought together to solve the aforementioned problems for direct and selective detection of bacteria towards PoC and LoC applications. First, a new selective method based on lytic enzymes was developed. Free of selective surface functionalization and centrifugation steps, the whole procedure is then simple, straightforward and reproducible. In short, once the sensor surface is totally covered with various bacteria, sensor levels before and after the application of specific lytic enzymes are compared; if different, some target bacteria have been lysed and the sample can be qualified positive. We demonstrate this concept with a selective detection of *S. epidermidis* in synthetic urine, also containing *Enterococcus faecium* as a negative control, to mimic urinary infections.

Second, CMOS-compatible microelectrodes, made in aluminum and covered with a very thin layer of atomic-layer-deposited (ALD) Al_2O_3 , have been used. The 30 nm-thick Al_2O_3 layer protects the underlying aluminum from corrosion and has a moderate impact on bacterial sensitivity, compared to a micrometer-sized oxide layer. As a 80 nm electrochemically-anodized Al_2O_3 layer has previously been demonstrated for bacterial detection in dried conditions [15], this paper extends the use of this type of passivated electrodes to detection in liquid.

Finally, an innovative setup combining simultaneous real-time optical and electrical monitoring of sensor was built. Compared to most studies where electrical shifts are interpreted without optical control, this original setup provides a way to accurately link electrical phenomena to surface events such as binding of bacterial cells and considerably decreases the risk of misinterpretations.

Materials and Methods

Micro-fabrication and encapsulation of the interdigitated microelectrodes (IDE)

Fig. 1 sketches the process flow. First, 3-in. Pyrex wafers were immersed in a freshly-prepared Piranha solution (H_2O_2 : H_2SO_4 , 2:5) during

10 min for cleaning, followed by two continuously renewed immersions in deionized (DI) water baths during 20 min. Afterwards, aluminum was evaporated in a rotate mode to deposit a 1 μm -thick layer. A first optical lithography with positive photo-resist then provides finger masking during the subsequent Al plasma etching. After removing resist, the whole wafer was covered with 33 nm of plasma-enhanced atomic layer deposited (ALD) Al_2O_3 at room temperature (RT) with trimethylaluminum and oxygen as precursor. The argon flow through plasma source was 200 sccm and the oxygen flow for the plasma step was 30 sccm during 20 s. Afterwards, a second optical lithography with positive photoresist was used to define pad area and etch the subsequent Al_2O_3 layer after 15 s immersion in IPA:HF 70% (3:1). Finally, a last optical lithography with negative photoresist KMPR1025 was used to define 30 μm -thick walls to support the microfluidic cap. The interdigitated microelectrodes (IDE) are 2 μm -wide, 4 μm -spaced, 1 μm thick and define a 300 μm -diameter circle (Fig. 2 a and b).

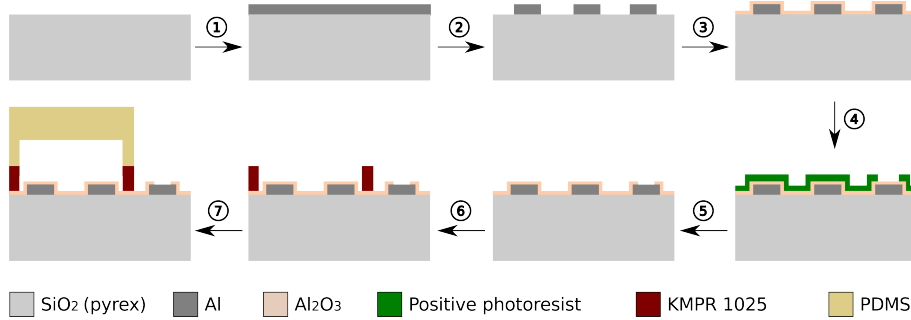


Figure 1: Sensor micro-fabrication steps: (1) aluminum deposition, (2) positive optical lithography followed by aluminum plasma etching, (3) deposition of an ALD Al_2O_3 layer, (4) positive optical lithography to open pads, (5) etching Al_2O_3 with IPA:HF 70% (3:1), (6) positive optical lithography to define thick KMPR walls and (7) PDMS cap pressure.

For the microfluidic cap, a 270 μm -thick KMPR1050 layer was patterned by optical lithography on a clean 3-in. silicon wafer. This mold was then covered with a thin hexamethyldisilazane (HMDS) layer to easily take off the polydimethylsiloxane (PDMS) cap subsequently. Inside a Petri dish, the PDMS was then flown on the mold wafer and incubated at 60°C overnight. To ensure the watertight sealing of the system, a transparent pressure tool was used (Fig. 2 c). As the PDMS cap is

pressed on the chip, the whole microfluidic channel including inlets and outlets is 1 mm-wide, 5 mm-long and 300 μm -thick, resulting in a total channel volume of 1.5 μL , while the immediate volume above the 300 μm -diameter sensor is 21 nL.

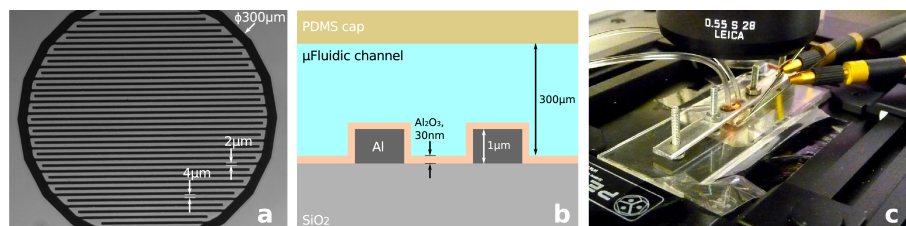


Figure 2: (a) Sensor microphotograph, (b) schematic cross-section of the encapsulated sensor and (c) photograph of the encapsulated sensor positioned on an inverted microscope and contacted through electrical probes.

Chemicals and reagents

Solutions and buffers used in this study were: TrypticSoyBroth (TSB), Tryptic Soy Agar (TSA), Lysogeny broth (LB), synthetic urine, poly-dopamine solution, 1 μM lysostaphin solution, 0.01 M phosphate buffer saline (PBS) and PBS diluted 1:10, PBS 1:100, PBS 1:1000 by volume in DI water, respectively. Detailed protocols to prepare these solutions are available in Supplementary information.

Bacterial samples

S. epidermidis ATCC 35984 and *E. faecium* ATCC 19434 were used as reference strains for this study. Detailed protocols for culture, resuspension and CFU counting are available in Supplementary information.

Experimental setup and impedance measurements

The device was positioned on an inverted microscope (DMI6000, Leica, Belgium) enabling real-time imaging of the sensor surface during electrical measurements (Fig. 2 c). Samples were flown through the microfluidic channel by a peristaltic pump (Gilson Minipuls). An impedance analyzer (LCR 4284A, Agilent, USA) was connected to electrical probes and remotely controlled through LabVIEW® to perform an automatic sweep from 100 Hz to 1 MHz, at voltage amplitude of 50 mV. Before

impedance measurement, an open calibration was performed by positioning electrical probes above aluminum pads, without any electrical contacts. Once electrical probes were put in contact with pads, the sensor was first rinsed and measured in PBS 1:1000 at 125 $\mu\text{L}/\text{min}$, during 10 min at RT. Then, the polydopamine solution was incubated during 30 min at dark condition, without flow and electrical measurements. Afterwards, the sensor was washed with PBS 1:1000 during 5 min at 250 $\mu\text{L}/\text{min}$ followed by 5 min at 125 $\mu\text{L}/\text{min}$ under electrical measurements to reach a constant value. At this point, the sensor is ready to be experimented with bacterial cells. But, as bacteria are contained in PBS 1:1000 or in synthetic urine, two different protocols must be considered and .

Real-time detection of *S. epidermidis* in PBS 1:1000

Before flowing bacterial suspensions, PBS 1:100, PBS 1:10, PBS and PBS 1:1000 solutions were successively flown at 125 $\mu\text{L}/\text{min}$ during 10 min and for each, reference measurements were performed. Suspensions from 10^6 to 10^9 CFU/mL of stationary-state *S. epidermidis* resuspended in PBS 1:1000 were then injected at 1 $\mu\text{L}/\text{min}$, each followed by the same washing procedure with sterile PBS 1:1000 as previously described. Finally, PBS 1:100, PBS 1:10 and PBS were flown again under electrical measurements to extract shifts in different conditions of salinity, but with exactly the same number of adherent *S. epidermidis* on the sensor surface, as verified by our optical setup.

Selective detection of *S. epidermidis* in synthetic urine, in the presence of *E. faecium*

For this experiment, the suspension of *E. faecium* (negative control) in synthetic urine was first introduced at 1 $\mu\text{L}/\text{min}$, followed by a 10 min wash with PBS 1:1000 as previously described. These two steps were repeated with exponential-state *S. epidermidis* (target) contained in synthetic urine, for experiments requiring both species on the sensor. Finally, lysostaphin was incubated 30 min at 1 $\mu\text{L}/\text{min}$, followed by a final wash with sterile PBS 1:1000. To assess reproducibility, three totally independent experiments were performed for both negative controls, i.e. sensors with *E. faecium* only, and target samples, i.e. sensors with *E. faecium* and *S. epidermidis*.

Automatic bacterial counting algorithm

Based on microscope images, a MATLAB s program was implemented to automatically count the number of attached bacteria on the sensor surface. A mask was first obtained after black and white (B&W) conversion and electrode dilatation, compensating the blur effect on IDE edges. Using the mask, the electrode region was set to a zero value and the whole image was converted to B&W with an optimal threshold to distinguish bacteria. Finally, the number of pixel clusters was automatically counted.

Results and Discussion

Impedance results are displayed in terms of the normalized admittance Y/ω [F], with Y [S] the sensor admittance, i.e., the inverse of impedance, and $\omega = 2\pi f$ [rad/s] the angular frequency. The capacitance denotation is not used because the impedance phase is slightly different from -90° . Before analyzing selectivity to *S. epidermidis* in synthetic urine, real-time detection of *S. epidermidis* resuspended in the low-salt buffer PBS 1:1000 is first investigated to optimize sensor performances. In all experiments, a polydopamine layer, known as biological glue [16] covers the sensor surface (Al_2O_3) to increase and homogenize bacterial binding.

Real-time detection of *S. epidermidis* resuspended in PBS 1:1000

Shifts and slopes in real-time

During the initial wash of the polydopamine-covered sensor with sterile PBS 1:1000, the normalized admittance Y/ω showed stability and exhibited no drift (Fig. 3 a, first ten minutes). Once samples of *S. epidermidis* resuspended in PBS 1:1000 were flown on the sensor, Y/ω systematically increased by three successive and different mechanisms highlighted in Fig. 3 a. The first is an immediate shift Δc [F], observed just after bacterial injection and attributed to the slight difference in ionic content, i.e., electrical conductivity, between sterile PBS 1:1000 and bacterial resuspension in PBS 1:1000. Indeed, centrifugation steps lead bacterial cells to release ions due to osmotic pressure and damaged cell walls [17]. Despite its dependence on bacterial concentration, two reasons make Δc unsuitable for bacterial sensing. First, Δc is strongly

affected by experimental procedures such as manipulation, contamination and temperature, all affecting the baseline sample conductivity (see the artifact on 10^6 CFU/mL in Fig. 3 a). The use of a reference conductimeter within the micro-fluidic channel could solve this problem, but increases the system complexity. Second, Δc is useless for real applications dealing with saline solutions, whose high electrical conductivity is hardly impacted by bacterial ion release. For these reasons, Δc -based sensing should be avoided.

During the next 20 min bacterial incubation, Y/ω was shown to continuously grow with increasing bacterial surface coverage, defining the real-time slope $s_{rt} = \delta(Y/\omega)/\delta t$ [F/min] (Fig. 3 a, dotted blue linear curve). Thanks to simultaneous optical and electrical measurements, the direct link between s_{rt} and the bacterial real-time binding was assessed (Fig. S1a!!!). Furthermore, a linear dependence between s_{rt} and the bacterial concentration is highlighted in Fig. 3 b. The related "real-time shift" Δ_{rt} [F] was extracted as the difference between Y/ω values after the incubation phase and after the initial conductivity peak. Its linear dependence on the bacterial density [number of cells/mm²] was quantified to 319 aF per adherent bacterium, resulting from the presence of both adherent and non-adherent, but close to the sensor surface, bacterial cells (Fig. 3 c). However, both s_{rt} and Δ_{rt} are strongly reduced in more saline bacterial samples for reasons described in .

After the bacterial incubation, a wash with sterile PBS 1:1000 flushed non-adherent bacteria and excessive ions away, so that Y/ω stabilized at a lower equilibrium value (Fig. 3 a). This level minus the pre-incubation Y/ω value in sterile PBS 1:1000 defines the "shift after wash" Δw [F]. A linear dependence between Δw and the bacterial surface coverage was experimentally evaluated to 101 aF per adherent bacterium (Fig. 3 c) and confirmed by simulations in Fig. S1!! b [18]. In contrast to Δ_{rt} , only the remaining adherent bacteria on the sensor affect Δw , justifying its smaller value. On the other hand, Δw is highly stable and reliable since it purely originates from dielectric properties of bacterial cells only. Indeed, as measured only at equilibrium and under flow with sterile PBS 1:1000, Δw is not subject to ionic release or contamination. In addition, wash procedures can be added as using saline bacterial samples to recover Δw . For these reasons, next discussions are only based on Δw .

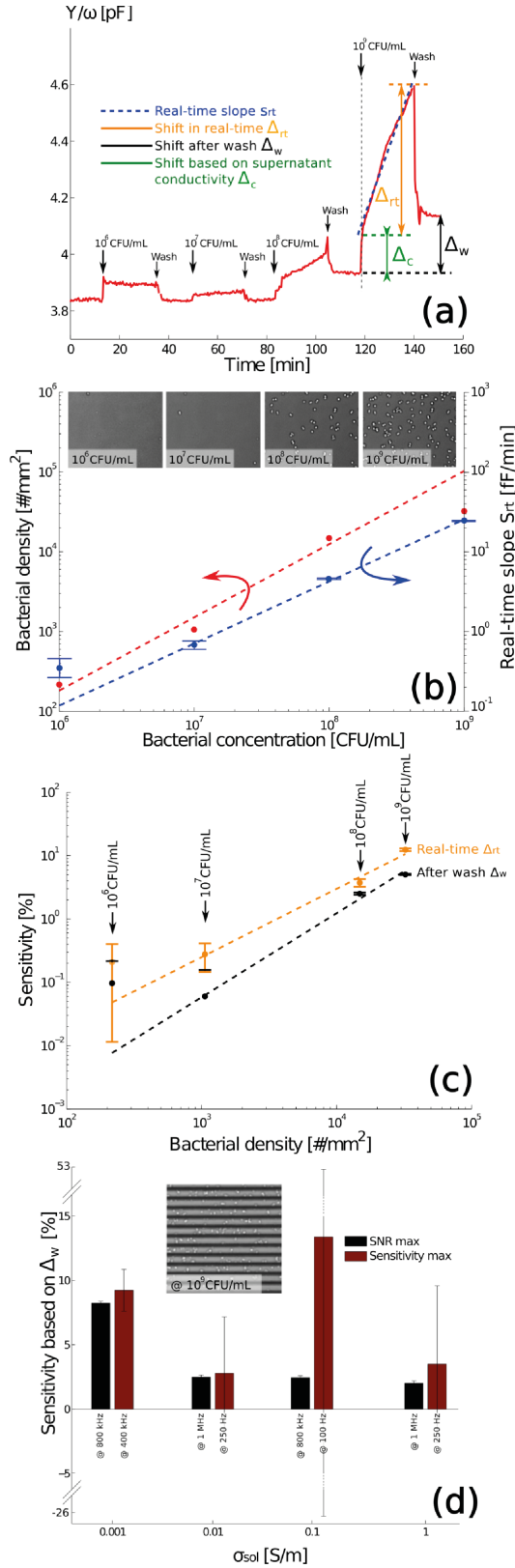


Figure 3: (a) Real time monitoring of the normalized admittance Y/ω at 1 MHz as increasing *S. epidermidis* concentrations from 10^6 to 10^9 CFU/mL in PBS 1:1000 are successively injected. (b) Dependence of the bacterial density measured optically after 20 min incubation and the 1 MHz admittance slope s_{rt} with the bacterial concentration. (c) Dependence of the sensitivity, expressed as the relative change of Y/ω in percent, with the bacterial coverage at 1 MHz for different conditions illustrated in (a). (d) Dependence of the maximal sensitivity and the sensitivity maximizing the SNR with the conductivity σ_{sol} of the buffer used for measurements, for the shift Δ_w and at a fixed bacterial coverage of 3×10^4 cells/mm². Error bars express temporal noise computed from at least 10 successive measurements of corresponding shifts at steady state.

Limit of detection

The intrinsic limit of detection (LOD) of surface-based sensors is defined as the minimum number of adherent bacteria required on the sensor surface to generate a signal Δw five times larger than the noise σ_n . It is possible to express the LOD in term of the bacterial concentration C_b [CFU/mL], which can be linked to the non-saturated bacterial density S_b [number of cells/mm²] by the following formula, for a 20 min incubation time: $\log_{10}(S_b) = -3.25 + 0.92 \log_{10}(C_b)$ (Fig. 3 b). As the noise source from the electrical readout was characterized to $\sigma_n = 1$ fF in optimal conditions, approximately 50 bacteria on the sensor surface are therefore needed to have $\Delta w > 5 \cdot \sigma_n$, corresponding to a bacterial density of 707 bacteria per mm², i.e., 0.06% of surface coverage. From the previous formula, the LOD is approximated to 10^8 (CFU/mL)*min, which means either 10^8 CFU/mL within 1 min of bacterial incubation, or 10^7 CFU/mL within 10 min, or equivalently 10^6 CFU/mL within 100 min.

Effect of buffer salinity

To quantify sensor performance in various conditions of salinity and at a fixed bacterial density of 3.10^4 cells/mm², pre- and post-incubation washes were performed with various dilutions of PBS, whose electrical conductivity spans from 1.4 mS/m to 1.4 S/m (see). For each PBS solutions, the sensitivity $S(\omega) = \Delta w / (Y_0/\omega)$ [%] was computed from Δw and from the initial normalized admittance Y_0/ω . Its mean $\overline{S(\omega)}$ and standard deviation σ_s were obtained through time averaging of at least 10 successive measurements in steady state. At the frequency maximizing the signal-to-noise ratio $\text{SNR} = 20 \cdot \log_{10}(\overline{S(\omega)}/\sigma_s)$ [dB], the sensitivity was shown to decrease from 9% to 2% as the ionic strength increases (Fig. 3 d, black bars). Indeed, the electric field gets more confined in surface and the insulator capacitance dominates (see for more explanation). In contrast, maximal sensitivities without SNR consideration comprise extremely large error bars (Fig. 3 d, red bars), unsuitable for accurate interpretation. In conclusion, the largest sensitivity is achieved with low-salt washing solutions.

Analytical model and simulations

In absence of bacteria, the sensor impedance can be modeled with the insulator capacitance C_{ins} [F], the double layer (DL) capacitance C_{DL} [F], the solution resistance R_{sol} [Ω] and the solution capacitance C_{sol}

[F] (Fig. 4 a). Since PBS 1:1000 has electrical conductivity $\sigma_{sol} \simeq 1.4 \text{ mS/m}$ and relative permittivity $\varepsilon_{r,sol} = 80$, the Debye length λ_D is approximately 32 nm, resulting in $C_D L \simeq 10.C_{ins}$ as the insulator thickness and relative permittivity are $t_{ins} = 30 \text{ nm}$ and $\varepsilon_{r,ins} = 9$, respectively. Therefore, $C_D L$ can be neglected in series and two cutoff frequencies govern, as confirmed experimentally (Fig. 4 b):

$$f_{c,1} = \frac{1}{2\pi R_{sol} \cdot C_{ins}} \approx \frac{1}{2\pi} \frac{t_{ins}}{d} \frac{\sigma_{sol}}{\varepsilon_0 \varepsilon_{r,ins}} \approx 21 \text{ kHz} \quad (1)$$

$$f_{c,2} = \frac{1}{2\pi R_{sol} \cdot C_{sol}} \approx \frac{1}{2\pi} \frac{\sigma_{sol}}{\varepsilon_0 \varepsilon_{r,sol}} \approx 225 \text{ kHz} \quad (2)$$

where d is the electrode gap [m] and ε_0 the vacuum permittivity [F/m]. For applied frequencies $f < f_{c,1}$, C_{ins} dominates while C_{sol} prevails at $f > f_{c,2}$. Between these two cutoff frequencies, the impedance is more resistive due to R_{sol} dominance.

With adherent bacterial cells on the sensor surface, the global impedance was experimentally shown to decrease, highlighting increasing capacitive behavior especially at large frequencies where the sensitivity $S(\omega)$ was maximized (Fig. 4 c). At such frequencies, the cytoplasm conductance dominates the bacterial impedance Z_b [Ω] and shortens electrical field lines, resulting in larger capacitance due to the smaller path. In contrast, the sensitivity is extremely small at low frequencies since C_{ins} is not affected by bacterial cells. As shown in Fig. 4 c, these analytical and experimental considerations were confirmed by finite-element simulations of the 2D-model comprising one bacterial cell (Fig. 4 a), modeled with a Gram-positive two-shell representation whose dielectric values are given in [19].

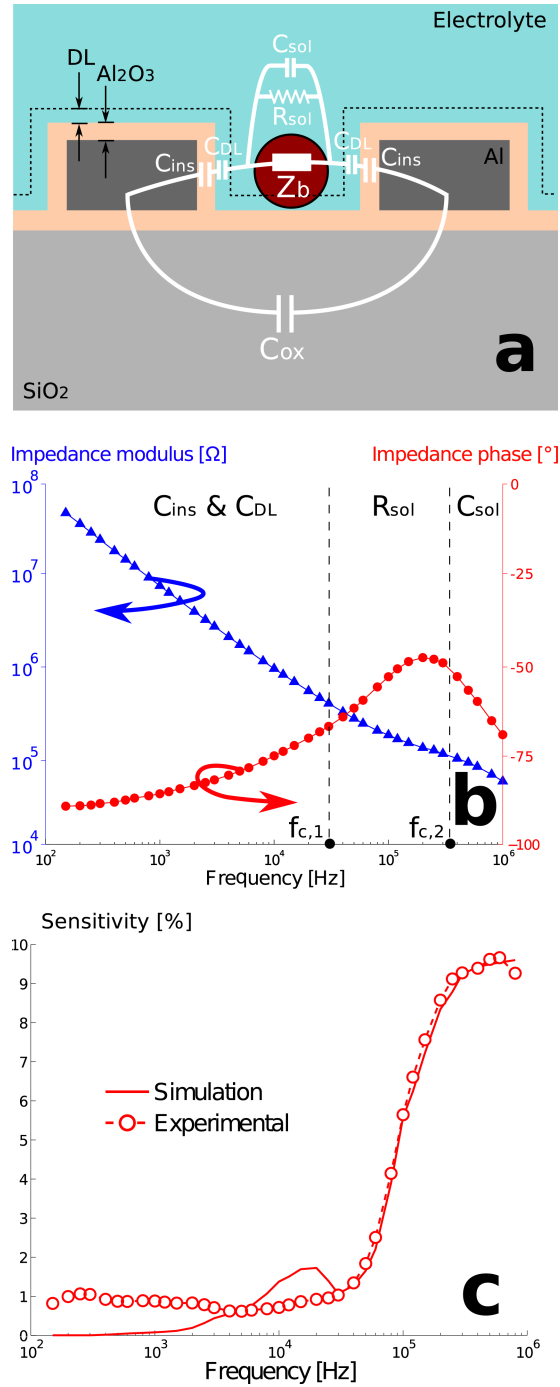


Figure 4: Sensor modeling: (a) Equivalent electrical circuit including bacterial cells, (b) experimental impedance modulus and phase in PBS 1:1000 without bacteria versus the applied frequency and (c) comparison between experimental and simulated sensitivity $S(\omega)$ to *S. epidermidis* versus the applied frequency.

Specific detection of *S. epidermidis* in synthetic urine

To provide bacterial selectivity in complex samples with lytic enzymes, our method involves five steps (Fig. 5 a). First, the matrix sample containing bacterial cells is flown during 20 min on the clean sensor covered with a polydopamine layer. After that, the sensor is washed with PBS 1:1000 to fully remove non-adherent species and enable sensitive and accurate measurements at 1 MHz (see for justification). Then, lytic enzymes are flown during 30 min atop the sensor and selectively lyse target bacteria, if present on the sensor surface. Then, a second wash with PBS 1:1000 is performed to sweep enzymes away before measuring the normalized admittance Y/ω again. A shift in Y/ω occurs between the two measurements only if target bacteria, if present on the sensor surface, have been lysed. An absence of shift means that the surface state with adherent bacteria is unchanged, i.e., target bacteria were missing from the sensor surface.

Fig. 5 b-c depicts the typical evolution of Y/ω at 1 MHz, for both the negative control, i.e., synthetic urine with only *E. faecium*, and the test sample, i.e., synthetic urine with both *S. epidermidis* and *E. faecium*. Only parts in PBS 1:1000 used for wash are shown since low-sensitivity occurs in saline solutions (see), whose out-of-range parts do not interfere with subsequent shifts $\Delta 1$, $\Delta 2$ and $\Delta 3$ measured at equilibrium in PBS 1:1000. As shown by $\Delta 1$ and $\Delta 2$ in Fig. 5 b-c, both adherent *E. faecium* and *S. epidermidis* on the sensor surface strongly affect the normalized admittance thanks to their dielectric properties at 1 MHz (see). Absolute shifts $\Delta 1$ and $\Delta 2$ are significant compared to the temporal noise computed on at least 10 successive measurements, but vary between the three independent sensors because of the different numbers of adherent bacteria (Fig. 6 a). As shifts are normalized by the number of bacteria counted on the sensor surface, *E. faecium* and *S. epidermidis* present reproducible normalized shifts spanning from 52 to 59 aF per bacterium and from 77 to 97 aF per bacterium, respectively (Fig. 6 b). The larger sensitivity to Staphylococci may be explained by their dielectric properties, probably increased by a higher ionic cytoplasmic content thanks to the heavily cross-linked cell wall.

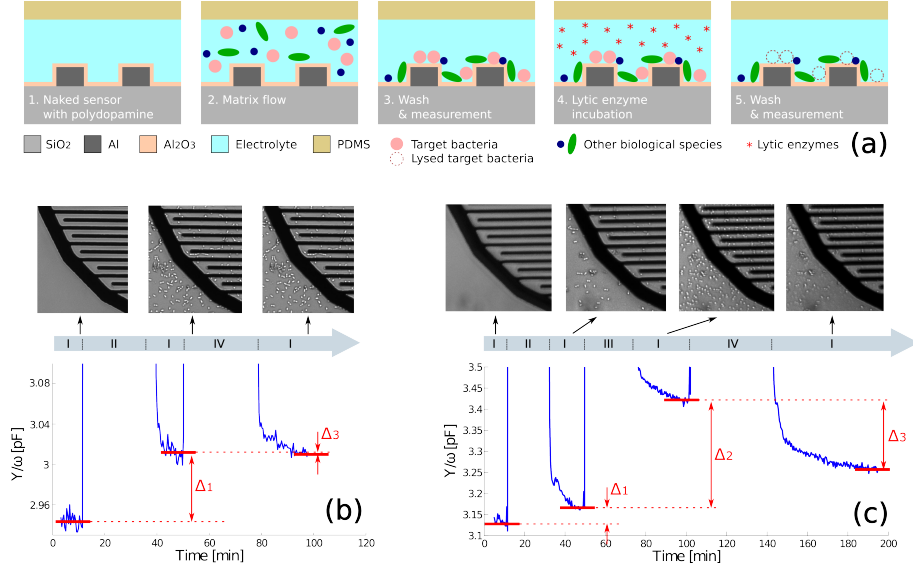


Figure 5: Selectivity method: (a) principle scheme, (b) real-time evolution of the normalized admittance Y/ω at 1 MHz for the negative control, *E. faecium* in synthetic urine, and (c) for the target sample, *S. epidermidis* and *E. faecium* in synthetic urine. The events mentioned in the timeline are: (I) washing with PBS 1:1000, (II) incubating 5.10^8 CFU/mL *E. faecium* in synthetic urine, (III) incubating 5.10^8 CFU/mL *S. epidermidis* in synthetic urine and (IV) incubating lysostaphin. Shifts after wash Δ_1 , Δ_2 and Δ_3 are evaluated after the addition of *E. faecium*, *S. epidermidis* and lytic enzymes, respectively.

After the incubation of lysostaphin, i.e., a lytic enzyme that specifically digests *Staphylococcus* spp., sensors with only *E. faecium* on the surface showed a slight decrease $\Delta_3 \in [2 \text{ fF}, 21 \text{ fF}]$ of the normalized admittance, despite intact *E. faecium* cells as observed optically (Fig. 5 b and 6 a). This slight drop can be attributed to enzymes binding onto the polydopamine layer, which typically sticks any biomolecules containing aromatic rings. On the other hand, sensors covered with *S. epidermidis* showed a significantly larger decrease $\Delta_3 \in [163 \text{ fF}, 299 \text{ fF}]$ of Y/ω (Fig. 6 a), whose larger variability results from the different amounts of lysed *S. epidermidis* on the sensor surface, for each experiment. As Δ_3 is divided by the number of lysed bacteria, a reproducible normalized shift spanning from 68 to 88 aF per lysed *S. epidermidis* is obtained. Despite the almost complete destruction of *S. epidermidis* cells, the sensor does not recover its initial impedance value with *E. faecium* only. Our interpretation is that cellular debris (DNA, proteins and peptidoglycan)

remains on the sensor surface after cell lysis and interacts with the electric field to cause measurable shifts in Y/ω .

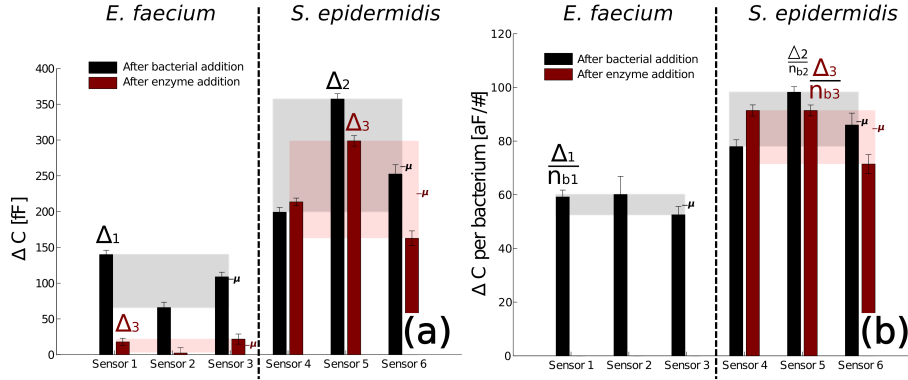


Figure 6: Comparison of impedance measurements on *E. faecium* (negative control) and *S. epidermidis* (target) at 1 MHz and for three different sensors in each case: (a) absolute capacitive shifts ΔC after *E. faecium*/*S. epidermidis* binding (in black) and after enzyme incubation (in red), (b) relative capacitive shifts normalized to the number of bound bacteria after incubation or to the number of destroyed bacterial cells after lytic enzyme incubation. Error bars at each block refer to temporal noise obtained after averaging at least 10 successive temporal measurements on one sensor in a steady-state window. Translucent boxes depict the measurement span in a given condition, whose μ corresponds to the mean value of the 3 sensors.

Advantages and limitations

The selective method of Section presents several advantages among others. First, the matrix can directly be flown on the sensor without any pre-treatment steps such as centrifugation or dilution. The possible presence of large cells (diameter $> 10 \mu\text{m}$) in the matrix is likely not a problem since they should be washed away by strong shear forces at flow rate of $250 \mu\text{L}/\text{min}$, as confirmed optically with large bacterial cell clusters. A filtration method, e.g., membrane at the channel inlet, could alternatively be added to filter them out.

Second, in contrast to affinity-based surface sensors, our selective method does not suffer from background noise due to non-specific binding of biomolecules on the sensor surface, which strongly facilitates its use in real matrix samples. Compared to antibodies, the use of lytic enzymes can be easily extended to all Gram-positive bacteria (using en-

dolysins) and strongly reduces cost at a large-scale production, since peptidoglycan hydrolases can be obtained by fermentation. The risk of false negatives due to mutation is also relatively low, because peptidoglycan hydrolases target highly conserved bonds in the bacterial cell wall.

Third, washing with low-conductivity media enables the stable and sensitive monitoring of intrinsic dielectric properties of adherent bacteria, avoiding time-consuming centrifugation required for ion release based techniques. Furthermore, the electrical conductivity of the bacterial sample does not need to be controlled, as it is preferably the case for ion release technique.

Finally, since the electrode materials ($\text{Al}/\text{Al}_2\text{O}_3$) are CMOS-compatible, the method can be extended to microelectrodes patterned in the last metal layer of a CMOS circuit, benefiting from system miniaturization and low manufacturing cost, both essentials for LoC applications.

On the other hand, our method exhibits several limitations. First, great care with PBS 1:1000 wash is needed since ionic contamination can easily occur and provide false positives. The fluidic setup must be optimized to avoid any cross contamination from saline to non-saline buffers.

Second, osmotic-sensitive bacteria may burst during an osmotic shock. In this case, the sensor would provide false positives during the rinsing procedure if burst bacteria are not those targeted. Due to their thick cell wall ($\sim 30 - 50$ nm), Gram-positive bacteria are less subject to this phenomenon than Gram-negative bacteria characterized by a 2-8 nm-thick cell wall. To resolve the two aforementioned issues, a higher ionic medium could be used for measurements, but the electrical frequency should be increased accordingly to keep the same sensitivity.

Third, reproducible cell adhesion on the sensor surface is crucial to avoid false negatives, in the case bacteria do not adhere to the surface. To address this problem, a polydopamine layer was used in this work to improve and homogenize bacterial binding in saline and non-saline buffers. Though other coatings could also provide adequate or better results, they should be investigated in a separate study. Furthermore, the proposed method could be combined with well-known concentration techniques such as dielectrophoresis [12] or magnetic beads [13], to enhance sensitivity and decrease detection time.

Finally, a comparison with other significant works dealing with impedimetric detection of bacteria in solution is provided in Table S1!! and supports our conclusions.

Conclusion

In this paper, an innovative selective method for impedimetric detection of bacterial cells was presented. It is based on the use of lytic enzymes to selectively destroy target bacteria, anchored on the sensor surface after the flow of a treatment-free sample matrix. This principle was demonstrated by selectively detecting *S. epidermidis* in synthetic urine samples also containing *E. faecium*, as a model for real urinary infections. This method is a step towards Lab-on-Chip (LoC) and Point-of-Care (PoC) systems, since it enables the direct flow of the sample matrix and the integration with CMOS readout circuit on the same chip. Furthermore, the selectivity principle can easily be extended to all Gram-positive bacteria using bacteriophage lysins, which have similar enzymatic activity as lysostaphin used in this paper. Finally, real-time detection of *S. epidermidis* resuspended in low-ionic buffer was also investigated, enabling key comprehension for sensor optimization. All electrical interpretations were confirmed by real-time simultaneous optical monitoring of the sensor surface thanks to an innovative setup.

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Supplementary Information

Protocols for chemical and reagents for section

PBS tablet, creatinine, Na_2HPO_4 , CaCl_2 , dopamine hydrochloride and lysostaphin were purchased at Sigma-Aldrich (Diegem, Belgium). Urea, KCl, MgSO_4 and Tris were purchased at Merck (Overijse, Belgium). NaCl, NaHCO_3 , NaCl and glycerol were purchased at VWR (Leuven, Belgium). $(\text{NH}_4)_2\text{SO}_4$ was purchased from UCB (Brussels, Belgium).

0.01 M phosphate buffer saline (PBS) at pH 7.4 was obtained by diluting one tablet in 200 mL of milliQ water. PBS 1:1000, PBS 1:100 and PBS 1:10 were prepared by adequately diluting PBS in milliQ water by volume. PBS and all PBS dilutions were autoclaved 15 min at 121°C before use. Tryptic Soy Broth (TSB), Tryptic Soy Agar (TSA) and Lysogeny broth (LB) were prepared standardly and autoclaved 15 min at 121°C .

Synthetic urine was made as follows (w/v): urea 2%, KCl 1.2%, creatinine 0.1%, $(\text{NH}_4)_2\text{SO}_4$ 0.1%, Na_2HPO_4 0.1 %, MgSO_4 0.01%, CaCl_2 0.01% and NaHCO_3 0.01%. These compounds were diluted in 1 L of milliQ water, the pH was then adjusted at 7 using 1M HCl. Synthetic urea was sterilized using $0.22\text{ }\mu\text{m}$ porosity Millipore filters in PES (Filter service, Eupen, Belgium) before use.

A Tris-NaCl buffer contains Tris 50 mM, NaCl 150 mM dissolved in milliQ water, adjusted to pH=9.2 with HCl 1 M and autoclaved prior to use. The polydopamine solution was prepared by dissolving 4 mg/mL of dopamine hydrochloride in the Tris-NaCl buffer. The solution was left 1 hour in the dark to polymerize before being injected in the microfluidic channel.

Commercialized lysostaphin, sold as 1 mg powder where 55% of the total mass is lysostaphin, was diluted in 1 mL of PBS supplemented with 30% of glycerol, and transferred into 50 μL aliquots stored at -20°C . Working solutions were prepared by adding 950 μL of PBS to 50 μL of stock solution (20 μM) yielding a 1 μM lysostaphin.

Protocols for bacterial strains, culture, suspension preparation

S. epidermidis ATCC 35984 and *E. faecium* ATCC 19434 (both purchased from LGC standards, Molsheim, France) were used as reference Gram-positive bacteria in this study. Following overnight culture on TSA plates at 37°C, liquid cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of TSB. Stationary phase cultures were obtained after overnight incubation at 37°C at 120 RPM.

Two 2mL tubes of stationary phase culture of *E. faecium* were centrifuged 10 min at RT at 5500 g. The supernatant was then discarded and both pellets were resuspended in 1 mL of synthetic urine. The centrifugation, supernatant removal and resuspension steps were repeated twice for both tubes. Suspensions from both tubes were pooled and the optical density (OD_{600nm}) was adjusted to $OD_{600nm}=1$ (5×10^8 CFU/mL). For stationary-state *S. epidermidis*, the same protocol was used but adjusted to $OD_{600nm} = 3$ ($\sim 10^9$ CFU/mL) to $OD_{600nm} = 0.003$ (10^6 CFU/mL), by adequately diluting the concentrated suspension in PBS 1:1000.

For exponential-state *S. epidermidis*, an exponential phase culture was obtained by inoculating 50 mL of fresh medium with 500 μ L of the stationary phase culture and incubating at 37°C, 120 RPM until an OD_{600nm} of 0.5 was reached. One 50 mL tube was then centrifuged 10 min at 4°C, 5500 g and the pellet was resuspended in 2 mL of synthetic urine. This step was repeated two times in two 2 mL tubes. The suspension was adjusted to an $OD_{600nm}=1$ (5×10^8 CFU/mL) in order to be injected into the setup.

The bacterial concentration was determined by plate count technique and expressed in colony forming units per milliliter (CFU/mL).

Simultaneous electrical and optical monitoring

As shown in Fig. S1 a, the simultaneous and real-time optical and electrical monitoring of surface events demonstrates a linear correlation between the bacterial surface coverage [number of cells/mm²] and the normalized admittance Y/ω [pF]. 3D simulations performed in [18] confirm this linear correlation, as shown in Fig. S1 b.

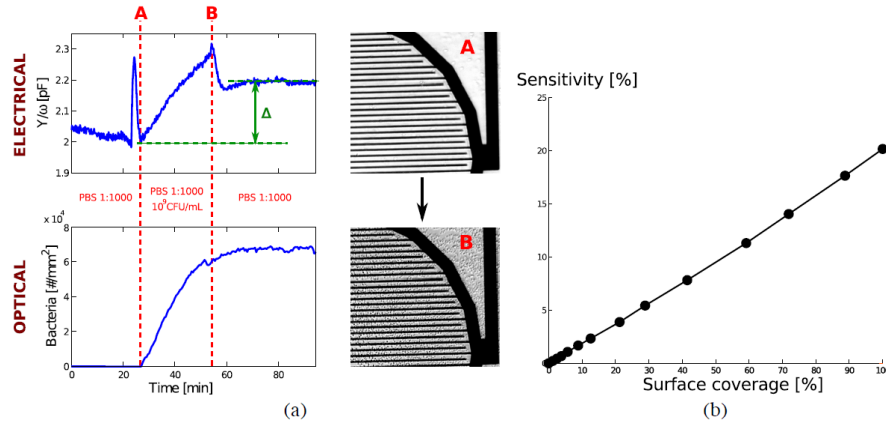


Figure S1: (a) Experimental simultaneous electrical and optical monitoring of *S. epidermidis* binding in real-time on the sensor surface. The events A and B correspond to the start and the end of the 10⁹ CFU/mL flow on the sensor surface, respectively. (b) Simulated sensitivity versus the bacterial surface coverage [18].

Comparison with state-of-the-art

A comparison with other significant works implementing growth-independent impedimetric detection of bacterial cells in solution is provided in Table S1. This summary strengthens previously mentioned advantages and limitations of the innovative method proposed in this work: first use of lytic enzymes as a selectivity means, working in mimicked matrix samples, CMOS compatibility, and capability to provide a LoC and PoC diagnostic tool.

Table S1 - Comparison of state of the art

	(Radke and Alodijia, 2005) [11]	(Vashiney, 2007) [13]	(Laczka et al., 2008) [20]	(Yang, 2008) [14]	This work
Bacterial species	<i>E. coli</i>	<i>E. coli</i>	<i>E. coli</i>	<i>Salmonella enterica</i>	<i>S. epidermidis</i> and <i>E. faecium</i>
Suspension medium	Romaine Lettuce	Ground beef	PBS	DI Water	Synthetic urine
Matrix pretreatment	Yes*	Yes*	Yes	Yes	No
Labeling	Label-free	Ab-coated magnetic nanoparticles	Label-free	Ab-coated magnetic beads	Label-free
Sample load and volume	Immersed (30 mL)	Pipetting (2 μ L)	Pipetting (1mL)	Closed chamber (25 μ L)	Microfluidic (27nL above sensor)
Selectivity	Ab on surface	Ab in volume	Ab in surface	Ab in surface	Lytic enzymes
Electrode material	Gold	Gold	Gold	Gold	Al ₂ O ₃
CMOS compatible?	No	No	No	No	Yes
LOD	10 ⁷ CFU/ml	8x10 ⁵ CFU/ml	5x10 ⁴ CFU/ml	3x10 ⁶ CFU/ml	10 ⁸ CFU/ml x min
Intrinsic sensitivity	NA	NA	NA	NA	~ 82 aF / bacterium
Total detection time**	70 min	52 min	50 min	46 min	(50 + 10 ⁸ / C ₀) min ***
Working range	NA	8x10 ⁵ to 10 ⁸ CFU/ml	5x10 ⁴ to 10 ¹⁰ CFU/ml	10 ⁶ to 10 ¹⁰ CFU/ml	10 ⁶ to 10 ⁹ , in 3 h

Abbreviations : Ab = Antibodies, DI = deionized, NA = Not-Available.

* Centrifugation, dilution and stomacher steps included

** Includes all pretreatment steps

*** 50 min is the necessary time for enzyme incubation and wash, C₀ refers to the bacterial concentration

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Ionic strength dependent aggregation of *Staphylococci*

In this chapter, a yet unreported type of aggregation of *Staphylococci* is presented. Several strains of *S. aureus* and *S. epidermidis* displayed fast and reversible intercellular adhesion that solely depends on the ionic strength of the external medium. Interestingly, an optimal ionic strength could be identified for each strain. These experiments demonstrate that *Staphylococci* can react to small variations of salt concentration in their environment. Besides, this ionic-strength dependent aggregation has been shown to significantly accelerate biofilm formation of *S. aureus*. Plausible mechanisms leading to aggregation are also discussed here. Finally, the Biostream device, a flow cell that was developed during this PhD thesis (see Appendix B), was used to highlight the consequence of the aggregative phenotype on biofilm formation by *S. aureus*.

An interesting question raised by this work is whether the observed phenomenon has an evolutionary purpose or if it is the undesired consequence of the composition of the bacterial cell wall.

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Come together: ionic strength modulates cell aggregation and biofilm formation in *Staphylococci*

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Abstract

Cell aggregation plays a key role in biofilm formation and pathogenesis of *Staphylococcus* species. Bacterial aggregates and biofilms are more resistant than isolated planktonic cells to antibiotics, disinfectants and host immune system. While the molecular basis of aggregation in *Staphylococci* has already been extensively investigated, the influence of environmental factors, like ionic strength, remains poorly understood. In this paper, we report a new type of cellular aggregation of *Staphylococci* that depend solely on ionic strength with a strain-dependent optimum. Seven strains out of 14, all belonging to Staphylococcal species, formed large cell clusters within minutes in buffers of ionic strength ranging from 1.5 to 50 mM whereas three other isolates belonging to Gram-positive species did not show the same phenotype. The optimal ionic strength for aggregation was found to be strain-dependent but in all cases, bacterial aggregates formed at an ionic strength of 1.5-50 mM were immediately dispersed in a solution of higher ionic strength, indicating reversibility of the cell aggregation process. In addition, this phenotype was shown to significantly promote biofilm formation. Indeed, *S. aureus* ATCC6538, a strain displaying the aggregative phenotype, displayed enhanced ability to form biofilms when grown in a medium (50 mM) rather than high (150 mM) ionic strength, indicating that ionic strength-dependent aggregation promotes biofilm formation. These findings suggest that some staphylococcal isolates can respond to ionic strength as an external stimulus to trigger rapid cell aggregation and biofilm formation in a way that has not yet been reported.

Importance

Staphylococci are amongst the leading pathogens causing nosocomial infections. During the development of such infections, these bacteria rely upon cell aggregation and biofilm formation to increase their virulence and especially their persistence inside the host. Understanding how environmental factors influence these processes is crucial to apprehend the behavior of the pathogens. In this work, we highlight the dramatic impact of ionic strength on intercellular adhesion in *Staphylococci* and its consequences on biofilm formation. At optimal ionic strength staphylococcal cells rapidly aggregate and revert back to a dispersed state as the ionic strength is modified. This suggests that the mechanism involves

electrostatic interactions between cell surfaces and not direct genetic regulation. The unique aggregative phenotype reported here provides an ecological advantage to *Staphylococci* as they would significantly accelerate the formation of mature biofilms.

Introduction

Staphylococcus spp. is a group of commensal bacteria colonizing animal skin and mucosa [1], among which the two notorious pathogens *S. aureus* and *S. epidermidis*. While the former is mainly responsible for acute infections [2] and wound inflammation [3], the latter, along with other coagulase negative *Staphylococci*, is often involved in nosocomial diseases associated with implanted devices [4], such as intravascular and urinary catheters [5] [6] or prostheses [7] [8]. In many cases, *Staphylococci* form biofilms, a lifestyle that increases their persistence in the host [9] [10]. Indeed, biofilms effectively shield bacteria from threats such as the host immune system [11] [12], antimicrobial peptides [13] or antibiotics [14].

Cell aggregation, whether reversible or irreversible, is crucial in the process of building up micro- and macro-colonies during biofilm formation [15]. Cell aggregation can be mediated by surface proteins that are involved in ligand-receptor recognition or in homophilic interactions between molecules present on the surface of two bacteria. For example, SasG and Aap proteins have been found to be responsible for Zn^{2+} -dependent intercellular aggregation by forming dimers of fibrils bridging two bacterial cells in *S. aureus* [16] and *S. epidermidis* [17] [18], respectively. Also, in *S. aureus*, the clumping factor A (ClfA) has been shown to trigger agglutination by catalyzing the transformation of fibrinogen into fibrin upon which bacterial cells aggregate in large clusters [19]. In addition, non-specific intercellular adhesion can also occur through attractive electrostatic or hydrophobic interactions, or through macromolecular bridging associated with cell surface polymers. The intercellular adhesin, also called PIA (Polysaccharide Intercellular Adhesin or poly-N-acetylglucosamine) has been shown to increase cell-cell stickiness and make cell aggregation irreversible [20] [21]. Another example is the *S. aureus* Biofilm-Associated Protein (Bap) and its homolog Bhp of *S. epidermidis*, a cell surface protein that plays a key role in the formation of biofilm in PIA deficient strains [22] [23].

Although progress has been made in unraveling the molecular basis of cell aggregation of *Staphylococci*, the impact of environmental parameters on intercellular adhesion remains largely unknown. In this paper, we report a novel phenomenon in staphylococcal cell aggregation in which ionic strength tightly modulates the aggregation phenotype. Several strains belonging to the *Staphylococcus* genus readily formed large cell clusters in response to changes in NaCl concentration. This aggregative phenotype was found to be reversible in a short time scale as the ionic strength was increased, pointing towards an electrostatic origin for the intercellular affinity. As a consequence, biofilms were found to develop significantly faster and displayed a more complex three-dimensional architecture. The impact of our findings on the development of nosocomial infections associated with implanted medical devices is also discussed.

Materials and Methods

Buffers

Cell aggregation experiments were conducted in Tris 0.1 mM (pH 7.4) with increasing concentrations in NaCl to obtain buffers with ionic strengths ranging from 0.1 to 150 mM. The buffer conductivity and pH were measured before performing the experiments as a control of the ionic strength and pH of the solutions, respectively.

Bacterial strains and culture

The bacteria used in this study were the reference strains *S. aureus* ATCC 6538, *S. epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, *S. epidermidis* HB [24], *Enterococcus faecium* ATCC 19434, and *Streptococcus salivarius* subsp. *thermophilus* ATCC 19258. The clinical strains *S. aureus* MRSA1, MRSA2 and MRSA3, *S. epidermidis* C1, C2, C3 and *Staphylococcus warneri* C4 were isolated from positive blood cultures (Brussels Saint-Luc University hospital, UCL, Woluwé-Saint-Lambert, Belgium). Three environmental isolates, all identified by 16S DNA sequencing, were also used in this study: *S. aureus* strains E1 and E2, both found in food products, *Staphylococcus hominis* E3 and *Micrococcus luteus* E4 originating from air sampling.

All strains were plated on Tryptic Soy Agar (TSA, BioRad) from frozen stock kept at -80°C and incubated at 37°C for 24 h. Liquid cultures were

carried out in 100 ml Erlenmeyer flasks containing 20 ml TSB (BioRad) inoculated from the plates and placed in a shaker-incubator at 37°C and 120 rpm for 16 h.

Quantification of cell aggregation by spectrophotometry

Cell aggregation was assessed by measuring the Optical Density at 600 nm (OD_{600}) of bacterial suspensions prepared in buffers with increasing ionic strength. A total of 7 ml of an overnight culture were recovered in 2 ml Eppendorf tubes after centrifugation at 4°C, 5000 g for 5 min. The successive supernatants were discarded and the pellets were resuspended in 1 ml of appropriate buffer. Centrifugation and resuspension steps were carried out three times to remove most components of the initial culture medium. The seven bacterial suspensions in the buffers of increasing ionic strength were transferred into PMMA cuvettes (VWR) and the OD_{600} was recorded using a spectrophotometer (Ultrospec 2100 pro). The cuvettes were incubated at 30 °C for 90 min and OD_{600} was measured again. This protocol was conducted in triplicates for the seventeen strains using independent overnight cultures.

The ratio R was defined as follows:

$$R = \frac{OD_{600}(t=90min)}{OD_{600}(t=0min)}$$

While a ratio of 1 (no visible aggregation) indicates the stability of the bacterial suspension, its reduction assesses the sedimentation of aggregated bacteria in the suspension. For strain ATCC 12228 of *S. epidermidis*, the kinetics of the aggregation phenomenon was followed by measuring OD_{600} every 10 min. This experiment was repeated three times with independent cultures.

Cell aggregation in parallel plate flow chamber

In order to assess the reversibility of the aggregative phenotype under changing ionic strength, flow cell experiments were carried out.

For these experiments, two bacterial suspensions of *S. epidermidis* ATCC 12228 were prepared in the following buffers: Tris 0.1 mM, NaCl 5 mM and in Tris 0.1 mM, NaCl 150 mM. Their bacterial concentrations, assessed by plate count of serial dilutions on TSA, were estimated to be ca. 5×10^8 CFU/ml.

A sterilized parallel plate flow cell (Biostream [25]) of 16 mm x 45 mm x 1 mm in size featuring a culture treated PolyStyrene (PS) slide was mounted on an inverted microscope (Leica DMI6000) at room temperature. Images of the surface of the PS slide were taken by a 40 x long working distance objective (numerical aperture=0.6, Fluotar, Leica) and a sensitive 1.4 MP cooled CCD B/W camera (Leica DFX 365). The flow cell system was filled with a Tris 0.1 mM, NaCl 150 mM buffer, using a peristaltic pump (Minipuls, Gilson) to remove the air from the tubing.

To conduct the experiment, the higher ionic strength inoculum was first injected for 5 min at a flow rate of 20 ml/h and a hydraulic retention time (Volume of the flow chamber [mm³]/Flow rate [mm³/s]) of 1.5 min. Rinsing was performed during 10 min with Tris 0.1 mM, NaCl 150 mM. Then, the lower ionic strength inoculum was injected in the flow cell, also for 5 min according to the same procedure. Loosely bound bacteria were removed by rinsing 10 min with the Tris 0.1 mM, NaCl 5 mM buffer at the same flow rate. Finally, the higher ionic strength Tris 0.1 mM, NaCl 150 mM buffer was flowed again in the flow cell for 10 min without changing the flow rate.

Atomic Force Microscopy (AFM)

The surface of the *S. epidermidis* strain ATCC 12228 was probed by AFM with a negatively charged tip in order to highlight the amount and the repartition of positive charges on the bacterial cell surface.

For chemical force microscopy (CFM) analyses, carboxyl-terminated AFM tips were prepared by immersing gold-coated cantilevers (OMCL-TR4, Olympus Ltd., Tokyo, Japan; nominal spring constant ~ 0.02 N m⁻¹) for 12 h in ethanol solutions containing 1 mM of (16-mercaptohexadecanoic acid) (Sigma) and (11-mercapto-1-undecanol) (Sigma) at a molar ratio of 10:90, and then rinsed with ethanol and finally dried with N₂. CFM measurements were performed at room temperature (20 °C), in TRIS buffer at three increasing ionic strengths (0.1, 5 and 150 mM NaCl), using a Nanoscope VIII Multimode AFM from Bruker Corporation (Santa Barbara, CA). The spring constants of the cantilevers were measured using the thermal noise method. Cells from the *S. epidermidis* strain ATCC 12228 were immobilized by mechanical trapping into porous polycarbonate membranes (Millipore, Billerica, MA) with a pore size similar to the cell size [26]. After filtering a cell suspension, the filter

was gently rinsed with buffer, carefully cut (1 cm x 1 cm), attached to a steel sample puck using a small piece of double face adhesive tape, and the mounted sample was transferred into the AFM liquid cell while avoiding de-wetting. Single cells were first localized using a bare tip, after which the tip was replaced with a carboxyl-terminated tip for force measurements. Force maps of 32 x 32 force-distance curves were recorded on areas of 1 μm^2 with a maximum applied force of 500 pN, a contact time of 100 ms, and an approach and retract tip velocity of 1,000 nm s⁻¹. Adhesion maps and histograms were obtained by calculating the largest adhesion force for each force curve.

Biofilm formation in parallel plate flow chamber

Biofilm formation of *S. aureus* strain ATCC 6538 was assessed in two media, with distinct ionic strengths but equal amount of nutrients. The tests were conducted in two Biostream flow cells, run in parallel, containing culture-treated PS slides (Ted Pella, Inc.). Media were prepared by diluting TSB 3-fold either with Tris 0.1 mM or Tris 0.1 mM, NaCl 150 mM, displaying conductivities of ca. 5.5 and 15.5 mS/cm, respectively. Two inocula were prepared by diluting bacterial suspensions, prepared in Tris 0.1 mM, 50 mM NaCl and Tris 0.1 mM, 150 mM NaCl, 100-fold in the same buffers. The conductivities of inocula were of ca. 5.5 and 15.5 mS/cm, respectively. The bacterial concentration of the inocula was ca. 5×10^7 CFU/ml, as estimated by plate count of serial dilutions on TSA. The two Biostream flow cell systems were put at 37°C and first filled with the buffer of appropriate ionic strength before the inocula were injected for 10 min at a flow rate of 20 ml/h. Then, the media were flowed each in one of the flow chambers for 6 h. To representatively assess biofilm growth, the surface of the PS slide was imaged in 6 randomly chosen zones in both flow cells, after 2, 4 and 6 h. After 4 h, the maximum height of micro-colonies, further referred to as biofilm thickness, was measured using the Z-stack function on the dedicated microscope software (Leica LAS AF). After 6 h, the flow of media was stopped and both flow cells were rinsed with Tris 0.1 mM, NaCl 150 mM for 15 min at the same flow rate. A 125 $\mu\text{g}/\text{ml}$ solution of Concanavalin A coupled to AlexaFluor350 (Invitrogen Life Technologies) was injected in both flow cells. This lectin marks polysaccharides of the biofilm EPS. The lectin stain was left to incubate without flow at room temperature, in the dark, for 15 min. A rinsing step of 15 min at a flow rate of 20 ml/h with a Tris 0.1 mM, NaCl 150 mM buffer was then performed. Finally, the flow cell

surfaces on which the biofilms grew were observed in bright-field, as well as in fluorescence microscopy with a 40 x long working distance objective, a metal-halide lamp for excitation, filters tailored for DAPI fluorescence (Leica A4 fluorescence cube). These experiments were repeated three times, with inocula prepared from independent cultures.

Statistical analysis

Standard deviation was computed for all data. When relevant, Student T-tests were performed to assess whether two mean values were significantly different, assuming normal distribution of our data and homogeneity of variance.

Results

NaCl concentration induces fast intercellular aggregation of some staphylococcal isolates

As shown in Fig. 1a, two suspensions of *S. epidermidis* ATCC 12228 after 90 min of incubation at 30 °C displayed strikingly different sedimentation depending on the ionic strength of the buffer. In the Tris 0.1 mM supplemented with 5 mM NaCl buffer, the suspension has significantly cleared and an accumulated biomass, referred to as pellet in Fig. 1, is visible at the bottom of the cuvette, whereas the suspension in the 150 mM NaCl buffer remained homogeneously dispersed. The kinetics of this behavior is shown in Fig. 1b, where the evolution of the OD₆₀₀ of *S. epidermidis* ATCC 12228 in Tris 0.1 mM buffers with 0, 5 and 150 mM of NaCl is reported. While the suspension in Tris 0.1 mM buffer with 5 mM NaCl started to clear after 20 min and continued until 90 min, the two other suspensions remained stable. The rapid clearance results from the formation of large cell clusters sedimentation, as observed microscopically with *S. epidermidis* ATCC 12228 suspensions (data not shown). Large aggregates of staphylococcal cells could be seen in the 5 mM NaCl buffer after 30 min whereas the cells in 150 mM remained well separated.

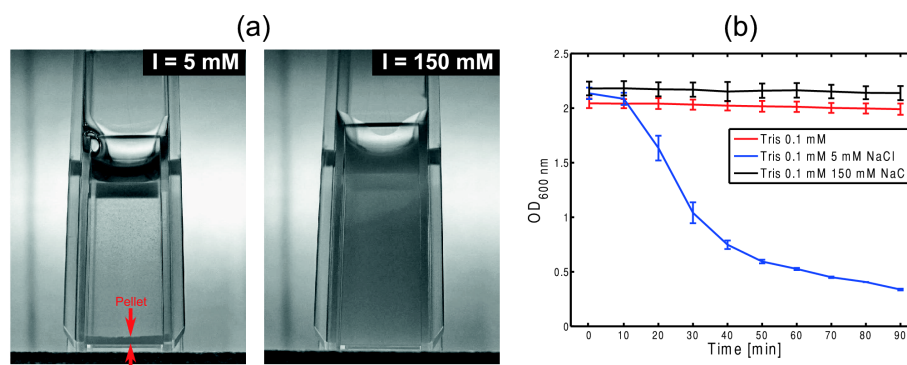


Figure 1: Macroscopic observation of the ionic strength (I) dependent aggregative phenotype. **(a)** The cuvettes contain *S. epidermidis* ATCC 12228 suspended in 0.1 mM Tris buffer with 5 mM NaCl ($I = 5$ mM, left) and in 0.1 mM Tris with 150 mM NaCl ($I = 150$ mM, right), respectively. Suspensions were initially adjusted to the same optical density and the images were taken after 90 min of incubation at 30°C. The suspension in the 5 mM NaCl buffer has sedimented, forming a 2 mm thick pellet at the bottom (arrows). On the contrary, on the right, the suspension in 150 mM NaCl remains homogeneously dispersed. **(b)** The OD_{600} of three suspensions of *S. epidermidis* ATCC 12228, in $I = 0.1$ mM (red line), $I = 5$ mM (blue line) and $I = 150$ mM (black line) buffers, were monitored every 10 min for 90 min. Turbidity of the suspension in $I = 5$ mM rapidly decreased unlike the suspensions in 0.1 mM and 150 mM. Error bars express the standard deviation obtained from the results of three independent experiments.

Occurrence of the ionic strength dependent cell aggregation

The occurrence of the observed aggregative phenotype was determined on a panel of 17 strains including: six *S. aureus*, four *S. epidermidis*, four other coagulase negative *Staphylococci*, one *E. faecium*, one *M. luteus* and one *S. salivarius*. Suspensions were prepared from stationary phase cultures, in seven Tris 0.1 mM buffers with ionic strength of 0.1, 0.5, 1.5, 5, 15, 50 and 150 mM (NaCl). The OD_{600} of these suspensions were measured when they were prepared ($t = 0$) and after 90 min of incubation at 30 °C. The results are expressed as a ratio R obtained by dividing the OD_{600} after 90 min by the initial OD_{600} of the suspension. All strains were tested in triplicates. Fig. 2a presents a graph of R measured for strain *S. epidermidis* ATCC 12228 in all seven Tris 0.1 mM buffers with increasing ionic strengths. The curve presents an optimum of ionic strength, at which R achieves a minimum value. At this opti-

mal ionic strength, intercellular aggregation took place and pellets could systematically be observed at the bottom the cuvettes (see Fig. 1a). At both high and low ionic strengths, R values were close to 1, meaning that the suspensions were stable and that only slow sedimentation occurred, with no or few cell aggregation.

In order to quantitatively assess the occurrence of the aggregative phenotype, two criteria were defined. The first is the range of ionic strengths in which aggregation is the most pronounced. It was defined as the ionic strengths in which minimal R values were noticed. In other words, this range corresponds to the spread of the minima in experimental curves, as shown in Fig. 2a. The second is the maximal amplitude (1-R) of the OD₆₀₀ drop after 90 min within the range of ionic strength previously defined. Fig 2b and 2c display the range of ionic strengths and the amplitude, respectively, for the seventeen strains tested in this study.

Out of a total of seventeen strains, seven strains (three *S. aureus*, three *S. epidermidis* and one coagulase-negative *Staphylococcus* - CoNS) were shown to dramatically aggregate at some ionic strengths. For these seven strains, an optimal ionic strength where aggregation occurs could always be identified. In buffers of 150 and 0.1 mM ionic strength, suspensions of these seven strains remained well dispersed as indicated by the R values close to 1, i.e. amplitude values close to 0.

The seven other isolates belonging to *Staphylococcus* spp., representing 50 % of staphylococcal strains tested in this work did not aggregate in these conditions. Three other Gram-positive cocci were also tested (*E. faecium*, *M. luteus* and *S. salivarius*) but did not present any sign of aggregation, in any of the buffers.

Amongst the seven staphylococcal isolates that positively reacted to the test, two facts can be noticed. First, all three *S. epidermidis* isolates significantly aggregate in a broad range of ionic strengths whereas the *S. aureus* and the CoNS strains were characterized by a single point minimum at which aggregation is optimal (Fig. 2b). Secondly, *S. aureus* ATCC 6538 showed an optimal aggregation in the buffer containing 50 mM NaCl, an ionic strength close to that of physiological buffers.

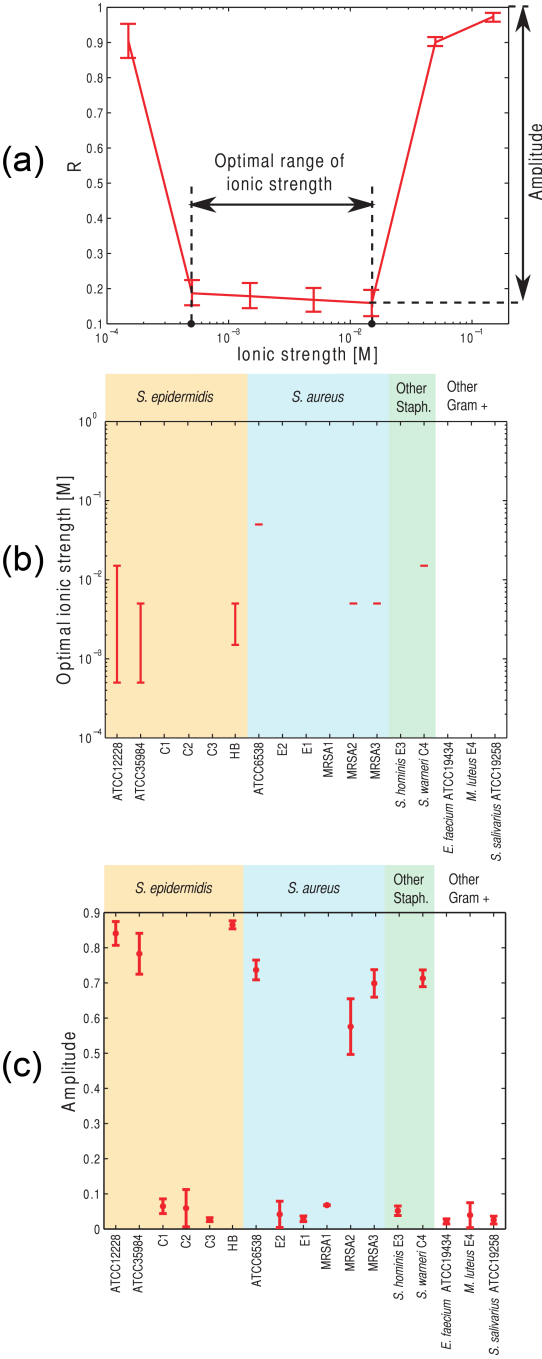


Figure 2: Dependence of the aggregative phenotype on the ionic strength. (a) Plot of the ratio $R = OD_{600}(t = 90 \text{ min}) / OD_{600}(t = 0 \text{ min})$ measured for suspensions of the strain *S. epidermidis* ATCC 12228 in seven buffers of increasing ionic strength, ranging from 0.1 to 150 mM. An ionic strength optimum of aggregation, characterized by low R values after 90 min, is present on the experimental curve. The maximal amplitude ($1-R$) and the optimal range of ionic strength in which the aggregation was the strongest have been defined as two relevant parameters. 16 other strains were tested according to the same protocol as *S. epidermidis* ATCC 12228, the amplitude of the phenotype for each strain is presented in (b). Error bars represent standard deviation ($n=3$). All strains displaying amplitude lower than 0.1 can be considered as not aggregating. For the seven strains that present significant amplitude values, the optimal ionic strength at which this amplitude was measured is plotted in (c).

Cell aggregation is reversible under changing NaCl concentration

Flow cell experiments were designed to assess whether the cell aggregation was reversible when the ionic strength was modified. Practically, a suspension of *S. epidermidis* ATCC 12228 in Tris 0.1 mM, NaCl 150 mM was first flowed in the chamber and then rinsed with the same buffer. Only single cells were immobilized on the PS surface. A suspension of the same strain in Tris 0.1 mM, NaCl 5 mM was then injected and rinsed with the same buffer. In this case, large bacterial aggregates were observed to bind to the surface. These aggregates remained fastened on the surface under flow until a Tris 0.1 mM, NaCl 150 mM buffer was flowed in the chamber again. At this moment, the immediate change in NaCl concentration caused the bacterial aggregates to be instantaneously dispersed and a massive release of separated cells was then observed, some of which adhering on the surface. Time-lapse images of this experiment were recorded and a representative selection is presented in Fig. 3.

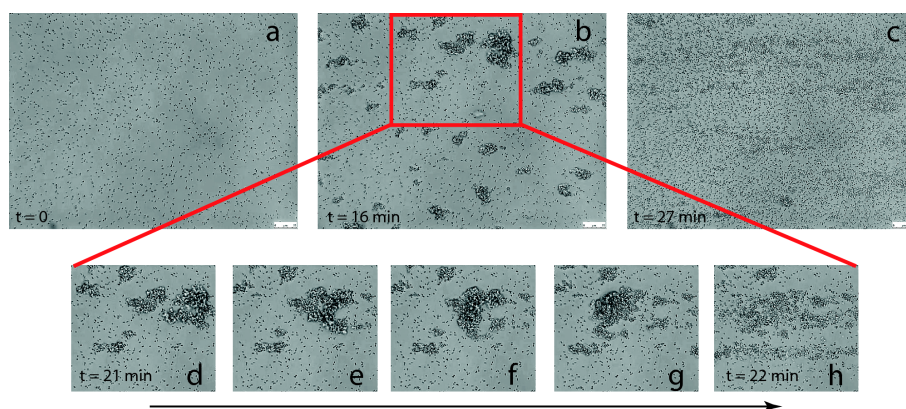


Figure 3: Reversible intercellular aggregation in real-time flow cell experiments. These experiments were performed with *S. epidermidis* ATCC 12228 during 27 min. In (a), a bacterial suspension (5×10^8 CFU/ml) in Tris buffer of 150 mM ionic strength was injected in the flow cell and the same buffer was used to rinse off the excess of unbound bacteria. Bound bacteria are visible as dark dots on the image. In (b), the surface of the flow cell is represented after that the same procedure as described for (a) was performed but with a buffer at $I = 5$ mM. Large cell clusters of aggregated bacteria are visible. In (c), the 150 mM buffer was flowed again and the aggregates were dispersed, as indicated by the increased density of adherent single cells. The transition from (b) to (c) is detailed in (d) to (h), zoomed images taken every 12 sec. Increasing ionic strength induces the immediate and massive release of single cells from the aggregates.

This experiment demonstrates the reversibility of the reported aggregative phenotype. Since the transition from the aggregated to the dispersed state occurred instantaneously, the cell-cell affinity is likely to be mediated by electrostatic interactions.

Influence of ionic strength on the bacterial cell surface charge

The results of the flow cell experiment described above led us to postulate the involvement of electrostatic forces in the ionic strength-dependent aggregation of Staphylococci. To investigate the effect of ionic strength on the cell surface charge, *S. epidermidis* ATCC 12228 were probed using atomic force microscopy (AFM) with chemically-functionalized probes. In this modality, known as chemical force microscopy (CFM, [27] [28] [29] [30]), the AFM probe is modified with hydrophobic or charged chemical groups, thereby enabling to probe cell surface hydrophobicity or

charge on the nanoscale. Fig. 4 shows the adhesion force maps, adhesion force histograms and representative force curves recorded at three ionic strength using COOH-terminated probes. At low ionic strength (Fig. 4a, 4d), most force curves (79 %) featured single adhesion force peaks of 205 ± 121 pN magnitude (mean $\pm$ s. d. from 4×1024 curves obtained on 4 different cells). As the probe is negatively charged at pH 7.4 (COO^-), these adhesion events were attributed to electrostatic attractive forces between the negative charges on the probe and positive charges on the cell surface. Adhesion maps revealed homogeneous distribution of adhesion events, without any evidence for clusters. Remarkably, increasing the ionic strength to 5 mM dramatically decreased the adhesion frequency and adhesion force (Fig. 4b, 4e), an effect that could be attributed to the screening of the probe and cell surface charges by surrounding ions. The same trend was observed at 150 mM (Fig. 4c, 4f).

The AFM experiments confirmed the impact of ionic strength on the presence of positive charges on the cell surface of *S. epidermidis* ATCC 12228. However, the data collected in the buffer of 5 mM ionic strength could not give additional insights on the reason for the rapid aggregation of cells in these conditions.

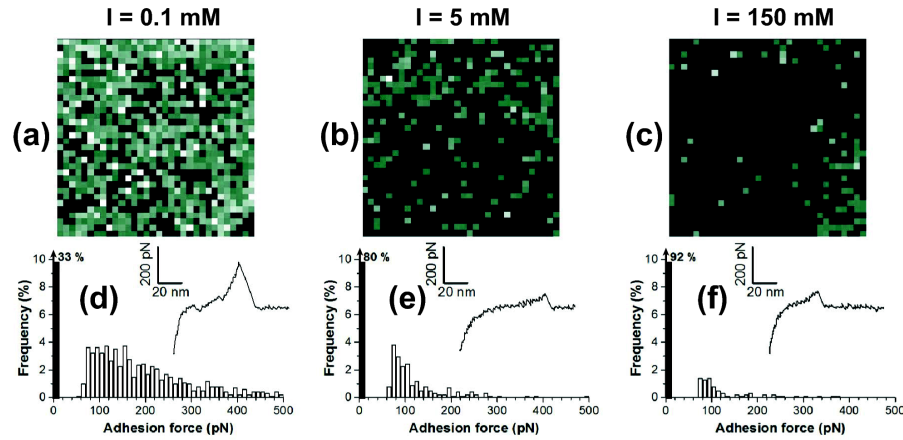


Figure 4: Probing of the bacterial cell surface of *S. epidermidis* ATCC 12228 with negatively charged AFM probes. (a - c). Adhesion force maps (500 nm x 500 nm; z range = 500 pN; bright pixels correspond to binding events). (d - f) Adhesion force histograms, and representative force curves obtained by recording spatially-resolved force curves on the cell surface using COOH-terminated probes ($n = 1024$ curves for each cell) in buffer at 0.1 mM (d), 5 mM (e) and 150 mM (f). All curves were obtained using a contact time of 100 ms, a maximum applied force of 250 pN, and approach and retraction speeds of $1,000 \text{ nm s}^{-1}$. Examples of a force-distance retraction curve recorded as the tip was being withdrawn are overlaid on histogram.

The aggregative phenotype has a major influence on biofilm formation

Intercellular aggregation is essential at all stages of the development of mature biofilms [6] [31]. To test whether the aggregative phenotype reported in this paper impacts the early stages of biofilm formation, the strain *S. aureus* ATCC 6538, known to be a strong biofilm former [32] [33] [34], was cultivated in flow cells during 6 h. Two experiments were conducted in parallel, the only difference being the ionic strength of the growth media. Both were prepared with a base of TSB diluted three-fold, one was diluted in Tris 0.1 mM buffer to obtain a medium with a conductivity corresponding to the optimal ionic strength for *S. aureus* ATCC 6538 aggregation (i.e. 50 mM, see Fig. 2b), the other in Tris 0.1 mM, NaCl 150 mM. Flow cells were inoculated for 10 min with two inocula of the same bacterial charge (ca. 5×10^7 CFU/ml) but suspended in 0.1 mM Tris buffer 50 and 150 mM NaCl, respectively. Then, the media were flowed in flow cells for 6 h at 37°C to allow biofilm growth.

The surface of flow cells were imaged in 6 positions, right after the inoculation and after 2, 4 and 6 h. Representative time-lapse pictures are presented in Fig. 5a.

Strikingly, the initial cell clumps were larger in the 50 mM ionic strength medium. That observation was also made for micro-colonies at every time points during growth (after 2, 4 and 6 h). In the more saline medium, bacterial development occurred horizontally as micro-colonies spread on the surface of the flow cell. In the medium of lower ionic strength, bacteria grew both vertically and horizontally to form denser aggregates. To quantitatively evaluate the biofilm development in these two media, the maximal height of micro-colonies (i.e. the biofilm thickness) after 4 h of growth in flow cells was recorded for three independent experiments (Fig. 5b). In the low salinity nutritive medium, micro-colonies were significantly thicker than those grown in the high salinity medium, reaching maximal mean height of 27.5 and 14.3 μm , respectively. These results confirmed the qualitative analysis of microscopic images that underlined the structural differences between biofilms of the same strain grown in media differing only by their ionic strength.

Additionally, at the end of 6 h of growth, both flow cells were rinsed with Tris 0.1 mM, NaCl 150 mM for 15 min. No significant morphological change could be observed in the biofilm grown in the medium of lower ionic strength, showing that the aggregation induced at a defined NaCl concentration is no more reversible after 6 h of growth. To assess the presence of the characteristic matrix of Extracellular Polymeric Substances (EPS) of a biofilm in the micro-colonies, both biofilms were stained with a fluorescent conjugate of Concanavalin A, a lectin that specifically binds polysaccharides (Fig. 5a, last micrographs). The intensity and the localization of the fluorescence in the biofilm grown with the medium of lower ionic strength indicate the presence of EPS located inside the well-developed micro-colonies. Using identical imaging parameters, the biofilm fed with the more saline medium displayed a weaker fluorescent signal (Fig. 5c). More abundant EPS in the micro-colonies is indicative of advanced maturity of the biofilm grown in the medium with a lower NaCl concentration.

Taken together, the experimental evidences presented here demonstrate the large influence of growth medium salinity on the biofilm development process. The reversible aggregative phenotype seems to enable

a fast buildup of micro-colonies and notably reduces the time needed to build a structured three dimensional biofilm. During biofilm formation, the secretion of the EPS matrix engulfs the bacterial cells together irreversibly.

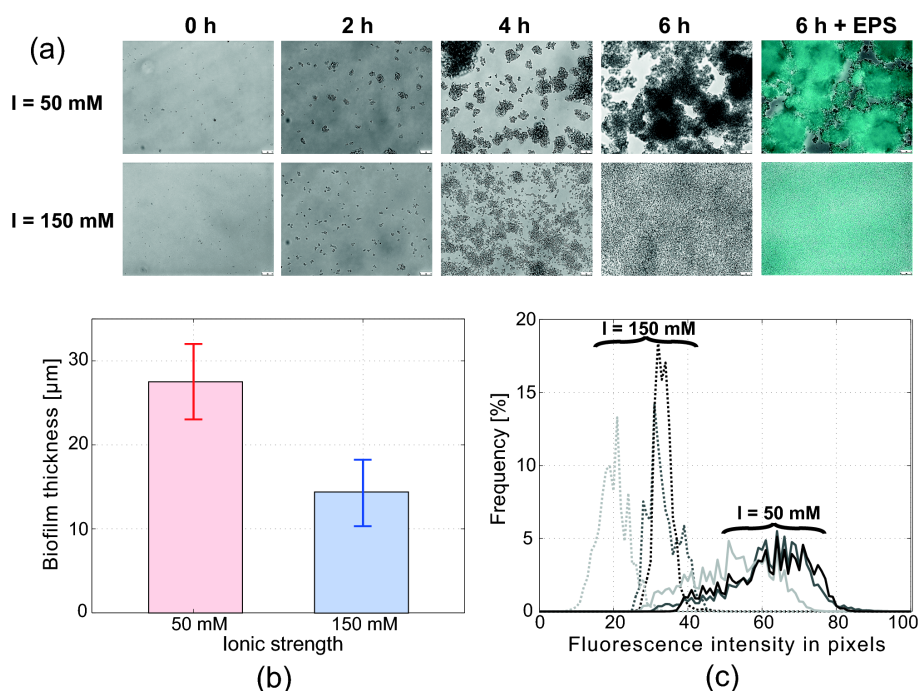


Figure 5: Early biofilm growth of *S. aureus* ATCC 6538 in flow cells in TSB-based media characterized by ionic strength of 50 mM and 150 mM. **(a)** Time lapse microscopic pictures of biofilms growing in the two media, only differing by their ionic strength, were taken 0, 2, 4 and 6 h after the inoculation. The far right images represent overlay of bright-field and fluorescence microscopy to image the presence of polysaccharides belonging to EPS by means of a tagged lectin (Concanavalin A). **(b)** A plot of the biofilm thickness versus the ionic strength of the medium in which the biofilms were grown. Biofilm thickness was assessed by measuring the height of micro-colonies after 4 h recorded by microscopy in 6 positions in flow cells. Error bars represent standard deviation computed on three independent experiments. **(c)** A graph of the intensity of fluorescence on lectin-marked biofilms recorded after 6 h. The *y*-axis represents the frequency (number of pixels at a given intensity/total number of pixels in the image) at which pixels of defined intensity (*x*-axis) occur in three images for both media. Pixel intensity scale ranges from 0 to 255, but was limited to 100 in the Figure to improve readability. Each condition represented by the three independent curves presents different shapes indicating that biofilms grown in 50 mM ionic strength medium contains significantly more EPS.

Discussion

In the present study, we demonstrated that the ionic strength directly modulates the intercellular aggregation of some *S. aureus*, *S. epidermidis* and one *S. warneri*. Several strains belonging to these species formed large cell clusters within minutes when they were suspended in buffers with ionic strengths ranging from 1.5 to 50 mM. In high (150 mM) and low ionic strength (0.1 mM) buffers however, these bacteria remained homogeneously dispersed in suspension. Within the 14 staphylococcal strains tested in this study, seven were shown to aggregate and were all clinical isolates. Amongst the strains that did not react to changes in ionic strength, four were also of clinical origin and three were collected in the environment or in food products. The absence of aggregation in strains of *E. faecium*, *S. salivarius* and *M. luteus* indicates that the phenotype could be limited to species of the *Staphylococcus* genus, though further investigations would be needed to confirm that the ionic strength aggregation is specific to Staphylococci and to analyze the distribution of this phenotype amongst this genus.

The intercellular aggregation observed in intermediate ionic strength buffers (i.e. 5 mM) was proved to be reversible on a short time scale in flow cell experiments. Indeed, bacterial aggregates were immediately dispersed as a high ionic strength buffer was injected (see Fig. 2). These results suggest that the forces involved in this particular type of aggregative phenotype are electrostatic. In general, the cell surface of bacteria is negatively charged at physiological pH [35].

In low ionic strength media, AFM results showed that forces retain a negatively charged tip on the bacterial cell surface, showing the presence of positive charges. However, in the same conditions, bacterial cells remained well dispersed in suspension, indicating that bacteria were at least non-attractive or even repulsive towards one another. Two phenomena could explain these contradictory results. First, in AFM, the tip is brought in contact with the surface without taking into account the electrostatic repulsion barrier. Our experimental setup does not give any information on the balance between positive and negative charges on the cell surface. In this case, we suggest that some proteins with an isoelectric point well above 7.4 and/or positively charged cell surface polymers, such as partially deacetylated PIA, could be responsible for this attractive interaction with the negatively charged probe. These

polymers harboring positive charges sensed by AFM could represent a minor percentage compared to negative ones. Second, due to the low concentration of ions in solution, the double ionic layer surrounding the cell is large, implying that the electrostatic repulsion between two globally-negative charged cells operates at larger distances from the cell wall. Besides, in these conditions, the cell wall anchored polymers are fully extended towards the external medium generating a soft layer at the cell surface. This was confirmed by AFM, the cell surface appeared granular as the cells were probed with a bare tip (data not shown). This layer of extended polymers accentuates the range of the electrostatic repulsion exerted between two bacterial cells. As a consequence, two approaching bacteria of net negative charge would repel one another before any contact could happen.

At high ionic strength, ions present in abundance in solution shield the electrostatic interactions between two cell surfaces. The thickness of the double layer is minimal, around a couple of tenth of nm [35]. Hence, the topographical smoothness of the cell surface upon probing with a bare AFM tip (result not shown) indicates that the soft layer of polymers around the bacteria is not forming. In these conditions, bacteria in suspension would remain dispersed as the electrostatic interactions are strongly dimmed by surrounding ions.

Concerning the cell aggregation occurring at intermediate ionic strength, we hypothesized that localized positively charged polymers (proteins with a high isoelectric point or PIA) interact with negatively charged polymers on the surface of another bacterium to induce cell aggregation. Such phenomena have already been highlighted among colloids with montmorillonite particles (negatively charged clay) that aggregate upon addition of polycations. In this case, the polycations form clusters at the surface of the clay and flocculation is mediated by electrostatic interactions [36]. As bacteria are different from mineral particles, two conditions should be met for this type of interaction to take place between bacterial cells: the soft layer of surface polymers should be moderately expanded and the shielding effect of ions forming the double layer should not be too pronounced. Both require moderate ionic strengths, where clusters of positive charges can efficiently interact with negative charges located on surface of other cells to cause cell aggregation. Clusters of charged polymer may result from defined sorting and anchorage of proteins on the cell surface [37] [38]. It is tempting to speculate that

a particular, strain dependent organization of the polymers on the bacterial cell surface is responsible for the ionic strength dependent cell aggregation. Unfortunately, AFM results collected in the 5 mM ionic strength buffer did not reveal any specific organization of charges upon the probed bacterial surface, which represents less than 10 % of the total bacterial surface.

Alternatively, conformational changes in cell surface proteins and other polymers can be induced by ionic strength [39] [40] [41] leading to modify some properties of bacterial surfaces. For example, it has been shown that cell hydrophobicity of *Lactobacillus acidophilus* and *Lactococcus casei* is higher at low ionic strength and reverted to a more hydrophilic state at high ionic strength [42]. Based on this study results, the reversibility of the aggregation observed in specific ranges of ionic strength could also be the result of a reaction of cell surface polymers to a sudden modification of NaCl concentration. The instantaneous reversibility of cell aggregation observed in flow cell experiments is compatible with the time scale of rearrangements in polymer conformation which can be extremely short, down to sub-milliseconds [43].

The aggregation of Staphylococci reported in this paper can have important consequences on the development of nosocomial infections. Indeed, in complex system such as body fluids, the electrical conductivity and the ionic strength are not linked by a simple relationship as it is the case for simple buffers, e.g. Tris-NaCl buffers. The presence of macromolecules or even cells strongly affects the electrical conductivity while the ionic strength remains constant. This has been illustrated for blood in which the presence of erythrocytes caused a drop in electrical conductivity proportional to their abundance. Visser has measured the conductivity of stationary blood plasma at around 15 mS/cm whereas the same blood plasma containing a normal quantity of 45 % of haematocrit (volume occupied by erythrocytes when these cells are separated from plasma by centrifugation in Ficoll) was found to have an electrical conductivity of 0.55 S/m. Furthermore, in the same study, the electrical conductivity of flowing blood was shown to be lower by up to 30 % than that of stationary blood [44]. This flowing blood electrical conductivity approximately corresponds to a solution at 50 mM ionic strength, i.e. the optimal condition in which the strain *S. aureus* ATCC 6538 aggregates. In this work, the impact of ionic strength and conductivity could not be clearly discriminated but it can be hypothesized that body fluids present

favorable conditions for the aggregation of *Staphylococci* to happen.

Staphylococcal biofilms are the most frequent cause of chronic infections related to implantable devices [45] [46]. The development of biofilms on surfaces highly depends on the ability of bacterial cells to promote intercellular aggregation, in order to ensure the cohesion of these sessile communities. To date, several molecules have been shown to play a key role in this process, including notably the PIA [20], and proteins such as Aap (or its homolog SasG) [47], Bap [22] and Embp [48]. We report that the ionic strength dependent cell aggregation phenomenon highlighted in this study impacts biofilm formation of *S. aureus* ATCC 6538 in terms of thickness, three dimensional development and EPS production, reducing the time needed to develop a structured biofilm. Mechanisms such as modulation of quorum sensing (QS) system, generally recognized to govern the genetic switch towards the biofilm lifestyle in *Staphylococci*, in particular by regulating the production of EPS [49], could be one of the reasons leading to a more structured biofilm at some given ionic strengths. Rapidly forming large aggregates in challenging environments would enable *Staphylococci* to fast track the formation of biofilms and decrease their vulnerability.

Based on our observations, the importance of the ionic strength of growth media and buffers used to develop biofilms of *Staphylococci in vitro* should not be overlooked. To date, most laboratory media feature a conductivity of about 15 mS/cm, equivalent to the ionic strength of a 150 mM NaCl solution, i.e. physiological solutions.

In conclusion, we report a novel phenomenon of *staphylococcal* intercellular aggregation that exclusively depends on ionic strength. Strains of the two notorious pathogens *S. aureus* and *S. epidermidis* displayed rapid and reversible cell aggregation on initially dispersed planktonic cells. For surface bound bacteria, intercellular aggregation induced faster and more structured biofilm formation. Biofilms are known to be the root cause of persistent infection on indwelling medical devices, notably thanks to host immune system evasion and increased tolerance to antibiotics. The mechanism underlying this aggregative phenomenon is yet unknown though evidences point towards electrostatic interactions. Further work is needed to elucidate the molecular determinants of this particular aggregative mechanism.

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Challenging biofilms with enzymes

This chapter brings up the issue of the control of mature biofilms. Given the increasing number of resistance to antibiotics and disinfectants, biofilms remain difficult to eradicate and novel strategies are needed to cope with them. Hereafter are presented the results of biofilm removal assays with several enzymes with lytic and matrix degrading activities. A statistical method called response surface methodology was chosen to assess the effect of individual enzymes as well as their potential synergies and inhibitions on mature biofilms of *S. epidermidis*. The enzymes displayed promising anti-biofilm properties that could prove useful in the management of staphylococcal biofilms.

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T. Vanzieleghem, F. Vande Cappelle, M. Schmelcher, M. Loessner, and J. Mahillon. Enzymatic degradation of *Staphylococcus epidermidis* biofilms.

Enzymatic degradation of *Staphylococcus epidermidis* biofilms

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Keywords : Biofilm removal, *Staphylococcus epidermidis*, lysins, cell lysis, response surface methodology

Abstract

S. epidermidis is a major nosocomial pathogen. This species ranks first in the causes of chronic infections associated with certain types of indwelling devices, especially catheters. The major reason why *S. epidermidis* is successful as a pathogen is its ability to develop biofilms. These surface-associated microbial communities provide improved resilience for bacteria in challenging environments, notably towards antibiotics and the host immune system. As a consequence, biofilms are difficult to treat with classical therapies and the need for alternatives to successfully eradicate them is present. This study provides demonstration that three lysins (peptidoglycan hydrolases) including lysostaphin, a bacterial exolysin originally isolated from *Staphyococcus simulans*, lysin K and Twort, two phage endolysins, and dispersin B analog degrade mature *in vitro* biofilms of *S. epidermidis*. In addition to the removal of the biofilms, lysostaphin and lysin K significantly lysed biofilm-resident cells during a 2 h treatment. The cumulated effect of biofilm removal and cell lysis yields an effective eradication of established biofilms. In the quest for novel anti-biofilm therapies, lysins and matrix-degrading enzymes represent interesting alternatives to antibiotics.

Introduction

Commensal bacteria of human skin and mucosa, *S. epidermidis* is now regarded as an opportunistic pathogen, encountered in about 5 % of all healthcare associated infections [1]. It is also the most frequently isolated pathogen in infection involving indwelling medical devices [2] [3] [4]. The virulence of this species is primarily due to its biofilm-forming ability [5] [6]. Biofilms continuously release planktonic cells, therefore causing acute infections [7]. Besides, these sessile communities display very little sensitivity to antibiotics as Minimal Biofilm Eradiction Concentration (MBEC) concentrations can be up to 100 to 1,000 times higher than the Minimal Inhibitory Concentration (MIC) established for planktonic cells [8] [9]. Even in synergy, antibiotics could reveal ineffective. Two phenomena may explain the increased tolerance of biofilms towards antibiotics. The first is the limitation of mass transfer in the core of biofilms due to the exopolymeric matrix. The second is the presence of persister cells, slow growing bacteria able to tolerate antibiotics [7] [10]

[11] [12]. This therapeutic dead-end underlines the need for novel strategies to tackle the issue of biofilm-related infections.

Currently, two main approaches can be distinguished: preventive strategies to avoid biofilm development and curative ones, designed to eradicate mature, well-established biofilms. On the one hand, prevention is primarily set up thanks to modified surfaces that aim to reduce bacterial adhesion and/or kill bacteria upon contact and best practices in hygiene [13] [14] [15] [16] [17]. On the other hand, the main curative strategies currently used rely on antibiotics. Given their limitations in combatting biofilms, new approaches have emerged. Firstly, nanoantibiotics (e.g. coated nano-particles or nano-encapsulated bactericidal molecules) could prove useful to improve the delivery of antibiotics in the core of biofilms [18] [19]. However, the question of nano-particles toxicity remains present and should be carefully considered [20]. Other leads include Anti-Microbial Peptides (AMP) and quorum sensing inhibitors also called quorum quenchers. Antimicrobials peptides, of natural or synthetic origin, selectively permeabilize bacterial membranes, and induce membrane depolarization, cell leakage, and eventually death [21] [22]. However, events of biofilms tolerance or resistance to AMP have been reported [5] [23] [24]. Although the inhibition of QS systems may lead to a physiological shift towards less virulence and impaired biofilm formation [25], in *Staphylococci*, activation of the Agr (Accessory gene regulator) QS system notably decreases production of adhesins and favors the expression of virulence factors [26] [27]. Eradication of biofilms has already been performed *in vitro* by activating Agr [28] [29]. However, this strategy could be detrimental *in vivo* regarding the release of planktonic cells and the enhancement of the expression of virulence factors [30].

So far, there is thus no panacea to prevent or treat biofilm-associated infections. In that sense, enzymatic control through the concerted action of lysins (peptidoglycan (PG) hydrolases) and enzymes targeting the biofilm matrix is a promising strategy in both preventive and curative treatments. PG hydrolases include bacterial lysins (exolysins, autolysins and lysozymes) and endolysins from phages [31]. Lysins can lyse Gram-positive bacteria when added exogenously to the medium due to the large osmotic pressure (up to 50 atm) of their cytosol compared to the environment [32] [33]. Besides, due to the variations in PG structure from one bacterial species to another [34], specific species can be targeted by using a dedicated lysin as bactericidal agent [35], avoiding the

disadvantages of the usual broad range antibiotics [36]. Concerning potential resistances, several mechanisms of resistance have been identified for bacterial lysins and lysozymes [37] [38] [39] but there is no report of resistance to phage endolysins [31]. Previous studies have shown that lysins (e.g. lysostaphin, LysK and LysH5) were able to remove staphylococcal biofilms *in vitro* [40] [41] and lysostaphin in particular was able to eradicate biofilm developed on catheterized mice [42] [43]. In addition, synergies between PG hydrolases and antimicrobial agents have also been reported [44].

In this study, the effect of a dispersin B analog (a β -hexaminidase, hereafter referred to as Dsp B*), lysostaphin (LST), and two lysins (PG hydrolases) from phages Twort and K (Tw and LysK) were tested on both biofilm removal and cell lysis activity within biofilms. All four enzymes proved efficient at degrading mature *S. epidermidis* biofilms although Tw and Dsp B* were inhibiting each other. Of interest, LST and LysK also displayed a lytic activity against biofilm-dwelling bacteria. These enzymes are thus promising candidates as anti-biofilm agents in the effort to control infectious biofilms.

Materials and Methods

Bacterial strains and cultures

The strain *S. epidermidis* ATCC 35984, known to be a strong biofilm former [45], was used in this study. The strain was plated from frozen stock (-80 °C) on Tryptic Soy Agar (TSA) plates and incubated at 37 °C for 24 h. The cultures were carried out in 250 ml Erlenmeyer flasks filled with 50 ml of Tryptic soy Broth (TSB) at 37 °C under 120 rpm agitation overnight.

For enzyme purifications, *Escherichia coli* XL1-Blue MRF' strains bearing constructs on plasmid pQE30 for the N-terminally His-tagged versions of lysin K (LysK) and Twort (Tw) were used as recently reported by Schmelcher and colleagues [46].

Enzymes

Four enzymes were used in this work: lysostaphin (LST, lyophilized powder, ≥ 500 units/mg protein, Sigma), an analog of dispersin B (DspB*),

and two endolysins from the staphylococcal phages Twort and K (Tw and LysK, respectively). The last three enzymes were purified as His-tagged versions from the *E. coli* strains carrying the appropriate constructs. The induction and purification procedures described in [46] were followed, except for the following steps: sonication was performed for 5 min (0.5 sec pulse and 0.5 sec rests) at 35 % of the maximal power of the sonicator (Sonopuls HD 2200, Bandelin) with HD73 probe, and purification was done in immobilized metal ion affinity columns (Ni-NTA Superflow 12.5 ml, QIAGEN). After filtration on 0.22 μ m, the purity of the eluate was assessed by SDS-PAGE using Amersham ECL gels (GE Healthcare Life Sciences) according to manufacturer instructions. The amount of proteins was measured with the Nanodrop (Thermo Scientific) by recording the absorbance at 280 nm and transformed into mg/ml as previously reported [46].

Standardisation of lysins activity

The bacteriolytic activity of LST, Tw and LysK were assessed on suspension of *S. epidermidis* ATCC 35984. Exponential phase cultures at an optical density at 600 nm of about 0.5 were stopped on ice for 30 min then centrifuged at 5,000 g for 5 min at 4 °C. The supernatant was removed and the pellet was resuspended in PBS (Tablets, pH 7.4, Sigma) to obtain a suspension at an optical density at 600 nm of 0.25. Lysins were added to the bacterial suspensions in PMMA cuvettes (Brand, VWR) and the optic density was recorded 10 min after the addition of enzymes. One unit of activity (U) is defined as the amount of enzyme necessary to cause a 50 % reduction of the optical density at 600 nm of a suspension from 0.25 to 0.125 in 10 min, assumed to be the result of cell lysis [47]. The following concentrations of lysins were determined as equivalent to 1 activity unit (U): lysostaphin, 10 nM, LysK, 2.5 nM and Tw, 7.5 nM.

Biofilm development in 96-well plates

Static biofilms were grown in culture-treated PolyStyrene (PS) 96-well plates (CellStar, Greiner Bio-One) according to the protocol described by [48]. Briefly, 190 μ l of TSB were added to the wells of the multiwell plates and inoculated with 10 μ l of an overnight culture of *S. epidermidis* ATCC 35984. The 96-well plates were then sealed with parafilm and incubated at 37 °C for 24 h.

Enzymatic treatment of mature biofilms and spectrophotometric quantification

Biofilms were rinsed twice with sterile PBS. Meanwhile, the enzymes mixes were prepared according to the indications summarized in Tables 1 and 2 by diluting enzyme stocks into PBS to reach the appropriate concentrations. These mixes were then transferred in the wells; each was tested four times in independent wells. The biofilms were incubated with enzymes during 2 h at 37 °C. After incubation, the Optic Density at 595 nm (OD_{595}) was recorded to quantify the extent of cell lysis in the biofilms. Then, the enzymes mixes were removed and the wells were rinsed once with sterile PBS. Subsequently, a staining procedure with safranin was used in order to quantify the biomass remaining in the biofilm after the 2h enzymatic treatment. Safranin was preferred to crystal violet for reproducibility reasons. The staining procedure was performed according to the protocol recently described by Wu et al. [49]. Briefly, the wells were incubated 1 h at room temperature with HCl 0.1 M, then with safranin 0.1 % w/v for 45 min. The excess of dye was thoroughly rinsed with sterile PBS. Finally, 100 μ l of NaOH 0.2 M were transferred in the wells and the 96-well plates were incubated at 57 °C for at least 1 h. During this time, the NaOH solution solubilizes the stained remnants of biofilms. The contents of the wells were then transferred to a new 96-well plate for measurement of A_{540} to quantify the amount of biofilm that remained in the wells after treatment with enzymatic cocktails. The results were expressed in percentage of reduction (% reduction) compared to untreated control biofilms.

Response surface methodology

In this work, a Response Surface Methodology (RSM) was applied, using an orthogonal Central Composite Design. The trials were defined by the statistical software JMP (SAS) and the experimental design is shown in Table 1. In the 30 trials, three types can be distinguished: 16 factorial points (from 1 to 16), 6 central points (from 17 to 22) and 8 star points (from 23 to 30). The factorial points allowed determining the effect of each enzyme individually and in combination with the others, two by two. The central points are replicated to assess the variability of the experiments. Finally, the star points indicate if the enzymes have a linear or quadratic dose-response effect on biofilms. The trials were split in three blocks, each corresponding to a 96-well plate. Each trial was repeated four times in different wells with the same enzyme mix.

The analyses were split in three blocks, each corresponding to a single 96-well plate. The "block" parameter allows taking into account the intra-experimental variability. This analysis is made possible because the 2 central points are repeated in each 96-well plate (i.e. block). In this case, possible slight loss of enzymatic activity occurring during the preparation of enzymatic cocktails are detected and accounted for in the equation of the regression model. The mean values of the four replicates of A_{540} and OD_{595} measured for the 30 trials (displayed in Table 1) were used to compute two multiple regressions, one for each experimental response. The multiple regression models should be validated according to four criteria. First, R^2 should be close to 1. Then, the p-value of the ANOVA test should be below 0.05, indicating that the model accounts for the variability in the data. Third, there should not be a "lack of fit", i.e. a lack of accordance between the multiple regression model and the experimental data. Finally, the residues (i.e. the difference between the values predicted by the model and the experimental values) should follow a normal distribution. The four criteria were verified for both models and are summarized in Fig. S1.

Results

Enzymatic degradation of mature *S. epidermidis* biofilms

Mature biofilms of *S. epidermidis*, grown in 96-well plates were challenged for 2 h at 37 °C with enzyme cocktails according to the trials indicated in Table 1, as defined by the central composite design. Two parameters were measured: OD_{595} at the end of the enzymatic treatment and A_{540} after a safranin staining protocol according to [49]. These data (Table 1) quantify the extent of the cell lysis in biofilms and the biomass remaining (i.e. biofilm) in the wells, respectively. A multiple regression analysis was performed on the results with the statistical software JMP and the results, the effect of each enzyme and their interactions on both responses (A_{540} and OD_{595}), are displayed in Table 3. The significance of the effects was classified in three levels according to the p-values.

Table 1 - Trials of the response surface methodology to assess the biofilm degradation activity of the four enzymes Tw, LST, DspB* and LysK. The concentrations are represented by ranges from -2 to 2 for which Table 2 gives the corresponding concentrations in nM. The column "Pattern" is a symbolic representation of each combination, "a" refers to -2, "-" to -1, "0" to 0, "+" to 1 and "A" to 2. The "Block" column, from 1 to 3, indicates in which of the three 96-well plates the trial was performed. The results for A_{540} and OD_{595} are presented in absolute values and in % of reduction compared to untreated controls in the far right columns. Mean values of A_{540} and OD_{595} are reported in the last line for the controls (n=12).

	Trials	Pattern	Block	Factors (Enzymes)				Responses		% reduction	
				Tw	LST	DspB*	LysK	A_{540}	OD_{595}	A_{540}	OD_{595}
	1	----	2	-1	-1	-1	-1	0.563	0.309	40.7	55.2
	2	+---	1	1	-1	-1	-1	0.292	0.240	69.3	65.2
	3	-+--	1	-1	1	-1	-1	0.304	0.153	68.0	77.8
	4	--+-	1	-1	-1	1	-1	0.203	0.307	78.6	55.5
	5	---+	1	-1	-1	-1	1	0.335	0.218	64.7	68.4
	6	++--	2	1	1	-1	-1	0.246	0.136	74.1	80.3
	7	+-+-	2	1	-1	1	-1	0.368	0.308	61.3	55.4
	8	+++-	2	1	-1	-1	1	0.365	0.210	61.6	69.6
	9	-++-	2	-1	1	1	-1	0.293	0.149	69.2	78.4
	10	-+++	2	-1	1	-1	1	0.282	0.156	70.3	77.4
	11	---++	2	-1	-1	1	1	0.345	0.179	63.7	74.1
	12	+++--	1	1	1	1	-1	0.161	0.146	83.1	78.8
	13	++++	1	1	1	-1	1	0.156	0.193	83.6	72.0
	14	++++	1	1	-1	1	1	0.171	0.223	82.0	67.7
	15	++++	1	-1	1	1	1	0.135	0.179	85.8	74.1
	16	++++	2	1	1	1	1	0.179	0.183	81.2	73.5
Repetitions	17	0000	1	0	0	0	0	0.149	0.149	84.3	78.4
	18	0000	1	0	0	0	0	0.170	0.162	82.1	76.5
	19	0000	2	0	0	0	0	0.182	0.139	80.8	79.9
	20	0000	2	0	0	0	0	0.188	0.157	80.2	77.2
	21	0000	3	0	0	0	0	0.271	0.160	71.5	76.8
	22	0000	3	0	0	0	0	0.248	0.155	73.9	77.5
	23	000a	3	0	0	0	-2	0.651	0.394	31.5	42.9
	24	000A	3	0	0	0	2	0.199	0.196	79.1	71.6
	25	00a0	3	0	0	-2	0	0.383	0.148	59.7	78.6
	26	00A0	3	0	0	2	0	0.259	0.155	72.7	77.5
	27	0a00	3	0	-2	0	0	0.478	0.494	49.7	28.4
	28	0A00	3	0	2	0	0	0.218	0.145	77.1	79.0
	29	a000	3	-2	0	0	0	0.377	0.215	60.3	68.8
	30	A000	3	2	0	0	0	0.222	0.171	76.6	75.2
Control				-2	-2	-2	-2	0.950	0.690	0	0

Table 2 - Concentration ranges (in nM) used for LST, Tw, LysK and DspB* in the response surface methodology trials reported Table 1.

Enzyme levels	-2	-1	0	1	2
Tw	0	250	500	750	1000
LST	0	25	50	75	100
DspB*	0	0,063	0,125	0,187	0,250
LysK	0	250	500	750	1000

Table 3 - The parameters of the multiple regressions for A_{540} and OD_{595} . They are classified in four categories: the effect of the enzymes alone, the quadratic terms that reflect the non-linearity of the dose-response curves in Fig. 1, the interactions, i.e. synergies or inhibitions, and finally the block effects that relate to the use of three distinct 96-well plates to perform the experiments. The p-values below 0.0001 were considered highly significant (***), between 0.0001 and 0.05, very significant (**), between 0.05 and 0.1, significant (*). NS stands for Not Significant. The following interactions were deemed NS for both A_{540} and OD_{595} and were eliminated from the regressions: TW x LysK, DspB x LysK, LST x DspB*, Block x DspB* and Block x Tw.

	Effects	A_{540}		OD_{595}	
		(Remaining biomass)		(Cell lysis)	
		p-value	Significance	p-value	Significance
Enzymes alone	LST	<.0001	***	<.0001	***
	LysK	<.0001	***	<.0001	***
	DspB*	0.0001	**	0.3834	NS
	Tw	0.0003	**	0.2232	NS
Quadratic terms	LST ²	0.0029	**	<.0001	***
	LysK ²	<.0001	***	<.0001	***
	DspB* ²	0.0197	**	0.0664	NS
	Tw ²	0.0859	*	0.2118	NS
Interactions	LST x LysK	0.7559	NS	<.0001	***
	Tw x LysK	0.6256	NS	0.0255	**
	Tw x DspB*	0.0330	**	0.1347	NS
Block	Block	<.0001	***	0.0022	**
	Block x LST	0.0366	**	0.0001	**
	Block x LysK	0.0004	**	0.0004	**

Biofilm removal

Table 3 shows that all four enzymes, LST, Tw, LysK and DspB*, were each active against mature *S. epidermidis* as determined by the associated p-values below 0.05. The slopes of the curves shown in Fig. 1 indicate the degree of activity of each enzyme. At first glance, LST, DspB* and LysK seem to remove biofilms better than Tw. A further analysis using Table 2 (concentrations of enzymes used in the experiments) shows that DspB* is active at subnanomolar concentrations, LST is most efficient around 50 nM while LysK is active only from several hundred nM, up to 1 μ M. The slope of the curves in Fig. 1 should thus be interpreted carefully, in light of the concentrations used.

Also in the curves of Fig. 1, it appears that LST, LysK and DspB* are characterized by non-linear dose-responses. This originates from the significance of the quadratic terms: LST^2 , $LysK^2$ and $DspB^2$ in the regression model (see Table 3). Significant quadratic terms imply that the biofilm removal activity is saturating at a certain level of concentration. In this case, the increment of biofilm removal provided by the addition of enzyme is dependent on the amount of enzyme already present. Of note, this effect is less marked for Tw, as confirmed by the significance of Tw^2 in Table 3.

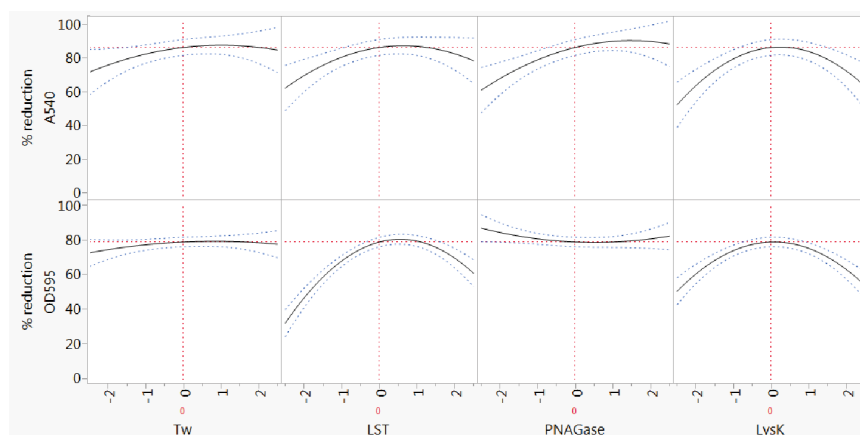


Figure 1: Prediction of the effect of Tw, LST, DspB* and LysK enzymes alone on the experimental responses represented on the y axes: % reduction in A_{540} (a) and % reduction in OD_{595} (b) (compared to untreated controls). Thick lines indicate the predicted effect of each enzyme on both experimental responses. Dotted lines represent the confidence intervals at 95 % computed by the model. The x axes indicate the concentration of all enzymes; please refer to Table 2 for the actual molar concentrations.

Finally, Fig. 2 (a, b) shows that DspB* and Tw negatively cross-influence one another. Indeed, in Fig. 2 (a), the biofilm degrading effect of Tw is clearly

marked when DspB* is present in low concentration (-1) while the presence of the highest concentration in DspB* (+1) annuls it, the curve is significantly flattened. The same goes for DspB* that has a strong biofilm removal activity in the presence of lower amounts of Tw whereas it shows no effect at all when Tw is present at high concentration (+1) as shown in Fig. 2(b). In the model, these effects translate in the significance of the interaction occurring between Tw and DspB* (Tw x DspB*, see Table 3). However, in this case, it is likely that this interaction appears significant due to saturation in the experimental response. Indeed, in the presence of a large concentration of Tw or DspB*, A₅₄₀ has already reached its minimal value and cannot be influenced by the addition of Tw or DspB*. Therefore, the nature of the interaction between Tw and DspB* is probably not an inhibition.

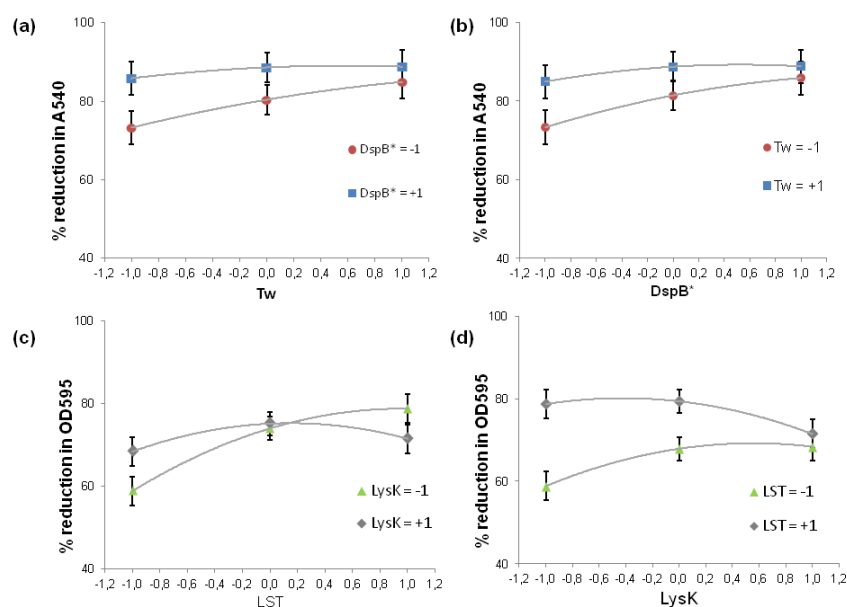


Figure 2: Interaction plots. The graphs display the influence of an enzyme (indicated in the top right corner) on the effect of another enzyme (x axis) on the biofilm removal (% reduction in A₅₄₀) or cell lytic activities (% reduction in OD₅₉₅) (left y axis) (a) Impact of DspB* on the biofilm removal effect of Tw; (b) Influence of Tw on DspB* biofilm removal activity; (c) Impact of LysK on the cell lysis caused by LST; (d) Inhibiting effect of LST on the lytic activity of LysK.

Cell lysis of biofilm-dwelling *S. epidermidis* cells

The cell lysis in biofilms after treatment with the enzymes was quantified by recording the OD₅₉₅. Fig. 1(b) indicates that LST and LysK are able to markedly lyse biofilm-dwelling *S. epidermidis* cells. These two enzymes are also classified as significant according to the multiple regression model (Table 3). Surprisingly, despite its lytic activity on *S. epidermidis* cells in suspension, Tw does not seem to participate to cell lysis, i.e. reduction of OD₅₉₅. This can be seen by the flatness of the curve in Fig. 1(b) as well as in Table 3, in which Tw appears as non-significant. Finally, DspB* does not play any role in cell lysis as expected from its enzymatic activity, i.e. N-acetylglucosamine hydrolase.

Also in Table 3, the interaction term involving both LysK and LST appears significant. Fig. 2 shows that LST and LysK interact in an inhibitive-competitive way. Indeed, Fig. 2(c) indicates a loss in LST lysis activity when LysK is present in higher concentration (+1). On the other hand, in a situation in which LysK is present in smaller amounts (-1), LST causes a decrease in OD₅₉₅. The cell lysis activity of LST is therefore strongly influenced by the presence of LysK in the medium. Similarly, Fig. 2(d) shows that LysK activity is impaired in the presence of larger amounts of LST. These results indicate that both enzymes inhibit each other in cell lysis.

Finally, as for biofilm removal, LST and LysK cell lytic activity do not follow a linear dose-response relationship. Instead, both are characterized by quadratic curves as confirmed by the significance of LST² and LysK² in Table 3.

Optimization of cell lysis and removal of biofilms

The regression model computed from the experimental data allows to predict how the enzymes LST, Tw, LysK and DspB* act on cell lysis and biofilm degradation for a 2 h treatment at 37 °C. Because of the particular geometry of the original composite central design, the domain of forecast is a sphere that expands from -1 to +1 for each enzyme (see Table 2 for concentrations in nM). Outside this sphere, the predictions would be inaccurate, even though the star points of the central composite design include concentration ranging from -2 to +2.

In Fig. 3, contour plots display the impact of varying concentrations of LST and LysK for defined quantities of DspB* and Tw (-1, 0, +1) on % reduction in A₅₄₀ (red lines) and % reduction in OD₅₉₅ (blue lines). The colored areas represent the zones in which prediction reduction in A₅₄₀ and in OD₅₉₅ are respectively above 85 and 80 %. These values correspond to experimental observations of an almost total degradation of the biofilm and a significant

clearance of the turbidity in the wells (i.e. an extensive cell lysis).

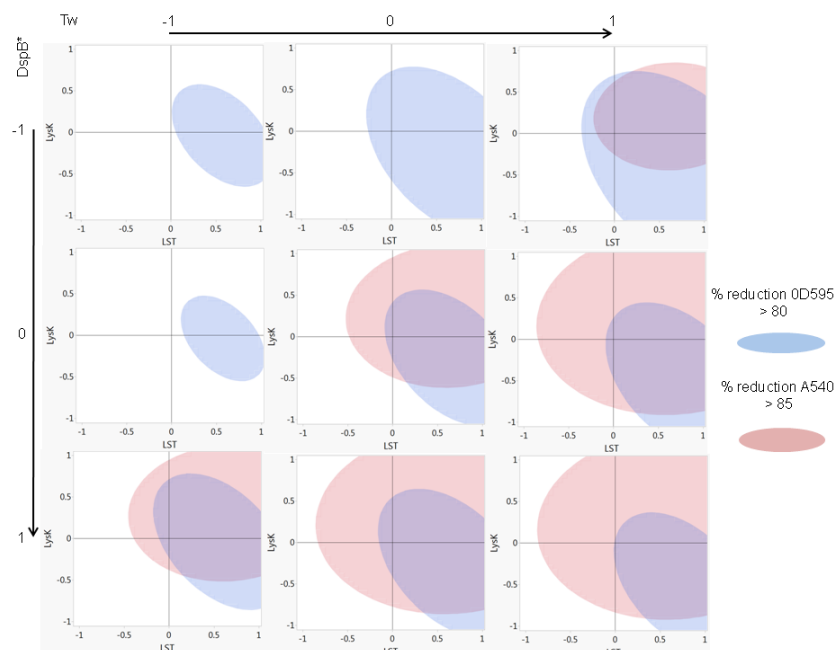


Figure 3: Optimization of Tw, LST, LysK and DspB* activity on mature *S. epidermidis* biofilms. Nine plots display areas in which more than 85 % reduction in A_{540} (in red) and 80 % reduction in OD_{595} (in blue) was achieved, for all concentrations of LST and LysK and for 3 fixed amounts of Tw (top x axis) and DspB* (left y axis). The overlap of blue and red zones represent the optimal combinations of LysK, LST, Tw and DspB*. Refer to Table 2 for the significance of -1, 0 and 1 in terms of concentrations (in nM).

Of note, the size and position of the blue zone, indicating the concentrations of LysK and LST at which cell lysis is optimal, is not significantly changing. This confirms that Tw and DspB* play a minor role in cell lysis within the biofilm, as monitored by the reduction of OD_{595} (see Table 3). On the contrary, the size and position of the red zone on the graph is strongly influenced by the concentration of Tw and DspB* that significantly affect A_{540} by removing the biofilm.

The main information indicated by these contour plots can be found in the overlap of the filled red and blue zones. These zones represent the numerous combinations of LST, Tw, LysK and DspB* concentrations that produce optimal levels of biofilm removal and cell lysis, i.e. 85 % reduction in A_{540} and 80 % reduction in OD_{595} compared to untreated control biofilms.

Discussion

With the intrinsic tolerance of biofilms to classical therapies and cleaning procedures it becomes urgent to develop new strategies to cope with biofilms that represent a threat to public health. In this article, the potential anti-biofilm activity of four enzymes, including three lysins (one bacterial and two from phages) and one matrix-degrading enzyme, was tested against biofilms grown in static conditions. The biofilm removal (A_{540}) and cell lysis (OD_{595}) activities of these enzymes were both monitored. Biofilm removal activity coupled with extensive cell lysis would provide an efficient way to eradicate biofilms.

In this work, a response surface methodology was implemented in order to assess the potential interactions between LST, LysK, Tw and DspB* on biofilms. It consists of a canonical set of experiments providing results that can be optimally treated with statistical models. Indeed, response surface methodology provides a predictive model that accounts for the effect of individual enzymes, as well as synergies. Such approaches have become increasingly popular for screening and optimization procedures in life sciences, notably because of the quantity of information gathered from the experiments [50]. For a review of advantages and limitations in using RSM for such purpose, see the work of Bas and co-authors [51].

Experimentally, the biofilms grown in 96-well plates were challenged for 2 h at 37 °C with cocktails of enzymes that are listed in Table 1 and 2. The choice was made to expose the biofilms for a relatively short period of time to avoid any growth to happen. After enzymatic treatment, a staining of biofilm remnants was performed with safranin, which was preferred to crystal violet for reproducibility reasons.

The interpretation of the results showed that LST and LysK were good candidates to remove *S. epidermidis* biofilms and lyse cells. However, if used together in a cocktail, they tend to inhibit each other with regard to their cell lysis activity. Tw was efficient at degrading biofilms but was found to be surprisingly inactive at lysing biofilm-dwelling cells. It is likely that the cell surface properties of *S. epidermidis* cells in biofilms impair the peptidoglycan hydrolase activity of Tw, resulting in protection from lysis. Also, this endolysin could be more sensitive to inactivation or denaturation by proteases or inhibitors present in the EPS matrix of biofilms or released by the lysis of other cells. Finally, DspB* was found to have a marked biofilm removal activity at subnanomolar concentrations, most probably by degrading the main component of the EPS matrix, poly-N-acetylglucosamine chains. Unfortunately, the lack of positive synergies involving DspB* with LST, LysK or Tw indicates that it does not facilitate the cell lysis of biofilm-dwelling *S. epidermidis* of these lysins.

The analysis also revealed an inhibition between LST and LysK on cell lysis activity. This observation is not in accordance with previous works that reported a synergistic action of these two enzymes to lyse *Staphylococci* in suspensions [52]. It seems that biofilm embedded cells do not favor a cooperative action of LysK and LST, despite the fact that they harbor distinct cut sites [31]. The biofilm lifestyle may induce protective modifications on the cell surface of *S. epidermidis* that hamper the activity of lysins.

Based on the results presented in this study, it was also possible to identify combinations of the four enzymes that maximize both the biofilm removal and cell lysis (see Fig. 3). It appeared that LST and LysK were good candidates for biofilm degradation that can be helped by either Tw or DspB* to achieve biofilm eradication.

These conclusions should however be put in perspective with the culture conditions *in vitro*. Indeed, the composition of biofilms can be strongly influenced by environmental factors such as, for instance, the presence of various carbon sources or fluid flow [53] [54]. Also, the presence of distinct species can have a dramatic influence on the structure and composition of the biofilms. In particular, the ratio between bacterial cells and EPS matrix as well as the proportions of the polymers in the matrix may vary [55]. As a result, the balance of each enzyme needed to effectively remove biofilms could be different from the combinations identified here. Further work to challenge the activity of LST, LysK, Tw and DspB* on multi-species biofilms grown in different conditions would provide additional insights on their potential as anti-biofilm agents.

This work provides direct demonstration that phage endolysins are a promising class of enzymes to combat bacterial biofilms. Two of these enzymes were found to actively degrade *in vitro* biofilms of *S. epidermidis*. Given the large pool of phages in nature [56] and the diversity of catalytic domains and structure of endolysins [31], there is a large untapped reservoir of potent active principle to target bacterial biofilms.

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Supplementary information

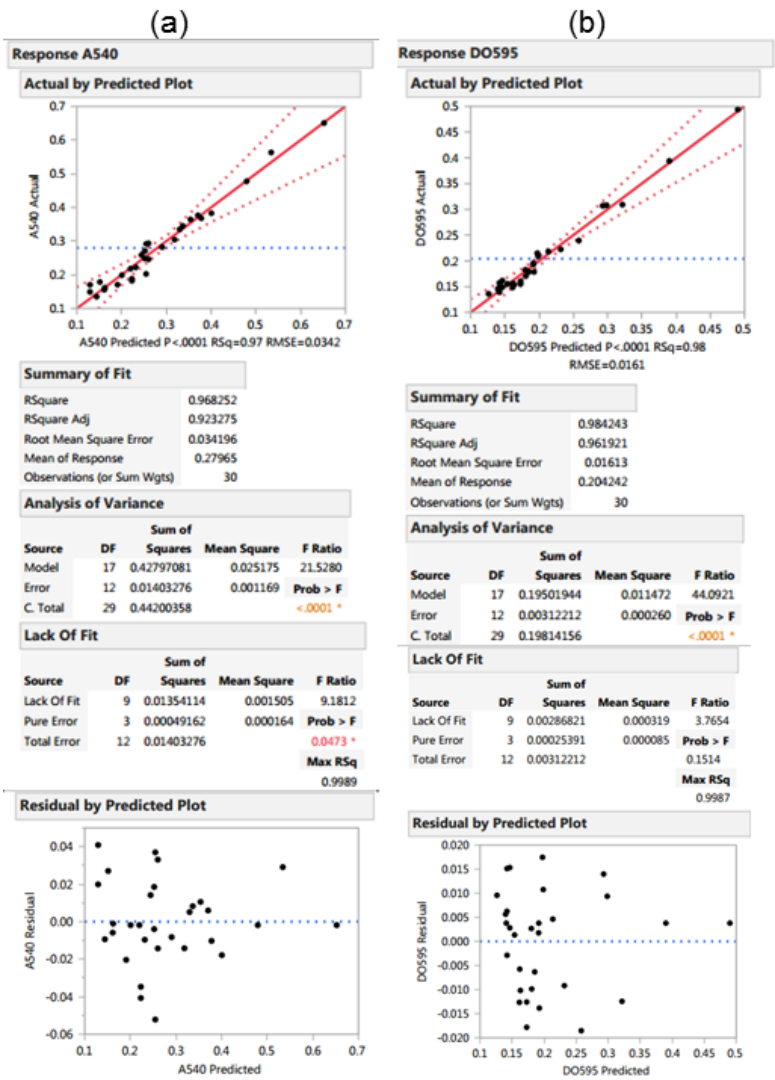


Figure S1 - Validation criteria for the two multiple regressions computed for A₅₄₀ (a) and OD₅₉₅ (b). From top to bottom, the following items are presented: the experimental values are plotted according to the predicted regression model, R², ANOVA, lack of fit and the distribution of the residues. See text for further explanation.

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Part III

Discussion and Conclusion

General discussion

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12.1 The onset of biofilm-associated nosocomial diseases : prevention and detection

Is *S. epidermidis* the next superbug?

The standards of modern medicine regarding hygiene in healthcare has led to the extensive use of disinfectants and antibiotics in hospitals. Over the years, these facilities have become very harsh environments for microorganisms to thrive in. The high selective pressure in hospitals results in only the fittest micro-organisms survive. For bacteria, evolution and adaptation to changing conditions can occur fast, through various mechanisms (Chapter 2). Challenging conditions can also be seen as opportunities to invade a niche in which the competitive microflora is considerably reduced. The past 70 years of antibiotics use in healthcare are the best illustration of this. Leading nosocomial pathogens such as *S. aureus* and *S. epidermidis* have been responding to each novel antibiotic molecules (penicillin, methicillin or vancomycin)

with resistance or tolerance mechanisms, acquired either by mutations, or through HGT. Besides antibiotic resistance, the ability of bacteria to develop in the form of biofilms also allow them to increase their resilience in healthcare facilities.

In this work, the focus was set on *S. epidermidis*, a ubiquitous bacterium in environments frequented by humans. For *S. epidermidis*, biofilm formation is recognized as a virulence factor [Otto 2009] [Christner 2010]. We questioned whether the biofilm forming ability could be influenced by exposure to clinical settings. In Chapter 7, a collection of 134 *S. epidermidis* strains originating both from the environment and hospitals (samples were taken from inert surfaces in rooms as well as from patients) was tested by PCR for the presence/absence of genes known to be involved in biofilm development. The functions include adhesion, aggregation, dispersal, QS (Agr) and IS elements that participate in the regulation of biofilm formation. These data were processed with a hierarchical clustering algorithm using the Ward method that provided a dendrogram (see Fig. 7.1).

Collectively, these results support the hypothesis that *S. epidermidis* reacts to the selective pressure omnipresent in hospitals by favoring the acquisition or retention of genes important for biofilm formation and antibiotic resistance. Besides, the strains isolated from infected patients appear to cluster apart from the majority of the strains collected from inert surfaces in hospitals, indicating that dwelling in patients body accentuates the need for bacteria to keep, maintain or acquire biofilm-related genes. These conclusions are in line with previous observations [Rohde 2004] [Kozitskaya 2004] [Schoenfelder 2010].

Despite the apparent lack of *bona fide* virulence factors, *S. epidermidis* relies on biofilm formation to invade the host, notably to hide from immune system [Fey 2010]. The mortality resulting from these diseases can greatly vary according to the patients age and the site of infection but is estimated to be between 1 - 25 % [Raad 2007] [O'Grady 2002]. These infections tend to leave medical staffs without options but to remove

implants or adopt long term antibiotic treatments. For these reasons, *S. epidermidis* has become a prominent cause of nosocomial, chronic infections on indwelling devices. It reaches about 0.1 % of the population in developed countries [Rogers 2009], representing a major burden for healthcare systems and a threat to patient safety [Otto 2012].

On the importance of bacterial adhesion to biomaterials

In the development of implant associated infections, the importance of bacterial adhesion to biomaterials is now widely accepted [Pavithra 2008] [Rodrigues 2011]. For example, it has been shown that bacteria adhering on the surface of biomaterials are already less susceptible to antibiotics and the immune system of the host compared to planktonic cells [Arciola 2001] [Arciola 2002] [Muszanska 2012].

The surface bound state, as recently proposed by Busscher and colleagues, can be delimited in three regimes [Busscher 2012]. The first is the lack of adhesion, when bacteria remain in planktonic state close to the surface. It is notably the case for surfaces coated with hydrophilic polymer brushes on which bacterial adhesion is compromised [Kaper 2003]. On the contrary, when bacteria bind the surface with intense forces, i.e. a negatively charged bacterium encounters a positively charged surface (for example coated with quaternary ammonia), excessive forces are exerted on the cell envelop causing cell death [Tiller 2001]. In the last regime, the interaction regime, bacteria adhere and thrive on the surface. In this configuration, a series of changes in the physiology of bacteria occur. These adaptations favor biofilm formation and strongly influence the fate of bacteria in the host [de la Fuente-Núñez 2013].

In the case of *S. epidermidis*, its ability to adhere massively to PDMS has been shown in static conditions in Chapter 8 and dynamic conditions in Appendix C. These results confirm previous reports of adhesion to a wide range of biomaterials such as silicone, polyethylene terephthalate (PET), PTFE and polyurethanes [Patel 2007] [Linnes 2012]

[Abusalim 2013]. However, these materials are readily coated by plasma proteins when implanted in the body, therefore changing their surface properties. The results of this study demonstrated the inhibiting effect of an adsorbed protein layer on bacterial adhesion of single cells and whole populations.

The inhibiting effect of adsorbed proteins can be explained by the DLVO theory [Hermansson 1999]. Indeed, on hydrophobic materials, adhesive hydrophobic forces occur between the bare material and the bacterial cell surface. Adhesion is therefore a thermodynamically favored event. However, a film of adsorbed proteins, most of which are negatively charged at physiological pH, tend to repel bacteria through repulsive electrostatic forces [Bos 1999]. In this case, the adhesion event is not thermodynamically favored, notably because the free energy of hydrophobic surfaces in contact with water has already been reduced by the proteins. To compensate for the absence of non-specific interactions with protein covered surfaces, *S. epidermidis* possesses several cell surface proteins, belonging to a class called MSCRAMMs [Clarke 2006]. They can mediate specific interactions with host factors. These molecules are thought to act as harpoons to fasten bacteria on surfaces on which they would not adhere if only non-specific interactions are at play. An example of MSCRAMM is SdrF, a surface protein that binds to Type I collagen in a ligand-receptor fashion [Arrecubieta 2007].

In this study, the interaction between SdrG, another member of MSCRAMMs family, and Fg (Chapter 8, sections 8.1 and 8.2) has been investigated. The binding mechanism of SdrG to Fg according to a "dock, lock and latch" model was elucidated some years ago. It involves a 15 amino-acid long peptide sequence in the Fg molecule, Fg(β 6-20). This peptide chain is stabilized between the N2 and N3 domains of SdrG by numerous hydrogen bonds [Ponnuraj 2003]. The dissociation rate of the SdrG-Fg interaction has been shown to be low, indicating that the bond is stable in time [Herman 2014]. The force that characterizes the SdrG-Fg bond was found to be about 2 nN [Herman 2014] and

confirmed in the results shown in Chapter 8. The magnitude of this force is unusually large, indicating the level of adaptation of SdrG to its substrate. As a comparison, specific interactions between human IgG and rat anti-IgG have been reported to be around 0.8 nN [Lv 2010], less than twice the force of SdrG-Fg binding. In the same vein, the results of this study also showed that a single SdrG-Fg interaction is stronger than the binding of whole *S. epidermidis* cells to a bare silicone surface, observed to be between 0.5 and 1.5 nN as a result of hydrophobic interactions.

An hypothesis for such a large force uniting SdrG to Fg could lie in the necessity for a bacterium to secure its adhesion to surface, even under high shear stresses, as they can be encountered in blood vessels [Papaioannou 2005]. It is critical for bacterial cells to ensure a lasting anchorage on the surface on which it thrives to avoid being removed. Detachment of *S. epidermidis* in a hostile context such as in the host (e.g. blood stream) would undoubtedly impair biofilm development and compromise its survival [Schoenfelder 2010].

In addition to these observations, the importance of SdrG abundance on the bacterial cell surface in the adhesion to Fg coated surfaces was investigated. Cell surfaces of four *S. epidermidis* strains were mapped by AFM with Fg-modified tips to highlight the presence of SdrG. It appeared that not all tested staphylococcal isolates displayed SdrG on their surface. SdrG was detectable in two strains and HB displayed significantly more SdrG on the cell surface than ATCC 12228. Despite the fact that it carries the *fbe* gene, encoding the SdrG protein, strain ATCC 35984 did not harbor any detectable amount of active SdrG molecules on its cell surface. It is likely that the *fbe* gene in the strain ATCC 35984 does not produce a fully functional SdrG adhesin. This hypothesis is based on the comparison of *fbe* genes in genomes of many *S. epidermidis* strains including those of ATCC 12228 and ATCC 35984 that revealed significant discrepancies in the length of the protein and the size of the SD (Ser-Asp) repeats (data not shown). Indeed, mutations, deletions or insertion in the sequence could have altered

the tertiary structure of the resulting SdrG. The spatial arrangement of N2 and N3 domains of SdrG, essential to the binding mechanism, could therefore be modified in this strain. Another possibility is that the expression of *fbe* is submitted to complex regulations, probably strain-dependent. Also, the *in vitro* conditions may not have favored the expression of SdrG. This is supported by reports that *fbe* expression increased after exposure to *in vivo* environments [Sellman 2008], indicating that the cells react to defined stimuli to trigger SdrG production. However, the mechanisms governing the genetic regulation of *fbe* remain still largely unknown.

To evaluate the impact of the bacterial surface density of SdrG on the adhesion to Fg-coated surfaces, adhesion assays were performed. They revealed a positive correlation between SdrG density on the cell surface and increased adhesion to Fg. That was shown both for single cells as well as for whole populations (Chapter 8, section 8.2). These results lead to believe that it is advantageous for *S. epidermidis* to massively express SdrG at its cell surface in order to maximize its chances to adhere to surfaces coated by Fg. This assumption is probably also valid for other MSCRAMMs that specifically recognize other host ligands such as fibronectin, vitronectin, elastin or collagen. It is reasonable to infer that, *in vivo*, the cell surface of *S. epidermidis* undergoes dramatic modifications in order to favor its adhesion to host tissues and coated biomaterials.

Approaches to tackle the issue of implant-associated infections

The incidence of nosocomial infections has diminished in the period 2002-2012 in the US, as estimates went from 1.7 million to 700.000 cases [Klevens 2007] [CDC 2014]. This is the result of several complementary strategies that are currently employed: best practises in hygiene, modified biomaterials (detailed in Chapter 4, section 4.1) or pressurized surgical rooms. But there is no silver bullet to completely avoid the risk of implant-associated infection to occur and despite these advances, the

matter of Healthcare Associated Infections (HAI) has become a priority for the World Health Organization (WHO) [Benedetta Allegranzi 2013]. The need for supplementary means of prevention is clearly present. From this work, two possible leads can be developed.

The first is based on the inhibition of *S. epidermidis* adhesion to Fg thanks to SdrG. As already mentioned, the expression of SdrG is dependent on environmental cues, probably sensed by a signalling pathway governing the expression of the *fbe* gene. Further work on the pathways involved in the expression of SdrG could lead to the identification of possible drug targets to limit its abundance on the bacterial cell surface. As it was shown in this work, *S. epidermidis* adhesion on biomaterials coated by plasma proteins would therefore be impaired. Bacteria unable to adhere on surfaces in the host would be more susceptible to antibiotics and to the immune system, limiting the risk of infection development. This type of strategy could also be used against other MSCRAMMs.

A second strategy consists in the development of a method for sensing bacteria on surfaces by means of impedance spectroscopy (Chapter 9). Briefly, micrometer wide interdigitated electrodes are submitted to a signal of low amplitude (50 mV) oscillating at a chosen frequency, ideally around 1 MHz. Bacterial adhesion on the area between electrodes modifies the properties of the electric field which translates in a change of impedance. This work was primarily focused on PoC applications, notably the detection of bacteria in a liquid but sensing surfaces could be integrated on the surface of implants to detect the adhesion and the proliferation of contaminating bacteria in real-time. Such smart implants would provide unique insights into the early stages of implant contamination, therefore decrease the time to diagnosis. [Paredes 2014]. However much effort and clinical trials are required to bring such smart implants on the market, the proof of concept shown in this work could be used to such purposes.

The prevention of HAIs requires multidisciplinary approaches that take into account the complexity of bacterial adhesion and biofilm development. Novel strategies will complement the measures already in place to tackle the issue and will hopefully diminish the risk for patients to acquire HAIs.

12.2 On the influence of the environment on biofilm formation by *Staphylococci*

Throughout the development of biofilms, from the initial adhesion to the dispersal phase, bacteria respond to environmental cues in order to adapt their physiological and metabolic states (for a complete review, see [Karatan 2009]). Such processes are essential to maintain fitness in changing environments. In the case of *S. epidermidis*, these gene regulations involve, for example, the production of EPS in the presence of glucose [Mack 2007], the response to fluid flow or shear stresses [Weaver 2012] or the modulation of expression of specific genes as previously mentioned for SdrG [Sellman 2008] (for more details, see Chapter 3, section 3.5).

Ionic strength has been reported to have major, though ambivalent, influences on biofilm formation in several bacterial species. In *Salmonella enterica* serovar Typhimurium, high salt concentration inhibits biofilm formation by downregulating *csgD*, a central regulator of biofilm related genes [Romling 1998]. The same effect was observed in *E. coli* for curli genes, known as virulence factor involved in the adhesion to surfaces [Jubelin 2005]. On the other hand, high salt concentration has also been shown to promote biofilm formation by upregulating genes encoding PNAG production in *E. coli* through the activation of a cation responsive protein NhaR [Goller 2006]. In *S. epidermidis* and *S. aureus*, the transcriptional regulator *rbf* was found to induce biofilm formation in high salt and glucose concentration but in an *ica*-independent manner [Lim 2004].

In Chapter 10, ionic strength was found to play an important role in intercellular adhesion in *Staphylococci*. In fact, at an optimal salt concentration, that was shown to be strain-dependent, self-aggregation of *S. aureus*, *S. epidermidis* and one other CoNS (i.e. *S. warneri*) could be observed. The auto-aggregative phenotype was noticed in 50 % of the strains tested indicating that it is not a general feature of *Staphylococci*. Besides, isolates from three other Gram-positive species did not display this phenotype.

This phenomenon happens rapidly as large cell clumps can be witnessed only a couple of minutes after the ionic strength of a suspension was modified. Interestingly, reverting back to high ionic strength (0.15 M) induced the immediate dispersion of cell clusters. Taken together, these results, and especially the time scale, led us to suggest that the impact of ionic strength in this phenomenon is not related to genetic regulation. The pursued hypothesis rather involves surface charges and the conformation and repartition of polymers on the bacterial cell surface (for complete discussion, see Chapter 10).

Aggregation plays a key role throughout the life cycle of biofilms and the direct consequence of the ionic strength dependent aggregation on biofilm formation was also demonstrated on one *S. aureus* strain. At optimal ionic strength, *S. aureus* ATCC 6538 developed significantly more structured micro-colonies and produced EPS faster than in an equally nutritive medium at a higher ionic strength. Interestingly, after 6 hours of growth in nutritive medium, the aggregation reaction was not reversible anymore, most likely due to the presence of EPS that engulfs bacteria in micro-colonies. These evidences show that some staphylococcal strains might have found an elaborated way to make use of changes in the external medium to increase cell-cell stickiness and accelerate their biofilm development.

Self-aggregation of *Staphylococci* was previously reported in a test referred to as the Salt Aggregation Test (SAT). Briefly, bacterial cells are suspended in 0.02 M phosphate buffer at pH 6.8 and mixed with

portions of solutions containing large concentrations of ammonium sulfate to achieve ionic strength from 0.3 M to 6 M. In conditions of increasing salt concentration, the cell surface of bacteria was progressively dehydrated as the salts affect the availability of water molecules [Hermansson 1999]. The addition of ammonium sulfate to a bacterial suspension was therefore used as a method to assess the surface hydrophobicity of bacterial cells, quantified by the extent of self-aggregation occurring in the suspension [Jonsson 1984] [Rozgonyi 1985]. The SAT employs solutions at very high ionic strength, from 0.3 M up to 6 M. In the present work, self-aggregation happened in a range of ionic strength comprised between 0.0004 M to 0.15 M, closer to physiological conditions and well below the ionic strength used to perform the SAT.

The reported phenotype of self-aggregation is most likely part of a broader strategy used by *Staphylococci* to modulate the degree of intercellular aggregation depending on the situation the bacteria find themselves in. At the initial adhesion step, intercellular cohesion should be maximized to favor the development of the community. In the dispersion phase however, aggregation should be more finely controlled or even abolished to allow the biofilm to release planktonic cells, also called colonizers.

12.3 What ways around the failure of antibiotics to eradicate biofilms?

The major reason why HAIs are such a burden for healthcare systems is the inability to properly treat the majority of them once they are diagnosed. The root cause of chronic HAIs are, in most cases, biofilms [Bryers 2008]. These are difficult to eradicate with classical antibiotic therapies. Indeed, biofilms are resilient and regrow, usually causing the symptoms to reappear shortly after the antibiotic treatment is stopped (see Fig. 3.9 for illustration).

Several factors explain the resilience of biofilms. First, as previously discussed, most bacteria causing HAIs display an arsenal of antibiotic resistance genes. The second is the presence of persister cells, tolerant to cidal antibiotics because they happen to be in a metabolical state close to dormancy. They are stochastically present in biofilms [Lewis 2005]. Then the EPS matrix plays an important role of passive protection by slowing down mass transfer to and from the biofilm. As a result, the diffusion of bactericidal molecules in the core of the biofilm is hampered, and the exposition of bacteria significantly reduced [Stewart 2008] [Flemming 2010]. The synergistic effect of these three distinct phenomena justifies the increased persistence of biofilms.

From a therapeutic point of view, there are often few options left but to act surgically, either by removing the medical device if there is one or cure the infected zone. To bring alternative solution to the problem, novel strategies have been explored, many of them have been described in Chapter 4. In this work, the use of matrix degrading and cell-lytic enzymes to degrade biofilms has been examined.

Enzymes as means to control mature biofilms

Four enzymes were selected for biofilm removal assays. The first is an analog of DspB, an endoglucanase cleaving the bond between two N-acetylglucosamine residues of the PNAG. PNAG is the major component of the matrix in the biofilm formed by strains of *S. epidermidis* that carry the *ica* locus.

The second and third are two phage endolysins, lysin K (LysK) and Twort (Tw) as a reference to the phage they were isolated from [Schmelcher 2014]. LysK and Tw are both composed of three modular domains, each having their own catalytic activity. In the laboratory, they have been purified from *E. coli* strains bearing a construct with a copy of the gene and a His-tag. Phage lysins are primarily used by bacteriophages to inject their DNA/RNA in the host cell and to trigger the release of viral particles at the end of the lytic cycle [Borysowski 2006].

Such enzymes have also the property to lyse Gram-positive bacteria from outside the cells, for example when they are added exogenously to a medium [Nelson 2012]. The susceptibility of *Staphylococci* to lysis by phage lysins is species- and sometimes even strain-dependent (data not shown).

The last enzyme used in this study is lysostaphin (LST), a bacterial lysin, also called exolysin, discovered several decades ago in *Staphylococcus simulans* [Sschindler 1964]. It is a glycyl-glycin endopeptidase, cleaving the interpeptide bridge of *Staphylococcus* peptidoglycan (PG). The ability of LST to degrade staphylococcal biofilms has already been reported elsewhere [Wu 2003] [Kokai-Kun 2009]. In this work, the focus was set on showing potential interactions between LST and the three other enzymes described above.

The effect of these enzymes on biofilms grown in 96-well plates were assessed with two parameters: first, after the enzymatic treatment, the optical density at 595 nm (OD_{595}) of the wells was measured, as an indicator of cell lysis in the biofilm. The second measurement relates to the quantity of biofilm remaining in the wells after the treatment. It is a way to quantify the biofilm removal activity of enzymes. Experimentally, the absorbance at 540 nm (A_{540}) was recorded after a staining procedure with safranin was performed. Though crystal violet was initially chosen to stain biofilms, the protocol proposed by [Wu 2014] was found to provide more accurate results and less variability. The combination of these two measurements offers more insights on the way enzymes degrade *S. epidermidis* biofilms.

The results displayed in Chapter 11 were obtained by using a Response Surface Methodology (RSM). Briefly, this methodology propose a canonical sets of experiments to analyse the effect of several factors on defined responses, in this case the biofilm removal and cell lysis (see Chapter 11 for complete design). The four factors of this work were the enzymes described above. The results were analysed with a statistical software (JMP, SAS) by means of multiple regression and

validated according to defined criteria (Chapter 11). The biofilm degrading effect of enzymes alone and in synergy could then be determined.

The interpretation of the results for the removal of mature *S. epidermidis* biofilms grown in static conditions from a surface indicate that all four enzymes, LST, Tw, LysK and DspB analog (DspB*), were able to significantly remove biofilms. The dose-response effects of LST and LysK, and to a lesser extent of DspB*, were found to be non linear (quadratic). This observation can be partly attributed to a saturation in the experimental response (A_{540}) from certain levels of enzyme concentrations.

For the results of cell lysis within the biofilms, the analysis revealed that LST and LysK were the only two active enzymes. As for the biofilm removal activity, their effect was found to be quadratic. Interestingly, Tw, that was effective at killing exponentially growing *S. epidermidis* cells in suspension, appeared not to be significantly contributing to cell lysis within the biofilm. This finding is particularly unsettling since Tw and LysK belong to the same group of endolysins, featuring the same type of catalytic domains. Besides, as mentioned above, they have the same molecular weight. The reason for this lack of activity might result from particular cell wall properties of *S. epidermidis* in biofilms. Also, it is possible that Tw diffuses more slowly into the core of the biofilm, due to apparent charge or hydrophobicity. For instance, charged nanoparticles (NPs) have been shown to diffuse much more slowly into biofilms than neutral NPs of the same size [Forier 2013]. Finally, the enzymes may display differential sensitivities to proteases in the matrix of the biofilms, causing a loss of activity over time.

Concerning the interactions between each of the four enzymes tested in this work, a negative synergistic effect of Tw and the analog of DspB on biofilm removal was noticed. Similarly, lysostaphin with LysK were cross-influencing one another cell lysis activity, contrary to other reports [Becker 2008]. On top of the evaluation of the interactions

of several compounds, RSM allowed the prediction of cell lysis and biofilm removal of the tested enzymes. Indeed, multiple combinations of enzymes concentrations that yield an optimal effect could be deduced from the multiple regression model.

Of note, the conclusions drawn from this study are only valid in the conditions in which it was performed. In this work, *S. epidermidis* ATCC 35984 biofilms were grown in static conditions (96-well plates). Biofilms of the same *S. epidermidis* strain grown in the Biostream flow cells (see Appendix B for description) were challenged with the optimal mix of enzymes predicted by the model. The cocktail failed to significantly remove the biofilm (data not shown), indicating that flow cell biofilms are more resistant to these enzymes than the ones grown in 96-well plates. It is likely that dynamic conditions provided by the Biostream setup had a major influence on the proportion of cells and EPS in biofilms compared to the situation in biofilms grown under static conditions. This underlines the importance of the experimental model chosen to perform screening experiments. In any case, the development of (combination of) anti-biofilm compounds should be confronted to an experimental model as close as possible from the real conditions in which biofilm thrive. Because such models may be costly and time consuming (i.e. flow cells, batch reactors or murine models) [Coenye 2010], experimental designs could prove useful to optimize screening procedures.

Pros and cons of using enzymes as means to eradicate biofilms

The use of enzymes to combat biofilms involved in specific types of HAIs is a promising strategy. Enzymes degrading the matrix of EPS of biofilms, for example DNAses or hexaminidases, could enhance the action of anti-microbial agents such as antibiotics by increasing the exposure of bacterial cells. On the other hand, they also induce a massive release of bacteria in the surroundings of the biofilm. In case of HAIs, a dissemination of planktonic cells from a biofilm would considerably augment the risk of developing secondary infections and potentially worsen

the situation [McDougald 2012]. For that reason, the use of matrix degrading enzymes is to be coupled with potent anti-microbial agents to kill bacteria liberated from the biofilm. These anti-microbial agents could be lytic enzymes, notably phage endolysins. Endolysins have been reported to significantly degrade biofilms of *S. aureus* [O’Flaherty 2005] [Sass 2007] [Kokai-Kun 2009] and more recently of *S. epidermidis* (see results in Chapter 11) [Gutiérrez 2014]. Even persister cells should be lysed by endolysins, although this has never been specifically reported.

The use of enzymes as potential therapeutic agents is not new and some successful examples have been reported, notably in the treatment of cancers [Holcenberg 1977]. However, each enzyme bound to become a drug has to go through clinical trials. Due to their proteinaceous nature, they could be eliciting adverse immune response from the patient, including the production of antibodies that could block their activity [Borysowski 2006]. Preliminary tests on murine models with a pneumococcal endolysin Cpl-1 indicated that mice produced antibodies against the enzyme but did not display any visible sign of anaphylaxis or adverse side effect. In the same work, the mice that were previously injected with Cpl-1 and control (naive) mice were challenged with pneumococci intravenously. The bacteremic titers (number of live pneumococci cells per blood volume) decreased about 2-3 logs shortly after both populations of mice were treated intravenously with 200 μ g of Cpl-1. These results show that Cpl-1 was still active *in vivo* despite the presence of anti-Cpl-1 antibodies [Loeffler 2003].

The potential to develop endolysin-based anti-biofilm drugs is enormous, given the diversity of phages and lysins in nature [Clokier 2011]. Besides, reports of bacterial resistance to endolysins are still rare [Nelson 2012], partially owing to the fact that these enzymes target highly conserved bonds in the PG. For the above cited reasons, endolysins with confirmed anti-biofilm potential might be part of the solution for HAIs that cannot be healed with currently available therapeutics.

Conclusion and perspectives

S. epidermidis has, over the last twenty years, emerged as a leading opportunistic pathogen. The success of *S. epidermidis* as a nosocomial pathogen is intimately linked to the increasing use of implantable medical devices. These provide an opportunity for this ubiquitous colonizer of human skin to settle in the host, and thrive in the form of biofilms. In most cases, it affects fragile people such as the neonates and the elderly who generally are immunocompromised. Recent statistics indicate that *S. epidermidis* ranks first in encountered pathogens in infections associated with central venous catheters, peritoneal dialysis catheters, neurosurgical devices (e.g. shunts and pressures devices), vascular grafts and prosthetic joints [Piette 2009]. Such chronic infections are very difficult to treat with antibiotics for two main reasons. First, biofilms are intrinsically tolerant to antibiotics due to the presence of persisters [Lewis 2010]. Second, most strains of *S. epidermidis* isolated in clinical settings carry various genes of resistance to commonly used antibiotics [Diekema 2001]. Besides, biofilms are known as "hot spots" for HGT, especially conjugation involving plasmids that carry antibiotic resistance genes [Savage 2013]. The clinical consequences of these nosocomial infections include significantly lengthened hospital stays, increased patient morbidity and mortality, usually resulting from septicemia [Eiff 2002].

This work was motivated by the crucial need to improve the understanding of the mechanisms underlying biofilm formation and to develop adequate strategies to control biofilms. Since for *S. epidermidis*, biofilm development is a major virulence factor, it was chosen as experimental

model.

The focus was first set on studying adhesion of *S. epidermidis* on surfaces coated with proteins and particularly Fg, a plasma protein that is notably involved in blood clotting and platelets recruitment. The specific role of SdrG, an adhesin described some years ago [Hartford 2001], has been questioned. At the beginning of this work, the mechanism by which SdrG is able to bind to Fg was known [Ponnuraj 2003] but the characteristics of the binding event were not yet elucidated. In particular, the extent of the force involved in the SdrG-Fg bond remained unclear. AFM force spectroscopy allowed to reveal that the binding strength of SdrG to its substrate was unusually large (about 2 nN). Also, it was shown to be influenced by contact time: the longer the two proteins were in contact, the more intense the force (see Chapter 8). Further work highlighted a correlation between the surface abundance of SdrG on the bacterial cell surface and the ability of individual cells to adhere to Fg-coated surfaces. Trends for whole bacterial populations adhesion levels were matching those of single cells, indicating that SdrG surface density directly influences the immobilization of *S. epidermidis* on surfaces covered with Fg.

In vivo, reports of increased expression of *fbe* to crowd the cell surface with SdrG [Sellman 2008] indicate that the opportunistic pathogen *S. epidermidis* tries to favor its establishment on implants or devices. Once bound to a surface, several lines of evidence suggest that bacteria undergo a physiological switch towards a so-called "surface-bound state". In this condition, bacteria "sense" that they are interacting with a substratum [Busscher 2012] and, through a network of regulatory pathways, induce the development of a biofilm [Hall-Stoodley 2004] [Mack 2007].

Given the likely importance of SdrG in the infective strategy of *S. epidermidis*, the quest for new strategies to prevent infections should focus on the genetic regulation of *fbe* expression or on blocking the ability of SdrG to bind to Fg, for example thanks to a synthetic

peptide that interferes with the "dock, lock and latch" mechanism. The idea of prophylactic vaccines targeting SdrG have also been proposed [Sellman 2008] but may not be well-suited since many of patients contracting such chronic infections are immunocompromised.

Several other MSCRAMMs and other cell surface polymers have been identified as key elements that facilitates the adhesion of *S. epidermidis* to various types of surfaces. For example, the protein Bhp has been shown to mediate binding on hydrophobic surfaces, teichoic acids can attach to fibronectin and SdrF can specifically recognize type I collagen [Otto 2012]. It is not likely that therapeutic agents silencing the functions of these polymers can easily be found and used in clinical settings to prevent the onset of indwelling device associated infections. Based on the findings reported in Chapter 9, another approach based on biosensors could be proposed. It involves the detection of surface-adhering bacteria using impedance spectroscopy. The developed biosensors are of small size (about 300 μm in diameter), a feature that would allow their integration on the surface of implants. So-called "smart" implants could provide a way to follow the contamination of device in a non-invasive fashion. Potential advantages of such devices are, among others, faster diagnosis (early detection of biofilms) and follow-up of the effect of therapeutic treatments on the biofilm. Incomplete eradication of biofilms could therefore be spotted early on, avoiding the reappearance of patients symptoms.

Beside the critical importance of bacterial adhesion, the development of biofilms relies heavily on intercellular adhesion, or aggregation. In fact, while growing, the lone surface-bound bacterial cells tend to form micro-colonies that are held together by several mechanisms including the production of an EPS matrix and the specific interaction of cell surface proteins (e.g. Aap for *S. epidermidis*). Alternatively, bacteria may also form cell clumps in suspension that can then adhere on a surface, partially short-cutting the formation of micro-colonies. The process of aggregation is known to be the second phase of biofilm formation. It is notably influenced by both active (e.g. genetic) and

passive (e.g. environmental influence) responses triggered by various stimuli such as the concentration of glucose, temperature, the presence of calcium or ethanol [Hall-Stoodley 2004].

In Chapter 10, the involvement of ionic strength in a yet unreported type of aggregation of *Staphylococci* was investigated. Indeed, several staphylococcal strains belonging to *S. aureus*, *S. epidermidis* and *S. warneri* species, were shown to rapidly form large cell clumps at an optimal ionic strength. In the experiments, the ionic strength was solely brought by NaCl which is not likely to induce aggregation in the way divalent ions such as calcium can. The reversibility of the aggregative phenotype led us to propose an hypothesis that involves electrostatic interactions of bacterial cell surface charges and deployment of cell surface polymers. Briefly, at an optimal ionic strength, the cell surface polymers are sufficiently contracted to diminish the repulsive radius of two negatively charged cells and allow localized positive charge clusters to interact with negative charges on another cell. Higher ionic strength would shield almost all electrostatic interactions and annul the phenomenon. AFM experiments gave indications that electrostatic interactions are at play but failed to fully validate the proposed hypothesis. Further work would be necessary to identify the root cause of this phenomenon. For instance, AFM force spectroscopy performed with tips displaying different chemistry, such as positive charges or hydrophobic groups (e.g. -CH₃ terminated groups) may reveal complementary information on the origin of the mechanism. Also, a comparison of the electrophoretic mobility of strains that aggregate and those that do not may give interesting hints related to surface potential of these strains. Alternatively, proton titration of bacterial suspension can also be performed to get additional insights [Poortinga 2002].

The impact of the ionic strength-dependent self-aggregation on biofilm formation of a *S. aureus* reference strain was demonstrated *in vitro*, in flow cells (see Chapter 10) and in microtiter plates (data not shown). This raise the question of the culture conditions of staphylo-

coccal biofilms *in vitro*. Indeed, the results presented in this study show that the structures of the biofilms grown at different ionic strength but with the same amount of nutrients are markedly distinct. This is to be put in perspective with other findings reported by [Lim 2004] that increased salt concentration, in combination with glucose, increase biofilm formation by inducing the production of PNAG. Ionic strength must now be considered as a parameter of importance of laboratory medium dedicated to biofilm growth. The impact of culture parameters on the properties and composition of mature biofilms should therefore not be overlooked.

Biofilms that have reached a certain degree of maturity display typical characteristics, notably provided by the presence of the EPS matrix and the various physiological states of the bacterial cells. One of their key features is the resilience in harsh environments, for instance in the presence of oxidative stresses, UV, dry conditions or high concentrations of antibiotics. Biofilms as bacterial communities make the most of unity and cooperation to survive threats that planktonic populations might not have withstood [Jefferson 2004]. This is one of the main reasons why they cause such troubles in the industry and in hospitals. In this study (Chapter 11), the potential of enzymes for the degradation of mature *S. epidermidis* biofilms was investigated. Four enzymes, including lysostaphin, Tw and LysK, two phage lysins, and an analog of DspB, were tested for anti-biofilm activity. The analog of DspB, an N-acetylglucosamine, displayed a strong biofilm removing effect and, as expected from its enzymatic activity, did not provoke cell lysis. On the contrary, both lysostaphin and LysK were deemed efficient at removing biofilm and killing bacteria. On the other hand, Tw, an endolysin of the same family as LysK, with a similar structure, did remove biofilm significantly but failed to lyse the cells in the biofilms. Preliminary tests with other phage endolysins (80 α and SH2) were not conclusive as they were not able to remove any biofilm and did not cause any detectable cell lysis. The anti-biofilm potential of phage endolysins thus does not seem to be a general characteristic of these enzymes. However, given the diversity of phages available in nature, carrying

endolysins with various features [Nelson 2012], this class of enzymes can still provide a largely untapped pool of anti-biofilm compounds.

In view of the development of potential therapeutic agents to target biofilms, it is essential to carefully choose the experimental model. Indeed, biofilms grown in diverse conditions, for instance in *in vitro* flow cells, or in *in vivo* murine models, will display different characteristics, notably in terms of proportions of EPS matrix and cells. This undoubtedly affects the potency of the active principle that degrade biofilms and lyse cells within these.

Controlling bacteria is a challenge. Nearly a century of struggle has taught us that the use of single anti-bacterial treatments almost always leads to the appearance of resistance mechanisms. In most cases, these resistance genes can be shared amongst bacterial populations and spread worldwide rapidly. A solution to counter this is to use combinations of two or more compounds to eradicate bacteria or biofilms. In this way, the likelihood for micro-organisms to survive becomes really low. In addition, one can also benefit from the synergistic action of the anti-bacterial principles. However, correctly determining the interactions, positive or negative, between the compounds can be time-consuming and difficult to properly quantify. In this study, RSM was implemented to this extent. These approaches are already used in analytical chemistry and tend to become more and more popular in biotechnological fields of research, for instance to assess the impact of temperature, pH and NaCl concentration on the activity of peptidases or the optimal culture conditions of *Streptomyces* spp. for the production of chitinase [Baş 2007]. These tools can be advantageous to spot the synergies between compounds that have proven efficient at degrading biofilms in order to propose potentially successful combinatory treatment of biofilms.

In conclusion, this work provides comprehensive insights in the biofilm formation process of *S. epidermidis*, in connection with the prob-

lem of device-related nosocomial infections. To tackle this issue, several leads for the development of preventive and curative strategies have also been proposed, including sensing surfaces and enzymatic control of mature biofilms.

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Scientific communications

A.1 Oral communications

- **Vanzieleghem T.**, Modrie P. and Mahillon J. (2011) Conjugation in *Bacillus cereus*: how does the biofilm lifestyle impact on transfer efficiency? IWA Biofilm Meeting 2011 - Shanghai (China)
- **Modrie P.**, Vanzieleghem T. and Mahillon J. (2011) Multi-species biofilm communities: from close proximity to enhanced cross-genus sexuality. IWA Biofilm Meeting 2011 - Shanghai (China)

A.2 Posters

- Modrie P., Timmery S., Castiaux V., **Vanzieleghem T.**, Beuls E., Deserranno C., Mahillon J. (2011) DNA exchange in the *Bacillus cereus* group in biofilms and under stress. Belgian Society for Microbiolohy (BSM) 2011 meeting - Brussels (Belgium)
- **Vanzieleghem T.**, Thiran E., Mahillon J. (2012) Genetic diversity in *Staphylococcus epidermidis* clinical and environmental isolates biofilm forming skills. Biofilm V Conference - Paris (France)
- **Vanzieleghem T.**, Nannan C. and Mahillon J. (2013) Intraspecies variation in *Staphylococcus epidermidis* adhesion onto coated and uncoated hydrophobic materials. BSM 2013 meeting and Belgian Interdisciplinary Biofilm Research (BIBR) 2013 meeting - Brussels (Belgium)
- **Vanzieleghem T.**, Nannan C., Herman P., Dupont C., Dufrene Y.F. and Mahillon J. (2013) Intraspecies variation in *Staphylococcus epidermidis* adhesion onto coated and uncoated hydrophobic materials, Eurobiofilm Meeting - Ghent (Belgium)

APPENDIX B

Biostream Patent

In this work, an efficient flow cell device was needed. Biostream was invented in order to respond to this demand. The UCL has pursued a patent application for this invention. The latter was published on the 13th of March 2014 under the publication number WO/2014/037479. A condensed version of the document with only the front page and the claims is presented in the next pages. A full version is freely accessible online on Espacenet : http://worldwide.espacenet.com/publicationDetails/originalDocument?CC=W0&NR=2014037479A1&KC=A1&FT=D&ND=3&date=20140313&DB=worldwide.espacenet.com&locale=fr_EP.

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[Continued on next page]

(54) Title: FLUIDIC DEVICE FOR STUDYING OF SURFACE-DWELLING MULTICELLULAR LAYERS AND MICROBIAL BIOFILMS

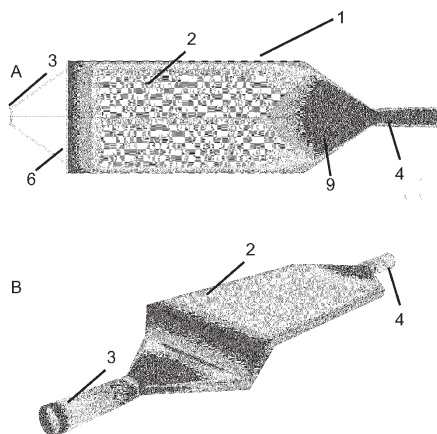


Figure 2

(57) Abstract: The present invention relates to a fluidic device that can be used for the analysis of surface-dwelling multicellular layers, some of which comprise biofilm and can be referred to as biofilms, and their formation under controlled dynamic conditions. More particularly, the surface in the fluidic chamber on which the multicellular layer is grown is detachable and/or removable from the fluidic chamber, thereby providing a highly versatile device.

WO 2014/037479 A1

CLAIMS

1. A fluidic device for the analysis of a surface comprising:
 - a fluidic chamber comprising one or more surfaces enclosing the fluidic chamber through which fluid can flow along a flow path, wherein said fluidic chamber is characterized by having a Young's Modulus [E] ranging between 500 kPa and 5 MPa;
 - an inlet port fluidically connected to said fluidic chamber through an inlet flow distributor for distributing the incoming fluidic flow from said narrow inlet port to said wider fluidic chamber;
 - an outlet port fluidically connected to said fluidic chamber optionally through an outlet flow distributor for distributing the outflowing fluidic flow from said wider fluidic chamber to said narrow outlet port;
- characterized therein that said fluidic chamber comprises at least one detachable and/or removable surface for analysis purposes.
2. A fluidic device according to claim 1, wherein said fluidic chamber is made from a polymeric material having Young's Modulus [E] ranging between 500 kPa and 5 MPa..
3. A fluidic device according to claim 1 or 2, wherein said fluidic chamber is made from a polymeric material chosen from PDMS, acrylate elastomers, fluoroelastomers and styrenic based elastomers.
4. A fluidic device according to any of claims 1 to 3, wherein said fluidic chamber comprises a bottom surface, two side surfaces and a top surface, wherein at least part of said top or bottom surface are detachable and/or removable.
5. A fluidic device according to any of claims 1 to 4, wherein at least part of said fluidic chamber is transparent for optical imaging, for microscopy, and/or for fluorescence imaging, thereby providing an imaging observation site.
6. A fluidic device according to any of claims 1 to 5, further comprising observation zones and/or sensors located on or in one or more surface enclosing said fluidic chamber, for observing and/or monitoring the formation and/or cultivation of the surface-dwelling multicellular layer on said detachable and/or removable surface.
7. A fluidic device according to any of claims 1 to 6, wherein said detachable and/or removable surface is made from or provided with an adherent surface material suitable for adhering a surface-dwelling multicellular layer, wherein said surface material models a surface likely to be involved in cell adhesion and/or formation of the multicellular layer.
8. A fluidic device according to any of claims 1 to 7, wherein said adherent surface material is chosen from the group comprising aluminum, stainless steel, silver, copper, hydroxyapatite, silicon, latex, urethane, PVC, ceramic, steel, gold, titanium, polyethylene,

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polysiloxanes, biocompatible glasses, poly-methylmethacrylate, Teflon (or PTFE), polypropylene, polystyrene, polyamides, polyethers, polyesters, coated block polymers of polyethylene oxide (PEO), polypropylene oxide (PPO), polybutylene oxide (PBO), hydrogels, food film polymers, polycarbonate filters and minerals.

- 5 9. A fluidic device according to any of claims 1 to 8, wherein said inlet flow distributor or part thereof slopes downward relative to the horizontal position of the fluidic chamber.
- 10 10. A fluidic device according to any of claims 1 to 9, wherein said inlet flow distributor comprises:
 - a first distributor region shaped as an isosceles trapezoid;
 - 10 - a second rectangular shaped distributor region;

wherein said first distributor region is fluidically connected to the narrow inlet channel through the smaller of the two parallel sides and fluidically connected to said second rectangular shaped distribution region through the larger of the two parallel sides of said isosceles trapezoid, wherein said second rectangular shaped distribution region is further fluidically
- 15 connected to said fluidic chamber, characterized therein that said second distribution region slopes downward relative to the horizontal position of said fluidic channel.
11. A fluidic device according to any of claims 1 to 10, wherein the depth of the fluidic chamber ranges between 0.1 and 5 mm.
- 20 12. A fluidic device according to any of claims 1 to 11, wherein said device comprises at least two fluidic chambers with corresponding inlet and outlet ports and distributors, wherein said two fluidic chambers are arranged adjacent to each other and are separated by detachable and/or removable surface, wherein said detachable and/or removable surface is preferably a membrane.
- 25 13. A method for performing an analysis between a surface of interest and a fluid of interest, comprising:
 - a) providing a fluidic device comprising:
 - a fluidic chamber comprising one or more surfaces enclosing the fluidic chamber through which fluid can flow along a flow path, said fluidic chamber comprising at
 - 30 least one detachable and/or removable surface of interest;
 - an inlet port fluidically connected to said fluidic chamber;
 - an outlet port fluidically connected to said fluidic chamber;
 - b) dispensing fluid of interest through said fluidic device, thereby contacting the fluid of interest with the surface of interest; and
 - 35 c) monitoring interaction between the surface of interest and a fluid of interest under varying conditions.

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14. A method according to claim 13, for cultivating and monitoring surface-dwelling multicellular layers, comprising:

a) providing a fluidic device comprising:

- a fluidic chamber comprising one or more surfaces enclosing the fluidic chamber through which fluid can flow along a flow path, said fluidic chamber comprising at least one detachable and/or removable surface of interest for the cultivating multicellular layers;

- an inlet port fluidically connected to said fluidic chamber;

- an outlet port fluidically connected to said fluidic chamber;

b) dispensing a fluid of interest through said fluidic device, said fluid of interest being a liquid growth medium, said liquid growth medium optionally comprising microorganisms, animal cells, plant cells or fungi cells, into said fluidic chamber, said growth medium flowing across said detachable and/or removable surface, thereby generating a surface-dwelling multicellular layer on said detachable and/or removable surface; and

c) monitoring the multicellular layer on said detachable and/or removable surface under varying conditions.

15. Method according to claim 13 or 14, wherein said varying conditions comprise different types of fluid media, different fluid flow rates, different temperatures, different compounds and/or combinations thereof.

Adhesion of *S. epidermidis* in flow cells

C.1 Aim

One of the main focus of this work is bacterial adhesion to surfaces. In this context, the adhesion of *S. epidermidis* strains in dynamic conditions was investigated on PDMS coated or not with proteins. The results presented in this Appendix were used to validate a model based on probabilities that accounts for bacterial adhesion under flow that is not presented in this work.

C.2 Material and methods

Bacterial strains and culture

Six *S. epidermidis* strains were used in this study: ATCC 12228, ATCC 35984, HB ([Nilsson 1998]), HB SdrG(-) in which the gene SdrG has been knocked out ([Hartford 2001]), DS1 and Bo19, two strains isolated from Mont-Godinne hospital in 2012, identified by 16S DNA sequencing or MALDI-TOF IVD (Bruker).

Bacteria were streaked on TSA plates from frozen stock and incubated for 24 h at 37°C. Liquid cultures were carried out in 250 ml Erlenmeyers containing 50 ml of TSB, incubated at 37°C for 16 h under 120 RPM agitation.

Preparation of bacterial suspension

Overnight cultures were centrifuged at 5000*g* for 15 min. The supernatant was removed and the pellet was resuspended in 20 ml of sterile PBS. The resulting suspension was filtered on 5 μm to remove any large aggregates. OD at 600 nm of the suspension diluted 10 times in PBS was adjusted to 0.4 to obtain a bacterial charge of ca. $1.5 \cdot 10^9$ CFU/ml. Plates counts of serial dilutions were performed on TSA to assess the number of CFU/ml in bacterial suspensions.

Spin coating of PDMS on glass surfaces

Glass coverslips (24 x 60 mm, 0.17 mm thick, Menzel Glazer) were cleaned in piranha ($\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$ 3:1 v:v) and thoroughly rinsed with distilled water at least five times. The clean coverslips were dried under a flow of gaseous nitrogen and placed in the spin coater (Laurell Technologies, model: WS-400B-6NPP/Lite). One ml of a previously prepared 1:2 solution of PDMS (Dow Corning, Sylgard 184) in hexane (VWR) was deposited on the glass surface and a cycle of 1 min at 3000 RPM was performed in the spin coater. After that, the PDMS coated slides were cured at 95°C for 1 h. The surfaces were kept in a sealed Petri dish until use.

Flow cell setup

Biostream flow cells (see Appendix B for detailed description of the devices) were assembled using PDMS coated slides. The input of each flow cell was connected to three recipients and the output to a waste bottle by 1.6 mm (inner diameter) silicone tubes. The whole system was autoclaved for 15 min at 121°C. Under a sterile atmosphere, PBS was added to the first recipient connected to flow cells input, a coating solution to the second and the bacterial suspension to the third. The coating solutions used were TSB, a solution of Fg (0.5 mg/ml in PBS, filter sterilized on 0.22 μm) or a solution of bovine serum diluted 10 times in PBS, filter sterilized on 0.22 μm .

Adhesion of *S. epidermidis* under flow

Adhesion experiments were performed at 37°C according to the following scheme. PBS was first flown in the flow cells for 10 minutes. Then, the coating solution was injected during 20 min at a flow rate of 0.5 ml/min, that was conserved for the whole experiment. Flow cells were rinsed with PBS for 20 min. To proceed to the adhesion experiment, bacterial suspensions were pumped through the flow cells for 20 min. A final rinsing step of 45 min with PBS was performed to remove any unbound or loosely bound bacteria.

Each strain was tested in triplicate on bare PDMS and on each coating.

Quantification of bacterial adhesion

Micrographs of the PDMS surfaces inside the flow cells were then taken in 2 rows, 7 positions in each row (a total of 14), using a 40x long working distance objective (0.6 numerical aperture, Fluotar, Leica) mounted on an inverted microscope (DMI 6000, Leica) equipped with a sensitive CCD black and white 1.4 MP camera (DFX365, Leica). The number of bacteria per image were determined with ImageJ using the 'Analyze particles' after a basic thresholding had been applied. Bacterial counts were converted to densities of adherent bacteria per square millimeter (bacteria/mm²).

Statistical analysis

An ANOVA was performed on the data of densities of adherent bacteria using JMP (SAS). Subsequently, a Tukey HSD (Honestly Significant Difference) was performed on the ANOVA results to discriminate the mean value of bacterial density on each coating for each strain. This test is conservative and assesses the difference between two values on the basis of the variance of the experiments provided by the ANOVA.

C.3 Results

Fig. C.3 presents the results of flow cell adhesion experiments. Globally, adhesion to PDMS was effective for all tested strains, only minor differences could be noticed for ATCC 12228 and HB. Interestingly, these two strains were the ones that showed significant adherence to Fg coated PDMS. These results are line with those presented in Chapter 8, section 8.2. The other strains displayed a low affinity for Fg coated PDMS. On serum coated PDMS, the strains HB and ATCC 35984 stick out while the adhesion of the rest of the strains was weak. Yet, the level of adhesion to the coating of bovine serum is clearly inferior to that of ATCC 12228 and HB on Fg. Finally, a coating of TSB does not impair bacterial adhesion to the same extent as bovine serum coating. The reference strains seemed slightly more affected than the other strains.

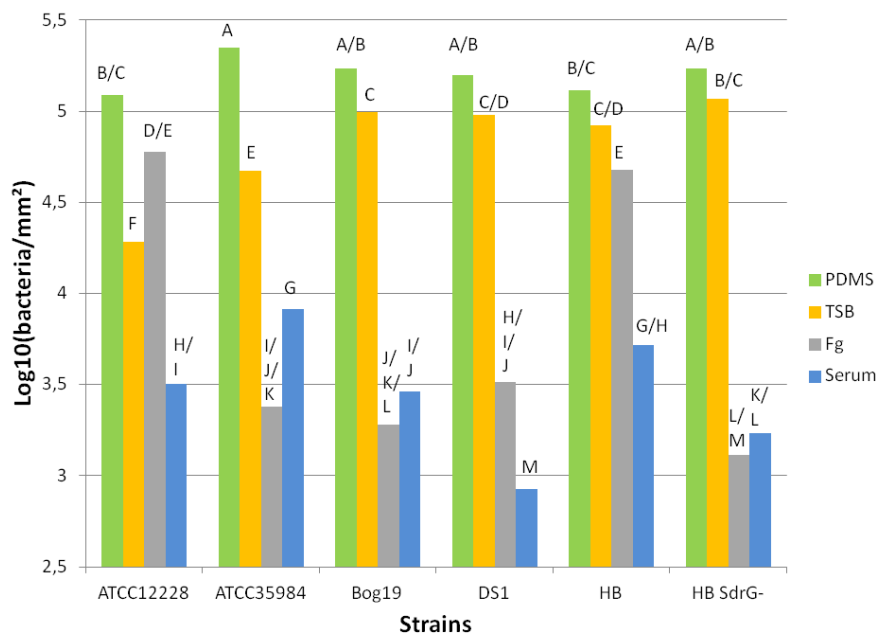


Figure C.1: Plot of the log10 of densities of adherent bacteria of six *S. epidermidis* strains on PDMS and PDMS coated with TSB, Fg and bovine serum. Two bars that are not connected by the same letter have been deemed significantly different by the Tukey HSD test.

C.4 Discussion

Hydrophobic PDMS surfaces promoted adhesion of all the strains tested in this study. According to the extended DLVO theory, it is likely that a sum of hydrophobic forces (attractive Lewis acid-base interactions) mediated by cell surface polymers strongly anchors *S. epidermidis* on PDMS [Hermansson 1999].

Bovine serum coated PDMS inhibited bacterial adhesion significantly. Albumin is the major compound present in bovine serum, it is likely that it accounts for the vast majority of the coating. Albumin is not recognized by specific MSCRAMMs, unlike Collagen or Fg, but has been shown to inhibit adhesion of *S. epidermidis* [Linnes 2012].

Our results are in line with this hypothesis. The strains ATCC 35984 adheres better than the other strains. This is probably the result of specific recognition of plasma proteins present in the coating, such as vitronectin or elastin. Indeed, genes coding for adhesins binding these proteins are present in the genome of *S. epidermidis*, notably in the strain ATCC 35984.

On TSB-coated PDMS, bacterial adhesion was globally reduced compared to bare surfaces but not to the extent of bovine serum coated PDMS. XPS analysis revealed that the surface conditioning layer forming in contact with TSB was less dense than that formed upon deposition of bovine serum. Water contact angle measurements confirmed that the drop in hydrophobicity compared to bare PDMS was far less than with bovine serum (Michel Thiel, Master thesis, June 2013). Our hypothesis is that adsorbed peptides from TSB were displaced by biopolymers of *S. epidermidis* cell surface upon adhesion. The surface affinity of larger biopolymers is often larger compared to peptides, notably thanks to conformational changes that occur to maximize the polymer-surface interactions.

Finally, on Fg-coated PDMS, two strains, ATCC 12228 and HB adhered significantly more than the other strains. In Chapter 8, the AFM results show that these two strains display SdrG in its active form on their surface while the mutant deficient for *sdrG* and the strain ATCC35984 did not. AFM analyses were not performed for DS1 and Bo19 but it is reasonable to believe that they harbor low amounts of SdrG on their cell surface, if at all.

Overall, these results confirm the trends presented in Chapter 8 and several reports in the literature [Linton 1999] [Patel 2006] [Heilmann 2011].