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VARIABILITY OF PHENOTYPIC, PROTEOMIC AND TRANSCRIPTOMIC EXPRESSION OF STAPHYLOCOCCUS AUREUS SURFACE ADHESINS

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VARIABILITY OF PHENOTYPIC, PROTEOMIC AND TRANSCRIPTOMIC EXPRESSION OF *STAPHYLOCOCCUS AUREUS* SURFACE ADHESINS

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Présenté à la Faculté de Biologie et Médecine de l'Université de Lausanne

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Lausanne, le 14 décembre 2012

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Antoine Guisan

à ma maman…

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Résumé

Staphylococcus aureus est un agent pathogène humain responsable d'une grande variété de maladies allant d'infections bénignes de la peau comme des furoncles, à des infections plus sévères telles que des septicémies ou des endocardites infectieuses. Il est également responsable d'intoxications alimentaires et du syndrome de choc toxique. Cette hétérogénéité des infections et la capacité de *S. aureus* à développer des résistances à la plupart des antibiotiques disponibles, reflète son extraordinaire capacité à s'adapter et à survivre dans une grande variété d'environnements. La pathogénie de l'infection à *S. aureus* implique un large éventail d'adhésines associées à la paroi cellulaire et de toxines extracellulaires qui favorisent la colonisation et l'invasion de l'hôte. De plus, *S. aureus* est extrêmement bien équipé de systèmes de régulation qui détectent les conditions environnementales et répondent de manière très fine par des modifications de l'expression des déterminants métaboliques et des facteurs de virulence. Les adhésines de surface ou MSCRAMMs (Composants de la Surface Microbienne qui Reconnaissent les Molécules d'Adhérence de la Matrice extracellulaire) permettent la liaison à la matrice extracellulaire de l'hôte ou à des composants du sérum, comme notamment le fibrinogène, la fibronectine, le collagène et l'élastine, afin de promouvoir la colonisation et l'invasion des tissus. Les principales MSCRAMMs incluent une famille de protéines de surface liées de manière covalente au peptidoglycane de la paroi cellulaire par l'intermédiaire d'un motif LPXTG conservé. Des analyses génomiques indiquent qu'il existe jusqu'à 22 protéines à LPXTG chez *S. aureus* qui peuvent agir individuellement ou en synergie pour favoriser l'infection.

Dans la première partie de cette étude, nous avons comparé les phénotypes d'adhérence au fibrinogène et la fibronectine de 30 isolats de *S. aureus* provenant de porteurs sains avec les phénotypes d'adhérence de 30 isolats d'endocardites infectieuses et de 30 isolats d'hémoculture. Dans l'ensemble, une grande variation des phénotypes d'adhérence a été obtenue *in vitro*, mais aucune différence significative n'a été observée entre les souches de porteurs sains et d'infections. Nous avons également étudié la relation entre l'adhérence *in vitro* et l'infectivité *in vivo* dans un modèle expérimental d'endocardite chez le rat, en utilisant 4 isolats qui présentaient soit des phénotypes d'adhérence extrêmement bas, soit très élevés. Contre toute attente, aucune différence n'a été observée entre le pouvoir infectieux *in vivo* des isolats qui étaient peu ou très adhérents *in*

vitro. Nous avons conclu que la variabilité d'adhérence au fibrinogène et à la fibronectine *in vitro* n'était pas corrélée avec le pouvoir infectieux *in vivo*, et que les différences de pouvoir infectieux entre les différentes souches ne pouvaient être exprimées qu' *in vivo*. Par conséquent, compte tenu de l'importance de l'expression des adhésines lors de l'infection, des analyses semi-quantitatives de la présence de ces adhésines à la surface bactérienne ont été réalisées par une approche protéomique.

Dans la deuxième série d'expériences, la présence physique des protéines à LPXTG a été mesurée à la surface cellulaire à différents temps au cours de la croissance bactérienne et dans différents milieux de culture. *S. aureus* Newman a été cultivé dans du bouillon de soja tryptique (TSB) ou du RPMI (Roswell Park Memorial Institut) et des échantillons de cellules ont été prélevés au cours de la phase exponentielle de croissance et jusqu'en phase stationnaire tardive. Les expériences ont été réalisées avec des mutants du régulateur global *agr*, ainsi que de la protéine A ou du facteur d'agglutination A, ClfA. Les peptides des adhésines ont été générés par une méthode de digestion enzymatique à la trypsine permettant un "rasage" de la surface des bactéries vivantes. Les analyses protéomiques semi-quantitatives ont été réalisées en tandem par chromatographie liquide et spéctrométrie de masse (LC-MS). Nous avons également déterminé en parallèle l'expression des ARNm correspondants par analyse de puces à ADN et le phénotype d'adhérence des bactéries au fibrinogène *in vitro*. Le protéome de surface obtenu était très complexe et contenait de nombreuses protéines qui théoriquement n'appartenaient pas à l'enveloppe bactérienne, y compris des protéines ribosomales et des enzymes métaboliques. Seize des 22 protéines à LPXTG connues ont été détectées, mais étaient exprimées de façon différentielle. Comme supposé, 9 protéines connues pour être régulées par *agr* (par exemple : Spa, FnBPA, ClfA, IsdA, IsdB, SasH, SasD, SasG et FmtB) ont augmenté jusqu'à la phase de croissance exponentielle tardive puis ont diminué en phase stationnaire. Cependant, cette expression "en cloche" a été annihilée avec le mutant *agr*-. Toutefois, seuls la protéine A et SasH ont modifié leur profil protéomique et ARNm en parallèle, dans la souche parental et le mutant *agr*-, tandis que toutes les autres protéines LPXTG ont modifié leurs profils protéomiques indépendamment de leur ARNm durant la croissance. De plus, ClfA a hautement été transcrit et actif dans les tests d'adhérence au fibrinogène *in vitro* en phase tardive de croissance (24h), alors qu'il a été très peu détecté en protéomique. Cette expression différentielle a aussi bien été détectée en milieu riche en fer dans le TSB qu'en milieu pauvre dans le RPMI. Les déterminants de surface du système régulés par le fer (*isd*), notamment IsdA, IsdB et IsdH étaient très peu exprimés

dans le TSB alors qu'ils ont augmenté leur expression par 10 dans le RPMI. Nous en avons conclu que des analyses protéomiques semi-quantitatives de protéines spécifiques étaient réalisables chez *S. aureus* et que les analyses protéomiques, transcriptomiques ainsi que les tests phénotypiques d'adhérence montraient des profils différentiels chez cette bactérie. De plus, les signatures peptidiques libérées par le rasage à la trypsine suggèrent des expositions différentielles des domaines de ces protéines dans des environnements différents, qui pourraient être pertinents pour l'établissement de vaccins anti-adhésines. Une compréhension approfondie de la physiologie de *S. aureus* doit donc intégrer toutes ces approches.

Summary

Staphylococcus aureus is a highly successful pathogen responsible of a wide variety of diseases, from minor skin infection to life-threatening sepsis or infective endocarditis, as well as food poisoning and toxic shock syndrome. This heterogeneity of infections and the ability of *S. aureus* to develop antibiotic-resistance to virtually any available drugs reflect its extraordinary capacity to adapt and survive in a great variety of environments. The pathogenesis of *S. aureus* infection involves a wide range of cell wall-associated adhesins and extracellular toxins that promote host colonization and invasion. In addition, *S. aureus* is extremely well equipped with regulatory systems that sense environmental conditions and respond by fine tuning the expression of metabolic and virulence determinants. Surface adhesins referred to MSCRAMMs – for Microbial Surface Component Recognizing Adherence Matrix Molecules – mediate binding to the host extracellular matrix or serum components, including fibrinogen, fibronectin, collagen and elastin, and promote tissue colonization and invasion. Major MSCRAMMs include a family of surface-attached proteins covalently bound to the cell wall peptidoglycan via a conserved LPXTG motif. Genomic analyses indicate that *S. aureus* contain up to 22 LPXTG surface proteins, which could potentially act individually or in synergy to promote infection.

In the first part of this study we determined the range of adherence phenotypes to fibrinogen and fibronectin among 30 carriage isolates of *S. aureus* and compared it to the adherence phenotypes of 30 infective endocarditis and 30 blood culture isolates. Overall there were great variations in *in vitro* adherence, but no differences were observed between carriage and infection strains. We further determined the relation between *in vitro* adherence and *in vivo* infectivity in a rat model of experimental endocarditis, using 4 isolates that displayed either extremely low or high adherence phenotypes. Unexpectedly, no differences were observed between the *in vivo* infectivity of isolates that were poorly and highly adherent *in vitro*. We concluded that the natural variability of *in vitro* adherence to fibrinogen and fibronectin did not correlate with *in vivo* infectivity, and thus that pathogenic differences between various strains might only be expressed in *in vivo* conditions, but not *in vitro*. Therefore, considering the importance of adhesins expression for infection, direct measurement of those adhesins present on the bacterial surface were made by proteomic approach.

In the second series of experiments we assessed the physical presence of the LPXTG species at the staphylococcal surface, as measured at various time points during growth in different culture media. *S. aureus* Newman was grown in either tryptic soy broth (TSB) or in Roswell Park Memorial Institute (RPMI) culture medium, and samples were removed from early exponential growth phase to late stationary phase. Experiments were performed with mutants in the global accessory-gene regulator (*agr*), surface protein A (Spa) and clumping factor A (ClfA). Peptides of surface proteins were recovered by "trypsin-shaving" of live bacteria, and semi-quantitative proteomic analysis was performed by tandem liquid-chromatography and mass-spectrometry (LC-MS). We also determined in parallel the mRNA expression by microarrays analysis, as well as the phenotypic adherence of the bacteria to fibrinogen *in vitro*. The surface proteome was highly complex and contained numerous proteins theoretically not belonging to the bacterial envelope, including ribosomal proteins and metabolic enzymes. Sixteen of the 21 known LPXTG species were detected, but were differentially expressed. As expected, 9 known *agr*-regulated proteins (e.g. including Spa, FnBPA, ClfA, IsdA, IsdB, SasH, SasD, SasG and FmtB) increased up to the late exponential growth phase, and were abrogated in *agr*-negative mutants. However, only Spa and SasH modified their proteomic and mRNA profiles in parallel in the parent and its *agr* negative mutant, while all other LPXTG proteins modified their proteomic profiles independently of their mRNA. Moreover, ClfA became highly transcribed and active in *in vitro* fibrinogen adherence tests during late growth (24h), whereas it remained poorly detected by proteomics. Differential expression was also detected in iron-rich TSB versus iron-poor RPMI. Proteins from the iron-regulated surface determinant (*isd*) system, including IsdA, IsdB and IsdH were barely expressed in iron-rich TSB, whereas they increased their expression by >10 time in iron-poor RPMI. We conclude that semi-quantitative proteomic analysis of specific protein species is feasible in *S. aureus* and that proteomic, transcriptomic and adherence phenotypes demonstrated differential profiles in *S. aureus*. Furthermore, peptide signatures released by trypsin shaving suggested differential protein domain exposures in various environments, which might be relevant for anti-adhesins vaccines. A comprehensive understanding of the S. aureus physiology should integrate all these approaches.

Abbreviations

AdsA	Adenosine synthase A
agr	*Accessory gene regulator*
AIP	Auto-Inducing Peptide
Aur	Aureolysin
Bbp	Bone sialoprotein-binding protein
BI	Bullous Impetigo
CA-MRSA	Community-Associated MRSA
CAPs	Cationic Antimicrobial Peptides
CFU	Colony Forming Units
Clf	Clumping factor
Cna	Collagen adhesin
Coa	Coagulase
CP	Capsular Polysaccharides
DTT	Dithiotreitol
Eap	Extracellular adherence protein
Ebps	Elastin-binding protein
Efb	Extracellular fibrinogen-binding protein
EF-Ts	Elongation factor TS
ET	Exfoliatin
FmtB	Formyl transferase B
FnBP	Fibronectin-binding protein
Fur	Ferric uptake repressor
GM17	Glucose M17 medium
Hla	α-hemolysin
Hlb	β-hemolysin
Hld	δ-hemolysin
IAA	Iodoacetamide
IgG	Immunoglobulin G
Isd	Iron-regulated surface determinant
kDA	kilo-Dalton
L. lactis	*Lactococcus lactis*
LTA	Lipotechoic Acids
LC-MS/MS	Liquid-Chromatography coupled to tandem Mass-Spectrometry
Map	MHC class II analog protein
MGEs	Mobile Genetic Elements
MgrA	Multiple gene regulator A
MHC	Major Histocompatibility Complex

MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMMs	Microbial Surface Components Recognizing Adherence Matrix Molecules
MSSA	Methicillin Sensible *Staphylococcus aureus*
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NEAT	NEAr Transporter
OD	Optical Density
PAMPs	Pathogen Associated Molecules Patterns
PBS	Phosphate Buffer Saline
PBP	Penicillin Binding Protein
Pls	Plasmin sensitive protein
PVL	Panton and Valentine Leucocidine
RB	Ribosome binding
Rot	Repressor of toxin
Rsb	Regulators of sigma B
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
Sar	Staphylococcal accessory regulator
Sas	*Staphylococcus aureus surface protein*
S. aureus	*Staphylococcus aureus*
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SD	Shine-Dalgarno
Sdr	Serine-aspartate repeat protein
SDS	Sodium Dodecyl Sulfate
Sdr	Serine-aspartate repeat protein
SERAMs	Secretable Expanded Repertoire Adhesive Molecules
SP	Signal Peptide
Spa	Protein A
Spj	Sureface protein in Japanese ST8 CA-MRSA
SrtA	Sortase A
Srap	Serin-rich adhesin for platelets
Ssp	Staphylococcal serine protéase
sRNA	Small non-codant RNA
SSSS	Staphylococcal Scaled-Skin Syndrome
TA	Techoic Acids
TCRS	Two Components Regulatory Systems
TNFR1	Tumor Necrosis Factor Receptor 1
TSB	Tryptic Soy Broth
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin 1

CHAPTER 1

General Introduction

I. General Microbiology of *Staphylococcus aureus*

S. aureus is a Gram-positive, non-motile, non-spore forming and facultative anaerobe spherical (coccus) bacterium, often found in grape-like clusters of 0.5 to 1.5 µm in diameter. Its genome consists of a circular chromosome of approximately 2800 Mb, which contains numerous mobile genetic elements (MGEs) such as prophages, transposons, pathogenicity islands and genomic islands (for review [1]). This MGEs are responsible for its great adaptability to various environmental conditions, including its capacity to colonize and infect mammal hosts. Comparative sequence analysis indicates that the core genome of *S. aureus* shares 50% homology with notoriously non-pathogenic *Bacillus subtilis*, suggesting that the two organisms originated from a common ancestor and diverged thereafter [2,3]. However, compared to *B. subtilis* and other non-pathogenic Gram-positive cocci, the genome of *S. aureus* harbors numerous surface-bound adhesins, secreted virulence factors, and antibiotic-resistance determinants that are primarily located on MGEs, and thus have been acquired by horizontal gene transfer [3-7]. Since gene exchange is a key player of bacterial evolution, this atypical genetic plasticity is a likely explanation for the capacity of *S. aureus* to behave both as a colonizer and disease-inducing microbe.

S. aureus is known to be a human commensal bacterium, but can become an opportunistic pathogen in certain circumstances. It is a permanent colonizer of the anterior nostrils and some other anatomical sites in 15-30% of the healthy human adult population, and can be recovered intermittently from up to 60% of other healthy individuals [8]. In addition, it can produce a wide array of infections, which are summarized in Figure 1. These extend from relatively benign skin infections (folliculitis and furunculosis) to life threatening conditions such as osteomyelitis, pneumonia, sepsis, and endocarditis [9]. While *S. aureus* is a leading cause of community-acquired infections, it has also become the first cause of nosocomial diseases, often due to multi-resistant strains [10,11]. Moreover, in addition to infections where the bacterium can be cultured from the infected site, *S. aureus* is also responsible for “distant” diseases that are mediated via toxin secretion, including well-known staphylococcal toxic shock syndrome and food poisoning [12,13]. Thus, the heterogeneity of these diseases and the unique ability of *S. aureus* to develop antibiotic-resistance reflect the extraordinary capacity of this organism to adapt and survive in a great variety of environments.

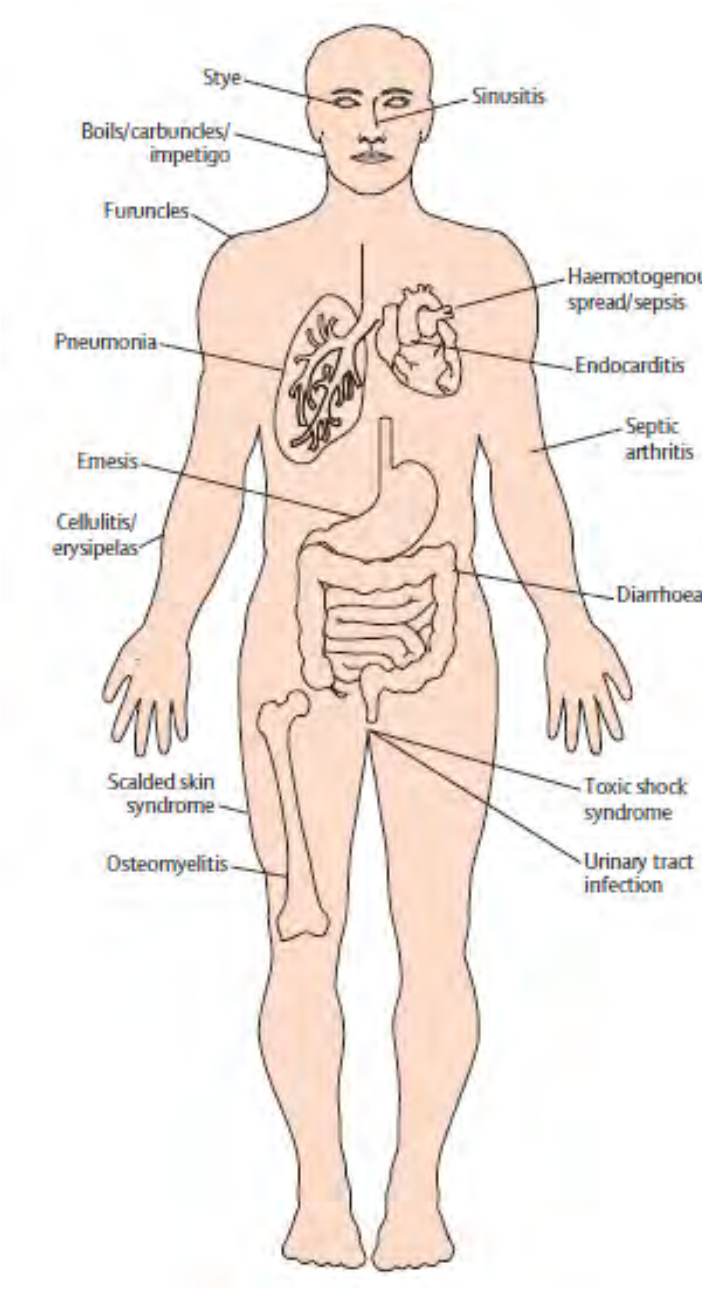

Figure 1: Large diversity in *S. aureus* infections [14].

Recently, technologies encompassing whole genome sequencing and high-throughput transcriptomics and proteomics have been widely used to better understand microbial evolution and cell physiology. The first sequenced genome of staphylococcus was published in 2001 [3]. Today, 31 *S. aureus* full sequenced genomes are available (http://www.ncbi.nlm.nih.gov/genomes/154) and there are many more ongoing projects. Over the last decade, molecular and genetic dissection of *S. aureus* has revealed a great number of surface adhesins and secreted enzymes and toxins. Cell-wall-associated surface adhesins, in particular proteins, play important roles in virulence. Indeed, the ability to bind to host extracellular matrix and plasma components allows staphylococci to attach to host tissues, invade epithelial and endothelial cell linings, and escape for host defense mechanisms [15-

17]. More recently, surface proteins have also been shown to play a role in biofilm formation [18]. However, while many of these features (described later in this chapter) were shown to play a role in specific *in vitro* or *in vivo* experimental models, there is, as yet, not much understanding of their integrated interplay during infection, either *via* protein-protein interactions (e.g., it was shown that domains of surface adhesins binding fibrinogen or fibronectin could synergize to produce diseases) [19], or *via* regulation of gene expression during time-course experiments. Given the number of determinants involved, and their differential regulation as a function of environmental stimuli, such approach appears crucial to better understand pathogenicity. To address this purpose, gene regulation of virulence factors is addressed in the next section.

II. Regulation and virulence determinants

S. aureus is extremely well equipped in surface factors and secreted proteins that mediate host colonization and pathogenicity. For instance, its cell envelope, which represents up to 50-60% of the dry weight of the bacterium, plays a major role in the interaction between the bacterium an its direct environment. This enveloppe consists of a sturdy peptidoglycan scaffold that is decorated with a variety of polyols such as teichoic acids (TA) and lipoteichoic acids (LTA), polysaccharides (extracellular capsule), and polypeptides that confer diverse properties such as resistance to phagocytosis (capsule), environmental sensing (proteins), and adherence to and invasion of host cell tissues (TA, LTA and protein adhesins). The adhesins are collectively referred to Microbial Surface Component Recognizing Matrix Molecules or MSCRAMMs [20]. Besides, *S. aureus* also produces a large number of virulence factors such as toxins, exo-enzymes and cell surface proteins implicated in pathogenesis, which will be detailed below. The expression of all these features is under the control of a serie of integrated gene regulatory systems that sense environmental conditions to respond by fine-tuning the expression of metabolic and virulence determinants [4]. These are shortly reviewed below.

II.1. Regulatory systems of virulence-determinant expression

There are at least three families of regulatory systems controlling gene expression in *S. aureus*: the two component regulatory systems (TCRS), the small regulatory RNAs, and the DNA-binding

proteins [4,21-23]. These regulatory systems are interdependent and the major part of virulence genes is directly or indirectly regulated by them.

II.1.1. The two component regulatory systems (TCRS) and the example of *agr (accessory gene regulator)*

TCRS serve as a basic stimulus-response coupling mechanism to allow organisms to sense and respond to changes in many different environmental conditions. They typically consist of a membrane-bound histidine kinase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response, mostly through differential expression of target genes. TCRS are widely present in prokaryotes, and often share homology [24], whereas only a few have been identified in eukaryotic organisms. TCRS appear to be major players in gene regulation and Table 1 shows the functions and implications of the most important found in *S. aureus*.

Table 1: Principal TCRS and functions in *S. aureus.*

TCRS	Function	References
Agr	Regulates many extracellular and cytoplasmic protein accessory genes	[4,21]
SaeRS	Repressor of extracellular proteases – Activator of biofilm formation	[25,26]
ArlSR	Regulator of virulence gene expression	[27,28]
SrrAB	Regulator of virulence factors under low-oxygen conditions (TSST-1, Spa)	[29,30]
VraSR	Regulator of cell-wall biosynthesis pathway – Activator of Pbp2	[31-33]
LytSR	Regulator of biofilm formation	[34]
YycFB	Regulator of cell permeability	[35,36]
GraSR	Regulator of CAMP resistance, stress response and cell wall metabolism signal transduction pathways	[37]

Among these, *agr* has been particularly well described (Figure 2). It is activated in a population density-dependent manner and controls the expression of more than 20 virulence factors, including adhesins, exo-enzymes and toxins [21,38]. It is basically composed of an auto-inducing peptide (AIP, encoded by AgrD), a transport protein (AgrB) that allows the export of AIP, a transmembrane receptor (AgrC) that recognized AIP, a response regulator (AgrA) that dephosphorylates when AIP contacts AgrC, and an RNA effector RNAIII. At low cell density (exponential growth phase) the P2 promoter, which activates the transcription of *agr*B-D-C-A (see Figure 2), is OFF and the operon is transcribed at a low basic level. As cell growth progresses, the concentrations of both bacteria and extracellular auto-inducing peptide (AIP) increase in the milieu, thereby augmenting the chance of AIP to make contact with its cognate AgrC receptor. Upon contact between AIP and AgrC, AgrC activates the response

regulator AgrA, a process that may involve AgrA dephosphorylation [4]. Activated AgrA is a DNA-binding protein that turns ON the transcription from both promoter P2 – generating a positive feed-back on the system – and also turn ON P3, which drives the transcription of δ-hemolysin and of an atypical effector called RNAIII. RNAIII has a reciprocal effect, activating the expression of most secreted proteins while downregulating the expression of surface-bound factors. RNAIII has a complex three-dimensional structure and a long half-life (up to 45 min) [39,40]. It is believed to regulate gene expression in several ways, including at the translational level by blocking the mRNA ribosome-binding site of the target genes [37,40-44].

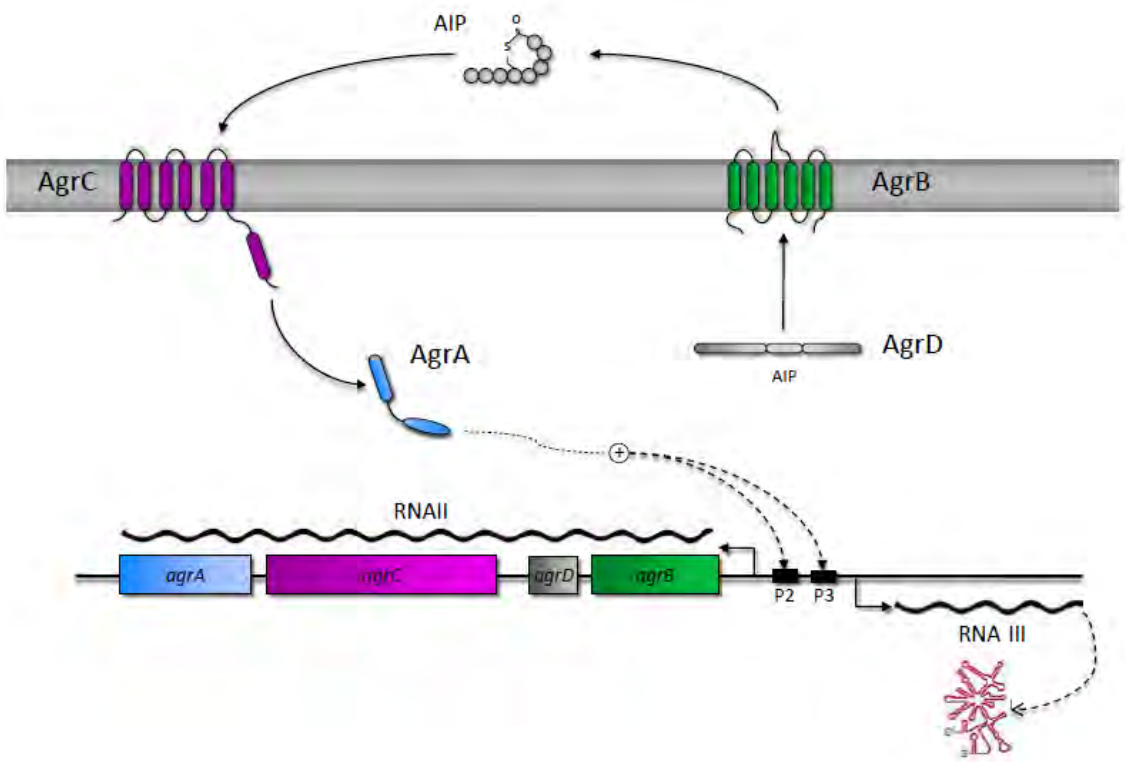

Figure 2: Organization of *agr* locus in *S.aureus* and functional model. The *agr* locus contains two divergent promoters, P2 and P3, which modulate transcription of two transcripts, RNAII and RNAIII, respectively. The promoter P2 drives the transcription of a series of components comprising a transmembrane protein AgrB, an auto-inducing peptide (AIP) precursor AgrD, which is processed and exported by membrane-spanning AgrB; a transmembrane sensor AgrC, which is the cognate receptor of the AgrD-derived AIP, and a transcription regulator AgrA that can be activated by AgrC. See text for functioning details. Adapted from [4,45].

Importantly, it has been largely accepted that *arg*-regulation was responsible for turning on the expression of surface protein genes (including MSCRAMMs) during the exponential phase of growth, while repressing them and turning on genes for secreted enzyme and toxins in the stationary phase of growth [4]. This has played an important conceptual role in understanding the sequential contribution of cell-surface and secreted determinants pathogenesis, i.e. first colonization via adherence and then

invasion via enzymes [4]. However, the expression of *agr* is itself modulated by a multiplicity of additional regulators [22,30,46,47], which are not all described in this introduction, but substantially complexify the study of pathogenesis in "mere" *agr* mutants. Therefore, experiments to understand pathogenesis using only *arg* mutants tend to simplify (maybe oversimplify) reality by first compartmentalizing of cell constituents or molecules, and then trying to reconstitute physiopathological processes. Trying to solve this issue is the purpose of one of the experimental approach described in the thesis dissertation.

There are at least seven other TCRS that have been described in *S. aureus* (Table 1). For instance:

- SaeRS is a regulatory system that activates the production of several exoproteins that would otherwise limit accumulation of critical proteins that contribute to biofilm formation [25,26].
- ArlS-ArlR is a parallel system that controls bacterial attachment to polymer surfaces by affecting secreted peptidoglycan hydrolase activity and modifying extracellular proteolytic activity [28]. In addition, the *arl* operon decreases the production of numerous virulence factor, such as α-hemolysin (Hla), β-hemolysin, lipase, coagulase, serine protease and especially protein A (Spa) by down regulating the transcription of their genes [27].
- SrrAB acts in the global regulation of virulence factors. This system down-regulates production of *agr* RNAIII, protein A, and toxic shock syndrome toxin 1 (TSST-1) and particularly under low-oxygen conditions [29]. SrrAB decreases virulence in the rabbit endocarditis model [30].
- VraSR was shown to be responsible for resistance to cell wall-damaging compounds, including β-lactam antibiotics and some cationic antimicrobial peptides (CAPs) [31-33].
- LytSR plays an important role in *S. aureus* biofilm development [34].
- Yyc plays a role in cell permeability [35].
- GraSR controls stress response and cell wall metabolism signal transduction pathways [37].

Thus, also *arg* came as an early paradigm in the regulation of genes encoding surface attached and secreted molecules, it is part of a broader network that may either directly interact with it (e.g. see SrrAB above), or act independently of it. As a result, conclusions drawn from studying mutants in only one of these systems are obligatory restrictive, and may provide only a limited view on the more complex reality.

II.1.2. Small regulatory RNAs: the example of RNAIII

Small non-coding RNA (sRNA) regulators in prokaryotes are a heterogeneous group of small molecules able to act by various mechanisms to rapidly regulate physiological or stress responses (e.g. temperature, pH, nutrients, etc). Moreover, in pathogenic bacteria, sRNA are often strategic elements for the regulation of virulence genes expression [48,49].

sRNAs typically act by modulating transcription, translation, mRNA stability or by gene silencing. It generally involves changes in RNA conformation, which eventually affect protein binding, DNA binding or antisense base pairing with mRNA targets [50]. In the latter case, positive regulation typically activates translation of proteins by making ribosome binding (RB) sites of mRNAs available, as in gene attenuation. In contrast, negative regulation involves sRNA-mRNA base-pairing interactions in order to sequester mRNA sequences and inhibits RB.

Over the last few years a great effort has been invested to understand regulation by non-coding sRNAs regulators in bacteria. Recently, Beaume *et al* reported up to 160 sRNA in intergenic regions of the *S. aureus* chromosome, of which 35 had cDNA antisense properties in reference strain *S. aureus* N315 [51]. They were principally localized in key metabolic regions or close to virulence genes or MGEs, as for instance in the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) island, which is responsible for methicillin resistance. Moreover, the expression of these sRNA appeared to be modulated along growth and in response to stress conditions [51].

All these elements suggest that sRNAs are precious for bacteria to adapt to variable environments. One potential advantage for the cells over other regulatory systems is the rapid response because of the small size and the absence of translation phase. Moreover, the action is reversible by the intervention of RNAse in the case of degradation. Thus, regulation by sRNAs provides an additional level of sophistication, which may intervene both upstream of more general regulatory systems, in order to modulate global responses, or downstream of them to better tune the expression of discrete subsets of specific genes. In any case, however, the best known and most thoroughly studied sRNA in *S. aureus* is RNAIII of *agr*, which was described almost two decades ago [4]. It is implicated in the control of exoproteins and virulence factors expression, such as cell wall-associated proteins, during growth [21]. RNAIII contains 517 nucleotides covering *hld* gene, which encodes the 26 amino-acids δ-hemolysin. Its 2D structure shows 14 hairpin arrangements (Figure 3). After transcription, RNAIII has a long half-life of up to 45 min [40].

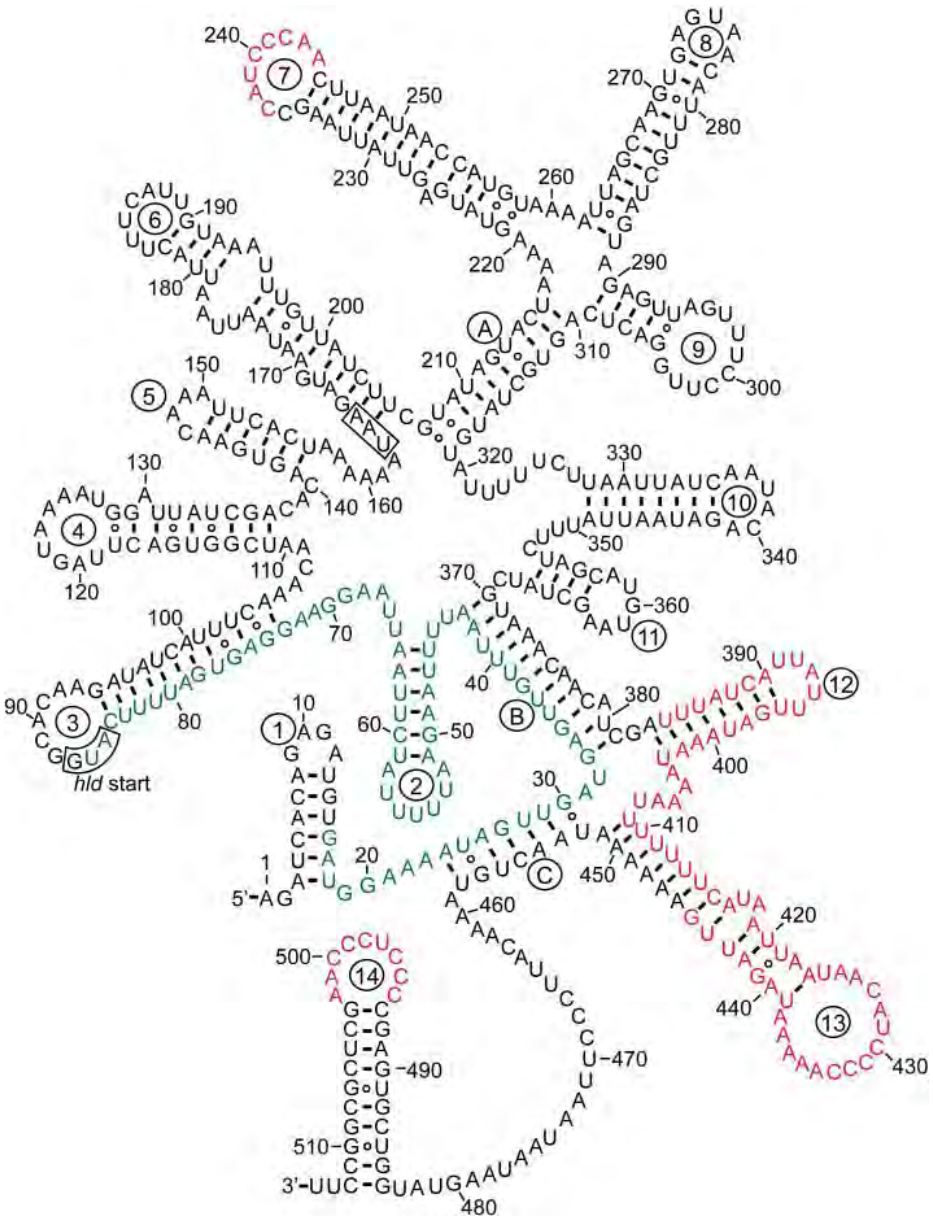

Figure 3: Secondary structure of RNAIII. RNAIII contains 514 nucleotides that form 14 hairpin motifs linked by unpaired nucleotides, which are numbered in the figure. The encoding region of δ-hemolysin is represented in green and the *hld* start and stop codons are framed. Nucleotides demonstrated to inhibit translation of target mRNAs are red. Adapted from [39,45].

In *S. aureus*, the *agr* system up-regulates the expression of some secretory proteins, whereas certain surface proteins are down-regulated [21]. RNAIII is the key effector of the *agr* system and can up-regulate the expression of certain target genes (e.g. *hla* and *map*), or down-regulate other genes (e.g. *spa*, *coa* and *rot*) at the level of translation by mechanisms of antisense base pairing (figure 4) [40-45,52]. In the first case, the sRNA transcript interacts with the target mRNA and leads to a conformational modification that opens a mRNA loop which hides the Shine-Dalgarno (SD) sequence, thus making it available for ribosomal access. In the second way, ribosomal access is prevented by double strand RNA forming at the SD domain and promoting RNaseIII cleavages (figure 4).

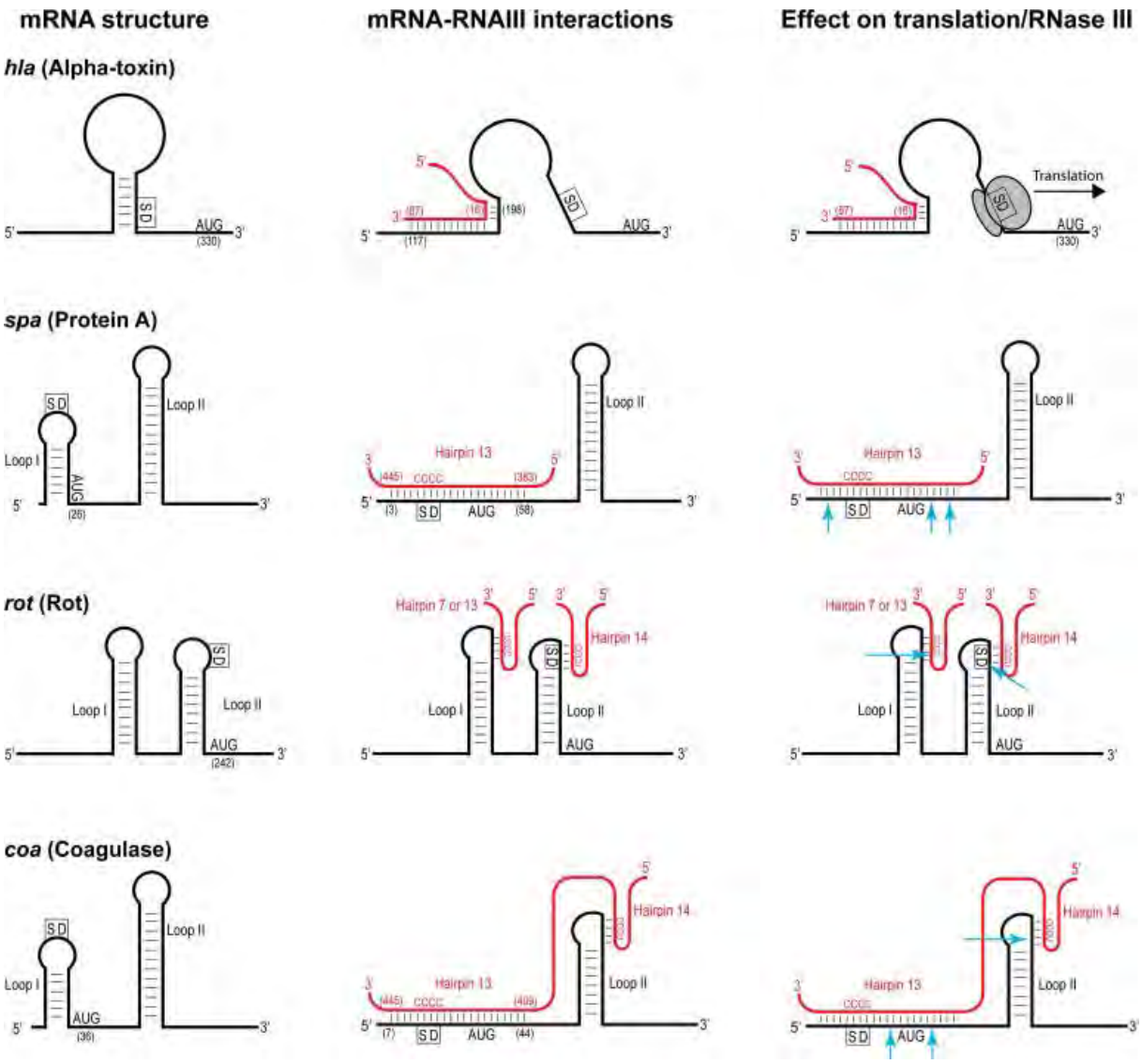

Figure 4: RNAIII mechanisms of post-transcriptional regulation on *hla*, *spa*, *rot*, and *coa* [45]. RNAIII activates *hla* translation by disrupting the stem-loop and allowing ribosomes to access the SD sequence. RNAIII inhibits the translation of genes (*spa*, *rot*, and *coa*) by basepairing with the mRNA, blocking recognition of the SD sequence, and promoting RNaseIII cleavage. Targeted mRNAs are shown in black while RNAIII sections are identified in red. Numbers reflect nucleotide positions from 5' to end of transcripts. Blue arrows indicate sites of cleavage by RNaseIII. From [45].

II.1.3 DNA-binding proteins and transcription factors

The virulence of *S. aureus* is essentially determined by cell wall associated proteins and secreted toxins that are regulated and expressed variously as a function of growth and environmental stresses. Gene expression is regulated by specific and sensitive mechanisms, which may act both at the transcriptional or post-transcriptional levels. Regulatory factors constitute numerous complex networks, driving specific interactions with target gene promoters. These factors are largely regulated by TCRS (previously described).

II.1.3.1. Sar family

The DNA-binding proteins or transcription factors are largely represented by the Sar family of proteins (Staphylococcal accessory regulator) and also regulate virulence factor expression (Table 2). The multiple pathways generated by these factors allow the staphylococci to adapt to environmental conditions rapidly and specifically, and to develop infection. Figure 5 summarizes the regulatory networks between Sar family proteins and *agr*.

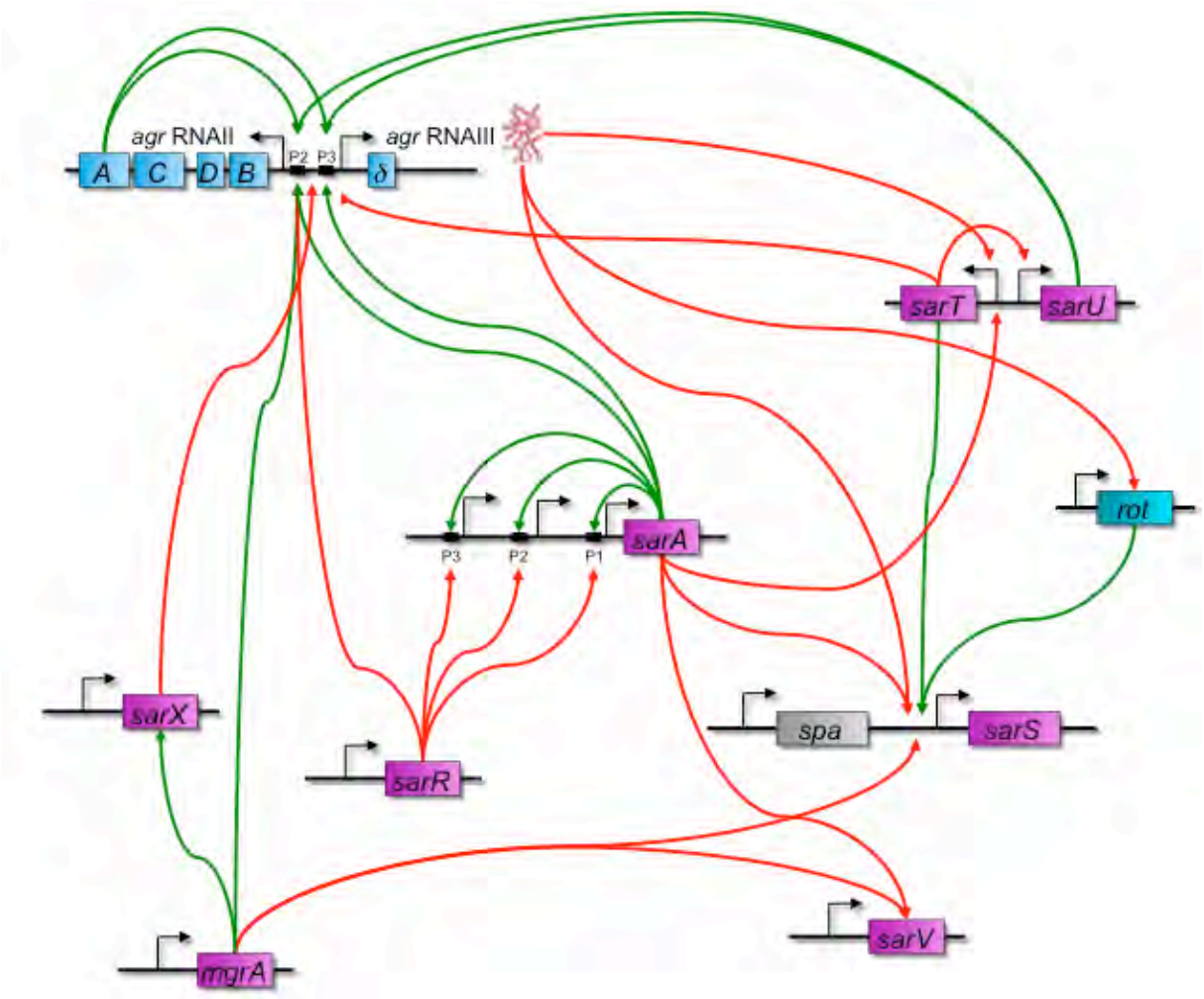

Figure 5: Regulatory network between Sar family DNA-binding proteins and *agr*. Each Sar type proteins identified can have an effect on one or more of these controlled systems, thus creating a network of very complex control on target genes. Green and red arrows represent activation and repression respectively. Gene promoters are represented by black boxes and denominated P1, P2 or P3. Adapted from [53].

The *sarA* operon was initially described in 1992 by Cheung *et al*. Random insertion of the Tn917 LTV1 transposon into the chromosome of *S. aureus* resulted in a pleiotropic effect on the expression of a number of extracellular and cell wall-associated virulence proteins [54]. The *sarA* operon consists

of three overlapping transcripts, with common 3' ends, and a transcriptional termination occurring upstream of a typical prokaryotic poly(T) termination signal. The three transcripts are controlled by three distinct promoters in a growth phase-dependent manner [55]. Manna *et al* reported that the P3 promoter of the *sar* locus was σ^B dependent and the three promoters were positively autoregulated by SarA [56]. SarA has been shown to be down-regulated by its homologue SarR [57], but the *sar* locus remains complex and may involve the *sar* gene product(s) and other regulatory protein(s) as well. Furthermore, due to the fact that σ^B activity peaks in the late exponential growth phase and diminishes in the stationary growth phase, the expression of *sarA* peaks during the late exponential phase [58]. However, western blot analyses made by Blevins *et al* [59], indicated that SarA was produced in indistinguishable amounts during both exponential and post-exponential growth phases, suggesting the SarA protein might be regulated at the post-translational level as well.

Table 2: Principal DNA binding proteins and functions in *S. aureus*

Gene	Function	References
sarA	Activation of *agr*. Transcription repressor of *sarS*, *sarT*, *sarV*. Activation of *fnbpA*, *fnbpB*, hemolysins, leucotoxins, enterotoxins, TSST-1 and *fmtA*. Repressor of *spa*, *cna* and *sspA*.	[39,54,55,59-68]
sarR	Repressor of *sarA* and *agr*. Activation of *aur* and *sspA*.	[57,69,70]
sarS	Activation of *spa* and repression of *hla*, *hld* and *ssp*.	[71,72]
sarT	Repressor of *rnaIII* and *sarU*. Activator of *sarS*.	[22,73]
sarU	Activation of *agr*.	[74,75]
Rot	Activation of *spa*, *sspB*, *sspC* and *clfB*. Repression of *hla* and *hlg* genes (*hlgB – hlgC*)	[76-78]
sarX	Repressor of *agr*	[79]
mgrA	Activator of *sarX* and *agr*. Repressor of *sarS* and *sarV*. Upregulation of exoproteins (*hla*) and down regulation of surface proteins (*spa*).	[22,80,81]
sarV	Control of autolysis	[22]
sarZ	Activator of *agr*	[22]
sigB	Repressor of *agr* and *sarX* and activator of *sarA*. Activation of stress resistance genes.	[56,58,82-89]

A homodimer of SarA was able to bind to a conserved A/T-rich recognition motif in the promoter regions of its target genes [61,90]. SarA was described as an activator of *agr*, where it directly binds to the P2 and P3 promoter regions and increases the levels of both RNAII and RNAIII, thus indirectly modifying the expression of *agr*-dependent virulence factors [60,62]. However, SarA can also modulate other target genes via *agr*-independent pathways. Indeed, an alignment of sequences from the promoter regions of *hla*, *spa*, *fnBPA*, *fnBPB*, and *sec* revealed an apparent conserved regions, termed Sar boxes, that shared homology with the SarA-binding site on the *agr* promoter [61]. Interestingly, it is well established now that SarA could promote the transcriptional expression of numerous targeted genes, as *fnBPA* and *fnBPB*, *agr*, hemolysins, leucotoxins (*LukED*, *LukSF*), enterotoxins, superantigen TSST-1 [58,60,63-67], and more recently *fmtA* (formyl transferase),

which encodes a low-affinity penicillin binding protein in *S. aureus* [68]. Conversely, SarA also acts as a repressor of other target genes, such as *spa*, *cna* and *sspA* (staphylococcal serine protease) [64,66,91].

Several *sarA* homologs have been identified in the *S. aureus* genome by sequence alignment, including *sarR, sarS, sarT, sarV, sarU, sarY,* and *rot* (repressor of toxins) [22,30]. The staphylococcal-specific SarA family of transcription regulators controls large numbers of target genes involved in virulence, autolysis, biofilm formation, stress responses and metabolic processes, and are recognized as potential therapeutic targets. One of them is *sarR*, which presented around 51% homology with *sarA*. The three *sarA* promoters are down-regulated by direct binding of the homolog SarR, which represses the expression of the SarA protein, especially in the late exponential and stationary phases [57]. Furthermore, SarR also binds to the P2 promoter of *agr*, in order to represse it [70], and activates *aur* (metalloprotease; aureolysin) and *sspA* [69].

Another homolog is SarS, a 29-kDa protein which has been first isolated by Tegmark *et al* as SarH1 (Sar Homolog 1). The expression of *sarS* is strongly repressed by *sarA* and *agr* [71]. SarS has the ability to bind to the promoter regions of various genes, like *hld*, *ssp*, and *hla* (repression) or *spa* (activation). The same protein was identified by Cheung *et al*. [72] at the same time, and was named SarS. The *sarS* gene is located upstream of *spa* in the *S. aureus* chromosome. Moreover, they conclude that the *agr* locus probably mediates *spa* repression by suppressing the transcription of *sarS*, an activator of *spa* expression whereas *sarA* repression acts by an independent *sarS* mechanism [72]. The SarS gene is transcribed from two distinct promoters controlled by an σ^A dependent promoter and the σ^B dependent stress response promoter [72].

Yet additional members of the Sar family is the 16,1 kDa SarT protein, which shares 35% of homology with the SarA sequence and 20% of homology with SarR. In addition, complex interactions exist between *sarA*, *sarT* and *agr*. The *sarT* gene is down-regulated either by SarA or *agr* but can also repress the transcription of *rnaIII* [73]. These interactions allow controlling the expression of *hla* by two distinct mechanisms. First, *hla* transcription could be activated directly by binding of *sarA* and indirectly by the repression of *sarT* via SarA. In contrast, SarT should repress the *hla* transcription by inactivating the *rnaIII* expression [73]. Moreover, the activation of *sarT* results in up-regulation of *sarS* [22], thus leading to *hla* repression and *spa* activation [71].

Adjacent but divergently transcribed from *sarT* is *sarU*, which is repressed by SarT. However, it is an activator of *agr* that promotes the expression of *agr*-targeted genes (*hla*) and decreases the expression of genes encoding cell wall-associated proteins like *coa* [91]. This mechanism may be an alternative pathway for the regulation of RNAIII and virulence factors independently of the quorum sensing regulation mediated by the auto inducible peptide AIP [75].

McNamara *et al* described Rot (repressor of toxins) as a homolog of the global regulators AgrA and SarA that represses toxins [78]. They also demonstrated that Rot could interact with targeted gene promoters during the logarithmic phase of growth. In 2003, GeneChips analyses revealed that Rot was not only a toxin repressor, but a global regulator with both positive and negative effects on the expression of 146 *S. aureus* genes. Rot and *agr* showed opposite effects on selected target genes: i.e. *spa*, *sspB* and *sspC* were up-regulated by Rot but down-regulated by *agr*, whereas *hla* and the γ-hemolysin genes (*hlgB* and *hlgC*) were down-regulated by Rot and upregulated by *agr* [76]. In 2006, Geisinger *et al* [44] suggested that the *agr* RNAIII molecule might interact with *rot* mRNA to inhibit Rot translation through a mechanism of base-pairing. More recently, Xue *et al* [77] demonstrated that Rot was an activator of *clfB* by directly binding to the *clfB* promoter during the early exponential growth phase, and that RNAIII could regulate *clfB* expression during the exponential phase via Rot.

SarX regulation has been investigated by Manna *et al* [79], who found that *sarX* transcription was growth phase-dependent and was expressed maximally during the stationary phase of growth. SarX acted also as a repressor of the *agr* locus and consequently on target genes regulated by the *agr* system [79].

MgrA (Multiple gene regulator A), another SarA homolog, regulates autolysis by a mechanism independent of other global regulators and inducts *agr* and *sarX* transcription but represses *sarS* and *sarV* [80]. The transcriptome of *S. aureus* Newman has been analyzed by microarrays and revealed that MgrA regulates 355 genes in all function categories. Accordingly, exoproteins are up-regulated and surface proteins are down-regulated by MgrA, suggesting that it acts in parallel with *agr* [80,81].

Others SarA homolog are SarV and SarZ [22]. The *sarV* gene seems to be repressed by MgrA and SarA in normal *in vitro* growth conditions, due to is significant enhancement in *mgrA* and *sarA* mutants. In contrary to *mgrA* and *sarA* mutants, the *sarV* mutants appeared more resistant to detergents or cell wall antibiotic-mediated lysis compared to the parent, while overexpression of *sarV*

rendered the parental strain more susceptible to lysis. Hence, *sarV* may be part of the common pathway by which *mgrA* and *sarA* control autolysis in *S. aureus* [22].

II.1.3.2. The sigma factor B, σ^B

RNA polymerase could involve σ factors to recognize specific promoter elements, leading to the initiation of transcription. One group of σ factors is required for the expression of housekeeping genes, and a second contains factors, like σ^B, that are involved in the regulation of the gene expression upon various stress conditions.

The σ^B factor is located within a chromosomal gene cluster with three other determinants: i.e. rsbU, rsbV and rsbW [82]. The regulation of σ^B is differentially modulated by the *rsb* (regulator of σ^B) factors, which are self-controlled between them. Gertz *et al* [83] have shown that numerous genes, most of which are involved in stress responses, are controlled by σ^B. Moreover, σ^B influences the expression of virulence factors such as enterotoxins B, *aur*, *hla*, *clfA*, *fnbA*, and numerous other surface-associated components, both *in vitro* and in *vivo* [84-89]. More generally, σ^B is responsible for the transcription of genes that can confer stress resistance to the vegetative cell.

In conclusion, *S. aureus* possesses a very complex network of regulatory systems and elements that interact together to allow the bacteria to express specific factors during adaptation to specific milieus or including the processes of active infection and persistence. Virulence factors involved in *S. aureus* infection are detailed in the next section.

II.2. Virulence factors

The bacterial components and secreted products that affect the pathogenesis of *S. aureus* infections are numerous and include surface-associated MSCRAMMs, antiphagoxytic polysaccharides, and exoenzymes or exotoxins. These allow staphylococci to adhere to eukaryotic membranes, lyse eukaryotic cells, resist opsonophagocytosis, and trigger the production of a cascade of host immunomodulating molecules. Because of the multifactorial nature of staphylococcal infections and the functional redundancy of *S. aureus* virulence factors, it has been difficult to sort out the role that individual virulence determinants play in the pathogenic process. The major *S. aureus* virulence factors are overviewed below.

II.2.1. Extracellular toxins and enzymes

S. aureus produces large numbers of secreted virulence factors including two major families of exoproteins, namely cytolysins and superantigens, which interfere with the immune system locally or systemically, respectively. Moreover, S. *aureus* secreted factors encompass various extracellular enzymes, including proteases and lipases that contribute to tissue invasion.

II.2.1.1. Cytotoxins or “pore forming toxins”

S. aureus cytotoxins, such as hemolysins and leukocidins are active on eukaryotic plasma membranes. By lysing target cells, they pave the way to polymer degrading proteases and lipases, which provide nutrients for the invading bacterium. They act either via channel forming proteins that punch holes in the target membranes, or through membrane degrading enzymes.

a) Hemolysins

Four types of hemolysins have been described and referred to as α-hemolysin (Hla), β-hemolysin (a sphyngomyelinase), γ-hemolysin, and δ-hemolysin [92]. They are present in most *S. aureus* isolates, are encoded on the chromosome and contain high-affinity structures that interact with the membranes of a variety of eukaryotic cells, including human platelets, monocytes and endothelia. Hemolysins are subject to *agr* regulation and *hla* is directly regulated by the RNAIII [41].

b) The Panton and Valentine leucocidin, PVL

The PVL is a homologue of γ-hemolysin and was first reported in 1932 by Panton and Valentine. The toxin is encoded by the *lukS* and *lukF* genes that are carried by the prophage φSLT. The prophage can be mobilized and transfer PVL to other strains [93]. PVL, is apparently regulated by *agr* [4], like other hemolysins, and is associated with community-associated methicillin resistant *S. aureus* (CA-MRSA) strains [94]. However, the genuine contribution of PVL to CA-MRSA pathogenesis remains controversial.

II.2.1.2. Superantigens

S. aureus can colonize mucosal surfaces, including the anterior nares and vagina. Moreover, due to their abilities to secrete a variety of virulence factors, *S. aureus* that are confined to mucosa, can cause distant illnesses by the production of exotoxins factors that cross the mucosal barrier and spread throughout the body. Such exotoxins are referred to as superantigens because they induce non-specific activation of the immune system [95]. Superantigens interact with antigen-presenting cells and T cells, by direct binding of the major histocompatibility complex (MHC) class II proteins and T-cell receptors, to induce T lymphocytes proliferation and massive cytokine production, thereby bypassing the normal antigen processing and presenting mechanism. To date, 19 different superantigens and related toxins have been described in *S. aureus* with some differences in structure and biological activity [96]. Corresponding genes are generally located on MGEs, such as pathogenicity islands, plasmids, and phages. The well-known toxic shock syndrome toxin (TSST-1) and enterotoxins belong to this class.

a) Staphylococcal enterotoxins, SEs

SEs function both as direct gastrointestinal toxins and as superantigens, two activities that are mediated by different domains on the protein [97]. SEs have compact structures that allow to be highly resistant to proteases, including trypsin, chymotrypsin, and papain, which are all found in the intestinal lumen. All enterotoxins seem to be regulated by *agr*, except SEA [21].

Several SEs have been described. For instance, SEA (encoded by the *entA* gene carried by a temperate bacteriophage [98,99]), is one of the major toxins implicated in staphylococcal food poisoning [13]. Its expression is not affected by the *agr* system [100]. SEA, like the majority of SEs, involves a Zn^{2+} coordination site to bind to the MHC receptor. Besides, SEB is the toxin most commonly associated with classical food poisoning. It has also been demonstrated to cause a non-menstrual toxic shock syndrome (TSS). The *entB* gene needs a functional *agr* for maximal expression [101]. Is it situated on the bacterial chromosome in most clinical isolates responsible for food poisoning, but may be carried on plasmids in other strains [102]. Ingestion of SEB produces profound gastrointestinal symptoms, including anorexia, nausea, vomiting, and diarrhea.

b) The toxic shock syndrome toxin, TSST-1

TSS leads to fever, rash, capillary leak and subsequent hypotension, the major symptoms of toxic shock syndrome. In the early 1980s a dramatic increase in the number of staphylococcal TSS cases occurred in young women who used high-absorbency tampons during menses [12]. This appeared to be due to colonization with TSST-1 positive *S. aureus*. TSST-1 is able to cross the mucosal membrane and disseminate throughout the body. TSST-1 activates directly epithelial cells and the innate immune system [103]. The toxin is also regulated by *agr* [21,92].

Non-menstrual TSS clinical forms do exist, especially following surgical wound dressing with hyper absorbent bandages. These cases, referred to surgical TSS, seems be association with SEB and SEC.

II.2.1.3. Enzymes and exotoxins

Numerous additional enzymes are implicated in *S. aureus* virulence and diseases. Diverse proteases, elastases, and hyaluronidases are able to degrade conjunctive tissues [104,105]. Some of these are described in some details below.

a) Coagulase

Coagulation is an innate defense mechanism against microbial pathogens that traps and immobilizes invading bacteria in a clot. However, coagulation is also the target of bacterial immune evasive strategies. For instance, inoculation of anticoagulated plasma or blood (with calcium chelators) with *S. aureus* results in rapid clotting in spite of anticoagulation [106]. This phenomenon is due to coagulase (Coa), a *S. aureus* protein of approximately 670 amino acids that triggers coagulation by direct activation of prothrombin [107]. The N-terminal part of Coa encompasses D1-D2 domains [108], which bind to the prothrombin [109]. The C-terminal domain is composed of 2–8 tandem repeats of a 27-residue peptide corresponding to the same fibrinogen-binding domain found at the C-terminal end of another staphylococcal fibrinogen-binding protein [108,110]. Thus, by interacting directly with both fibrinogen and prothrombin, Coa mediates clotting of plasma or blood [111].

b) Staphylococcal serine protease, Ssp

SspA (or V8 protease) was one of the first secreted enzymes of *S. aureus* to be purified and characterized in detail [112]. Using sequence analysis of the *S. aureus* strain RN6390 genome, Rice *et al* [113] found that *sspA* was closely followed by an open reading frame encoding an extracellular cysteine protease, *sspB*. They described an operon, which also includes a third open reading frame *sspC*, of unknown function. Moreover, they also described a cascade pathway of proteolytic activity, where metalloproteases were required for maturation of SspA, which then processes SspB and controls cell wall autolytic activity. Ssp can degrade the bacterial cell surface FnBPs and affect the overall surface protein composition that confers a controlling role in microbial adhesion [113,114]. Moreover, Karlsson *et al* [115] demonstrated that SspA was also able to degrade Spa and FnBPs at the cell surface. Thus, although serine proteases (e.g. V8 protease) may intuitively be associated to pathogenicity and invasion, these enzymes may primarily carry housekeeping functions, including regulation of the functional expression of surface proteins.

At the level of regulation, *agr* was shown to positively regulate the *ssp* operon [113,116], whereas SarA acted as a repressor of protease activity [66].

c) The aureolysin, a metalloprotease family member

Aureolysin is encoded by the *aur* gene that is up-regulated by *agr* at the end of exponential phase and repressed by SarA [49,66]. Different studies suggested that not only coagulase, but also other extracellular proteolytic enzymes could stimulate plasma clotting [117]. Aureolysin, in addition to V8 protease, was shown to modify cell surface proteins of *S. aureus*, for instance by inactivating ClfB dependent of fibrinogen binding activity of the bacteria when they enter into the stationary phase. It was suggested that this mechanism could facilitate the detachment of bacterial cells in certain conditions, in order to promote their dissemination to other colonization sites within the host [118].

d) Exfoliatins, ETs

ETs promote superficial skin disorder that varies from local blistering (bullous impetigo or BI) to impressive generalized scalding (staphylococcal scalded-skin syndrome or SSSS). It was first described in young children in 1878. SSSS generally occurs in newborns and infants less than one

year old and rarely in adults, but BI can occur at any age [119]. It is typically the result of mucosal or skin colonization (e.g., umbilical cord) with a toxigenic *S. aureus* that produces either exfoliatin toxin A (ETA) or ETB, encoded by the *eta* and *etb* genes, which are located on a phage inserted in the bacterial chromosome and a plasmid, respectively. ETA is generally associated with BI whereas ETB is associated with generalized SSSS [120]. Two additional isoforms of SSSS toxins, ETC and ETD, were isolated through pathologic observations in animals and with genome screening [121-123]. ETA expression was reported to be regulated by *agr* in exponential growth phase [124].

II.2.2. Bacterial envelope and surface determinants

II.2.2.1. Capsule

The microbial envelope is not an amorphous scaffold that merely ensures bacterial shape. It is a sophisticated structure indispensable to mediate adherence, sensing and growth in complex environments. From the outermost layer of the bacterium, one first finds the polysaccharidic capsule. The majority of clinic isolates causing invasive diseases produce a polysaccharidic capsule (Figure 6). This extracellular structure mostly prevents the access of phagocytes to phagocytosis-triggering molecules such as pathogen associated molecular patterns (PAMPs) that trigger innate immunity, or antibody or complement-mediated phagocytosis. The production of capsule by *S. aureus* was first described in 1931 by Gilbert [125]. Since 1985, eleven polysaccharide serotypes have been characterized in *S. aureus* [126].

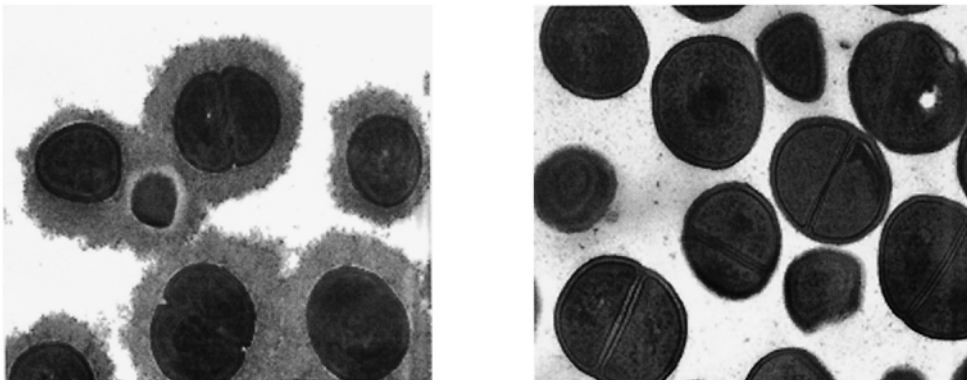

Figure 6: Transmission electron micrographs of *S. aureus* cells cultured on agar plates. Prior to fixation, both strains were incubated with rabbit CP5-specific antibodies to stabilize and visualize the capsule. On the left, CP5-producing strain Reynolds; on the right, an acapsular *S. aureus* mutant. From [127].

Most clinical isolates of *S. aureus* produce either CP5 (Capsular Polysaccharides 5) or CP8 (75%) and are often referred to as microencapsulated. Colonies of serotype 5 and 8 strains of *S. aureus* are not mucoid (hence, they are distinguishable from mucoid serotype 1 or 2), and their colony morphology is usually indistinguishable from that of acapsular isolates. The expression of the CP5 and CP8 capsules *in vitro* is highly sensitive to various environmental signals and is probably influenced by the *in vivo* environment as well [128-132]. Importantly, the expression of capsular polysaccharides inhibits *S. aureus* ClfA-mediated binding to fibrinogen and platelets. This is most likely due the fact that the thick capsules masks the access to ClfA, a phenomenon which has unforeseen implications for vaccine development [133]. In 1995, Soel *et al* [134] revealed that CP5 and CP8 were also able to bind to human epithelial cells, endothelial cells, and monocytes. They concluded that capsules were bacterial adhesins with immunomodulatory effects for human cells.

A*gr* positively regulates CP5 and CP8 production (with a minimal similar impact of SarA) *in vitro* [135,136]. Likewise, Van Wamel *et al.* [137] reported that *agr* positively regulates *cap5* expression both *in vitro* and *in vivo* in a rabbit model of experimental endocarditis, suggesting that it might be important for invasive diseases. Conversely, capsule-negative *S. aureus* induced more promptly chronic experimental mastitis in mice [138], suggesting that the loss of CP5 or CP8 expression might favor the persistence of staphylococci in the mammary glands of chronically infected hosts. This is consistent with the fact that the number of *S. aureus* isolates from patients with chronic infections that do not express capsular polysaccharides is significantly higher than in isolates from acute infections, suggesting again that loss of capsule expression is associated with *S. aureus* with persistence. Thus, is appears that the capsule may promote bacterial colonization and persistence in various conditions.

II.2.2.2. Peptidoglycan

The peptidoglycan is a highly conserved constituent of both the Gram-positive and Gram-negative envelopes. It is constituted of glycan chains made of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) disaccharide subunits, in which the N-acetylmuramate moiety is linked to highly conserved pentapeptide or tetrapeptide stems (L-alanine – D-isoglutamine – L-lysine – D-alanine – [D-alanine]) (Figure 7) [2].

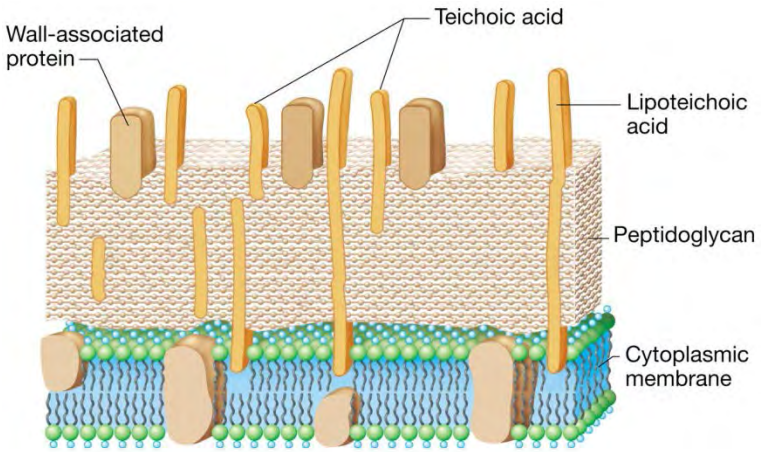

Figure 7: Schematic representation of Gram positive bacteria cell envelope (http://202.204.115.67/jpkch/jpkch/2008/wswx/chapter%203.htm).

In Gram-positive bacteria the thick peptidoglycan (50-100 nm in width) [139] functions as a surface organelle that ensures the contact between the external environment and the inside of the cell. It also serves to convey a number of functional proteins to the bacterial surface. In order to be targeted to bacterial envelope, proteins must carry the specific sequence YSIRK/GS [140,141]. The peptidoglycan is the major scaffold for anchoring most MSCRAMMs and other molecules. In that way it plays a key, yet indirect role in pathogenesis. Concomitantly, it is recognized by the innate immune system and triggers cytokine release and inflammation, through components such as LTA [142]. Thus, it is important for the microorganisms to hide these structures from host recognition, an objective that can be achieved by producing antiphagocytic components such as a capsule, or protein A.

Because peptidoglycan is a critical cell structure, its assembly is the target of antibiotics such as β-lactams and glycopeptides. Accordingly, modification of peptidoglycan synthesis is a response of resistant staphylococci to cell wall-active antibiotics [143].

II.2.2.3. Teichoic acids and lipoteichoic acids

TA represent up to 50% of the dry weight of purified staphylococcal walls. It is constituted of polyribitol-phosphate polymers crosslinked to NAM residues of the peptidoglycan (Figure 7) and decorated with D-alanine and NAG residues [144]. TA play an important physiologic role in the cell wall metabolism, and are likely to be a site of attachment of cell wall active enzymes and other proteins. They have been involved in adherence to nasal epithelia [145], but their role in invasive infection and host inflammatory response is unclear [142].

LTA are the plasma membrane-bound counterparts of TA. They share a common general structure but contain polyglycerol-phosphates and are linked to a diacylglycerol moiety that serves as a plasma membrane anchor. LTA have been implicated in inflammation by triggering the release of cytokines by macrophages and other players of the innate immune system. In particular, it was shown that the stereochemistry of the D-alanine decorations as well as the presence of the diacylglycerol lipid anchor were critical for host recognition and subsequent inflammation [144].

While LTA may facilitate bacterial recognition by host innate immunity, they also protect the microbes from killing by cationic antimicrobial peptides (CAPs) that are produced by professional phagocytes. Native LTA are polyanionic and may attract CAPs, which may be detrimental for the bacterium. To circumvent this problem, LTA become decorated with D-alanyl residues by the *dltABC* gene products, which render the structure more positively charged and thus repulses CAPs. Mutants impaired in *dltABC* are less adherent to endothelial cells and less able than wild type staphylococci to produce experimental endocarditis in rabbits [146].

II.2.2.4. Cell surface loosely and strongly associated proteins

Colonization is an important factor in the initiation of *S. aureus* infection. In order to colonize host tissues, *S. aureus* is capable of binding to extracellular host matrix or serum components, including fibrinogen, fibronectin, collagen, elastin, laminin, vitronectin, bone sialoprotein and thrombospondin, to mention some of them.

These proteins either remain associated with the surface, like MSCRAMMs, or may be released into the culture supernatant, like SERAMs (for Secretable Expanded Repertoire Adhesive Molecules). Both types of proteins are involved in colonizing host tissues and in evading the host immune

response [20,147]. Table 3 summarizes the main known species of these two groups. Individual species of the two groups are also described below.

Table 3: *S. aureus* surface proteins and secreted proteins that contribute to adherence to extracellular matrix components and serum proteins.

Full Protein name	Gene	Binding ligand - Function	Ref
SERAMs			
Extracellular adherence protein	*eap*	Fibrinogen (α-chain), fibronectin, prothrombin, endothelial cells	[148,149]
Extracellular fibrinogen binding protein	*efb*	Fibrinogen (α-chain), C3b, platelets aggregation	[150-154]
Coagulase	*coa*	Fibrinogen, prothrombin	[107-109,111]
MSCRAMMs			
Protein A	*spa*	IgG, TNFR1, Von Willerbrand factor, platelets activation, superantigen	[155-161]
Fibronectin binding protein A	*fnbpA*	Fibronectin, fibrinogen, elastin	[110,162-165]
Fibronectin binding protein B	*fnbpB*	Fibronectin, Elastin	[162-166]
Clumping factor A	*clfA*	Fibrinogen (γ-chain), fibrin	[167]
Clumping factor B	*clfB*	Fibrinogen (α/β-chain), Cytokeratin I-10	[168,169]
Iron-regulated surface determinant A	*isdA*	Heme	[170,171]
Iron-regulated surface determinant B	*isdB*	Hemoglobin, heme	[170-172]
Iron-regulated surface determinant C	*isdC*	Heme	[170,171]
Iron-regulated surface determinant	*isdH*	Heme	[171,172]
Serine-aspartate repeat protein C	*sdrC*	Unknown	[173]
Serine-aspartate repeat protein D	*sdrD*	Unknown	[173]
Serine-aspartate repeat protein E	*sdrE*	Unknown	[173]
Serine-rich adhesin for platelets	*srap*	Platelets	[174]
Staphylococcus aureus surface proteins C	*sasC*	Biofilm formation, cell aggregation	[175]
Staphylococcus aureus surface proteins D	*sasD*	Unknown	[176]
Staphylococcus aureus surface proteins F	*sasF*	Unknown	[176]
Staphylococcus aureus surface proteins G	*sasG*	Biofilm formation, nasal epithelial cells	[177-180]
Staphylococcus aureus surface proteins H	*sasH*	Unknown	[181]
Staphylococcus aureus surface proteins K	*sasK*	Unknown	[181]
Formyl transferase B	*fmtB*	Penicillin resistance, cell wall synthesis	[182,183]
Collagen binding protein	*cna*	Collagen	[184,185]
Plasmin sensitive protein	*pls*	Preventing adherence	[186]
Surface protein in Japanese ST8 CA-MRSA	*spj*	Unknown	[187]
Elastin binding protein	*ebps*	Elastin	[188,189]
Bone sialoprotein binding protein	*bbp*	Bone sialoprotein, fibrinogen	[190,191]

a) SERAMs

Extracellular adhesive proteins, Eap

SERAMs are not structurally related, but share a common functionality. A major studied SERAM, Eap, binds to multiple matrix proteins, including fibrinogen, fibronectin, prothrombin, and vitronectin. After secretion, it mediates agglutination by acting as a bridge between bacteria and host cell, either by Eap–Eap interaction or via other components of the bacterial surface [148]. Recently, Edwards *et al* [149] demonstrated that S. *aureus* Eap promoted attachment to endothelial cells via Protein A and generated more severe infections in a murine bacteremia model, thus demonstrating its

role in invasive diseases. Moreover, *S. aureus* strains that fail to produce Eap, exhibited a significantly decreased affinity for eukaryotic tissue binding and lacked the ability to colonize and invade host tissues [192-194].

Extracellular fibrinogen binding, Efb

As a prototype of SERAMs, Efb is an innate immune evasion molecule which was reported to inhibit complement activation [150] and to block platelet aggregation *in vitro* and *in vivo* [151,154]. Moreover, Efb was shown to bind to fibrinogen [150,152] with an unusually high affinity [153]. Interestingly, it has been show that such a high-affinity binding resulted in an inhibition of neutrophil adherence to immobilized fibrinogen, suggesting Efb could be powerful evader of human host defenses[153].

b) MSCRAMMs

Protein MSCRAMMs are encoded on the chromosome and have single or multiple binding properties for ligands. In Gram-positive bacteria many protein MSCRAMMs are covalently attached to the peptidoglycan via conserved mechanism (Figure 8A).

This mechanisms involves a conserved protein archtecture that starts with a N-terminal secretory signal peptide, required for Sec-dependent secretion and ends with a positively-charged C-terminal extension, which ensures that the protein is not secreted. Just proximal to the positively-charged C-terminal is a transmembrane-spanning domain, which is itself preceded by a conserved LPXTG motif that is indispensable for further anchoring the protein into the peptidoglycan. This anchoring involves a transpeptidation mechanism, which is mediated by an enzyme called sortase. The principal *S. aureus* sortase is encoded by the *srtA* gene [195]. It is a membrane protein which cleaves the MSCRAMMs between the threonine and the glycine of its LPXTG motif, and catalyzes the formation of an amide bond between the carboxyl-group of threonine and the amino-group of peptidoglycan penta-glycine cross-bridges (Figure 8B) [196]. Mutants lacking *srtA* are defective in surface proteins and are attenuated in animal infection models [197].

At least 22 genes encoding surface proteins belonging to the LPXTG family have been identified by *in silico* analysis of six *S. aureus* genome sequences [176,187,196]. The domain architecture, repeat regions and common signal peptide motif suggest that the evolution of these

LPXTG proteins may have arisen in a modular fashion [176]. These adhesins are described in more details below.

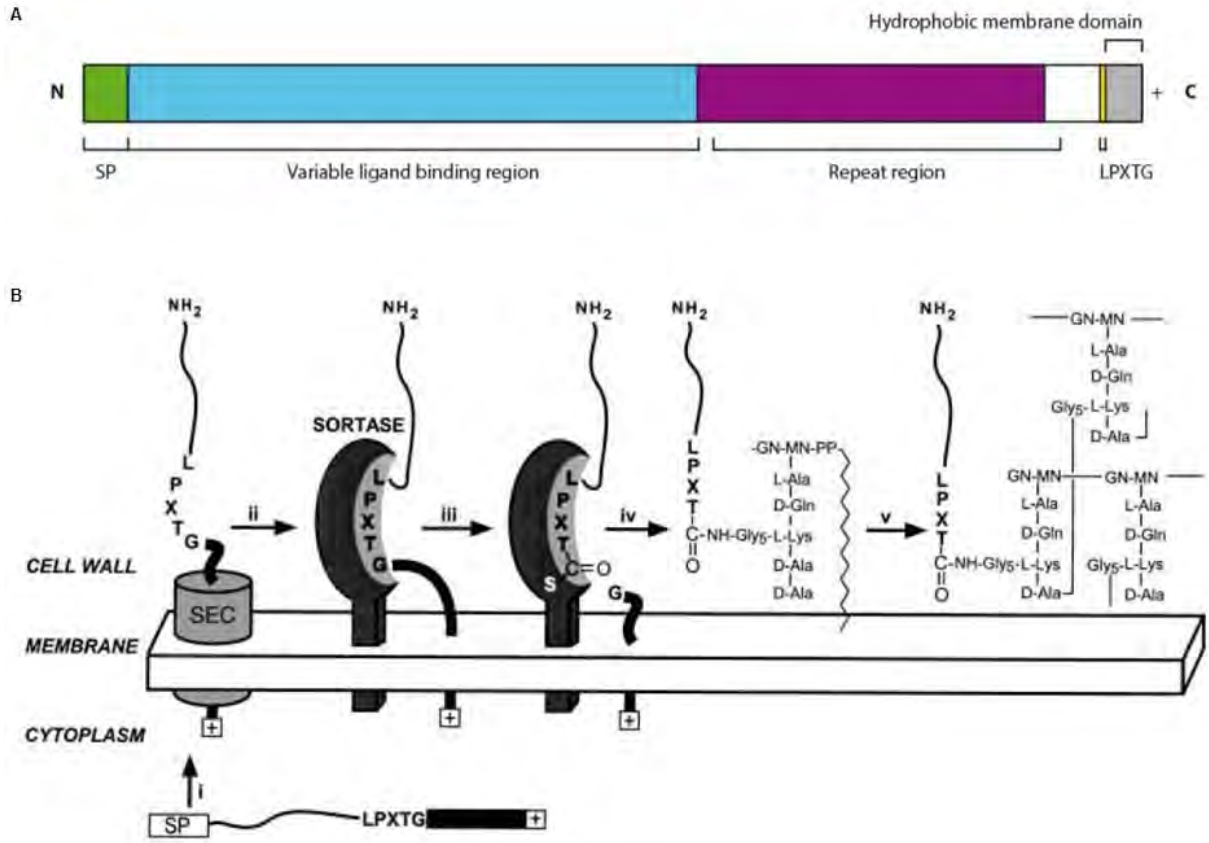

Figure 8: A, General structure of MSCRAMMs B, Surface protein anchoring in *S. aureus*. (i) Export. (ii) Retention. (iii) Cleavage. (iv) Linkage. (v) Cell wall incorporation. From [196].

Protein A, Spa

Protein A is encoded by the *spa* gene and is a major surface-attached protein of *S. aureus*. Dedent *et al* demonstrated that Protein A was unevenly distributed over the bacterial surface and that this distribution was related to the process of cell wall synthesis [156].

Protein A carries several important functions in pathogenesis. First, it interferes with opsonisation by catching immunoglobulin G (IgG) by their Fc portion, and thus hiding their opsonizing domain to professional phagocytes [155,157]. Second, Protein A may trigger pulmonary epithelial cell inflammation by attracting polymorphonuclear cells into respiratory tract, via TNFR1 (Tumor Necrosis Factor Receptor 1) activation [158]. Thus, it seems to play a role in the pathogenesis of pneumonia. Third, Protein A may be implicated in endothelial damages via binding to Von Willebrand factor, which is a multimeric glycoprotein involving platelet adhesion [159], and to a platelet complement receptor

(gC1qR/p33) [160]. Finally, Protein A can act as a superantigen and trigger non-specific inflammation in response to staphylococcal infection [161].

As mentioned above, numerous global regulators interact between themself to regulate *spa* expression during the bacterial growth (Figure 5). The first regulator identified was *agr,* which acts via RNAIII as a repressor at the post-transcriptional level during the late exponential and stationary growth phases (Figure 4) [21,40]. Spa expression is also controlled by the transcription regulators SarA, SarS, SarT and Rot. SarS seems to be the key effector of this regulation. SarS is localized just before *spa* in the *S. aureus* genome and directly active the *spa* transcription [71,72]. SarT and Rot activate indirectly *spa* via SarS [76,198].

Fibronectin binding protein, FnBP

FnBPs expression and ability to bind fibronectin are found in most clinical isolates of *S. aureus* [162]. *fnBPA* and *fnBPB* genes are located in tandem on the chromosome and corresponding proteins are crucial for invasion mechanisms in eukaryotic cells [163,164]. Figure 9 exposes the structural organization of *S. aureus* Fnbp.

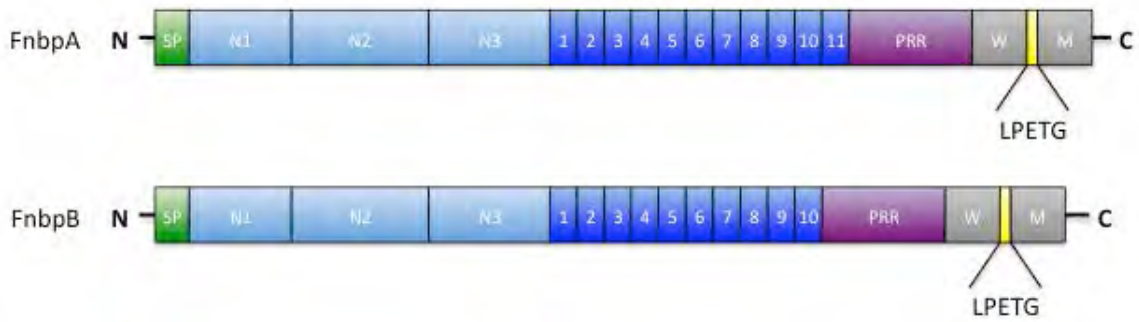

Figure 9: Structural organization of *S. aureus* FnbpA and FnbpB. The newly defined segments containing putative fibronectin-motifs binding with the F1 modules of fibronectin are represented in dark blue [199]. The N1, N2 and N3 domains in light blue represent the fibrinogen and elastin-binding domains. The signal peptide (SP, in green), the proline rich repeat domain (PRR, in purple), the cell wall-spanning W region (in grey), the membrane-spanning M region (in grey), and the LPETG cell wall anchor motif (in yellow) are also indicated. Adapted from [166,200].

One of the pathogenic properties of *S. aureus*, that has been difficult to assess *in vivo*, is the role of host-cell invasion during infection. Recent *in vitro* studies have provided convincing evidences that staphylococci can invade non-professional phagocytes, including epithelial and endothelial cells, fibroblasts, osteoblasts and keratinocytes [201-204]. The fibronectin-binding proteins were identified as major factors in initiating the internalization of *S. aureus* [19,200]. Furthermore, evidences from

several experimental models suggest that interfering with the ability of the bacteria to associate with fibronectin attenuates *S. aureus* virulence [205].

Additional studies investigating interactions between more than one surface protein help clarify the complex interplay between different virulence factors, or even between distinct domains within single proteins. They also clearly provided *in vivo* demonstration that Fnbp expression is sufficient to confer an invasive phenotype when heterogeneously expressed in non-pathogenic *L. lactis* [19]. Additional clinical evidence that cell invasion by *S. aureus* occurs *in vivo* came from a recent study in which intracellular *S. aureus* were detected in the endonasal mucosa of patients suffering from recurrent rhino-sinusitis [206]. Taken together, these studies clearly underline the importance of *S. aureus* host cell invasion for infection *in vivo*. Moreover, these findings support the concept that an "intracellular life-style" protects *S. aureus* from attacks by the immune system and shelters it from the action of antibiotics.

It has been recently reported that some MRSA strains formed a novel type of biofilm that was promoted by FnBPA and FnBPB under mildly acidic growth conditions. MRSA biofilm phenotype appeared by a secondary and indirect interaction mechanism of intercellular accumulation. However, this seems not to be the case in MSSA (methicillin sensible *S. aureus*) strains [165]. Although rather similar, polymorphism does exist between FnBPA and FnBPB in *S. aureus* isolates and should be considered for the development of vaccines or immunotherapeutics that target FnBP [166].

Clumping factors, Clf

ClfA and ClfB are members of the Sdr (Serine-aspartate repeat protein) family characterized by the presence of an R region composed largely of repeated SD dipeptides (more details in Sdr paragraph below) [173]. They exhibit a comparable structural organization including an N-terminal secretory signal sequence followed by a ligand-binding A region, the dipeptide R region, and the LPXTG cell wall-anchoring motif (W). However, the Sdr proteins are not closely related in sequence, with only 20 to 30% identical amino acid residues in the ligand-binding A domain, suggesting different roles in *S. aureus* pathogenesis [207]. A hydrophobic membrane-spanning domain (M) and a short positively charged cytoplasmic domain are located at the C-termimus of the native protein, but these regions are cleaved upon LPXTG- attachment on the peptidoglycan.

ClfA is the major fibrinogen-binding protein on the surface of cells in stationary phase of growth [167,168]. ClfB binds not only to fibrinogen [167], but also to human type I cytokeratin 10 (CK10) expressed on squamous epithelial cells, which plays a key role in establishing human nasal colonization [168,169]. Likewise, studies have shown that immunization of mice with ClfB reduced nasal colonization [208]. Binding to fibrinogen is expected to be significant in platelet activation and aggregation and might contribute to the pathogenesis of experimental endocarditis in rats [209,210].

The Rot and *agr* systems have no significant effect on *clfA* expression [77]. On the other hand, Rot and *agr* exhibited consistent regulatory effects on *clfB* transcription and bacterial fibrinogen-binding ability. Thus, the Rot and *agr* systems might affect bacterial fibrinogen-binding ability mainly through the regulation of *clfB* transcription [77].

Iron regulated surface determinants, Isd

The *isd* locus of *S. aureus* encodes four surface proteins, one lipoprotein, one membrane transporter, one cytoplasmic protein, and one sortase (*srtB*) [171]. The figure 10 shows a schematic structure representation of the Isd surface proteins, and Table 4 provides additional information on them. The *isdH* gene is not located on the *isd* locus. All these proteins, except IsdC are covalently bound to peptidoglycan by a sortase A system. IsdC is attached by the sortase B.

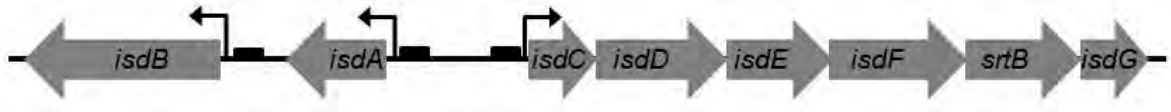

Figure 10: Organisation of the *isd* locus of *S. aureus*. Black boxes represent Fur boxes. Genes are represented in grey arrows. Black arrows indicate the initiation site of transcriptional units (adapted from [171]). The three transcriptional units encompass a ferric uptake repressor (*fur*) box, which inhibits transcription when iron concentration is high. Free iron is low in most environments including blood (approximately 10^{-18}M). *S. aureus* uses the specific Isd system as its primary heme-iron uptake pathway to promote iron acquisition in such limitating conditions [26].

IsdA, IsdB, IsdC and IsdH are cell wall associated proteins. Each Isd surface protein contains a secretion signal to be export, a sortase anchoring domain for SrtA or SrtB and one to three copies of a conserved NEAT (NEAr Transporter) domain [211]. NEAT domains are members of the immunoglobulin (Ig) superfamily.

Table 4: Localization and function of the Isd system components [170]

Protein	Localisation	Function	Ref
IsdA	Cell wall	Cell wall LPXTG anchored surface receptors	[170]
IsdB	Cell wall	Cell wall LPXTG anchored surface receptors	[170]
IsdC	Cell wall	Cell wall NPQTN anchored surface receptors	[170]
IsdD	Membrane	Unknown	[170]
IsdE	Membrane	Heme binding lipoprotein	[170,212]
IsdF	Membrane	Iron/heme permease – ABC transporter	[171]
IsdG	Cytoplasmic	Heme degrading enzyme	[171,213]
IsdH	Cell wall	Cell wall attached surface receptors	[172]
IsdI	Cytoplasmic	Heme degrading enzyme	[213]
SrtB	Cell wall	Attachment of IsdC NPQTN cell surface receptor	[170]

A schematic representation of the Isd surface receptors is presented of figure 11.

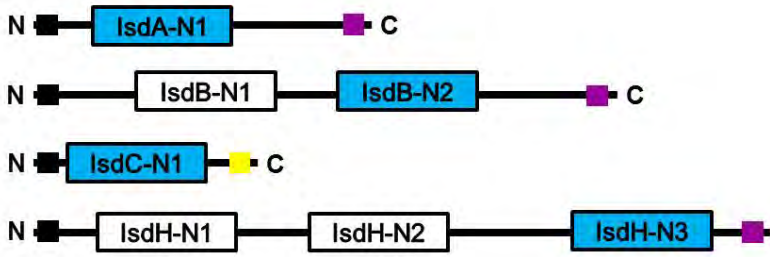

Figure 11: Schematic representation of the Isd surface proteins. All proteins contain a secretion signal represented by a black box, a sortase recognition sequence (in purple square for sortase A processing and in yellow square for sortase B), and at least one NEAT domain. NEAT domains are identified by IsdX-Ny where "x" indicates the unique protein and "y" the order of the NEAT domain. Blue boxes represent the heme-binding NEAT domains. Adapted from [172].

Figure 12 summarizes the heme transport from the environment to the bacterial cytoplasm. Heme transport starts with the capture of heme after binding to hemoglobin at the surface of the bacterial cells by surface proteins IsdB or IsdH [171,172]. First, IsdH transfers its cargo to IsdB, which is then transported more deeply inside the peptidoglycan by IsdA or IsdC [171]. After crossing the wall, the heme molecule is relayed to the membrane binding protein IsdE that interacts with the membrane transporter IsdF. Once in the cytoplasm, IsdG and IsdI degrade the heme to liberate the central iron atom and make it available for the bacteria [171,213].

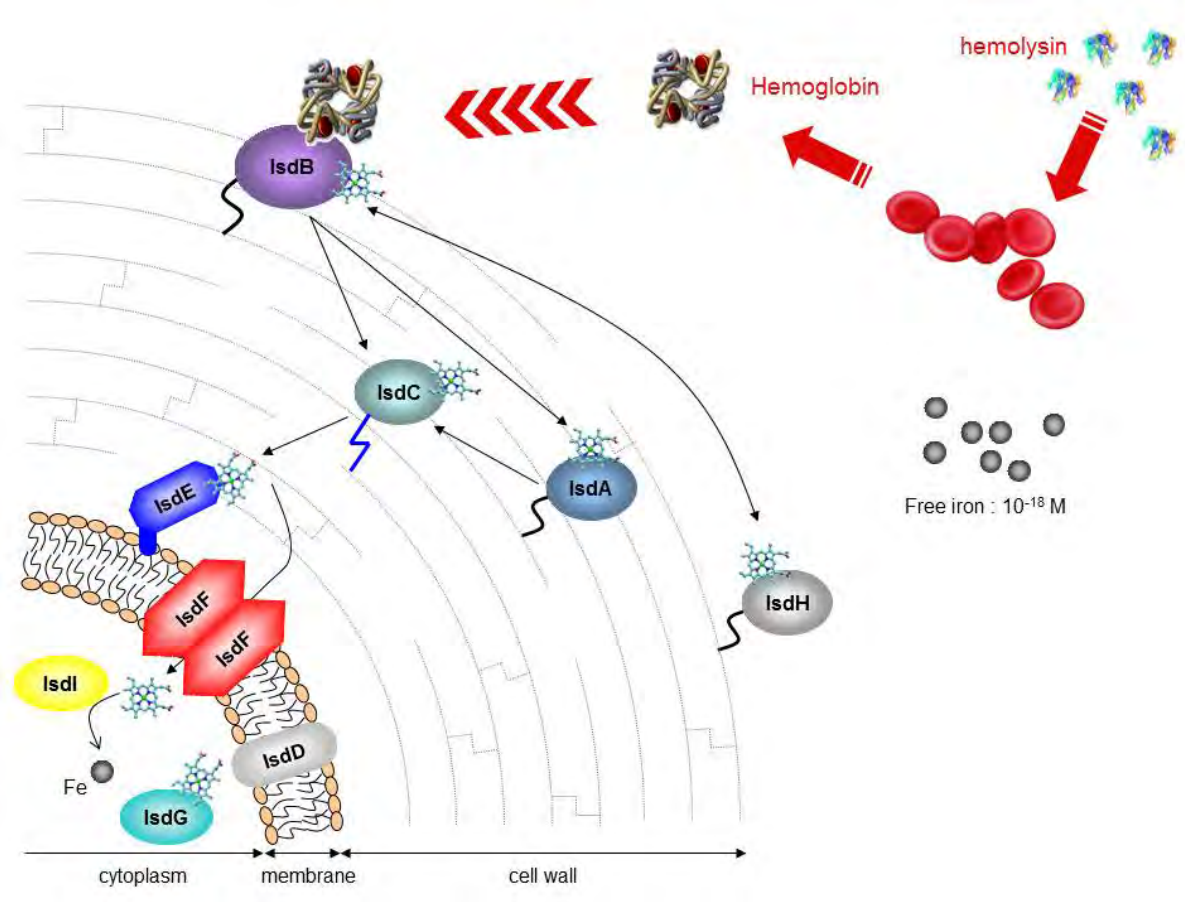

Figure 12: Schematic representation of the Isd heme transport pathway. In blood, hemolysin acts by disrupting the red blood cell membrane in order to free its hemoglobin content. IsdA, IsdB, IsdC and IsdH, which are covalently bound to the peptidoglycan, are capture iron sources ant transfer it to the membrane receptor IsdE, which function is as yet unknown. Then, heme or iron could cross the membrane using the permease IsdF. In the cytoplasm, IsdG binds to the heme and IsdI reduces it in free iron. Adapted from [172].

Serine-aspartate (SD) repeat proteins, Sdr

The Sdr proteins are a subset of putative staphylococcal MSCRAMMs, covalently anchored to the cell wall and characterized by a R segment composed of repeated serine-aspartate (SD) dipeptides. The Sdr proteins are encoded by *sdrC*, *sdrD* and *sdrE* tandemly present in the *sdr* locus

[173]. They have similar structural organizations, where the N-terminal ligand-binding A region can be further divided into three subdomains N1, N2, and N3. The N2 and N3 subdomains adopt IgG-like folds.

The ligand-binding A region is similar in size, but is not closely related between the different members of the *sdr* family (with only 20 to 30% of similar amino acid residues), as well as with ClfA and ClfB, suggesting that Sdr proteins have different roles in pathogenesis [173]. The B-domains binds to Ca^{2+} and promotes the maintenance of structure to modulate the distance between the interactive A region and the bacterial cell surface. The C-terminal domain of Sdr contains the SD repeats followed by the cell wall anchoring LPXTG motif.

Serine-rich adhesin for platelets, Srap

The N-term of Srap contains an atypical putative signal peptide, followed by a short serine-rich region, a non-repeat region, a second serine-rich region and a cell wall anchoring motif LPDTG [174]. SraP promotes bacterial aggregation and binding to their own non-repeat domains via the serine-rich repeat domain located within its N-terminal region. Moreover, SraP appears to be a direct virulence determinant in endovascular infection [174].

Staphylococcus aureus surface proteins, Sas

Analyses of primary sequences of the Sas family of proteins indicated that they present a similar structural organization and a common signal sequence with a characterized LPXTG motif of *S. aureus* and other Gram-positive cocci. SasC represents a novel *S. aureus* protein implicated in cell aggregation and biofilm formation, which may play an important role in colonization during infection [175]. SasD and SasF harbour a single residue variation in the fourth position of the LPXTG motif: LPXAG [176] and not the YSIRK/GS motif, which addresses proteins to the cellular pole [141]. SasK is absent from the majority of *S. aureus* genomes [181].

SasG is an important mediator of biofilm formation in virulent strains of *S. aureus* [178,179]. It contains an N-terminal A domain and repeated B domains. Geoghegan *et al* [180] demonstrated that biofilm formation due to SasG accumulation is a process that requires a physiological concentration of Zn(2+) and is supported by the B domains. The *sasG* and *sasH* genes were positively and independently associated with disease isolates [177].

Factor affecting methicillin resistance and triton X-100 suseptibility, FmtB (formyl transferase)

The β-lactam antibiotic resistance of *S. aureus* is mainly mediated by penicillin-binding protein 2A (PBP2A), which is active in the presence of methicillin and other β-lactam antibiotics [214]. A transposon insertion in *fmtB* demonstrated its implication in methicillin resistance in MRSA strain COL [183]. In the presence of the non-ionic detergent triton X-100, MRSA were shown to be more sensitive to methicillin-like antibiotics by a mechanism implicating autolysis [182]. The *fmtB* gene codes for a cell wall anchored protein of 263 kDa that contains 17 tandem repeats of 75 amino acids and a C-terminal LPDTG cell wall-sorting motif [183]. Moreover, FmtB contained two of the three conserved motifs shared by PBPs and β-lactamases, suggesting that FmtB may be involved in cell wall synthesis [182].

Collagen binding protein, Cna

Cna was described as a specific *S. aureus* adhesin capable of binding collagen and collagenous tissues [184,185]. The structural organization of Cna follows the standard MSCRAMMs features. However, Ryding *et al.* [215] demonstrated that binding to collagen was possible despite the absence of *cna*, but with a lesser affinity.

Plasmin sensitive protein, Pls

The structure of Pls revealed three distinct repeated regions, of which one was made of serine-aspartate dipeptide repeats similar to those found in the Clf and Sdr families [186]. Pls is sensitive to proteolysis by plasmin. It is a surface protein only present in MRSA, because it is located in the methicillin-resistance genomic island SCC*mec*. Unless it is cleaved by plasmin, Pls inhibits bacterial adhesion to immobilized fibronectin, fibrinogen, laminin, and immunoglobulin G as well as invasion of host cells [186]. This suggests that Pls could play a role in preventing adhesion at some stages of the infection process. However, the mechanism of the anti-adhesive and anti-invasive effect of Pls is unclear.

Surface protein in Japanese ST8 CA-MRSA, spj

The *spj* gene is a 4.8 kb novel open reading frame (ORF), encoding for a large LPXTG protein in a major ST8 CA-MRSA. This new MSCRAMMs encompass a typical LPXTG protein [187].

Elastin binding proteins, Ebps

EbpS is a cell surface integral membrane protein that is not covalently attached to the peptidoglycan [189]. It mediates binding to the extracellular matrix protein elastin *in vitro*, but its function in virulence *in vivo* remains unclear [188].

Bone sialoprotein-binding protein, Bbp

Bbp mediate binding to bone sialoprotein, a glycoprotein of bone and belongs to the *sdr* family [190]. The *S. aureus* Bbp protein may be important in the targeting of bacteria towards bone tissues, and thus might be of relevance in the pathogenicity of osteomyelitis. Recently, a study has shown that Bbp was also able to bind fibrinogen [191], implying that Bbp may have two independent functions: an important factor in the colonization of bone tissue (i.e. sialoprotein binding) and a contributing factor in *S. aureus* hematologic diseases (i.e. fibrinogen binding), such as sepsis [191].

III Aim of the study

The description summarized above highlights the multiplicity of *S. aureus* pathogenic features, as well as the complexity of their regulation. Thus, although useful to dissect out the system, any simplistic approach to understand the function or regulation of *S. aureus* virulence factors is bound to provide only a limited view of reality, and may lead to conclusions that do not much the natural history of infection. This is particularly true for surface proteins, which undergo a complex ride with potentially multiple modifications at both the intracellular and extracellular levels (a minimal example is the cleavage of the signal peptide).

This thesis work tried to address these issues by a two-steps logical experimental approach, first phenotypic and second transcriptomic and proteomic. In the first part of the thesis we asked a seemingly very simple question, i.e. is the *in vitro* adherence phenotype of *S. aureus*, for instance to fibrinogen or fibronectin, predictive of its ability to infect humans or animals? The premises of this question was that, based on studies of specific adhesins [19,77], adherence to fibrinogen or fibronectin should promote adherence to endovascular tissues and thus be more pronounced in *S. aureus* isolates recovered from patients with bacteremia or endocarditis than in isolates recovered from healthy carriers. The first study presented below addressed this very question (Chapter 2).

However, unexpectedly the results contradicted the hypothesis. *S. aureus* isolates recovered from a variety of environments, including nasal cultures from healthy carriers and blood isolates from patients with bacteremia or endocarditis, demonstrated a wide array of adherence phenotypes *in vitro*, which did not correlate with any particular colonization of disease status. Even more, *S. aureus* isolates that were highly adherent to fibrinogen and fibronectin *in vitro* where not necessarily more prone to induce experimental endocarditis in rats than poorly adherent strains [216]. This was in apparent contradiction with the paradigm that fibrinogen and fibronectin-binding were necessary and sufficient for endovascular infection [19,79].

However, the interpretation of these results might be subtler than just concluding that adherence was not involved in disease. Indeed, it could be that the tested *S. aureus* isolates did just not express their surface adhesins identically when grown *in vitro* than when exposed to *in vivo* conditions, and that differential gene regulation in these two conditions obscured the interpretation. To try better understand these features, we run a second serie of studies, presented in the second paper of this thesis dissertation (Chapter 3). Here we specifically enquired about the physical presence of surface protein MSCRAMMs in time-course experiments in *S. aureus* grown in different milieu, and compared these results to their transcriptomes and *in vitro* adherence phenotypes. We found a number of differences between the expected protein expression based on previous gene regulation literature [4] and the physical presence of these factors on the bacterial surface. Moreover, proteomic analyses performed by trypsin shaving of live bacteria also revealed that the same protein, although present, could hide certain of their domains in the complex peptidoglycan-polysaccharide meshwork of the bacterial envelope, thus making them unavailable for trypsin digestion [217]. This has a potentially important consequence in terms of disease mechanisms and also vaccine development. Indeed, whether transient or not, steric hindrance of specific proteins domains may make them (temporarily) available or unavailable for adherence purposes, in case of disease mechanism, or recognition by antibodies in case of host defenses. This type of behavior implicates an additional, sometimes underestimated, type of regulation, i.e. conformation plasticity or induced-fitness.

While this new question arose from the present work, the proteomic experiments also revealed other as yet unknown features of the *S. aureus* proteome, as exemplified by a new post-translational modification described in a collaborative study with P. Waridel (Annexe 1) [218].

Taken together, the present work provides a threefold answer to the original question regarding the correlation or not between *in vitro* and *in vivo* phenotypes in *S. aureus* isolates:

1. No, the *in vitro* adherence phenotypes do not correlate or predict the *in vivo* disease capacity.
2. Yes, differential *in vitro* and *in vivo* gene and protein regulation may explain these differences.
3. In addition, alternative regulation mechanisms of protein function, via steric modification or induced-fitness, are likely to operate in medium-dependent phenotypic differences.

IV. References

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V. Introduction to Chapter 2

Chapter 2 describes the natural variability of *in vitro* adherence to fibrinogen and fibronectin of carriage isolates and the correlation with *in vivo* infectivity of *S. aureus* in an endocarditis model in rat.

Carriage of *S. aureus* is a significant risk factor for infection and studies of colonization offer a potential way to disease prevention [1]. Although there is some evidence that certain *S. aureus* genotypes are more virulent than others, it is generally accepted that all colonizing strains are potentially infectious.

This study investigates the use of infectivity profiles of different carriage isolates of *S. aureus* to predict and classify the risk of disease development in permanent or intermittent carriers. To do this, we compare the ability of adherence to immobilized fibronectin or fibrinogen *in vitro* of carriage strains with strains from endocarditis and blood culture isolates. We demonstrate that no matter the source of bacteria, the profile of adhesion to either ligand was very heterogeneous.

Next, we choose two pairs of carriage strains that have opposing abilities to adhere to fibronectin and fibrinogen *in vivo* in order to compare their infectivities in an endocarditis model in rats. The results show that regardless of the profile of adhesion, all strains are able to infect rats despite their inability to adhere *in vitro*.

CHAPTER 2

Natural variability of *in vitro* adherence to fibrinogen and fibronectin does not correlate with *in vivo* infectivity of *Staphylococcus aureus*

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Author Contributions: O. Sakwinska, P. Moreillon and M. Ythier designed research; M. Ythier and J. M. Entenza performed research; J. Bille, M. Bes and F. Vandenesch furnished strains; M. Ythier, O. Sakwinska and J. M. Entenza analysed data; M. Ythier, P. Moreillon and O. Sakwinska wrote the paper.

I. Abstract

Adherence to fibrinogen and fibronectin plays a crucial role in *Staphylococcus aureus* experimental endocarditis. Previous genetic studies have shown that infection and carriage isolates do not systematically differ in their virulence-related genes, including genes conferring adherence, such as *clfA* and *fnbA*. We set out to determine the range of adherence phenotypes in carriage isolates of *S. aureus*, to compare the adherence of these isolates to the adherence of infection isolates, and to determine the relationship between adherence and infectivity in a rat model of experimental endocarditis. A total of 133 healthy carriage isolates were screened for in vitro adherence to fibrinogen and fibronectin, and 30 isolates were randomly chosen for further investigation. These 30 isolates were compared to 30 infective endocarditis isolates and 30 blood culture isolates. The infectivities of the carriage isolates, which displayed either extremely low or high adherence to fibrinogen and fibronectin, were tested using a rat model of experimental endocarditis. The levels of adherence to both fibrinogen and fibronectin were very similar for isolates from healthy carriers and members of the two groups of infection isolates. All three groups of isolates showed a wide range of adherence to fibrinogen and fibronectin. Moreover, the carriage isolates that showed minimal adherence and the carriage isolates that showed strong adherence had the same infectivity in experimental endocarditis. Adherence was proven to be important for pathogenesis in experimental endocarditis, but even the least adherent carriage strains had the ability to induce infection. We discuss the roles of differential gene expression, human host factors, and gene redundancy in resolving this apparent paradox.

II. Introduction

Staphylococcus aureus is a human commensal, but at the same time it is one of the most important bacterial pathogens that cause community-acquired and nosocomial infections. It can produce a wide variety of diseases, from benign skin infections, such as folliculitis or furunculosis, to life-threatening conditions, like osteomyelitis, septic arthritis, sepsis, pneumonia, and endocarditis [2]. About 20% of humans carry *S. aureus* permanently in their noses, and another 60% are intermittent carriers [3]. The association between *S. aureus* nasal carriage and staphylococcal disease has been reported for several decades [4,5]. More recently, it has been unambiguously shown that carriers have a higher risk of infection, at least when they are hospitalized [1,6]. The pathogenicity of *S. aureus* involves a wide range of cell wall-associated adhesins and extracellular toxins. Surface adhesins, referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), bind to the host extracellular matrix and thus promote tissue colonization and infection [7]. The major MSCRAMMs involved in *S. aureus* pathogenesis are particular surface proteins that are covalently bound to the cell wall peptidoglycan via a conserved LPXTG motif [8]. Genomic analyses indicated that the *S. aureus* genome contains up to 21 such LPXTG surface proteins [9]. In addition to their multiplicity, these proteins often have redundant functions, as exemplified by clumping factors A and B (ClfA and ClfB), which bind fibrinogen [10,11], and fibronectin-binding proteins A and B (FnBPA and FnBPB), which bind fibronectin [12], fibrinogen [13], and elastin [14]. It has been unambiguously demonstrated that in *S. aureus* ClfA and FnBPA are key pathogenicity factors, at least in infective endocarditis. This has been achieved by expressing these adhesins in bacteria lacking the rest of the *S. aureus* surface features [15]. Using a variety of truncated and chimera constructs of these proteins, Que et al. observed that fibrinogen-binding domains were necessary and sufficient for colonization of damaged valves in experimental endocarditis, but not for persistence and invasion, whereas fibronectin-binding domains of FnBPA were unable to initiate infection but mediated aortic cell invasion and microbial persistence. Thus, the two adherence functions were necessary for progressive infection [16]. Despite the great effort to establish whether there are specific genetic determinants that distinguish carriage and invasive infection strains, the answer was largely negative [17,18]. Genetic studies revealed that many *S. aureus* wild-type strains lack some of the genes coding for LPXTG motif proteins, but there is no overall difference between carriage and infection isolates [19]. However, *fnbA*

and *clfA* are nearly always present in carriage and clinical isolates, confirming their pivotal role. Moreover, sequence analysis of functional regions of the two proteins has shown that there is a high degree of conservation in sporadic and epidemic isolates of *S. aureus* [20]. On the other hand, the presence of the genes does not imply that there is efficient expression of a protein on the cell surface. It is entirely possible that either the carriage isolates express FnBPA and ClfA at a lower level or the adherence to the host matrix is less efficient. To our knowledge, a comparison of the adherence phenotypes of infection and carriage isolates has never been conducted. Here we determined the levels of adherence to fibrinogen and fibronectin of 133 carriage isolates. We compared these isolates to 30 infective endocarditis and 30 blood culture isolates. In addition, we compared the infectivities of isolates displaying extreme adherence phenotypes in a rat model of experimental endocarditis.

III. Material and methods

III.1. Bacterial strains and growth conditions

The carriage isolates used in this study were described in detail elsewhere [21]. Briefly, 133 *S. aureus* isolates were collected from 406 healthy adults in western Switzerland in 2005 and 2006. The blood culture isolates originated from patients at a tertiary care hospital in western Switzerland, and they came from the same catchment area as the carriage isolates and were collected over the same time period. These isolates were consecutive isolates received by the clinical microbiology laboratory of the university hospital and thus represented both community and acquired episodes of bacteremia that were associated or not associated with intravenous catheter colonization. Infective endocarditis isolates were collected between January and December 1999 during a population-based study conducted prospectively in six regions of France [22]. *S. aureus* was grown at 37°C without agitation in tryptic soy broth (Difco Laboratories, Detroit, MI). *S. aureus* NCTC 8325-4 was used as a control strain to monitor the overall quality of the assays. Strains DU5883 (*fnBPA*-, *fnBPB*-), a mutant of NCTC8325-4 [23], and DU5852 (*clfA*-) [24], were used as negative controls for adherence to fibronectin and fibrinogen, respectively.

III.2 Bacterial adherence to solid-phase fibrinogen and fibronectin

We modified a previously described staphylococcal adherence assay [25] to measure the ability of *S. aureus* to adhere to low levels of surface-adsorbed fibrinogen and fibronectin. Serial 2-fold dilutions of fibrinogen (Sigma) or fibronectin (Sigma) in phosphate-buffered saline (PBS) were placed in 96-well plates (Nunc-Immuno plates; MaxiSorp surface; Nalge Nunc International). PBS without a ligand was placed in the last well as a negative control. The plates were incubated at 4°C for 16 h. Then they were washed three times with PBS, and 200 µl of 2% bovine serum albumin (Fluka) in PBS was added to each well to block nonspecific sites. The plates were incubated for 1.5 h and then washed three times with PBS. Bacterial cultures were harvested in the mid-logarithmic phase of growth (optical density at 600 nm [OD_{600}], 0.6). After centrifugation for 10 min at 3,000 rpm, cells were frozen. Shortly before the test, the cells were resuspended in PBS, and the concentration was adjusted to 5 x10^9 CFU/ml. Portions (50 µl) of the cell suspension were applied to individual wells (2.5 x 10^8 CFU per well) and incubated for 2 h at 37°C. Then the wells were washed with PBS and fixed at 55°C for 30 to 45 min. Bound bacteria were detected by staining with crystal violet, and the OD570 was determined with an enzyme-linked immunosorbent assay plate reader. For the initial screen of 133 carriage isolates, the assay was carried out once with 11 ligand concentrations starting with same initial concentration. For all subsequent tests the measurements were repeated twice with five concentrations using independently grown bacterial cultures. The adherence score was calculated by subtracting the control value (no ligand) from the area under the curve. The optical densities for five substrate concentrations were added, and from the resulting value we subtracted the value for the well containing no ligand multiplied by 6 (Figure 1).

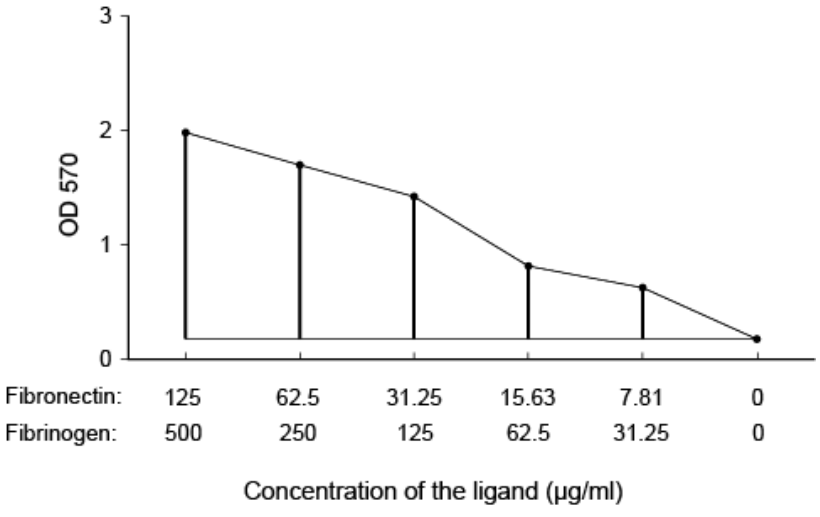

Figure 1: Relationship between ligand concentration and adherence measured using optical density. The adherence value was calculated as an approximation of the area under the curve as follows. The optical density values for all concentrations were added, and the control value (which represented the optical density when no ligand was present) was multiplied by six and subtracted from the sum.

III.3. Rat model of infective endocarditis

All animal experiments were carried out according to Swiss federal and cantonal regulations (authorization 879-6). Sterile aortic vegetations were produced in female Wistar rats as described previously [26]. Groups of animals were inoculated with 10^3 or 10^4 CFU from cultures in the exponential growth phase. These inoculum sizes were used because clinical isolates have been shown to induce 50% and 90% experimental endocarditis in this model [27-29]. Rats were sacrificed 24 h after inoculation, quantitative vegetation and spleen culture analyses were performed, and bacterial densities were expressed in $\log_{10}$ CFU/g. Median bacterial titers in the vegetations were compared by using the nonparametric Kruskal-Wallis test with Dunn's correction for multiple comparisons. The chi-square test with Yates correction was used to detect differences among infection rates. Differences were considered significant if the P value was < 0.05, using two-tailed significance levels.

IV. Results

The initial screen of 133 carriage isolates originating from healthy carriers showed that there was great variation in adherence to fibrinogen and fibronectin (Figure 2). The two adherence scores were significantly correlated (Spearman's rank correlation, 0.52; P < 0.0001).

Twenty-eight carriage isolates were randomly chosen further investigation. Similar to carriage isolates, both types of infection isolates displayed variability in adherence to fibronectin and fibrinogen (Figure 3). The adherence to both ligands was the same for the three groups of isolates. However, individual strains differed significantly in adherence to fibrinogen (Kruskal-Wallis chi-square test, 123; df, 87; P = 0.006). The differences in adherence to fibronectin were not significant (Kruskal-Wallis chisquare test, 105; df, 87; P = 0.08). Strain NCTC 8325-4 exhibited relatively strong adherence to both substrates.

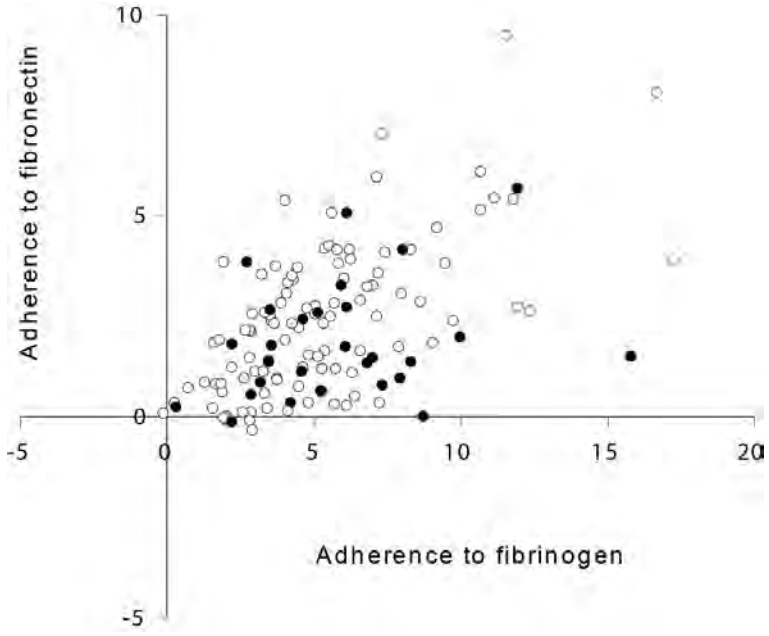

Figure 2: Adherence to fibrinogen and fibronectin of 133 carriage isolates. Each circle represents an isolate (one assay). The axes indicate adherence scores as described in Material and Methods. The isolates indicated by filled circles were subsequently used for comparison with infection isolates.

We chose four carriage isolates which exhibited very low (two isolates) and very high (two isolates) levels of adherence to fibrinogen and fibronectin to test infectivity in the rat model of experimental endocarditis. The abilities of all isolates to induce endocarditis were similar for both inoculum sizes tested (Figure 4).

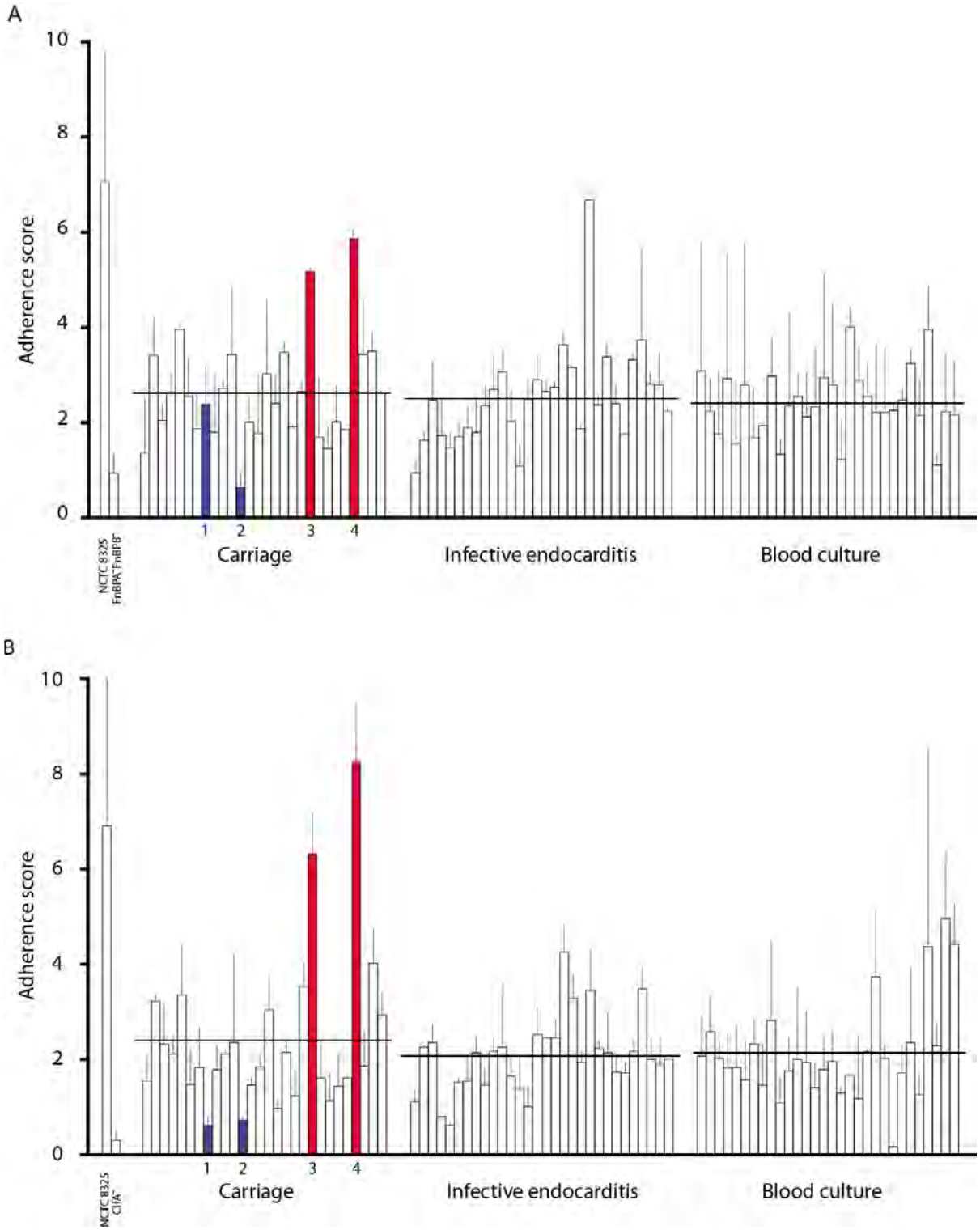

Figure 3: Adherence of *S. aureus* carriage and infection isolates to fibronectin (A) and fibrinogen (B). The bars and error bars indicate the average and standard deviation for each isolate (two assays per isolate). The average for each category is indicated by a horizontal line. Isolates which were used for testing in the experimental endocarditis model are indicated by filled bars (high adherence) and striped bars (low adherence) and numbers.

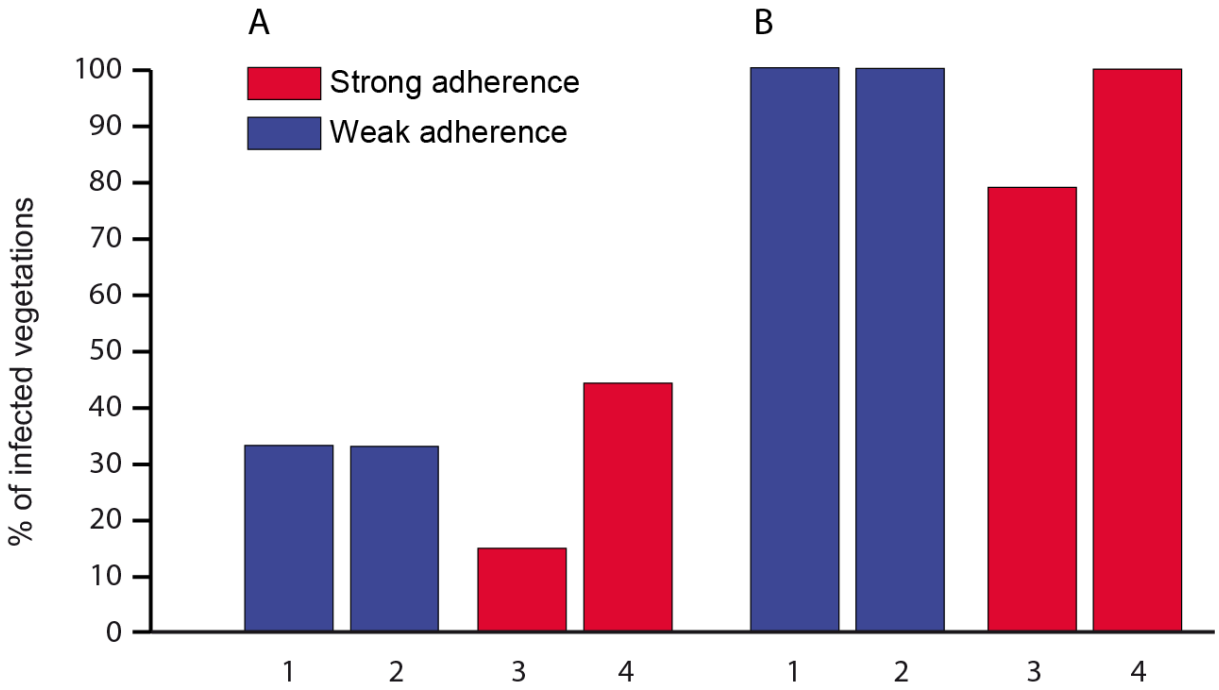

Figure 4: Incidence of endocarditis in rats challenged with 10^3 (A) or 10^4 (B) CFU of *S. aureus* carriage isolates exhibiting a low (strains 1 and 2) or high (strains 3 and 4) level of adherence to fibrinogen and fibronectin.

In addition, all animals had 80 to 100% infected spleens and similar densities of bacteria in vegetations and spleens irrespective of the challenge strain (Table 1). There were no differences among the strains in any of the variables measured.

Table 1: Bacterial counts in infected spleens and vegetations

			Inoculum countaning 10^3 CFU			Inoculum countaning 10^4 CFU		
				Bacterial counts (log10 CFU/g)			Bacterial counts (log10 CFU/g)	
Location	Adhesion	Strain	n	Median	Range	n	Median	Range
Spleens	Weak	1	9	2.21	1.56–4.71	9	4.73	3.79–5.71
		2	7	2.57	1.60–4.67	9	4.70	3.28–5.80
	Strong	3	8	2.18	2.02–4.65	10	4.71	3.48–6.67
		4	9	3.65	2.91–4.53	10	5.73	4.72–6.78
Vegetations	Weak	1	3	6.32	4.59–7.76	10	9.05	4.64–9.66
		2	3	7.90	7.68–7.98	8	8.59	7.96–9.29
	Strong	3	1	6.28		8	7.58	5.25–9.39
		4	4	8.47	4.41–8.84	9	8.50	4.23–9.63

V. Discussion

V.1. No difference in adherence between infection and carriage isolates

The present study showed that a prominent aspect of the *S. aureus* phenotype, adherence to fibrinogen and fibronectin, does not correlate with the source of isolates. The levels of adherence to fibrinogen and fibronectin, as measured in in vitro assay, were very similar for isolates obtained from healthy carriers and members of two different groups of infection isolates. Given the prominent role of adherence to fibrinogen and fibronectin conferred by ClfA and FnBPA, which was demonstrated in an experimental endocarditis study [16], stronger adherence could have been expected for infective endocarditis isolates than for carriage isolates. Our phenotypic observations complement the results of previous studies, which did not identify factors associated with increased virulence in *S. aureus* at the genetic level. It was demonstrated previously that all lineages (clonal complexes) of *S. aureus* were equally likely to cause an infection [17]. Likewise, Lindsay *et al.* [19] were unable to identify any genes overrepresented in infection isolates, further confirming the lack of simple genetic determinants of staphylococcal infection. This suggested that most, if not all, natural isolates of *S. aureus* are capable of infection. We have not tested whether the *clfA* and *fnbA* genes are present in the isolates used in this study, because previous studies demonstrated that these genes were nearly universally present in human infection and carriage isolates [20,30]. However, a recent study reported a tendency toward specific features for methicillin-resistant *S. aureus* strains responsible for persistent bacteremia compared with the strains responsible for transient bacteremia [31]. Persistent bacteremia isolates were phenotypically more adherent to fibrinogen, fibronectin, and endothelial cells in vitro. Nevertheless, although persistence was associated with a phenotypic and molecular signature, the two types of strains were equally able to induce endocarditis in rabbits, which suggests that a disease-inducing capacity is universal in *S. aureus* strains. Similarly, it has been shown that certain genotypes (clonal complexes) might be associated with more severe disease [32]. However, these findings appear to be due, at least in part, to the association of certain genotypes, predominantly ST36, with methicillin resistance. While no specific genetic makeup correlated with invasive infection, specific genes are indeed related to specific disease syndromes, such as the genes encoding superantigens

(e.g., toxic shock syndrome toxin and enterotoxins) or exfoliative toxins. Human host factors are crucial for *S. aureus* infection. The most important risk factor is illness, as shown by the nearly 100-fold increase in the risk of invasive staphylococcal infection in humans who are ill, from 0.03% in the general population (or approximately 30 cases/100,000 people [33]) to 5.7% during hospitalization (80 cases/14,000 people [6]). Various medical conditions and interventions, such as diabetes, dialysis, surgery, and drug and alcohol abuse, have been identified as important risk factors [33]. Nasal carriage was shown to increase the risk of staphylococcal bacteremia 3-fold but not the risk of death inhospitalized nonsurgical patients [6]. Specific genetic predispositions do not seem to play a major role, with the exception of male gender and a few very rare inherited immune deficiencies. Nevertheless, whether host predisposition is a unique factor promoting *S. aureus* infection remains to be determined.

V.2. Wide range of adherence phenotypes but no correlation with infectivity

A wide range of adherence phenotypes was observed for all types of isolates, including infective endocarditis isolates displaying very low adherence. In particular, the carriage isolates displaying very low adherence might be expected to show lower infectivity in experimental endocarditis. However, even these isolates were capable of inducing experimental valve infection. Human endocarditis isolates were tested previously on multiple occasions using this model, and they exhibited infectivities very similar to those reported here [27-29,34,35].

This was clearly shown by the minimum inoculum size necessary to infect 90% of the animals, which was 10^4 CFU for both the isolates used in the present study and endocarditis isolates tested in previous experiments.

V.3. Proven role of adherence in infection but no difference in infectivity between strongly and weakly adherent isolates

Although adherence to fibrinogen and fibronectin has been proven to be important for pathogenesis in experimental endocarditis, even the least adherent carriage strains were able to induce infection in this model. Several explanations can be offered for this apparent paradox. The first

explanation is the possible inability of the in vivo endocarditis model to detect relevant differences. It is always questionable to use specific models, in this case experimental endocarditis, to examine general characteristics, such as infectivity. Moreover, induction of valve infection proceeds via at least two steps; it starts with tissue colonization, which may be reversible if the bacterium is susceptible (and accessible) to host defense mechanisms, and this is followed by invasion and persistence, when the microbes settle further, invade local tissues, and spread to distant sites.

These two steps may involve different virulence factors that might be regulated differently to achieve optimal infectivity (see below). The present study addressed primarily induction of infection (with relatively early sacrifice 24 h after inoculation), because this is the unavoidable primum movens for further invasion. Moreover, it attempted to correlate adherence to fibrinogen and fibronectin with the capacity to promote experimental endocarditis, because the adhesins mediating these phenotypes were specifically associated with valve infection in previous studies [16,29,36], whereas other adhesins were not [37]. Therefore, the system model is pertinent to the questions asked. The second putative explanation is adhesin redundancy. Indeed, our observations are reminiscent of the situation where knockout mutations of major adherence-promoting genes had very modest effects on infectivity in experimental endocarditis, despite marked decreases in adherence to the specific ligands [29,38]. *S. aureus* possesses a wide array of virulence factors, including up to 21 LPXTG cell wall-anchored adhesins [9]. Moreover, many isolates contain only some of these factors but are still infectious [19], suggesting that there is great functional overlap among the factors. This issue was recently studied by expressing 18 of the 21 *S. aureus* LPXTG proteins in surrogate lactococci and testing the recombinant organisms to determine their capacities to induce experimental endocarditis [37]. ClfA and FnBPA significantly increased the ability of the recombinants to produce experimental valve infection, but other proteins (including Cna, SdrC, SdrD, SdrE, and Pls) had a marginal effect, suggesting that they might cooperate to cause infection as well. Hence, it is likely that an isolate of *S. aureus* needs only some of these proteins to display the full functionality necessary for an infection.

The third hypothesis concerns differential gene expression in various milieus. The difference between infection isolates and carriage isolates might be manifested only during actual infection and not under standard in vitro conditions. A seminal example is microbial crowding and quorum sensing via agr (accessory gene regulator), which promotes expression of surface adhesins during the exponential phase of growth and production of exoproteins in the stationary phase [39,40]. Apart from

cell density, *agr* is highly dependent on environmental factors [41]. For example, standard adherence experiments, including our experiments, are conducted under static conditions, whereas flow conditions were shown to have a notable effect on adherence phenotypes [42]. There has been increasing interest in how the regulation via *agr* works *in vivo* [43], and remarkable differences in regulation, and consequently in the expression of virulence factors, between experimental and human infection and in vitro conditions have been found [44-47]. In particular, a recent study showed that the differences between in vitro and in vivo conditions involve the regulation of FnBPA by both the global regulon genes *saeRS* and the *sigB* gene [48]. Moreover, many other regulators, which often interact with agr, have been described (e.g., *sarA*, *sarR*, *sarS*, *sarT*, *sarV*, *sarU*, *sarY*, *rot*, and alternative sigma factors) (for reviews, see references [44] and [43]). Because of the complexity of gene regulation in *S. aureus*, which is not fully understood yet, the differences in regulation, and consequently the differences in expression of virulence factors, undoubtedly present among natural isolates of *S. aureus* cannot be captured at the genetic level. Although in vitro profiles of adherence (in this case adherence to fibrinogen and fibronectin) did not correlate with in vivo infection in humans and experimental endocarditis in rats, the laboratory tests that have been performed cannot capture differential gene expression at the infection site. Hence, appraising the importance of adhesins expression for infection requires direct measurement of the adhesins present in infected tissues compared to the adhesins present under *in vitro* conditions. Such measurement requires more specific molecular approaches, such as in situ hybridization or proteomic techniques (29).

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VIII. Introduction to chapter 3

The virulence of *S. aureus* is essentially determined by cell wall associated proteins and secreted toxins that are regulated and expressed according to growth phases and/or growth conditions.

Chapter 3 describes the results of correlations between three different approaches to study the presence and function of LPXTG-proteins in *S. aureus* Newman, using variants carrying different regulatory mutations and grown in variable conditions. We determined the protein and mRNA expression of the 18 LPXTG-proteins presents in *S. aureus* Newman in time-course experiments and their relation to fibrinogen binding *in vitro*. We used an isogenic mutant of *S. aureus* Newman deleted for *agr* in order to examine the impact of this global regulator on adhésines profiles. Moreover, we made parallel cultures in two different media, one rich in iron and the other poor in iron, to explore the influence of iron on adhésines regulation.

The results indicate first that proteomic, transcriptomic and adherence phenotypes demonstrated different profiles in *S. aureus* Newman. Moreover, peptide released by trypsin treatment for proteomic profiling indicated that exposure of protein domains on the bacterial surface may change in various environments as well as between different strains. This differential surface display may be critical for antibody recognition, and thus might be important to take into account for further vaccine strategies.

CHAPTER 3

Proteomic and transcriptomic profiling of *Staphylococcus aureus* surface LPXTG-proteins: correlation with *agr* genotypes and adherence phenotypes

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I. Abstract

Staphylococcus aureus infections involve numerous adhesins and toxins, which expression depends on complex regulatory networks. Adhesins include a family of surface proteins covalently attached to the peptidoglycan via a conserved LPXTG motif. Here we determined the protein and mRNA expression of LPXTG-proteins of *S. aureus* Newman in time-course experiments, and their relation to fibrinogen adherence *in vitro*. Experiments were performed with mutants in the global accessory-gene regulator (*agr*), surface protein A (Spa) and fibrinogen-binding protein A (ClfA), as well as during growth in iron-rich or iron-poor media. Surface proteins were recovered by trypsin-shaving of live bacteria. Released peptides were analyzed by Liquid-Chromatography coupled to Tandem Mass-Spectrometry (LC-MS/MS). To unambiguously identify peptides unique to LPXTG-proteins, the analytical conditions were refined using a reference library of *S. aureus* LPXTG-proteins heterogeneously expressed in surrogate *Lactococcus lactis*. Transcriptomes were determined by microarrays. Sixteen of the 18 LPXTG-proteins present in *S. aureus* Newman were detected by proteomics. Nine LPXTG-proteins showed a bell-shape *agr*-like expression that was abrogated in *agr*-negative mutants – including Spa, fibronectin-binding protein A (FnBPA), ClfA, iron-binding IsdA and IsdB, immunomodulator SasH, functionally uncharacterized SasD, biofilm-related SasG and methicillin resistance-related FmtB. However, only Spa and SasH modified their proteomic and mRNA profiles in parallel in the parent and its *agr*- mutant, while all other LPXTG-proteins modified their proteomic profiles independently of their mRNA. Moreover, ClfA became highly transcribed and active in fibrinogen-adherence tests during late growth (24h), whereas it remained poorly detected by proteomics. On the other hand, iron-regulated IsdA-B-C increased their protein expression by >10-time in iron-poor conditions. Thus, proteomic, transcriptomic and adherence-phenotype demonstrated differential profiles in *S. aureus*. Moreover, trypsin peptide signatures suggested differential protein domain exposures in various environments, which might be relevant for anti-adhesin vaccines. A comprehensive understanding of the *S. aureus* physiology should integrate all three approaches.

II. Introduction

S. aureus is a highly successful opportunistic pathogen that can produce a wide variety of diseases [1]. Moreover, it has a unique ability to develop antibiotic resistance, which reflects its extraordinary capacity to adapt and survive in a great variety of environments. Over the last decades molecular and genetic dissections of *S. aureus* have revealed a great number of surface adhesins, secreted enzymes, and toxins that might be implicated in pathogenesis [2-5]. In particular, cell-wall-associated surface adhesins – referred to as Microbial Surface Components Recognizing Adherence Matrix Molecules or MSCRAMMs [5] – mediate binding to extracellular matrix and plasma components, enabling staphylococci to colonize and invade host tissues and cells, as well as to escape immune defenses [6-8]. Surface proteins are also implicated in biofilm formation [9], which promotes chronic infections and helps bacteria to escape antibiotic-induced killing.

MSCRAMMs encompass several surface components including proteins, teichoic acids, lipoteichoic acids, and maybe polysaccharidic capsules, which have been implicated in tissue colonization and disease to various extents [5,10,11]. Important surface proteins include polypeptides covalently attached to the peptidoglycan via a conserved anchoring mechanism. After membrane translocation, a transpeptidase called “sortase” cleaves the exported protein at a specific LPXTG motif present at its C-terminus, and attaches its penultimate threonine to a side-chain of the peptidoglycan stem peptides, i.e. a pentaglycine in the case of *S. aureus* [12]. Twenty-one genes encoding LPXTG-proteins have been identified by *in silico* analysis of *S. aureus* genomes [13]. Experimental deletion or heterologous expression of these proteins helped identify their physiological functions and infer their roles in diseases [14,15]. However, while highlighting the multiple facets of *S. aureus* pathogenesis, none of these experiments provided a comprehensive view of the infection process. For instance, none of the gene inactivation experiments could entirely abrogate the *S. aureus* disease capacity, suggesting that infection is a multi-factorial process. Moreover, experimental results may be difficult to interpret, due to the complex regulatory gene network operating in *S. aureus* (e.g. *agr*, *sae*, *srrAB*, *arlS*, *sarA sarR*, *sarS*, *sarT*, *sarV*, *sarU*, *sarY*, and *rot*) [3,16-22]. As an example, the TSST-1 toxin is positively regulated by the “global accessory gene regulator” *agr* when bacteria are grown *in vitro*. However, the *agr*-regulation pathway may become over-ruled by other regulators *in vivo*, as TSST-1 can be expressed in animals even in the absence of *agr* [23].

Several approaches have been used to understand the pathogenic behavior of *S. aureus*. These include genomics, transcriptomics, and more recently proteomics [15,24-29]. In particular, proteomics might provide the most realistic picture of the infective process, since it detects the very end-products of gene biosynthetic pathways, which may eventually determine a biological phenotype. Moreover, post-translational protein regulation (or modification) may affect the stability and function of proteins independently of their upstream transcriptional or translational regulation – e.g. proteins may persist longer than their encoding mRNAs. Therefore, in addition to transcriptional and translational regulation, understanding the behavior of an organism requires both qualitative and quantitative assessment of its protein equipment over time.

Here we describe a semi-quantitative proteomic approach based on trypsin digestion (i.e. trypsin shaving) of surface-exposed proteins and spectral counting of resulting peptides. This technique was applied to time course analysis in order to determine the level of LPXTG-proteins expressed in a variety of conditions known to affect the expression of their corresponding mRNAs. Transcriptomic controls were performed in similar conditions using microarrays. Experimental conditions included mutants inactivated in the global regulator *agr*, which promotes expression of adhesin mRNAs in post-exponential growth phase, and shuts it off in the stationary phase [16,30,31], as well as growth of bacteria in iron-poor or iron-rich media, promoting or repressing the expression of mRNAs of LPXTG siderophore proteins, respectively [32,33]. Eventually, the adherence phenotype to host matrix proteins was determined. The results show that the time course profiles of LPXTG-proteins detected on the bacterial surface do not systematically follow the time course profile of their encoding mRNAs [16,30,31], and that some of these proteins can be functionally very active, e.g. in case of adherence to fibrinogen, in spite of the fact that they are poorly detected *in vitro*. The results also suggest that surface proteins may adopt different conformations and expose different portions on the surface of different bacteria. Indeed, trypsin digestion released different sets of peptides when LPXTG-proteins were expressed in parent *S. aureus* or surrogate *L. lactis*, as exemplified by protein A (Spa), clumping factor A (ClfA), clumping factor B (ClfB) and fibronectin-binding protein A (FnBPA). Our work extends previous proteomic studies in *S. aureus* [27,34,35] and adds a level of subtlety in the continuum of transcriptional to post-translational protein regulation. Notably, the differences in domain exposure in various bacterial backgrounds might have unforeseen implications in vaccine development.

III. Material and methods

III.1. Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table1. Staphylococcal strains included the well-described *S. aureus* Newman, one isogenic mutant (*S. aureus* ALC355) deleted in the global regulator *agr* [36], one isogenic mutant (*S. aureus* DU5873) deleted in the protein A gene (*spa*) [37] and one isogenic mutant (*S. aureus* DU5852) deleted in the clumping factor A gene (*clfA*) [38]. Lactococcal recombinants were used for method validation and included the18 previously described *L. lactis* constructs, each expressing a different staphylococcal LPXTG-protein [39-41] (see below).

Table 1 : strains used in this study

Strains	Relevant genotype or phenotype	References
Lactococcus lactis		
Pil 253	*L. lactis* subsp. *cremoris* 1363 with pIL253 vector	[42]
pOri23-spa	*L. lactis* strain expressing staphylococcal proteinA	[41]
pOri23-clfA	*L. lactis* strain expressing staphylococcal ClfA	[39]
pOri23-clfB	*L. lactis* strain expressing staphylococcal ClfB	[41]
pOri23-fnbpA	*L. lactis* strain expressing staphylococcal FnBPA	[43]
pOri23-fnbpB	*L. lactis* strain expressing staphylococcal FnBPB	[41]
pOri23-isdA (sasE)	*L. lactis* strain expressing staphylococcal IsdA	[41]
pOri23-isdB (sasJ)	*L. lactis* strain expressing staphylococcal IsdB	[41]
pOri23-isdH (sasI)	*L. lactis* strain expressing staphylococcal IsdH	[41]
pOri23-sdrC	*L. lactis* strain expressing staphylococcal SdrC	[41]
pOri23-sdrD	*L. lactis* strain expressing staphylococcal SdrD	[41]
pOri23-sdrE	*L. lactis* strain expressing staphylococcal SdrE	[41]
pOri23-sasD	*L. lactis* strain expressing staphylococcal SasD	[41]
pOri23-sasF	*L. lactis* strain expressing staphylococcal SasF	[41]
pOri23-sasG	*L. lactis* strain expressing staphylococcal SasG	[41]
pOri23-sasH	*L. lactis* strain expressing staphylococcal SasH	[35]
pOri23-sasK	*L. lactis* strain expressing staphylococcal SasK	[41]
pOri23-cna	*L. lactis* strain expressing staphylococcal Cna	[41]
pOri23-pls	*L. lactis* strain expressing staphylococcal Pls	[41]
Staphylococcus aureus		
Newman	ClfA-producing *S. aureus* strain	[44]
DU5873	*S. aureus* Newman defective in proteinA	[37]
ALC355	*S. aureus* Newman defective in *agr*	[36]
DU5852	*S. aureus* Newman defective in ClfA	[38]

Staphylococci were grown at 37°C either in tryptic soy broth (TSB, Becton Dickinson, NJ, USA) or agar, or in Roswell Park Memorial Institute culture medium 1640 (RPMI, Life Technology, NY, USA), without agitation. Lactococci were grown at 30°C in GM17 broth (M17 medium containing 0,5%

glucose, Becton Dickinson) supplemented with 5 µg/ml of erythromycin (Sigma-Aldrich, MO, USA) without agitation, or on GM17 agar.

Growth was followed by colony counting on plates and OD_{600nm} measurements of the different cultures using a spectrophotometer (Ultrospec 500 pro, GE Healthcare, NJ, USA). Bacterial stocks were kept frozen at -80°C in 20% (v/v) glycerol.

III.2. Bacterial cell preparation for proteolysis of surface proteins

Three different protocols were tested including (i) bacterial cell wall purification [35] prior to proteolysis, (ii) bacterial cell wall purification followed by teichoic acid removal with hydrofluoric acid for 48h [45] prior to proteolysis, and (iii) trypsin surface shaving of live bacteria according to a slightly modified described methods [34]. Protocols (i) and (ii) (described in supplemental experimental procedures) appeared too stringent and resulted in the loss of up to 2/3 of the released peptides. Therefore, trypsin-shaving was used and is described here. In brief, bacteria were grown in 300 ml liquid cultures in the different media described above. At various times of the logarithmic or stationary growth phases, samples (between 10 and 100 ml depending on the cell density) were removed, immediately chilled at 4°C, and harvested by centrifugation. Pellets were washed 3-times with ice-cold PBS and finally resuspended in 1ml of the same buffer. To allow semi-quantitative comparisons between the proteomes of different samples, cell concentrations were adjusted to $1x10^9$ bacteria/ml in all samples. Cell counts were validated by optical microscopy (Neubauer cell) and viable colony counts on nutrient agar. There were<0.5 $\log_{10}$ differences between the Neubauer cell and viable counts, indicating that the large majority of cells were alive. Samples were then shaved for 1 h with 1 µg/ml (final concentration) of trypsin (Promega, Madison, USA) at 37°C, after which they were chilled at 4°C and bacterial cells removed by centrifugation for 10 min at 4,000 rpm and 4°C. Supernatants containing trypsin-shaved peptides were filtered (0,22 µm) and freeze-dried until further use.

III.3. Peptide preparation for LC-MS/MS analysis

The freeze-dried shaved peptides were re-diluted in 100 µl of 100 mM ammonium bicarbonate, reduced with 10 µl of 45 mM DTT (Sigma-Aldrich) for 30 min at 60°C, and alkylated with 10 µl of 100 mM IAA (Sigma-Aldrich) for 30 min at RT in the dark. The resultant mixtures were digested a second time at 37°C with 1 µg of trypsin (Promega) for 4h. The digested peptides were desalted through Sep-Pak tC18 cartridges (Waters, MA, USA) following the manufacturer's recommendations and eluted

with 1 ml of 60% (v/v) and 1 ml of 30% (v/V) acetonitrile (Merck, NJ, USA). Solutions of purified peptides were pooled, dried under vacuum, and kept at -20°C.

III.4. Liquid Chromatography-MS/MS analysis and protein identification

Samples were analyzed on a hybrid linear trap LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) interfaced via a TriVersa Nanomate (Advion Biosciences, Norwich, UK) to an Agilent 1100 nano HPLC system (Agilent Technologies, Waldbronn, Germany). Solvents used for the mobile phase were 95:5 H2O:acetonitrile (v/v) with 0.1 % formic acid (solvent A) and 5:95 H2O:acetonitrile (v/v) with 0.1 % formic acid (solvent B).

Solutions of purified peptides were loaded onto a trapping microcolumn ZORBAX 300SB C18 (5 mm x 300 µm ID, 5 µm, Agilent) in H_2O:acetonitrile 97:3 (v/v) + 0.1 % formic acid at a flow rate of 10 µl/min. After 5 min, they were back-flush eluted and separated on a reversed-phase nanocolumn ZORBAX 300SB C18 column (75 µm ID x 15 cm, 3.5 µm, Agilent) at a flow rate of 300 nl/min with a 7-step gradient from 5 to 85 % acetonitrile in 0.1% formic acid as following: 1) 5 min at 0 % of solvent B, 2) from 0 to 25 % of B in 35 min, 3) from 25 to 50 % B in 15 min, 4) from 50 to 90 % in 5 min, 5) 90 % B during 10 min, 6) from 90 to 0 % in 5 min and finally 7) 15 min at 0 % (total time: 90 min).

For spraying, a 400 nozzle ESI Chip (Advion Biosciences) was used with a voltage of 1.65 kV, and the mass spectrometer capillary transfer temperature was set at 200°C. In data-dependent acquisition controlled by Xcalibur 2.0 software (Thermo Scientific), the four most intense precursor ions detected in the full MS survey performed in the Orbitrap (range 350-1500 m/z, resolution 60000 at m/z 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 10’000 counts, carried out at relative collision energy of 35 %, and with isolation width of 4.0 amu. Only precursors with a charge >1 were selected for CID fragmentation and fragment ions were analyzed in the LTQ linear trap. The m/z of fragmented precursors was then dynamically excluded, with a tolerance of 0.01 amu-from any selection during 120 s. From raw files, MS/MS spectra were exported as mgf files (Mascot Generic File, text format) using the extract_msn.exe script from Thermo Scientific.

MS/MS spectra were analyzed using Mascot 2.2 (Matrix Science, London, UK). Mascot was set up to search the UNIPROT database (SWISSPROT + TrEMBL, www.expasy.org) restricted to Other Firmicutes taxonomy (database release used was 13.2 of April 8th 2008, 527’426 sequences after taxonomy filter). For time course experiments, a sub-set database of UniProt was used (2’594

sequences), which contained only proteins of *S. aureus* strain Newman, as well as sequences of *S. aureus* LPXTG-proteins expressed in *L. lactis* clones. Trypsin (cleavage at K,R, not before P) was used as the enzyme definition. Mascot searches were done with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10 ppm. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine, and oxidation of methionine were specified as variable modifications.

Scaffold (version Scaffold_2_06_02, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications, and to perform dataset alignment. Peptide identifications were accepted if they could be established at a probability >90.0% as specified by the Peptide Prophet algorithm [46]. Protein identifications were accepted if they could be established at a probability >95.0% and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [47]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Relative quantification of proteins between samples was based on spectral counting [48]. Spectral counts were normalized by Scaffold (semi-quantitative values) to take into account variations of protein amounts between samples.

III.5. Microarrays

Total RNAs from 2 independent triplicates of 100 ml bacterial cultures of *S. aureus* Newman and its isogenic *agr*- mutant were harvested at OD_{600nm} of 0.2, 0.6, 1.8, and 2.2 by centrifugation at 4000 rpm at 4°C for 10 min and processed as follows. Resuspended bacterial cells were first lysed in 100µl TE containing 800 µg/ml lysostaphin (Sigma-Aldrich, Saint Louis, USA) for 1h at room temperature. Total RNA were further purified and stabilized using the RNeasy Protect Bacteria mini kit (Qiagen) following the manufacturer's recommendations. All RNA quantities were assessed by NanoDrop®ND-1000 spectrophotometer and the RNA quality was assessed using RNA 6000 NanoChips with the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, USA). Triplicates were equitably pooled to obtain at least 10 µg of RNA. For each sample, 10µg of total RNA were reverse transcribed using dUTP for enzymatic fragmentation; 2µg of the resulting sense cDNA was fragmented by UDG (uracil DNA glycosylase) and APE 1 (apurinic/apyrimidic endonuclease 1) and biotin-labelled with TdT (terminal deoxynucleotidyl transferase) using the GeneChip® WT Terminal labelling kit (Affymetrix Cat.no. 900671, Santa Clara, USA). Affymetrix GeneChip *S. aureus* Genome Array (Affymetrix, Cat.

No. 900514) were hybridized with 1.8 µg of biotinylated target, at 45°C for 16 hours washed and stained according to the protocol described in Affymetrix GeneChip® Expression Analysis Manual (Fluidics protocol FS450_0007).

The arrays were scanned using the GeneChip® Scanner 3000 7G (Affymetrix) and raw data was extracted from the scanned images and analyzed with the Affymetrix Power Tools software package (Affymetrix).

All statistical analyses were performed using the free high-level interpreted statistical language R and various Bioconductor packages (http://www.Bioconductor.org). Hybridization quality was assessed using the Expression Console software (Affymetrix). Normalized expression signals were calculated from Affymetrix CEL files using RMA normalization methods. Differential hybridized features were identified using Bioconductor package "limma" that implements linear models for microarray data [49]. The *p* values were adjusted for multiple testing with Benjamini and Hochberg's method to control the false discovery rate (FDR). Probe sets showing a FDR < 0.05 were considered significant.

III.6. Bacterial adherence to solid-phase extracellular matrix compounds

We used a previously described *in vitro* adherence assay to measure the ability of *S. aureus* to adhere to increasing concentrations of surface-adsorbed fibrinogen, fibronectin, and collagen [50]. Briefly, 96-well plates (Nunc-Immuno plates; MaxiSorp surface; Thermo Fisher Scientific) were filled with 100 µl of 2-fold serial dilutions of fibrinogen (1 mg/ml initial concentration; Sigma-Aldrich), fibronectin (250 µg/ml initial concentration; Sigma-Aldrich) and collagen I and VI (20 µg/ml initial concentrations; Sigma-Aldrich). The last well served as a negative control and was filled with 100 µl of PBS without ligand. After washing, bovine serum albumin (Sigma-Aldrich) was added to each well to block non-specific binding sites. Bacterial cultures were harvested at different times during growth by centrifugation (4000 g at 4°C for 20 min). Cells were re-suspended in PBS and bacterial cell concentrations were adjusted to 5.10^9 CFU/ml. Fifty microliters (i.e. $2,5.10^8$ cells) were added to each well. Plates were incubated for 1.5 h at 37°C, after which wells were washed with PBS and fixed at 55°C. Adherent bacteria were detected by staining with crystal violet, and the OD_{570nm} was determined with an enzyme-linked immunosorbent assay plate reader [50].

IV. Results

IV.1. Trypsin-shaving of live cells

As mentioned in Experimental procedures, initial attempts to recover LPXTG-proteins from purified staphylococcal cell walls resulted in too much contamination with non-wall proteins, and/or poor recovery of LPXTG-proteins (described in supplemental Experimental procedures). In contrast, trypsin-shaving decreased contamination with non-wall proteins by ≥5 times and reproducibly released similar sets of peptides from individual LPXTG-proteins. Figure 1 indicates that the recovery of peptides during trypsin treatment of live staphylococci was time-dependent, and that 1 h of treatment appeared optimal. This duration was experimentally amenable for serial extractions during time course experiments and thus was used in all subsequent experiments. The decrease in peptide recovery after longer incubation periods is not explained, but could be due to concomitant proteolysis by intrinsic *S. aureus* proteases (see Discussion section).

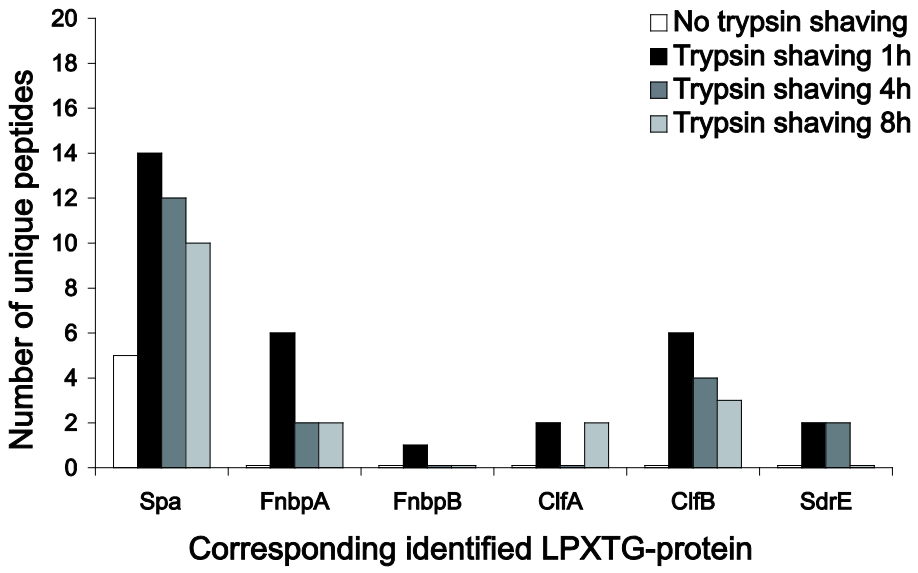


Figure 1: Kinetics of unique peptides released during trypsin-shaving of *S. aureus* Newman. Liquid cultures of *S. aureus* Newman were grown to the early stationary growth phase, harvested by centrifugation, and bacteria were shaved with 1 µg/ml of trypsin for various periods of time (1h, 4h and 8h). Released peptides were processed as described in Experimental procedures and identified by LC-MS. Studied LPXTG-proteins are indicated at the bottom of the graph. Columns indicate the numbers of unique peptides (nonredundant) recovered. Since a maximum of unique peptides were detected after 1h, this incubation time was further used in all experiments of trypsin-shaving.

IV.2. Construction of a reference peptide library of *S. aureus* LPXTG-proteins expressed in lactococci

An important prerequisite to this study was to unambiguously identify the trypsin peptide signatures of *S. aureus* LPXTG-proteins using the LC-MS/MS system described herein. This was achieved thank to a preliminary analysis of each of these proteins expressed in *L. lactis*, which does not carry *S. aureus* proteins [35]. This permitted to alleviate certain ambiguities regarding to different adhesin denominations in UniProt, due to redundancies or isoforms. It also allowed verifying if an observed set of peptides could be attributed to a unique parent protein, or whether peptides were found in different proteins showing sequences similarities.

Table 2: Number of unique peptides released after trypsin-shaving of the surface of *L. lactis* strains expressing *S. aureus* recombinant LPXTG-proteins.

		Lactococci recombinant expressing staphylococcal LPXTG-proteins																	
		Spa	ClfA	ClfB	FnbpA	FnbpB	IsdA	IsdB	IsdH	SdrC	SdrD	SdrE	SasD	SasF	SasG	SasH	SasK	Cna	Pls	Pil
Number of unique peptides released from single LPXTG proteins	Spa	17																	3	
	ClfA		9																	
	ClfB			20																
	FnbpA				10															
	FnbpB					5														
	IsdA						4													
	IsdB							0												
	IsdH							4	28											
	SdrC									20										
	SdrD										49	3								
	SdrE									3	4	59								
	SasD												5							
	SasF													13						
	SasG														20					
	SasH															0				
	SasK																5			
	Cna																	3		
	Pls																		8	

The presence of two numbers for certain lactococci expressing a single LPXTG-species (i.e. SdrC, SdrD, and SdrE) indicates the existence of redundant peptides that are shared with other LPXTG-species.

To address these questions, we reinvestigated the 18 lactococcal clones successfully expressing unique *S. aureus* MSCRAMMs (Table 1) [35] using the shaving technique, and assigned the obtained peptides to the corresponding proteins. Table 2 shows that the shaving procedure generated sets of peptides (between 3 and 59 peptides) for 16 out of the 18 LPXTG-proteins studied. The great majority of the detected peptides was specifically assignable to a unique parent LPXTG-protein (Table 2). Only few peptides were redundant between more than one protein species, for instance between IsdB and IsdH, Spa and Pls, and between SdrD, SdrE, and SdrC. No peptides were detected for IsdB and SasH when expressed in lactococci (Table 2). Possible explanations could be either poor expression in this particular organism, or poor detectability of these peptides by LC-MS/MS.

This allowed constructing a concordance table between UniProt protein nomenclatures (shown in Table 3) as well as a dedicated sequence database specific for *S. aureus* Newman (supplementary Table 1). Specifically, Table 3 also presents the number of unique peptides and the percentage of peptide coverage of each of the LPXTG-proteins detected in recombinant *L. lactis*. Coverage varied from 6% to 60% (median 31%).

Table 3 : UniProt nomenclature, numbers of unique peptides, and sequence coverage of LPXTG wall-associated protein obtained in lactococcal recombinants shaving experiments

Protein Name	Accession Number	UniProt Protein Name	Number of uniques peptides	Sequence Coverage (%)
ClfA	A3F6G7_STAAU	Clumping factor A	9	31
	A3F6G8_STAAU	Clumping factor A		
ClfB	CLFB_STAAE	Clumping factor B precursor - Newman	20	41
	A5IW57_STAA9	LPXTG-motif cell wall anchor domain precursor - JH9		
	A6U514_STAA2	LPXTG-motif cell wall anchor domain precursor - JH1		
	A7X714_STAA1	Clumping factor B - Mu3 / ATCC 700698		
	CLFB_STAA8	Clumping factor B precursor - NCTC 8325		
	CLFB_STAAC	Clumping factor B precursor – COL		
	CLFB_STAAM	Clumping factor B precursor - Mu50 / ATCC 700699		
	CLFB_STAAN	Clumping factor B precursor - N315		
FnBPA	FNBA_STAA8	Fibronectin-binding protein A precursor - NCTC 8325	10	19
FnBPB	A8YYQ2_STAAT	Fibronectin-binding protein B - USA300 / TCH1516	5	15
	Q2FE04_STAA3	Fibronectin binding protein B - USA300		
	Q2G1T5_STAA8	Fibronectin binding protein B, putative - NCTC 8325		
	Q53682_STAAU	Fibronectin binding protein B		
	Q5HD53_STAAC	Fibronectin binding protein B - COL		
Cna	A2PZA0_STAAU	Collagen adhesion	3	6
	Q6GDB2_STAAR	Collagen adhesin - MRSA252		
Pls	PLS_STAAC	Putative surface protein SACOL0050 precursor – COL	8	8
	PLS_STAAU	Surface protein precursor		
	Q9LC00_STAAU	Putative uncharacterized protein		
SasD	A6QDB8_STAAE	Putative uncharacterized protein – Newman	5	39
	A8YZ60_STAAT	Cell wall surface anchor protein - USA300		
	Q2FKC5_STAA3	Cell wall surface anchor family protein - USA300		
	Q2G260_STAA8	Putative uncharacterized protein - NCTC 8325		
	Q2YUU8_STAAB	Surface protein - bovine RF122		
	Q5HJN4_STAAC	Cell wall surface anchor family protein - COL		
IsdA	ISDA_STAAE	Iron-regulated surface determinant protein A precursor – Newman	4	12
	A5IS16_STAA9	LPXTG-motif cell wall anchor domain precursor - JH9		
	A6U0U7_STAA2	LPXTG-motif cell wall anchor domain precursor - JH1		
	A7X148_STAA1	Cell surface protein - Mu3 / ATCC 700698		
	A8Z1R0_STAAT	Iron - USA300 / TCH1516		
	ISDA_STAA3	Iron-regulated surface determinant protein A precursor - USA300		
	ISDA_STAA8	Iron-regulated surface determinant protein A precursor - NCTC 8325		
	ISDA_STAAC	Iron-regulated surface determinant protein A precursor – COL		
	ISDA_STAAM	Iron-regulated surface determinant protein A precursor - Mu50 / ATCC 700699		
	ISDA_STAAN	Iron-regulated surface determinant protein A precursor - N315		
	ISDA_STAAS	Iron-regulated surface determinant protein A precursor - MSSA476		
	ISDA_STAAW	Iron-regulated surface determinant protein A precursor - MW2		
SasF	A6QKD5_STAAE	Putative uncharacterized protein – Newman	13	26
	Q6G628_STAAS	Putative surface anchored protein - MSSA476		
	Q8NUK1_STAAW	Putative uncharacterized protein MW2567 - MW2		
	Q6T1N1_STAAU	Surface protein SasF		
SasG	A6QJY2_STAAE	Putative uncharacterized protein - strain Newman	20	16
	Q5HD57_STAAC	Cell wall surface anchor family protein - strain COL		
	SASG_STAA8	Surface protein G precursor - strain NCTC 8325		
IsdH	A6QHR4_STAAE	Haptoglobin-binding surface anchored protein – Newman	28	47
	A8Z2P9_STAAT	Cell wall surface anchored protein - USA300 / TCH1516		
	ISDH_STAA3	Iron-regulated surface determinant protein H precursor - USA300		
	ISDH_STAAC	Iron-regulated surface determinant protein H precursor - COL		

Protein Name	Accession Number	UniProt Protein Name	Number of uniques peptides	Sequence Coverage (%)
IsdB	ISDB_STAAE	Iron-regulated surface determinant protein - Newman	0	0
SasK	A7X6X3_STAA1	Putative uncharacterized protein - Mu3 / ATCC 700698	5	31
	Q7A3B0_STAAN	Putative uncharacterized protein SA2381 - N315		
	Q99R43_STAAM	Putative uncharacterized protein - Mu50 / ATCC 700699		
SdrC	SDRC_STAAE	Serine-aspartate repeat-containing protein C precursor - Newman	20	31
	SDRC_STAA3	Serine-aspartate repeat-containing protein C precursor - USA300		
	SDRC_STAA8	Serine-aspartate repeat-containing protein C precursor - NCTC 8325		
	SDRC_STAAC	Serine-aspartate repeat-containing protein C precursor - COL		
	A8YZQ9_STAAT	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrC - USA300 / TCH1516		
SdrD	SDRD_STAAE	Serine-aspartate repeat-containing protein D precursor - strain Newman	49	53
	A8YZR0_STAAT	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrD - USA300 / TCH1516		
	SDRD_STAA3	Serine-aspartate repeat-containing protein D precursor - strain USA300		
	SDRD_STAA8	Serine-aspartate repeat-containing protein D precursor - NCTC 8325		
	SDRD_STAAC	Serine-aspartate repeat-containing protein D precursor - COL		
SdrE	A8YZR1_STAAT	Ser-Asp rich fibrinogen/bone sialoprotein-binding protein SdrE - USA300 / TCH1516	59	60
	SDRE_STAA3	Serine-aspartate repeat-containing protein E precursor - USA300		
Spa	A6QD95_STAAE	Immunoglobulin G binding protein A – Newman	17	42
	A1KDX8_STAAU	Immunoglobulin G binding protein A		
	A1KDY8_STAAU	Immunoglobulin G binding protein A		
	A1KE04_STAAU	Immunoglobulin G binding protein A		
	A1KE29_STAAU	Immunoglobulin G binding protein A		
	A1KE57_STAAU	Immunoglobulin G binding protein A		
	A1KE58_STAAU	Immunoglobulin G binding protein A		
	A1KE61_STAAU	Immunoglobulin G binding protein A		
	A1KE85_STAAU	Immunoglobulin G binding protein A		
	A1KEA2_STAAU	Immunoglobulin G binding protein A		
	A4L7T5_STAAU	Immunoglobulin G binding protein A precursor		
	A5INY2_STAA9	LPXTG-motif cell wall anchor domain precursor - JH9		
	A6TXP6_STAA2	LPXTG-motif cell wall anchor domain precursor - JH1		
	A7XW80_STAAU	Immunoglobulin G binding protein A		
	A8YZ36_STAAT	Immunoglobulin G binding protein A - USA300 / TCH1516		
	Q2FKE8_STAA3	Immunoglobulin G binding protein A - USA300		
	Q2UW16_STAAU	Immunoglobulin G binding protein A		
	Q2UW21_STAAU	Immunoglobulin G binding protein A		
	Q2UW30_STAAU	Immunoglobulin G binding protein A		
	Q2UW31_STAAU	Immunoglobulin G binding protein A		
	Q2UW33_STAAU	Immunoglobulin G binding protein A		
	Q2UW54_STAAU	Immunoglobulin G binding protein A		
	Q2UW59_STAAU	Immunoglobulin G binding protein A		
	Q5HJQ8_STAAC	Immunoglobulin G binding protein A – COL		
	Q6GD14_STAAS	Immunoglobulin G binding protein A - MSSA476		
	Q8NYT0_STAAW	Immunoglobulin G binding protein A - MW2		
	SPA1_STAA8	Immunoglobulin G-binding protein A precursor - NCTC 8325		

IV.3. Profiling of LPXTG-proteins in *S. aureus* Newman and its *agr*-mutant in various growth conditions

Time course profiling of the surface proteome of *S. aureus* was performed during growth from early logarithmic (OD_{600nm} = 0.2) to late stationary (OD_{600nm} = 2.2) phases (Figure 2). At each time point, the proteomic analysis assessed the relative quantity of proteins in $1x10^9$ bacterial cells. This semi-quantification was based on spectral counting [48] normalized to take into account the variations of protein amounts between samples (n = 3 to 4). Spectral counting measures the number of times that a peptide is selected for fragmentation during a LC-MS/MS analysis, and is correlated to abundance of specific peptides and proteins [48]. As dynamic exclusion has a direct impact on

spectral counting, a value of 120 s was chosen as a compromise between redundant peptide fragmentations requested for better quantitation accuracy and the need of selecting low abundant peptides for a higher proteome coverage.

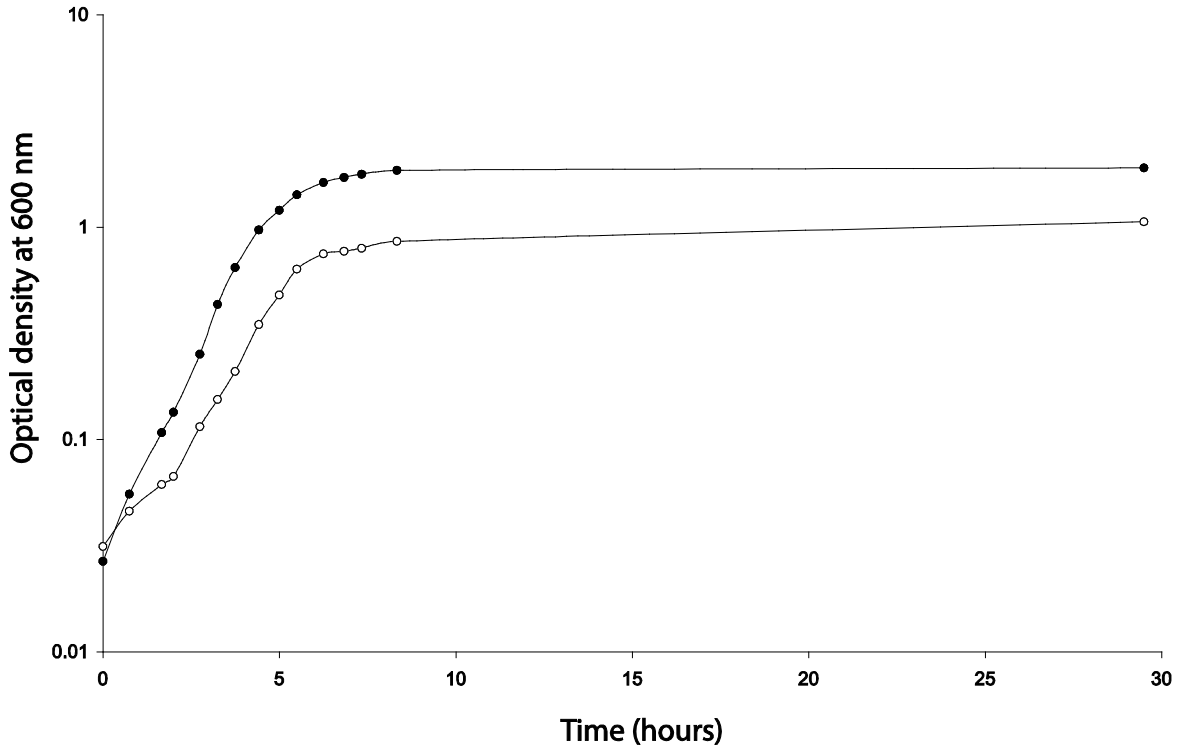


Figure 2: Growth curves or S. aureus Newman in iron-rich (TSB) or iron-poor (RPMI) liquid media. Bacteria were grown at 37°C without agitation in in either TSB (closed circles) of RPMI (open circles).and optical densities at a wavelength of 600nm (OD600nm) were followed. Samples were removed at OD600nm values of 0.2; 0.6; 1.8; and 2.2 in TSB and 0.2; 0.5; 0.8 and 1.2 in RPMI, and then processed for proteomic and transcriptomics analysis, as shown in Figure 3 and 4 and for adherence phenotype in Figure 5.

IV.3.1. Overall protein profiling:

Figure 3 depicts the results obtained for the wild-type *S. aureus* Newman and its *agr*- mutant grown either in iron-rich TSB (Figure 3A and 3C, respectively) or iron-poor RPMI (Figure 3B and 3D, respectively). Overall, 16 of the 21 putative LPXTG-proteins described in *S. aureus* [13] were successfully identified in our tests. Three (i.e. Cna, Pls and SasK) of the 5 undetected species had no gene counterparts in the genome of *S. aureus* Newman (GenBank Accession Number AP009351) [51] and thus were not expected to be found, and 2 (Srap and SasC) remained undetected, maybe because of poor detectability of their corresponding peptides by LC-MS/MS. As a negative control, no Spa was detected at the surface of the *spa*-negative mutant DU5873, while the profile of the other LPXTG-proteins remained unaffected in this mutant (data not shown).

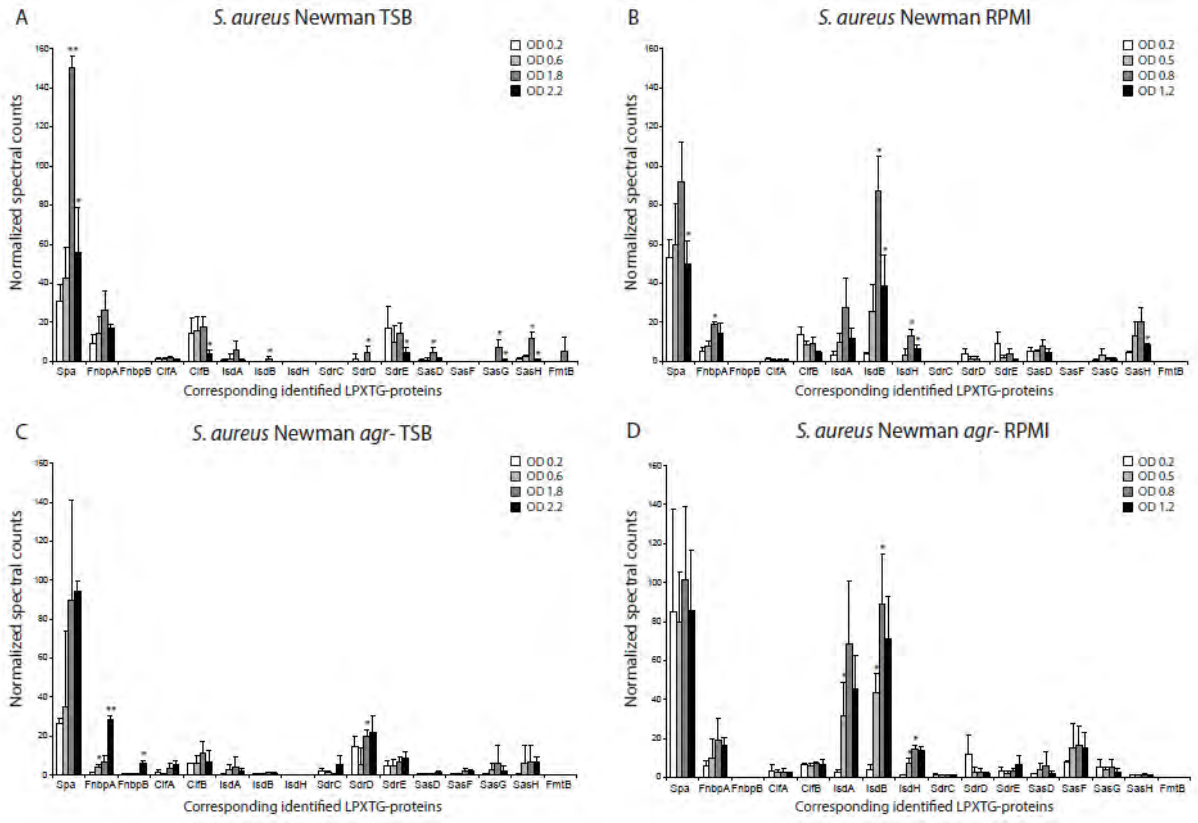


Figure 3: Semi-quantitative assessment of *S. aureus* Newman LPXTG proteins in time course experiments. The parent *S. aureus* Newman and its *agr*-negative mutant were grown in TSB (A and C) or RPMI (B and D) liquid medium. Samples were collected at four different time points during the exponential and stationary growth phases, adjusted to $1x10^9$ bacterial cell/ml, shaved for 1h with trypsin, and peptides released from LPXTG-proteins were quantified by LC-MS as described in Experimental procedures. Columns and error bars indicate the mean $\pm$ SD of normalized spectral counts of 3 to 4 independent experiments for each detected protein. Statistical analysis was performed by pairwise comparisons with Student's t test and asterisks above columns indicate significant differences with the sample collected at the previous time point (*, $p<0.05$; **, $p<0.001$).

IV.3.2. Effect of *agr*:

Figure 3 indicates that the amounts of several LPXTG-proteins depended on *agr* integrity and growth conditions. When wild type *S. aureus* Newman was grown in TSB, 9 of the 16 LPXTG-proteins (i.e. Spa, FnBPA, ClfA IsdA, IsdB, SasD, SasG, SasH and FmtB) showed a time-dependent *agr*-like bell-shape expression, with an increase in abundance during late logarithmic growth followed by a decrease up to the late stationary phase (Figure 3A). Although there were some variations between individual adhesins, as well as few relatively unexpected findings (e.g. poor detection of ClfA, see below), such a time-dependent expression pattern is in general agreement with proteins regulated by *agr* [16,30,31]. On the other hand, ClfB was present quite early during growth, and was rather stable until it disappeared in late stationary phase, and SdrD and SdrE reproducibly presented a biphasic

expression pattern. Iron-regulated IsdH was not detected in TSB grown staphylococci, most probably because this rich medium provides ample iron for growth (see below).

Strikingly, this *agr*-like pattern was abrogated when the isogenic *agr*-negative mutant of *S. aureus* Newman was tested in similar conditions (Figure 3C). In this case, the bell-shape pattern was replaced by a continuing increase of protein quantities into the late stationary phase for all nine proteins mentioned above. Moreover, several proteins became either detectable or became more expressed in the *agr* mutant, including FnBPB, ClfA, SdrC, SdrE, SasF, and SasG. This is compatible with the loss of *agr*-mediated down-regulation of surface protein synthesis during stationary growth phase [16]. Besides, some protein decreased (e.g. FmtB), while the atypical patterns of ClfB, SdrD and SdrE persisted.

IV.3.3. Effect of iron:

The experiments were repeated in the iron-poor medium RPMI. Of note, the growth rate of strains was substantially slower than in TSB (Figure 2). Nevertheless, when wild type *S. aureus* Newman was tested in this condition (figure 3B), the global expression profile was very similar to that in TSB, except for the sharp increase in iron-regulated surface determinants IsdA, IsdB and IsdH [32,33]. The IsdC determinant of the iron-capturing system was not analyzed herein. IsdC is processed by Sortase B and associated to the peptidoglycan via a NPQTN module, and its expression should increase as well [32,33]. Aside from these major changes, minor differences were also observed, notably increase in the presence of iron of SasD and SasH, and decrease of SdrE (Figure 3A and 3B).

When the isogenic *agr*-negative mutant was examined in RPMI (figure 3D), the loss of the *agr* bell-shape pattern was much less striking than in TSB. Nevertheless, some obvious modifications occurred such as a significant ($p < 0.05$) increase of the detection of SasF and a decrease for SasH.

IV.4. Transcriptome analysis

In order to assess the relationships between the profiles of time course expression of LPXTG-proteins and their mRNAs, we determined the parallel time course transcriptomes of the parent *S. aureus* Newman and its *agr*- mutant grown in TSB. Transcriptomic results indicated that the two organisms segregated very well at the level of their global transcriptomes. In addition, all duplicated

microarray experiments clustered together, using all 7668 Probe sets, indicating high reproducibility and consistency of the data (supplemental Figure 1). Figure 4 presents the dynamics of the relative changes in mRNA amounts for specific transcripts in the parent *S. aureus* Newman and its *agr*- mutant. Note that these are relative changes – not absolute mRNA quantities – with regard to a basal value arbitrarily fixed at 1 for the first time point of the growth curve, i.e. at OD_{600nm} = 0.2. Therefore, the relative dynamics of proteomic and transcriptomic profiles (Figure 3 and 4, respectively) can be compared.

Considering *agr*-related genes, the *agr*+ parent demonstrated a linear increase (by 1.6 fold) of the RNAIII transcript over the whole growth duration. Conversely, the *agr*- mutant did not show any hybridization to the structural genes of the *arg* locus (i.e. *argA*, *agrB*, *argC* and *argD*), as well as a >300 fold decrease in hybridization to RNAIII and hemolysin δ as compared to the parent strain (supplemental Table 2). Thus, the transcription of *agr* was genuinely silenced in the mutant (supplemental Table 2). As an additional control, the mRNA of the gene of protein A (*spa*), which is typically regulated by *agr*, followed an *agr*-like bell-shape profile in the parent strains whereas this profile was flattened in the mutant (figure 4), as previously described [30]. One additional LPXTG-protein genes *sasH* adopted an *agr*-like bell-shape pattern in the parent, which was modified in the *arg*- mutant. Moreover, the transcription of *sasD* and *fmtb* showed statistically significant modifications (p<0.001) in the late growth phase, i.e. at OD_{600nm} of 2.2, in the *agr*- mutant. Of note, the transcript of *clfA* showed a sharp increase in the late growth phase, i.e at OD_{600nm} of 1.8 (p<0.001) and 2.2 (p<0.05), in both the parent and the *agr*- mutant (Figure 4), an observation that comes in support to recent observations of *clfA* regulation using the RNAseq technology [52]. Thus, at least four of the LPXTG-protein mRNAs (*spA*, *sasD*, *sasH* and *fmtB*) showed clear modifications between the *agr*+ and *agr*- strains, and two of them (*spA* and *sasH*) had a clear *agr*-like profile. On the other hand, most of the other LPXTG-protein genes adopted various mRNA profiles that were essentially not affected by inactivation of *agr*.

When comparing mRNA and proteomic profiles, the expression of Spa clearly followed an *arg*-like profile at both the transcriptional and translational levels, which was abrogated in the *agr*- mutant. SasH followed a relatively similar pattern. On the other hand, FnBPA and to a lesser extend FnBPB, ClfA, ClfB, IdsB, SdrC SdrE, and SasF modified their protein expression patterns between the two mutants, but not their mRNA profiles. Only SasG did not modify its proteins and mRNA profiles in both

parent strains. Therefore, while the transcriptome profile was remarkably predictive of the LPXTG-protein profile in some cases, it appears that additional factors were interfering with the physical presence – or access to trypsin – of several adhesins at the bacterial surface. Of note, Srap was detected at the transcriptional but not at the protein level.

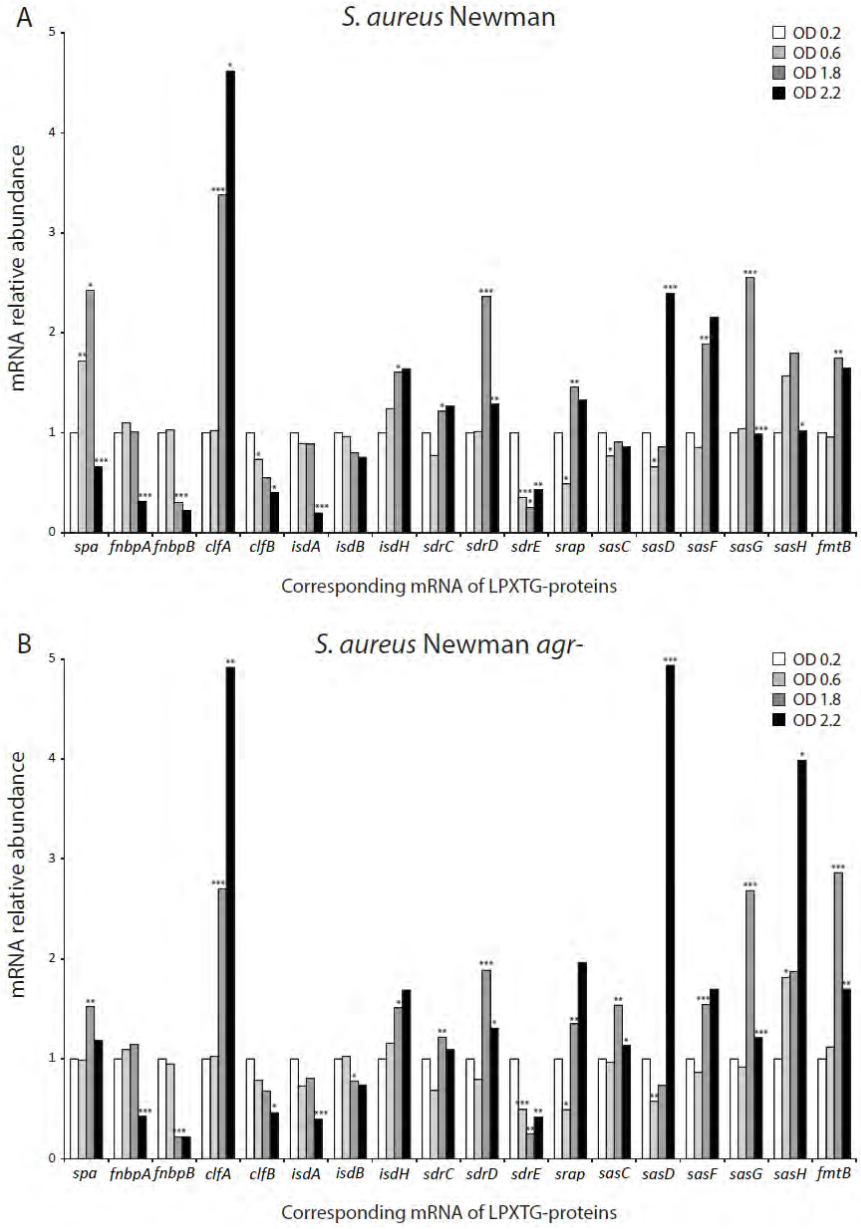


Figure 4: Expression profiles of mRNA from LPXTG-proteins in time course experiments. The parent *S. aureus* Newman and its *agr*- mutant were grown in TSB, and harvested at four different time-points (i.e. at OD_{600nm} of 0.2, 0.6, 1.8 and 2.2,) before being processed for RNA extraction. The transcriptomes were analyzed by microarray as described in Experimental procedures. The amounts of mRNA at the different time points are represented as fold changes compared to a value arbitrarily fixed at 1 for the first time point (i.e. at OD_{600nm} = 0.2). Thus, all measures are reported as relative values. The results represent the mean of $\geq$ 2 determinations on 2 separate chips, with relative variations between individual values of $\leq$ 15%. Asterisks above the columns indicate that the values are statistically significantly different (*p<0.05; ** p<0.01 and *** p<0.001) from the previous time point. *P* values were adjusted for multiple testing with Benjamini and Hochberg's method to control the false discovery rate (FDR).

IV.5. Correlation between proteomic expression profiles and *in vitro* adherence phenotypes

Figure 3A shows that 9/16 LPXTG proteins detected in *S. aureus* Newman (i.e. Spa, FnBPA, ClfA, IsdA, IsdB, SasD, SasG, SasH and FmtB) followed an *agr*-like expression pattern. Therefore, we tested whether the *in vitro* adherence profile of this organism followed a similar pattern when grown in the same conditions. Figure 5 indicates that adherence to fibrinogen adopted differential profiles depending on both the growth medium and the presence or not of an intact *agr*.

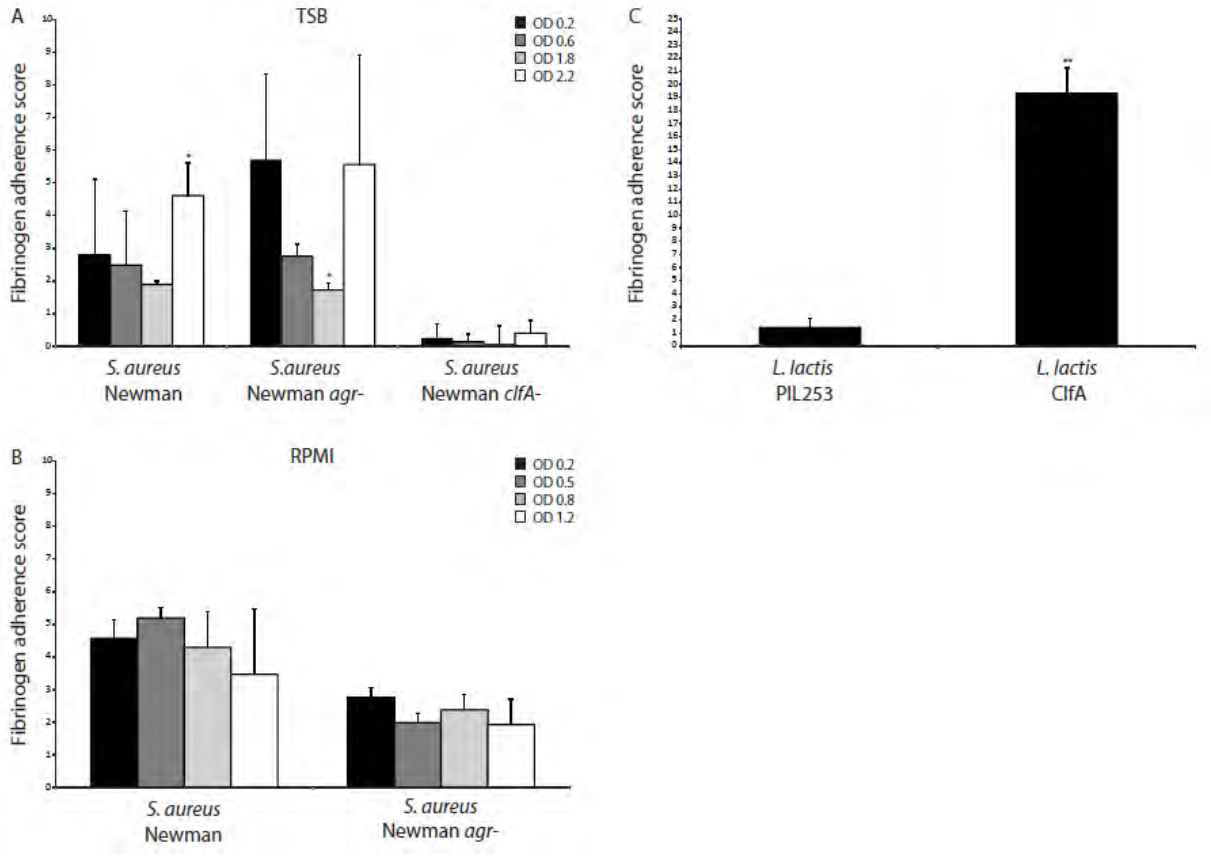


Figure 5: Adherence of parent *S. aureus* Newman and its *agr*-negative mutant to immobilized fibrinogen. The parent *S. aureus* Newman, its *agr*-negative mutant and a *clfA*-negative (but *agr*+) mutant were grown in TSB (A) or RPMI (B). Samples were removed at various times during the exponential and stationary growth phases (OD_{600nm} values are indicated on the graph; last sampling time was 24h), titrated to identical concentrations of cell bodies, and tested for their ability to stick to immobilized fibrinogen. Negative and positive controls included *L. lactis* carrying the empty expression vector Pil253 and *L. lactis* carrying the same vector expressing ClfA, respectively (C). Columns and error bars indicate the mean $\pm$ and SD of 3 independent determinations for each isolate. Statistical analysis was performed by pairwise comparisons with Student's t test: asterisks above columns indicate significant differences with the previous sample (*, $p<0.05$; **, $p<0.001$).

In TSB (Figure 5A), binding of the parent *S. aureus* Newman to fibrinogen was more pronounced during exponential growth and decreased in the early stationary phase, thus obeying an *agr*-like pattern. As a control, the *clfA*- mutant was virtually unable to bind to immobilized fibrinogen, indicating that ClfA was largely responsible of the phenotype. The early stationary phase drop was even more pronounced in the *arg*- mutant, in spite of the fact that adhesins are supposed to be more expressed during late growth in the *agr*- mutant [16,30,31]. Finally, adherence increased again after 24 h in both strains, a phenotype that did not correlate with the proteomic detection of ClfA (Figure 3), but correlated well with the *clfA* gene transcription profile (Figure 4). In consequence, ClfA-mediated binding to fibrinogen did not strictly follow an *agr* pattern in these experimental conditions, and senescent bacteria were still able to bind fibrinogen to a substantial extent.

Conversely, binding to fibrinogen adopted an *agr*-like bell-curve in RPMI (Figure 5B) and this bell-curve was abrogated in the *agr*-inactivated mutant. Besides, binding to fibronectin and collagens was quasi null (data not presented), which is coherent with the fact that in *S. aureus* Newman, the genes encoding for fibronectin binding and collagen binding are either truncated (for *fnA* and *fnB*) or absent (for *cna*).

IV.6. Trypsin releases different sets of peptides from LPXTG-proteins expressed on the surface of *S. aureus* or *L. lactis*

S. aureus had a much lower adherence score to fibrinogen than ClfA-positive *L. lactis* in *in vitro* adherence tests (Figure 5). This difference could result from a lower expression of ClfA on the surface of *S. aureus* than on the surface of *L. lactis*, or from differences in the accessibility to ClfA-binding domains when the protein is exposed on the surface of *S. aureus* versus *L. lactis*, or from both reasons.

Figure 6 compares the ClfA, ClfB, Spa and FnBPA-specific sets of peptides released by trypsin shaving of the surface of recombinant lactococci or *S. aureus* Newman. In case of ClfA, 9 peptides were released from lactococci expressing ClfA and 8 from the surface of *S. aureus*. Thus, the peptides numbers were quite similar. However, among these, three peptides were specific of lactococci and 2 were specific of *S. aureus*. Therefore, while the majority of the released peptides were similar (i.e. 6/9 in lactococci and 6/8 in *S. aureus*), some were specific of the host bacteria, suggesting that different

portions of the protein were accessible to trypsin digestion on the surface of the two microorganisms. Details on these peptides are presented in supplemental Table 1.

This small difference in peptide numbers was also true for ClfB and Spa (Figure 6). Regarding to ClfB, amongst 20 and 21 released peptides 3 and 4 were specific for *L. lactis* and *S. aureus*, respectively. For Spa, amongst 23 and 29 released peptides, 0 and 5 were specific for *L. lactis* and *S. aureus*, respectively. Moreover, in these cases, some peptides, which were recovered in the same LC-MS runs, displayed redundancies between partial and complete hydrolysis (see overlapping black boxes in Figure 6). Partial hydrolysis could result from a too short duration of trypsin digestion. However, extending the length of digestion to more than 1h did not yield more peptides (Figure 1). Therefore, partial trypsin hydrolysis of LPXTG-proteins might depend on other factors, possibly including protein conformation and trypsin accessibility.

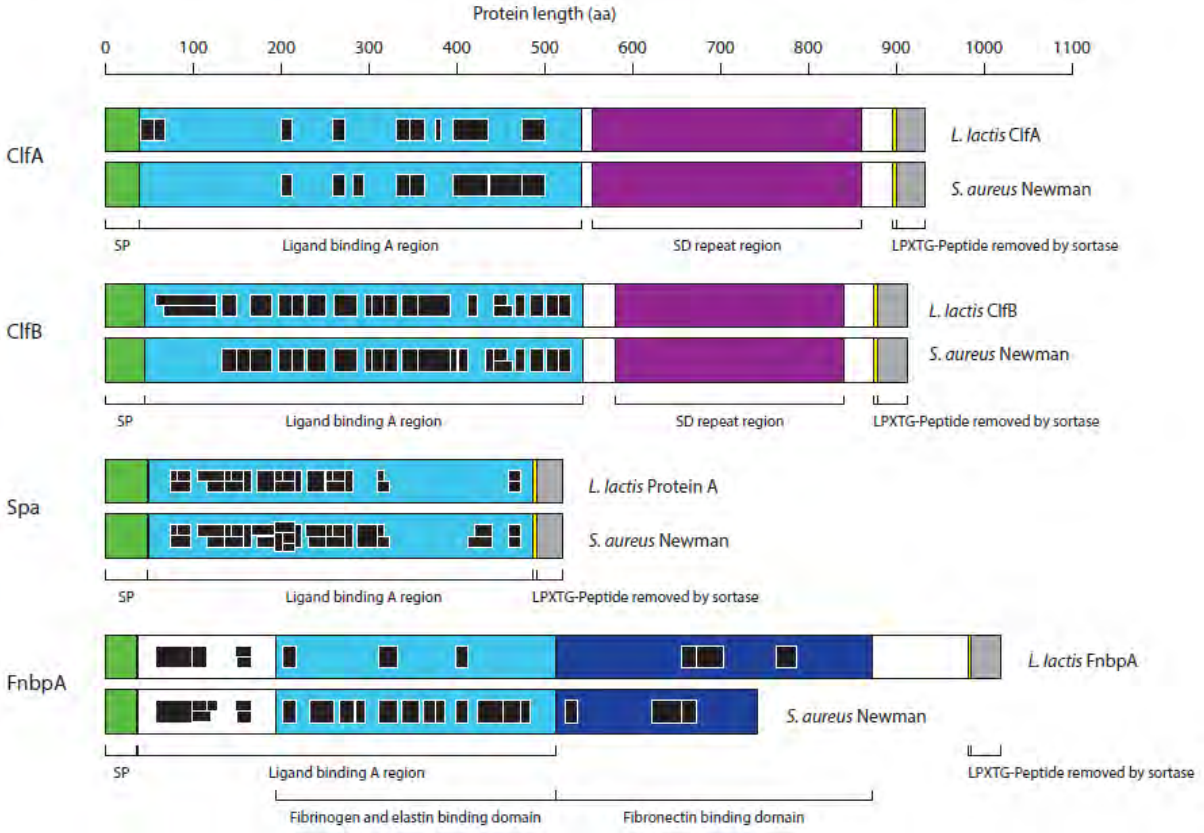


Figure 6: Comparison of the sets of peptides released by trypsin digestion of several LPXTG-proteins expressed on the surface of recombinant *L. lactis* or *S. aureus* Newman. Sets of unique peptides of ClfA, ClfB, Spa, or FnBPA released by trypsin shaving of recombinant *L. lactis* or *S. aureus* Newman are shown. Data were extracted from the experiments presented in Table 2 and Figure 3. Major protein domains and gross amino acid numbering are indicated. Precise amino acid numbering of the peptides is presented in supplemental Table 1. Trypsin-released peptides are represented by the inserted black boxes. Note that some peptides displayed both completely and partially digested species simultaneously (indicated by overlapping boxes). LPXTG motifs are indicated by thin yellow bars and the peptide removed by sortase in grey. SP stands for signal peptides, which are indicated as green boxes. The ligand binding domains are highlighted in blue and SD repeat regions in purple.

Finally, an unexpected observation was that twice as many peptides were released from FnBPA expressed in *S. aureus* than from FnBPA-positive *L. lactis* (18 versus 9 peptides, respectively). This observation is interesting since the *fnbpA* gene of *S. aureus* Newman carries a stop codon, which leads to a premature arrest of the transcription and the translation of a protein devoid of the C-terminal LPXTG anchoring domain. Hence, this truncated protein could be free-floating in the cell envelope of *S. aureus* Newman and thus more accessible to trypsin digestion. Such a possibility would support the differential trypsin accessibility of other surface proteins expressed in either of the two tested bacteria, as suggested above.

V. Discussion

S. aureus produces a plethora of virulence determinants [53], which are regulated by a complex network of two-component regulatory systems, DNA-binding proteins, and small RNAs [3,16,21,30,54,55]. This explains why there is no simple approach to assess the presence or absence of each individual pathogenic feature along the successive steps of infection. Previous experiments in which specific genes were inactivated were sometimes difficult to interpret, particularly when bacteria were equipped with multiple genes encoding redundant or complementary functions [13,40,43]. Moreover, gene regulation may vary between *in vitro* and *in vivo* conditions [23].

To integrate this multilevel information, experimental systems should allow appraising quantitative snapshots of global macromolecule expression in both *in vitro* and *in vivo* conditions. While this is possible at the level of mRNA [11,29,31], its equivalent at the protein level is as yet less developed [27-29]. Individual proteins can be quantitatively evaluated by western-blotting or *in situ* hybridization. However, these methods are not amenable to evaluate multiple proteins simultaneously, due to the need of numerous different antibodies and the limited number of dyes that can be used together in a single experiment. Here, we attempted to bypass this limitation by using a proteomic approach. Since we previously contributed to the understanding of the role of *S. aureus* surface adhesins using heterologous gene expression [13,39-41,43,56], we intentionally concentrated our efforts on the analysis of the time course detection of the 21 known *S. aureus* LPXTG surface proteins.

Initial analyses indicated that the whole proteome of purified cell walls was much more complex than expected, revealing numerous proteins that were not anticipated to be found in the peptidoglycan

and its appendages. The unexpected presence of these species was considered as contamination, at least in the setting of crude bacterial walls purified after mechanical cell breakage. However, this kind of contamination persisted both after harsher purification (e.g. removal of teichoic acids), and in the trypsin-shaving protocol, which was performed on >99,9 % integral cells as assessed by microscopy and colony counts. Therefore, the question as to whether some cytoplasmic proteins might be constitutive parts of the normal wall environment, as also suggested by others [27,34], remains open.

We previously showed that LPXTG-proteins from *S. aureus* could be heterogeneously expressed in *L. lactis* and individually detected by LC-MS/MS in the recombinant lactococci [35,41]. On this basis we constructed a peptide library specific to each of these LPXTG-proteins. This library was indispensable to further quantify LPXTG-proteins in the more complex *S. aureus* environment. With some exceptions, the amounts of LPXTG-proteins in *S. aureus* increased up to the early stationary growth phase, and decreased thereafter. This bell-shape behavior is reminiscent of *agr*-regulated surface proteins such as protein A, which is expressed during logarithmic growth and repressed in stationary phase [3,30]. In the present experiments, comparisons between proteomic and transcriptomic profiles confirmed this parallelism for protein A, which is in accordance with previous studies [29,31,57]. Moreover, we identified at least one additional LPXTG-protein,SasH, that demonstrated similar profile modifications between protein and mRNA detection in the parent strain and its *agr*- mutant, suggesting that it was also under tight control by *agr*. Conversely, however, several LPXTG-proteins modified their proteomic profiles between the parent and the mutant in spite of the fact that transcriptomic profiles remained unchanged. This suggests that, in addition to mRNA, protein expression was further affected by additional factors at the post-transcriptional level, e.g. via interference with mRNA, or post-translational level via protein modification [58] or protease degradation [29,31,57].

This was particularly relevant when comparing proteomic profiles with adherence phenotypes. Taking fibrinogen binding as a model, the present results show that adherence was indeed affected by both *agr* integrity and growth conditions, but did not follow an absolute *agr* paradigm. For instance, in TSB, adherence decreased in the early stationary phase of growth and re-increased later on (at 24h) without a clear correlate with measured amounts of surface ClfA, but with a clear correlate with increasing *clfA* mRNA. Likewise, in RPMI adherence tended to decrease over time, without a good proteomic correlate either (mRNA was not measured in this condition). This seeming incoherence

most likely reflects our lack of understanding of the subtlety of the wall environment, which implicates additional factors that may affect the phenotype. Indeed, the Gram-positive envelope is not a static peptidoglycan scaffold merely decorated with protein, polyols (teichoic and lipoteichoic acids) and polysaccharide appendages. It rather acts as a dynamic interface between the environment and the intracellular milieu. For instance, the *S. aureus* envelope is constantly traversed by secreted molecules including $\geq$ 10 different proteases [59], among which some were shown to regulate LPXTG-proteins by protein degradation (e.g. ClfB) [29,31,57]. These could be responsible for the progressive decreases in recovery of unique peptides over time, as observed in Figure 1.

Alternatively, mutual interactions between various wall polymers may influence the exposure of protein binding domains to the extracellular milieu. This was recently exemplified with recombinant ClfA, where artificial lengthening or shortening of the spacer region (R-repeats) between the proximal wall anchor and the outermost binding domains increased or decreased adherence to fibrinogen, respectively, because they modified the exposure of distal binding domains to their ligand fibrinogen [60]. These authors reported similar variations in the presence of absence of an expo-polysaccharide capsule. Therefore, the bacterial surrounding may influence the access of exogenous ligands or proteases to LPXTG-protein domains, a phenomenon that observed with trypsin herein.

Apart from these differences, some other proteins also demonstrated differential regulation between TSB and RPMI, including genes of the iron-capturing *isd* locus, as well as sizable increases in SasD and SasH in iron starvation. The increase in *isd* genes is expected in low iron medium [32,33]. On the other hand, the reason for the increase in SasD and SasH is more difficult to interpret. While the physiological role of SasD is as yet unclear, SasH (recently renamed AdsA [61]) is a cell wall associated adenosine synthase that converts adenosine-monophosphate into adenosine, a strong immunomodulator helping staphylococci to escape phagocyte-induced killing. Hence, SasH (or AdsA) could well be co-regulated with the siderophore locus *isd*, which expression is induced in experimental *S. aureus* nasal colonization [11]. In this setting, expression of *isd* could be required for survival in the low-iron mucosal environment, while SasH could be required to damper host defenses and promote bacterial persistence. Eventually, the mRNA of Srap was detected but its encoded protein was not, suggesting the possible lack of access to trypsin shaving.

The present study yielded other interesting observations. First, FnBPA was detected in both purified walls and trypsin-shaving experiments, in spite of the fact that it lacks the LPXTG-anchoring

module and the entire cell wall proximal D-W regions in *S. aureus* Newman, due to the presence of a stop codon [62]. This is also true for its FnBPB counterpart, which in contrast was barely detected at all our experiments. One possibility for this difference is that the 741 (out of 1018) amino acids of truncated FnBPA is enough for non-specific wall attachment, whereas the 678 (out of 940) residues of truncated FnBPB is too short. Alternatively, the two proteins could have been differentially expressed in the present experimental conditions, a possibility which has yet to be demonstrated. Another noteworthy observation was the fact that trypsin released different sets of peptides from LPXTG proteins expressed in *S. aureus* or in recombinant *L. lactis*. Thus, some kind of differential domain hindrance or exposure must have taken place in the two bacterial backgrounds, as suggested by others [27,34]. In the same line, a few LPXTG-proteins were not detected at all (i.e. Srap and SasC) in *S. aureus* Newman, although they were detected by LC-MS/MS in recombinant lactococci [35,41]. This raises the question of their conditional expression, as observed in the present experiments and by others for *isd* genes [32,33], or of differential access of trypsin digestion.

Taken together, two sets of conclusions can be drawn from these results. First, from the biological point of view, they indicate that some LPXTG-proteins followed an *agr*-like regulation, which was abrogated in *agr*-negative mutants and correlated with the mRNA transcription profile in the parent strain and its *agr*- mutant. On the other and, several LPXTG-proteins varied their expression without a good mRNA correlate, and remained functionally active for prolonged periods of time, such as, for instance, for fibrinogen-binding. This study revises somewhat the dogma that surface adhesins are essentially active during the exponential growth phase, in order to colonize new sites, and shut off after colonization, to facilitate bacterial detachment and colonization of other sites. Moreover, it also reveals that the bacterial wall environments are different in *S. aureus* and *L. lactis*, potentially resulting in different exposure of LPXTG-proteins at the bacterial surface, which in turns might lead to a differential accessibility for trypsin digestion. Whether this altered access has functional consequences for bacterial adherence remains to be determined. Likewise, whether this could influence the protective efficacy of blocking antibodies might be relevant for vaccine development.

Second, from the technical point of view, the results open the way to semi-quantitative and time course proteome analysis of multiple *S. aureus* pathogenic polypeptides simultaneously. Hence, they add to other recently published proteomic analyses [27,29,34]. One theoretical limitation of trypsin-shaving is that it peptide-release is limited to the trypsin-accessible proteins. Thus, it may

underestimate proteins buried deeper in the multi-polymeric wall. However, preliminary purification of cell walls or removal of teichoic acids further decreased the recovery of LPXTG-protein peptides, suggesting that trypsin-shaving was a good compromise in this complex surrounding. Another limitation is strain-dependency, which may require re-characterization of each singular organism. However, the same remark is valid for any physiologic or phylogenic characterization of any isolates. Finally, the method could help determining the real-time behavior of numerous bacterial adhesins not only *in vitro*, but possibly also *in vivo*. For the latter case, targeted mass spectrometry techniques based on selected reaction monitoring [63,64] could be easily developed to specifically detect and quantify bacterial surface molecules in complex matrices such as those obtained from animal models or clinical samples.

VI. Supplemental Experimental procedures

VI.1. Preparation of bacterial cells walls

Bacterial cell walls were purified as previously described [35], in order to remove all possible contaminants from the cytoplasm as well as proteins non-covalently attached to the peptidoglycan. In brief, bacteria were grown in 300 ml batch cultures to the late exponential phase, chilled on ice, and centrifuged for 10 min at 6,000 rpm at 4°C. Bacterial pellets were suspended and washed in phosphate buffer saline (PBS) before being boiled for 10 min in 8% (final concentration) of sodium dodecyl sulfate (SDS, Sigma) to denature proteolytic enzymes and remove soluble materials. SDS-treated cells were washed by suspension-centrifugation 2 times with 1 M NaCl and several times with MiliQ water, until no more foaming was observed. Washed pellets were resuspended in 1 ml of water. Bacterial sacculi were broken by shaking with ceramic beads (FastPrep apparatus, Qbiogene, Morgan Irvine, CA, USA; 3 cycles for 45 seconds at 6,5m/s). The extent of cell breakage (>99.9%) was assessed by phase-contrast microscopy. Unbroken cells were removed by low speed centrifugation. Broken cells were recovered by centrifugation for 20 min at 16,000 rpm at 4°C, resuspended in water and stored at -20^{o}C before processing for trypsin digestion.

VI.2. Bacterial cell wall purification followed by teichoic acid removal with hydrofluoric acid for 48h

In certain experiments purified walls were further treated with hydrofluoric acid (48 %) for 48h at 4°C to remove the covalently bound teichoic acids [45]. Indeed, considering the high complexity of the proteome recovered from purified walls (see Results section), it was reasoned that physico-chemical treatment such as removal of the charged teichoic acids could decrease contamination by proteins attached to the walls via non-specific (e.g. ionic) binding. Teichoic acid removal was assessed by loss of >80% of the phosphate content of the samples as described [65].

VII Suplemental Tables and Figure

Supplemental Table 1 : Protein sequences and position of peptides detected by MS/MS in *S. aureus* and *L. lactis* protein sequences

Protein	Sequence	Position (aa)	Peptides detected in *S. aureus*	Peptides detected in *L. lactis*
ClfA	MNMKKKEKHAIRKKSIGVASVLVGTLIGFGLLSSKEADASENSVTQSDSASNESKSNDSSSVSAA	40-55		SENSVTQSDSASNESK
	PKTDDTNVSDTKTSSNTNNGETSVAQNPAQQETTQSSSTNATTEETPVTGEATTTTTNQANTP	56-67		SNDSSSVSAAPK
	ATTQSSNTNAEELVNQTSNETTFNDTNTVSSVNSPQNSTNAENVSTTQDTSTEATPSNNESAP	200-212	DVVNQAVNTSAPR	DVVNQAVNTSAPR
	QSTDASNKDVVNQAVNTSAPRMRAFSLAAVAADAPAAGTDITNQLTNVTVGIDSGTTVYPHQ	259-271	LNYGFSVPNSAVK	LNYGFSVPNSAVK
	AGYVKLNYGFSVPNSAVKGDTFKITVPKELNLNGVTSTAKVPPIMAGDQVLANGVIDSDGNVIY	282-293	ELNLNGVTSTAK	
	TFTDYVNTKDDVKATLTMPAYIDPENVKKTGNVTLATGIGSTTANKTVLVDYEKYGKFYNLSIKG	331-345	ATLTMPAYIDPENVK	ATLTMPAYIDPENVK
	TIDQIDKTNNTYRQTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIKVYKVDNAADLSES	347-363	TGNVTLATGIGSTTANK	TGNVTLATGIGSTTANK
	YFVNPENFEDVTNSVNITFPNPNQYKVEFNTPDDQITTPYIVVVNGHIDPNSKGDLALRSTLYGY	375-381		FYNLSIK
	NSNIIWRSMSWDNEVAFNNGSGSGDGIDKPVVPEQPDEPGEIEPIPEDSDSDPGSDSGSDSNS	396-434	QTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIK	QTIYVNPSGDNVIAPVLTGNLKPNTDSNALIDQQNTSIK
	DSGSDSGSDSTSDSGSDSASDSDSASDSDSASDSDSASDSDSASDSDSDNDSDSDSDSDSDSDS	438-473	VDNAADLSESYFVNPENFEDVTNSVNITFPNPNQYK	
	DSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSD	474-500	VEFNTPDDQITTPYIVVVNGHIDPNSK	VEFNTPDDQITTPYIVVVNGHIDPNSK
	SDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDS			
	DSASDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSESDSDSESDSDSDSDSDSDSDS			
	DSDSDSDSASDSDSGSDSDSSSDSDSESDSNSDSESGSNNNVVPPNSPKNGTNASNKNEAKDS			
	KEPLPDTGSEDEANTSLIWGLLASIGSLLLFRRKKENKDKK			
ClfB	MKKRIDYLSNKQNKYSIRRFTVGTTSVIVGATILFGIGNHQAQASEQSNDTTQSSKNNASADSE	57-125		NNASADSEKNNMIETPQLNTTANDTSDISANTNSAN

KNNMIETPQLNTTANDTSDISANTNSANVDSTTKPMSTQTSNTTTTEPASTNETPQPTAIKNQ
ATAAKMQDQTVPQEANSQVDNKTTNDANSIATNSELKNSQTLDLPQSSPQTISNAQGTSKPS
VRTRAVRSLAVAEPVVNAADAKGTNVNDKVTASNFKLEKTTFDPNQSGNTFMAANFTVTDKV
KSGDYFTAKLPDSLTGNGDVDYSNSNNTMPIADIKSTNGDVVAKATYDILTKTYTFVFTDYVNN
KENINGQFSLPLFTDRAKAPKSGTYDANINIADEMFNNKITYNYSSPIAGIDKPNGANISSQIIGV
DTASGQNTYKQTVFVNPKQRVLGNTWVYIKGYQDKIEESSGKVSATDTKLRIFEVNDTSKLSDS
YYADPNDSNLKEVTDQFKNRIYYEHPNVASIKFGDITKTYVVLVEGHYDNTGKNLKTQVIQENV
DPVTNRDYSIFGWNNENVVRYGGGSADGDSAVNPKDPTPGPPVDPEPSPDPEPEPTPDPEPS
PDPEPEPSPDPDPDSDSDSDSGSDSDSGSDSDSESDSDSDSDSDSDSDSDSESDSDSESDSDSD
SDSDSDSDSDSDSDSDSDSDSDSDSDSDSESDSDSESDSESDSDSDSDSDSDSDSDSDSDSD
SDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDS
DSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSDSD
SDSDSDSDSDSDSDSRVTPPNNEQKAPSNPKGEVNHSNKVSKQHKTDALPETGDKSENTNATL
FGAMMALLGSLLLFRKRKQDHKEKA

66-125		VDSTTKPMSTQTSNTTTTEPASTNETPQPTAIK NNMIETPQLNTTANDTSDISANTNSANVDSTTKPMS TQTSNTTTTEPASTNETPQPTAIK
133-149	MQDQTVPQEANSQVDNK	MQDQTVPQEANSQVDNK
150-164	TTNDANSIATNSELK	
165-191	NSQTLDLPQSSPQTISNAQGTSKPSVR	NSQTLDLPQSSPQTISNAQGTSKPSVR
197-211	SLAVAEPVVNAADAK	SLAVAEPVVNAADAK
212-225	GTNVNDKVTASNFK	GTNVNDKVTASNFK
229-250	TTFDPNQSGNTFMAANFTVTDK	TTFDPNQSGNTFMAANFTVTDK
261-286	LPDSLTGNGDVDYSNSNNTMPIADIK	LPDSLTGNGDVDYSNSNNTMPIADIK
296-303	ATYDILTK	ATYDILTK
304-316	TYTFVFTDYVNNK	TYTFVFTDYVNNK
317-331	ENINGQFSLPLFTDR	ENINGQFSLPLFTDR
337-354	SGTYDANINIADEMFNNK	SGTYDANINIADEMFNNK
355-391	ITYNYSSPIAGIDKPNGANISSQIIGVDTASGQNTYK	ITYNYSSPIAGIDKPNGANISSQIIGVDTASGQNTYK
392-399	QTVFVNPK	
402-411	VLGNTWVYIK	
412-423		GYQDKIEESSGK
442-456	LSDSYYADPNDSNLK	LSDSYYADPNDSNLK
442-463	LSDSYYADPNDSNLKEVTDQFK	LSDSYYADPNDSNLKEVTDQFK

		433-441	IFEVNDTSK	
		466-477	IYYEHPNVASIK	IYYEHPNVASIK
		484-498	TYVVLVEGHYDNTGK	TYVVLVEGHYDNTGK
		502-515	TQVIQENVDPVTNR	TQVIQENVDPVTNR
		516-529	DYSIFGWNNENVVR	DYSIFGWNNENVVR
Protein A	MMTLQIHTGGINLKKKNIYSIRKLGVGIASVTLGTLLISGGVTPAANAAQHDEAQQNAFYQVLN MPNLNADQRNGFIQSLKDDPSQSANVLGEAQKLNDSQAPKADAQQNNFNKDQQSAFYEILN MPNLNEAQRNGFIQSLKDDPSQSTNVLGEAKKLNESQAPKADNNFNKEQQNAFYEILNMPNL NEEQRNGFIQSLKDDPSQSANLLSEAKKLNESQAPKADNKFNKEQQNAFYEILHLPNLNEEQR NGFIQSLKDDPSQSANLLAEAKKLNDAQAPKADNKFNKEQQNAFYEILHLPNLTEEQRNGFIQS LKDDPSVSKEILAEAKKLNDAQAPKEEDNNKPGKEDNNKPGKEDNNKPGKEDNNKPGKEDGN KPGKEDNKKPGKEDGNKPGKEDNKKPGKEDGNKPGKEDGNKPGKEDGNGVHVVKPGDTVN DIAKANGTTADKIAADNKLADKNMIKPGQELVVDKKQPANHADANKAQALPETGEENPFIGT TVFGGLSLALGAALLAGRRREL	74-81	NGFIQSLK	NGFIQSLK
		74-96	NGFIQSLKDDPSQSANVLGEAQK	NGFIQSLKDDPSQSANVLGEAQK
		82-96	DDPSQSANVLGEAQK	DDPSQSANVLGEAQK
		105-134	ADAQQNNFNKDQQSAFYEILNMPNLNEAQR	ADAQQNNFNKDQQSAFYEILNMPNLNEAQR
		115-134	DQQSAFYEILNMPNLNEAQR	DQQSAFYEILNMPNLNEAQR
		135-142	NGFIQSLK	NGFIQSLK
		135-156	NGFIQSLKDDPSQSTNVLGEAK	NGFIQSLKDDPSQSTNVLGEAK
		143-156	DDPSQSTNVLGEAK	DDPSQSTNVLGEAK
		157-165	KLNESQAPK	KLNESQAPK
		166-192	ADNNFNKEQQNAFYEILNMPNLNEEQR	
		173-192	EQQNAFYEILNMPNLNEEQR	EQQNAFYEILNMPNLNEEQR
		193-200	NGFIQSLK	NGFIQSLK
		193-214	NGFIQSLKDDPSQSANLLSEAK	NGFIQSLKDDPSQSANLLSEAK
		201-214	DDPSQSANLLSEAK	DDPSQSANLLSEAK
		201-215	DDPSQSANLLSEAKK	

		215-223	KLNESQAPK	KLNESQAPK
		228-250	FNKEQQNAFYEILHLPNLNEEQR	
		231-250	EQQNAFYEILHLPNLNEEQR	EQQNAFYEILHLPNLNEEQR
		251-258	NGFIQSLK	NGFIQSLK
		251-272	NGFIQSLKDDPSQSANLLAEAK	NGFIQSLKDDPSQSANLLAEAK
		259-272	DDPSQSANLLAEAK	DDPSQSANLLAEAK
		273-281	KLNDAQAPK	KLNDAQAPK
		286-308	FNKEQQNAFYEILHLPNLTEEQR	
		289-308	EQQNAFYEILHLPNLTEEQR	
		309-316	NGFIQSLK	NGFIQSLK
		309-323	NGFIQSLKDDPSVSK	NGFIQSLKDDPSVSK
		413-440	EDGNKPGKEDGNGVHVVKPGDTVNDIAK	
		421-440	EDGNGVHVVKPGDTVNDIAK	
		459-471	NMIKPGQELVVDK	NMIKPGQELVVDK
		459-472	NMIKPGQELVVDKK	NMIKPGQELVVDKK
FnBPA	MKNNLRYGIRKHKLGAASVFLGTMIVVGMGQDKEAAASEQKTTTVEENGNSATDNKTSETQT TATNVNHIEETQSYNATVTEQPSNATQVTTEEAPKAVQAPQTAQPANIETVKEEVVKEEAKPQ VKETTQSQDNSGDQRQVDLTPKKATQNQVAETQVEVAQPRTASESKPRVTRSADVAEAKEAS NAKVETGTDVTSKVTVEIGSIEGHNNTNKVEPHAGQRAVLKYKLKFENGLHQGDYFDFTLSNN VNTHGVSTARKVPEIKNGSVVMATGEVLEGGKIRYTFTNDIEDKVDVTAELEINLFIDPKTVQTN	57-97	TSETQTTATNVNHIEETQSYNATVTEQPSNATQVTTE	TSETQTTATNVNHIEETQSYNATVTEQPSNATQVTTE
			EAPK	EAPK
		98-114	AVQAPQTAQPANIETVK	AVQAPQTAQPANIETVK
		98-119	AVQAPQTAQPANIETVKEEVVK	
		115-127	EEVVKEEAKPQVK	

GNQTITSTLNEEQTSKELDVKYKDGIGNYYANLNGSIETFNKANNRFSHVAFIKPNNGKTTSVTV
TGTLMKGSNQNGNQPKVRIFEYLGNNEDIAKSVYANTTDTSKFKEVTSNMSGNLNLQNNGSY
SLNIENLDKTYVVHYDGEYLNGTDEVDFRTQMVGHPEQLYKYYYDRGYTLTWDNGLVLYSNKA
NGNGKNGPIIQNNKFEYKEDTIKETLTGQYDKNLVTTVEEEYDSSTLDIDYHTAIDGGGGYVDG
YIETIEETDSSAIDIDYHTAVDSEAGHVGGYTESSEESNPIDFEESTHENSKHHADVVEYEEDTNP
GGGQVTTESNLVEFDEDSTKGIVTGAVSDHTTIEDTKEYTTESNLIELVDELPEEHGQAQGPIEEI
TENNHHISHSGLGTENGHGNYGVIEEIEENSHVDIKSELG

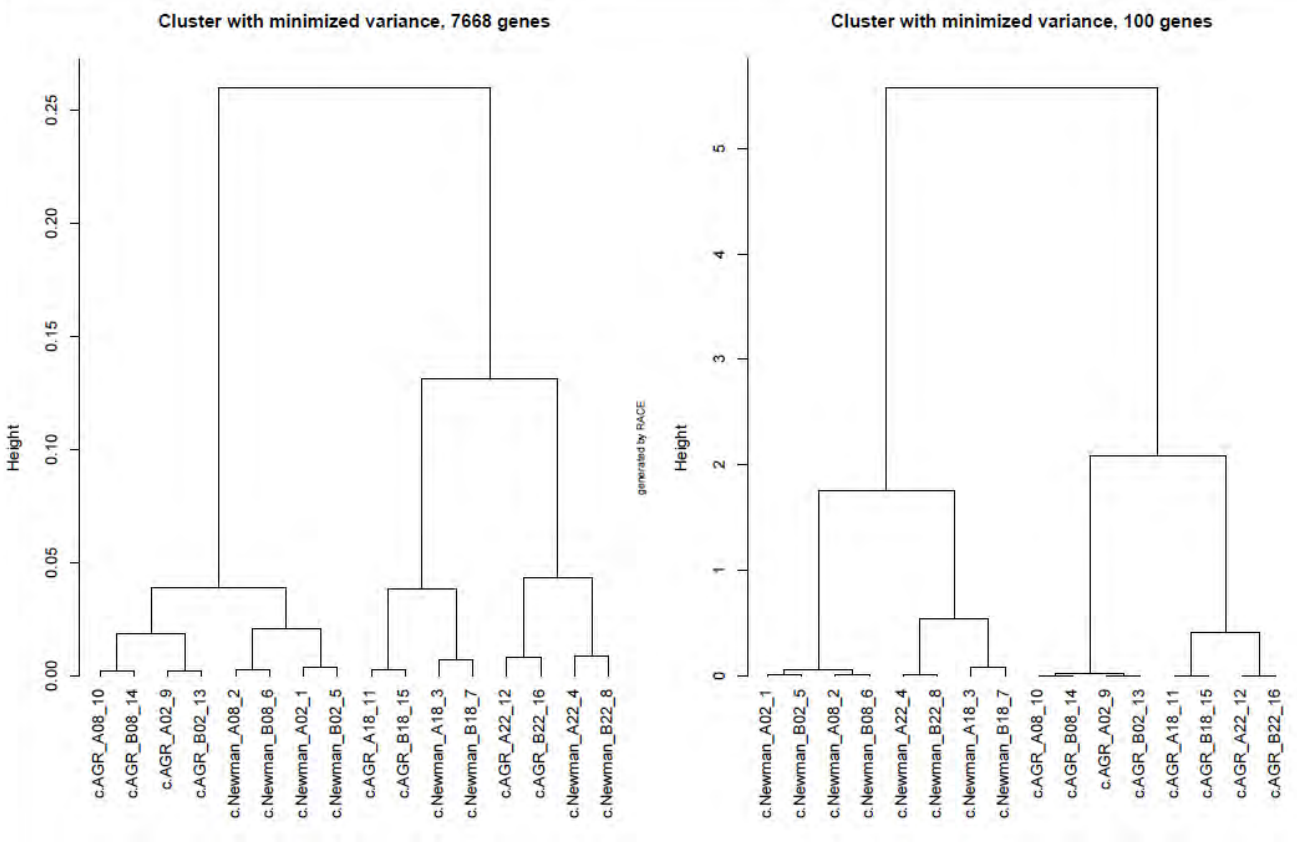
148-165	KATQNQVAETQVEVAQPR	KATQNQVAETQVEVAQPR
149-165	ATQNQVAETQVEVAQPR	ATQNQVAETQVEVAQPR
201-216	VTVEIGSIEGHNNTNK	VTVEIGSIEGHNNTNK
233-260	FENGLHQGDYFDFTLSNNVNTHGVSTAR	
267-282	NGSVVMATGEVLEGGK	
285-294	YTFTNDIEDK	
311-331	TVQTNGNQTITSTLNEEQTSK	TVQTNGNQTITSTLNEEQTSK
337-357	YKDGIGNYYANLNGSIETFNK	
339-357	DGIGNYYANLNGSIETFNK	
362-374	FSHVAFIKPNNGK	
375-386	TTSVTVTGTLMK	
399-411	IFEYLGNNEDIAK	IFEYLGNNEDIAK
423-451	FKEVTSNMSGNLNLQNNGSYSLNIENLDK	
452-471	TYVVHYDGEYLNGTDEVDFR	
472-483	TQMVGHPEQLYK	
524-537	EDTIKETLTGQYDK	
622-655	HHADVVEYEEDTNPGGGQVTTESNLVEFDEDSTK	
656-672	GIVTGAVSDHTTIEDTK	GIVTGAVSDHTTVEDTK
673-702		EYTTESNLIELVDELPEEHGQAQGPVEEITK
764-786		YEQGGNIVDIDFDSVPQIHGQNK

Supplemental Table 2 : Fold change of *agr* locus RNA expression in *S. aureus agr-* mutant versus its parent Newman in TSB.

Locus tag	Gene ID[a]	Gene name	Function	OD 0.2[b]	OD 0.8[b]	OD 1.8[b]	OD 2.2[b]
NWMN_1946	5331721	agrA	Transcription regulator	-360.4 ***	-367.6 ***	-484.5 ***	-422.3 ***
NWMN_1943	5331201	agrB	Transmembrane protein	-575.8 ***	-664.9 ***	-762.1 ***	-969.8 ***
NWMN_1945	5331722	agrC	Transmembrane sensor	-331.6 ***	-422.8 ***	-479.5 ***	-661.2 ***
NWMN_1944	5331720	agrD	Auto-inducing peptide precusor	-711.1 ***	-772.9 ***	-730.2 ***	-828.4 ***
		rnaIII	Small RNA regulator	-562.5 ***	-696.6 ***	-883.1 ***	-875.5 ***

[a] GeneID in *S. aureus* Newman

[b] the asterix indicate significant changes of expression between *S. aureus* Newman *agr* mutant and *S. aureus* Newman grown in TSB at various OD during growth, as determined by false discovery rate control, FDR; *, $p<0.05$; **, $p<0.01$; ***$p<0.001$.



Supplemental Figure 1: Hierarchical clustering analysis using all 7668 Probe sets of the different microarray runs. The left panel depicts the clustering according to the variations in all 7688 probes, and the right panel according to variation in 100 randomly probes. The figure shows the high reproducibility between replicates as biological repeats always cluster together. cAGR stands for the agr- mutant and cNEeman for the parent. A and B stands for the first and the second chip testes in parallel. The additional values of 02, 08, 18 and 22 stand for the different optical densities of the sampling.

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CHAPTER 4

Conclusion and Perspectives

The series of experiments presented above shed new light on the subtlety with which *S. aureus* fine tunes the expression of its gene end products, i.e. its proteins, and on our lack of ability to predict it. Until recently, molecular microbiologists have concentrated on understanding specific structures and mechanisms of molecules, alone or in combination, including specific regulatory of systems (e.g. transcriptional units, operons and regulons), interactions between specific systems, and consequences of alterations of systems (via mutations) on the bacterial phenotype in various environments. These were indispensable and powerful tools that helped unravel the myriads of intertwined functional elements allowing a bacterium to grow and divide, to put in the most global sense of life sciences.

Moreover, in the more specific context of *S. aureus* colonization and invasion of the mammal host, experiments aimed at dissecting the individual function of each of the 21 (recently 22) MSCRAMM LPXTG-proteins and their role in experimental infection. For instance, they demonstrated the critical implication of protein-protein and protein domains cooperation (e.g. between fibrinogen-binding and fibronectin-binding) in colonization and invasion of endovascular tissues.

With such premises, it was tempting to try setting up a new *clinical microbiology* tool that would screen *S. aureus* strains on the basis of their *in vitro* adherence phenotype, and tag them as more or less prone to produce severe infection or innocuous nasal colonization. However, this maybe too naïve hope was turned down by the first series of experiments presented above (Chapter 2, [1]), which demonstrated a total lack of correlation between *in vitro* adherence phenotypes, e.g. to fibrinogen and to fibronectin, and the ability of clinical isolates of *S. aureus* to induce severe endovascular infection rather than mere colonization. Even more, some isolates that were very adherent *in vitro* were less infectious *in vivo* than isolates that were very poorly adherent, thus contradicting the working hypothesis.

These paradoxical phenotypes raised the critical issue of contextualizing the experimental settings when trying to draw general conclusions. Indeed, before concluding that molecular mechanisms revealed by functional dissection of protein domains were wrong, it was important to first know whether the paradoxical results described above were due to differential regulation of genes or gene products in different environments. The Introduction chapter (Chapter 1) of the present thesis dissertation offers a glimpse at what might be expected from gene regulation, including the knowns and (certainly underestimating) the unknowns. Among the knowns is the seminal discovery of the *agr*

global regulatory system in the early 90s, which has rapidly set the widely accepted paradigm that *S. aureus* produce adhesins during the logarithmic phase of growth, and switch to the production of toxins in the stationary growth phase. However, additional work progressively showed that while *agr* was indeed a kind of a regulatory hub, its activity was largely positively and negatively modulated by up to 10 parallel systems that were also interconnected by stimulatory or inhibitory interactions, and responded to different signals.

Just considering these individual systems, it is relevant that the effect of each of them on the others were mostly tested in deleted mutants, and the effect of their loss of activity was monitored on other systems individually or globally by microarray. However, although instructive, none of these approaches provides integrative information on the functioning of the whole system, which should include the sum of all the regulatory elements together. Moreover, comparing ON / OFF responses between parent cells and deleted mutants does not provide information on the effect of intermediate stages of gene modulation (rather than radical ON / OFF responses), which is much more likely to occur in nature. Therefore, already at the level of gene expression there is an urgent need to understand regulatory circuitries at a more systemic level, which is emerging thank to the approach of systems biology.

Moreover, aside from transcriptional and post-transcriptional regulation, there is even more unknowns on post-translational protein regulation, because several of these aspects were less systematically studies, at least in *S. aureus*. For instance, while we know how proteins are exported, how they may be retained in the plasma membrane, covalently attached to the peptidoglycan, or loosely attached to extra-membrane structures of the cell envelope, not much is known on the dynamics of their functional regulation, for instance by protease degradation (except for ClfB), including what could affect such regulation during growth or when facing peculiar environmental conditions. Likewise, little is known on the functional consequences of protein-protein interaction, including steric hindrance, domain exposure, or interactions with other structures of the cell envelope or ligands in the environment. Yet, salient examples of such phenomena do exist, for instance when the production of capsular polysaccharides manage to obscure fibrinogen-binding proteins, resulting in phenotypically fibrinogen-binding-negative staphylococci. Likewise, capsule production can also obscure Protein A, which is one of the most important immune-escape features of *S. aureus*. Hence, the functional expression of important fibrinogen-binding proteins and Protein A can be modulated by

capsule expression, which is strain-dependent and regulated by *agr*. Moreover, another example is the capping of fibrinogen- and fibronectin-binding proteins by plasmin-sensitive LPXTG-protein Pls, which is found only in certain MRSAs, because it is encoded on certain types of SCC*mec* cassettes. Pls is cleaved when it encounters activated plasmin, i.e. in the vicinity of blood clots, and this cleavages restores fibrinogen- and fibronectin-binding. Therefore, here the functional expression of bacterial adhesins depends on the host.

These (at least) two aspects of protein regulation, i.e. proteins half-life and functional “aptitude” could well explain the paradoxical observation described in the first published article presented in this thesis. Therefore, this question was further addressed in a second series of experiments investigating the correlation between proteomic and transcriptomic profiles, and adherence phenotypes, in time course experiments during growth in various conditions. The experiments concentrated on LPXTG-proteins and revealed a few noticeable points that are very briefly summarized here. First, while the physical presence of proteins on the bacterial surface followed, in several cases, an *agr*-like regulatory pattern, this was by far not the case for all of them. Second, this relatively poor correlation with *agr* was also true for a number of LPXTG-protein mRNAs, indicating that they were not under strict control of *agr*; this information was new for most of the studies proteins and mRNAs. Third, some proteins (e.g. Protein A) could persist on the surface of the bacteria for very prolonged periods of time, indicating that they did not undergo protease degradation. Fourth, some LPXTG-proteins (e.g. ClfA) were poorly detected by trypsin shaving, but yet very active in phenotypic adherence tests to fibrinogen *in vitro*. Finally, this paradox was likely explained by the fact that some LPXTG-proteins could adopt conformations that allowed them to hide some of their domains to trypsin shaving, most probably by interactions with other molecules on the polysaccharidic meshwork of the outermost microbial envelope.

These experiments confirmed that the regulation of the *S. aureus* surface proteome was *de facto* complex, multilevel, medium and environment dependent, and that phenotypes of live bacterial were difficult to predict form knowledge on individual determinants tested separately. While this more global appraisal did not help understand the individual role of each of the factors within the system, it allowed setting a new paradigm for future work, i.e. next generation studies will need to integrate both traditional approaches and systems biology. Indeed, cross-fertilization between these zoom-in / zoom-out views will be the only way to progress.

Just take the example of anti- *S. aureus* vaccination, which has failed in human so far. While antibodies can be raised against a number of *S. aureus* surface determinants, and while virtually every human individuals carry many such antibodies due to previous exposure to *S. aureus*, these antibodies do not confer protective immunity. The second article presented in this dissertation (Chapter 3, [2]) proposes at least two further thoughts regarding this issue. First concerning protein domain availability. If we do carry antibodies targeted against a given *S. aureus* surface determinant that can be detected in *in vitro* tests, it does not necessarily mean that the same determinant will be available for recognition *in vivo* as well. It could be that these antibodies were raised by the host against dead rather than against live bacteria, but that live bacteria hide these specific determinants because they are sensible. As suggested above, *S. aureus* do have a number of ways to hide surface determinants, for instance fibrinogen-binding proteins and Protein A via capsule production.

Second, we may not target the right proteins domain(s) when choosing bacterial determinants that are expressed during growth in *in vitro* conditions, because these may become hidden *in vivo*.

Then, how would the bacterium use a specific adhesin *in vivo* if it keeps it hidden to the immune system? The issue is speculative, but one could imagine some solutions. One would be that hidden surface components reveal themselves only transiently when making physical contact with their target protein or target membrane. The physical contact, for instance with endothelial cells, would trigger a conformational change of the staphylococcal protein that would engage with the ligand and lock with it in a transient structure conformation that is different from its native domains. This “deadly kiss” type of mechanism would imply induced-fitness, and be elusive enough to remain undetected by the host. Therefore, future vaccine strategies should take into account not only protein domains, but protein interacting with other molecules, and variations in macro- and micro-environments.

Taken together, the experiments presented herein have conducted us from paradoxical phenotypes to new insights in the global staphylococcal life style. From this very point of view, we envision future work progressing toward both (i) proteomic and transcriptomic analyses of surface proteins in infected tissues, (ii) analyses of specific peptide availability in such conditions, and (iii) generating and testing preventive antibodies directed against hidden domains revealed by experiments (i) and (ii).

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ANNEXES

This annexe section is composed of two published manuscripts to which I contributed during the course of my thesis:

- Evidence for a new post-translational modification in *Staphylococcus aureus*: hydroxymethylation of asparagine and glutamine.

  Waridel P, **Ythier M**, Gfeller A, Moreillon P, Quadroni M.

  Journal of Proteomics. 2012 Mar 16;75(6):1742-51. Epub 2011 Dec 20

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Annexe 1

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Evidence for a new post-translational modification in *Staphylococcus aureus*: Hydroxymethylation of asparagine and glutamine

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen whose infectious capacity depends on surface proteins, which enable bacteria to colonize and invade host tissues and cells. We analyzed "trypsin-shaved" surface proteins of *S. aureus* cultures by high resolution LC-MS/MS at different growth stages and culture conditions. Some modified peptides were identified, with a mass shift corresponding to the addition of a CH_2O group (+30.0106 u). We present evidence that this shift corresponds to a hyxdroxymethylation of asparagine and glutamine residues. This known but poorly documented post-translational modification was only found in a few proteins of *S. aureus* grown under specific conditions. This specificity seemed to exclude the hypothesis of an artifact due to sample preparation. Altogether hydroxymethylation was observed in 35 peptides from 15 proteins in our dataset, which corresponded to 41 modified sites, 35 of them being univocally localized. While no function can currently be assigned to this post-translational modification, we hypothesize that it could be linked to modulation of virulence factors, since it was mostly found on some surface proteins of *S. aureus*.

1. Introduction

The Gram-positive bacterium *Staphylococcus aureus* is a highly successful opportunistic pathogen that can induce a wide variety of diseases [1]. Over the last decades molecular and genetic dissection of *S. aureus* has revealed numerous virulence factors that might be implicated in pathogenesis [2]. Among these are proteins involved in adhesion, invasion and spreading in host cells, others enabling bacteria to avoid host immune response, as well as proteins allowing the bacteria to utilize nutrients from the host cells [3]. The majority of virulence factors are secreted in the extracellular medium or attached to the cell surface [4]. Proteins exposed on the surface — the "surfaceome" — include membrane, cell wall bound and secreted proteins, and can be specifically studied by various proteomics methods [5,6]. In one of them — named surface shaving — cells are incubated with a protease and the peptides cleaved from surface proteins are analyzed by LC-MS for protein identification [7,8]. Several groups used variants of this method — with soluble or immobilized trypsin, alone or in combination with proteinase K — to study the surfaceome of different *S. aureus* strains [9,10,11]. This technique was also used in combination with a

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biotinylation approach and supernatant precipitation for a more general profiling of the outer sub-proteome fraction [12] of bacteria. In a quantitative comparison of *S. aureus* proteome between growing (exponential phase) and non-growing cells (stationary phase), Becher et al. also used surface shaving as a part of a sub-proteomic fractionation strategy to characterize an extensive fraction of the bacterial proteome, covering about 80% of all expressed genes [13].

As stressed by Dreisbach et al. in their recent review [5], post-translational modifications of surface proteins have been scarcely studied so far, although they could be important for cell wall binding, surface exposure, modulation of virulence factors and evasion of immune response. Ravipaty and Reilly observed several modifications, such as oxidation, acetylation, formylation, loss of methionine and various processing events — removal of signal peptide, proteolytic processing — in *S. aureus* surface and secreted proteins [14]. Data reported from *S. aureus* and other bacteria suggest also that glycosylation of surface proteins may play a role in pathogenesis and antigenicity, for example through its contribution to antigen diversity or modulation of adhesion [15,16,17]. Recent studies also showed that phosphorylation is important in the central metabolic processes of *S. aureus*, but it does not seem to play a direct role in bacterial virulence through modulation of adhesion and invasion of host cells [18]. Nevertheless, the function of protein phosphorylation in staphylococcal pathogenesis still needs further investigation to be clarified.

In this paper we report the LC-MS/MS analysis of surface proteins by "trypsin-shaving" of *S. aureus* cells (strain Newman) in time course experiments between the exponential and the late stationary growth phase. Our investigation was focused on unexpected post-translational modifications (PTM) and uncovered an unusual PTM, hydroxymethlyation of asparagine and glutamine, in some proteins expressed in *S. aureus* grown in an iron-poor medium. We show evidence that this is a genuine PTM and not a sample preparation or analytical artifact, and we argue for the revision of its actual status, as it has been considered so far as dubious.

2. Material and methods

2.1. *Bacterial strains and growth conditions*

Staphylococcal strain used was *S. aureus* Newman. Staphylococci were grown at 37 °C either in tryptic soy broth (TSB, Becton Dickinson, USA), or in Roswell Park Memorial Institute culture medium 1640 (RPMI — Gibco, USA), without agitation. Growth was followed by colony counting and by determining the culture optical densities using a spectrophotometer (OD 600 nm, Ultrospec 500 pro, Amersham Biosciences). Bacterial stocks were kept frozen at −80 °C in 20% (vol/vol) glycerol.

2.2. *Preparation of bacterial cells for proteolysis of surface proteins*

Whole live cells were "trypsin-shaved" following a slightly modified version of a recently described protocol [8]. Bacteria were grown in 300 ml batch cultures. At various times during exponential growth or stationary phase, aliquots (depending on the cell density) were removed, immediately chilled, harvested by centrifugation, resuspended and washed 3× with PBS before being resuspended in 1 ml of the same buffer. To ensure the quantitative comparability of the proteomes analyzed from samples taken at various growth times, each sample was adjusted to the same number of cell bodies (1×10^9 cells/ml) as assessed by optical microscopy, using a Neubauer cell. These samples were immediately processed for trypsin shaving as described below.

2.3. *Proteolytic digestion and sample preparation for mass spectrometry*

Bacterial bodies were adjusted to a final concentration of 1×10^9/ml and predigested ("shaved") with 1 μg of porcine trypsin (Promega, Madison, USA) for 1 h at 37 °C without agitation. Treated samples were immediately chilled and bacterial bodies were removed by centrifugation for 10 min at 4000 rpm at 4 °C. Supernatants were filtered (0.22 μm) and freeze-dried before being prepared for LC-MS analysis.

Pre-digested peptides from shaving were resuspended in 100 mM ammonium bicarbonate buffer, reduced with 45 μM 1,4-dithio-dl-threitol (DTT, Sigma) for 30 min at 60 °C and alkylated with 100 mM iodoacetamide (IAA, Sigma) for 30 min in the dark. The resulting mixture was digested for 4 h with 1 μg of modified sequencing-grade porcine trypsin (Promega, Madison, USA). The digested peptides were desalted through Sep-Pak tC18 cartridges (Waters, Milford, USA) as described by the manufacturer and consecutively eluted with 1 ml of 60% and 1 ml of 30% acetonitrile (Merck, Germany). Solution of purified peptides were pooled, dried under vacuum, and kept at −20 °C.

2.4. *Liquid chromatography-tandem mass analysis and protein identification*

Samples were analyzed on a hybrid linear trap LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) interfaced via a TriVersa Nanomate (Advion Biosciences, Norwich, UK) to a Agilent 1100 nano HPLC system (Agilent Technologies, Waldbronn, Germany). Solvents used for the mobile phase were 95:5 H_2O:acetonitrile (v/v) with 0.1% formic acid (A) and 5:95 H_2O:acetonitrile (v/v) with 0.1% formic acid (B).

Peptides were loaded onto a trapping microcolumn ZORBAX 300SB C18 (5 mm × 300 μm ID, 5 μm, Agilent) in H_2O:acetonitrile 97:3 (v/v) + 0.1% formic acid at a flow rate of 10 μl/min. After 5 min, they were back-flush eluted and separated on a reversed-phase nanocolumn ZORBAX 300SB C18 column (75 μm ID × 15 cm, 3.5 μm, Agilent) at a flow rate of 300 nl/min with a gradient from 5 to 85% acetonitrile in 0.1% formic acid: 5 min at 0% of solvent B, from 0 to 25% of B in 35 min, 25 to 50% B in 15 min, 50 to 90% in 5 min, 90% B during 10 min, 90 to 0% in 5 min and 15 min at 0% (total time: 90 min).

For spraying, a 400 nozzle ESI Chip (Advion Biosciences) was used with a voltage of 1.65 kV, and the mass spectrometer capillary transfer temperature was set at 200 °C. In data-dependent acquisition controlled by Xcalibur 2.0 software (Thermo Scientific), the four most intense precursor ions

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detected in the full MS survey performed in the Orbitrap (range 350–1500 m/z, resolution 60 000 at m/z 400) were selected and fragmented. MS/MS was triggered by a minimum signal threshold of 10 000 counts, carried out at relative collision energy of 35% (CID), with isolation width of 4.0 amu. Only precursors with a charge higher than one were selected for CID fragmentation. Fragment ions were analyzed at low resolution in the LTQ linear trap, or at high resolution (7500) in the Orbitrap for some analyses. The m/z of fragmented precursors was then dynamically excluded, with a tolerance of 0.01 amu, from any selection during 120 s. For confirmatory analyses, some samples were also reanalyzed on a LTQ-Orbitrap Velos instrument (Thermo Scientific, Bremen, Germany) interfaced to an Ultimate 3000 RSLC nano HPLC system (Dionex, Olten, Switzerland), using high resolution CID and HCD MS/MS spectra acquisition. From raw files, MS/MS spectra were exported as mgf files (Mascot Generic File, text format) using the extract_msn.exe script from Thermo Scientific.

MS/MS spectra were analyzed using Mascot 2.3 (Matrix Science, London, UK). Mascot was set up to search both a subset of UniProt database (release 15.8), which contained only proteins of *S. aureus* strain Newman (2594 sequences), and a custom-built database containing the sequences of usual contaminants (enzymes, keratins, etc). Trypsin (cleavage at K, R, not before P) was used as the enzyme definition allowing up to 1 missed cleavage. Mascot was searched with a parent ion tolerance of 10 ppm and a fragment ion mass tolerance of 0.50 Da, or 0.02 Da with high resolution MS/MS data. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Deamidation of asparagine and glutamine and oxidation of methionine were specified as variable modifications. In a second pass search, hydroxymethylation of asparagine and glutamine was also added as variable modification, and the corresponding configuration file modified to include the neutral loss of water in the Mascot scoring.

Scaffold (version Scaffold_3_09, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications, and to perform dataset alignment. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm, using high mass accuracy scoring [19]. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm [20]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principle of parsimony.

2.5. *Control experiment with Protein A*

To determine if a potential hydroxymethylation activity was present in the RPMI medium, Protein A standard coupled to sepharose beads (Protein A Sepharose CL-4B, GE Healthcare) was incubated at 37 °C overnight either in H_2O or in standard RPMI 1640 medium (Invitrogen, Carlsbad, CA). Samples were then digested with trypsin, and the resulting peptides were analyzed by LC-MS/MS as described above. MS/MS spectra were searched against the UniProt database (release 2011_03) restricted to *S. aureus* taxonomy and the identification results were validated with Scaffold.

3. Results and discussion

Surface proteins ("surfaceome") of cultured *S. aureus* Newman cells were recovered by proteolysis ("trypsin-shaving") of live bacteria at 4 different time points between early exponential (~2 h) and late stationary (24 h) growth phases. These growth kinetics experiments were realized in triplicates in iron-rich TSB or in iron-poor RPMI medium. After tryptic digestion, the resulting peptides were analyzed by LC-MS/MS, and proteins were identified using Mascot as database search software. Based on spectral counting, we observed an increase of various surface and membrane proteins between exponential and early stationary phases, such as immunoglobulin G binding protein (Protein A, A6QD95), 65 kDa membrane protein (A6QIG2: OMP7_STAAE), extracellular matrix protein-binding protein (A6QF98: EMP_STAAE), fibronectin-binding protein A (A6QJY9). Additionally a specific increase in the iron-regulated surface determinants IsdA (A6QG31), IsdB (A6QG30) and IsdH (A6QHR4) were seen with the RPMI medium, as expected in low iron growth conditions [12,21]. Additional results regarding kinetics of adhesins in these experiments will be discussed elsewhere, while we focus here on the discovery of post-translational modifications.

Looking for unexpected PTMs, we performed an error-tolerant search on some selected data of staphylococci grown in the RPMI medium. In this mode, Mascot searches database entries found by a first pass classical MS/MS search with relaxed enzyme specificity, while iterating through a comprehensive list of chemical and post-translational modifications, together with a residue substitution matrix. As the statistical significance of error-tolerant search results is difficult to evaluate, these must be interpreted very cautiously, but nevertheless may allow pinpointing some potentially interesting PTMs. In our case, several peptides of a few proteins appeared to be modified with a shift of 30.011 u, which was matched by Mascot to either hydroxymethylation of asparagine (Fig. 1), or a substitution of glycine or alanine by respectively a serine or threonine residue, both modifications being isomeric and thus isobaric. As we found no indication in the UniProt database of alanine or glycine substitution in the proteins considered here, we decided to investigate the potential presence of a hydroxymethyl modification.

Although an addition of a hydroxymethyl group to peptides is already described as PTM of Asn in the RESID database (www.ebi.ac.uk/RESID), this modification is poorly documented. We reanalyzed our data with Mascot, specifying hydroxymethylation of asparagine and glutamine as variable modification. Although the hydroxymethylation of glutamine is not documented in the RESID reference, it was included here by analogy. With this new database search, several peptides were observed with an apparent hydroxymethylation of asparagine or glutamine residues, which motivated further investigation. Selected samples in which putative hydroxymethylated peptides appeared to have the highest concentrations were thus reanalyzed by LC-MS/MS, using high resolution MS/MS for a more confident interpretation of tandem mass spectra. These analyses were carried out either on an LTQ-Orbitrap XL or LTQ-Orbitrap Velos instrument, using data-dependent acquisition or inclusion lists.

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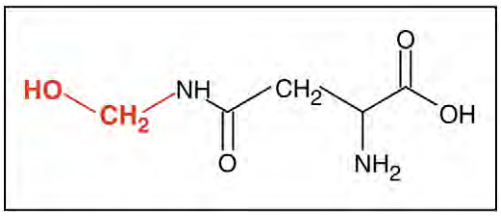

Fig. 1 – Structure of N4-hydroxymethylasparagine according to RESID database (AA0236, www.ebi.ac.uk/RESID). The net mass difference relative to asparagine is +30.0106 u.

3.1. *Evidence for hydroxymethylation of asparagine and glutamine*

The first step in the characterization of hydroxymethylation as a PTM was to determine if it was a genuine modification or the result of an artifact, for example the combination of a methylation and an oxidation, or a point mutation. As shown in Fig. 2, displaying the MS/MS spectra of native and modified peptide ADSYVPYTIAVNGTSTPILSK (from protein OMP7), the 30.01 shift is clearly localized either on N12 or on G13. Although a substitution of the glycine by a serine is theoretically possible as discussed above, it seems here to be an unlikely event as it would not explain the disappearance of the b12 and y9 ions around the modification site in the MS/MS spectrum of the modified peptide. Indeed, we observed such a change in fragmentation pattern — i.e. absence of the fragments corresponding to the modified residues — in most of the spectra assigned to hydroxymethylated peptides, which often made the exact localization of the modification quite difficult. Furthermore, in all samples analyzed the unmodified peptides with the native sequence were clearly detected and identified with a strong signal, making the presence of mutants unlikely. An addition of a hydroxymethyl group on the asparagine seems therefore a better explanation for the observed shift. More importantly, no "intermediate" methylated or oxidized form of the peptide was observed in the MS data, which undermines the hypothesis of methylation plus oxidation on two adjacent residues, as such a complete double modification would be very unlikely. Interestingly we observed only a small retention time shift of 20 s between the unmodified and the hydroxymethylated peptide. This seems to be compatible with the modification proposed here. On one hand a supplementary methylene group should make the sequence more hydrophobic, on the other hand the modified peptide would become more hydrophilic because of the presence of a free hydroxyl group, the resulting retention time shift being probably small.

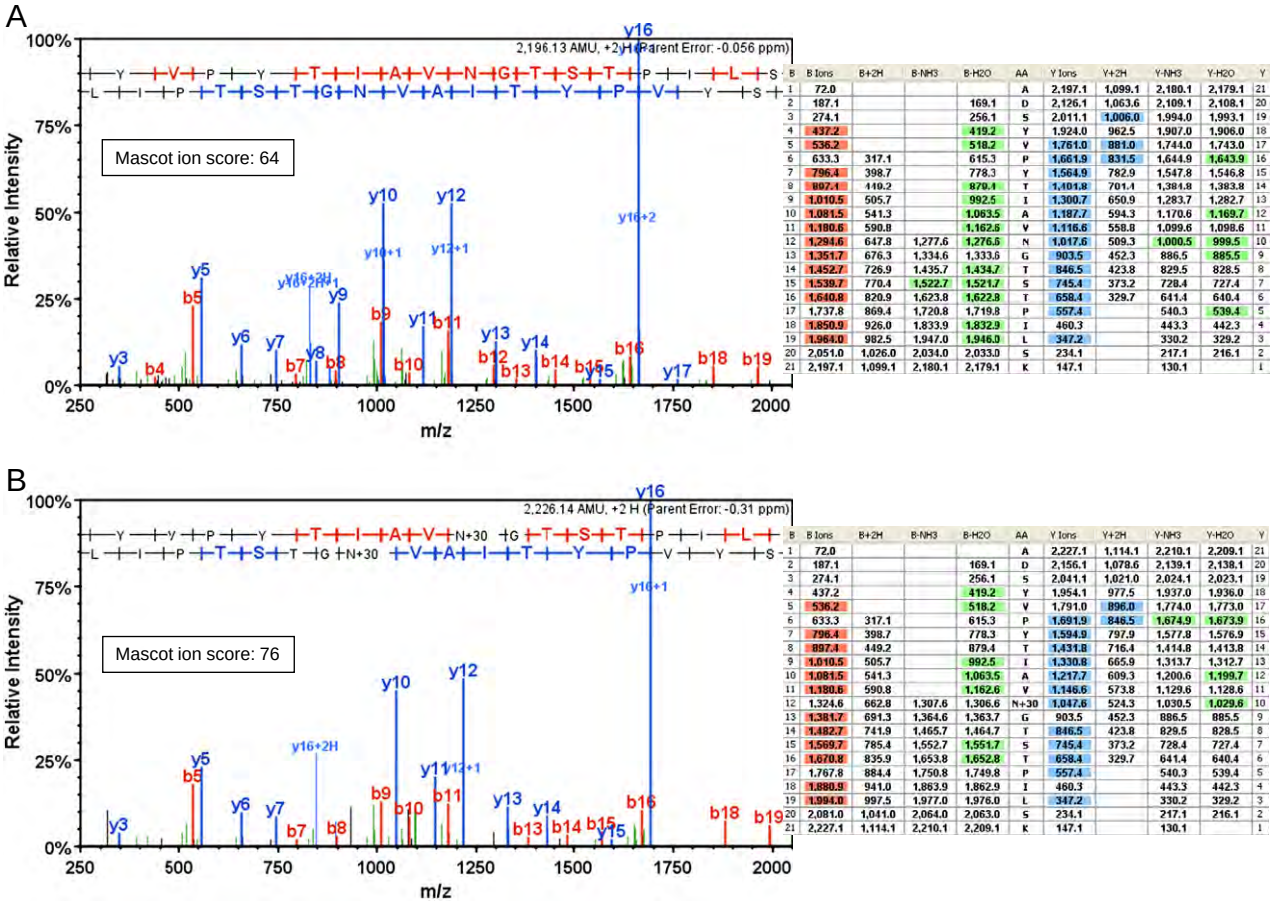

Fig. 2 – Tandem mass spectra of peptide ADSYVPYTIAVNGTSTPILSK (A) from protein OMP7 and its modified version (B). The shift of 30.01 u at position N12 corresponds to a hydroxymethylation. Spectra were acquired on a LTQ-Orbitrap XL at resolution 7500, from peptides fragmented by CID, and annotated with the software Scaffold.

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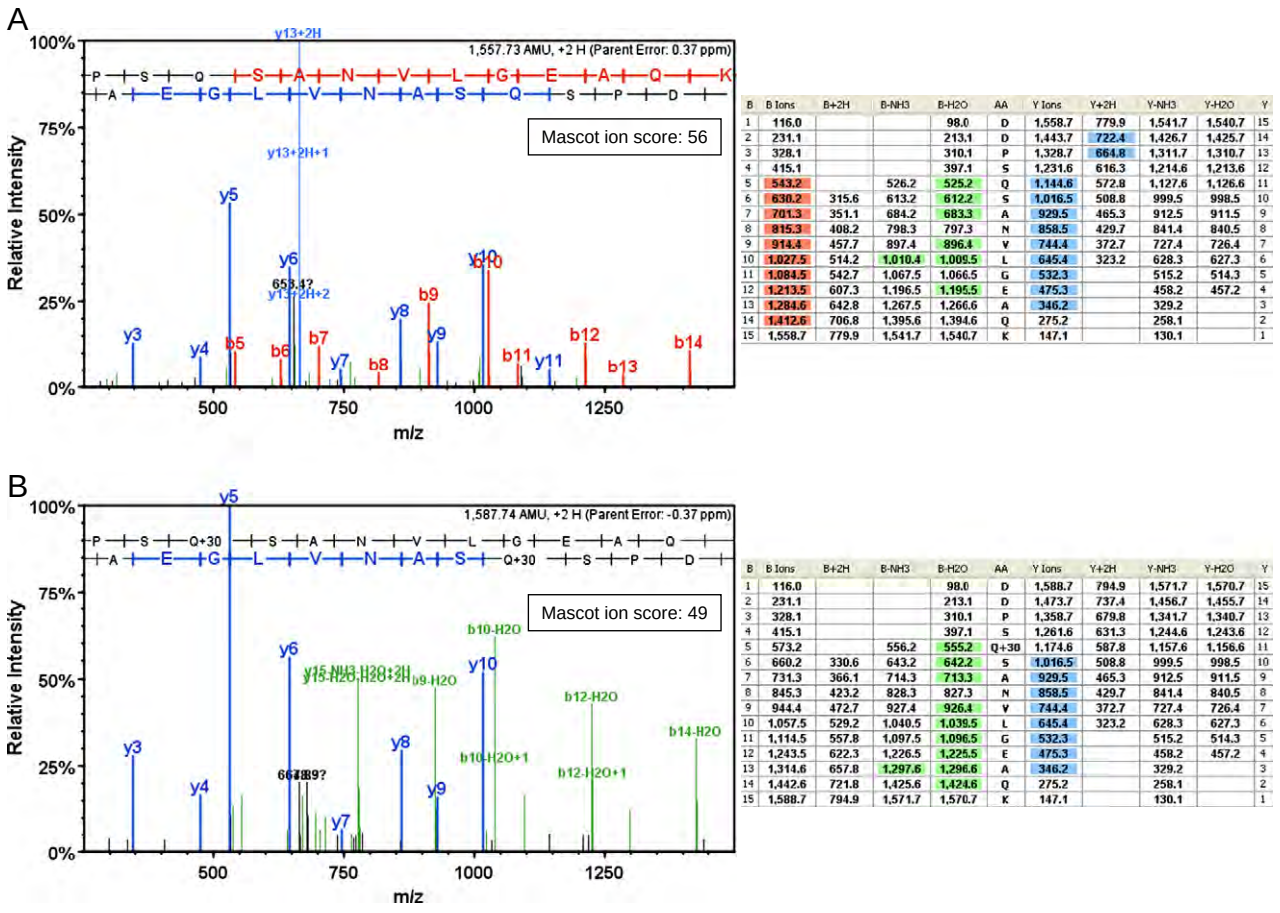

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	116.0			98.0	D	1,558.7	779.9	1,541.7	1,540.7	15
2	231.1			213.1	D	1,443.7	722.4	1,426.7	1,425.7	14
3	328.1			310.1	P	1,328.7	664.8	1,311.7	1,310.7	13
4	415.1			397.1	S	1,231.6	616.3	1,214.6	1,213.6	12
5	543.2		526.2	525.2	Q	1,144.6	572.8	1,127.6	1,126.6	11
6	630.2	315.6	613.2	612.2	S	1,016.5	508.8	999.5	998.5	10
7	701.3	351.1	684.2	683.3	A	929.5	465.3	912.5	911.5	9
8	815.3	408.2	798.3	797.3	N	858.5	429.7	841.4	840.5	8
9	914.4	457.7	897.4	896.4	V	744.4	372.7	727.4	726.4	7
10	1,027.5	514.2	1,010.4	1,009.5	L	645.4	323.2	628.3	627.3	6
11	1,084.5	542.7	1,067.5	1,066.5	G	532.3		515.2	514.3	5
12	1,213.5	607.3	1,196.5	1,195.5	E	475.3		458.2	457.2	4
13	1,284.6	642.8	1,267.5	1,266.6	A	346.2		329.2		3
14	1,412.6	706.8	1,395.6	1,394.6	Q	275.2		258.1		2
15	1,558.7	779.9	1,541.7	1,540.7	K	147.1		130.1		1

B	B Ions	B+2H	B-NH3	B-H2O	AA	Y Ions	Y+2H	Y-NH3	Y-H2O	Y
1	116.0			98.0	D	1,588.7	794.9	1,571.7	1,570.7	15
2	231.1			213.1	D	1,473.7	737.4	1,456.7	1,455.7	14
3	328.1			310.1	P	1,358.7	679.8	1,341.7	1,340.7	13
4	415.1			397.1	S	1,261.6	631.3	1,244.6	1,243.6	12
5	573.2		556.2	555.2	Q+30	1,174.6	587.8	1,157.6	1,156.6	11
6	660.2	330.6	643.2	642.2	S	1,016.5	508.8	999.5	998.5	10
7	731.3	366.1	714.3	713.3	A	929.5	465.3	912.5	911.5	9
8	845.3	423.2	828.3	827.3	N	858.5	429.7	841.4	840.5	8
9	944.4	472.7	927.4	926.4	V	744.4	372.7	727.4	726.4	7
10	1,057.5	529.2	1,040.5	1,039.5	L	645.4	323.2	628.3	627.3	6
11	1,114.5	557.8	1,097.5	1,096.5	G	532.3		515.2	514.3	5
12	1,243.5	622.3	1,226.5	1,225.5	E	475.3		458.2	457.2	4
13	1,314.6	657.8	1,297.6	1,296.6	A	346.2		329.2		3
14	1,442.6	721.8	1,425.6	1,424.6	Q	275.2		258.1		2
15	1,588.7	794.9	1,571.7	1,570.7	K	147.1		130.1		1

Fig. 3 – Tandem mass spectra of peptide DDPSQSANVLGEAQK (A) of immunoglobulin G binding protein A and its modified version (B). The shift of 30.01 u at position Q5 corresponds to a hydroxymethylation. Spectra were acquired on a LTQ-Orbitrap Velos at resolution 7500, from peptides fragmented by CID, and annotated with the software Scaffold.

In Fig. 3, displaying the MS/MS spectra of native and modified peptide DDPSQSANVLGEAQK (from protein A6QD95), we show an example of a glutamine hydroxymethylation (Q5). More strikingly than in the previous example, the modification changed the fragmentation pattern. Fragment ions of y series corresponding to the putative hydroxymethylated glutamine and those N-terminal of the modified site were not observed in the MS/MS spectrum, preventing the unambiguous localization of the hydroxymethyl on glutamine, based on this ions series. This phenomenon was best illustrated by the disappearance of the base peak corresponding to fragment ion $y13^{(2+)}$ in the spectrum of the modified peptide. Furthermore, only b ions with neutral loss of H_2O appeared in the modified spectrum, while a clear b ion series was observed with the unmodified peptide. Incidentally this b-H_2O ions series allowed a clear localization of the modification on Q5, confirming the presence of a hydroxymethylated glutamine. When the Mascot fragment matching settings were changed to include neutral loss of water in the scoring of the hydroxymethylation, the score of the modified peptide increased from 20 to 49. Similar to the first example discussed above, no "intermediate" methylated or oxidized form was observed in the MS data, and the retention times of the modified and unmodified peptide were very close (about 7 s difference). These data further support the idea that the mass shift observed is due to a hydroxymethyl group. The data also suggest that the modification considered is not a methoxy group (isomeric with hydroxymethyl), as shown by the similar retention time and the easy loss of H_2O during the fragmentation of the peptide. Indeed, the increased neutral loss of water in the modified peptide can be explained by the presence of a terminal hydroxyl group.

Similarly, in Suppl. Fig. 1 showing the spectrum of peptide VNVATNNPASQQVDK (from protein EMP) hydroxymethylated on asparagine N2, only b ions with loss of H_20 were observed while the unmodified peptide produced a clear b series after CID fragmentation. Including water neutral loss in Mascot scoring of hydroxymethyl modification increased the ion score from 49 to 72. Modified and native peptides almost co-eluted, with a difference in retention time of about 10 s.

3.2. *Characterization of hydroxymethylated proteins*

The dataset of staphylococci grown in iron-rich RPMI medium, including triplicate analyses of *S. aureus* surfaceome at 4 different time points, was searched again with hydroxymethylation

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Table 1 – Hydroxymethylated peptides identified in *S. aureus* Newman grown in RPMI medium. Aliquots of staphylococci were taken at various times between exponential growth and stationary phase, based on optical density (DO) measurements. Growth experiments were carried out in triplicates. Tandem mass spectra were acquired in the LTQ trap of a LTQ-Orbitrap XL instrument, while precursor masses were measured at high resolution in the Orbitrap. Hydroxymethylated peptides with an asterisk (*) were confirmed by high resolution MS/MS spectra. Protein accession IDs are from UNIPROT (www.uniprot.org).

Protein description/UniProt name (accession number)	Peptide sequence [a]	Highest Mascot Ion score	Calculated hydroxymethylated Peptide Mass (AMU)	T1: ~3 h DO=0.2	T2: ~5 h DO=0.5	T3: 6–7 h DO=0.8	T4: 24 h D0=1.0–1.3	Protein region
				Number of modified spectra[b]				
Immunoglobulin G binding protein A (Protein A)	DDPSQSANLLSEAK *	39	1503.7	2(2)		1(1)	2(2)	B3 repeat
A6QD95_STAAE (A6QD95)	DDPSQSANVLGEAQK *	53	1587.7		1(1)	2(2)	1(1)	B1 repeat
MW: 57 kDa	NGFIQSLKDDPSQSANLLAEAK *	53	2375.2	1(1)	1(1)	1(1)	2(2)	B4 repeat
	NGFIQSLKDDPSQSTNVLGEAK *	44	2377.2			4(2)	2(1)	B2 repeat
	NGFIQSLKDDPSQSANLLSEAK *	64	2391.2	2(2)	2(2)	2(2)	1(1)	B3 repeat
	NGFIQSLKDDPSQSANVLGEAQK	61	2475.2			1(1)	2(2)	B1 repeat
	DQQSAFYEILNMPNLNEAQR *	85	2410.1	1(1)	5(2)	5(2)	2(2)	B2 repeat
	EQQNAFYEILHLPNLTEEQR	22	2501.2	1(1)				B5 repeat
	EQQNAFYEILNMPNLNEEQR *	59	2509.2	2(2)	3(2)	2(2)	1(1)	B3 repeat
	EQQNAFYEILHLPNLNEEQR	29	2514.2		2(2)			B4 repeat
	ADNNFNKEQQNAFYEILNMPNLNEEQR	45	3312.5	1(1)	1(1)	2(2)	2(2)	B3 repeat
	ADAQQNNFNKDQQSAFYEILNMPNLNEAQR	33	3540.6		1(1)	2(2)		B2 repeat
	Sum of modified spectra (% of total spectra)			10 (6.1%)	16 (8.1%)	22 (8.5%)	15 (9.0%)	
	Total number of spectra (sum of triplicates)			164	197	258	167	
Iron-regulated surface determinant protein B	APETKPVANAVSVSNK	22	1640.9		1(1)			
ISDB_STAAE (A6QG30)	ATNNTYPILNQELR *	40	1675.9				2(2)	
MW: 72 kDa	SAITEFQNVQPTNEK	42	1734.8		1(1)	2(2)	2(2)	
	YMoxVMoxETTNDDYWK	36	1756.7			1(1)		NEAT 2 domain
	YVVYESVENNESMMDTFVK	48	2313.0			2(1)	1(1)	NEAT 2 domain
	Sum of modified spectra (% of total spectra)				2 (2.5%)	5 (2.2%)	5 (4.4%)	
	Total number of spectra (sum of triplicates)			10	79	230	113	
65 kDa membrane protein	SGIYTANLINSSDIK *	56	1624.8		2(2)	2(2)		MAP2 repeat
OMP7_STAAE (A6QIG2)	AGIYTADLINTSEIK	38	1637.9		2(2)	2(2)		MAP1 repeat
MW: 66 kDa	YVPYTIAVNGTSTPILSDLK *	65	2181.2				3(3)	MAP4 repeat
	ADSYVPYTIAVNGTSTPILSK *	99	2226.1				3(3)	MAP5 repeat
	Sum of modified spectra (% of total spectra)				4 (4.9%)	4 (3.5%)	6 (6.1%)	
	Total number of spectra (sum of triplicates)			24	81	114	99	
Iron-regulated surface determinant protein A	FYNANNQELATTVVNDNK *	59	2084.0		1(1)	2(2)	1(1)	NEAT domain
ISDA_STAAE (A6QG31)	FYNANNQELATTVVNDNKK *	51	2212.1			2(2)	2(2)	NEAT domain
MW: 39 kDa	Sum of modified spectra (% of total spectra)				1 (3.3%)	4 (5.1%)	3 (7.5%)	
	Total number of spectra (sum of triplicates)			9	30	79	40	

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Glyceraldehyde 3-phosphate dehydrogenase	**Q**DVTVEQVNEAMK	25	1519.7		1(1)			
A6QF81_STAAE (A6QF81)	TIVFNTNH**Q**ELDGSETVVSGASCTTNSLAPVAK	44	3476.7	1(1)				
MW: 36 kDa	Sum of modified spectra (% of total spectra)			1 (1.7%)	1 (1.6%)			
	Total number of spectra (sum of triplicates)			60	63	32	43	
Fructose-bisphosphate aldolase	I**N**VNTENQIASAK	63	1430.7	1(1)				
A6QIW9_STAAE (A6QIW9)	Sum of modified spectra (% of total spectra)			1 (4.8%)				
MW: 31 kDa	Total number of spectra (sum of triplicates)			21	23	7	13	
Extracellular matrix protein-binding protein emp	V**N**VATNNPASQQVDK *	53	1613.8			2(2)	2(2)	
EMP_STAAE (A6QF98)	Sum of modified spectra (% of total spectra)					2 (4.9%)	2 (5.7%)	
MW: 38 kDa	Total number of spectra (sum of triplicates)			6	16	41	35	
Fibronectin binding protein A	AV**Q**APQTAQPANIETVK	71	1794.9		1(1)	2(2)	1(1)	Ligand-binding A region
A6QJY9_STAAE (A6QJY9)	Sum of modified spectra (% of total spectra)				1 (3.7%)	2 (4.3%)	1 (2.1%)	
MW: 81 kDa	Total number of spectra (sum of triplicates)			15	27	47	47	
Leukocidin/hemolysin toxin family S subunit	TISY**N**QQNYDTIASGK	52	1831.9				2(2)	
A6QIL8_STAAE (A6QIL8)	Sum of modified spectra (% of total spectra)						2 (8.3%)	
MW: 40 kDa	Total number of spectra (sum of triplicates)			16	22	36	24	
Major cold-shock protein CspA	SLEEG**Q**AVEFEVVEGDR	36	1921.9	1(1)				
A6QGV3_STAAE (A6QGV3)	Sum of modified spectra (% of total spectra)			1 (12.5%)				
MW: 7 kDa	Total number of spectra (sum of triplicates)			8	10	5	2	
Probable transglycosylase isaA	LSNG**N**TAGATGSSAAQIMAQR	90	2035.0	1(1)				
ISAA_STAAE (A6QK59)	Sum of modified spectra (% of total spectra)			1 (3.4%)				
MW: 24 kDa	Total number of spectra (sum of triplicates)			29	25	23	11	
30S ribosomal protein S16	IIEQIGTY**N**PTSANAPEIK	42	2088.1	1(1)				
RS16_STAAE (A6QGD8)	Sum of modified spectra (% of total spectra)			1 (11.1%)				
MW: 10 kDa	Total number of spectra (sum of triplicates)			9	7	5	6	
Elongation factor Tu	**N**GDSVAQSYDMIDNAPEEK	64	2111.9		1(1)			
EFTU_STAAE (A6QEK0)	Sum of modified spectra (% of total spectra)				1 (0.8%)			
MW: 43 kDa	Total number of spectra (sum of triplicates)			112	121	43	195	
50S ribosomal protein L1	VSFTDE**Q**LIENFNTLQDVLAK	62	2453.2				1(1)	
RL1_STAAE (A6QEJ0)	Sum of modified spectra (% of total spectra)						1 (4.5%)	
MW: 25 kDa	Total number of spectra (sum of triplicates)			17	14	9	22	
Putative uncharacterized protein	DG**N**FYQTNVDANGVNHGGSEMVQNK	44	2724.2		1(1)			
A6QDB8_STAAE (A6QDB8)	Sum of modified spectra (% of total spectra)				1 (6.3%)			
MW: 26 kDa	Total number of spectra (sum of triplicates)			14	16	19	15	

[a]In blue, site(s) of the modification with the highest Mascot ion score; in red, alternate sites to which were assigned other MS/MS spectra with lower Mascot score. When the sites could be clearly localized, they are displayed in bold.
[b]Sum of triplicates experiments. In brackets, number of replicates where the modified peptide was observed. The sum of modified spectra per protein and time point across all replicates was divided by the corresponding total number of spectra assigned to each protein to calculate the "% of total spectra".

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of asparagine and glutamine as variable modification, including neutral loss of water in Mascot scoring. As these analyses were realized with CID fragmentation and spectra acquisition in the LTQ part of the LC-MS system, higher sensitivity was expected for finding hydroxymethylation, but also a higher false positive identification of the modified peptides because of the lower resolution and accuracy of MS/MS spectra. We therefore applied a stringent filtering and identification of peptides was only accepted if it could be established at greater than 95.0% probability based on Peptide Prophet algorithm built-in Scaffold software [19], giving a peptide FDR below 1.0%. As an additional filter, peptides that were only observed in the hydroxymethylated form (and not in unmodified form) were discarded.

Using these criteria, hydroxymethylation was observed in 35 peptides from 15 proteins in the RPMI dataset (Table 1, Suppl. Figs. 2 and 3), which corresponded to 41 modified sites (25 N, 16 Q), 35 of them being unambiguously localized. Some variability in occurrence of hydroxymethylation was observed between replicates: while a similar number of modified peptides appeared in two replicates, only 2 hydroxymethylated peptides (in protein OMP7) were seen in the third replicate experiment. Because of the quite low stoichiometry of the modification, in most cases below 10% based on spectral counting, hydroxymethylated peptides were mostly seen in abundant proteins such as immunoglobulin-binding protein A, or when proteins were expressed at higher abundance during growth of staphylococci, as IsdB and OMP7 at stationary phase. As notable exceptions, some proteins of low abundance (30S ribosomal protein S16, major cold-shock protein CspA) were also found to be modified. Altogether only a small subset of the 321 proteins present in this dataset contained a hydroxymethylation. The modification appeared thus not to be randomly widespread in correlation with protein abundance, but on the contrary showed some selectivity. More than 80% of modified sites were localized on membrane, secreted or cell wall associated proteins, while some abundant cytoplasmic proteins found in our dataset were only marginally modified — Elongation factor Tu (A6QEK0), Glyceraldehyde 3-phosphate dehydrogenase (A6QF81) (see Table 1) — or not modified — Elongation factor G (A6QEJ9) and Pyruvate kinase (A6QHN2) (data not shown). Selectivity of hydroxymethylation is also suggested by the modification of peptides of similar sequence from different regions in Protein A (B repeat, predicted by homology with SPA_STAAU) [22] and OMP7 (MAP (Eap) repeat) [23], and by the modification of peptides belonging to the NEAT domain [24] in ISDA and ISDB proteins (Table 1). This seems to single out hydroxymethylation as a genuine PTM, and not as an artifact of sample preparation. Indeed, staphylococci grown in iron-rich TSB medium were analyzed in the same conditions and after identical sample preparation and no hydroxymethylated peptides were observed in any of the 381 proteins identified in this dataset. In particular no modification appeared in immunoglobulin protein A (see example of XICs in Suppl. Figs. 4 and 5), although it was the most abundant protein.

As a supplementary control to test for a potential hydroxymethylating activity in the RPMI medium itself, a sample of Protein A standard was incubated overnight at 37 °C either in H_2O or in RPMI, digested with trypsin and analyzed by LC-MS/MS (Suppl. Table 1). While similar sequence coverage as in *S. aureus* samples was observed, only one and two putatively modified peptides, each one matched by a unique spectrum, were identified in the H_2O and RPMI samples respectively. Concomitantly, the unmodified versions of the same peptides were clearly identified with respectively 35 (H_2O) and 39 (RPMI) spectra matched for both peptides. Thus, despite using a fairly large amount of material, only weak evidence for hydroxymethylated peptides was found in this standard. More importantly, no significant difference was observed between samples incubated in H_2O and RPMI.

3.3. *Hydroxymethylation of trypsin*

Although hydroxymethylation seemed to be an authentic post-translational modification, two peptides from the digestion enzyme trypsin appeared to be also modified (Suppl. Fig. 6) in the same samples. The abundant trypsin peptide LGEHNIDVLEGNEQFINAAK in particular was identified as hydroxymethylated, mostly on N5, in several replicate experiments at different time points of *S. aureus* grown in RPMI medium (Suppl. Table 2). Relative to its unmodified counterpart, the peptide showed changes in fragmentation pattern and elution behavior similar to what observed for other hydroxymethylated peptides. This suggests that it is not a specific artifact of trypsin, for example due to some isobaric combination of modifications. Indeed, such putative trypsin modified peptides were never observed in the TSB dataset, or in other unrelated samples from different origin analyzed in our laboratory using the same brand of trypsin for protein digestion. If we accept that these trypsin peptides are genuine hydroxymethylated peptides only observed when *S. aureus* was grown in RPMI medium, we could explain this surprising result by postulating the modification of trypsin during the pre-digestion ("shaving") step of sample preparation. During this step, trypsin could be exposed to some active enzyme responsible for hydroxymethylation on the *S. aureus* surface. Such a localization of this activity would be compatible with the preferential modification of membrane and surface proteins observed in our data. It should be noted here that in our experiments trypsin was not exposed to RPMI medium, since the bacteria were washed with buffer before the shaving procedure. This, in addition to the Protein A standard test discussed above, rules out any specific activity of the medium itself.

3.4. *Hydroxymethylation: a new post-translational modification ?*

Hydroxymethylation of asparagine is annotated in the RESID database (http://www.ebi.ac.uk/RESID, AA0236) as dubious (see also Unimod database: www.unimod.org, accession number 414). Indeed, its first "identification" in cyanobacteria of the genus *Anabaena* was unclear and based on low resolution MS data [25]. Furthermore, to our knowledge this modification has not been found again in these bacteria or in any other organism. Nevertheless, our results indicate that the status of Asn and Gln hydroxymethylation should be revised. Although it is not possible to completely exclude that some of the modifications found in our data could be explained by the presence of unknown protein isoforms containing amino acids

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substitutions — such as Gly→Ser and Ala→Thr — this cannot account for all our observations given the very diverse amino acid composition of the detected modified peptides. The extent of hydroxymethylation in proteins like Protein A and the variation in the presence of the modification depending on *S. aureus* growth conditions support the notion of hydroxymethylation being a true post-translational modification. It can appear surprising that this PTM has not been mentioned in the literature since the first report 25 years ago. One possible reason could be that high resolution/high accuracy mass measurements are necessary to distinguish it from other PTMs with similar mass increase. Furthermore, hydroxymethylation seems to occur only in specific culture conditions, at least in the case of *S. aureus*, and preferentially on surface proteins. It is thus certainly easier to detect it when specific sample preparation methods are used for surface protein enrichment before proteomics analyses. Based on our data no sequence consensus was found, although in many cases hydroxymethylation seemed to appear preferentially in regions rich in asparagine or glutamine.

4. Conclusions

In this study we identified hydroxymethylation in various proteins of *S. aureus* and demonstrated that the status of this modification, previously considered as dubious, should be revised. We also presented evidence suggesting that it is an authentic PTM and not an artifact due to sample preparation or analytical conditions. Its structure could not be unambiguously characterized, although our data tend to rule out the isomeric methoxy form. Structural analysis, such as NMR, would be necessary to confirm the structure shown in the RESID database. Tandem MS analyses of synthetic peptides could also allow determining more precisely the influence of hydroxymethylation on CID fragmentation of modified peptides, as discussed above, and in this way help to clarify the structure.

The function of this PTM is for now fully unknown. As it was mostly found on some surface proteins of *S. aureus*, we can hypothesize that it has a function in the modulation of staphylococci virulence. To further assess its function, it would be very useful to identify the enzyme responsible for the hydroxymethylation activity. There is currently no information on a possible candidate, although there is some indication that this enzyme, too, could be localized on the surface of the bacteria. In UniProt database of *S. aureus* Newman proteins there are presently 2078 sequences which have been only automatically annotated (UniProtKB/TrEMBL), including 930 proteins noted as "putative uncharacterized protein". This means that there is still a large part (about 80%) of this proteome which is poorly known and could indeed contain an enzyme with a specific activity explaining the modifications observed in our data.

Altogether, further work will be necessary for fully assessing hydroxymethylation from both the analytical and biological points of view. We hope that data presented here will generate interest among microbiologists, and that this study will contribute to expand the landscape on post-translational modifications in bacteria.

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Annexe 2

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Penicillin-Binding Protein Gene Alterations in *Streptococcus uberis* Isolates Presenting Decreased Susceptibility to Penicillin[▽]

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Streptococcus uberis **is an environmental pathogen commonly causing bovine mastitis, an infection that is generally treated with penicillin G. No field case of true penicillin-resistant *S. uberis* (MIC > 16 mg/liter) has been described yet, but isolates presenting decreased susceptibility (MIC of 0.25 to 0.5 mg/liter) to this drug are regularly reported to our laboratory. In this study, we demonstrated that *S. uberis* can readily develop penicillin resistance in laboratory-evolved mutants. The molecular mechanism of resistance (acquisition of mutations in penicillin-binding protein 1A [PBP1A], PBP2B, and PBP2X) was generally similar to that of all other penicillin-resistant streptococci described so far. In addition, it was also specific to *S. uberis* in that independent resistant mutants carried a unique set of seven consensus mutations, of which only one ($Q_{554}E$ in PBP2X) was commonly found in other streptococci. In parallel, independent isolates from bovine mastitis with different geographical origins (France, Holland, and Switzerland) and presenting a decreased susceptibility to penicillin were characterized. No mosaic PBPs were detected, but they all presented mutations identical to the one found in the laboratory-evolved mutants. This indicates that penicillin resistance development in *S. uberis* might follow a stringent pathway that would explain, in addition to the ecological niche of this pathogen, why naturally occurring resistances are still rare. In addition, this study shows that there is a reservoir of mutated PBPs in animals, which might be exchanged with other streptococci, such as *Streptococcus agalactiae*, that could potentially be transmitted to humans.**

Penicillin resistance has been particularly well studied for the human pathogen *Streptococcus pneumoniae* (5, 15). In this organism, resistance occurs through modifications of penicillin-binding proteins (PBPs), leading to a decreased affinity for the drug. These modifications include mutations and/or mosaics in PBP2X and PBP2B, as well as in PBP1A for the highly resistant isolates (20). Several studies also indicated that full expression of resistance necessitates mutations both in the *pbp* genes and in other genes, of which only a few have been determined, namely, in the *ciaRH*, *cpoA*, and *murMN* loci (8, 9, 14). The same mechanism has also been found in viridians streptococci, which are occasionally responsible for human infections (18, 26). Very recent reports also demonstrated the implication of PBP mutations in *S. agalactiae* presenting a decreased susceptibility to penicillin (4, 17, 21). In contrast, the highly pathogenic *Streptococcus pyogenes*, which has been widely exposed to penicillin for decades, is apparently incapable of acquiring clinical resistance *in vivo* (22), even though laboratory mutants presenting decreased susceptibilities were reported (11). Thus, the capacity to develop penicillin resistance varies among different bacteria belonging to the same genus.

S. pneumoniae is primarily a human pathogen, while *S. agalactiae* infects humans and sometimes animals. In contrast, *S. uberis* and *Streptococcus suis* are primarily distributed among cattle and pigs, respectively, in which they may be responsible for serious and life-threatening diseases. For instance, *S. uberis* is commonly responsible for clinical and subclinical bovine mastitis, a type of infection that causes major economic loss in the dairy industry worldwide. Recent surveys in England and in France demonstrated that this bacterial species was implicated in about 20% of clinical and subclinical mastitis (2, 3). In addition, *S. uberis* can also be found in the environment surrounding the animal.

Currently penicillin remains one of the first-line antibiotics for prophylaxis and treatment of such pathologies. Moreover, despite several decades of widespread use of this molecule, it is still commonly believed that *S. uberis*, as well as other *Streptococcus* spp. implicated in animal intramammary infections, are susceptible to this drug. However, although it is true that no high-level penicillin resistance (MIC > 16 mg/liter) has yet been described for these bacteria, isolates with intermediate resistance (MICs ranging from 0.5 to 16 mg/liter) have been reported (10, 24).

The present study shows that decreased susceptibilities to penicillin can be achieved in *S. uberis* after repeated exposures to increasing drug concentrations in the laboratory, despite its phylogenetic link with *S. pyogenes* (27). Decreased susceptibility correlated with specific mutations in selected PBP genes. Moreover, similar PBP mutations were also detected in clinical isolates displaying decreased penicillin susceptibility that were collected in France, Holland, and Switzerland. These results highlight the reality of a reservoir of mutated PBPs in animals and suggest that resistance development in *S. uberis* might follow a quasiobligatory pathway.

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TABLE 1. Primers used for PCR amplification[a]

Gene	Forward sequence	Reverse sequence	Size (bp)
pbp1A	5′-G_{572}GCGACATCTGGATGAAAAT	5′-G_{1289}CCATTGTTCCAACATAATCA	718
pbp1B	5′-C_{1167}TTTGGCGGTTTGCTAGATG	5′-G_{2056}GATGGCGTTGGCTAGATTA	890
pbp2A	5′-A_{1275}GGGCTTGTTGGTCGTGTTA	5′-C_{2008}GGTCTTGTTAAAACCGATCC	734
pbp2B	5′-C_{971}TATGTCGGGCTTGTCTCGT	5′-T_{1849}GGCAACAGCTACTTCAGGA	879
pbp2X	5′-T_{936}GCAGATACTTTAGAAGGCTTGAA	5′-G_{1739}GAACCATTGCTACGACTGAG	804
murM	5′-T_{-27}GCATTACGTCAGTGGGTTT	5′-T_{+55}CAACTGATTCAGAAAAGGTTTCA	1,306
murN	5′-C_{-25}GCAAACAATTAAGGAGTAAACA	5′-G_{+21}CTGAAGCACTTGGTTTTTGA	1,279
fruA	5′-C_{901}CATTTGTTATTGGTGGTGGT	5′-G_{1790}CACCAGTTAAGGCAGAACC	890
pepC	5′-G_{136}CAACCGCTGATCAAGATTT	5′-C_{998}AGTCATCAGAGGCCACAAA	863

[a] −, number of nucleotides upstream of the start codon; +, number of nucleotides downstream of the stop codon.

(Part of this work was presented at the 1st ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Food-borne Pathogens, Copenhagen, Denmark, 2008 [abstract C121].)

MATERIALS AND METHODS

Microorganisms and growth conditions. *S. uberis* ATCC 19436 and two penicillin-susceptible *S. uberis* strains isolated from cow mastitis cases were used as model organisms for the selection of laboratory-cycled penicillin-resistant mutants. These were compared to seven *S. uberis* isolates recovered from mastitis cases in France, Holland, and Switzerland and displaying decreased penicillin susceptibilities (MICs of 0.25 to 0.5 mg/liter). All strains were grown at 37°C either in brain heart infusion broth (BHI) (AES, Combourg, France) without aeration or on Columbia agar (Oxoid, Dardilly, France) supplemented with 3% of blood. Bacterial stocks were stored at −80°C in broth supplemented with 10% (vol/vol) of glycerol.

Antibiotics and chemicals. Penicillin G was purchased from Sigma (Saint Quentin Fallavier, France). All other chemicals were reagent grade, commercially available products.

Antibiotic susceptibility. The MICs of penicillin were determined by a previously described broth macrodilution method (1) with a final inoculum of *ca*. 10^6 CFU/ml. The MIC was defined as the lowest antibiotic concentration that inhibited visible bacterial growth after 24 h of incubation at 37°C.

Selection for penicillin resistance by successive cycling. Selection for penicillin-resistant mutants was performed in broth cultures by exposing bacteria to stepwise-increasing concentrations of antibiotics (7). The whole cycling experiment was followed over a minimum of 50 cycles and repeated with 3 independent isolates (strains ATCC 19436, 8749, and 9529). Individual mutants were isolated by picking single colonies from agar plates, regrown in broth, and stored at −80°C for further study.

Identification of PBP genes in *S. uberis*. The DNA sequences of genes encoding putative PBP1A, PBP1B, PBP2A, PBP2B, and PBP2X of *S. uberis* were determined by homology searches from its nonannotated sequence, available at http://www.sanger.ac.uk. BLAST searches were performed using the corresponding amino acid sequences from *S. pyogenes* (accession numbers NP_269695 [PBP1A], NP_268494 ′PBP1B[, NP_270001 [PBP2A], and NP_269705 [PBP2X]) and *S. pneumoniae* (NP_359110.1 [PBP2B]).

DNA preparation and genetic strategies. Molecular techniques were carried out by using standard methods (25) or by following instructions provided with commercially available kits and reagents. Genomic DNA was extracted using the Qiagen DNeasy tissue kit (Qiagen, Courtaboeuf, France). Resistance mutations were sought in the transpeptidase domains of *pbp* genes and in the *murMN* operon. These genes, as well as *fruA* and *pepC*, which were used as negative-control genes, were amplified by PCR on a thermocycler (Bio-Rad, Richmond, CA). Primers were purchased from Sigma and are presented in Table 1. Cycling conditions consisted of 30 cycles at 94°C for 30 s, 52°C for 45 s, and 72°C for 1.5 min, followed by a 10-min delay period at 72°C after the last cycle. Amplicons were sequenced in both directions (Genome Express, Meylan, France). Sequences were analyzed using the LALIGN program on the Infobiogen website (www.fr.embnet.org).

Visualization of PBPs with Bocillin FL. First, membrane-enriched proteins were extracted from one liter of bacterial culture in the late exponential phase of growth (optical density at 620 nm [OD_{620}] of *ca*. 0.7) as described previously (12). The concentration of membrane proteins were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA), with bovine serum albumin as a standard. Then, 10 μg of membrane proteins were mixed with 15 μM Bocillin FL (Invitrogen) and incubated at 37°C for 45 min. The reaction was stopped by adding 4 μl of a rinsing solution (unlabeled penicillin [60 mg/ml] and the detergent Sarkosyl [10%]), followed by 15 min of incubation at room temperature before the samples were resuspended in loading buffer (1:1 ratio; 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.1% bromophenol blue in 50 mM Tris-HCl [pH 6.8], and 100 mM dithiothreitol) and heated at 95°C for 3 min. The labeled PBPs were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 6% gels and were visualized directly by fluorography using a Typhoon Trio+ variable-mode imager (excitation at 488 nm and emission at 520 nm; GE Healthcare). Images were analyzed using the ImageQuant TL software program (GE Healthcare). Gels were then stained with Coomassie brilliant blue to visualize the standard and all membrane proteins.

RESULTS

***S. uberis* PBPs.** Five PBPs were detected *in silico*, which were named according to their pneumococcal homologues. The presence of these five high-molecular-weight PBP genes was confirmed in *S. uberis* by PCR, and the expression of their protein products was assessed by gel separation of Bocillin-FL labeled membranes (Fig. 1).

Selection for penicillin resistance. The MIC of the *S. uberis* ATCC 19436 reference strain was 0.032 mg/liter. When the strain was cycled with increasing concentrations of penicillin G, the MIC increased from the wild-type level up to 2 mg/liter over 30 cycles (Table 2), representing a 60-fold increase. The 2-mg/liter level constituted a plateau, since despite continuous cycling (up to 50 cycles) the MIC did not increase any more. According to the recommendations of either the Antibiogram

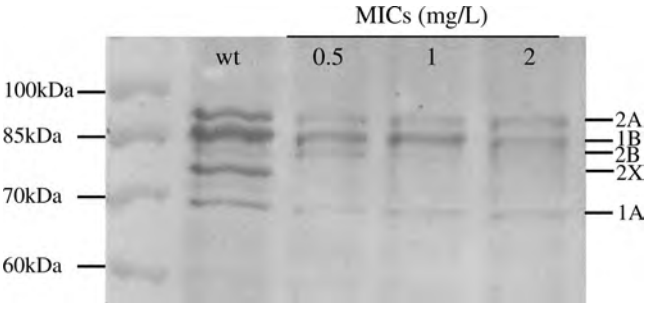

FIG. 1. PBP profiles of *S. uberis* ATCC 19436 wild type and its derivatives, presenting increasing MICs. Proteins were separated by SDS-PAGE, and Bocillin-PBP complexes were visualized by fluorography. Molecular mass markers are indicated at the left of the fluorogram, and PBP numbers, named after their homologues in *S. pneumoniae*, are at the right. The wild type (wt) and cycled mutants with increased penicillin MICs are indicated at the top.

TABLE 2. PBP mutations in laboratory-evolved *S. uberis* strains

Isolate	No. of cycles	MIC (μg/ml)	Mutations		
			PBP1A	PBP2B	PBP2X
ATCC 19436	0	0.032	—[a]	—	—
ATCC 19436	15	0.5	$A_{227}T$	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
ATCC 19436	18	1	$A_{227}T$	$N_{366}I/T_{402}I$	$E_{381}K/D_{524}A/Q_{554}E$
ATCC 19436	30	2	$A_{227}T/G_{386}R$	$N_{366}I/T_{402}I$	$E_{381}K/D_{524}A/Q_{554}E$
8749	0	0.016	—	$N_{366}I/T_{402}I/V_{570}A/P_{575}S$	$A_{492}E$
8749	16	0.5	$R_{273}H$	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
8749	28	1	$A_{227}T/G_{386}R$	$N_{366}I/T_{402}I$	$E_{381}K/D_{524}A/Q_{554}E$
8749	37	2	$A_{227}T/G_{386}R$	$N_{366}I/T_{402}I$	$E_{381}K/D_{524}A/Q_{554}E$
9529	0	0.128	—	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
9529	11	0.5	$R_{273}P$	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
9529	28	1	$R_{273}P$	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
9529	39	2	$A_{227}T/G_{386}R$	$N_{366}I/T_{402}I$	$E_{381}K/D_{524}A/Q_{554}E$

[a] —, no detected mutation.

Committee of the French Society for Microbiology (CA-SFM; sensitive, ≤0.25 mg/liter; resistant, >16 mg/liter) or of the CLSI (sensitive, ≤0.12 mg/liter; resistant, >2 mg/liter), these mutants are considered intermediately resistant.

The whole cycling experiment was repeated with two additional isolates tested independently. One of them had a basal MIC of 0.016 to 0.032 mg/liter (strain 8749), and one had an increased basal MIC of 0.128 mg/liter (strain 9529), which would correspond to intermediate resistance according to CLSI criteria. Interestingly, this strain already carried four consensus resistance mutations in PBP2X and PBP2B (see details in the next section).

In all three strains (ATCC 19436, 8749, and 9529), the MIC increased step by step at a steady rate. The rapidity with which the different steps were reached differed slightly between the isolates, but the general pattern remained unchanged and reproducible.

Resistance mutations in penicillin-resistant derivatives from a single culture. Mutations were first sought in the transpeptidase domains of PBP2B, -2X, and -1A of the three *S. uberis* cycled isolates (ATCC 19436, 8749, and 9529). The relevance of the detected mutations was validated by the absence of alterations in the two control genes, *fruA*, which is not implicated in the penicillin-dependent pathway, and *pepC*, which is located close to PBP1A but does not belong to the same operon, both in the wild-type strains and their resistant derivatives. Table 2 presents the mutations found in the parent strains and at MIC levels of 0.5, 1, and 2 mg/liter. The specific locations of all PBP mutations are schematically represented in Fig. 2.

At baseline, no sequence differences were observed between the analyzed PBP domains of the ATCC 19436 wild-type strain and the nonannotated genome available on the Sanger website (www.sanger.ac.uk/Projects/S_uberis/). In this strain, the first PBP mutations were detected at an MIC of 0.5 mg/liter, and

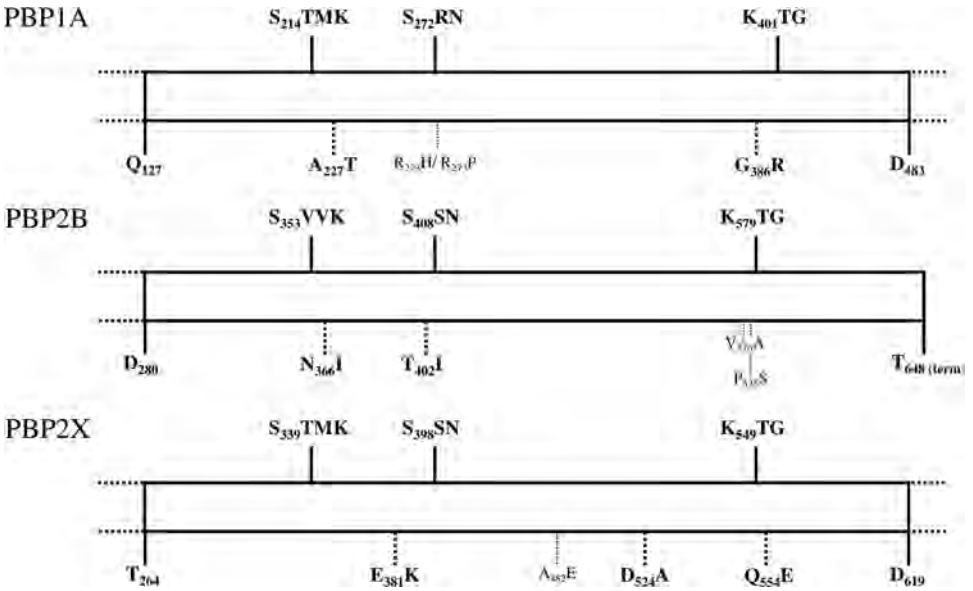

FIG. 2. Schematic representation of the transpeptidase domains of PBP1A, PBP2B, and PBP2X. The three conserved motifs of the active site (SXXK, SXN, and KXG) are indicated at the tops of the schemes. The mutations detailed in Table 2 are indicated at the bottoms of the schemes. Bold characters correspond to mutations detected in all laboratory-evolved isolates.

TABLE 3. PBP mutations in *S. uberis* isolates naturally presenting decreased susceptibility to penicillin

Isolate	Origin	MIC (μg/ml)	Mutation(s)		
			PBP1A	PBP2B	PBP2X
ALP8092	Switzerland	0.25	$G_{386}R$	—[a]	$E_{381}K/Q_{554}E$
ALP 8144	Switzerland	0.25	—	—	$E_{381}K/Q_{554}E$
BL246	Switzerland	0.25	—	$N_{366}I/T_{402}I$	$E_{381}K/Q_{554}E$
1.11	Holland	0.25	$G_{386}R$	$T_{593}A$	$E_{381}K/Q_{554}E$
20568	France	0.5	$G_{386}R$	$N_{366}I/T_{402}I/V_{570}A/P_{575}S$	$E_{381}K/Q_{554}E$
20592	France	0.5	—	—	$E_{381}K/Q_{554}E$
20618	France	0.25	$G_{386}R$	—	$E_{381}K/Q_{554}E$

[a] —, no detected mutation.

three PBPs were affected. First, class B PBP2X, which is a main determinant of penicillin resistance in *S. pneumoniae*, carried the $E_{381}K$ and $Q_{554}E$ mutations, which were both located close to a conserved motif (Fig. 2). Second, class B PBP2B also carried two substitutions ($N_{366}I$ and $T_{402}I$) that were located in the vicinity of conserved domains. Third, class A PBP1A already carried an $A_{227}T$ mutation at this early resistance step, although it is reputed to be mutated only in pneumococci with high-level resistance.

At the second step of resistance (MIC of 1 mg/liter), these five mutations were conserved, and an additional one was detected in PBP2X ($D_{524}A$). At the last step (MIC of 2 mg/liter), a second substitution in PBP1A appeared ($G_{386}R$), which was also located in the vicinity of conserved motifs (Fig. 2). Both PBP1A modifications led to the conversion of simple hydrophobic amino acids (alanine or glycine) into a more complex hydrophilic threonine or basic arginine.

This progressive accumulation of mutations paralleling the step-by-step increase in the MIC was a feature of each strain, and most interestingly, the same seven consensus mutations were systematically detected in all of them at the highest level of MIC (at 2 mg/liter). Nevertheless, slight differences occurred in between, as shown by the appearance of transient mutations that disappeared at higher resistance levels. For example, isolate 8749 already carried two PBP2B mutations at the basal MIC level, in addition to three other transitory alterations. This strain was cycled in triplicate to test whether these transient mutations ($V_{570}A$ and $P_{575}S$ in PBP2B and $A_{492}E$ in PBP2X) might be deleterious for penicillin resistance development. Yet in all triplicates, they were always detected both at the wild-type level and at an MIC of 0.5 mg/liter, suggesting that whereas they are not necessary for penicillin resistance development, they are not impeaching it either. Similarly, isolate 9529, which had an increased MIC (0.128 mg/liter) and already carried four mutations in PBP2B and PBP2X at the basal level (Table 2), also displayed a mutation in PBP1A ($R_{273}P$) that disappeared after it had reached an MIC of 2 mg/liter and developed its whole set of consensus mutations.

Mutations were also sought in the *murMN* operon, at the basal level and at an MIC of 2 mg/liter. In all three strains, two mutations were detected in the *murN* gene in comparison to the Sanger sequence, both at the basal level and in laboratory-evolved mutants. Two additional substitutions in *murM* were also transiently present in the noncycled ATCC 19436 strain. However, these modifications were not linked to penicillin resistance development, since they were already present at the basal level.

Impact of mutations on the affinity for penicillin. The PBPs of wild-type ATCC 19436 and the two associated mutants presenting increasing MICs to penicillin were analyzed on Bocillin-FL-labeled gels (Fig. 1). A progressive fading of all PBPs could be observed, but the ones corresponding to nonmutated PBP2A and -1B seemed less impacted. On the contrary, the bands corresponding to PBP2B and -2X had already completely disappeared in the mutant presenting an MIC of 1 mg/liter.

Resistance mutations in environmental *S. uberis*. The transpeptidase domains of PBP1A, -2B, and -2X of seven noncycled strains isolated from bovine mastitis cases and presenting a decreased susceptibility to penicillin (MICs of 0.25 to 05 mg/liter) were sequenced. No mosaic PBPs were identified, as assessed by the correct PCR amplification of their transpeptidase domains and the absence of internal fragments presenting highly diverging sequences compared to both the nonannotated Sanger sequence and the sequences of the other isolates tested in this study. On the other hand, all mastitis isolates carried ≥2 of the consensus resistance mutations found in the laboratory-cycled strains (Table 3). The two alterations ($E_{381}K$ and $Q_{554}E$) in PBP2X were systematically detected. Mutations in PBP1A and -2B were not found in all isolates, but whenever present, they were in conformity with the consensus ($G_{386}R$ in PBP1A, $N_{366}I$ and $T_{402}I$ in PBP2B). Consequently, very similar PBP mutations could be observed between laboratory-evolved mutants and the natural strains with decreased penicillin susceptibility.

DISCUSSION

In this study, the capacity of *S. uberis* to develop penicillin resistance was questioned, first, because *S. uberis* is one of the principal causes of streptococcal mastitis; second, because penicillin is widely used to treat intramammary infections in animals; and third, because field cases of *S. uberis* strains presenting decreased susceptibility to this drug were reported to our laboratory. In addition, *S. uberis* is phylogenetically linked to *S. pyogenes*, which is reputedly incapable of developing penicillin resistance. The results obtained *in vitro* demonstrated that a step-by-step increase in the MIC can occur for *S. uberis*. This was exemplified by the 60-fold increase in the MIC that progressively developed over 30 cycles of penicillin exposure in the

reference strain ATCC 19436 and in the two other strains studied.

PBP alterations have been extensively described as the major mechanism leading to penicillin resistance in *S. pneumoniae* (5). Additionally, PBP alterations were shown to be implicated in decreased susceptibility in *Streptococcus gordonii* and more recently in *S. agalactiae* (13, 17). Here the development of laboratory-evolved mutants, which presented MICs that were increasing concomitantly to the accumulation of mutations in both class A and class B PBPs, demonstrates that *S. uberis* shared the same resistance mechanism common to all streptococci. In PBP2B, two recurrent mutations were detected in the vicinity of the SVVK and SSN domains, both generating an isoleucine residue that might play a central role in protein stability because of its critical importance for binding of ligands to proteins. In PBP2X, all mutants systematically harbored three mutations at an MIC of 2 mg/liter, among which was the $Q_{554}E$ alteration. This substitution is characteristic of all penicillin-resistant streptococci described so far (4, 13, 19, 21) and was shown to be one of the major genetic determinants of pneumococcal resistance (23). Interestingly, two PBP2X mutations and two PBP2B mutations were already found in the wild-type 9529 isolate, which displayed an MIC of 0.128 mg/liter. Conserved mutations were also observed in PBP1A, close—but not inside—the STMK and KTG motifs. In coherence with the presence of point mutations in class B and class A PBPs, a likely decrease in the affinity of the proteins for penicillin was evidenced with Bocillin FL-labeled gels. Thus, all of these mutations were probably responsible for the resistance of the isolates, though to different extents.

All mutations were highly conserved between laboratory-evolved mutants, apart from a few marginal alterations that were transiently detected, as already observed in *S. gordonii* (data not published) and probably reflecting the high mutability of these PBP proteins upon selection by penicillin. Additionally, the same mutations were present in isolates from bovine mastitis that presented decreased susceptibilities to penicillin. More precisely, two PBP2X mutations were systematically found ($E_{381}K$ and $Q_{554}E$), alone or in association with PBP2B or PBP1A substitutions. On the contrary, no mosaic proteins were detected in these natural isolates, since their transpeptidase domain could be amplified by PCR and presented sequences quasi-identical to that of the wild-type ATCC reference strain. This high conservation between mutations from unrelated isolates (laboratory and environmental strains from diverse origins) seems to be specific to *S. uberis*. Indeed, in pneumococci, as in other *Streptococcus* spp., high-level MICs of penicillin are generated by a diversity of different patterns of mutations (6, 13, 16, 21). This specificity of *S. uberis* might argue for a strong pressure of selection on very precise regions of the protein and probably represents a quasiobligatory pathway for the development of decreased susceptibility to penicillin in this particular species. Moreover, constraint seems particularly strong considering the fact that all the resistant environmental isolates from France, Holland, and Switzerland carried the same types of mutations. Such a stringent selection mechanism, combined with the ecological niche on a usually sterile mammary gland, which decreases the chances of horizontal gene transfer with other species, might explain why no *S. uberis* penicillin-resistant strains have been reported yet.

In addition to PBP-related mutations, a few other genes have been implicated in penicillin resistance (8, 9, 14). Here the *murMN* operon was sequenced, but the only detected alterations were either already present in the wild-type strain or transient, which indicates that they were most probably not related to resistance development. However, the fact that the same set of mutations can be found in two successive laboratory-evolved mutants with different MICs (strains 8749 and 9529, MICs of 1 mg/liter and 2 mg/liter; Table 2), as well as the existence of environmental isolates with the same pattern but different MICs (ALP 8144 and 20592; Table 3) or with different patterns but the same MIC (20568 and 20592), indicates that other still-unknown genetic determinants have an important function.

Potential genetic exchanges are undoubtedly less frequent for bacteria living in the open environment or on the normally sterile mammary gland than for an organism living in the oral cavity, like *S. pneumoniae*, in close contact with thousands of other species. Yet each step toward decreased susceptibility might be a risk for the emergence of new resistant phenotypes. Consequently, attention should be paid to any shift of population toward progressively increased MICs.

An ultimate practical question relates to the phenotypic and genotypic definition of resistance. In *S. uberis*, a simultaneous $E_{381}K/Q_{554}E$ substitution in PBP2X reliably correlated with a penicillin MIC of $\geq$0.125 mg/liter, while partner mutations could differ at this stage. Furthermore, a set of 7 consensus mutations in PBP1A, -2B, and -2X was reproducibly observed at an MIC of 2 mg/liter. Although this correlation needs to be confirmed with larger numbers of isolates, it could provide a genetically based marker for newly defined intermediate resistance (MIC $\geq$ 0.125 mg/liter) and high-level resistance (MIC $\geq$ 2 mg/liter), which would conform with CLSI criteria, compared to the CA-SFM criteria (MIC $\geq$ 0.25 mg/liter and MIC $\geq$ 16 mg/liter for intermediate and full resistance, respectively). Moreover, it would also help in rationalizing the conceptual differences between epidemiological cutoff values, which are defined to differentiate a wild-type population from a diverging one, and clinical breakpoints, which are related primarily to the therapeutic chances of success.

In conclusion, the present work demonstrates that *S. uberis* can develop decreased susceptibilities to penicillin. This development involves mechanisms that are in part common to all streptococci (accumulation of PBP mutations, leading to a decreased affinity for the drug) and in part specific to *S. uberis* (accumulation of highly conserved alterations). Numerous and precise mutations related to penicillin resistance acquisition were described, which might contribute to the pool of resistance determinants exchangeable between streptococci—for instance, between *S. uberis* and *S. agalactiae*, which are both causative agents of intramammary infections in cattle. Yet the use of penicillin as a first-line therapeutic choice can still be recommended in veterinary medicine. But the use of epidemiological cutoff values that would be more stringent, combined with molecular tools, seems mandatory for the survey of the evolution of resistance in *S. uberis* strains causing bovine mastitis and for decreasing the contribution of animals to the pool of penicillin-resistant streptococci shared by animals and humans.

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