The GTP-dependant pleiotropic repressor "CodY" regulates biofilm formation in Staphylococcus aureus

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THÈSE
Présentée à la Faculté des sciences de l'Université de Genève pour obtenir le grade de Docteur ès sciences, mention biologie

Par

Ludwig STENZ
De
Künten (AG)
(Suisse)

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La Faculté des sciences, sur le préavis de Messieurs P. LINDER, professeur ordinaire et codirecteur de thèse (Faculté de médecine – Département de microbiologie et médecine moléculaire), J. SCHRENZEL, professeur associé et codirecteur de thèse (Faculté de médecine – Département de médecine interne), D. SHORE, professeur ordinaire et codirecteur de thèse (Département de biologie cellulaire) et Madame C. WOLZ, professeure (Universität Tübingen – Institut für Medizinische Mikrobiologie und Hygiene – Tübingen, Deutschland), autorise l'impression de la présente thèse, sans exprimer d'opinion sur les propositions qui y sont énoncées.

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Abbreviations

(Aaa) Multifunctional autolysins/adhesins

(*agr*) Accessory gene regulator

(AIP) auto-inducing peptide

(Atl) Autolysins

(Bap) Biofilm associated protein

(BCAA) Branched chain amino acid

(CDC) Centers for Disease Control and prevention

(ChIP) Chromatin immunoprecipitation

(ClfA & ClfB) Clumping factors A & B

(Cna) Collagen adhesin

(CP8) type 8 capsular polysaccharide

(Dsg1) desmoglein 1

(EAP) Extracellular adherence protein

(Ebh) Extracellular matrix-binding homologue

(eDNA) Extracelullar DNA

(Emp) Extracellular matrix binding protein

(EPS) Extracellular polymeric substance

(Ets) exfoliative toxins

(FnBP) Fibronectin binding protein

(HTLV-1) Human T cell leukemia virus type 1

(IsdA, IsdB, IsdC, IsdH) iron-regulated surface determinants

(MLVA) Multi-locus variable number of tandem repeat analysis

(MRSA) Methicillin-resistant *Staphylococcus aureus*

(MSCRAMM) Microbial surface components recognizing adhesive matrix molecules

(NCBI) The National Center for Biotechnology Information

(NDM-1) New Delhi metallo-beta-lactamase 1

(ORF) Open reading frames

(ORSA) Oxacillin-resistant *S. aureus*

(PbPs) Penicillin binding proteins

(PIA) Polymeric intercellular adhesin

(Pls) Plasmin-sensitive adhesin

(PUFA) Free polyunsaturated fatty acids

(PVL) Panton-Valentine leukocidin

(Rot) repressor of toxins

(RNAP) RNA polymerase

(SCCmec) Staphylococcal chromosome cassette *mec*

(SD) Shine-Dalgarno sequence

(Sdr) Serine-aspartate repeats proteins family

(Sec) Secretion pathway

(SraP) Serine-rich adhesin for platelets

(SrtA) Sortase A

(SrtB) Sortase B

(SSSS) staphylococcal scalded-skin syndrome

(TcaR) Teicoplanin-associated locus regulator

(TSST-1) Toxic shock syndrome toxin-1

(VISA) Vancomycin-intermediated *S. aureus*

(VRSA) Vancomycin-resistant *S. aureus*

(vWF) von Willebrand factor

(WHO) World Health Organization

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Résumé

Le sujet d'étude de la présente thèse effectuée en vue de l'obtention du titre de docteur ès science mention biologie/médicine porte sur l'analyse des fonctions du gène appelé *codY* dans la formation de biofilm chez la bactérie *Staphylococcus aureus*. De manière générale, les bactéries sont en effet

capables selon les circonstances d'adhérer à une surface et de produire une matrice extracellulaire collante, ce que l'on appelle un biofilm. Les biofilms à *S. aureus* sont responsables de nombreuses infections associées aux implants et autres types de matériels médicaux, le biofilm adhérant au matériel implanté. Ces types d'infection sont caractérisés par une difficulté de traitement, une chronicité de l'infection (=persistance), une résistance aux antibiotiques inhérente au mode de vie en biofilm, et donc la fréquente nécessité d'extraire le matériel implanté afin de traiter localement l'infection puis éventuellement de réopérer s'il convient de déposer un nouvel implant. Cela représente un coût conséquent à la fois économique, mais également humain. Pour toutes ces raisons, les biofilms sont de plus en plus étudiés en maladies infectieuses.

Le gène *codY* est présent chez les bactéries gram-positifs et à faible contenu en GC tel que *S. aureus*. Il code pour une protéine capable de s'activer en fixant les acides aminés branchés et le GTP. CodY est capable de réprimer l'expression d'une multitude d'autres gènes en se fixant sur des séquences palindromiques présentes dans l'ADN de la bactérie. Une première étude du laboratoire d'accueil a mis en évidence l'implication de ce gène dans la formation de biofilm chez une souche particulièrement productrice de biofilm, appelée S30.

Afin d'étudier plus en profondeur les mécanismes moléculaires en liens avec CodY qui affectent la formation de biofilm, différents gènes ont été inactivés, soit par disruption, soit par substitution, et les conséquences de ces altérations de gènes sur la production de biofilm ont été mesurées.

Les résultats obtenus ont notamment montré une régulation complexe de CodY sur un opéron appelé *icaADBC*. Cet opéron est responsable de la production d'un polymère extracellulaire appelé PIA, composant principal des matrices des biofilms à *S. aureus* ainsi qu'à *S. epidermidis*.

Le séquençage de la région régulatrice de cet opéron chez la souche S30 qui forme anormalement beaucoup de biofilm a permis l'identification d'une mutation probablement naturelle dans le répresseur de l'opéron appelé *icaR*. L'ajout artificiel d'un allèle normal d'*icaR* dans cette souche sur un plasmide entraîne une diminution drastique de la quantité mesurée de biofilm produit alors que le plasmide lui-même n'affecte pas la formation de biofilm. Nous en déduisons que la mutation d'une paire de base de l'ADN sur un génome qui en contient environ 2,9 millions a probablement été la cause principale de la forte production de biofilm chez S30, même si la preuve formelle nécessite la restauration de la copie normale du gène dans le génome lui-même, ce qui est techniquement plus difficile à réaliser. Cette souche de *S. aureus* a été responsable d'une infection associée à un cathéter posé sur un bébé aux Hôpitaux Universitaires de Genève, au début des années 1970. De manière surprenante, il s'est avéré que l'inactivation du gène *codY* dans cette souche cause une très forte

diminution du biofilm alors que dans la souche UAMS-1, qui produit au départ beaucoup moins de biofilm, la mutation de ce même gène entraîne un effet opposé sur la quantité de biofilm produit. Afin d'expliquer ce paradoxe, les génomes des souches ont été séquencés. Les résultats ont montré que la souche UAMS-1 est elle aussi affectée par une mutation naturelle qui se situe de manière fort intéressante sur un répresseur de *ica*R appelé *rbf*. Ainsi, deux mutations dont les effets sur le contrôle de l'opéron *icaADBC* sont supposés opposés d'après les connaissances rapportées dans la littérature, sont présentes individuellement dans deux souches différentes, et pourraient permettre d'expliquer l'effet opposé de la mutation du gène *codY* sur le biofilm produit par ces deux souches.

Deux aspects sont mis en lumière dans ce travail concernant l'effet de CodY sur les biofilms de *S. aureus*. D'abord, le contrôle transcriptionel complexe de l'opéron *icaADBC* par CodY implique que cet opéron est régulé en fonction de l'état nutritionnel de la bactérie, lui-même mesuré partiellement par les taux de GTP et d'isoleucine. De plus, deux sites d'attachement pour CodY ont été découverts, *in vivo* par ChIP et *in silico* par prédiction bioinformatique, dans *ica*R et dans *icaB*. Si CodY se fixe dans *icaB* ou *ica*R, son effet devrait être opposé sur l'expression de l'opéron. La subsitution de la guanine en position 292 en adénine a provoqué un codon stop à la place d'un codon glutamine dans *ica*R chez S30. Cette mutation est localisée à une distance de 22 paires de base (pb) en aval (selon le sense de lecture d'icaR) de la séquence AATTTTCTGGAAATA. Cette séquence correspond à deux substitutions près à la séquence consensus AATTTTCWGAAAATT, reconnue comme le site d'attachement de CodY. Nos résultats de ChIP chez S30 montrent que CodY se fixerait plutôt sur les deux sites, entraînant un effet dominant au niveau de la régulation d'*icaADBC* comparé à IcaR.

De plus, par une régulation du système appelé *agr*, impliqué dans le contrôle balancé de l'expression des protéines adhésives de surface et des toxines sécrétées, CodY pourrait interférer avec la communication entre les bactéries (quorum sensing) et affecter l'expression des protéines adhésives et donc de l'adhésion des bactéries aux surfaces toujours en fonction de l'état nutritionnel de la bactérie, ce qui devrait affecter sa capacité à former des biofilms. Finalement, si le travail devait continuer, je proposerais de complémenter de manière propre les gènes naturellement mutés dans les souches étudiées, afin d'observer si cela entraîne un effet reproductible de la mutation de *codY* selon les souches sur les biofilms produits. Enfin, j'imaginerais traiter une infection à biofilm de *S. aureus* créée dans un modèle animal ou sur un tissu *in vitro*, par l'injection d'isoleucine dans le site d'infection, en espérant activer CodY par une augmentation de la concentration d'isoleucine dans les bactéries. Ainsi, si les bactéries manquent d'acides aminés essentiels, mais si l'isoleucine reste élevée,

CodY devrait être activé. La conséquence devrait en être un arrêt de la croissance de la bactérie et donc un contrôle de l'infection, cela restant à démontrer.

Abstract

The subject of the present study, performed in order to obtain a PhD in science under the mention biology & medicine, consists in the analysis of the function of the gene *codY* for biofilm formation in the bacterium *Staphylococcus aureus*. In general, bacteria are capable to attach to surfaces and to produce a sticky extracellular matrix, referred to a biofilm. *S. aureus* biofilms are responsible for numerous infections associated to implants and other kinds of medical materials. Such kinds of infections are difficult to treat, are persistent and cause chronic infection, are resistant against antibiotic treatments inherent to the biofilm mode of growth, necessite to operate the patient in order to extract the implanted material to cure the local infection, and then to re-operate the patient to implant a new device. That is highly costly, both economically and from a human standpoint. For all of these reasons, biofilms are more and more often studied in the field of infectious diseases.

The *codY* gene is present in gram positive and low GC containing bacteria such as *S. aureus*. It encodes for a protein able to become active when bound to branched chain amino acids (BCAA) or GTP. CodY is capable to repress the expression of a multitude of genes by binding to some palindromes present in the DNA of the bacterial genome. A preliminary study performed in the host laboratory revealed that *codY* was implicated in biofilm formation within a particularly highly biofilm producing strain of *S. aureus* called S30.

In order to study in more details the molecular mechanisms related to CodY and its function in the formation of biofilms, different genes were inactivated either by disruption or by replacement, and the consequences of these genetic alterations were analyzed on the formation of biofilm.

The results showed a complex regulation carried out by CodY on the so-called *icaADBC* operon. This operon is responsible for the production of PIA, an extracellular polymeric sugaric compound present in matrices of *S. aureus* and *S. epidermidis* biofilms.

Sequencing the regulatory region of the *icaADBC* operon in the hyper-biofilm producing strain S30 revealed a naturally-acquired mutation located in the *ica*R specific repressor of the *icaADBC* operon. The addition of a normal *ica*R allele in S30 on a plasmid resulted in a drastic decrease in the quantity of biofilm produced whereas the presence of the plasmid without the *ica*R insert was not affecting biofilm production. We deduced that the mutation consisting of one base pair (bp) substitution in a genome that contains approximately 2.9 millions bp was probably the cause of the hyper-biofilm

producing phenotype of S30, even if the formal proof for that statement would imply restoring the normal allele in the genome, which is technically more difficult in this clinical isolate. That strain (S30) was recovered from a catheter-related infection in a baby admitted to Geneva University Hospitals in the beginning of the seventies. Compared to S30, another clinical isolate, UAMS-1, produced by far less biofilm. Interestingly, the mutation in the *codY* gene in UAMS-1 resulted in an opposite effect regarding the quantities of biofilm produced. In order to explain this paradox, the genomes of these two strains were sequenced. The results showed that UAMS-1 is also naturally affected by a mutation located in another repressor of *ica*R called *rbf*. Thus, two mutations, whose effects on the *icaADBC* operon should be opposite according to the scientific literature are individually present in two different strains, putatively explaining the opposite effects observed in the production of biofilm.

Two different aspects are highlighted in the present work regarding the effects of CodY on the formation of biofilm in *S. aureus*. First, the complex transcriptional control of the *icaADBC* operon by CodY suggests that this operon is regulated in function of the nutritional state of the bacterium, in part by GTP and BCAA sensing. Moreover, two different attachment sites for CodY were discovered both *in vivo* by ChIP and *in silico* by bioinformatics predictions, one located in the *ica*R and the other one in *icaB*. According to the sites bound by CodY, the effects of that fixation on the expression of *icaADBC* should be opposite. The ChIP results suggest that CodY attaches to both sites, triggering a dominant effect on the regulation of *icaADBC* as compared to IcaR.

Second, by regulating the *agr* system involved in the balance of expression of surface-attached adhesins and secreted toxins, CodY could interfere with bacterial intercommunication (quorum sensing) and with the adhesin-mediated bacterial adhesion to surfaces, which should affect the formation of biofilms as a function of the nutritional status of the bacteria. Finally, for the continuation of this work, I would propose to complement strain-specific altered genes, expecting a reproducible effect on biofilms regarding *codY* mutations in the different strains. Lastly, I would suggest treating a *S. aureus* biofilm-related infection generated in an animal model or in an *in vitro* tissue model by injecting isoleucine to the site of infection, expecting to activate CodY expression by increasing the concentration of isoleucine in the bacteria. Thus, if bacteria are missing essential amino acids under high levels of isoleucine conditions, CodY should be activated even in conditions favoring its inactivation. The consequence should be a growth arrest and thus the control of the infection. This remains to be proven.

PART I INTRODUCTION

A. *Staphylococcus aureus*

Staphylococcus aureus was described for the first time in Scotland in 1880, by microscope-assisted observations of pus from surgical abscesses by Sir Alexander Ogston [253]. He described micrococci of spherical shape of similar sizes estimated between 0.7 and 1.3 µm. He injected pus from abscess into guinea pigs and mice in order to demonstrate a causal relationship between the presence of micrococci and the formation of abscesses. Results have shown that pus coming from cold abscesses, characterized by the absence of micrococci, was not able to produce abscesses when injected in animals, whereas injections of pus containing micrococci led to the formation of abscesses containing similar micrococci and always localized at the injection sites. These experiments led to the conclusion that the cause of abscess formation was the presence of these micrococci, in agreement with the postulates established by Robert Koch [253]. The Koch's postulates are not always effective today, particularly when the etiological agent is not a microorganism, such as the pathogenic form of the prion protein causing -among other- the Creutzfeldt-Jakob disease [153]. The experiments performed by Sir Ogston resulted in the identification of a new bacterial pathogen, named *Staphylococcus aureus* (*S. aureus*), able to infect both humans and animals [353]. Forming part of the name of that organism, the term "*Stapyhlo*" derives from an old Greek origin meaning clusters of grapes, and was used to describe the property of the organism, whereas "coccus" is associated to any kind of spherical bacteria and "aureus" characterizes the golden appearance due to the endogenous production of carotenoids [219].

The genome of *S. aureus* consists of a circular double stranded chromosome and it may contain one or several plasmids. Different research groups sequenced various genomes of *S. aureus* and the entire information is now freely available, for example on the website of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Today, 14 complete genome sequences of various *S. aureus* strains are listed on the NCBI website, with the adition of two recent sequences not reported in the table 1 [145]. Genomes of *S. aureus* consist in approximately 2'851 ± 49 kilobase pairs (kbp). 82 ± 1 % of that double stranded DNA is encoding for 2'760 ± 92 genes, whereas these genes are encoding for 2'650 ± 94 proteins and 81 ± 10 structural RNAs (tRNA & rRNA), see Table 1. Non-coding DNA remains to be studied in further details. Additionally, in average, one plasmid is found in each strain. Note that, in order to predict for the presence of a

gene, genome-scanning algorithms require a minimal sequence length that is not containing any stop codon and that typically measures at least 80-100 nucleotides, thus potentially missing shorter open reading frames (ORF). These shorter ORFs could correspond to small RNAs and small –yet undetected- proteins. Technological improvement of sequencing allowed characterizing small RNAs at the genome scale in *S. aureus* [18]. These molecules are recognized as important regulators mediating virulence and adaptation to environmental changes [18 357].

TABLE 1: COMPLETED SEQUENCE GENOMES OF STAPHYLOCOCCUS AUREUS

strains	year*	gene	Protein coding	Structural RNAs	bp	% coding	pseudogenes	% GC	plasmid
MRSA252	2001	2839	2650	101	2902619	81	88	32	0
Col	2001	2723	2612	72	2809422	82	39	32	1
Mu50	2001	2774	2696	77	2878529	83	0	32	1
N315	2001	2664	2583	79	2814816	83	1	32	1
MW2	2002	2704	2624	80	2820462	83	0	32	1**
RF122	2005	2663	2509	76	2742531	80	76	32	0
MSSA476	2006	2715	2571	104	2799802	81	40	32	1
USA300 FPR3757	2006	2648	2560	70	2872769	82	18	32	3
NCTC 8325	2006	2969	2892	77	2821361	84	2	32	0
USA300 TCH1516	2007	2802	2657	88	2872915	80	58	32	2
JH1	2007	2870	2747	81	2906507	83	43	32	1
JH9	2007	2816	2697	81	2906700	83	33	32	1
Mu3	2007	2768	2690	78	2880168	83	0	32	0
Newman	2007	2687	2614	73	2878897	83	0	32	0
	MEAN	**2760**	**2650**	**81**	**2850536**	**82**	**28**	**32**	**1**
	SD	**92**	**94**	**10**	**49164**	**1**	**30**	**0**	**1**

* Year when the genome was completed.

** A plasmid in strain MW2 was identified by sequencing without being reported to NCBI.

Approximately 75% of the genome of *Staphylococcus aureus* (the 'core' genome) is highly conserved between strains, whereas the remaining 25% (the 'accessory' genome) is composed of mobile genetic elements (MGEs), containing virulence and resistance genes [100]. Note that insertions, deletions and mutations occur in both, the core and the accessory part of the genome. These mutations can be either silent or can affect the protein sequence. Therefore, even the core genome has its own variability. For example, experiments with *S. aureus* Newman strain were performed in order to characterize the functions of fibronectin-binding protein A & B (FnBPAB) for virulence in rabbits and these experiments led to negative results considering *S. aureus* adhesion to fibronectin and invasion of mammalian cells [81]. Then, the discovery of natural mutations located inside the *fnBAB* genes in Newman leading to a loss of their function was discovered, explaining the previously obtained negative results [128]. Additionally, a recent work using different *S. aureus* strains showed the presence of mutational alterations in one or more regulators among all tested strains [139]. Therefore,

genomes of *S. aureus* strains represent many variations that can explain strain-specificity. Recent improvements in bacterial genome sequencing could therefore be useful for explaining strain-specificity.

A.1. Microbiological characteristics

Staphylococcus aureus is a Gram positive non-motile bacterium of approximately 1 µm diameter, able to form clusters of bacteria, to grow either in the absence or in the presence of oxygen (=facultative anaerobe), not able to produces spores but able to ferment glucose and mannitol [261,356]. The genus *Staphylococci* is characterized by positivity in catalase tests except for the anaerobic species *Staphylococcus saccharolyticus* and *S. aureus* subsp. *anaerobius*, whereas the *aureus* species is characterized by coagulase positive tests, (figure 1) [274]. First, the Staphylococci catalase enzymes (*kat*) are responsible for the conversion of H_2O_2 into water and oxygen [274;320]. However, cases of *S. aureus* strains negative for the catalase test were identified and explained by missense mutations or deletions occurring inside the *katA* gene [274]. Second, the coagulase tube test was previously the gold standard for *S. aureus* identification at the species level, (figure 1) [320]. In that test, the bacteria are inoculated in presence of plasma. The clumping factors of *S. aureus* (ClfAB, see part B.1.d.) bind a soluble plasma glycoprotein called the fibrinogen, leading to agglutination of bacteria and fibrinogen in the tube [287].
At the species level, additional procedures have been developed in order to discriminate *S. aureus* from coagulase-negative staphylococci (*Staphylococcus epidermidis* and others) [102]. For example, the Pastorex® Staph-Plus agglutination test, developed by Sanofi-Pasteur, presents a specificity for *S. aureus* identification reaching around 98.6 %, but decreasing to 95.1 % in case of oxacillin-resistant *S. aureu*s (ORSA), and remains a good standard for the identification of *S. aureus* [112]. That test consists of a mixture of latex particles coated with fibrinogen, immunoglobulin G and monoclonal antibodies against serotype 5 and 8 capsular polysaccharides of *S. aureus* [112]. The test allows the identification of *S. aureus* based on the detection of the clumping factors, the protein A, and the capsular polysaccharides, resulting to naked-eye visible agglutination as a positive result [64]. Indeed, clumping factors of *S. aureus* bind the fibrinogen, the protein A binds to the IgG whereas the capsular polysaccharides binds to its specific antibodies (see part B.1.c and B.1.d.). Additionally, PCR amplification and DNA sequencing of 16S rRNA, *hsp60*, *tuf*, *soda, nuc* and *rpoB* molecular targets were used for identification of *S. aureus* [35,95,221,277]. Furthermore, a high-density DNA probe arrays was designed in order to identify Staphylococci, resulting to 92 % of valuable identifications at the species level [72].

FIGURE 1: CLASSICAL MICROBIOLOGY OF *STAPHYLOCOCCUS AUREUS*

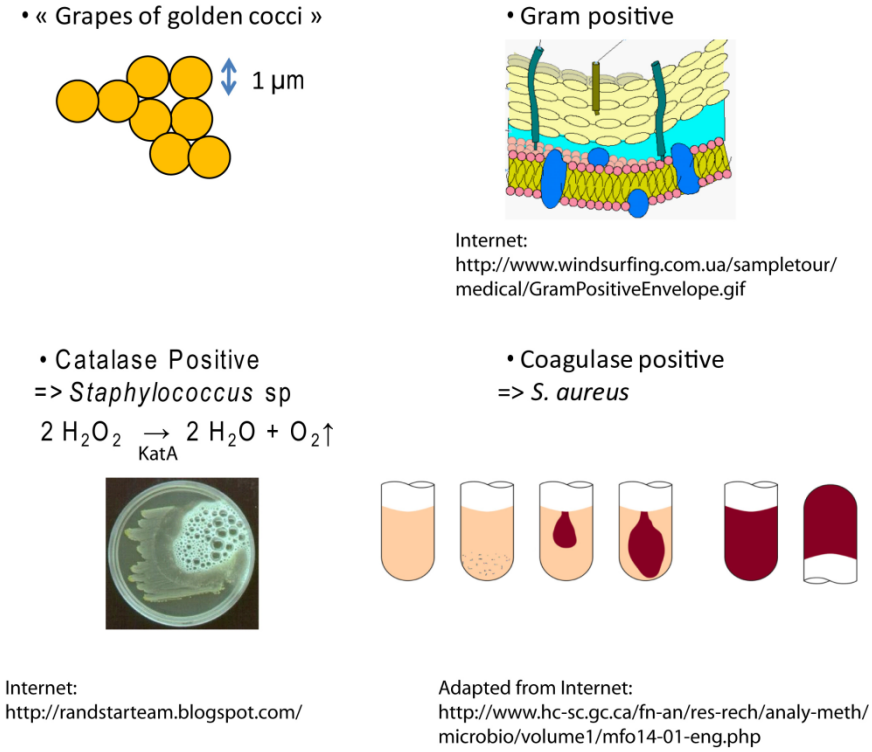


Figure 1 shows different microbiological properties of *S. aureus* bacteria. Top left, *S. aureus* bacteria of ~1 µm diameter presenting a golden color and forming clusters or grapes of cocci. Top right, *S. aureus* is a Gram-positive bacterium containing an outside cell wall connected to the unique membrane. Bottom left, the catalase test shows the degradation of H_2O_2 to H_2O and O_2 by the catalase of *S. aureus* or other staphylococci, with the visible production bubbles that correspond to oxygen. Bottom right, the tube coagulase test used to identify *S. aureus* at the species level using rabbit plasma. A firm clot that does not move when the tube is tipped is considered as a positive reaction. The reaction is mediated by the coagulase enzyme reacting with the prothrombin and the fibrinogen from the rabbit plasma.

A.2. Antibiotics and bacterial resistances

Various epidemic waves of antibiotic-resistant clones of *S. aureus* infecting patients in hospitals have been documented [50]. The story begins with penicillin, the first antibiotic then described as probably the most efficacious life-saving drug in the world. Penicillin, produced by the mold called *Penicillium notatum* (also called *Penicillium chrysogenum*), was able to kill bacteria as discovered by Fleming in August 1928 [106]. For that discovery, Fleming received the Nobel Prize in 1945 [106]. Penicillin affects the production of the peptidoglycan, involved in the synthesis of the cell wall and responsible for the resistance of the bacteria to the intracellular pressure as well as for giving the bacteria its cell shape [299]. The first successful scientific report of penicillin treatment to cure human patients was

published in 1940 by Chain and Florey [352]. It was the beginning of a massive use of penicillin for curing infected patients despite Fleming predicted early that "*the greatest possibility of evil in self-medication is the use of too small doses so that instead of clearing up the infection, the microbes are educated to resist penicillin and a host of penicillin-fast organisms is bred out*" [205]. In 1944, Kirby reported the first description of penicillinase-producing strains of *S. aureus* and thus penicillin resistance [180]. The resistant determinant was a plasmid-encoded penicillinase, which hydrolyses the beta-lactam ring of penicillin that is essential for its antimicrobial activity [50]. Penicillinase-producing *S. aureus* strains were first rare and only isolated from hospitalized patients, but today, most hospital isolates are resistant to penicillin and even strains circulating in the community appear also resistant to penicillin [49]. Then, beta-lactamase inhibitors (clavulanate, sulbactam, and tazobactam) were introduced before the emergence of resistance to the combinations of beta-lactam-beta-lactamase inhibitor [96]. New antibiotics were needed in order to treat infections due to penicillin-resistant bacteria.

A new kind of penicillin resistant to penicillinase called methicillin (prev. Celebin or BRL 1241) also affecting the cell wall synthesis, has been introduced in 1961 [190]. But resistance appeared less than 1 year after with a methicillin-resistant *Staphylococcus aureus* (MRSA) reported in London [160]. All MRSA carry a mobile genetic element of variable size and composition called staphylococcal chromosome cassette *mec* (SCCmec), today known to be of various major types (I to VIII and likely expanding), always inserted at the 3' end of the same gene called *orfX* and carrying among others the gene *mecA* responsible for the resistance against methicillin [49,155]. The SCCmec was not present in *S. aureus* before the apparition of MRSA and should be therefore coming from another unknown organism. Some researchers proposed that the *mecA* gene in MRSA may come from *Staphylococcus sciuri*, because a very close *mecA* homologous gene called *pbpD* was encountered in that organism, conferring similar phenotypes when introduced into the MSSA strain COL-S in laboratory conditions [7]. On the other side, the mobile SCC cassette without the *mecA* gene is thought to come from *Staphylococcus hominis*, which carries a similar cassette in term of structure called SCC_{12263}, encoding similar recombinases as those present in the SCCmec (*ccrA* & *ccrB*) and allowing site-specific excision and integration of the mobile element in a comparable manner as for the SCCmec cassette [171]. A transfer of the SCCmec type IVa from methicillin-resistant *S. epidermidis* (MRSE) to MRSA was additionally proposed based on sequence comparison resulting to 99.99 % identity between the SCCmec IVa from CA-MRSA strain USA300-FPR3757 as compared to SCCmec IVa from MRSE strains BCB-57 and BCB-F63 [15]. Thus, the exact origin of the various SCCmec remains not fully understood. In medical practice, methicillin was replaced by oxacillin (5-methyl-3-phenyl-4-isoxazolyl penicillin),

because oxacillin was more stable in presence of acid allowing better absorption after oral administration when passing the stomach and showing a better activity *in vitro* against staphylococci. Oxacillin-resistant *S. aureus* strains (ORSA) also appeared, sometimes probably miscalled MRSA instead of ORSA for historical reasons [181]. Indeed, methicillin, whose production was stopped, is today rarely tested in U.S. laboratories which preferentially test the oxacillin, whereas in Europe, the reverse is true [37]. Additionally, oxacillin in discs is less labile than methicillin but oxacillin is less resistant to hydrolysis by staphylococcal β-lactamases leading to confusing assessments regarding phenotypical characterizations of MRSA and ORSA, very often considered as synonymous [228] .

Additionally, vancomycin, a glycopeptide antibiotic also affecting the bacterial cell wall, introduced around 1956, worked well during 40 years before the apparition of *S. aureus* resistance in so-called vancomycin-intermediated *S. aureus* (VISA) in Japan in 1997 with strains resisting to 4-8 μg/ml of vancomycin [142]. Then, strains of *S. aureus* presenting higher level of resistances against vancomycin were discovered; low-level-resistant vancomycin resistant *S. aureus* (LLR-VRSA) resiste to 30-60 μg/ml of vancomycin, high-level-resistant VRSA (HLR-VRSA) resiste up to 250 μg/ml [142 272]. 11 VRSA strains were reported from United States, India and Iran, reviewed in 2009 [272]. Their high levels of resistance against vancomycin but also against another glycopeptide (teicoplanin) were attributed to the *vanA* operon located on a transposon (Tn*1546*) [272]. The transposon is thought to have inserted itself from its first location on an *Enterococcus* plasmid to a secondary location on an endogenous plasmid of *S. aureus*, suggesting a breach in the *S. aureus* restrictions barrier [272]. Normally, restriction enzymes of *S. aureus* digest foreign DNA, but some clinical MRSA strains are deficient in their restriction systems, allowing horizontal transfer of foreign DNA as demonstrated *in vitro* in our laboratories [67]. Additional evidence supports the hypothesis for the successful co-transfer of vancomycin and other resistance genes from *Enterococcus faecalis* NCTC 12201 to *Staphylococcus aureus*, by the co-isolation of both a VRSA and a glycopeptide-resistant clone of *Enterococcus* from the same patient [245,272,313].

Thus, introduction of new antibiotics has led to the emergence of new bacterial defense mechanisms in the last century. Today, some *S. aureus* strains are able to resist to any known antibiotic [50]. Moreover, the pool of sequences conferring antibiotic resistances seems able to be distributed among different bacteria, as more or less accepted for the *van* operon transferred from *Enterococcus* to *Staphylococcus aureus* and presumably for the SCCmec cassette from some coagulase-negative staphylococci. As the production of new antibiotics remains poorly investigated, bacterial antibiotic resistances emerge rapidly and can be propagated between different bacterial genera, future control

of bacterial infections will be challenging to the point that some researchers allude to the post-antibiotic area [4].

A.3. Epidemiology

Human and mammals are natural reservoirs of *S. aureus* and they can carry the pathogen without being infected and without being disturbed by the presence of the bacteria (i.e. they are referred to as healthy carriers) [314]. In human, the bacterium is mainly found in the anterior nostrils of the nose and more generally on mucosal and skin surfaces [291]. In order to estimate the rate of carriage without associated infections, random sampling, effective detections methods and statistically sufficient number of samples representative of the human community have to be performed. But *S. aureus* can persist within human cells and therefore lead to false negative detections. Nevertheless, it was estimated that 30 to 50 % of human are colonized by *S. aureus* without being infected [46,208,244]. More precisely and according to a recent review, 10–35% of individuals carry *S. aureus* persistently, 20–75% carry intermittently, and 5–50% never carry *S. aureus* in the nose [187]. Furthermore, the number of *S. aureus* cells in the nose is significantly higher in persistent carriers than in intermittent carriers [187]. These carriers can transmit *S. aureus* notably by skin contact, whereas nasal, axillary, or inguinal colonization with *Staphylococcus aureus* generally precedes invasive infection. Colonization by MRSA was estimated to present a 4 fold higher risk for infection as compared to colonization by MSSA [291]. As an example of transmission, football players seem subject to skin contact during play and skin injury, thereby increasing their risk for infections [93,122]. Interestingly, a highly conserved community-associated MRSA (CA-MRSA) clone caused abscesses among professional football players playing in the same team [173]. Once an infection occurs, *Staphylococcus aureus* can be responsible for highly diverse diseases ranging from benign skin infections to deadly invasive infections depending on the toxins produced by the strain, the host defenses, the site of infection, and additional parameters. *S. aureus* is also well-known for being involved in hospital-acquired infections (the so-called nosocomial infections) and is more and more frequently associated with antibiotic resistance and persistent infections. In the hospital, the hands of health care workers seem important for the transmission of *S. aureus* as well as other pathogens. Various hospitals adopted hand-hygiene programs, and both the Centers for Disease Control and prevention (CDC) and the World Health Organization (WHO) published guidelines for hand washing [2,74,198,208,340]. Infections with *S. aureus* occur in hospitals but also in the community once a barrier of the human body is broken, as for example a wounded skin, or

even after an insect bite, whereas sometimes false diagnostic of spider bites were in fact the result of a MRSA infection [91].

Today, *S. aureus* is considered as the leading cause of hospital-associated infections and considerable amount of epidemiological data exist regarding cases of *S. aureus*-related infections occurring in a continent, in a country, in a hospital, in a healthcare unit and across different periods of time [89]. A global estimation at the level of the planet for the morbidity, the mortality, the incidence and the prevalence of *S. aureus* infections could be produced by compiling different data coming from different sources with a high level of difficulty [349]. Reports of MRSA-related infections are well covered in North America, Europe, Japan, Australia, but remain under-estimated or not-estimated in South America (no nation-wide studies), in Africa (despite data for Nigeria) and in China (no national surveys) [349].

A prospective nationwide surveillance study performed in USA over a 7-year period from March 1995 through September 2002 reported 24'179 cases of nosocomial bloodstream infections occurring in 49 different hospitals [367]. That study showed that 0.6 % of hospitalized patients developed a nosocomial bloodstream infection, whereas 20 % of these infections were due to *S. aureus*. The mortality rate of all kind of *S. aureus* related infections, exceeding 80 % before the discovery of penicillin, has decreased between 20 % and 30 % today (12'000 deaths per year in USA), with arguable variations related to the kind of *S. aureus* (MRSA or MSSA), but probably related to epidemic outbreaks of antibiotic-resistant clones [32,179,234,266,293,336,363]. Number of outbreaks of *S. aureus* infections were reported among hospitals and healthcare units throughout the world [3,16,66,71,98,103 130,163,193,202,223,231,281,283,286,289,305 327].

The development of strain-typing techniques allowed to observe strains of *S. aureus* circulating inside hospitals and to compare them to clones found outside hospitals. Today, hospital-acquired MRSA (HA-MRSA) and healthcare-associated MRSA (HCA-MRSA) are distinguished from community-acquired MRSA (CA-MRSA), that appeared in the community at the end of the nineties, such as the spread of CA-MRSA USA300 in the United States [115 316,368].

In hospitals, a correlation was observed between the number of beds and the number of MRSA; 15 % of isolates were MRSA in hospitals containing less than 200 beds raising to 38 % in hospitals with 500 or more beds in the USA for a long-term study performed between 1975 and 1991 [266]. The annual incidence of CA-MRSA representing new infections varied between 25.7 cases per 100'000 population in Atlanta and 18 cases per 100'000 population in Baltimore, with a recent decreasing trend [115]. To summarize, approximately five to six hospital-acquired infections per 100 admissions

occur, in which *S. aureus* remains of major concern. Moreover, outbreaks of *S. aureus* resistant clones are recurrent and some strains are more dangerous than others. Finally, animals are also reservoirs of *S. aureus* and animal workers could be more susceptible to develop *S. aureus* infections, such as it was reported for pig-farmers [104].

A.4. Toxins and clinical impacts

Different toxins and virulence factors are present in a strain-specific manner in *S. aureus* and have been implicated in various clinical pictures as shortly summarized thereafter.

First, some exotoxins, enterotoxins and the toxic shock syndrome toxin-1 (TSST-1) produced by *S. aureus* are called superantigens, because picomolar concentrations of these toxins are sufficient to activate T cells polyclonally [191]. These potent activators of the immune system cause a variety of diseases in humans, ranging from food poisoning to shock [191]. They bind in a non-specific manner to both, MHC class II molecules and specific regions of the T cell receptor, leading to the activation of both antigen-presenting cells and T lymphocytes, resulting to excessive production of pro-inflammatory cytokines and T cell proliferation causing clinical symptoms that include fever, hypotension, and shock [191].

Second, the exfoliative toxins (Ets) implicated in the staphylococcal scalded-skin syndrome (SSSS) allow the bacteria to invade mammalian host through their skin barriers by cleavage of keratinocyte cell-cell adhesion [243]. More precisely, three isoforms of ETs (ETA, ETB, and ETD) are glutamate-specific serine proteases that specifically and efficiently cleave a single peptide bond in the extracellular region of human and mouse desmoglein 1 (Dsg1), a desmosomal intercellular adhesion molecule belonging to the family of cadherin.

Third, the Panton-Valentine leukocidin (PVL), present in 2-3 % of *S. aureus* strains, was responsible for necrotizing pneumonia and invasive skin and soft tissue infections [90,166,331]. The association between PVL and necrotizing pneumonia leads to a trans-Atlantic virulent scientific debate without reaching a consensus today [195,350]. The PVL is composed of two proteins encoded by the *lukS* and *lukF* genes. The two proteins bind together to form an active pore-forming exotoxin targeting cells of the immune system such as polymorphonuclear neutrophils (PMNs) [166,331]. The corresponding genes are carried by phages (ΦPVL, ΦSLT, ΦSa2mw, ΦSa2USA300, ΦSLT-USA300_TCH1516, Φ108PVL, Φtp310-1 and Φ2985PVL) and are present in phage-infected *S. aureus* strains (USA300, USA400, strains of linage ST80 and the Southwest Pacific clone) [365].

Other *luk* genes encode additional *S. aureus* virulence factors but few data are available concerning the relationship between the production of these toxins and the pathology of staphylococcal infections [331].

Fourth, *S. aureus* produces four classes of hemolysins (alpha, beta, delta, and gamma). Nutrient agar supplemented with a 5% concentration of defibrinated sheep blood is commonly used to analyze the hemolytic phenotypes of *S. aureus* [188]. Alpha hemolysin is able to lyse a broad range of mammalian cells and is especially effective when observed on rabbit erythrocytes; it is positively regulated by *agr*, *sarA*, and *sae*, and negatively regulated by *rot* and *sarT* [90,373]. The alpha-hemolysin was crystallized showing a pore-forming structure in the cytoplasmic membranes of targeted cells [319]. Alpha-hemolysin is an important virulence factor for *S. aureus* pneumonia, sepsis, septic arthritis, brain abscess and corneal infections [38,44,148,176,242,267]. Beta-hemolysin is a sphingomyelinase produced by some *S. aureus* strains that is able to lyse sheep but not rabbit erythrocytes [41,90]. The activity of that hemolysin is enhanced after incubation below 10 °C ("hot-cold" hemolysin) [278]. Delta-hemolysin (*hld*) lyses many mammalian erythrocytes, is produced by almost every strains of *S. aureus*, is able to lyse also membrane-bound organelles [41,90]. The delta hemolysin (*hld*) is encoded within the *agr* locus and is positively controlled by *agr* [158]. Gamma-hemolysin lyses erythrocytes from human and other mammalian species [90].

Fifth, *S. aureus* can produce 22 different enterotoxins (SEA to SEE, SEG to SEI, SER to SET, and SElJ to SElQ, SElU to SElW) resistant to temperature and that can lead to nausea, violent vomiting and food poising even if the bacteria are killed, notably in meat that was not kept correctly frozen [254]. Enterotoxins are also superantigens and their corresponding genes can be identified by a PCR-based method [105]. SEA is the most common *S. aureus* enterotoxin responsible for food-poisoning, is carried by a bacteriophage, and is not regulated by the *agr* in contrast to SEB, SEC and SED [12,25,143]. SEB is chromosomal in clinical isolates of *S. aureus* from food-poisoning cases but was found also onto a 750 kb plasmid [303,304]. SEC contains three subtypes (SEC1, SEC2, and SEC3) divergent in the N-termini which determine the subtype-specific antigenic epitopes, while the conserved C-terminal regions determine biological properties and cross-reactive antigenic epitopes shared with other pyrogenic toxins [12]. Interestingly, *S. aureus* isolated from different animal species produce a unique host-specific SEC [218]. SED is the second most common serotype associated with food poisoning and can form dimers in the presence of zinc [51].

To summarize, very different toxins are produced by *S. aureus*, sometimes in a strain-dependant manner, sometimes by every strain, leading to different clinical pictures somehow related with the

toxins properties. Among the most pathogenic toxins of *S. aureus*, the PVL seems associated with necrotizing pneumonia, the enterotoxins are associated with food-poisoning, the exfoliative toxins are associated with SSSS, and the TSST-1 lead to toxic shock.

A.5. *Staphylococcus aureus* biofilm related infections and persistence

S. aureus biofilm-related infections are mainly associated to surfaces of artificial devises that are introduced in the human body, such as vascular or orthopedic prostheses. It was estimated that 80% of all bacterial infections involve biofilm formation [150]. They are characterized by difficulties to treat the related persistent infections. Both *Staphylococcus epidermidis* and *S. aureus* are the most frequent bacteria causing nosocomial infections and infections on indwelling medical devices, probably because staphylococci are frequent commensal bacteria on the human skin and mucous surfaces [259]. They characteristically involve biofilms. Thus, staphylococci are among the most likely bacteria to infect any medical device inserted during surgery [351]. Catheter-associated urinary tract infections remain the most common nosocomial infection [321]. When central venous or urinary catheters are deposited for a long-term period, bacterial infections seem inevitable [321]. In order to decrease such infections, the Nosocomial Infections Surveillance System has published guidelines for prevention of catheter-associated urinary tract infections in 2009, available on the website of the Centers for Disease Control (www.cdc.gov). Less than 6 per cent of patients who undergo pacemaker insertion develop a bacterial infection probably resulting from skin contamination at the time of surgery. 75 per cent of such infections are due to staphylococci; *Staphylococcus aureus* causes most infections occurring within 2 weeks after surgery, while *S. epidermidis* typically causes delayed infections. The need to remove infected pacemakers was controversial [136]. The overall incidence of prosthetic valve endocarditis (PVE) ranges from 0.98 to 4.4 per cent. Coagulase-negative staphylococci are responsible for 43 per cent of infections occurring early after the deposition of prosthetic valves [136].
Approximately one million cases of nosocomial infections associated with indwelling devices occur each year in USA [80]. *Staphylococcus aureus* has been isolated from biofilms on artificial hip prostheses, central venous catheters, intrauterine devices, prosthetic heart valves and cochlear implants [92,189]. Rates of device infection vary in function of prosthetic type (42 % of orthopedic devices and 93 % of long-term catheters) according to a prospective study which selected 298 hospitalized patients possessing a prosthetic device and infected with a *S. aureus* [58]. 58 % of these patients were re-operated and the 12-week mortality reached 27 %. Additionally, the cost ($ 40'000 to $ 70'000) of *S. aureus* infections occurring in prosthetic devices is larger as compared to infections occurring in

devices-free patients (~$ 30'000) [58,288]. Infections associated with surgical implants remain difficult to manage because they require prolonged periods of antibiotic therapy as well as repeated surgical procedures [78,80,361]. The increasing lifespan in humans and the development of prosthetic devices are probably responsible for the increasing number of implanted prostheses in developed countries [292,293]. For example, the rate of knee replacement for patients aged 65 years and higher increased 46 percent between 2000 and 2006, whereas the rate doubled among those aged 45-64 years during the same time period. Each year in the USA 700'000 joint replacements were performed and more than 348'000 cardiac pacemakers and 60'000 valvular implants were inserted [85,293,348]. Arthritis and direct physical trauma to a joint are the causes of damages resolved by prosthetic-joint deposited in order to improve mobility and alleviate pain [376]. Unfortunately, bacterial biofilm can form on prosthesis after surgery leading to persistent infections resistant to antibiotic treatments and serious complications [70]. Therefore, antibiotic treatments are highly recommended in prevention during surgery [79]. Bacterial infections occur in 0.8 to 1.9 % of knee arthroplasties and in 0.3 to 1.7 % of hip arthroplasties, whereas Staphylococci account for half of cases in these infections [57,86,157,271,273,279,338]. The difficulty to kill bacterial biofilms by antibiotic treatment, as compared to their planktonic counterpart, is well documented today. Recently, diffusion of some antibiotics into *S. aureus* and *S. epidermidis* biofilms was analyzed by growth inhibition resulting to significantly reduced diffusion of oxacillin, cefotaxime and vancomycin into both biofilms, whereas the penetration of amikacin and ciprofloxacin was not affected by the formation of biofilms [315].

B. Biofilms

The term "biofilm" is strictly defined in the present text as being "a community of micro-organisms attached to a surface and producing an extra-cellular matrix surrounding them and conferring cell-to-cell attachment", in accordance with the current definitions found in scientific publications [68,70,99]. A shorter definition could be: "surface-associated communities of microbes encompassed by an extracellular matrix" [150]. Van Leeuwenhoek first observed microorganisms on tooth surfaces and can be credited with the discovery of microbial biofilms [92]. The surface of attachment can be organic, inorganic, an interface between two different states of matter (e.g. solid-liquid) or between two different matters in the same state (e.g. liquid-liquid) [68]. When the microorganisms producing the biofilm and localized inside the biofilm belong to the same species, the corresponding biofilm is defined as "monospecies biofilm", whereas more than one species is present in "multispecies" or "mixed" biofilm, without clear difference in the use of the two terms [325,355]. The "matrix" consists of

the extracellular material surrounding the attached cells whereas the biofilm includes both the matrix and the cells.

In nature, biofilms are mainly composed of highly structured multispecies communities [325]. Fungi and bacteria can co-exist in mixed-species biofilms [335]. A recent paper discovered that the Human T cell leukemia virus type 1 (HTLV-1), responsible for cases of human leukemia, is able to form a "biofilm" when infecting T-lymphocytes [262]. That assertion could trigger polemics because viruses do not contain the cell machinery and are therefore not considered as an entire form of live. In fact, the HTLV-1 is a retro-virus that requires cell-to-cell contact between the already infected T lymphocytes and with the future infected T lymphocyte for its propagation, in a similar manner as HIV-1, implying the apparition of so-called "virological synapses" [275]. By studying aspect of that synapses at the Pasteur Institute, a research group showed that these synapse structures localized at the surface of the infected lymphocytes were composed of viral particles surrounded by an extracellular matrix composed of carbohydrates, collagen, agrin and cellular linker proteins. These structures adhere to other cells by contact and are composed of an extracellular matrix induced by the virus, even if produced by the cell machinery of the host [262]. Underestimated by scientists in terms of occurrence, biofilms appeared universal in the microorganism's world, and reflect probably an ancestral adaptation to the accumulation of nutrients in the bottom of ocean, where live on Earth is supposed to have appeared [152,325,330]. The formation of biofilms is a dynamic complex process composed of different distinct phases illustrated in the section "developmental phases" of the present introduction [257].

Biofouling, plaques, slime, mucus and flocculation were used in the past to described biofilms. The term "biofouling" deriving from industries means "the undesirable accumulation of living organisms on wetted structures". Biofouling is not restricted to microorganisms, whereas "microfouling" is analogue to "biofilm" by opposition to macrofouling [73]. Various systems such as transporting pipelines and storage tanks filled with liquid or gas can be confronted to biofouling, such as the industries dealing with waters [62], the food and beverage industry [371], the oil & gas industry [235], etc. Biofilms or biofouling formed onto industrial material can trigger bio-corrosion, flow-related problems, can be of public health concern, whereas dispersing biofilms is neither easy nor safe [62 168]. Note that microbially-influenced corrosion was used to distinguish bacterial biofilm mediated corrosion occurring on implants in patients from corrosion triggered by host cells [19]. Most probably, the first important studies performed on the formation of biofilms were conducted by industries trying to solve the problems of corrosion and flow due to the formation of biofilms in liquid

systems [285]. Some researchers used even recently the terms "slime" or "mucoid" when confronted to biofilms, and glycocalyx instead of extracellular matrix [17]. The well-known tartar found on teeth is in fact the result of a mineralization of the dental plaque corresponding to a bacterial biofilm, whereas caries results from the demineralization of the tooth subsequent to a bacterial fermentation of sugars. In case of oral biofilm studies and cariology (i.e. the study of the formation of caries), the term "plaque" is increasingly replaced today by the term "biofilm" [186].

When pathogenic bacteria grow in biofilms, and as compared to their free-floating forms, they usually appear more resistant against antibiotic treatment, host's immune system, disinfectant solutions and thus frequently leading to persistent infections [70]. Therefore, biofilms produced by pathogenic bacteria begin to be an active field of research in infectious diseases. Several theories based onto different observations try to explain the phenomenon of biofilm-related resistance which may well be multi-factorial. First, the extracellular matrix was thought to physically restrict the diffusion of antimicrobial agents, but this does not seem to be a predominant mechanism for biofilm-acquired resistances according to a recent review [268]. More probably, according to the same review, nutrient and oxygen depletion within the biofilm could cause some bacteria to enter a non-growing state, in which they are less susceptible to growth-dependent antimicrobial killing [268]. This is coherent with the observation that oxygen limitation and low metabolic activity in the interior of the biofilm, and not poor antibiotic penetration, were correlated with antibiotic tolerance of *P. aeruginosa* biofilm [354]. Second, a subpopulation of microorganisms might differentiate into a phenotypically resistant state [175]. Third, some organisms in biofilms were shown to express biofilm-specific antimicrobial resistance genes that are not required for biofilm formation [347]. Fourth, transmission electron micrographs of clinical biofilms show no penetration of inflammatory cells into the biofilm, suggesting that biofilms exhibit resistance to host phagocytes defenses [196]. Finally, the charge of polymers component of matrix may repulse or bind antibiotic [354].

Staphylococcus aureus biofilms can be distinguished from other biofilms, essentially based on the involvement of specific *S. aureus* adhesins and matrix components. The principal implants that can be compromised by staphylococcal biofilm-associated infections are central venous catheters, heart valves, ventricular assist devices, coronary stents, neurosurgical ventricular shunts, implantable neurological stimulators, orthopedic prostheses, fracture-fixation devices, inflatable penile implants, breast implants, cochlear implants (see publication 2), intraocular lenses and dental implants [69].

B.1. Adhesion & surfaces

To be formal with the physicists' terminology, the adhesion of bacteria occurs more specifically on interfaces, such as between solid and liquid, between liquid and liquid and between liquid and gas [68]. In *Pseudomonas aeruginosa*, a glucose-rich polysaccharide named "Pel" was found essential for the formation of biofilms at air–liquid interfaces [43,107].

Considering solid surfaces, biofilms can form on a plethora of different materials, but some matters are more easily colonized by biofilms than other. Finding a material that prevents (or minimizes) biofilm formation remains an active field of research with a major economical potential. Titanium alloys (Ti) are the preferred material for orthopedic applications [317] and silicon coating is used for modern cochlear implants [189]. In general, rough surfaces, above a threshold of 0.2 μm of roughness, could favor biofilm formation as compared to smooth surfaces [334]. The surface free energy, or the measurement of the contact angle (i.e. the angle between the surface of a liquid droplet and the plan of the solid surface) that reflects surface hydrophobicity, seems to be a parameter of importance for predicting biofilm development [334]. Thus, physicochemical properties of solids probably influence the initial attachment of bacteria. In the experiments I have performed in the laboratory, *S. aureus* biofilms were produced on solid-liquid interfaces between the medium and the surface of polystyrene or polypropylene plates, as well as on glass coverslips used for microscopy. Other kinds of surfaces were not tested for *S. aureus* biofilm formation.

B.1.a. Translocation of adhesins and anchoring to the cell wall

Adherence of *S. aureus* needed for biofilm formation is mediated by at least 22 proteins present at the surface of the bacteria called adhesins and belonging to the so-called microbial surface components recognizing adhesive matrix molecules (MSCRAMM) [109]. Note that other substances such as extracellular DNA (eDNA) and polymeric intercellular adhesin (PIA) can also trigger adhesion of *S. aureus* to surfaces. These proteins share a common signal sequence for secretion and another for anchoring to the cell wall, as detailed below. Moreover, a large majority of adhesins or MSCRAMM shows strain-specific repetitions in the peptide sequence, and are therefore useful for strain typing techniques such as multi-locus variable number of tandem repeat analysis (MLVA) [113]. The present sections emphasize general considerations of the MSCRAMM in *S. aureus*. The natural ligands of the *S. aureus* adhesins are the fibronectins, the fibrinogen and the collagen present in the extracellular matrix of host tissues, that should not be confused with the extracellular matrix of the

biofilms [109]. An extensive functional overlap was reported between adhesins: they are typically able to bind various molecules even if they are generally more specifically binding to one of them [59]. The adhesin proteins are mainly covalently anchored to the cell wall of *S. aureus*, and are implicated in the binding to the bone matrix and to bone implant biomaterials coated with host plasma constituents [109,146]. These proteins have to be exported from the inner part of the bacteria and have to be attached to the surface of the bacteria. This process involves the sec pathway (ubiquitous in bacteria) and the sortase (specific to gram-positive) in *S. aureus* [224,263,310]. Most probably, the sec pathway exports actively uncoiled proteins recognizing the signal peptide YSIRK localized at the N-terminal region of such proteins, in a process called translocation, using the molecular motor SecA, a process using energy [307]. Then, the uncoiled protein passes through a channel embedded in the membrane and composed of SecY, SecE, and SecG [309]. During that process, the YSIRK signal peptide is cleaved and finally the protein localizes outside of the bacteria. Mutational analysis combined to protein localization studies indicate that the YSIRK motif is dispensable for the cell wall anchoring of the Protein A [10]. In *S. aureus*, an accessory secretion pathway composed of at least SecA2 and SecY2 has been involved in the secretion of the adhesin SraP, and was involved in the adhesion to platelets [310]. Many translocated proteins from *S. aureus* are covalently bound the cell wall by the "sortase" system [224]. The sortase A (SrtA) recognizes a LPXTG motif (X means any amino acid), localized at the C terminal regions of proteins [224]. In a similar manner, an additional sortase B (SrtB) present in *S. aureus* recognizes a C terminal NPQTN motif [24]. 22 known proteins are covalently attached to the cell wall of *S. aureus* by sortase A or B [59,224]. Sortase A cleaves the polypeptides between the threonine and the glycine of the LPXTG motif and catalyses the formation of an amide bond between the carboxyl-group of threonine and the amino-group of peptidoglycan cross-bridges, leading to a cell wall anchored protein [24].

B.1.b. Fibronectin binding proteins (FnBP)

Two different adhesins of *S. aureus* named FnBPA & FnBPB are expressed in most clinical strains and are specifically involved in the adhesion to fibronectin (an extracellular high molecular weight glycoprotein produced by the host) [39]. Moreover, FnBPAB are involved in the attachment to prosthetic devices causing biofilm-related infections [39]. The strain Newman displays the particularity to carry an amber mutation inside *fnbPA* & *fnbPB* localized in the LPXTG motif, leading to the release of the FnBPAB into the growth medium instead of being anchored by the sortase to the cell wall [128]. Before this discovery, different reports showed the absence of functionality of the FnBPAB

using Newman strain and the conclusions of these articles should be carefully reconsidered today. In a recent work, different *S. aureus* strains were constructed to express either one FnBP, both FnBPAB, or none of them. Then, these strains were tested for their adherence to prosthetic devices that are coated with the host fibronectin using atomic force microscopy. Results showed that one FnBP is sufficient for the binding of the strain to fibronectin-coated prosthetic devices and that strains lacking both FnBPAB were not able to attach to the coated device [39]. Importantly, different adhesins other than FnBPs are able to bind fibronectin, such as the **i**ron-regulated **s**urface **d**eterminant **A** (IsdA) [61], the **e**xtracellular matrix-**b**inding **h**omologue (Ebh) [60], the **e**xtracellular **m**atrix binding **p**rotein (Emp) [151], the autolysins (Atl) [59] and the multifunctional autolysins/adhesins (Aaa) [135]. Parallel bonds may form between fibronectin and FnBPs [39], and the structure of these interacting molecules was solved recently [26]. Seven different isoforms of fibronectin-binding proteins were recently detected in *S. aureus*, differing in the "A" domain, without disturbing their ligand-binding functions, but affecting their antigenicity [40]. Additionally, FnBPAB are negatively regulated by the *agr* system of *S. aureus* but appear also additionally regulated by an *agr*-independent pathway [296]. Finally, even if the *fnBPAB* genes are located in tandem on the DNA, they are transcribed separately [59,125].

B.1.c. Protein A

Staphylococcus aureus expresses the well-known Protein A encoded by the *spA* gene. It was the first surface protein identified in *S. aureus* [59]. This protein is used for typing the strains by counting the number of 24 bp repeats in the *spA* gene, a method called *spA* typing [165,308]. The Protein A, expressed at the surface of *S. aureus*, is able to bind the F_c fragment of antibodies of different organisms as well as the variable region of the F_{ab} heavy chain of IgM located on B cells, probably to partially escape the immune response [59]. More precisely, Protein A binds to the F_c gamma of immunoglobulin (Ig) and to the F_{ab} portion of V(H)3-type B cell receptors [177]. Protein A is also able to bind the blood glycoprotein von Willebrand factor (vWF) involved in platelet adhesion [134] and a receptor for the tumor-necrosis factor-α (TNFR1) [121]. Because of its particular immune properties, Protein A was and remains used in different molecular techniques involving the binding of antibodies, such as the purification of antibodies by chromatography or by immunoprecipitation [6,13]. The *spA* gene is regulated by the topology of the DNA, by the two-component system ArlS-ArlR, and is also negatively regulated by the *agr* system [111,332]. Finally, the Protein A may well be a component of the proteinaceous biofilms of *S. aureus* [123].

B.1.d. Clumping factor A & B

The two **cl**umping **f**actors of *S. aureus* (ClfA & ClfB) contain extensive serine-aspartate dipeptide repeats characteristic of the Sdr protein family among which additional adhesins are found (SdrC, SdrD, SdrE) [164]. Both ClFAB bind fibrinogen [227,241]. ClfA also interacts with the immune system of the host, more specifically with the complement factor I, by increasing the cleavage of C3b on the bacterial surface of *Staphylococcus aureus*, and by decreasing complement-mediated phagocytosis [131]. Moreover, ClfA enhances staphylococcal virulence in animal infection models [232,369], mediates the adherence to polyethylene or polyvinylchloride tubes [345], and is implicated in the proteinaceous matrix of biofilms [123]. Clumping factors might be involved as matrix constituents in the adhesion process, when considering the biofilm formation in *S. aureus* [59]. Additionally, ClfA is an important virulence factor needed in infectious arthritis and enhancing immunostimulatory activity [59]. ClfB is involved in the colonization of the nose of both human and mouse [108,251,300]. ClfA was more efficient in binding platelets as compared to ClfB [59,250,312]. ClfB enhances adherence of *S. aureus* to desquamated nasal epithelial cells, putatively by binding to cytokeratin 10 [251].

B.1.e. Other adhesins

The extracellular adherence protein (EAP), also called Map or p70, can bind at least seven different proteins of the human plasma and is able to attach back to the cell surface of the bacteria after secretion [133]. EAP can trigger the agglutination of *S. aureus* without the need of endogenous expression, is able to form homo-oligomers as well as to bind to epithelial and endothelial cells, to fibroblasts, to the extracellular matrix present in tissues and it is also able to modulate the immune system of the host, probably by stimulating the humoral response in addition to the production of interleukin 4 [133]. EAP is regulated by Sae, Agr, and to a lesser extent also by SarA [162].

The collagen adhesin (Cna) mediates adherence of *S. aureus* to the cartilage [269]. Cna is a virulence factor in experimental models of septic arthritis, osteomyelitis and it is also required for the maintenance of endocarditis [101,141,270]. The plasmin-sensitive (Pls) adhesin mediates adherence to lipids and gangliosides notably found in keratinocytes membranes [147].

The four iron-regulated surface determinants (IsdA, IsdB, IsdC, IsdH) are involved in the adhesion to iron-containing proteins (transferrin, haemin, haemoglobin) [97,225,333].

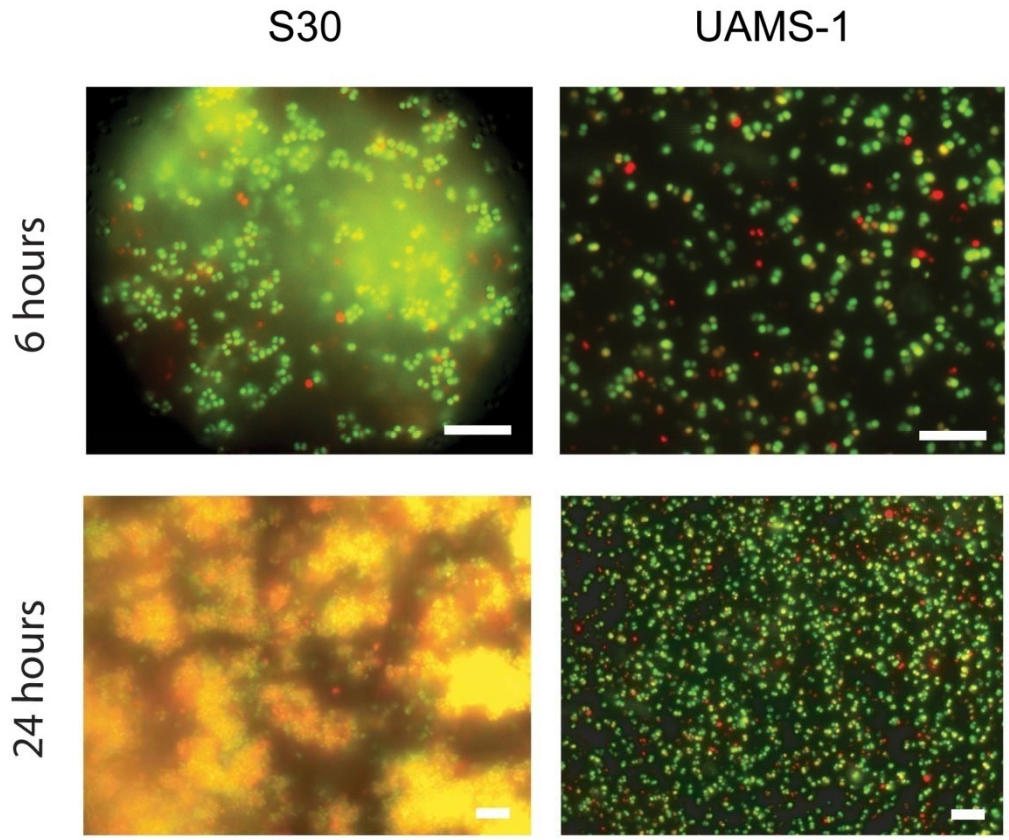
The serine-rich adhesin for platelets (SraP), present in different clinical isolates, is a virulence factor in infective endocarditis and can bind platelets [311]. All the previous adhesins usually are covalently

attached to the cell wall by the sec-sortase pathway, but other adhesins bind to the cell wall by ionic interactions. These adhesins are the extracellular matrix-binding protein homologue (Ebh), the extracellular matrix protein-binding protein (Emp), the autolysins (Atl, AtlE, Aas, AtlC) also involved in adhesion, Enolase, the elastin-binding protein (EbpS) [59]. Finally, the wall teichoic acids (WTA) composed of sugaric and phosphate compounds were also involved in adhesion [59,359].

B.2. Extracellular matrix

The matrix, also called extracellular polymeric substance (EPS) or slime, may account for 90 % of the dry mass of biofilms [107]. The main constituent of the matrix remains water, representing 97% of the total mass [328]. The matrix stabilizes mechanically the biofilm by forming a cohesive network, interconnecting and immobilizing bacteria; it also mediates adhesion to surface and provides an external digestive system by keeping enzymes close to the embedded bacteria [107]. The constituent of the matrix are polysugaric compounds, proteins, extracellular DNA and lipids. All of these constituents are illustrated in the following sections [107]. Matrices trigger the architecture of the biofilm in three dimensions, leading after maturation to fungi-like structures, open channels and release of flocs (bacterial biofilm released aggregates) during dispersion, see Figure 2 [107].

FIGURE 2 LIVE DEAD STAINING ASSAY OF *S. AUREUS* BACTERIA ADHERING TO GLASS SURFACES.



S30 *ica*R-deficient hyper-biofilm producer (left) and UAMS-1 *rbf*-deficient weak biofilm producer (right) were grown overnight and equilibrated to OD_{540} nm = 1.5 before being diluted 50 fold in TSB medium. The inoculated media were deposited onto glass cover slips and incubated during the indicated times (Top: 6 hours, bottom: 24 hours). Then, the washed adherent bacteria were stained with LIVE/DEAD® BacLight™ Bacterial Viability Kit before being observed with a fluorescent microscope. The images were acquired with a camera incorporated into the microscope. (□) Scale bars represent 10 µm length. Red bacteria are stained with Propidium iodide DNA inter-calling agent that penetrates only the dead fraction of bacteria presenting destabilized membranes, whereas the Syto-9 dye emitting green fluorescence penetrates all bacteria.

B.2.a Sugaric compounds

Exopolysaccharides are described as the major fraction of the matrix and are formed by polymers of modified sugars outside the bacteria [364,372]. They are necessary for many different biofilms [77,210,358]. In mixed biofilms, an exopolysaccharide produced by one species can also be used by the other species [107]. These molecules can be branched or linear, composed of neutral or/and charged residues, are usually of heavy weight with an order of magnitude corresponding to approximately 10^6 daltons, are

strain-specific and can contain organic or inorganic substituents [107]. Heteropolysaccharides contain both neutral and charged residues whereas homopolysaccharides contain only one of the two types [107]. Different types of exopolysaccharides were characterized chemically; the β-1,6-linked N-acetylglucosamine constituting the polymeric intercellular adhesin (PIA) produced by Staphylococci that will be discussed in more details, the 1,4-linked uronic residues of β-D-mannuronate and α-l-guluronate constituting Alginate produced by *Pseudomonas aeruginosa*, repetition of pentasaccharide composed of D-mannose and D-glucose and L-rhamnose constituting Psl, also produced by *Pseudomonas aeruginosa* [107]. Usually, bacteria contain enzymes able to digest the exopolysaccharides they produce or the enzymes used to synthesize them can act in both directions. These enzymes remain of great interest to degrade plastics produced by industries or biofilms formed on prostheses [168,306]. The PIA from *S. aureus* can be digested by meta-periodate treatment or by dispersin B [185]. Dispersin B is a N-acetyl-β-hexosaminidase (encoded by *dspB*), which is produced by the periodontal pathogen *Actinobacillus actinomycetemcomitans*, and the recombinant enzyme is now commercially available [169]. The degradation of the polymer is involved in the process of biofilm dispersion and maturation [297,298]. Exopolysaccharides are degraded mainly by hydrolases and lyases [107,170]. There is no single enzyme or simple enzyme mixture that can degrade all of the polysaccharides in a biofilm matrix [107]. The PIA can be detected and quantified using specific antibodies notably in dot blot experiments [341].

B.2.b. Protein compounds

Although exopolysaccharides were seen and described as the most important and often essential compounds of biofilm matrices, recent evidence suggests that surface and extracellular proteins are probably as important as exopolysaccharides. They can be involved as enzymes implicated in the biofilm maturation process and/or as structural elements in the matrix [107]. Therefore, proteases can have anti-biofilm properties [29,30,220,252,339]. Recently, researchers described a new large protein of 2,276 amino acids called biofilm-associated protein (Bap), with homologues in different organisms [75,199]. Note that the MSCRAMM previously discussed could be incorporated in proteinaceous biofilms, such as the Protein A inducing bacterial aggregation in the absence of antibodies and serum compounds [59,230].

Lectins are extracellular proteins bound to sugaric compounds and to bacterial surfaces [107]. Other ubiquitous proteinaceous components of the matrix are amyloids [107]. These compounds have been defined as orderly repeats of protein molecules arranged as fibres of indefinite length in a cross-β

structure, in which the β-strands are perpendicular to the fibre axis [260]. The proteinaceous matrices can be digested with proteinase K [185]. The amyloids can be specifically stained with Thioflavin T and specific antibodies targeting amyloid fibrils [197]. Bacteria growing onto Congo red agar can be discriminated for biofilm formation, Congo red agar binds amyloids [178].

Amyloids are involved in adhesion to inanimate surfaces and host cells, with subsequent invasion of the host cells, and they also function as cytotoxins for both plant cells and bacteria [107,260]. Structural protein of pili, fimbriae and flagella can also act as structural elements by interacting with other EPS components of the biofilm matrix such as eDNA [107,343]. Some proteins such as AltA, FmtA, GraS, RsbU and RsbV are important in *S. aureus* biofilm formation [27,30,138,200,341].

B.2.c. eDNA

Extracellular DNA (eDNA) was described recently as a component of the matrix in *S. aureus* biofilms and was also implicated in adherence, maturation and dispersion of biofilms [156,201,212,284,360]. *S. aureus* lysis mediated by CidA and inhibited by lrgAB resulted in the release of DNA [212]. This eDNA can be then incorporated into the matrix [212]. Moreover, the beta-toxin of *S. aureus* cross-links with eDNA, thereby putatively forming the skeletal framework upon which staphylococcal biofilms are established [150]. The content of eDNA can be estimated by comparing biofilm recorded quantitites between DNAse digested biofilms and biofilms not digested by DNAse [154]. *S. aureus* contains thermostable nucleases able to degrade the eDNA, putatively promoting biofilm maturation and dispersion [156,212]. Carbohydrates, proteins and nucleic acids were identified in the matrix of the marine photosynthetic bacterium *Rhodovulum sulfidophilum*. Interestingly, flocs of that organism were dissolved by DNAse treatment, whereas both polysaccharide-degrading and protein-degrading enzymes had no effect [5,107,329]. Moreover, *Rhodovulum sulfidophilum* produces structural extracellular RNAs (mainly tRNAs and rRNAs) localized in the matrix and not distinguishable from intracellular RNAs [5,107,329]. This is the first report of structural analyses of bacterial extracellular RNAs. Because RNAs are not stable, their implication as structural element in the matrix of biofilm was not expected.

B.2.d. Lipids

In general, biofilm matrices are described as mainly composed of hydrophilic polysaccharides, but recent evidences have demonstrated important variability in matrices with the additional presence of hydrophobic compounds. Indeed, some matrices of bacterial biofilms contain insoluble lipid

compounds [65,107]. For example, serrawettin was identified in a matrix produced by *Serratia marcescens* [222], rhamnolipids were identified in matrix of *Pseuomonas aeruginosa* [82] and lipopolysaccharides were crucial for the adherence of *Thiobacillus ferrooxidans* to pyrite surfaces [295]. It could be that extracellular lipids could be involved in biofilms formed at liquid-gas interfaces, because the lipids can probably form a thin pellicle on the aqueous phase able to support bacteria, even if there is yet no evidence to support this in the scientific literature. Finally, even if lipids are found in matrices, different free lipids show anti-bacterial and anti-biofilm properties [87].

Interestingly, *Pseudomonas aeruginosa* produces cis-2-decenoic acid, which is capable of inducing the dispersion of established biofilms from various origins and also of inhibiting biofilm development [84]. It is possible that unsaturated fatty acids may inhibit more strongly biofilm formation than saturated fatty acids would do [194]. Additionally, the cacao bean husk containing both oleic and linoleic acid decreases the *in vivo* production of *Streptococcus mutans* biofilm formed on human teeth and was suggested to be implicated in the formation of caries [256]. Free polyunsaturated fatty acids (PUFA) may interfere with microbial adhesion to intestinal surfaces through unknown mechanisms [167]. Contradictory results were obtained regarding the effects of oleic acid with respect to the formation of *S. aureus* biofilms. This could probably be explained by a phase-dependant opposite effect [45,322]. Indeed, oleic acid may well inhibit primary adhesion but stimulate the already formed biofilms. Oleic acid presents also an efficient inhibitory effect on the glucosyltransferase enzyme [370]. Finally, 1 hour of exposition to 5 % tea tree oil leads to the complete eradication of *S. aureus* biofilm [34].

B.3. Developmental phases

Today, the formation of biofilm by bacteria is seen as a dynamic process involving successive steps that are summarized in the following sections. This begins with the initial attachment of bacteria to a surface and ends with dispersion of bacteria from the mature biofilm (see Figure 3).

B.3.a. Initial attachment

First, *Staphylococcus aureus* free floating bacteria passively diffuse in a liquid according to Brownian movements (40 μm/h in water), sediment to the bottom of any liquid-containing recipient due to gravity, whereas the velocity of *S. aureus* increases in function of the turbulent flow and decreases in function of the viscosity of the liquid [264,362]. When getting closer to a surface, i.e. at a distance corresponding to less than 50 nm (1/20 of the *S. aureus* size), the bacteria attaches to the surface by two different mechanisms [334]. First, specific interactions occur between surface adhesins and the

recognized components coating the surface [59]. Second, non-specific interactions occur between the bacteria, the solid and the liquid [11,42,88]. Non-specific interactions depend on the physical and chemical properties of both surfaces from the solid and the bacteria as well as the behavior of the liquid. The physical and chemical parameters known to be involved in that process are hydrophobicity or surface free energy, roughness, electrostatic charges or van der Waals interactions and elasticity, all of them being related to the surfaces of both, the bacteria and the solid. Additionally, shearing induced by the movement of the fluid is also an important parameter [11,88,325]. The interactions occurring between the bacteria and the surface are weak and reversible during the initial attachment [334]. Interestingly, good correlations were reported between the relative negative charge on the bacterial surface and the initial attachment to lean beef muscle on the one side, and between bacterial hydrophobicity and initial attachment to fat tissue on the other side [88]. However, the initial attachment of bacteria to a surface remains not fully understood. This step initiates the eventual formation of a biofilm [325].

B.3.b. Irreversible attachment

After reversible attachment of bacteria to surfaces, an irreversible attachment phase precedes the maturation of the future biofilm [325]. The transition between reversible and irreversible adhesion involves permanent bonding which is usually mediated by extracellular polymers produced by the bacteria and able to bind to a broad range of surfaces [325]. This step occurs within 15 minutes after the initial attachment of *Pseudomonas aeruginosa* and is correlated with the production of its alginate polymer [83]. In *S. aureus* and *S. epidermidis*, the PIA could be - at least partially - responsible for the irreversible attachment phase [118,123,325]. During this step, bacteria are "cemented" to the surface [325]. Note that even planktonic *S. aureus* bacteria not released by a biofilm are able to produce the PIA (not shown).

B.3.c. Maturation

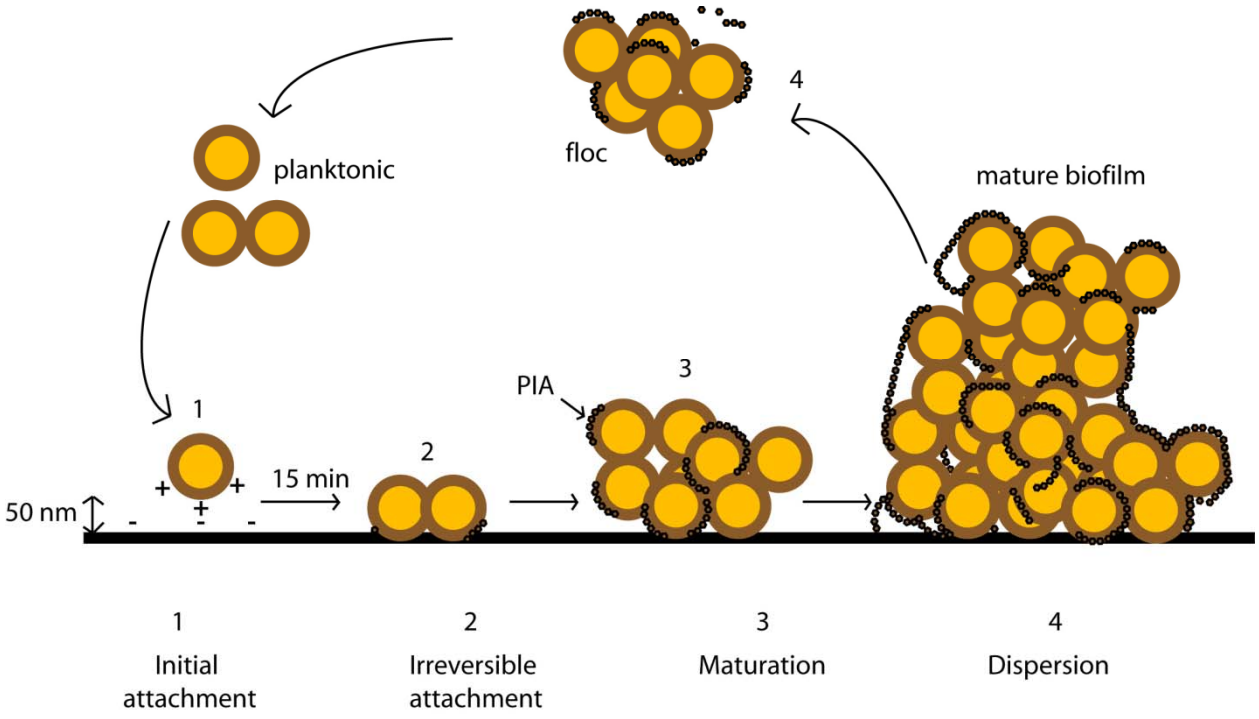
Once bacteria are irreversibly attached to a solid surface, the maturation process of the biofilm begins [132,325,326]. The expression of genes at the genome level changes, according to transcriptomic experiments obtained by comparing transcripts produced between adherent and planktonic sub-populations [21,203]. The results of this phase can lead to complex architecture of the biofilm with channels and pores formed perhaps by detachment of bacteria or by local inhibition of bacterial growth and fungi-like structure corresponding to matrix-surrounded bacteria growing in three

dimensions [325]. Additionally, quorum sensing, cell-cell communications, genetic exchanges and heterogeneity of sub-populations in terms of growth, transcription of genes and expression of proteins occur during the maturation process [132,325,326]. Chemical gradients appear in complex structured biofilms and are probably responsible for the different metabolic states of bacteria as a function of their location within the biofilm, due for example to gradients of oxygen and nutrients [323].

B.3.d. Dispersion

In mature biofilms, single bacteria and cluster of bacteria named "flocs" are able to detach from the biofilm by both active and passive processes. This is the last step of the biofilm cycle ending with planktonic bacteria able to colonize new surfaces [116,132,290,324-326]. The detachment is active when involving endogenous processes and passive when due to external perturbations such as increased fluid shear [324,326]. The active process could be related to endogenous enzymatic degradation and/or to the release of EPS or surface-binding proteins [33,168-170,204].

FIGURE 3: *S. AUREUS* BIOFILM DEVELOPMENT PHASES



C. Genetic regulations involved in biofilm formation

Staphylococcus aureus adapts the expression of virulence genes in function of environmental changes, stress, host response and quorum sensing, thus enhancing its chances for survival [52]. Notably, the expression of approximately 40 different extracellular and cell wall proteins are coordinated with infection stages probably by sensing environmental cues [9,52]. In general, adhesins are expressed early during colonization whereas toxins are expressed later [52]. These changes in gene expression suggest the existence of global regulator and signal transduction systems (two-component systems recognizing and transmitting a signal from the environment to the DNA) [52,246]. The next sections of the text will summarize recent knowledge on global regulators of virulence in *S. aureus*. Note that different regulators may interact by a direct or indirect regulatory effect and their respective targets may overlap.

C.1. The accessory gene regulator (*agr*)

The *S. aureus* locus called *agr* is an operon (*agrBDCA*) transcribed as the so-called RNAII. Deficiency in this locus results in a reduction of virulence of various *S. aureus* strains in mice models of endophthalitis, septic arthritis and osteomyelitis as well as in either mice or rabbit models of endocarditis [1,31,53,120]. In fact, the accessory gene regulator (*agr*) system regulates the expression of virulence factors in response to cell density (quorum sensing) by sensing the concentration of a strain-specific extracellular thiolactone-containing pheromone peptide, whereby the concentration of this peptide depends on the density of the bacteria [258,282]. This peptide results from the transcription, the translation and post-translational modifications of *agrD* into an auto-inducing peptide (AIP). Four different subgroups of *agr* (type I, II, III, and IV) were defined according to amino acid sequences of both peptides and their receptors reflecting bacterial interference [114,161]. Indeed, the autoinducing peptide secreted in the medium showes autologous *agr* activation and cross-activation between strains belonging to the same *agr* subgroup, whereas the AIP of a strain belonging to one *agr* subgroup inhibits the expression of the *agr* of a strain belonging to another subgroup [161]. These bacterial interferences regarding respective *agr* types could be correlated with the exclusion of other strains by a resident strain from infection or/and colonization sites [161]. The *agr* system is composed of two divergent transcripts (RNAII & RNAIII) being under the control of two divergent promoters called P2 and P3 [246,248,282]. RNAIII is a 514 nucleotides long RNA molecule regulating the expression of some genes at the translational levels, whereas RNAII carries the auto-inducing part of

the system [110,258,282]. Note that RNAIII carries a small ORF encoding the δ-hemolysin (*hld*) whereas the non-coding part of RNAIII is implicated in regulatory functions [246]. This system (RNAII/RNAIII/*hld*/*agrBDCA*) is referred to the *agr* system in the present text and does not consist of the *agrBDCA* operon alone.

First, translated from the polycistronic *agrBDCA* mRNA, AgrD is secreted and processed by the transmembrane protease AgrB [247,280]. Then, the processed extracellular AgrD peptide binds and activates the membrane receptor histidine kinase and signal receptor AgrC [207]. Activated AgrC activates then AgrA by a transfer of a phosphate group from AgrC to AgrA [247]. When activated, the AgrA response regulator presents an enhanced binding affinity for repeated DNA sequences in the P2 and P3 promoters located between RNAII and RNAIII [184]. AgrA binding results in an increased transcription of both RNAII and RNAIII, whereas the P2-based auto-activation preceedes the P3-based expression of the RNAIII regulator [184]. Finally, the RNAIII acts as antisense RNA that forms a duplex with targeted mRNAs preventing translation of the mRNA and leading to degradation of the duplex [28]. It is probable that RNAIII C-rich sequences forming stem-loops could base-pair with Shine-Dalgarno (SD) sequences of target mRNA, thus preventing ribosome binding, whereas an additional endoribonuclease III participates probably in the degradation of the targeted transcript [23,149]. Known targets repressed by RNAIII are the protein A (*spa*) [23], the repressor of toxins (*rot*) [28], the staphylocoagulase (*coa*) [55] and a fibrinogen-binding protein (SA1000) [247]. Known targets positively regulated by the RNAIII are the α and β hemolysins (*hla, hlb*) [233,249] as well as indirectly the virulence factors repressed by ROT.

C.2. The SarA family

The SarA family consists of at least 10 DNA-binding paralogues of the first characterized member of that family of regulators called SarA, sharing between 20 to 45 % identity and named SarR [213], SarS [54], SarT [301], Rot [229], SarU [214], SarV [216], MgrA [209] and SarX [215,217]. First, the so-called repressor of toxins (*rot*) is a target of RNAIII and is therefore repressed at the post-transcriptional level by the *agr* system, as mentioned above [28]. Interestingly, the generation of an *agr rot* double mutant and comparison of its transcriptome with the transcriptome of the *agr* mutant showed that targets regulated by Rot are inversely regulated by the *agr* system [294]. The SarR regulatory protein binds DNA in the promoters of *sarA* and consequently represses the transcription of SarA [213]. SarS (SarH1) binds the *spa* promoter and activates the transcription of *spa* [54]. *sarT* is negatively regulated by both SarA and RNAIII, and SarT represses *hla* [301]. SarU is a positive activator of *agr* [214]. SarR and

SarU are antagonists towards the *agr* expression [214,301]. *sarV* is repressed by SarA and MgrA, and may be part of the common pathway by which MgrA and SarA control autolysis in *S. aureus* [216]. MgrA is a pleiotropic transcriptional regulator, activating the expression of type 8 capsular polysaccharide (CP8) and nuclease but repressing the expression of alpha-toxin, coagulase, protease, and protein A [209]. Additionally, Mgr affected the production of extracellular proteins. SarX is maximally expressed during the stationary phase of growth, is repressed by MgrA, represses the *agr* [215].

Finally, the SarA DNA-binding regulator is required for maximum expression of the *agr* system in some growth media [137]. SarA binds to conserved DNA sequences and may positively (e.g. *hla*) and negatively (e.g. *spa*) regulate the expression of genes [56]. SarA regulates additionally the expression of *agr* and *sec* (enterotoxin C) [56]. Mutation of *sarA* in *Staphylococcus aureus* results in a reduced capacity to form a biofilm, but the mechanism for this remains unknown [20]. Previous transcriptional profiling experiments identified a number of genes that are differentially expressed both in a biofilm and in a *sarA* mutant including genes involved in acid tolerance and in the production of nucleolytic and proteolytic exoenzymes [339]. Thus, SarA seems necessary for the production of the extracellular matrix involved in biofilm formation, probably by affecting matrix degrading enzymes. Interestingly, SarA and the *agr* system present an epistatic relationships for the production of biofilm in *S. aureus* [22]. Δ*sarA* mutants lose the ability to form biofilms as compared to parents whereas Δ*agr* mutants present increased biofilm production [22]. Most probably, whereas the *agr* system represses the expression of adhesins, SarA represses the expression of matrix degrading enzymes [22,339].

C.3. *σB*

σB is an alternative sigma factor present in different Gram-positive bacteria, activated by the signaling cascade resulting from stress and redirecting the RNA polymerase to the promoters of genes involved in stress tolerance [344]. The various stresses able to activate SigB are heat, high osmolarity, high ethanol concentrations, high and low pH, as well as oxidizing agents [344]. The RNA polymerase (RNAP) consists of different subunits forming together a holoenzyme able to synthesize RNA from a DNA template by specific adjunction of nucleotides after unwinding DNA [124]. The sigma factors are dissociable subunits of the RNA polymerase that bind to -35 and -10 regions of promoters in order to initiate with other factors the formation of the holoenzyme RNAP to the DNA strand for the initiation of transcription [127]. The mRNA *sigB* level is thought to be constant whereas the activity of SigB varies according to physical interactions between co-transcribed

staphylococcal RsbU-RsbV, RsbV-RsbW, and RsbW-SigB, seen as a partner-switching mechanism in the sigma(B) activation cascade [302].

SigB probably acts on multiple pathways involved in biofilm formation in *S. aureus*. SigB acts on the production of PIA needed for matrix as well as on exoproteins that digest proteins associated to the matrix. SigB acts on the *agr* system, controlling the production of adhesins [144,182,183,302]. SigB may be implicated in the repression of *ica*R promoting the production of PIA in *Staphylococcus epidermidis* notably in response to ethanol and salt stresses [182,183]. Deleting SigB resulted in a drastic reduction of biofilm production in *S. aureus*, whereas deleting *agr* in the *sigB* mutant compensates the biofilm decrease [200]. Additionally, SigB represses the *agr* system by targeting the P3 promoter and therefore the expression of RNAIII [200]. A high level of RNAIII was associated with anti-biofilm effects whereas *agr* mutations in the *sigB* mutant restored biofilm formation [200]. Additionally, active SigB decreases the levels of the exoproteins SspA and Hla, probably mediated by reduced expression of *agr* [144]. Inactivation of aureolysin and Spl extracellular proteases as well as treatment with protease inhibitors (phenylmethylsulfonyl fluoride) and (alpha-macroglobulin) restored biofilm capacity in a *sigB* mutant [144]. The lack of SigB activity results in increased RNAIII expression, thus elevating extracellular protease levels and altering the murein hydrolase activity profile, suggesting that SigB is an essential regulator of *S. aureus* biofilm maturation [200]. Laboratory strains of *S. aureus* derived from NCTC8325 present an 11-bp deletion in the *rsbU* gene and are therefore probably naturally deficient in SigB signaling, a process needing *rsbU* [139].

C.4. *tcaR*

The teicoplanin-associated locus regulator (TcaR) is a weak negative regulator of transcription of the *icaADBC* operon [159]. TcaR up-regulates *sarS* and thus *spa* transcription, attenuates full-length transcription of *sasF*, thus producing a truncated transcript lacking the 3' terminus cell wall anchor motif [226]. TcaR is likely to decrease the amount of the surface-associated protein SasF and to increase that of the surface-associated Protein A. Laboratory strains derived from NCTC8325 were found to be natural, truncated mutants of *tca*R, therefore expressing very low levels of *sarS* [226].

C.5. *rbf*

A gene required for biofilm formation (*rbf*) seems to be a member of the AraC/XylS family of regulators, according to an amino acid consensus region signature extending over a 100-residue stretch, and constituting the DNA binding domain of the family members [117,206]. Rbf is required for

glucose and NaCl mediated induction of biofilm formation but not for ethanol-mediated induction [206].

Rbf was found to repress *ica*R transcription with a concomitant increase in *icaADBC* expression and increased PIA and biofilm production [76]. Moreover, Rbf was needed for multicellular aggregation stage in biofilms formed by *S. aureus*, whereas the PIA allowed intercellular adhesion [123,206]. Strain UAMS-1 presents a 2-bp insertion affecting the 50th codon of the *rbf* open reading frame [76]. Therefore, deletion of *rbf* in UAMS-1 does not affect the expression level of the *icaADBC* operon whereas over-expression of a correct copy of *rbf* in trans has increased *icaADBC* mRNA levels [76]. Note that purified Rbf does not bind to the *ica*R or *icaA* promoter regions, suggesting that *rbf* controls the expression of an unknown factor(s) that represses *ica*R [76].

C.6. *spx*

The suppressor of *clpP* and *clpX* ATP-dependant proteases (*spx*) was mainly studied in *Bacillus subtilis* but its regulatory function seems conserved across bacteria [236-240]. Spx is a transcriptional regulator that is normally degraded by the ClpXP proteases [236,237,240]. Spx blocks the interactions between transcriptional activators and RNAP by binding to the α subunit of the RNAP, but Spx can control either negatively or positively the initiation of transcription, putatively depending on its conformation [238,239]. SpX responds to various stresses, notably the redox status, the temperature, the osomolarity or the presence of hydrogen peroxide [265]. Proteome comparison between *S. aureus spx* mutant (not growing in stressed condition) and its parent revealed major differences in protein expression, notably for an essential thioredoxin reductase (TrxB) [265]. Inactivation of *spx* also enhanced the production of the *S. aureus* biofilm [265]. Finally, SpX probably activates the transcription of *ica*R, controlling the production of PIA as a response to various stress conditions [265].

D. The CodY pleiotropic repressor controls virulence in Gram-positive pathogens

D.1. Introduction

This text describes current knowledge as well as the most recent discoveries published since the last review covering the topic was published in 2005 by Sonenshein and colleagues, reporting on the functions of CodY among Gram-positive pathogens [318]. There are medical interests regarding the studies of CodY functions, notably because this pleiotropic repressor seems to control virulence mechanisms across different pathogens, such as *Bacillus anthracis*, *Clostridium difficile*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Streptococcus pneumoniae, S. mutans* and *S. pyogenes*. These pathogens are responsible for anthrax, diarrhea, listeriosis, nosocomial infections, pneumonia, tooth decay, endocarditis, scarlet fever and numerous pyogenic infections. But CodY remains also important in non-pathogenic Gram-positive bacteria, being involved in the acquisition of foreign DNA, sporulation mechanisms, production of bacteriocins and motility. In *S. aureus*, CodY represses the *agr* system as well as the *icaADBC* operon, thus affecting the expression of virulence factors and biofilm formation. CodY activity can be relatively easily modified by the addition or depletion of CodY co-factors. A revolutionary approach could consist in converting an infecting pathogen into a non-pathogenic one, by targeting CodY activity. These approaches, sparing antibiotics (or more likely used on top of antibiotics) will have to be explored for the future post-antibiotic area. Because new antibiotics are not developed sufficiently as compared to the apparition and selection of bacterial resistant strains, because already known antibiotics are used massively (app. 200'000 tons per year worldwide [366]), the humanity faces the risk to be confronted more and more often to situations where bacteria infecting people are no longer amenable to therapy with traditional antibiotics, and therefore could result in death. As an illustration, the new carbapenemase gene called New Delhi metallo-beta-lactamase 1 (NDM-1) is a new genetic support for antibiotic multi-resistance, probably coming from various origins. It was discovered in India at the end of 2009 and bacteria containing that gene were circulating between India, Pakistan and United Kingdom in 2010, conferring resistance to bacteria against almost all known antibiotics [192] (resistance to non beta-lactam antibiotics is due to the parallel transfer of other independent gene resistance determinants). Indeed, NDM-1 has conferred very efficient resistances when testing the following beta-lactamines; ampicillin, piperacillin, cephalothin, cefoxitin, cefotaxime, cefuroxime, ceftazidime, aztreonam,

cefepime, ertapenem, imipenem and meropenem [374]. Finally, as CodY controls also biofilm-related mechanisms, this gene could be also targeted for treatment against bacterial persistence in chronic biofilm-related infections. My contribution to this work was to write entirely the manuscript.

D.2. Review:

The CodY pleiotropic repressor controls virulence in gram-positive pathogens

The CodY pleiotropic repressor controls virulence in gram-positive pathogens

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Abbreviations

(EMSA) Electrophoretic mobility shift assay

(GAF) cGMP-specific and –stimulated phosphodiesterases, Anabaena adenylate cyclases and *Escherichia coli* FhlA

(BCAA) Branched chain amino acids: leucine, valine and isoleucine

(DIGE) Two-dimensional (2D) differential gel electrophoresis

Abstract

The highly conserved pleiotropic repressor CodY is involved in the post-exponential adaptive response to starvation in at least 30 different low G+C gram-positive bacteria. After dimerization and activation by co-factors, CodY binds to a consensus palindromic DNA sequence leading to the repression of over hundred of genes corresponding approximately to 5 % of the genome. CodY binds in front of its native promoter creating a negative feed-back control also called auto-repression. CodY represses the transcription of target genes when bound to DNA by competition with the RNA polymerase (RNAP) for the promoter binding or by interference with transcriptional elongation as a roadblock. CodY displays enhanced affinity for its DNA target when bound to GTP and/or branched chain amino acids (BCCA). Upon nutrient limitation occurring in post-exponential growth phase, a decrease of intracellular levels of GTP and BCCA causes a deactivation of CodY and decreases its affinity for DNA, leading to the induction of its regulon. By decreasing the levels of GTP, the stringent response could also independently de-repress the CodY regulon. CodY-regulated genes trigger adaptation of the bacteria to starvation by highly diverse mechanisms, such as secretion of proteases coupled to expression of amino acid transporters, or by promoting survival strategies like sporulation or biofilm formation. Additionally, in pathogenic bacteria, several virulence factors are also regulated by CodY. Thus, in function of its nutrient status, pathogenic gram positive bacteria express virulence factors in a *codY*-dependant manner, as for example the anthrax toxins of *Bacillus anthracis* or the hemolysins of *Staphylococcus aureus*. The purpose of the present review is to illustrate CodY-regulated mechanisms with a special emphasis on virulence in major gram-positive pathogens.

Introduction

The main problem for bacterial growth is the limitation of nutrients. According to the nutrient status, the bacterium has to adjust its transcriptome. For example, there is no need to express genes implicated in the division process if the environment is not enough rich in nutrients for feeding the "mother" bacteria. This adaptation to the nutritional status is triggered in gram-positive bacteria by CodY. The *codY* gene has been identified for the first time in 1993 in *B. subtilis* [82]. Homologues of *codY* were found exclusively among other low

G+C gram-positive bacteria. Putative GTP binding motifs were detected in the CodY peptide sequence that may be involved in GTP binding. The binding of this nucleotide induces increased DNA-binding properties of CodY. CodY is able to dimerize [8,46,47]. The dimer interface is formed by highly conserved residues across CodY homologues, (figure 1) [47]. Each monomer of CodY is able to bind one BCAA co-factor [46]. Two major domains in CodY peptide sequence are responsible for two distinct physical interactions. One interaction involves the binding of branched chain amino acids (BCAA) to the GAF ("cGMP-stimulated phosphodiesterases, adenylate cyclises and a bacterial transcription regulator FhlA") domain of CodY localized at the N-terminal region of the protein with, as a consequence of the binding, a conformational change activating DNA-binding property and leading to the formation of a hydrophobic pocket surrounding the bound BCAA, (figure 1) [46]. The second interaction involves a helix-turn-helix (HTH) motif localized at the C-terminal region of the CodY. The HTH of CodY binds to a consensus DNA palindromic sequence leading to the repression of genes localized downstream of the binding site [5,6,20,26,30,31,60,71,79]. The *B. subtilis* CodY HTH sequence -**AS**KI**AD**RV [Helix 1], **GITR** [turn], **SVIVNALR** [Helix 2]- is extremely well conserved (conserved amino acids are depicted in **bold**) suggesting that CodY homologues recognize and bind target promoters in a similar manner [34,47].

CodY senses the intracellular nutrient status of bacteria by BCAA binding

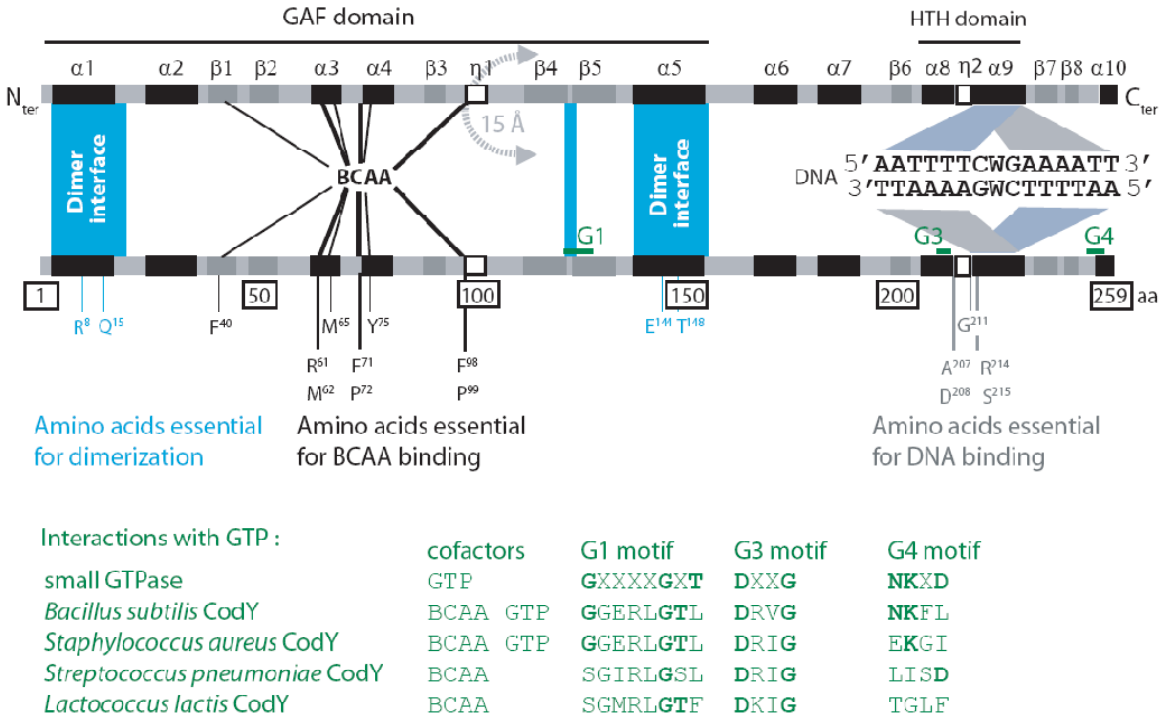
Bacteria take up nutrients from the environment to survive and multiply. When nutrients become limiting, bacteria stop dividing and the population remains stationary. A hallmark of this transition is the decrease of intracellular levels of BCAA [22,29,48,49,57,63,84;87]. The decreasing concentration of BCAA in the starved bacteria causes a transcriptional de-repression of the direct targets of CodY linked to a detachment of the inactivated dimers from DNA binding sites. CodY seems to act as a nutritional sensing molecular system leading to bacterial adaptation by newly expressed genes in starved low G+C gram-positive bacteria. Mechanistically speaking, binding of BCAA occurs to the N-terminal GAF domain of CodY and induces a conformational change that is thought to be translated to the C-terminal DNA binding domain, thereby increasing the affinity of CodY for its binding DNA box [18,46,47]. GAF domains are present in numerous signalling and sensory proteins, in transcription factors, and in the c-di-GMP signalling mechanism [46,47,97]. The combination of HTH and GAF domains within a single polypeptide chain was not previously reported in other proteins among all sequenced organism; it seems to be specific to CodY and restricted of low G+C gram-positive bacteria [47]. The common characteristics of all GAF domains present in proteins belonging to all the three kingdoms of life, are a β-sheet and two α-helices forming super-imposable tri-dimensional structures [47]. The crystal structure, the putative GAF domain of *B. subtilis* CodY was super-imposable with the GAF domain of the YKG9 protein found in *Saccharomyces cerevisiae*. This superposition validated that a GAF domain is present in CodY [47].

In order to investigate which BCAA is needed for CodY activity, *L. lactis* CodY was tested for *ilvD* promoter affinity with gel retardation assays performed in the presence of leucine, valine and isoleucine [27]. Only the presence of isoleucine enhances significantly CodY binding whereas leucine and valine showed lower and insignificant increase of DNA binding [27]. Isoleucine seemed also to be the major cofactor for CodY in *S. aureus* [69]. Interestingly, excess of isoleucine in the medium leads to a situation where CodY is abnormally activated, causing a specific growth inhibition. This growth inhibition might be due to the block of metabolic pathways involved in amino acid synthesis [27]. The specific isoleucine-induced and CodY-related growth inhibition was observed in *L. lactis* and *S. aureus* [14,27,69]. But, isoleucine is not the only BCAA able to activate CodY homologues. Valine was also able to bind *B. subtilis* CodY leading to 18 fold higher affinity for DNA [47]. The best description of CodY-isoleucine binding comes from X-ray crystallography with *B. subtilis* CodY and showing that the isobutyl side chain of isoleucine is enveloped in the hydrophobic pocket formed by the GAF domain of CodY whereas the carboxyl groups of isoleucine present polar interactions with nearby residues [47]. The conformational changes resulting from the binding of BCAA to CodY can affect residues far removed in space from the co-repressor binding site [47]. Finally, an additive effect of GTP and BCAA on the binding of *B. subtilis* CodY to DNA was reported in the promoter region of *B. subtilis ylmA* [5,29,80].

CodY senses the intracellular energetic status of bacteria by GTP-binding

As reported for BCAA, the intracellular concentration of GTP also decreases in starved bacteria entering the stationary phase of growth. For example, intracellular GTP concentration was estimated to be between 2 and 3 mM during the exponential phase of growth in *B. subtilis* and decreases to 300 µM during the early-stationary phase [29,85]. By binding GTP, CodY directly senses the energetic status of the cell and may control the expression of developmental genes implicated in morphological differentiation. Sporulation or expression of flagella were reported in starved *B. subtilis*, leading to different bacterial adaptation mechanisms: adopting a tougher survival form or swarming towards healthier environment, respectively [6]. The discrimination of genes repressed by BCAA-CodY or GTP-CodY is not fully understood yet. Indeed, the binding site of GTP in the CodY protein remains unknown but occurs definitely not in the GAF domain [29,46,47]. Three motifs encountered in small GTPase proteins, called G1, G3 and G4, are conserved among CodY homologues and were proposed to be implicated in GTP binding (Figure 1) [29,71].

Interestingly, in the G1 motif of *L. lactis* CodY, a homologue that lacks the ability to be activated by GTP, the first conserved guanine residue (G) is replaced by a serine residue (S) (Figure 1) [68].



Interactions with GTP :	cofactors	G1 motif	G3 motif	G4 motif
small GTPase	GTP	GXXXXGXT	DXXG	NKXD
Bacillus subtilis CodY	BCAA GTP	GGERLGTL	DRVG	NKFL
Staphylococcus aureus CodY	BCAA GTP	GGERLGTL	DRIG	EKGI
Streptococcus pneumoniae CodY	BCAA	SGIRLGSL	DRIG	LISD
Lactococcus lactis CodY	BCAA	SGMRLGTF	DKIG	TGLF

Figure 1: Structure of *Bacillus subtilis* CodY and physical interactions.

The picture presents the secondary structure of the 259 amino acids of *B. subtilis* CodY with α-helices (■), β-sheets (■) and η-turns (□) as determined by crystal structure analysis [46,47]. The interface region between two CodY homodimers is presented in blue as well as the amino acids important for the dimerization process. In green is depicted the G1, G3 and G4 putative GTP binding motifs. The lines above the GAF and HTH domains show their location on the peptidic sequence of CodY. Black lines illustrate amino acids in the CodY sequence that interact with BCAA according to their position, also shown in black. In gray are depicted the amino acids needed for DNA-binding. *Dimerization:* Arg[8], Glu[144], Gln[15] and Thr[148] amino acids are important for CodY dimerization according to crystal structure and sequence alignments [47]. *HTH motif:* The *B. subtilis* CodY helix-turn-helix (HTH) motif is located in the C-terminus between residues 203 to 226 according to crystallography studies, and between residues 200 to 224 by comparison of HTH regions of characterized transcription factors [21,34,47]. Ala[207], Asp[208], Arg[214] and Ser[215] amino acids are essential for DNA-binding properties of CodY according to site-directed mutagenesis combined with DNA-binding experiments [34]. *GAF domain:* in *B. subtilis*, the binding of BCAA to CodY triggered extending refolding of the binding site loops linking β3 and β4 sheets with up to 15 angstroms movement. This refolding appeared responsible for the formation of a hydrophobic binding pocket in the GAF domain of CodY. The core and its dimerisation interface are unchanged during the refolding process [46]. More precisely in the GAF domain, F[40], F[71] and F[98] amino acids are essential for CodY-BCAA binding, according to site-directed mutagenesis combined with *in vitro* CodY-DNA footprinting experiments [92]. *GTP-binding:* Comparison of putative GTP binding motifs in CodY homologues leads to the characterization of three conserved motifs with a total of 10 amino acids highly conserved; motif G1 (GXXXXGXT), motif G3 (DXXG) and motif G4 (NKXDTQ), where X is a non-conserved amino acid [71;29]. These motifs are typically found within a single domain in small GTPase proteins and are dispersed in CodY, the G1 motif being localized in the N-terminal moiety of CodY whereas G3 and G4 are localized in the C-terminal part of the protein [47]. The implication of these motifs for cofactor binding to CodY remains controversial [29].

Moreover, substituting a glycine in the sixth position of the G1 motif of CodY to asparagine greatly decreased CodY affinity for GTP [71,83]. Small GTPase proteins hydrolyse GTP whereas CodY probably does not. Indeed, non-hydrolysable analogs of GTP (guanosine 5'-[γ-thio]triphosphate, guanosine 5'[βγ-imido]triphosphate) have been able to activate CodY at similar concentrations than those at which GTP was able to activate CodY for DNA binding [29]. An extensive study was performed by Handke *et al* to investigate the ability of molecules resembling GTP to activate CodY of *B. subtilis* for DNA binding [29]. GTP and dGTP are able to induce CodY binding to the *ilvB* promoter suggesting that CodY does not discriminate between ribose and desoxyribose forms of GTP [29]. But because GTP concentration is much higher than dGTP concentration within cells, as reported for example in *Salmonella typhimurium*, GTP is probably the physiological substrate of CodY [9]. Intriguingly, ATP is able to compete against GTP for binding to CodY -in contrast to CTP and UTP- but without increasing the affinity of CodY for DNA [29,71]. Finally, a synthetic additional ppppG molecule was able to activate CodY binding to DNA even more efficiently than GTP [29].

In parallel to CodY, another regulatory system is triggered whenever the intracellular GTP levels decrease. This adaptation is called "**s**tringent **r**esponse" (ST) and leads to global adjustment of gene expression [23,29,45,80,86,87]. Upon nutrient starvation, the intracellular pool of amino acids decreases thus leading to increased accidental binding of uncharged tRNA (tRNA without amino acid) to the ribosome. Then, the ribosome-associated protein RelA mediates the synthesis of two different alarmones, called magic spots, the guanosine pentaphosphate (pppGpp) and the guanosine tetraphosphate (ppGpp). These alarmone molecules are the products of enzymatic phosphorylation of GTP and GDP, taking phosphate groups from ATP hydrolysis. The reaction is mediated by RelA and SpoT in gram-negative bacteria and **R**elA/**S**poT **h**omologs (RSH) in gram-positive bacteria [50,56,58,93]. Subsequently, ppGpp binds to the RNA polymerase (RNAP) and redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival [86]. In other words, genes implicated in macromolecular biosynthesis are down-regulated whereas genes involved in amino acid biosynthesis and stress tolerance are up-regulated [50]. Therefore, in response to starvation, macromolecular stocks of energy and nutrients are mobilised and metabolism of amino acids is activated, as well as stress tolerance mechanisms [50]. The stringent response decreases intracellular GTP levels and also decreases GTP synthesis by inhibiting the inosine monophosphate dehydrogenase [86]. Therefore, the stringent response is related in gram-positive bacteria to the de-repression of the CodY regulon. However, the alarmones pppGpp and ppGpp are not able to bind and activate CodY directly [29]. In summary, CodY is also activated by GTP cofactors which probably bind inside motifs resembling those found in small GTPases, and the stringent response can de-repress the CodY regulon by decreasing the intracellular GTP levels.

DNA fixation sites and conserved targets of CodY

HTH motifs of DNA-binding proteins are supposed to function in a comparable manner [66]. Generally, helix 1 has a stabilizing role and sits above the major groove of DNA whereas helix 2, the so-called recognition helix, forms specific interactions with the major groove of DNA, both helices being placed at an angle of 120° [34,66]. Through its HTH motif, CodY binding to the AATTTTCWGAAAATT palindromic DNA sequence was established first in *L. lactis* when searching for a *de novo* motif in front of CodY-regulated genes, and then across a variety of CodY-containing bacteria [7,17,19,26,27,51,76,77]. It is probable that each monomer of CodY recognizes half of the symmetrical DNA binding site, as reported for other DNA-binding proteins such as the well known oncogenic transcription factors Fos and Jun, many restriction enzymes and the telomerase [24,73]. In addition, investigation based on protein stitchery revealed the design superiority of palindromic DNA sequences for protein recognition [67]. Experiments performed with substitutions of DNA base pair showed, first, that the two primary GG remain important for DNA-binding, and second, that increasing or decreasing the similarity between CodY binding sites to the consensus recognized sequence increases (or decreases, respectively) the binding of CodY to DNA [5;27]. Third, varying the location of the artificial CodY box by 5 nucleotides (half helical turn of DNA) from the correct position erases CodY regulation, suggesting that CodY-mediated repression might be helix-face-dependant [19]. Fourth, inserting an artificial CodY-binding site in front of a gene that is not regulated by CodY leads to an artificial CodY-mediated regulation of that gene, whereas removing the binding site of a CodY-regulated gene leads to the loss of CodY regulation [19]. Altogether, these experiments prove that the AATTTTCWGAAAATT palindromic DNA sequence is "an independent functional motif" responsive to CodY protein *in vivo*, and therefore, it is generally accepted that a gene de-repressed in the absence of CodY and possessing a CodY box in its promoter should be a direct target of CodY [5,19,27,30]. Surprisingly, the AATTTTCWGAAAATT sequence is not present in the genome of *B. subtilis*, but appears to harbour homologues containing up to five mismatches [5,42]. A CodY box containing 2 mismatches compared to the consensus is present in the promoter region of *B. subtilis ylm*. Base substitution leading to only one mismatch resulted in CodY-DNA binding both in absence and presence of BCAA and GTP. Thus, the regulation of the targeted gene by cofactors sensing is not possible, suggesting that *B. subtilis* CodY displays too high affinity for the consensus binding motif [5]. Note that sometimes, CodY-DNA binding can interfere with other regulators, as for example in the promoter region of *dpp* (dipeptide permease operon) where CodY binds to a DNA sequence that overlaps with the binding region of another regulator called AbrB [5]. The strength of CodY-DNA binding has been estimated in 3 independent studies and results by similar K_d values in the nano-molar range. Since sub-nanomolar dissociation constants usually result from covalent binding interactions, CodY-DNA interaction is therefore quite strong [30,34,79]. Additionally, increasing CodY concentrations *in vitro* can lead to additional CodY binding sites displaying various affinities for CodY, as in the promoter region of *L. monocytogenes argC* [6;34].

Hence, among low GC gram-positive bacteria, the CodY sequence, its cofactors, the recognised DNA palindrome and the dimerization process are all relatively well conserved. Therefore, it is likely that some CodY-regulated genes could also be conserved across species, thereby illustrating a probable basic and ancestral function triggered by CodY. CodY regulons, mainly determined by microarray experiments comparing wt and *codY* mutant, have been determined in *S. aureus*, *B. subtilis*, *L. lactis*, *L. monocytogenes* and *Streptococcus pneumoniae*. Similarly, searches for CodY DNA-binding motifs were performed in 12 different genomes belonging to *Lactococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, *Listeria*, *Enterococcus* and *Clostridium* species, and resulted to the discovery of a consensus sequence (containing up to three nucleotide mismatches) upstream of microarray-based differentially expressed genes encoding for BCAA synthesis, secreted proteases and oligopeptide transporters. Thus, the ancestral function of CodY probably aims at increasing the pool of intracellular BCAA by different mechanisms [6,27,30,60].

CodY exerts a feedback control on the pathways involved in the biosynthesis of its direct effectors or cofactors [27]: it affects operons involved in the synthesis of isoleucine, leucine and valin (*ilv-leu*) as well as the oligopeptide permease (*opp*), all recurrently identified as targets of CodY [5,6,8,20,29,34,47,51,51,52,60,69,71,76-80,82,83,86,91,92]. In general, CodY regulons are composed of hundreds of genes that usually overlap across bacterial species when concerning genes implicated in the amino acid metabolism. However, and not unexpectedly, CodY-regulated virulence mechanisms remain highly organism-specific [6,27,30,60]. Finally, the major metabolic regulators CodY, the catabolite control protein A (CcpA) and the transcriptional nitrogen regulator A (TnrA) control together the transcription of the *ilv-leu* operon, that can be seen as a major intersection of metabolic pathways, and a way to link nitrogen and carbon metabolism in gram-positive bacteria [25,80,88].

Regulation and physiology of CodY

Numerous studies attempted to reveal how CodY regulates the expression of genes, but the regulation of CodY itself remains still poorly understood. From a mechanistic point of view, there is no necessity to regulate CodY because the presence of co-factors could be sufficient to influence the activity of CodY. Nevertheless, CodY boxes have been detected in the promoter region of *codY* genes in bacteria belonging to *Lactococcus*, *Streptococcus*, *Enterococcus*, *Staphylococcus* and *Clostridium* genera [27], thus permitting negative feedback regulation. The most convincing evidence for an auto-regulation of *codY* expression comes from experiments performed in *L. lactis*, where CodY is able to bind to a box located upstream of its ORF, in the presence of isoleucine [19]. Replacement of that box with an unrelated sequence prevents CodY binding to its promoter. Moreover, *lacZ* fused in frame to disrupted *codY* resulted in an increased of β-galactosidase activity [19]. These results are coherent with a negative feedback regulation of *codY* gene by CodY itself [19]. The auto-regulation of *codY* has also been validated experimentally in *Streptococcus pyogenes* and *Streptococcus pneumoniae* [32];[19,30]. Thus, during the exponential growth, activated CodY should be expressed at low levels due

to auto-repression whereas when nutrients become limiting and CodY inactivated, its expression should increase.

Interestingly, CodY is sometimes expressed independently or co-transcribed with other genes (*pncA*, *aat*, *xerC*, *clpQ* and *clpY*), and in some cases both the transcript of *codY* alone and co-transcript of *codY* and another ORF were detected. But the biological significance of the co-transcription is not known. For example, *codY* is probably co-transcribed with downstream *pncA,* encoding a pyrazinamidase/nicotinamidase in *Streptococcus mutans* [45]. In *S. pyogenes*, *codY* is expressed both monocistronic and together with a downstream *aat* gene encoding an aspartate aminotransferase. Interestingly, only the monocistronic mRNA of *codY* appeared to be negatively auto-regulated [51]. Finally, in *Staphylococcus aureus*, *codY* is co-transcribed with a tyrosine recombinase and two ATP-dependent proteases in a long transcript but also in a monocistronic form [54,69]. In cases of co-transcription, there is a need to control that the observed phenotype is not due to polar effect, as done in the study on *S. aureus* using Northern blot to probe the transcripts of genes surrounding *codY* [54]. Finally, the genomic context surrounding *codY* differs amongst organism as well as the *codY* sequence itself. But the homology of *codY* sequences is not related to the similarity of the genomic context surrounding *codY* [69].

CodY in pathogenic gram-positive bacteria

CodY regulates genes involved in the primary metabolism in order to adapt the stressed bacteria to starvation. Additionally, virulence mechanisms appear also CodY-regulated in various pathogenic gram-positive bacteria. The purpose of the following sections is to summarize recent discoveries of CodY-regulation on the virulence of gram-positive human pathogens.

***Bacillus anthracis* and anthrax disease**

Anthrax is an acute and lethal disease of mammals caused by inhalation, ingestion or contact with wounded skin of *Bacillus anthracis* spores [91]. Once inside the host, spores germinate giving vegetative cells that multiply in host tissues. Both a tripartite toxin encoded by *pagA*, *lef* and *cya* genes, as well as a poly-y-D-glutamate capsule encoded by the *cap* operon of *B. anthracis* are needed for host tissue invasion and immune system fighting during proliferation of *B. anthracis* vegetative bacteria [59]. The toxin component genes are located on pXO1, a 182-kb plasmid [64]. The biosynthetic enzymes for capsule production are encoded by the *capBCADE* operon located on plasmid pXO2 [13]. The presence of pXO1 plasmid is required for toxicity of *B. anthracis* but both plasmids are necessary for full virulence. The regulation of virulence in *B. anthracis* is complex and involves a lot of factors: among them, the anthrax toxin activator gene (*atxA*) that is also located on pXO1 seems to play a central role [16]. Experimental observations support the idea that an additional unknown factor is important for controlling *B. anthracis* toxin genes [16]. Interestingly, the production of toxin components in *B. anthracis* is low during the exponential growth and reaches maximal levels during entry in the stationary phase [81]. Recently, complete abolition of the expression of the three

virulence factors (*pagA*, *lef*, *cya*) needed for proliferation of *Bacillus anthracis* in mammals has been reported in a *codY* mutant as assessed by a gene-*lacZ* fusion assay and confirmed with immuno-blotting [91]. The authors suggested that CodY probably promotes the AtxA posttranslational accumulation needed for the expression of *pagA*, *lef* and *cya*, instead of directly repressing the toxins [91]. Finally, the disruption of *codY* completely abolishes virulence of subcutaneously injected *B. anthracis* spores in OF1 mice, (figure 2). Although CodY-regulated virulence in *B. anthracis* is clearly established, mechanisms of action and regulation remain an open question.

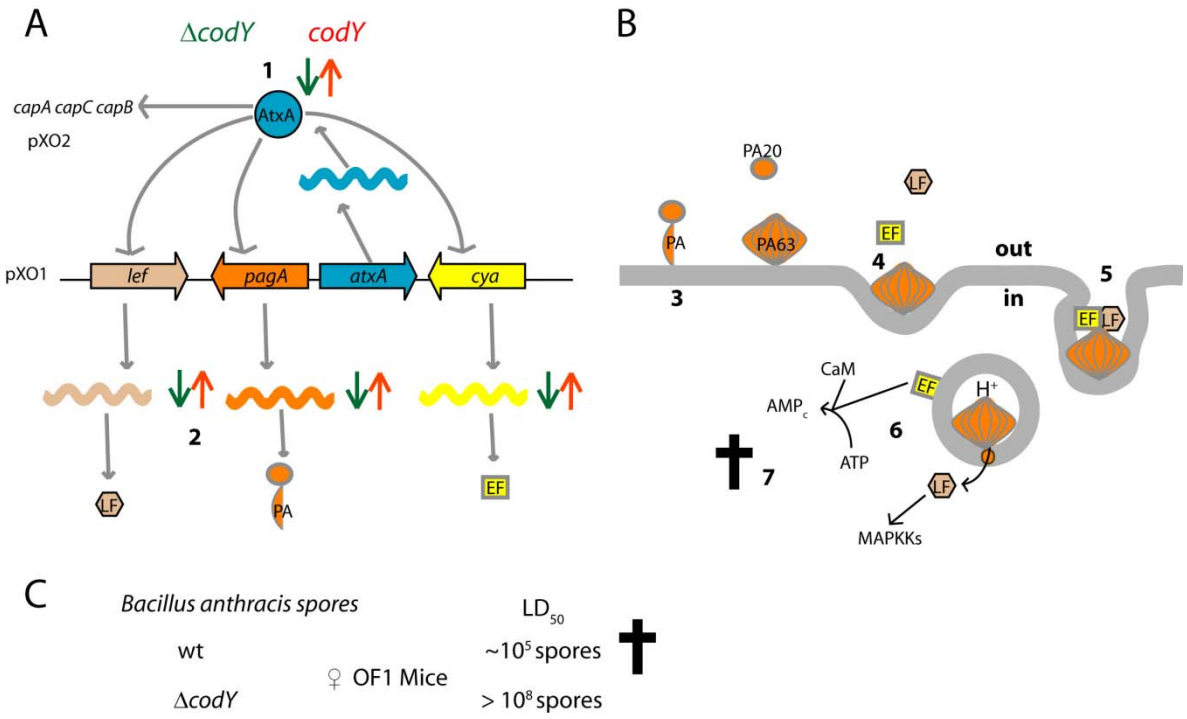


Figure 2. CodY is required for anthrax toxin production in *Bacillus anthracis*.
A. Schematic representation of the genes encoding anthrax toxins lethal factor *lef* (brown), the protective antigen *pagA* (orange), the edema factor *cya* (yellow) and the toxin regulator *atxA* (blue) on the pXO1 plasmid. **1.** AtxA activates the expression of *lef*, *pagA* and *cya* as well as the capsular genes located on pXO2. **2.** Both the toxins mRNA level and the protein level of AtxA decreased in absence of CodY. **B.** Cellular model of action of anthrax toxins. **3.** The mature protective antigen (PA) binds to a not-shown receptor present in many cell types. PA is cleaved by host cell proteases on the surface of the targeted cell or in the serum of the host releasing PA20 whereas PA63 oligomerizes into a ring-shaped heptamer. The cleavage of PA is needed to expose binding sites for EF and LF. Without cleavage, the process is not toxic for the targeted cell. **4.** Oligomerization triggers receptor-mediated endocytosis of the anthrax complex. **5.** LF and EF bind to the complex. **6.** Acidification of the endocytosolic vesicle allows the translocation of EF and LF, EF remaining associated to

the vesicle whereas LF is released into the cytosol, both reaching their targets. EF, the adenylate cyclase edema factor converts intracellular ATP into cAMP implying also host calmodulin. LF, the zinc protease lethal factor cleaves mitogen-activated protein kinases (MAPKKs) of the MAPK pathway that relays environmental signals to transcriptional machinery. There are also putative additional targets of LF. **7.** The decrease of intracellular ATP and the cleavage of MAPKKs leads to cell death. **C.** Spores of *Bacillus anthracis* 7702 wild-type and *codY* mutant were injected subcutaneously into females OF1 mice in order to estimate the half-lethal dose. Results showed that injection of $3.6*10^5$ spores of wild-type kills half of infected mice whereas even at 10^3 fold higher doses ($>10^8$) of spores of the *codY* mutant did not kill infected mice.

Clostridium difficile and antibiotic-associated diarrhea

Clostridium difficile is the principal agent of antibiotic-associated diarrhea leading to the potentially lethal pseudomembranous colitis [20,37]. Today, *C. difficile* is considered to be one of the most important causes of health care-associated infections [74]. Antibiotic therapy alters the microflora allowing colonization of the intestinal tract by *C. difficile* [37] or simply permitting its growth. CodY is a repressor of toxin gene expression in *C. difficile* [20]. At the molecular level of *C. difficile* toxicity, *tcd*R gene encodes a sigma factor allowing the expression of two toxins TcdA (enterotoxin A) and TcdB (cytotoxin B) responsible for gastrointestinal damages and the antibiotic-associated pseudomembranous colitis [35,36]. Both toxins act by glycosylating members of the Rho family of small GTPases in host cells leading to the disaggregation of the microfilament cytoskeleton [35,36]. The initial observation that isoleucine may play an important role in toxin production by *C. difficile* suggests a putative CodY-mediated expression of toxins not reported by the authors [33]. The mRNA levels of *tcd*R, *tcdB*, *tcdE*, *tcdA* and in a less impressive manner the one of *tcdC* (all of which being co-localized on a pathogenicity locus) are all de-repressed in a *codY* mutant of *C. difficile,* both during the exponential and the stationary phase [20]. CodY is able to bind the promoter region of *tcd*R and the binding increases in the presence of BCAA and GTP in a gel shift. Footprinting experiments show three protected regions in the promoter of *tcd*R. One is localized at -281 to -309 and shows the highest affinity for CodY, the second at -348 to -382 and finally the last at -40 to -58 from the start codon [20]. The transcriptional start of *tcd*R has not been characterized during the study. Observation of increased mRNA levels and CodY binding suggest CodY-regulated toxin production in *C. difficile* by direct binding to the *tcd*R promoter [20]. Thus, CodY indirectly represses the virulence factors encoded in a pathogenicity island through repression of the activator *tcd*R (Figure 3).

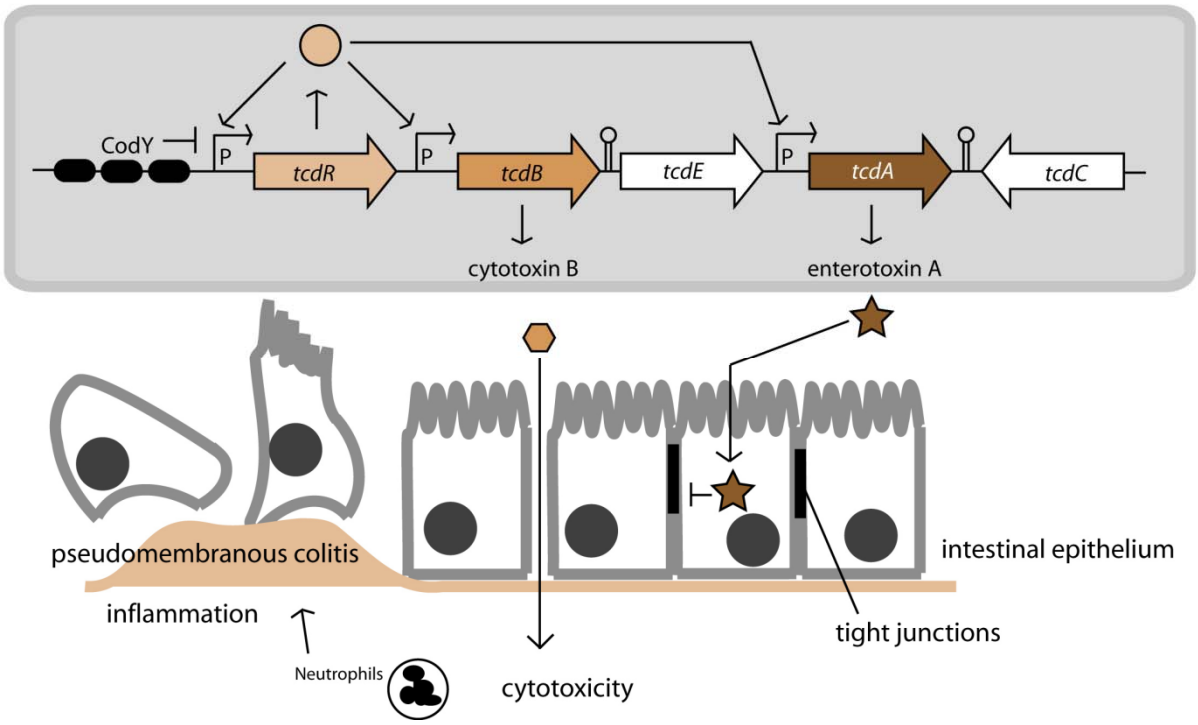


Figure 3. Schematic representation of toxins expression in *Clostridium difficile* in the intestine and the function of its CodY-regulated toxins in pseudomembranous colitis appearing notably after antibiotic treatment.

The bacterium is presented as a gray rectangle on the picture and possesses a pathogenic island beginning with three CodY binding motifs and ending with the *tcdC* gene. Three promoters (P) and two terminators (⍑) are located in the pathogenicity island (not drawn to scale). Upon starvation, CodY detachment from the boxes located in front of the *tcd*R gene activates the transcription of *tcd*R, a positive regulator for toxins encoding genes, and TcdC, a negative regulator. By TcdR-mediated activation, *C. difficile* expresses both TcdA and TcdB. TcdA, also called enterotoxin A, is able to enter apically the intestinal epithelial cells and disrupt the tight junctions. The created opening between cells allows TcdB or cytotoxin B to attack cells located below the epithelium. Both toxins mediate the production by the host of tumour necrosis factors and proinflammatory interleukins, leading to increased vascular permeability, recruitment of immune cells (neutrophils and monocytes), apoptosis, detachment of epithelial cells and inflammation. For the human host, this process leads to pseudomembranous colitis. The function of TcdE is not clear but could be related to the export of toxins from the bacteria to the environment.

Listeria monocytogenes and listeriosis

Listeria monocytogenes is a gram-positive facultative intracellular bacteria and the causative agent of human listeriosis characterized by meningitis, septicaemia and foetal death [55]. CodY in *L. monocytogenes* is able to regulate both carbon and nitrogen assimilation, both responding to GTP and BCAA [6]. A mutation of *relA*, the gene responsible for the production of alarmones during the stringent response, leads to a decreased virulence phenotype in a murine infection model, whereas mutations of *codY* in that *relA* minus mutant can restore virulence [6]. Moreover, *relA* mutations prevent the de-repression of CodY regulon during the early

stationary phase [6]. These results suggest that RelA probably renders CodY inactive by decreasing intracellular levels of GTP and that CodY represses virulence of *L. monocytogenes in vivo*; yet the deletion of *codY* leads to a similar virulence as in the wild-type, but with a slightly delayed onset of disease [6]. No differences occur between *relA*, *codY* and *relA-codY* mutants as compared to the wild-type during the escape from the phagosome as monitored by transmission electron microscopy [6]. The links between CodY and RelA have been demonstrated in different organisms. But how they influence virulence in *L. monocytogenes* remains poorly understood. Finally, CodY activates the *agr*-like system of *L. monocytogenes*, a two component regulatory system responsive to auto-inducing peptide well studied in *Staphylococcus aureus* [6]. *S. aureus agr* is a highly complex regulatory circuit allowing the bacteria to sense the presence of neighbours, phenomena that has been named quorum sensing. *Agr*-like systems refer to two component signalling system presenting high level of homology with the *agr* system of *S. aureus*, as the one of *L. monocytogenes* [2,72].

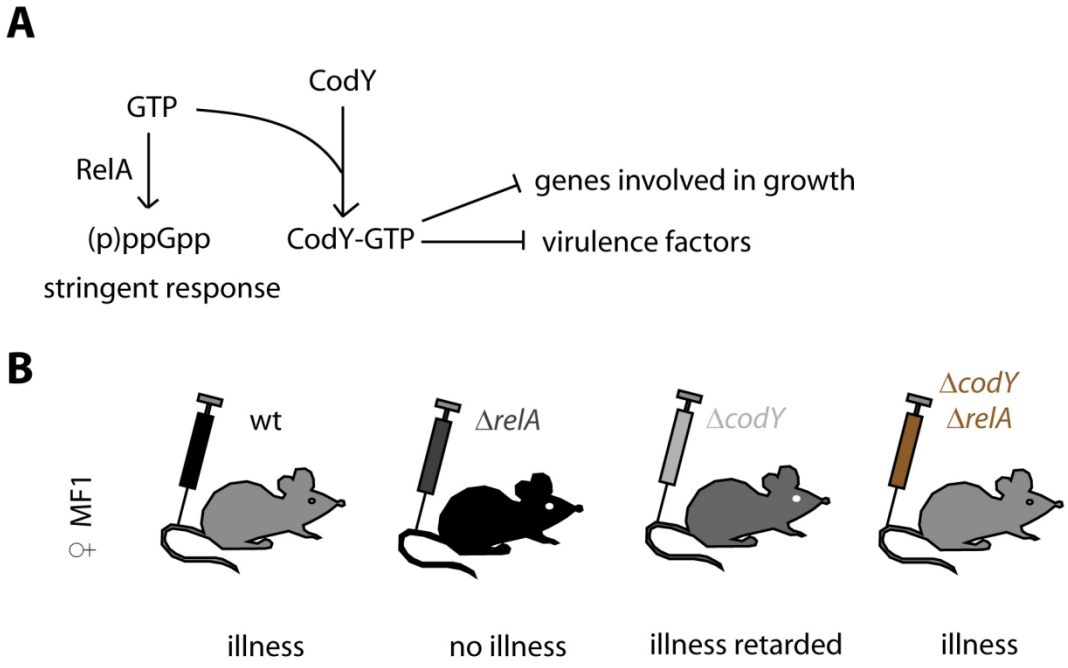


Figure 4. CodY and RelA control virulence in *Listeria monocytogenes*.
A. Schematic representation of RelA-dependant formation of alarmones in connection with GTP-dependant CodY activation regulating growth involved genes and virulence factors in *L. monocytogenes*. **B.** $5*10^5$ *L. monocytogenes* wild-type, *relA* mutant, *codY* mutant and *codY relA* double mutant were injected intravenously into females of MF1 mice. After injection, mice were observed during 96 hours for signs of illness. Results show that signs of illness were retarded when the *codY* mutant was injected, no illness occurs after injecting the *relA* mutant not able to produce a stringent response, whereas illness was restored in the double mutant. Results of CFU recorded from spleens and livers followed the same tendency as the recording of illness signs. The interpretation of these results suggested that the avirulence of the *relA* mutant could in part be explained by the continued repression of the CodY regulon whereas *codY* mutants independently of *relA* present a derepression of virulence.

Staphylococcus aureus and its virulence systems

Staphylococcus aureus is usually found on skin and in the anterior nostrils of healthy human carriers. It can be responsible for highly diverse diseases varying from benign skin infections to deadly invasive infections depending on the strain, the host defences, the site of infection, and additional parameters. *S. aureus* is also well-known for being involved in hospital-acquired infections (the so-called nosocomial infections) and is more and more frequently associated with antibiotic resistance and persistent infections. Different toxins and virulence factors are sometimes present in a strain-specific manner in *S. aureus* and were implicated in different kinds of clinical pictures such as superantigens inducing toxic shock syndrome, exfoliative toxins implicated in the staphylococcal scalded-skin syndrome, the bi-component phage-related Panton-Valentine leukocidin responsible for necrotizing pneumonia and invasive skin and soft tissue infections, various haemolysins responsible for the lysis of erythrocytes and other host cells, and so on. The expression of these toxins is highly regulated and some of the key global regulators are the accessory gene regulator (*agr* system) and the staphylococcal accessory regulator (*sarA*). According to recent studies, *S. aureus* CodY seems to be an upstream major regulator of the global regulators *agr* and *sarA*, with numerous interactions and interconnections taking place between these different systems. Both *sarA* and *agr* system are targets of CodY repression according to a pull-down assay, and CodY represses the *agr* system as evidenced by three independent studies [51,52,69]. Indeed, deletion of *codY* in strains SA564 and UAMS-1 de-represses the levels of mRNA for the haemolytic alpha-toxin (*hla*) and delta-toxin (*hld*), as well as the accessory gene regulator (*agr*) (RNAII and RNAIII) [52]. Resulting from that de-repression, the haemolytic activity of the culture supernatant of a *S. aureus codY* mutant towards rabbit erythrocytes increases compared to that of the wild-type, but can be restored by trans-complementation [52]. These results suggest a CodY-mediated repression of the *agr*-dependant haemolytic activity during the exponential growth phase. CodY negatively regulates *hla* and RNAIII transcription whereas RNAIII positively regulates *hla* on a post-transcriptional level [51]. The de-repression of the CodY regulon and the Agr activation appear when cells enter the stationary phase: CodY because of nutrient depletion whereas the Agr system is induced by the high concentration of bacteria (quorum sensing effect) and by the de-repression from inactive CodY. Under conditions of isoleucine limitation, the *agr* is prematurely activated by a de-repression of CodY, probably reflecting an escape mechanism for the bacteria becoming more virulent under nutrient limiting conditions [69]. The *agr* system of *S. aureus* does not influence the transcript levels of CodY [69].

Additional virulence factors appear CodY-regulated, such as the secreted lipase encoded by the *geh* gene, the catalase encoding gene *katA*, the haemolysin D *hld*, and the superoxide dismutases *sodM* or *sodA,* as identified in two independent transcriptomic experiments from two different laboratories working with two different *S. aureus* backgrounds [51,69]. The capsular polysaccharides (CP) were shown to enhance staphylococcal virulence in numerous animal models of infection [90]. The *cap* operon is responsible for the synthesis of the capsule in

S. aureus. Deletion of *codY* results in a de-repression of the *cap* operon, whereas a mutation of *agr* leads to the over-expression of *cap,* as reported by two independent laboratories [51,69]. Direct binding of CodY to the promoter of the *cap* operon and within the *agrA* ORF were detected [51]. Secondly, *fnbA* and *spa* are activated by CodY independently of *agr* during the post-exponential phase [69]. Both are cell-wall associated proteins implicated in the adhesion process needed for the early biofilm formation. In *S. aureus*, contradictory results have been reported for the effect of CodY on biofilm formation depending on the strain background [52,89].

RelA/**S**poT **h**omologs (RSH) are essential and conditional *rsh* mutants were generated in *S. aureus* [23]. *S. aureus* bacteria submitted to deprivation condition for valine and leucine responds to the resulting stress with the induction of RSH-regulated gene. RSH dependant repression occurred independently of CodY (*infB*), whereas genes activation by RSH (*ilvDBC-leuABC-ilvA*, *ilvE*, *brnQ1*, SAHSC_02932) depended of BCAA-CodY. Thus, the interaction between CodY and RSH is required to activate some genes involved in the response to amino acids deprivation, connecting CodY to the RSH-involved stringent response in *S. aureus* [23]. Interaction between CodY and RSH was also been studied in the context of virulence in animal models by injecting $3*10^7$ CFU of *S. aureus* HG001 wild-type, *codY* mutant, *rsh* mutant, and *rsh/codY* double mutants into the tail vein of female BALB/c mice before recording the *S. aureus* colony forming units (CFU) obtained from mouse homogenised kidneys [23]. Less CFU/kidney were obtained when mice were infected with *S. aureus rsh* mutant compared to mice infected with the other strains (figure 5). Thus, the decrease of CFU/kindey observed with the *rsh* mutant was suppressed in the *codY rsh* double mutant. To conclude, in *S. aureus*, CodY represses different virulence factors such as the *cap* operon and hemolysins as well as a broad range regulator of virulence (*agr* system), and behaves as a suppressor of *rsh* for mouse kidney colonisation.

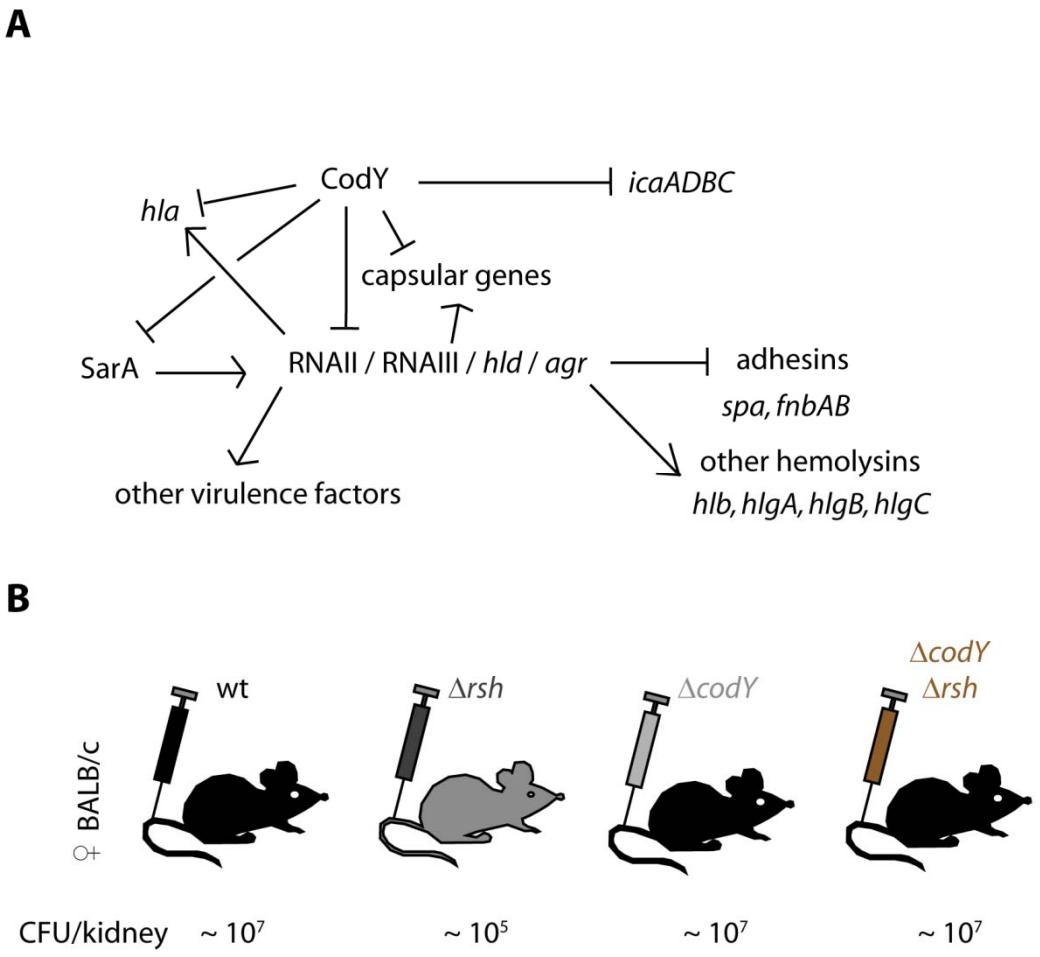


Figure 5. *Staphylococcus aureus* CodY regulates virulence mechanisms.
A. Simplified schematic representation of CodY regulating virulence pathways in *S. aureus*. Arrows indicate positive regulation and lines ending in bars denote negative regulation. CodY, when activated by GTP and/or BCAA, represses the *icaADBC* operon involved in the production of the polymeric intercellular adhesin (PIA), a component of the biofilm matrix. CodY also represses capsular genes, hemolysin A (*hla*), the staphylococcal accessory regulator A (*sarA*) and the *agr* operon as well as *hld* translated from RNAIII. By repression of RNAII and RNAIII, CodY also indirectly affects quorum sensing regulated targets such as other hemolysins and adhesins that are positively regulated by RNAIII. **B.** Schematic representation of experiments performed in an animal model. $3*10^7$ CFU of *S. aureus* HG001 wild-type, *codY* mutant, *rsh* mutant and *rsh*/*codY* double mutant were injected into the tail of vein of female BALB/c mice using 10 mice for each isogenic strains resulting to the colonization by *S. aureus* of mice kidney [23]. CFU/kidney reported showed lower CFU after injections of *rsh* mutant compared to wild-type without significant effects for *codY* mutant and *codY*/*rsh* double mutant. Thus, *rsh* is required for kidney colonization of mice after artificial *S. aureus* infections occurring only in presence of CodY.

Streptococcus pyogenes and its virulence systems

Streptococcus pyogenes is considered as the most pathogenic member of the genus *Streptococcus*. It is a human-specific pathogen causing high morbidity in many types of skin and upper respiratory tract infections. It is responsible for "strep throat", scarlet fever, pyoderma, streptococcal toxic shock, septicaemia, necrotizing fasciitis [32,53]. The organism possesses a plethora of toxins differentially expressed and putatively explaining the great diversity of diseases caused by this pathogen. Despite years of study, many unanswered questions remain regarding the pathogenesis and virulence of group A streptococci. *S. pyogenes* is able to switch phenotypes (e.g. immune evasion, adherence, internalization, persistence) when the organism responds to different environmental cues (e.g. elevated CO_2, iron limitation, increased temperature, pressure, atmospheric conditions, blood components, reactive oxygen species) [41].

Recently, *S. pyogenes* CodY was found to be involved in the regulation of virulence factors and these results could explain its adaptive behaviour in terms of virulence. In a recent approach, 51 arbitrary chosen genes were quantified by q-RT-PCR and compared between *S. pyogenes* strain NZ131 and its *codY* disrupted mutant when grown in a defined medium and in human blood [53,54]. Results showed differences of CodY-mediated gene regulation when comparing both experiments, suggesting the action of biological cues which were missing in the laboratory medium, and adaptation through gene regulation according to the environment [53]. Interestingly, the *S. pyogenes covRS* and *sptRS* two-component regulatory systems appeared to increase in the absence of CodY when grown in human blood [53]. In case of *covRS*, the up-regulation decreases time-dependently suggesting the adaptation of *S. pyogenes* to human blood [53]. CodY and CovR are required for the transcription of the dipeptide transporter *dppA*. The authors suggested that CodY may acts as a co-activator of CovR, but a *codY covR* double mutant remains needed for deeper analysis of *dppA* transcriptional control [28]. The *S. pyogenes* activator *fasX* gene is responsible for aggressiveness towards the human laryngeal epithelial cell line HEp-2 (ATCC, CCL23) by promoting adherence, internalization, cytokine expression and release, and finally cell apoptosis [39]. This pleiotropic activation was down-regulated in a *codY* mutant of *S. pyogenes* strain NZ131 grown in human blood suggesting that CodY activated *fasX* indirectly [53]. The pleiotropic virulence factor regulator Mga activates the virulence factors *emm49* (a membrane-associated protein used for sequence typing in *S. pyogenes*), streptococcal collagen-like surface protein *(scl*), C5a peptidase genes (*scpA*) and a fibronectin-binding protein (*sof*) [32]. Both, the *mga* regulator and the previously mentioned virulence factors are under-expressed in the absence of CodY when growing *S. pyogenes* in human blood (Figure 6A) [53]. In Todd-Hewitt medium, CodY regulates positively and in a growth-phase dependant manner the virulence-regulatory systems encoded by *pel/sagA* and *mga* as well as the virulence factors *scl*, *prts* and *scpA* [54]. SagA is the streptolysin S precursor peptide responsible for lysis of red cells in blood-agar plates [54,61]. CodY also represses the *S. pyogenes* virulence factors *grab* which regulated proteolysis at the bacterial surface [70]. To conclude, the powerful toxic arsenal of *S. pyogenes* seems mainly regulated by CodY and the ability of the bacteria to switch its phenotypes

depending on environmental cues seems related to CodY and to CodY-connected two component systems *covRS* and *sptRS*.

- ***Streptococcus pneumoniae*** **and pneumonia**

  Streptococcus pneumoniae is a leading human pathogen responsible for pneumonia, but also meningitis and otitis media in young children, elderly people and immuno-compromised patients [10]. Each year, 1 million children younger than 5 years die from pneumonia and invasive disease [62]. Depending on age, 30–60% of survivors of meningitis develop long-term sequel including hearing loss, neurological deficits, and neuropsychological impairment [40]. CodY has been studied in *S. pneumoniae* and appeared to bind only to BCAA as cofactors and not GTP [30].

  In a recent study, nine week old CD-1 mice infected with *S. pneumoniae* D39 and its *codY* mutant revealed that *codY* is needed for nasopharynx and lung colonization by *S. pneumoniae* D39, an observation that is also confirmed when testing the adherence of *S. pneumoniae* to human nasopharyngeal cells (ATCC CCL-138) [30]. The authors suggest that a direct target of CodY named *pcpA* (a choline binding protein) is implicated in the adhesion process of nasopharyngeal epithelium. Indeed, *S. pneumoniae pcpA* is CodY-regulated based on microarray studies and contains an AT-rich sequence resembling the CodY box (AATTTATAAAATGTA) [30,75].

  Colonization of the nasopharynx is mediated by the adherence of *S. pneumoniae* to the human epithelial cells [30]. Encapsulated *S. pneumoniae* strains adhere at lower extent compared to un-encapsulated strains [11]. The implication of CodY and *pcpA* in the process of adhesion of *S. pneumoniae* to human pharyngeal epithelial cells *in vitro* was tested with Δ*codY* and Δ*pcpA* mutations in a Δ*cps* (type 2 capsule locus) deficient background [30]. Both, Δ*codY* and Δ*pcpA* mutations were associated with decreased numbers of adherent bacteria (figure 6B) [30]. The strain that displayed the lowest ability to adhere on epithelial cells was the *codY*/*pcpA* double mutant [30]. Additionally, a *S. pneumoniae* colonisation model in mouse shows that less bacteria are needed for persistent colonisation in the *codY* mutant compared to the wild-type [30]. The *ami* locus containing six ORFs encoding oligopeptide ABC transporters has been associated with adhesion of *S. pneumoniae* to eukaryotic cells [15]. Nevertheless, it is possible that the transported molecule and not the transporter itself is directly implicated in adhesion. Interestingly, *amiA*, *amiC* and *amiD* have been 1.5 to 3 times de-repressed in the Δ*codY* mutant according to microarrays performed with *S. pneumoniae* D39 [30]. Similar observations were performed with *aliA*, also encoding an oligopeptide ABC transporter and also de-repressed in the Δ*codY* mutant [30]. *aliA*, *aliB* or *ami* were also implicated in the nasopharyngeal colonization but not in the invasive disease [38]. Therefore, in *S. pneumoniae*, CodY seems to regulate the expression of *ali* and *ami* permeases as well as the choline-binding protein *pcpA*, all involved in the adhesion of the bacteria to the epithelial cells, a critical step for colonization prior to the infection.

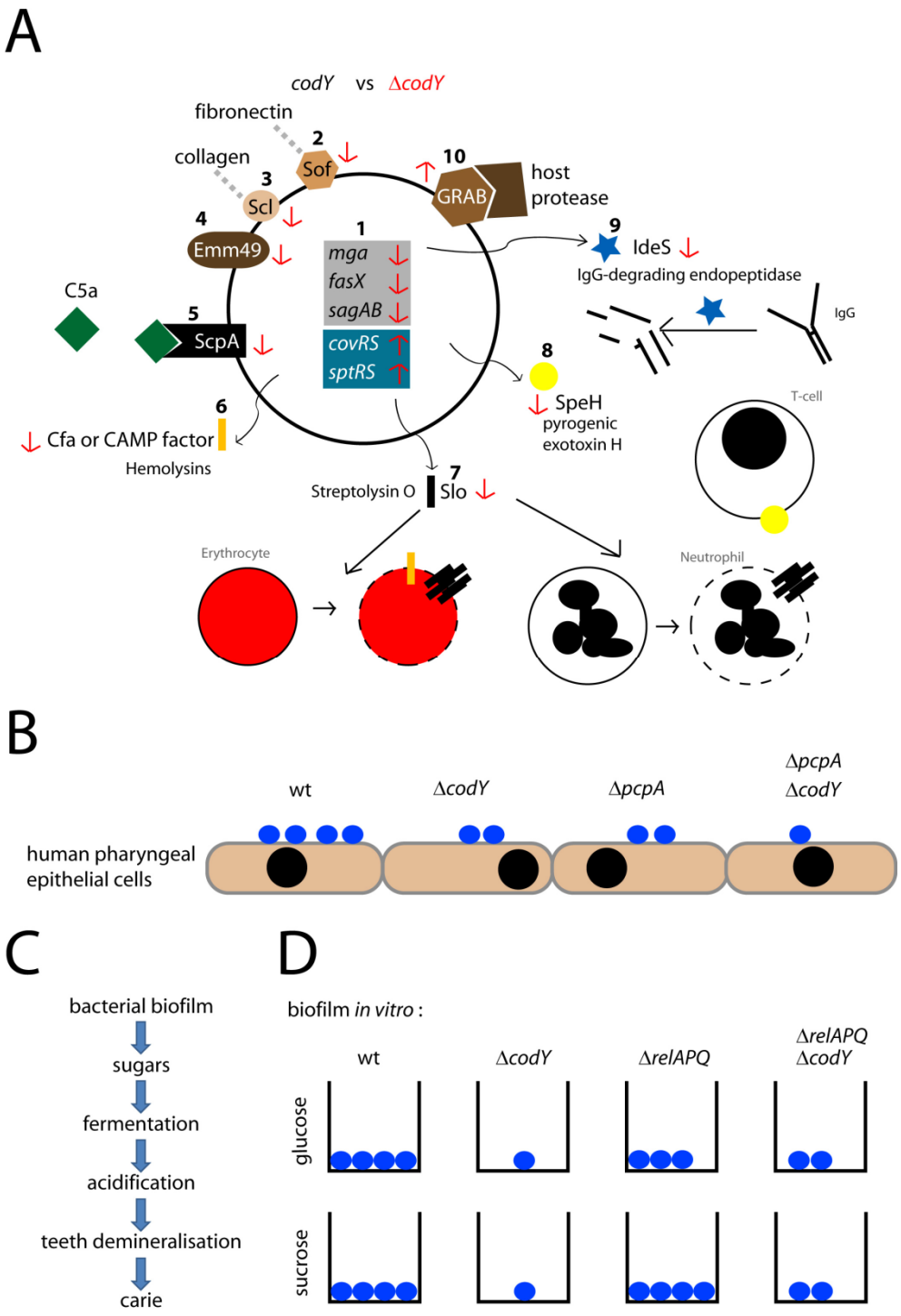


Figure 6. CodY in Streptococci.

A. In vitro CodY-regulated virulence factors in *Streptococcus pyogenes*.

Schematic representation of expression of virulence factors of *Streptococcus pyogenes* according to microarray comparing NZ131 wild-type to *codY* mutant that were grown in human blood [53]. Red arrows indicate up or down expression in the *codY* mutant compared to wild-type. Note that even if some factors in the picture are

shown at the protein level, the over or down expression has been estimated at the mRNA level. **1.** Regulators of virulence (the multigene regulator in group A streptococci *mga*, the regulatory RNA *fasX* stimulating streptococcal aggressiveness towards host cells by promoting host cell apoptosis, the streptolysin S associated proteins *sagAB*) are down-expressed in absence of *codY*, whereas two component systems (*covRS* and *ptRS*) are stimulated. **2.** The fibronectin-binding protein (*sof*), **(3)** the collagen binding protein (*scl*), **(4)** the M-like protein used for genotyping (*emm49*), and **(5)** the C5a peptidase gene anti-immune-chemotaxis (*scpA*) all of them were down-regulated in absence of CodY. **(6)** The CAMP factor (*cfa*) responsible for light hemolysis and **(7)** the streptolysin O (*slo*) responsible for lysis of various cell types were down regulated in absence of *codY*. **(8)** The pyrogenic exotoxin H (*speH*) down expressed in absence of *codY* is able to bind receptors present at the surface of T-cell leading to T-cell activation and production of interleukins and cytokines. **(9)** The IgG-degrading endopeptidase (*ideS*) is also down regulated in absence of *codY*. **(10)** The protein **G**-**r**elated α_2 macroglobulin **b**inding protein (*grab*), up-regulated in absence of *codY*, is able to inactivate host proteases protecting *S. pyogenes* surface proteins. Thus, many important virulent factors in *S. pyogenes* are CodY-regulated. **B. CodY and PcpA needed for adhesion of *Streptococcus pneumoniae*.** Colonization of the nasopharynx is mediated by adherence of *S. pneumoniae* to the human epithelial cells *in vivo*. D39 wild type strain was compared to Δ*codY* mutant, Δ*pcpA* mutant and Δ*codY* Δ*pcpA* double mutant. The *codY* or *pcpA* mutants showed a decrease ability of *S. penumoniae* to adhere to nasopharyngeal cells, a process occurring *in vivo* during the development of pneumonia due to *S. pneumoniae*. CodY is thought to regulate the expression of pcpA, whereas the cell wall associated choline binding protein (*pcpA*) is needed in the process of adhesion of *S. pneumoniae* to human pharyngeal epithelial cells. C & D**. CodY needed for in vitro biofilm formation in *Streptococcus mutans*. C.** Schematic representation of the process leading to the formation of caries. Bacteria live in biofilm on teeths. *S. mutans* is the most important bacterium in the formation of dental caries. Sugars provided by the alimentation are fermented by the bacteria producing lactic acid, but can also be used to produce polymeric matrix for the biofilm. The production of lactic acid results in an acidification of the surface of the teeth which leads to a demineralisation of the teeth resulting in caries. **D.** Schematic representation of *in vitro* biofilm formation of *S. mutans* (blue round shape) in presence of glucose and sucrose comparing wild-type strain UA159 to Δ*codY*, Δ*relAPQ* and Δ*codY* Δ*relAPQ* mutants. Mutation of *codY* drastically decreased the quantity of biofilm, suggesting that CodY is needed for *S. mutans* to produce biofilm *in vitro*.

- ***Streptococcus mutans* and dental caries**

Streptococcus mutans is a predominant bacteria associated to oral biofilms and implicated to the formation of caries (figure 6C) and to the development of subacute infective endocarditis [43,45]. The bacterium in the oral cavity has to adapt to low pH conditions, nutrient source fluctuations and low levels of free amino acids [4,43]. *S. mutans* colonizes the tooth surface first by a sucrose-independent mechanism during the initial attachment to the enamel pellicle and then by sucrose-dependant pathways involving the production of extracellular polymeric and sugaric compounds formed through the action of glycosyltransferase enzymes [3,45,65]. *In vitro* experiments comparing *S. mutans* strain UA159 to its Δ*codY* mutant have shown a reduced capacity of the mutant to form biofilms in the presence of both glucose and sucrose (figure 6D), and a decreased resistance to low pH [45]. These results suggest that CodY is probably needed in *S. mutans* for caries formation, but this hypothesis has to be tested directly on teeth.

In *S. mutans*, in addition to RelA, RelP and RelQ enzymes are responsible for the production of (p)ppGpp alarmones [44,45]. RelA is the major enzyme controlling the stringent response, whereas RelP produces (p)ppGpp basal levels under unstressed conditions and the action of RelQ is poorly understood [44,45]. A recent study revealed that in *S. mutans*, RelP and RelQ are

required for the synthesis of basal levels of (p)ppGpp needed to grow under leucine and valine starvation conditions, whereas deletion of *codY* abolished grow deficiency, suggesting an inter-connection of the stringent response and the CodY-regulon. A Δ*relAPQ* mutant of *S. mutans* unable to produce alarmones and stringent response does not grow in minimal medium lacking leucine and valine, but can grow if isoleucine is also omitted. Interestingly, deletion of *codY* in that mutant restores growth, suggesting that the growth-defect is linked to CodY and that basal levels of alarmones are required for growth, in particular in amino acids depleted media [45].

- **Concluding remarks and outlook**

  The striking importance of CodY in low GC gram-positive bacteria for adaptation to starvation conditions and for the control of virulence brings the question of which factor could replace CodY function in gram-negative bacteria? CodY has been compared to **L**eucine-responsive **r**egulatory **p**rotein (Lrp) of the gram-negative *Escherichia coli* regarding the aspects of DNA binding, dimerization, repression of operons involved in branched-chain amino acid biosynthesis, and amino acid binding [27]. Indeed, Lrp is able to repress *ilvIH* operon by binding to DNA, upstream of the *ilvIH* [94]. But, whereas CodY affinity for DNA increases after cofactor binding, Lrp is removed from DNA in the presence of leucine. Moreover, Lrp and CodY do not share any structural similarity [27,60,79]. Regarding structural aspects, CodY has been compared to *Agrobacterium tumefaciens* TraR, a quorum-sensing protein that responds to a homoserine lactone pheromone [47]. The structure of TraR has been solved in complex with its cofactor and with a targeted DNA box [96]. But TraR binds homoserine lactone co-factor and belongs to a two-component system whereas CodY binds BCAA and GTP and is not involved into a two-component system to our knowledge, rendering the comparison valuable only for some structural aspects [47].

  Whereas the activation of CodY by BCAA binding and dimerization process is well understood on a mechanistic and structural level, corresponding data for GTP binding and DNA binding are missing, leading to partial or hypothetical explanations of CodY-GTP binding and CodY-DNA binding [46,47]. It is not known whether each CodY monomer recognizes half of the symmetrical DNA binding site, whether GTP effectively binds G1, G3 and G4 motifs, whether transcription factors are able to regulate *codY* expression and how *codY* is expressed during time-course studies.

  Finally, the recently-acquired knowledge of CodY regulation of virulence opened new putative approaches for therapy: for example the use of BCAA as additive drugs against infections by gram-positive pathogens could putatively keep the bacteria in a non-pathogenic status. Investigation of clearance of gram-positive pathogens from infected animal models with BCAA injected to the infection site may lead to promising future therapeutic approaches, notably in conjunction with antibiotic treatment. Using antibiotics to treat bacterial infections has decreased human mortality significantly as compared to the pre-antibiotic area. To have an idea of the order of magnitude, the use of antibiotic worldwide has been estimated between 100'000 to 200'000 tonnes for the year 2002 [95]. The biological

cost of the massive use of antibiotic is the apparition and selection of resistances against antibiotics in the bacterial world. Today, bacterial infections remains more and more often associated with resistances against antibiotics, with first the apparition of resistant clone, then multi-resistance and finally pan-resistance. In parallel, development of new antibiotics slows down and remains hard today. Now, it is possible to be infected with a bacterium resistant against all known antibiotics. But maintaining resistance also has a cost for the bacterium in term notably of growth rate [1]. Therefore, reducing the use of antibiotics could reverse the selection of clones carrying antibiotic resistance. But physicians need alternative therapeutic approaches. The post-antibiotic area begin with the lost of systematic usage of antibiotics and with the development of new therapeutic approaches. If a virulent bacterium is kept in a non-pathogenic status, there is no reason to kill it. After all, human body contains more bacteria then their own cells. It has already been proposed to supplement nontoxic BCAA in the mucosa against *Staphylococcus aureus* colonisation targeting the regulation of CodY activity [12].

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PART II RESULTS

I. Immuno-detection of *Staphylococcus aureus* biofilm on a cochlear implant

I.A. Introduction

This paper studied the case of a patient, carrying a cochlear implant to improve his hearing, who is admitted to the Geneva University Hospital because he complained from retro-auricular pain. Samples taken from the suspicious site revealed the presence of *Staphylococcus aureus*. Despite treatment with three different antibiotics (amoxicillin/clavulanate, then a combination of ciprofloxacin and rifampin), the infection, co-localized with the cochlear implant, persisted. The medical decision to remove the implanted material was made. We received the explanted cochlear implant in the research laboratory on July 2007 for analysis. We decided to observe the material by microscopy, and detected *S. aureus* as well as PIA on the implant using fluorescent microscopy and anti-PIA antibodies. The strain responsible for the infection was named "Coch" for cochlear and was easy to grow *in vitro*. The PIA produced by Coch was compared to genetic controls for PIA production *in vitro*. Results strongly suggested that the patient was infected by a *S. aureus* biofilm localized on its cochlear implant. This story illustrates the difficulties physicians encounter when a patient is infected by a bacterial biofilm on a device. The infection could not be cured by antibiotics using state-of-the art approaches. Worldwide, only two published cases of cochlear implant removal due to persistent infections by *S. aureus* were clearly associated to biofilms. In the previous cases, identification was performed using scanning electron microscopy by observation of micro-structures. We suspected that the biofilm probably established from the removable magnet pocket, because there were no report on biofilm-related infections on cochlear implants prior to the apparition of the removable magnet pocket on such implants and because detection of bacteria was stronger in this part of the implant. A reviewer from the journal Infection has cited William Cowper English poet as a feed-back: "Absence of proof is not proof of absence", to underline the fact that absence of reported cases in the past could not exclude biofilm-related infections. My contribution

to that article consisted in performing all the experiments on the explanted material and to write the manuscript.

I.B. Article:

Immuno-detection of *Staphylococcus aureus* biofilm on a cochlear implant

Immuno-detection of *Staphylococcus aureus* Biofilm on a Cochlear Implant

M.I. Kos, L. Stenz, P. François, J.-P. Guyot, J. Schrenzel

Abstract

Case presentation: A 46-year-old man suffering from progressive deafness since childhood received a Clarion 90 K cochlear implant with the HiRes® preformed electrode in his left ear in October 2006. A persistent *Staphylococcus aureus* infection failed to be treated with corticoids, amoxicillin/clavulanate, ciprofloxaxin, and rifampin. The processor was removed on July 2007.

Interventions: The removed cochlear implant processor was treated with different reagents, with the aim of detecting a *S. aureus* and *S. aureus* biofilm: (1) fluorescein-coupled Fc of anti-human serum, (2) polyclonal anti-polysaccharide intercellular adhesion antibodies coupled to Alexa Fluor 568 goat anti-rabbit immunoglobulin (Ig)G, (3) crystal violet, (4) methylene blue, (5) acridine orange, (6) Gram stain, and (7) live/dead fluorescent stain.

Results: *S. aureus* and the major constituent of the *S. aureus* biofilm, the polysaccharide intercellular adhesion, were detected on the surface of the implant. *S. aureus* was isolated after a simple contact between the implant and a solid growth medium. The ability of the isolated *S. aureus* strain to produce biofilm *in vitro* was confirmed.

Interpretation: *S. aureus* biofilm was documented on the implant. Initial bacterial colonization could be related to the pocket of the removable magnet. Colonies of *S. aureus* without biofilm were found attached to the electrode wire.

Conclusion: We report one case of a *S. aureus* biofilm infection documented on a cochlear implant, as assessed by immuno-microscopy. The biofilm was likely responsible for the persistent infection which manifested for many months after the implant surgery and could explain the unusual bacterial phenotypic resistance against administered antimicrobial agents.

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Introduction

Until recently, medical complications associated with cochlear implants were mainly related to flap necrosis, incision dehiscence, and post-operative wound infections [4, 11, 23]. The incidence of such complications was low, and they were usually successfully managed with antibiotics and/or plastic and middle ear surgery. Cases of persistent infection requiring the removal of the processor have been rare, and some have even been attributed to a primary immunodeficiency of the recipient [23]. Similarly to what has been observed on other types of implants [5], reports of biofilm formation on the surface of cochlear implants started to appear in print in 2004 [1, 17, 20]. Clinically, biofilms are complex bacterial communities that adhere to the surface of implanted biomaterial or mucosa [9, 18] and produce an extra-cellular matrix [3], leading to increased bacterial resistance against the host's immune defenses and to antibiotics [22]. Since 1985, 200 patients have been implanted at the Geneva Cochlear Implant Centre. Different types of implants have been used and, until recently, not a single case of wound dehiscence, flap necrosis, or infection of the processor has been observed. In 2007, we were confronted with an atypical and persistent infectious case that required removal of the processor, a Clarion 90 K cochlear implant with the HiRes® preformed electrode, which was then submitted to microbiological analysis.

Case History

In October 2005, a 46-year-old man suffering from progressive deafness since childhood received a Clarion 90 K cochlear implant with the HiRes® preformed electrode in his left ear.

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Surgery was performed according to the minimally invasive technique described by*O'Donoghue and Nikolopoulos* [19]. The processor was placed in a muscle pouch and attached to the bone. The patient was given intravenous ceftriaxone during the surgery, followed by amoxicillin/clavulanate (orally) for 1 week post-surgery. Healing was uneventful. Two months after cochlear implantation the patient had achieved a very good performance and was wearing his implant daily. 17 months after the surgery, however, he felt an increasing retro-auricular pain, and the site of the processor became swollen. We suspected that a hematoma had developed inside the muscle pouch containing the processor and treated the symptoms with corticoids and amoxicillin/clavulanate orally. The swelling and pain disappeared in 15 days, but reappeared 6 weeks later. Puncture removed 3 cc of a citrin liquid, and subsequent culture of the liquid showed the growth of *Staphylococcus aureus* sensitive to all tested antibiotics with the exception of penicillin G. The patient received a treatment of ciprofloxacin and rifampin for 8 weeks, but the swelling, redness, and pain persisted. Surgical drainage was performed. The surrounding tissue was debrided, and the muscle pouch and the processor were irrigated with antibiotics. Culture showed again *S. aureus*. The wound healed, but 2 weeks later liquid had again collected. The processor was removed in July 2007. The electrode wire was sectioned at the level of the cochleostomy, and the electrode array was left inside the cochlea. The wound healed in 2 days. Three months later the patient received a new cochlear implant, which was switched on 2 weeks after surgery. The patient has since reached the same performance levels with the new implant as he had with the original one.

Material and Methods

Microbiological Sampling, Cultures, and Identification of the Strain of *S. aureus*

During the removal surgery, samples were collected from the inguinal, axillary, retro-auricular, and external ear canal skin, from nasal and throat mucosa, and from fragments of the surrounding tissue. The identification of a strain of *S. aureus* was performed according to Clinical and Laboratory Standards Institute (CLSI) recommendations and included Pastorex agglutination (Bio-Rad, Hercules, CA) and the DNAse production test. A real time-PCR amplification procedure [8] was performed for confirmation.

S. aureus growth was achieved by culturing the removed processor on Mueller Hinton agar (MHA; Bio-Rad, Marnes-La-Coquette, France). *S. aureus* strain SA113 (ATCC 35556) and its *ica* mutant (*Δica::tet*) were used as control for immuno-detection of polysaccharide intercellular adhesion (PIA) [10]. MHA and trypticase soy broth (TSB; Becton Dickinson, Le Pont de Claix, France) supplemented with 1% glucose (TSBgluc) were used for bacterial growth. The *in vitro* formation of the biofilm was tested on a strain grown in TSBgluc medium during a 15-h culture. A glass coverslip was added to the well prior to culture for the detection of PIA.

Pre-Treatment of the Processor

The processor body was divided into two parts. The silicon part harboring the removable magnet pocket after the magnet was removed was snap-frozen in liquid nitrogen and conserved at 80 °C. Before testing, the device was washed with a PBS solution (Invitrogen, Carlsbad, CA) and sliced into five pieces, which were deposited in separated wells on a six-well plate. The other part, which included the electrode wire, was washed with PBS, immediately fixed for 1 h in 2% v/v glutaraldehyde (Fluka, Chemica, Germany) in PBS, and conserved in a PBS solution at 4 °C. Before testing, this part and the electrode wire were sliced into five pieces each.

Crystal Violet Staining Assay for Initial Evaluation of the Presence of the Biofilm

Glutaraldehyde-fixed implant sections and the*in vitro* heat-fixed bacteria biofilm were stained for 10 min with 1% (w/v) crystal violet (CV) stain freshly diluted twofold in 1% ethanol/distilled water, as previously described [21]. The stained material was then washed three times with PBS and inspected with the naked eye and by white light microscopy.

Immuno-Detection of PIA

Sections of the processor and *in vitro* biofilm from the *S. aureus* grown on a circular glass coverslip (diameter 25 mm) were washed twice with PBSAT (PBS containing 0.02% azide and 0.05% Tween. 20) with slow shaking for 5 min. Cross-reactions with *S. aureus* protein A were blocked by incubating the material for 2 h with 1:1,000 normal donkey serum (Jackson Immuno Research, West Grove, PA). For specific detection of PIA, the material was incubated for 1 h with 1:3,000 α-PIA rabbit polyclonal anti-PIA antibody [15], then washed twice with PBSAT. The binding of specific antibodies was revealed after incubation with 1:3,000 dilution of Alexa Fluor 568 goat anti-rabbit IgG (H + L) (Molecular Probes, Eugene, OR) as secondary antibody. The material was washed with a PBS solution (PBSAT) containing 1% albumin (ZLB Behring AG, Bern, Switzerland) and 0.1% tween-20 (Fluka).

Immuno-detection of *S. aureus* Through Binding to Cell-Wall Protein A

Processor sections and the slices of the electrode wire were incubated for 30 min with PBSAT and for 30 min with 1:1,000 fluorescein-coupled to the Fc fragment of goat anti-human serum (Jackson Immuno Research). The slices were then washed five times with PBSAT before being observed under the microscope.

Immuno-Fluorescent Microscopy

All incubations were performed in PBSAT. A humid chamber was prepared for antibody incubations, consisting of a six-well plate that was hermetically sealed with Parafilm and light-protected with aluminium foil. Images were acquired by an Axiocam color camera (Zeiss, Iena, Germany) on an Axioskop 2 microscope (Zeiss). Ultraviolet excitation for fluorescent imaging and white light microscopy were performed both separately and in combination. Filter set 09 (Zeiss; excitation BP 450–490, emission LP 515) was used for PIA immuno-detection through Alexa Fluor detection (emission 603 nm), whereas Filter set 02 (Zeiss; excitation G 365, emission LP 420) was used to detect fluorescein isothiocyanate (FITC; emission 530 mm ± 15 nm), indicating the presence of *S. aureus* through the binding of the Fc fragments to bacterial protein A. Scaling was performed automatically with the AxioVision software (Zeiss) according to the objective in use.

Results

Bacterial Recording

Staphylococcus aureus was identified in the external ear canal and retro-auricular swabs, in the tissue fragments, and in the liquid surrounding the implant collected during the removal surgery. Identification was performed

according to CLSI recommendations. Identification of the bacterium was based on a positive by Pastorex agglutination and DNAse production tests. Confirmation was obtained using a previously described duplex PCR amplification. *S. aureus* was also detected by the direct growth of the bacterium following culture of the explanted implant on a solid medium in a petri dish, yielding a pure culture. The isolated *S. aureus* strain was named ''Coch''.

S. aureus Biofilm Identification on the Cochlear Implant

The biofilm was detected on the glutaraldehyde-fixed part of the implant containing the magnet pocket (Figure 1a). Cochlear implant pieces were successfully stained with CV in different zones visible to the naked eye. These colored zones were less abundant than areas reacting with the methylene blue stain. White-light microscopic observations of CV-stained zones showed an association with cocci (Ø = 1 μm) (Figure 1b). Presence of adherent *S. aureus* on the implant was confirmed by the immunodetection of protein A using a FITC-coupled immunoglobulin (Figure 1c). The presence of the biofilm was postulated in these CV-stained zones and confirmed using specific antibodies raised against *S. aureus* PIA (Figure 1d). Surface PIA-positive zones were less abundant than CV-stained zones.

In vitro PIA-based Biofilm Formation

Coch strain produced PIA *in vitro* on glass coverslips at amounts quite similar to those of the control laboratory strain SA113 (Figure 2). Surface colonization to be appeared homogeneous for strain SA113 *in vitro*, whereas strain Coch showed punctual aggregates. The *ica* mutant was negative for PIA-specific fluorescence (Figures 1, 2).

Binding of Fluorescent Fc Fragments on the Electrode Wire

Immuno-detection performed on the electrode wire was positive for *S. aureus* on the first five proximal sections (corresponding to a 4.4-cm length from the implant side) as revealed by Fc-FITC binding (not shown). The three most distal fragments studied were negative.

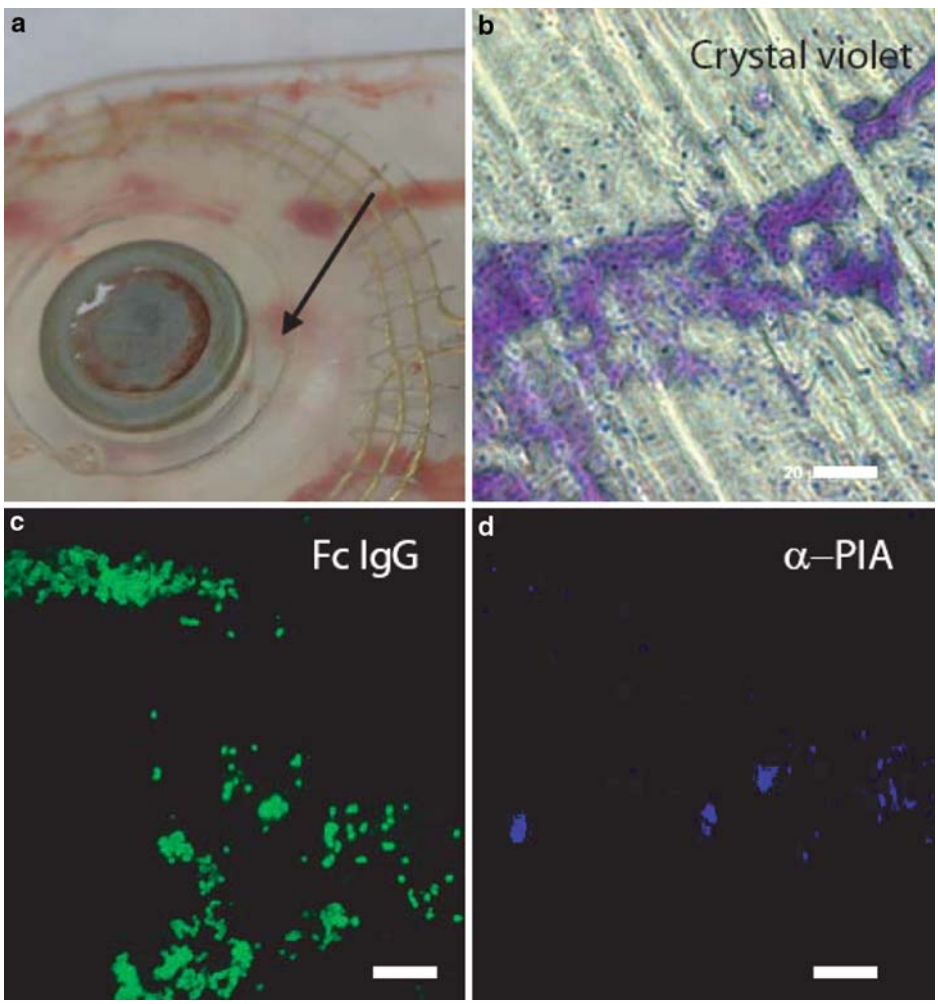

Figure 1. Microscopy imaging of the removed cochlear implant. a) Magnet pocket of the explanted Clarion 90 K cochlear implant. The successfully stained zones depicted on panels b, c, d are localized near the recess surrounding the magnet (Ø = 8 mm, see arrow). b) Crystal violet-stained zone associated with cocci-like structures. c) Immunofluorescence microscopy showing the presence of *S. aureus* protein A. d) Immuno-fluorescence microscopy showing the presence of biofilm-associated polysaccharide intercellular adhesion (PIA). Scale bars: 20 μm.

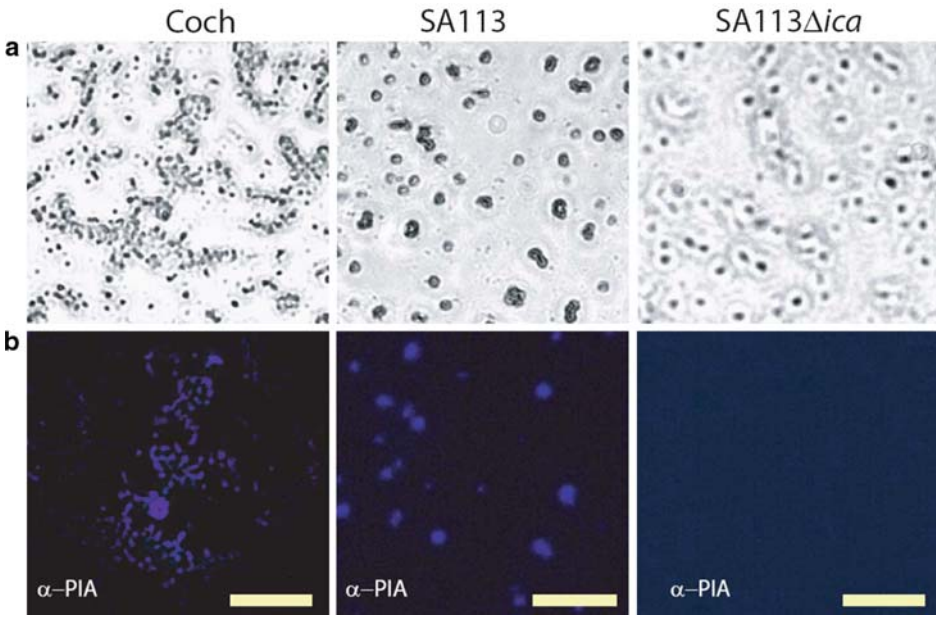

Figure 2. *In vitro* production of PIA. Microscopic pictures of *in vitro* biofilms formed on glass coverslips of Coch (left column), SA113 (middle column), and SA113*Δica* (right column). Row A: Adherent bacteria visible using white light microscopy. Row B: Pictures of PIA immuno-detection on the corresponding surfaces. Scale bars: 20 μm.

Discussion

Worldwide, only two cases of implant extrusion due to persistent infections by *S. aureus* have been clearly associated to biofilms. In these cases, identification was performed using scanning electron microscopy [1, 20]. We report here a third case of *S. aureus* biofilm infection of a Clarion HiRes 90 K cochlear implant. The microscopic appearance of the biofilm-related PIA matrix produced by the isolated *S. aureus* strain, denoted here as Coch, differed between the *in vitro* and *ex vivo* experiments. CV staining is an easy indirect procedure that can be used to quantify biofilm *in vitro* [7, 21], but it has never been used for *ex vivo* biofilm detection. Our case is therefore the first time this procedure has been applied to detect bacterial biofilm on *ex vivo* materials as an initial evaluation of the surface of the implant. Cocci-like structures (Φ approx. 1 μm) were clearly visible in the microscopic observations. Taken together, PIA immuno-detection and CV staining confirmed the presence of a *S. aureus* biofilm on the surface of the implant inside the pocket of the removable magnet. Analysis of the magnet itself was impossible for technical reasons. We speculate that *S. aureus* contaminated first a hematoma inside the muscle pouch made to hold the processor and subsequently the processor itself. Even though a treatment of amoxicillin/clavulanate followed by ciprofloxacin and rifampin was given as soon as a biofilm infection was suspected, the processor had to be ultimately explanted. This case illustrates how efficiently the bacterial community resisted the host immune response and the antibiotics that are effective when the same bacteria are in their planktonic form [3] and confirms that biofilm contamination of an implant often requires removal of the latter [5]. *S. aureus* had already been detected on the culture of the puncture carried out at the early stage of the disorder. There have been published cases of redness and tenderness around the processor followed by flap necrosis or dehiscence and, ultimately, rejection of the processor. These cases were suspected to be caused by an allergy to one of the implant components [16] because no germs could be detected. However, even in such cases, biofilm infections should not be excluded. Sub-clinical biofilm infections can persist for many years before they manifest [11]; therefore, a biofilm infection cannot be ruled out based on a negative culture of the material [14].

Antibiotic prophylaxis is recommended for surgeries involving implants, but the postoperative use of antibiotics is not [2]. This latter practice has, since this case, been abandoned by our center. Although we consider the case reported here to be unique among all the procedures carried out at our center, representing 0.5% of our implants, the post-operative use of antibiotics could have contributed to increasing the resistance of the infecting bacteria and the formation of biofilm.

The first processors implanted in the 1980s were sealed in a smooth ceramic case [13]. The contribution of bacterial biofilm-related infection to the frequency of implant removal is clearly under-reported in the literature as biofilm presence has not been not systematically assessed. In 2000, new processors constructed out of flexible silastic and containing a removable magnet pocket were developed by Cochlear® and by Advanced Bionics®. Two recent studies in which this type of implant was used have reported the presence of biofilm scattered over the entire the surface of the devices [1, 20]. The authors of another report observed a higher abundance of biofilm and biofilm-associated bacteria inside the depressions on the body processor [17]. In the latter study, *Loeffler* et al. [17]

also reported higher counts of bacteria on implants harboring an empty magnet pocket as compared to models without a magnet pocket.

As reported previously by other authors [1, 17, 20], we cannot confirm the presence of biofilm around the electrode wire. The tests performed after the removal of the processor revealed that there was *S. aureus* attached to the electrode wire up 4.4 cm distally from the body of the processor, but these are not specific for the detection of biofilm. In our case, during the removal surgery, the length of the wire that extended from the mastoid to the cochleostomy appeared to be normal. We arbitrarily decided to cut the wire at the level of the cochleostomy and leave the electrodes array inside the cochlea to avoid obliteration by fibrous tissue that could prevent a new implant.

Modern processors equipped with a removable magnet represent a major improvement for patients suffering from chronic disease that requires regular follow-up with MRIs. The ability to remove the magnet removes the risk of magnet mobilization or demagnetization in patients requiring radiological examination. Unfortunately the pocket designed to encase the magnet seems to favor biofilm formation, and the possibility that this pocket is involved in biofilm formation should not be ignored. Technical developments are under way to avoid the formation of bacterial biofilms, such as surface treatment with antimicrobial molecules [6]. Clearly, such developments need careful evaluation in the clinical context [6, 12].

In conclusion, biofilm can cause resistant infections of cochlear implants that manifest many months after the surgery. The pocket of the removable magnet could be one niche facilitating biofilm, formation although colonization of the electrode wire is not excluded.

Acknowledgments

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II. Impact of oleic acid (cis-9-octadecenoic acid) on bacterial viability and biofilm production in *Staphylococcus aureus*

II.A. Introduction

We were interested to interfere with the formation of biofilm by *S. aureus* using a compound in the medium, instead of manipulating genes in the bacteria present in biofilms. Indeed, we wanted to be able to compare biofilm incorporated and planktonic bacteria using the same strain of *S. aureus*, growing bacteria in a condition that prevents or promotes the formation of biofilm. Interestingly, it was reported in 1986 that the presence of oleic acid increases biofilm production when *S. aureus* is grown in absence of oxygen [45]. Later, it was reported that the production of carotenoids confers resistance against oleic acid killing probably by protecting the bacterial membrane [48]. In 2002, a review on biofilm in *Staphylococcus* mentioned putative ionic interaction of the positively charged PIA with the negatively charged oleic acid [123]. Then, *S. aureus σB* stress response regulator was shown to be implicated in the synthesis of carotenoids, whereas the *asp23* mRNA level was correlated with active SigB level [119,172]. For all of these reasons, we decided to test the effects of oleic acid on the formation of biofilm using *S. aureus* UAMS-1. Therefore, we emulsified six different logarithmic concentrations of oleic acid ranging from absence to 1 % v/v presence in the growth medium (TSB) before inoculating UAMS-1 and performing subsequent biofilm-related crystal violet staining assays. Results showed a clear dose-dependent inhibition of biofilm formation linked to oleic acid concentrations visible with naked eyes in multititer plates. But when we asked if oleic acid was able to dissolve an already established biofilm, we were surprised to observe opposite effects: oleic acid resulted in increased biofilm production. This could be explained by the fact that genes are differentially expressed if the bacterium is "passively swarming" or attached to a surface, and by the fact that the primary adhesion process involves electrostatic interactions, which is different when compared to interactions occurring between adhesins and the surface in an already formed biofilm.

We then asked if oleic acid could induce or activate the expression of *sigB* needed for resistance against that fatty acid. Therefore, we quantified the mRNA relative levels of both *sigB* and *asp23* in function of oleic acid presence after extraction of mRNA from both adherent and planktonic populations of UAMS-1 in the presence and absence of oleic acid. But neither the mRNA of *asp23* nor that of *sigB* were affected by oleic acid. The caveat of our work was that we do not have a clear explanation of the effect of oleic acid on *S. aureus* bacteria notably at the molecular level, whereas the

principal asset was the strength of alteration of biofilm phenotypes related to oleic acid presence, the reproducible effects in every tested strain, and an interest in the field of biofilm studies to find chemicals able to prevent biofilm formation. My contribution to the work was to perform the work and to write the manuscript.

II.B. Article:

Impact of oleic acid (cis-9-octadecenoic acid) on bacterial viability and biofilm production in *Staphylococcus aureus*

RESEARCH LETTER

Impact of oleic acid (*cis*-9-octadecenoic acid) on bacterial viability and biofilm production in *Staphylococcus aureus*

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Keywords
Staphylococcus aureus; oleic acid; biofilm; viability; planktonic; adherent bacteria.

Abstract

Staphylococcus aureus is responsible for a broad variety of chronic infections. Most *S. aureus* clinical isolates show the capacity to adhere to abiotic surfaces and to develop biofilms. Because *S. aureus* growing in a biofilm is highly refractory to treatment, inhibition of biofilm formation represents a major therapeutic objective. We evaluated the effects of oleic acid on primary adhesion and biofilm production in eight genotypically different *S. aureus* strains as well as in the biofilm-negative *Staphylococcus carnosus* strain TM300. Oleic acid inhibited primary adhesion but increased biofilm production in every *S. aureus* strain tested. *Staphylococcus aureus* strain UAMS-1 was then selected as a model organism for studying the mechanisms triggered by oleic acid on the formation of a biofilm *in vitro*. Oleic acid inhibited the primary adhesion of UAMS-1 dose dependently with an IC_{50} around 0.016%. The adherent bacterial population decreased proportionally with increasing concentrations of oleic acid whereas an opposite effect was observed on the planktonic population. Overall, the total bacterial counts remained stable. Macroscopic detachments and clumps were visible from the adherent bacterial population. In the presence of oleic acid, the expression of *sigB*, a gene potentially involved in bacterial survival through an effect on fatty acid composition, was not induced. Our results suggest a natural protective effect of oleic acid against primary adhesion.

Introduction

Most pathogens involved in human infections are able to produce biofilms (Costerton *et al.*, 1999). The chemical nature of bacterial biofilms is either polysaccharidic or proteic. It constitutes a matrix enclosing bacteria following their attachment to a surface (Costerton *et al.*, 1978). Extracellular DNA may also be necessary for biofilm formation (Whitchurch *et al.*, 2002; Rice *et al.*, 2007). The adherent bacteria display gene patterns that differ profoundly from their planktonic counterparts (Sauer *et al.*, 2002; Beenken *et al.*, 2004). *Staphylococcus aureus* biofilms have been isolated and characterized on a plethora of different medical devices, causing major medical problems usually unsolved even after adapted and prolonged antibiotic treatments (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2004). Biofilm-related infections usually require removal of the implanted material to avoid a persistent infection (Costerton *et al.*, 1999). Furthermore, *in vivo* detection of a biofilm is challenging and remains particularly difficult (Hall-Stoodley *et al.*, 2004; Trampuz *et al.*, 2007). Development of modified surfaces or screening for substances able to inhibit the formation of biofilm is an active field of research (Rodrigues *et al.*, 2006; Zhang *et al.*, 2006; Donelli *et al.*, 2007). Oleic acid has been reported to be the predominant bactericidal unsaturated fatty acid naturally present in staphylococcal abscesses and on the skin surface (Speert *et al.*, 1979; Dye & Kapral, 1981). The production of carotenoids in *S. aureus* has been found to confer resistance against oleic acid (Xiong & Kapral, 1992). Interestingly, production of staphyloxanthin, the major stationary-phase carotenoid (Marshall & Wilmoth, 1981),

is dependent on the activated form of the alternative sigma factor (*sigB*) (Katzif *et al.*, 2005), which in turn regulates the alkaline shock protein 23 (*asp23*) (Gertz *et al.*, 1999). In *S. aureus*, *sigB* is involved in the biogenesis of carotenoids (Kullik *et al.*, 1998; Giachino *et al.*, 2001; Katzif *et al.*, 2005). Oleic acid was reported previously to induce an *in vitro* 'slime production' under hypobaric oxygen conditions, as demonstrated in the case of a strain isolated from a sputum sample (Campbell *et al.*, 1986). Here, we investigated the effects of oleic acid on primary adhesion and biofilm production in eight unrelated strains of *S. aureus*. The viability of the planktonic or the adherent populations, as well as mRNA levels of *asp23* and *sigB* were evaluated under oleic acid stress in a model organism.

Materials and methods

Strains

Staphylococcus aureus strain UAMS-1 was isolated from a patient suffering from osteomyelitis and was shown previously to produce a biofilm in an experimental model of osteomyelitis (Smeltzer *et al.*, 1997) as well as *in vitro* (Beenken *et al.*, 2004). This strain and its *sigB* mutant have been described previously (Beenken *et al.*, 2004; Cassat *et al.*, 2006). The other strains used in this study are listed in Table 1. The identification of *S. aureus* Coch was based on Pastorex agglutination (Bio-Rad) and DNAse tests, and was further confirmed using a real-time PCR amplification procedure (Francois *et al.*, 2003).

Media and growth conditions

Staphylococcus aureus strains were grown in trypticase soy broth (TSB, Becton Dickinson, Le Pont de Claix, France) supplemented with 1% glucose. Oleic acid (free acid cell culture tested, *cis*-9-Octadecenoic acid, Sigma-Aldrich, Basel, Switzerland) was emulsified with TSBgluc (TSBglucOleic) by overnight agitation at 220 r.p.m. in a Lab-Shaker at 37 °C. Biofilm development was performed in TSBgluc (or TSBglucOleic emulsions) with 20 μL of overnight cultures mL^{-1} of medium. Bacterial colonies were counted on Mueller–Hinton agar plates (MHA, Bio-Rad, Marnes-La-Coquette, France).

Crystal violet biofilm assay

Biofilm staining assays were performed as described previously (O'toole & Kolter, 1998). Briefly, after bacterial growth, microtiter plates (Multiwell™ 6 well, Becton Dickinson) were washed twice with phosphate-buffered saline (PBS), fixed for 20 min at 80 °C and stained for 10 min with 1% (w/v) crystal violet solution freshly diluted twofold in 1% (v/v) ethanol/distilled water. Plates were then washed with water and photographed. The crystal violet was dissolved in dimethyl sulfoxide (DMSO) for 1 h before $OD_{600\,nm}$ measurements (Tu Quoc *et al.*, 2007). Half inhibitory concentration IC_{50} of biofilm inhibition was determined by curve fitting in Microcal™ Origin® (version 6.1, Northampton, MA).

Bacterial sample preparation and counting methods

UAMS-1 planktonic bacterial population was recovered from the supernatant, whereas the adherent bacterial population was detached from the surface with a scraper (Cell Scraper, BD Falcon™, Becton Dickinson) after addition of 0.5 M EDTA. Each sample was sonicated (Branson 2200, Geneva, Switzerland) and microscopically observed to exclude aggregation before bacterial measurements. $OD_{540\,nm}$ was used for indirect cell counting. Direct counting was performed in Neubauer chambers following the instructions of Marienfeld (http://www.marienfeld-superior.com/pgr06_info_e.htm). CFU determination was performed on MHA using a Countermat Flash colony counter (IUL, RB Scientific, Southampton, England).

Live/dead staining

A circular glass coverslip (diameter: 25 mm) was deposited on the microtiter plate before initiating bacterial culture. Live/dead staining was performed on the UAMS-1 planktonic population after centrifugation, allowing replacement

Table 1. Bacterial strains used in this study

Strain	Description	Sources/references
UAMS-1	Osteomyelitis isolate producing a proteinaceous and extracellular DNA-related biofilm	Gillaspy *et al.* (1995) Rice *et al.* (2007)
Newman	Sequenced strain	Baba *et al.* (2008)
N315	Sequenced strain	Kuroda *et al.* (2001)
Coch	PIA-positive strain isolated from a cochlear implant.	This laboratory
SA113	Restriction-deficient laboratory strain ATCC35556 producing an *ica*-dependent biofilm	Kristian *et al.* (2004)
S30	Pediatric isolate and strong PIA-dependent biofilm producer	Tu Quoc *et al.* (2007)
ISP479r	Laboratory strain derivative of RN8325 with restored *rsbU*. Strong biofilm producer	Pattee (1981), Toledo-Arana *et al.* (2005)
SA564	Recent human isolate	Somerville *et al.* (2002)
S. carnosus TM300	Biofilm-negative *icaADBC* and PIA-negative *S. carnosus* strain	Wagner *et al.* (1998)

of the supernatant by the staining mixture. The L7012 live/dead® BacLight™ bacterial viability kit (Invitrogen) was used to stain bacteria according to the manufacturer's protocol. Bacteria were then observed under an Axioskop 2 microscope using different filter combinations allowing detection of PI and SYTO 9. Pictures (resolution 2600 × 2600 pixels) were acquired with an Axiocam color camera (Zeiss).

Relative *sigB* and *asp23* mRNA quantification

Transcription of *S. aureus sigB* and *asp23* genes was monitored using quantitative real-time reverse-PCR (qPCR) from planktonic and adherent UAMS-1 (wt) and its Δ*sigB* mutant following the procedure described previously (Renzoni *et al.*, 2004; Garzoni *et al.*, 2007). qPCR was performed in a StepOne Real-Time PCR system (Applied Biosystems, Foster City, CA) using the ThermoScript Reaction mixture (Invitrogen). The following oligonucleotides were designed using PRIMEREXPRESS software (Applied Biosystems). Forward 5′-TCGCGAACGA GAAATCATACAA, reverse 5′-ACCGATACGCTCACCTGT CTCT and the MGB probe 5′-TACGTTTATTGAGGGTTT-GAG (coupled with FAM and dark quencher) were used to quantify *sigB* gene expression. The transcript levels of *asp23* were determined as described previously (Renzoni *et al.*, 2004), except the forward primer 5′-GTTAAGCCACCTTT CATGTCTAAGATAC.

Results

Effect of oleic acid on *S. aureus* primary adhesion and biofilm formation

All tested strains were able to produce a biofilm in various amounts, except the nonbiofilm producer *Staphylococcus carnosus* strain TM300 that was used as a negative control (Fig. 1, column A). The presence of oleic acid during primary adhesion drastically affected biofilm production and resulted in the absence of crystal violet incorporation (Fig. 1, column B). The presence of oleic acid on an already formed biofilm showed a slight increase in crystal violet incorporation (Fig. 1, column C). These results were similar in the eight independent genetic backgrounds tested, whereas the absence of crystal violet incorporation was consistently observed for the biofilm-negative *S. carnosus* TM300.

Dose-dependent inhibition of primary adhesion in UAMS-1

Increasing oleic acid concentrations (ranging from 0.001% to 0.1%) led to a dose-dependent decrease of UAMS-1 biofilm detection. The curve fitted with a logistic equation revealed an IC50 of 0.016% (Fig. 2). The addition of 0.1% v/v oleic acid before primary adhesion and during bacterial growth precluded the formation of a detectable biofilm.

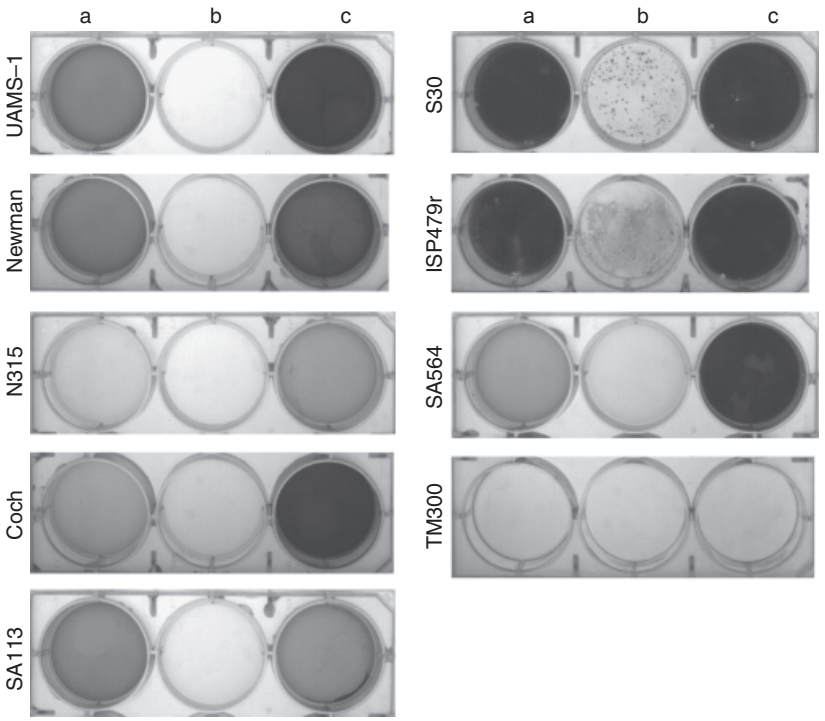

Fig. 1. Effect of oleic acid on primary adhesion and biofilm production in *Staphylococcus aureus*. Pictures of crystal violet staining assays used to quantify biofilms produced by eight genotypically different *S. aureus* strains as well as the biofilm-negative *Staphylococcus carnosus* strain TM300. Strains were incubated for 2 h on a first medium, which was replaced by a second bacterial-free medium for another 2 h. (a) Left wells show 4-h-old biofilms formed in TSBgluc without oleic acid. (b) Central wells show 4-h-old biofilms formed in TSBgluc in the presence of oleic acid. (c) Right wells show biofilm formation after primary adhesion for 2 h in TSBgluc, followed by a 2-h exposure to oleic acid. The oleic acid stress consisted of 0.1% v/v oleic acid emulsified in TSBgluc.

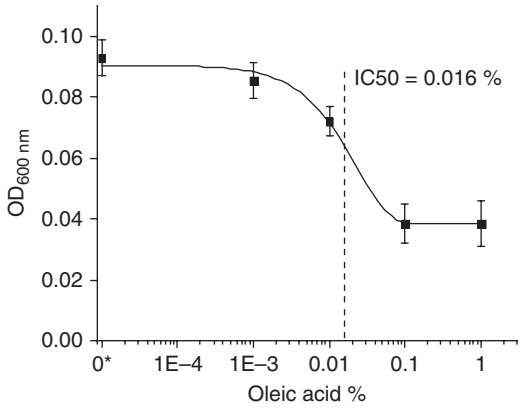

Fig. 2. Oleic acid inhibits primary adhesion of UAMS-1 dose-dependently. Spectrophotometric determination of crystal violet incorporated into a UAMS-1 biofilm after growth in the presence of different concentrations of oleic acid. Values are mean of three independent determination ± SEs. Exponential decay fit shows an IC50 value of 0.016% (dashed line). 0*; the 0% oleic acid concentration has been shifted artificially to 10^{-5}.

Effect of oleic acid on bacterial viability

The three different microbiological counting methods used in this study showed a dose-dependent decrease of the adherent population with increasing concentrations of oleic acid (Fig. 3a). An opposite effect was observed on planktonic cells but the total number of bacteria (i.e. adherent plus planktonic cells) remained stable across the different oleic acid concentrations and growth times tested in our study.

Live/dead fluorescence assay revealed that the proportion of living UAMS-1 cells remained stable under oleic acid conditions when grown as planktonic cells (Fig. 3b). In the planktonic population of the UAMS-1 *sigB* mutant, the proportion of living bacteria decreased from 83% to 14% in the presence of 0.1% oleic acid. In the adherent phenotype, both wild-type UAMS-1 and its *sigB* mutant revealed a marked decrease in viability in the presence of 0.1% oleic acid. Microscopic observations of adherent UAMS-1 revealed a viability of 81% in the absence of oleic acid and a drastic reduction to 19% in the presence of 0.1% oleic acid, whereas the *sigB* viability was 76% and 0%, respectively. Taken together, these results suggest a specific killing effect of oleic acid against all adherent bacteria but also against the UAMS-1 *sigB*-deficient mutant when grown in a planktonic phenotype.

Active SigB and *asp23* mRNA respective abundance under oleic acid stress conditions

UAMS-1 and its isogenic *sigB* mutant were plated on MHA to evaluate colony pigmentation. UAMS-1 clearly developed pigments visible through a yellowish coloration whereas *sigB* mutant colonies appeared white (not shown). Despite the presence of oleic acid, the relative transcript levels of *asp23* were similar in adherent and planktonic populations of UAMS-1 (not shown).

Discussion

Little is known about *S. aureus* biofilm formation in the presence of oleic acid, excluding an increased biofilm production reported under hypobaric oxygen conditions and in the presence of oleic acid (Campbell *et al.*, 1986). This lack of knowledge created the need for further test of oleic acid effects on UAMS-1 biofilm formation both under aerobic and under anaerobic conditions. In aerobic and anaerobic conditions, oleic acid stress led to the lack of detectable biofilm when added before primary bacterial adhesion. However, when oleic acid was added later, i.e. after primary adhesion, biofilm formation was found to have increased significantly under anaerobic conditions as compared with aerobic conditions. The previously described stimulating effect of oleic acid on *S. aureus* biofilm (Campbell *et al.*, 1986) could result from an ionic interaction of the positively charged polysaccharide intercellular adhesin (PIA) with the negatively charged oleic acid (Gotz, 2002) or from the specific response of an *S. aureus* strain to a peculiar chemical environment (Campbell *et al.*, 1986). Our *in vitro* studies show that oleic acid inhibited dose-dependently *S. aureus* UAMS-1 adhesion to polystyrene surfaces, resulting in a limited incorporation of crystal violet. However, once primary adhesion was developed, oleic acid stimulated biofilm formation. This effect was consistently observed across a panel of eight genotypically diverse *S. aureus* strains, ruling out a strain-specific effect.

Oleic acid is modifiable by a bacterial enzyme named fatty acid-modifying enzyme (FAME), ensuring bacterial survival in tissues (Mortensen *et al.*, 1992). *In vivo* FAME could esterify cholesterol with oleic acid, thus leading to a non-bactericidal product. There is no cholesterol in our assay and some strains of *S. aureus* did not produce FAME. Invasiveness defined by the authors as 'the ability either to survive within intraperitoneal abscesses or to multiply in the kidneys' was associated with FAME production in *S. aureus* strains P78, PG114, 18Z and TG, whereas strains 18Z-H, P78-22, 689 and 303 did not produce FAME; 5 other strains were not tested for FAME production (Mortensen *et al.*, 1992). The theoretical proteome of UAMS-1 (Hernandez *et al.*, in preparation) contains four putative esterases, but the FAME gene could not be identified.

Oleic acid has been reported previously as a killing agent for *S. aureus*, presumably through a mechanism involving bacterial lipids (Chamberlain *et al.*, 1991). Previous reports demonstrated that different staphylococci species are not capable of metabolizing oleic acid (James *et al.*, 2004) and

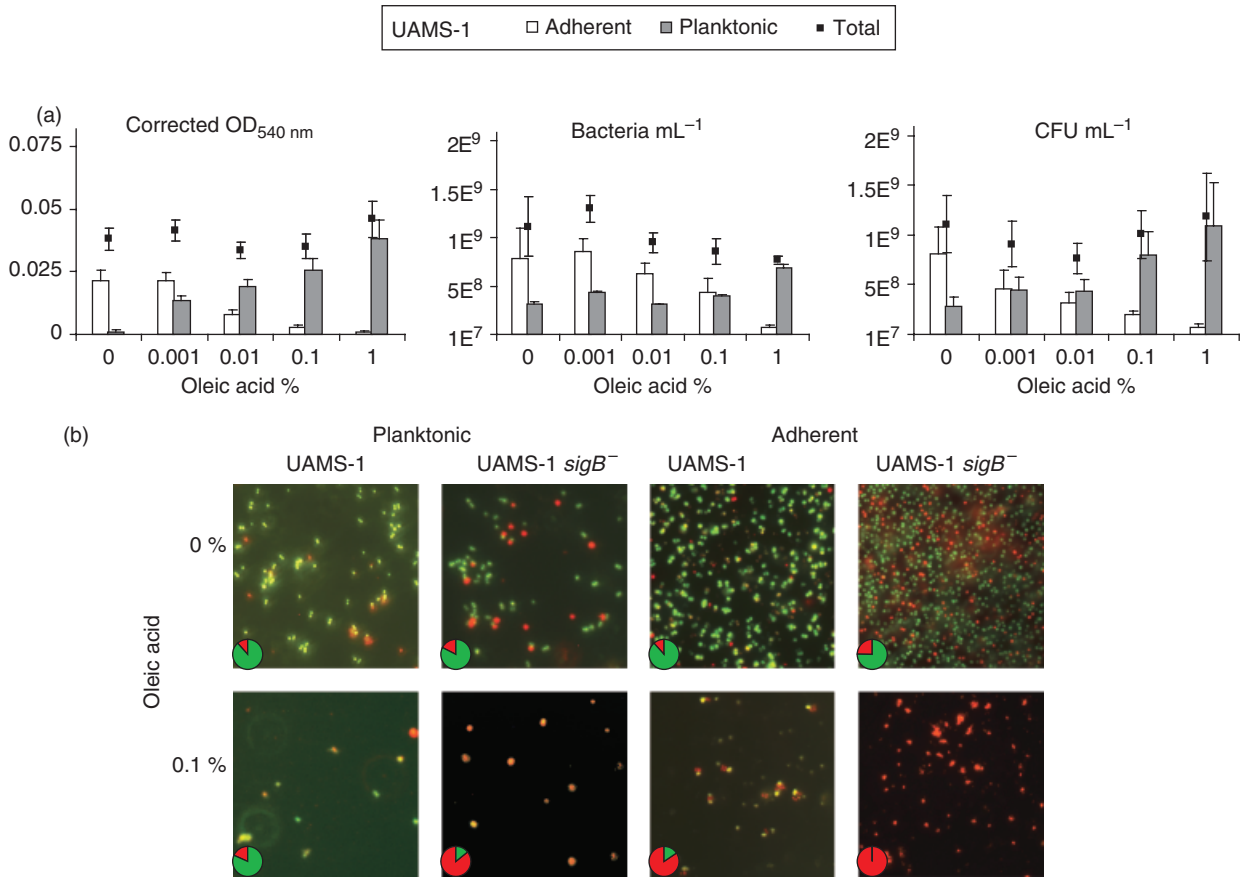

Fig. 3. Effect of oleic acid on planktonic and adherent bacterial cells. (a) Left, planktonic and adherent bacterial counts as measured by $OD_{540\,nm}$. Values are mean ± SEs of two independent determinations. Middle, direct counts of adherent and planktonic cells determined in a Neubauer chamber. Average values and SEs were calculated on six different squares for the dilutions 10e-3 and 10e-4. Right: CFUs on MHA Petri dishes were counted automatically using a Countermat Flash instrument. Average values and standard errors were calculated based on dilutions ranging from 10e-5 to 10e-7 on planktonic and adherent populations. The total bacterial count (i.e. adherent plus planktonic cells) is plotted as a square on each graph. b. Live/dead staining assay was performed on both adherent and planktonic UAMS-1 ± *sigB*. Pie charts represent the proportion of living bacteria in green and dead bacteria in red, as determined by counting bacteria on the live/dead staining images.

that oxidation compounds resulting from their oxidation are highly toxic for bacterial cells (Clarke *et al.*, 2007). This compound is present in abscesses and in exudates from cystic fibrosis patients at a concentration of *c.* 12 nmol mL^{-1} (Meyer *et al.*, 2000). This concentration appears to be lower than the IC_{50} determined in this study (*c.* 500 nmol mL^{-1}). Despite several *in vitro* studies that analyzed the effects of oleic acid on *S. aureus* (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993), the potential of oleic acid as a bactericidal agent on *S. aureus* remains controversial. Owing to the plasticity of the *S. aureus* genome, which contributes to environmental adaptation, these observations are possibly strain dependent. Indeed, some reports suggest an adaptation of staphylococci to oleic acid (Speert *et al.*, 1979), whereas others report an *in vitro* bactericidal effect (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993). The strains studied (18Z and 303) appeared to be most susceptible to oleic acid killing during the exponential phase of growth (Xiong *et al.*, 1993). The authors concluded that the *in vitro* killing property of oleic acid on *S. aureus* 18Z strain resulted from an increased membrane permeability and was indirectly correlated with the production of carotenoids (Chamberlain *et al.*, 1991). As carotenoids were shown to have a direct protective effect against oleic acid killing in *S. aureus* (Xiong & Kapral, 1992), this raises the question of a potential influence of oleic acid on *sigB* signaling.

In agreement with previous studies showing the effects of oleic acid on planktonic bacteria (Chamberlain *et al.*, 1991; Xiong & Kapral, 1992; Xiong *et al.*, 1993), our live/dead experiments performed with planktonic UAMS-1 and its Δ*sigB* mutant suggest a *sigB*-dependent resistance against oleic acid killing. However, the adherent bacterial population was affected by oleic acid independently of *sigB* expression as shown by the transcript levels of *asp*23 used to indirectly quantify the active form of SigB (Senn *et al.*,

2005). Thus, expression levels of *asp23* that are correlated to the abundance of protective compounds such as bacterial carotenoids were not induced by the presence of oleic acid during biofilm development in *S. aureus* strain UAMS-1.

During our biofilm development experiments in the presence of oleic acid, we observed the release of large bacterial clumps from already constituted biofilms. These clumps, containing mainly living bacteria, probably contributed to the overestimation of planktonic bacterial counts. The release of clumps was described previously during biofilm maturation (Stoodley *et al.*, 2001), probably creating free spaces that become parts of water channels contributing to the free circulation of metabolites in the biofilm (Stoodley *et al.*, 2002). The presence of free-floating bacterial cell clumps remains to be evaluated *in vivo* as it should be considered as a risk of systemic bacterial dissemination (Hall-Stoodley *et al.*, 2004).

In conclusion, identification or development of compounds able to inhibit biofilm formation or to initiate its release must be evaluated with the concern of bacterial viability. As suggested recently (Donelli, 2006), this issue appears to be crucial considering the release of viable clumps from biofilm-colonized surfaces and the risk of systemic bacterial dissemination. The different technical issues addressed in this study allowed testing potential inhibitors of surface-associated biofilm development and simultaneously evaluating detachment properties and impact on bacterial viability.

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III. CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression

III.A. Introduction

CodY in *S. aureus* was shown to repress the *agr* system, the hemolytic activity, the formation of biofilm as well as the production of PIA in two *S. aureus* strains, whereas other functions of CodY in *S. aureus* remain to be studied [211]. In the present paper, both *codY* and *agrA* genes were altered in different *S. aureus* strains, in order to distinguish *agr*-dependent from *agr*-independent functions of CodY. Therefore, the *codY* gene was first replaced by a tetracycline resistant cassette (*tetM*) in strain RN4220 after homologous recombinations using a plasmid-based strategy and resulting in strain RN4220-21. This mutation was then transduced into different strains using Φ11-mediated transduction and chloramphenicol resistance selection [276]. Second, the Tn551 transposon carrying an erythromycin resistance cassette was integrated into the *agrA* gene of strain RN450 leading to RN6112, both strains deriving from the NTCT8325 lineage [174,337]. In the case of *agrA*, strain RN6112 was used as donor of the *agrA::tn551* mutation. This mutation was transferred into wild-types and *codY::tetM* mutants of different *S. aureus* strains using Φ11-mediated transduction and erythromycin resistance selection. Note that *agrA* being the response regulator for quorum sensing activating both RNAII and RNAIII, mutations in *agrA* were shown to block the hemolytic activity and were also supposed to block the quorum sensing mechanism [184].

The different obtained constructions, positive and negative for expression of both *agrA* and *codY*, were grown in different amino acid containing defined media and were compared for their growth. Additionally, the respective transcriptomes of Newman ± *agrA* ± *codY* were determined using microarray experiments. Results showed that the threonine synthesis pathway was derepressed in the absence of *codY,* probably explaining why only the *codY* mutants were able to grow in the absence of threonine, as compared with parental strains. Another interesting observation was that an excess of isoleucine in the medium inhibited the growth of the wild-type but not that of the *codY* mutant. In such cases, isoleucine is probably a cofactor for CodY, as observed previously in *L. lactis* [47,129]. Indeed, an excess of isoleucine in the medium leads to a situation where CodY is abnormally

activated and represses the metabolic pathways involved in amino acid synthesis even when the other amino acids are depleted, resulting in growth inhibition [129]. Another aspect of the work was to identify the localization of the putative CodY-DNA binding sites among *S. aureus* genomes of strains Newman, Col and N315, all of them being genome-sequenced strains. Therefore, a bioinformatic approach was performed, allowing up to two different mismatches in the AATTTTCWGAAAATT consensus sequence that was determined originally in *Lactococcus lactis*. The ORFs present within 1000 bp downstream of the box were recorded. Plotting the number of detected boxes in function of the distances to the ORFs resulted in a maximum number of sequences localized between 50 to 300 bp upstream of the different translational starts, around 70 CodY boxes were detected per genome and they were mainly detected in intergenic regions. Resulting from to microarrays experiments, 124 genes (5% of the genome) showed differential expression in the *codY* mutant compared to its parent. The majority of these genes was involved in amino acid metabolism and was probably directly repressed by CodY in an *agr*-independent-manner, whereas the second most represented pool of genes consisted in those involved in nucleotide metabolism, probably indirectly up-regulated by CodY. Another important functional class represented among differentially regulated genes was the one encoding for virulence factors. Even if some virulence factors were regulated by CodY in an *agr*-independent manner, the majority of virulence factors were *agr*-dependent.

Combining microarrays and bioinformatic resulted in the identification of 16 genes. All of them were reported as processing a CodY box in front of their respective translational start and were derepressed in Newman *codY* mutant as compared to the wild type. Some of them were well-known direct targets of CodY as reported in other organisms, such as the isoleucine operon (*ilv*) and the branched-chain amino acid transport system (*brnQ1*). The regulation of these genes by CodY was then confirmed by measuring their transcript levels in the wt and the *codY* mutants with Northern blots. Finally, as *codY* is part of an operon with *xerC* and *clpQY*, mutating *codY* could putatively affect the other cistrons. Northern results showed, first, a slight but reproducible increase in both *xerC* and *clpQY* co-transcripts in the absence of *codY*, suggesting an auto-repressing activity of CodY towards its operon, as already shown in other genetic contexts (see the regulation part of the review onto CodY), and second, that two different sized transcripts are generated from the operon, one only with *codY* and the second with all the four ORFs.

My contribution to that work was to analyze the microarray results and to combine these results with the CodY boxes, to read the manuscript, to give my feedback and to create the supplementary

Figure 1 illustrating the occurrence of CodY boxes as a function of the distance to the translational start sites. This work characterized for the first time a putative CodY regulon in *S. aureus*, unraveling *agr*-mediated regulation and demonstrating that CodY principally regulates the amino acid metabolism and additional virulence factors by targeting the *agr* system, thus linking virulence with metabolism.

III.B. Article:

CodY in *Staphylococcus aureus*: a regulatory link between metabolism and virulence gene expression

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CodY in *Staphylococcus aureus*: a Regulatory Link between Metabolism and Virulence Gene Expression▽†

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The repressor CodY is reported to inhibit metabolic genes mainly involved in nitrogen metabolism. We analyzed *codY* mutants from three unrelated *Staphylococcus aureus* strains (Newman, UAMS-1, and RN1HG). The mutants grew more slowly than their parent strains in a chemically defined medium. However, only *codY* mutants were able to grow in medium lacking threonine. An excess of isoleucine resulted in growth inhibition in the wild type but not in the *codY* mutants, indicating that isoleucine plays a role in CodY-dependent repression. Prototypic CodY-repressed genes including the virulence regulator *agr* are repressed after up-shift with isoleucine. The CodY-dependent repression of *agr* is consistent with the concomitant influence of CodY on typical *agr*-regulated genes such as *cap*, *spa*, *fnbA*, and *coa*. However, some of these virulence genes (e.g., *cap*, *fnbA*, and *spa*) were also regulated by CodY in an *agr*-negative background. Microarray analysis revealed that the large majority of CodY-repressed genes were involved in amino acid metabolism; CodY-activated genes were mainly involved in nucleotide metabolism or virulence. In summary, CodY in *S. aureus* not only acts as a repressor for genes involved in nitrogen metabolism but also contributes to virulence gene regulation by supporting as well as substituting for *agr* function.

Staphylococcus aureus asymptomatically colonizes the nares of healthy individuals but also causes a variety of infections in humans. Regulatory loci are necessary for the adaptation of the organism to the different nutrient limitations and stress conditions encountered in vivo. This allows the pathogen to survive and/or multiply in different compartments during colonization and infection processes. However, knowledge of the environmental conditions encountered in vivo is still incomplete, and the interaction of regulatory circuits leading to metabolic adaptation and differential expression of virulence factors remains poorly understood. The in vitro expression of most virulence factors is tightly related to the growth phase. For instance protein A (encoded by *spa*), fibronectin-binding proteins (encoded by *fnbA* and *fnbB*), and coagulase (encoded by *coa*) are expressed during the exponential growth phase, whereas most secreted proteins (e.g., hemolysins, enterotoxins, and proteases) and the capsule (enzymes encoded by the *capA-capP* operon) are expressed mainly during the postexponential phase (22, 37). In many bacteria, the transition to postexponential growth is accompanied by a profound reprogramming of gene expression. Several underlying mechanisms are thought to be involved in such a transition. Quorum sensing allows the bacteria to detect their own density. Basically, bacteria secrete small diffusible molecules (autoinducers) which are also effectors of their own synthesis. Upon passing a critical concentration threshold, the autoinducers activate specific transcriptional regulators, leading to the differential expression of target genes. The *agr* locus of *S. aureus* is a prototypic quorum-sensing system mainly involved in the regulation of virulence genes (37). At high cell densities, the regulatory RNAIII is expressed, leading to the inhibition of *spa*, for instance, and to the activation of genes encoding secreted virulence factors and the capsular polysaccharide. Besides quorum sensing, additional mechanisms have to be triggered for the growth phase transition in *S. aureus* since the expression of certain virulence factors remains growth phase dependent in *agr* mutants (42, 51, 53).

In *Bacillus subtilis*, the CodY repressor has been described as a central regulator important for the transition to stationary phase and sporulation (40, 46). Homologs of *codY* could be identified in the genome of most gram-positive species, including pathogenic staphylococci and streptococci (23, 46). In *B. subtilis*, GTP reaches its highest concentration during the exponential phase, when it binds to CodY and leads to the repression of late gene expression. This mechanism is linked to the stringent response since the synthesis of (p)ppGpp by Rel leads to a lowering of the GTP pool (28). The central role of GTP in gene regulation was further proven by the use of decoyinine, an inhibitor of GMP synthetase (40). Depletion of the GTP pool by this inhibitor leads to the activation of typical late genes already in the exponential phase. CodY represses genes which are primarily involved in nitrogen metabolism (proteases, oligopeptide transporters, and genes for amino acid synthesis) and activates transcription of genes of the carbon overflow pathway (46, 47). Interestingly, in *Lactococcus lactis* CodY does not bind GTP. Here, branched-chain amino acids (BCAAs) are bound by CodY and constitute the

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
Strains		
TOP10	Competent *E. coli* for plasmid transformation	Invitrogen, Karlsruhe. Germany
CYL316	RN4220 harboring pYL112Δ19 and L54 *int* gene; r^-	31
RN6112	RN6390 *agr*A::Tn*551*	Richard Novick
RN4220	Restriction-deficient *S. aureus* strain	29
RN4220-21	RN4220 Δ*codY*::*tet*(M)	This work
Newman	Wild type	15
Newman-21	Newman Δ*codY*::*tet*(M)	This work
Newman-agr	Newman *agrA*::Tn*551*	This work
Newman-21/-agr	Newman *agrA*::Tn*551* Δ*codY*::*tet*(M)	This work
UAMS-1	Osteomyelitis isolate	19
UAMS-1-21	UAMS-1 Δ*codY*::*tet*(M)	This work
UAMS-1-agr	*agrA*::Tn*551*	17
UAMS-1-21/-agr	UAMS-1 *agrA*::Tn*551* Δ*codY*::*tet*(M)	This work
RN1HG	*rsbU* restored RN1	This work
RN1	8325	NARSA strain collection[a]
RN1HG-21	RN1HG Δ*codY*::*tet*(M)	This work
Plasmids		
pALC2073	*E. coli-S. aureus* shuttle vector with tetracycline-inducible promoter	3
pMAD	Shuttle vector for gene replacement mutagenesis	1
pCG29	pMAD with cloned *clpY-tet*(M)-*rpsB* fragment for *codY* mutagenesis	This work
pCG30	pALC2073 with *codY* integration via EcoRI restriction site	This work

[a] NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

primary signals for *codY* repression (23, 39). The difference between the two species is probably due to sequence variations in the proposed GTP binding domain of CodY (39). It has been shown that CodY of *B. subtilis* can also use BCAAs in addition to GTP as signaling molecules (39, 45). A conserved CodY-binding site (AATTTTCWGAAATT) was first described for *L. lactis* (12, 24) and later confirmed in *B. subtilis* (5).

The metabolic regulatory cascades for gram-positive pathogens are only partly understood, and knowledge about nitrogen metabolism and amino acid availability during infection remains limited. However, there is growing evidence that CodY is an important regulatory link between metabolism and virulence gene expression in pathogenic bacteria (6, 14, 26, 32, 35, 46). The complete set of genes necessary for amino acid synthesis has been predicted to be present in the *S. aureus* N315 genome (25). Nevertheless, *S. aureus* usually requires a complex mixture of amino acids for growth, probably because of repression of the corresponding pathways (25). It can be assumed that during infection the bacterium recruits at least some of the amino acids from the host. Interestingly, in a whole-genome screen, genes coding for oligopeptide transporters were shown to be essential for infection or to be specifically activated during infection (10, 36). *S. aureus* is also equipped with several genes coding for proteases, whose activity could provide amino acids or peptides during infection. The analysis of other gram-positive organisms together with our own preliminary data led to the hypothesis that in *S. aureus* CodY may play a central role not only in metabolic adaptation but also in virulence gene expression. Indeed, it was recently shown that a *codY* mutant shows enhanced expression of the *agr* effector molecule RNAIII (34). Additionally, *codY* influences biofilm formation, albeit with opposite effects depending on the strain analyzed (16, 34). Our results obtained at the transcriptome level clearly demonstrate that CodY is involved in the regulation of metabolic genes and also influences gene expression of virulence genes in an *agr*-dependent and -independent manner.

MATERIALS AND METHODS

Strains and growth conditions. Strains and plasmids are listed in Table 1. *S. aureus* strains were grown in tryptic soy broth, CYPG (10 g/liter Casamino Acids, 10g/liter yeast extract, 5 g/liter NaCl, 20% glucose, and 1.5 M phosphoglycerate), or in a chemically defined medium (CDM). For strains carrying resistance genes, antibiotics were used only in precultures at the following concentrations: kanamycin, 50 μg/ml; erythromycin, 10 μg/ml; and tetracycline, 5 μg/ml. CDM was composed as follows (final concentration in mg/liter in brackets): group 1 amino acids from a 10× stock consisting of L-tryptophan (100), L-tyrosine (100), and L-phenylalanine (100); group 2 amino acids from a 10× stock consisting of L-cysteine (50), L-histidine (100), and L-methionine (100); group 3 amino acids from a 100× stock consisting of L-glutamine (200), L-glutamic acid (100), glycine (100), and L-proline (100); group 4 amino acids from a 100× stock consisting of L-isoleucine (100), L-leucine (100), L-threonine (200), and L-valine (100); group 5 amino acids from a 100× stock consisting of DL-alanine (100), L-arginine (100), L-aspartic acid (100), L-lysine (100), hydroxy-L-proline (100), and L-serine (100); group 6 vitamins from a 50× stock consisting of *p*-aminobenzoic acid (0.2), biotin (0.2), folic acid (0.8), niacinamide (1), β-NAD (2.5), pantothenate calcium salt (2), pyridoxal (1), pyridoxamine dihydrochloride (1), riboflavin (2), thiamine hydrochloride (1), and vitamin B_{12} (0.1); group 7 nucleotides from a 100× stock (predissolved in 2 N HCl) consisting of adenine (20), guanine hydrochloride (20), and uracil (20); group 8 and 9 salts from a 50× stock consisting of K_2HPO_4 (200) and KH_2PO_4 (1,000); group 9 consisting of NaH_2PO_4 (3,195), $MgSO_4$ (700), and $CaCl_2$ (10); group 10 consisting of a 100× stock of Na_2HPO_4 (9,214); and group 11 carbohydrate from a 20× stock of glucose (10,000). Single amino acids were omitted in some experiments, as indicated in the figure legends. The pH of the medium was buffered to 7.0.

Bacteria from an overnight culture were diluted to an initial optical density at 600 nm (OD_{600}) of 0.05 in fresh medium without antibiotics and grown with shaking (222 rpm) at 37°C to the indicated OD_{600}. In the complemented strains, *codY* was induced with anhydrotetracycline (IBA GmbH, Göttingen) (0.05 μg/ml). For up-shift experiments, strains were grown in CDM without isoleucine to an OD_{600} of 0.4. The cultures were divided into aliquots and supplemented with isoleucine and grown for 1 h.

Construction of mutant strains and complementation. The *codY* locus was replaced by a tetracycline resistance cassette [*tet*(M)]. Briefly, two fragments flanking *codY* and the *tet*(M) gene were amplified and annealed by overlapping PCR using oligonucleotides (see Table S2 in the supplemental material). The

amplicon was cloned into pMAD using the BamHI/BglII restriction sites of pMAD to gain pCG29. Mutagenesis of strain RN4220 was performed as described previously (1). The obtained *codY* gene replacement mutant strain (RN4220-21) was verified by PCR (for oligonucleotides, see Table S1 in the supplemental material). *codY* and *agr* mutants of different *S. aureus* strains were obtained by transduction using ϕ11 lysates of strains RN4220-21 and RN6112, respectively. Transductants were verified by PCR and pulsed-field gel electrophoresis. All *agr* mutants were negative for δ-hemolysin and RNAIII expression. For complementation, *codY* was amplified with oligonucleotides (see Table S1 in the supplemental material) containing EcoRI sites and cloned in the EcoRI site of the tetracycline-inducible vector pALC2073, yielding plasmid pCG30. The plasmid was used to transform strain RN4220, from which it was transduced, into the *codY* mutant strains. Strain RN1HG is an *rsbU*-restored derivative of *S. aureus* strain RN1 (8325) obtained by site-directed mutagenesis using pMAD *rsbU* as described previously (50).

RNA isolation, Northern blot hybridization, and real-time reverse transcription-PCR. RNA isolation and Northern blot analysis were performed as described previously (20). Briefly, bacteria were lysed in 1 ml of Trizol reagent (Invitrogen Life Technologies, Karlsruhe, Germany) with 0.5 ml of zirconia-silica beads (0.1 mm-diameter) in a high-speed homogenizer (Savant Instruments, Farmingdale, NY). RNA was isolated as described in the instructions provided by the manufacturer of Trizol. Digoxigenin-labeled probes for the detection of specific transcripts were generated using a DIG-Labeling PCR Kit following the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). Oligonucleotides used for probe generation are as described previously (48) or are listed in Table S1 in the supplemental material.

Microarray manufacturing and microarray design. The microarray was manufactured by in situ synthesis of 60-base-long oligonucleotide probes (Agilent, Palo Alto, CA), selected as previously described (8). The array covers >98% of all open reading frames (ORFs) annotated in strains N315 and Mu50 (30), MW2 (2), COL (18), NCTC8325 and USA300 (13), and MRSA252 and MSSA476 (27), including their respective plasmids.

Preparation of labeled nucleic acids for expression microarrays. Total RNA was purified from bacteria grown in CDM to an OD_{600} of 0.5. For each strain RNA of three independently grown cultures was analyzed. After additional DNase treatment, the absence of remaining DNA traces was confirmed by quantitative PCR (SDS 7700; Applied Biosystems, Framingham, MA) with assays specific for 16S rRNA (41, 43). Batches of 5 μg of total *S. aureus* RNA were labeled by Cy3-dCTP using SuperScript II (Invitrogen, Basel, Switzerland) following the manufacturer's instructions. Labeled products were then purified onto QiaQuick columns (Qiagen).

Purified genomic DNA from the different sequenced strains used for the design of the microarray was extracted (DNeasy; Qiagen), labeled with Cy5 dCTP using the Klenow fragment of DNA polymerase I (BioPrime, Invitrogen, Carlsbad, CA) (8), and used for the normalization process (49). Cy5-labeled DNA (500 ng) and a Cy3-labeled cDNA mixture were diluted in 50 μl of Agilent hybridization buffer and hybridized at a temperature of 60°C for 17 h in a dedicated hybridization oven (Robbins Scientific, Sunnyvale, CA). Slides were washed, dried under nitrogen flow, and scanned (Agilent, Palo Alto, CA) using 100% photon multiplier tube power for both wavelengths.

Microarray analysis. Fluorescence intensities were extracted using Feature Extraction software (version 8; Agilent). Local background-subtracted signals were corrected for unequal dye incorporation or unequal load of the labeled product. The algorithm consisted of a rank consistency filter and a curve fit using the default LOWESS (locally weighted linear regression) method. Data consisting of two independent biological experiments were expressed as log 10 ratios and analyzed using GeneSpring, version 8.0 (Silicon Genetics, Redwood City, CA). A filter was applied to select oligonucleotides mapping ORFs in the Newman genome, yielding approximately 92% coverage. Statistical significance of differentially expressed genes was calculated by analysis of variance (9, 43) using GeneSpring, including the Benjamini and Hochberg false discovery rate correction of 5% (*P* value cutoff, 0.05) and an arbitrary cutoff of twofold for expression ratios.

Microarray data accession number. The complete microarray data set has been posted on the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE12340 for the platform design and GPL7137 for the original data set.

RESULTS

Molecular organization of the *codY* operon. To characterize the CodY regulon, *S. aureus* gene replacement mutants were constructed in three clonally distinct *S. aureus* strains (Newman, RN1HG, and UAMS-1). In the mutant strains, *codY* was replaced by a *tet*(M) resistance cassette, as verified by Southern hybridization and PCR. To evaluate whether the mutation exerted a polar effect on the surrounding genes, Northern analysis was performed using probes specific for *codY* as well as for the neighboring genes (Fig. 1A). *codY* is part of a polycistronic operon encompassing *xerC*, *clpQ* (*hslV*), *clpY* (*hslU*), and *codY* (4.1 kb) (Fig. 1B). An additional 1.4-kb transcript codes for *codY* only. In the mutant, *codY* was replaced by *tet*(M), leading to termination of the transcript (*xerC* and *clpQY*) in front of *tet*(M). The expression levels of *xerC* and *clpQY* in the mutant were similar to those in the wild-type strains. However, there was a slight, but reproducible increase in the *xer* and *clpQY* transcript levels in the *codY* mutant. Microarray analysis also suggests that *xerC* is slightly affected by the mutation although the difference was not significant. *rpsB* located downstream of the *codY* operon was not affected in the mutant. Overall, the *codY* mutation showed minimal polar effects on the surrounding genes. Thus, for complementation analysis only, the *codY* ORF was cloned into an inducible vector (pCG30).

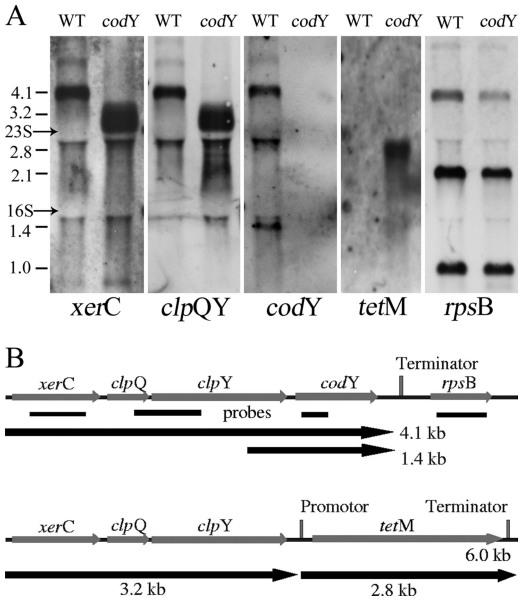

FIG. 1. Genetic organization of the *codY* locus. (A) Total RNA from strain Newman and its *codY* mutant Newman-21 was hybridized with probes specific for *xerC*, *clpQY*, *codY*, *tet*(*M*), and *rpsB*. Transcript size was estimated by comparison with an RNA marker. Note that slight hybridization bands below the ribosomal rRNA are probably due to comigration of the mRNA with rRNA and were not taken as distinct mRNA species. (B) Scheme of the genetic organization of the *codY* operon. Probes used for Northern blot analysis are indicated with black lines. Putative transcriptional units are indicated by black arrows in the wild type (upper part) and the *codY* mutant (lower part). WT, wild type.

Effect of *codY* on selected genes with putative CodY binding motifs. For other organisms, it has been shown that CodY functions as a repressor via binding to a proposed CodY-binding motif (AATTTTCWGAAAATT) (5, 12, 24). We performed a stringent search for the occurrence of the proposed motif (two mismatches accepted) within 1,000 bp upstream of

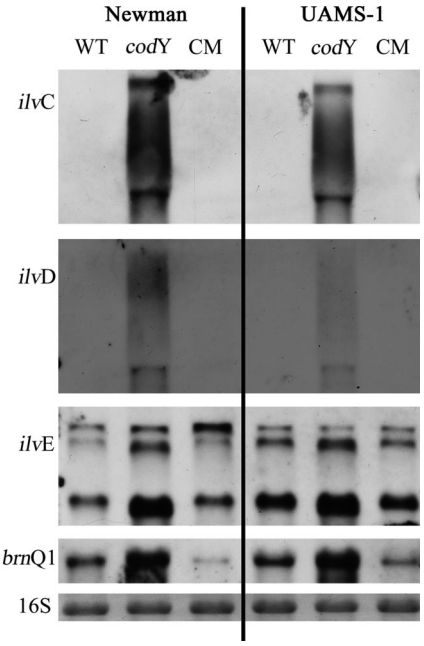

FIG. 2. Detection of *codY*-regulated operons: *ilvDBC-leuABC-ilvA* (CodY box in front of *ilvD*, detected with *ilvD* and *ilvC*), *ilvE*, and *brnQ1* (MWMN_0180, BCAA transport system II carrier protein). RNA was isolated from strains Newman and UAMS-1, their *codY* mutants (Newman-21 and UAMS-1-21), and the complemented strains ([CM] Newman-21/pCG30 and UAMS-1-21/pCG30). Bacteria were grown to an OD_{600} of 0.5 in CDM. The 16S rRNAs detected in the ethidium bromide-stained gels are indicated as loading controls in the lower lane. WT, wild type.

putative ORFs in three *S. aureus* genomes (Col, N315, and Newman) (http://xbase.bham.ac.uk/pattern.pl?id=1327). A total of 68, 76, and 71 genes preceded by a putative CodY box were identified in strains Col, N315, and Newman, respectively. The CodY boxes were mainly localized 50 to 300 bp upstream of the translational start site (see Fig. S1 in the supplemental material). Many of the identified genes code for enzymes involved in amino acid metabolism or transport. We selected four genes containing CodY boxes for Northern blot analysis: *ilvC* and *ilvD* (part of the *ilvDBC-leuABC-ilvA* operon, with the CodY box in front of *ilvD*), *ilvE*, and *brnQ1* (MWMN_0180, branched-chain amino acid transport system II carrier protein). It could be shown that all four genes were strongly upregulated in the *codY* mutants of strains Newman and UAMS-1 in comparison to the wild-type or *codY*-complemented mutant strains (Fig. 2). These results suggest that CodY in *S. aureus* binds to the same conserved motif as described for *B. subtilis* and *L. lactis*.

Effect of *codY* on growth in CDM. In a first attempt to characterize the *codY* phenotype, we performed growth analysis in CDM containing all three BCAAs (100 mg/liter each) and threonine (200 mg/liter). In this medium *S. aureus* strains did not grow without glucose, suggesting that amino acids were not being used as the primary carbon source. This is further supported by the lowering of the pH after prolonged growth, which also is indicative of glucose consumption. No growth of wild-type or mutant strains occurred if any of the five groups of amino acids was omitted from the medium. Growth analysis revealed that *codY* mutants grew more slowly in complete CDM than the wild-type strains (Fig. 3A and B). The *codY* mutants were also delayed in growth in complex medium such as CYPG (data not shown). Thus, one may assume that gene repression by CodY might be favorable for the organism under conditions of amino acid surplus.

Next, growth in CDM that had been depleted either of one of the BCAAs (valine, leucine, or isoleucine) or of threonine was analyzed. Wild-type strains and *codY* mutants failed to grow in medium lacking either valine or leucine (data not shown). In medium lacking isoleucine, there was no growth difference between the wild type and *codY* mutants in contrast to growth in CDM containing 100 mg/liter of isoleucine. We hypothesized that isoleucine may be a major signal for CodY regulation. If isoleucine is indeed a natural ligand of CodY, then maximal repression of CodY-regulated genes should occur under isoleucine-rich conditions. Thus, an excess of isoleucine may lead to growth inhibition, e.g., if some amino acids become limiting but the biosynthetic pathways remain repressed. In fact, a high concentration of isoleucine (500 mg/liter) in the medium resulted in diminished growth in the wild type in comparison to growth in medium with moderate isoleucine (100 mg/liter) (Fig. 3C). This growth inhibition is CodY dependent since in the *codY* mutant no effect of isoleucine excess on growth was observed. Similar results were obtained with strain UAMS-1 and strain RN1HG (data not shown). A predominant role of isoleucine in comparison to other BCAAs for signaling is emphasized by the observation that an excess of valine or leucine did not result in growth inhibition of the wild-type strains (data not shown).

Interestingly, in medium without threonine the *codY* mutants were able to grow but not the wild type (Fig. 3D). This indicates that, under these growth conditions, CodY represses genes necessary for threonine synthesis. The growth of the mutant in medium lacking threonine was characterized by a typical lag phase. Thus, the mutant is obviously able to adapt to the CDM conditions. When the *codY* mutant was subcultured from the exponential growth phase (OD_{600} of 0.5) into fresh medium, growth continued without lag (Fig. 3D), indicating adaptation to the medium. However, when the mutants were subcultured in complete CDM and again inoculated into medium without threonine, the typical lag phase was again apparent. Thus, the adaptation of the *codY* mutants to threonine-depleted medium seems to be due a regulatory adaptation in metabolism and is not mediated by the generation of suppressor mutations in genes of the biochemical pathways.

Influence of isoleucine on CodY target genes. To gain further insight into putative CodY ligands in *S. aureus*, we analyzed whether isoleucine in the medium affects the transcription of *codY* target genes (Fig. 4). Strains were precultured in CDM without isoleucine and then supplemented with isoleucine for 1 h. Isoleucine resulted in a dose-dependent repression of the CodY target gene *ilv*C (Fig. 4B). The addition of 5 μg/ml isoleucine was already sufficient to cause a severe downregulation of *ilvC*. The repression is mediated by CodY since the *codY* mutants were not responsive to isoleucine under the tested conditions (Fig. 4A). However, the *ilvC* level observed in the wild type grown without isoleucine was lower than that in the *codY* mutant. This may be due to a baseline level of isoleucine produced by the bacteria or by other putative signaling

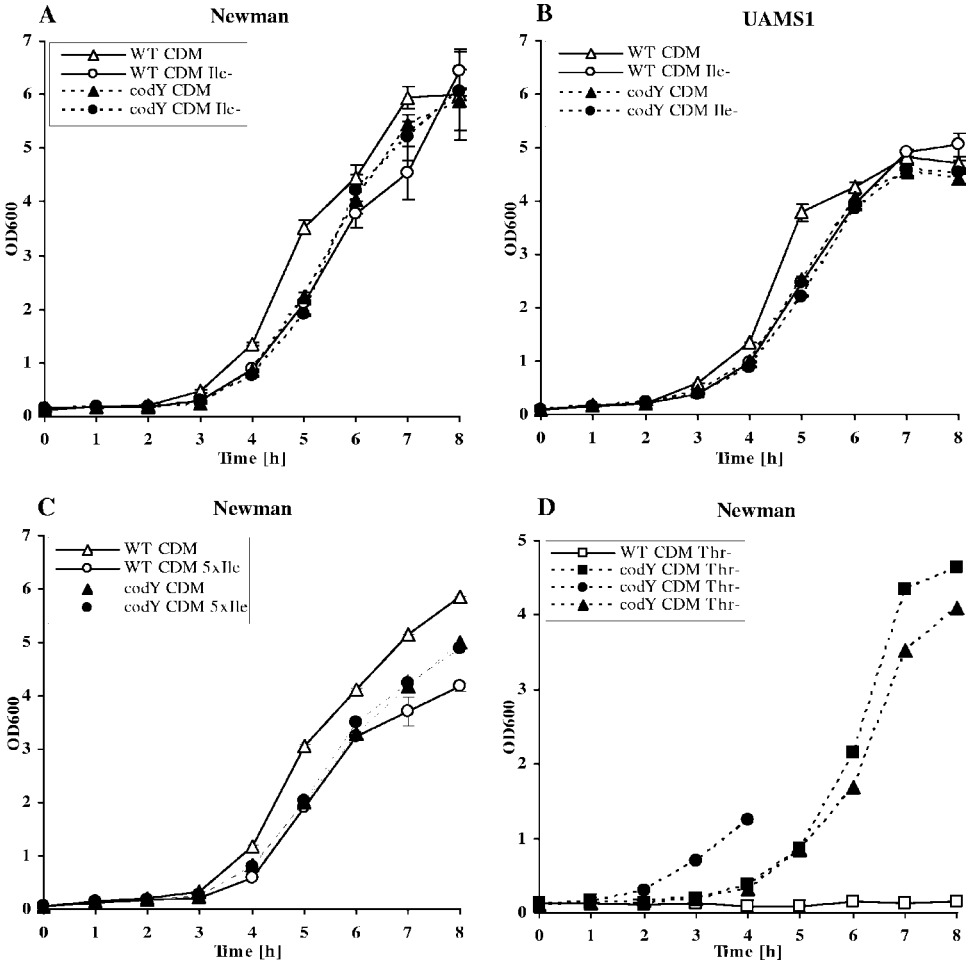

FIG. 3. Growth analysis of wild-type and *codY* mutants in CDM. (A and B) Growth of *S. aureus* strain Newman and the *codY* mutant Newman-21 (A) and of UAMS-1 and the *codY* mutant UAMS-1-21 (B) in CDM and in CDM lacking isoleucine (Ile^-). (C) Growth of *S. aureus* strain Newman and the *codY* mutant Newman-21 in CDM and CDM with an excess of 500 μg/ml isoleucine (5× Ile). (D) Newman-21 was grown in medium lacking threonine (Thr^-; triangle). For analysis of the observed lag phase, the *codY* mutant was subcultured from threonine-depleted CDM. Bacteria from CDM without threonine grown to the exponential phase (OD_{600} of 1) were then subcultured in medium lacking threonine (square). In addition, the bacteria were recultured in complete CDM overnight and again inoculated in CDM without threonine (circle). The lag phase was omitted after preadaptation of the bacteria to threonine depletion but was restored after intermittent growth in medium with threonine. WT, wild type.

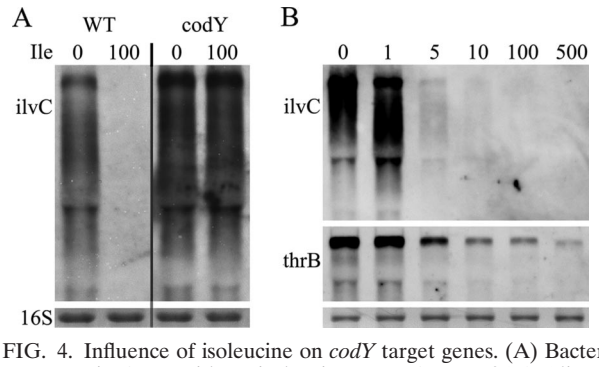

FIG. 4. Influence of isoleucine on *codY* target genes. (A) Bacteria were grown in CDM without isoleucine to an OD_{600} of 0.4. Aliquots were then supplemented with isoleucine (200 mg/liter) and grown for another 1 h. (B) Strain Newman was grown without isoleucine to an OD_{600} of 0.4. Aliquots were then supplemented with increasing concentrations of isoleucine and further grown for 1 h. RNA were hybridized with a probe specific for *ilvC* or *thrB*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as a loading control in the lowest panel. WT, wild type.

molecules such as GTP. Since only the *codY* mutant was able to grow in medium lacking threonine, we also tested whether genes involved in threonine biosynthesis are similarly repressed via CodY. We could show that *thrB* transcription is also dependent on the isoleucine concentration. However, repression of *thrB* by isoleucine was less pronounced than that of *ilvC*.

CodY as a virulence regulator in *S. aureus*. The repressor CodY may be essential not only for the regulation of metabolic genes but also for the fine-tuning of virulence-associated genes, as recently proposed by Majerczyk et al. (34). Indeed, the transcriptional pattern of RNAIII of the virulence regulator *agr* was identical to that of the prototypic CodY-repressed *ilvC* in strain Newman: repression after growth was observed with isoleucine in the wild-type and complemented strains but not in the *codY* mutant (Fig. 5). In contrast, genes coding for the cell surface proteins *fnbA* and *spa* were upregulated by isoleucine in a *codY*-dependent manner. No significant effect

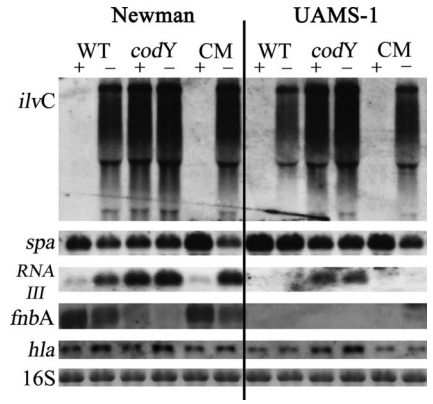

FIG. 5. Influence of isoleucine on virulence gene expression. RNA from strain Newman and UAMS-1, their *codY* mutants (Newman-21 and UAMS-1-21, respectively) and the complemented strains ([CM] Newman-21/pCG30 and UAMS-1-21/pCG30, respectively) isolated from bacteria grown to an OD_{600} of 0.5 in CDM with (+) or without (−) isoleucine. They were then hybridized with probes specific for *ilvC*, *spa*, *agr* (RNAIII), *fnbA*, and *hla*. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as a loading control in the lowest panel. WT, wild type.

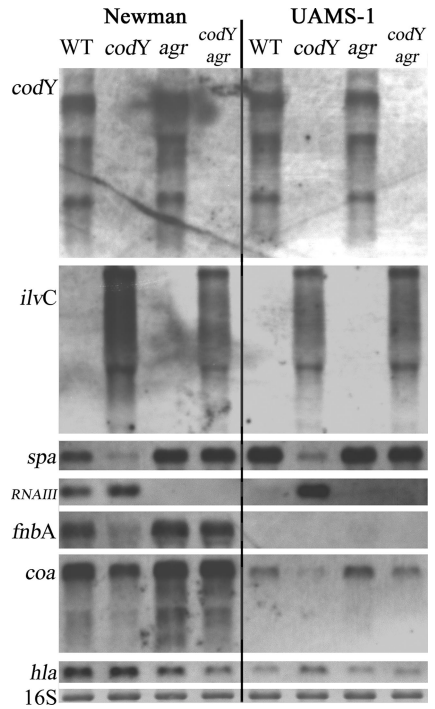

FIG. 6. Influence of CodY and *agr* on virulence gene expression in bacteria from exponential growth phase. RNA from the wild type (WT), *codY*, *agr*, and *codY agr* mutants was isolated from bacteria grown in CDM to the exponential (OD_{600} of 0.5) growth phase. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the lowest panel.

on *hla* expression was observed in strain Newman under these growth conditions. The diminished expression of *spa* and enhanced expression of RNAIII in the *codY* mutant compared to the wild type were also evident in strain UAMS-1. However, there were some differences between the results obtained from strain UAMS-1 and those from strain Newman. First, *fnbA* transcription was not detectable, which agrees with the results from genome sequencing showing that *fnbA* is not present in strain UAMS-1 (unpublished observation). Second, the effects of isoleucine in the wild-type and complemented strains were less pronounced. In addition, in this genetic background, the expression of the *agr*-regulated gene *hla* was enhanced in the *codY* mutant compared to the wild-type and complemented strains.

Effect of *codY* on virulence gene expression in *agr*-negative background. The virulence gene *hla* is known to be activated by RNAIII, whereas *spa*, *coa*, and *fnbA* are inhibited by RNAIII (37, 52, 53). To clarify whether the effect of CodY on virulence genes is solely due to RNAIII upregulation in the *codY* mutant, we analyzed virulence gene expression in *agr* and *codY agr* double mutants (Fig. 6). Interestingly, *codY* expression was not affected in the *agr* mutants of *S. aureus*. This is in contrast to *Staphylococcus epidermidis*, in which *agr* leads to elevated *codY* transcription (4). As expected in the *agr*-negative background, increased levels of *spa*, *fnbA*, and *coa* transcripts were detectable. In the *agr*-negative background, no significant effect of CodY was observed on these genes in bacteria during the exponential growth phase. However, when we analyzed bacteria from the postexponential phase, it could be shown that *fnbA* and *spa* were activated by CodY, independently of *agr* (Fig. 7). The lack of *spa* transcription in the *agr*-positive background is due to the strong repressive effect of RNAIII on *spa* transcription. Surprisingly, growth in CDM also allowed transcription of *fnbA* in the late growth phase. This is usually not seen using complex medium, in which *fnbA*, like *coa*, is repressed during postexponential growth independently of *agr* (42, 53).

Global effect of *codY* on gene expression in *agr*-positive and *agr*-negative backgrounds. In order to acquire a more comprehensive understanding of the *codY* regulon, we performed microarray analysis. On the basis of the results obtained up to that point, we expected to detect the most pronounced effects of CodY in bacteria grown with isoleucine during the expo-

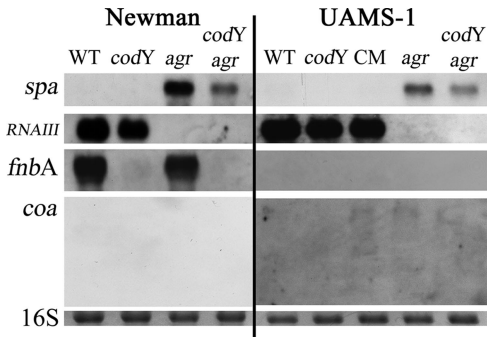

FIG. 7. Influence of CodY and *agr* on virulence gene expression in bacteria from postexponential growth phase. RNA from wild-type (WT) strains (Newman or UAMS-1) and their *codY*, *agr*, and *codY agr* mutants was isolated from bacteria grown in CDM with an excess of isoleucine (500 mg/liter) to the postexponential phase. The 16S rRNA detected in the ethidium bromide-stained gels is indicated as loading control in the lowest panel.

nential phase. Under these conditions, CodY should be saturated with its ligand(s) and thus display full repression.

First, we determined the *codY* regulon by comparing gene expression in the parental strain with that in the *codY* mutant. A total of 124 genes (5% of the genome) showed differential expression in the *codY* mutant compared to its parent (Table 2). These genes were predicted to be contained in 71 operons (operon structure was predicted by the public database http://www.microbesonline.org). For each gene with significant difference in gene expression, the neighboring genes within the operons were also analyzed (see Table S3 in the supplemental material). In most cases genes that were predicted to be located in an operon were coregulated although this correspondence did not always reach the level of significance.

The vast majority of targets (106/124) appeared to be repressed by CodY (upregulated in the mutant). Sixteen of these genes were preceded by a CodY box as predicted above (see Table S2 in the supplemental material). Fifty-eight of the *codY*-repressed genes encompassed genes involved in amino acid transport and metabolism, e.g., the *ilv* operon and the peptide transporters *opp* and *brnQ1* encoding proteins involved in the metabolism or the transport of BCAAs. Besides *agr* only one other regulatory gene was significantly affected by CodY. The transcription activator of the glutamate synthase operon *gltC* is repressed by CodY, suggesting that the CodY-repressed genes *gltB* and *gltD* are indirectly influenced via *gltC*.

Only 18 genes seemed to be activated by CodY (downregulated in the mutant). None of these genes contained a putative CodY binding motif, supporting the hypothesis that upregulation of these genes is probably not related to a direct interaction with CodY. Interestingly, six CodY-activated genes were predicted to be involved in nucleotide transport and metabolism.

Genes encoding virulence and defense factors were either up- or downregulated by CodY. For instance, MWMN_1084, which codes for phenole-soluble moduline, appeared to be strongly downregulated. The *cap* operon coding for enzymes in capsular biosynthesis and genes for katalase (*katA*) and superoxide dismutase (*sodM*) were also downregulated. In contrast, expression of genes coding for the cell surface proteins *fnbA*, *sasG*, and *coa* were positively influenced by CodY.

We next asked which of the 124 *codY*-dependent genes determined in the *agr*-positive background are still *codY* regulated in an *agr*-negative background. To determine this, we compared gene expression of the *agr* mutant versus the *agr codY* double mutant. Ninety-six of the 124 genes appeared to be regulated by *codY* independent of the *agr* background, and most of these were involved in amino acid metabolism and transport. In contrast, many of the genes categorized as virulence and defense factors were not significantly influenced by CodY in the *agr*-negative background. However genes within the *cap* operon, *katA* and *sodM*, were still *codY* repressed independent of *agr*. These results confirm the results of Northern blot analysis indicating that CodY acts in an *agr*-independent and *agr*-dependent manner on virulence genes, whereas the influence on metabolic genes is mostly *agr*-independent.

DISCUSSION

CodY has been described as a conserved repressor of genes involved in the biosynthesis and transport of amino acids in several gram-positive species. Here, we analyze the role of CodY in the human pathogen *S. aureus*. In *S. aureus codY* is cotranscribed with three genes located upstream of *codY* (*xerC*, *clpQ*, and *clpY*). *xerC* codes for tyrosine recombinase, and *clpQY* codes for the ATP-dependent heat shock protease HslVU. The same gene order is present in the genome of *B. subtilis*, *Listeria monocytogenes*, and *Enterococcus faecalis*, indicating a physiological link between CodY and the heat shock protease Hs1VU in these bacteria. This must remain speculative, however, since little is known about the function of Hs1VU in stress response or pathogenesis (7). In streptococcal species, *L. lactis* or *Clostridium difficile*, *codY* is located in a different genetic context. Alignments of the CodY sequences (http://www.ebi.ac.uk/Tools/clustalw/) from a selected set of gram-positive bacteria revealed two clusters: cluster one containing CodY from *S. aureus*, *B. subtilis*, *L. monocytogenes*, and *C. difficile* and cluster two with CodY from *E. faecalis*, streptococci, and lactococci. Thus, the evolution of the genetic context is not congruent with sequence differences within CodY. For instance, *E. faecalis* resembles *S. aureus* with respect to the gene context, whereas from the CodY amino acid sequence, *E. faecalis* is very similar to the streptococcal species.

From studies of other bacteria, it was postulated that BCAAs are major ligands of CodY, resulting in severe repression of target genes. We could show that in *S. aureus* isoleucine, although not required for growth, resulted in strong repression of CodY target genes. Furthermore, an excess of isoleucine resulted in growth inhibition in wild-type bacteria. This suggests that certain target genes that are needed for optimal growth are repressed under these conditions. Since an excess of leucine or valine had no effect on *codY* target gene repression or growth, we conclude that isoleucine is the major ligand in *S. aureus* as proposed for CodY from *L. lactis* (11).

Interestingly, only *codY* mutants but not wild-type *S. aureus* strains were able to grow without threonine, indicating that threonine synthesis requires enzymes whose transcription is efficiently repressed by CodY. Indeed, *thrC* (coding for threonine synthase) was shown to be repressed by CodY (shown by microarray analysis and Northern analysis). Since threonine auxotrophy was also seen under conditions without isoleucine, it can be assumed that besides isoleucine, other ligands (or CodY without ligand) are sufficient to mediate the CodY-dependent repression of the threonine biosynthetic genes.

Functionally, there are also clear differences between different species in the GTP-binding capacity of CodY (39). CodY from *S. aureus* is similar to its homolog in *B. subtilis* with respect to a proposed GTP-binding motif derived from structure and sequence analyses (33). For an in-depth analysis of the role of GTP as a signaling molecule, mutants with defects in internal GTP synthesis and/or the stringent control are currently being examined.

Overall, it can be assumed that CodY of *S. aureus* functions in a manner similar to that which has been shown for other gram-positive organisms. This is probably also true for the proposed CodY binding motif since we were able to detect CodY target genes based on a stringent motif search allowing

TABLE 2. The *codY* regulon of *S. aureus*

Newman ORF[a]	Description	Gene	Differential expression in the indicated strains (*n*-fold)[b]		Category
			Newman vs *codY* mutant	*agr* mutant vs *agr codY* mutant	
NWMN_0128	*N*-Acetylglutamate gamma-semialdehyde dehydrogenase	*argC*	0.39	NS	Amino acid transport/metabolism
NWMN_0130	Branched-chain amino acid transport system II carrier protein	*brnQ1*	0.33	0.44	Amino acid transport/metabolism
NWMN_0144	Oligopeptide ABC transport. permease		0.03	0.15	Amino acid transport/metabolism
NWMN_0145	Peptide ABC transporter. permease		0.15	0.33	Amino acid transport/metabolism
NWMN_0146	RGD-containing lipoprotein	*rlp*	0.31	0.4*	Amino acid transport/metabolism
NWMN_0147	Gamma glutamyltranspeptidase	*ggt*	0.12	0.21	Amino acid transport/metabolism
NWMN_0348	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	*metE*	0.06	0.06	Amino acid transport/metabolism
NWMN_0349	Methylenetetrahydrofolate reductase protein	*metH*	0.03	0.04	Amino acid transport/metabolism
NWMN_0350	*trans*-Sulfuration enzyme family protein		0.36	0.06	Amino acid transport/metabolism
NWMN_0351	Cys/Met metabolism PLP-dependent enzyme		0.02	0.06	Amino acid transport/metabolism
NWMN_0425	Cystathionine gamma-synthase	*metB*	0.36	0.48	Amino acid transport/metabolism
NWMN_0436	Glutamate synthase large subunit	*gltB*	0.08	0.14	Amino acid transport/metabolism
NWMN_0437	NADH-glutamate synthase small subunit	*gltD*	0.09	0.07	Amino acid transport/metabolism
NWMN_0516	Branched-chain amino acid aminotransferase	*ilvE*	0.47	0.52*	Amino acid transport/metabolism
NWMN_0831	Argininosuccinate lyase	*argH*	0.24	0.46	Amino acid transport/metabolism
NWMN_0855	Oligopeptide transport system permease protein	*oppB*	0.15	0.15	Amino acid transport/metabolism
NWMN_0858	Oligopeptide transport ATP-binding protein	*oppD*	0.16	0.19	Amino acid transport/metabolism
NWMN_0860	Hypothetical protein		0.12	0.17	Amino acid transport/metabolism
NWMN_0883	Na^+/alanine symporter family protein		0.03	0.04	Amino acid transport/metabolism
NWMN_1239	Aspartate kinase		0.06	0.06	Amino acid transport/metabolism
NWMN_1240	Homoserine dehydrogenase	*metL*	0.05	0.06	Amino acid transport/metabolism
NWMN_1241	Threonine synthase	*thrC*	0.06	0.07	Amino acid transport/metabolism
NWMN_1245	Amino acid permease		0.5*	0.5*	Amino acid transport/metabolism
NWMN_1277	Prephenate dehydrogenase	*tyrA*	0.50	0.44	Amino acid transport/metabolism
NWMN_1279	Anthranilate synthase component I		0.11	0.23	Amino acid transport/metabolism
NWMN_1280	Anthranilate synthase component II	*trpG*	0.15	0.21	Amino acid transport/metabolism
NWMN_1281	Anthranilate phosphoribosyltransferase	*trpD*	0.16	0.16	Amino acid transport/metabolism
NWMN_1282	Indole-3-glycerol phosphate synthase	*trpC*	0.10	0.11	Amino acid transport/metabolism
NWMN_1283	Phosphoriborylanthranilate isomerase	*trpF*	0.08	0.16	Amino acid transport/metabolism
NWMN_1284	Tryptophan synthase subunit beta	*trpB*	0.10	0.09	Amino acid transport/metabolism
NWMN_1304	Aspartate kinase	*lysC*	0.12	0.19	Amino acid transport/metabolism
NWMN_1305	Aspartate semialdehyde dehydrogenase	*asd*	0.12	0.09	Amino acid transport/metabolism
NWMN_1306	Dihydrodipicolinate	*dapA*	0.09	0.09	Amino acid transport/metabolism
NWMN_1307	Dihydrodipicolinate reductase	*dapB*	0.15	0.07	Amino acid transport/metabolism
NWMN_1308	Tetrahydrodipicolinate acetyltransferase	*dapD*	0.10	0.11	Amino acid transport/metabolism
NWMN_1311	Diaminopimelate decarboxylase	*lysA*	0.29	0.23	Amino acid transport/metabolism
NWMN_1348	Threonine dehydratase	*ilvA*	0.12	0.13	Amino acid transport/metabolism
NWMN_1616	Aminotransferase, class V		0.08	0.10	Amino acid transport/metabolism
NWMN_1617	D-3-Phosphoglycerate dehydrogenase	*serA*	0.07	0.10	Amino acid transport/metabolism
NWMN_1749	Glutamine transport ATP-binding protein		0.30	0.55*	Amino acid transport/metabolism
NWMN_1750	Extracellular glutamine-binding protein		0.5*	NS	Amino acid transport/metabolism
NWMN_1960	Dihydroxy acid dehydratase	*ilvD*	0.03	0.09	Amino acid transport/metabolism
NWMN_1961	Acetolactate synthase large subunit	*ilvB*	0.02	0.03	Amino acid transport/metabolism
NWMN_1962	Ketol acid reductoisomerase	*ilvC*	0.01	0.02	Amino acid transport/metabolism
NWMN_1963	2-Isopropylmalate synthase	*leuA*	0.01	0.02	Amino acid transport/metabolism
NWMN_1964	3-Isopropylmalate dehydrogenase	*leuB*	0.02	0.04	Amino acid transport/metabolism
NWMN_1965	Isopropylmalate isomerase large subunit	*leuC*	0.02	0.04	Amino acid transport/metabolism
NWMN_1966	3-Isopropylmalate dehydratase small subunit	*leuD*	0.03	0.02	Amino acid transport/metabolism
NWMN_2347	Glycine betaine/L-proline transport	*opuCA*	0.47	0.27	Amino acid transport/metabolism
NWMN_2500	Amino acid permease family protein		0.10	0.11	Amino acid transport/metabolism
NWMN_2501	4-Aminobutyrate aminotransferase		0.09	0.13	Amino acid transport/metabolism
NWMN_2571	Imidazole glycerol phosphate synthase subunit	*hisF*	0.20	0.21	Amino acid transport/metabolism
NWMN_2572	Phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase	*hisA*	0.18	0.14	Amino acid transport/metabolism
NWMN_2573	Imidazole glycerol phosphate synthase	*hisH*	0.20	0.31	Amino acid transport/metabolism
NWMN_2574	Imidazoleglycerol phosphate dehydratase	*hisB*	0.11	0.03	Amino acid transport/metabolism
NWMN_2577	ATP phosphoribosyltransferase	*hisG*	0.18	0.31	Amino acid transport/metabolism
NWMN_2370	Putative transport protein		0.19	0.24	Amino acid transport/metabolism
NWMN_0859	Oligopeptide transport ATP-binding protein	*oppF*	0.33	0.30	Amino acid transport/metabolism
NWMN_0428	ABC transporter		0.10	0.18	Inorganic ion transport/metabolism
NWMN_1950	Ammonium transporter	*nrgA*	0.23	NS	Inorganic ion transport/metabolism
NWMN_0423	**Sodium-dependent symporter protein**		**3.03**	**3.92**	**Inorganic ion transport/metabolism**
NWMN_2288	**Nitrite transport protein**	*narK*	**2.28**	**NS**	**Inorganic ion transport/metabolism**
NWMN_0016	**Adenylosuccinate synthase**	*purA*	**2.65**	**NS**	**Nucleotide transport/metabolism**
NWMN_0379	**Xanthine permease**	*pbuX*	**2.51**	**NS**	**Nucleotide transport/metabolism**
NWMN_1110	**Uracil permease**	*pyrP*	**2.23**	**2.1***	**Nucleotide transport/metabolism**
NWMN_1111	**Aspartate carbamoyltransferase catalytic subunit**	*pyrB*	**2.35**	**2.2***	**Nucleotide transport/metabolism**
NWMN_1112	**Dihydroorotase**	*pyrC*	**2.09**	**2.1***	**Nucleotide transport/metabolism**
NWMN_1249	**GMP oxidoreductase**	*guaC*	**2.33**	**2.1***	**Nucleotide transport/metabolism**
NWMN_0163	Formate acetyltransferase activating enzyme	*pflA*	0.40	0.12	Energy production/conversion
NWMN_0162	Formate acetyltransferase	*pflB*	0.27	0.16	Energy production/conversion
NWMN_0979	Pyruvate carboxylase	*pycA*	0.37	0.31	Energy production/conversion
NWMN_1325	Dihydrolipoamide acetyltransferase	*sucB*	0.45	0.55*	Energy production/conversion
NWMN_2294	**Putative nitrate reductase gamma chain**	*narI*	**2.44**	**NS**	**Energy production/conversion**
NWMN_0167	**Acetyl-coenzyme A acetyltransferase**	*fadA*	**3.04**	**NS**	**Energy production/conversion**

Continued on following page

TABLE 2—*Continued*

Newman ORF[a]	Description	Gene	Differential expression in the indicated strains (*n*-fold)[b]		Category
			Newman vs *codY* mutant	*agr* mutant vs *agr codY* mutant	
NWMN_0435	Transcription activator of glutamate synthase operon	*gltC*	0.32	NS	Regulation
NWMN_1943	Accessory gene regulator B	*agrB*	0.42	NS	Regulation
NWMN_1946	Accessory gene regulator A	*agrA*	0.44	NS	Regulation
NWMN_0077	Superoxide dismutase	*sodM*	0.26	0.49*	Defense/Virulence factor
NWMN_0095	Capsular polysaccharide synthesis enzyme CapA	*capA*	0.32	0.33	Defense/Virulence factor
NWMN_0096	Capsular polysaccharide synthesis enzyme CapB	*capB*	0.29	0.19	Defense/Virulence factor
NWMN_0097	Capsular polysaccharide synthesis enzyme CapC	*capC*	0.35	0.16	Defense/Virulence factor
NWMN_0098	Capsular polysaccharide synthesis enzyme CapD	*capD*	0.30	0.36	Defense/Virulence factor
NWMN_0099	Capsular polysaccharide synthesis enzyme CapE	*capE*	0.32	0.31	Defense/Virulence factor
NWMN_0100	Capsular polysaccharide synthesis enzyme CapF	*capF*	0.33	NS	Defense/Virulence factor
NWMN_0101	Capsular polysaccharide synthesis enzyme CapG	*capG*	0.30	NS	Defense/Virulence factor
NWMN_0102	Capsular polysaccharide synthesis enzyme CapH	*capH*	0.34	0.45	Defense/Virulence factor
NWMN_0103	Capsular polysaccharide synthesis enzyme CapI	*capI*	0.41	0.50	Defense/Virulence factor
NWMN_0104	Capsular polysaccharide synthesis enzyme CapJ	*capJ*	0.32	NS	Defense/Virulence factor
NWMN_0105	Capsular polysaccharide synthesis enzyme CapK	*capK*	0.31	0.64	Defense/Virulence factor
NWMN_0107	Capsular polysaccharide synthesis enzyme CapM	*capM*	0.45	NS	Defense/Virulence factor
NWMN_0108	Capsular polysaccharide synthesis enzyme CapN	*capN*	0.43	NS	Defense/Virulence factor
NWMN_0262	Truncated glycerol ester hydrolase	*geh*	0.35	NS	Defense/Virulence factor
NWMN_0525	Bone sialoprotein-binding protein	*sdrE*	0.50	1.88*	Defense/Virulence factor
NWMN_1084	Antibacterial protein (phenole-soluble moduline)		0.06	NS	Defense/Virulence factor
NWMN_1246	Catalase	*katA*	0.36	0.36	Defense/Virulence factor
NWMN_1872	MHC class II analog protein	*eap*	0.48	NS	Defense/Virulence factor
NWMN_nd	Delta-hemolysin	*hld*	0.30	NS	Defense/Virulence factor
NWMN_0166	**Staphylocoagulase precursor**	*coa*	**2.49**	**NS**	**Defense/Virulence factor**
NWMN_0394	**Auperantigen-like protein 7**	*set7nm*	**3.34**	**NS**	**Defense/Virulence factor**
NWMN_2392	**Cell wall-anchored protein**	*sasG*	**2.85**	**0.5***	**Defense/Virulence factor**
NWMN_2399	**Fibronectin binding protein**	**FnBPA**	**2.01***	**NS**	**Defense/Virulence factor**
NWMN_0071	Acetoin reductase	*butA*	0.19	0.17	Miscellaneous
NWMN_0429	*N*-Acetylmuramoyl-L-alanine amidase		0.50	NS	Miscellaneous
NWMN_0721	Sigma 54 modulation protein		0.45	0.47	Miscellaneous
NWMN_1309	Hippurate hydrolase		0.14	0.19	Miscellaneous
NWMN_1618	Haloacid dehalogenase-like hydrolase		0.43	0.31	Miscellaneous
NWMN_2097	Tagatose-6-phosphate kinase	*lacC*	0.20	NS	Miscellaneous
NWMN_2448	ATP-dependent Clp protease	*clpC*	0.38	0.37	Miscellaneous
NWMN_0028	**Metallo-beta-lactamase superfamily protein**		**2.91**	**NS**	**Miscellaneous**
NWMN_0322	**Ascorbate-specific phosphotransferase system enzyme IIC**	*ulaA*	**2.35**	**NS**	**Miscellaneous**
NWMN_0220	Hypothetical protein		0.20	0.47	Function unknown
NWMN_0404	Hypothetical protein	*lpl2nm*	0.5*	0.25	Function unknown
NWMN_0667	Hypothetical protein		0.5*	0.47	Function unknown
NWMN_0896	Hypothetical protein		0.21	0.20	Function unknown
NWMN_0901	Hypothetical protein		0.07	0.24	Function unknown
NWMN_0902	Hypothetical protein		0.11	0.07	Function unknown
NWMN_1243	Hypothetical protein		0.27	0.27	Function unknown
NWMN_2221	Hypothetical protein		0.30	0.42	Function unknown
NWMN_2222	Hypothetical protein		0.10	0.16	Function unknown
NWMN_2230	Hypothetical protein		0.21	0.14	Function unknown
NWMN_2470	Hypothetical protein		0.11	0.09	Function unknown
NWMN_2578	Hypothetical protein		0.06	0.25	Function unknown
NWMN_0027	**Hypothetical protein**		**3.66**	**4.07**	**Function unknown**
NWMN_0401	**Hypothetical protein**		**2.38**	**NS**	**Function unknown**

[a] Based on the publically available Newman genome sequence. Boldface, genes that are repressed in the *codY* mutant.
[b] Fold changes are indicated for each comparison and displayed for genes showing statistically significant differential expression. Values correspond to expression ratios, i.e., averaged expression levels from two independent replicate experiments ($P < 0.05$). NS, not significantly different; *, limit of significance.

only two mismatches within the consensus sequence. Lowering the stringency to four or five mismatches resulted in >1,000 putative sites within the genome, which appears of little informative value. However, the high stringency may have caused us to miss several real CodY boxes as a previous study in *B. subtilis* showed that even up to five mismatches within the CodY box consensus could result in a functional element (5). In fact, a CodY box was predicted for only a subset of the *codY*-dependent genes identified by our microarray analysis. When we allowed three mismatches within 200 bp upstream of ORFs, a reasonable number (222) of additional genes with a putative *codY* box were found. For instance, in the upstream sequence of the *cap* operon and the *fnbA* gene, a *codY* motif could now be identified. Interestingly, the CodY box preceding the *cap* operon overlapped with the mapped −10 region (38). The putative CodY box in front of *fnbA* was located between a sigma B binding motif and the transcriptional start site. Thus, these two virulence-associated genes may be direct targets of CodY regulation. Direct binding assays such as chromatin immunoprecipitation with microarray analysis or gel retardation assays using purified CodY are needed to clarify this topic in the future.

From microarray analysis it became clear that, overall, the *codY* regulon is conserved between gram-positive species with regard to profound and mostly direct repression of genes involved in amino acid biosynthesis and transport. Genes that are activated via CodY are much less conserved, and a direct interaction of CodY with this activated target gene could be

shown only for *ackA* of *B. subtilis* (44). In our screen, several genes involved in nucleotide synthesis and transport were activated by *codY*. Similarly, in *L. monocytogenes*, *guaA* and *guaB*, which encode the enzymes involved in biosynthesis of GMP, which encode, were also found to be activated by *codY* (6). Thus, there may be at least an indirect link between CodY and the intracellular nucleotide pool.

The most comprehensive data on the CodY regulon and function are based on the analysis of the nonpathogenic bacteria *B. subtilis* and *L. lactis*. There is emerging evidence that CodY also has an impact on virulence gene expression in gram-positive pathogens (6, 14, 26, 32, 35, 46). Interestingly, in *S. aureus* and *L. monocytogenes*, the quorum-sensing *agr* system is affected by CodY in opposite directions: in *L. monocytogenes* CodY leads to *agr* activation, while in *S. aureus* it leads to *agr* repression (6, 34). Although a putative CodY box with three mismatches is located upstream of *agrB* in *S. aureus*, this box overlaps with *hld* and is not in proximity to the transcriptional start site or the binding site of the sensor histidine kinase ArgA. Thus, the mechanism leading to *agr* repression remains unclear. Nevertheless, repression of the *agr* system by CodY presumably enhances the tight growth phase-dependent activation of this quorum-sensing system which by definition is activated at higher cell densities. Thus, under conditions of isoleucine limitation (or in *codY* mutants), the *agr* system is prematurely activated. Consequently, *agr*-activated genes like toxins and the *cap* operon also become activated, whereas genes known to be downregulated by the *agr* system are switched off. This can be seen as an escape mechanism for the bacteria under limiting conditions. A CodY-dependent repression of *hla* and the *ica* operon (coding for the enzyme generating the polysaccharide intercellular adhesin) was shown recently by Majerczyk et al. (34). In our microarray analysis, *hla* and the *ica* operon were not significantly affected in the *codY* mutants. Both genes are known to be poorly expressed in strain Newman. However, increased expression of *hla* in the *codY* mutant could be confirmed for strain UAMS-1. Genes of the *ica* operon were below the threshold in our analysis but also showed a clear tendency toward higher expression in the *codY* mutant, as further confirmed by reverse transcription-PCR in both genetic backgrounds (strain Newman and UAMS-1) (data not shown).

Genes for cell-associated proteins such as *fnbA*, *spa*, and *coa* are activated by CodY. The role of CodY in the activation of these virulence genes might be primarily via *agr*. However, direct activation through CodY binding can be presumed for *fnbA* activation because of the presence of a putative CodY box and the observation that *fnbA* is still CodY dependent in an *agr*-negative background. Additional regulatory mechanism(s) may act primarily at the posttranscriptional level on some of the genes since, besides *agr*, no other virulence regulatory gene was differentially expressed between wild-type and mutant strains.

A link to the situation during infection or colonization is hard to draw since several studies have shown that *agr* is not activated during chronic infections and/or colonization (20, 21), and little is known about the growth conditions in vivo. However, our analysis of the *codY* regulon in an *agr*-negative background clearly shows that CodY also regulates some of the virulence factors independently of *agr*. We and others have shown elsewhere that *hla* can be transcribed independently of *agr* in vivo (21, 54). Thus, CodY may contribute to virulence gene regulation in vivo not only by supporting but also by replacing *agr* function: under limited conditions (low isoleucine) the capsule and several secreted enzymes and toxins are derepressed, whereas some of the cell surface molecules are downregulated. Overall, the impact of *codY* mutation on *S. aureus* needs to be assessed in vivo as some *codY* targets may contribute to cell adhesion and survival in hostile environments.

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Fig. S1, positions of CodY boxes related to start codons in *S. aureus* genomes

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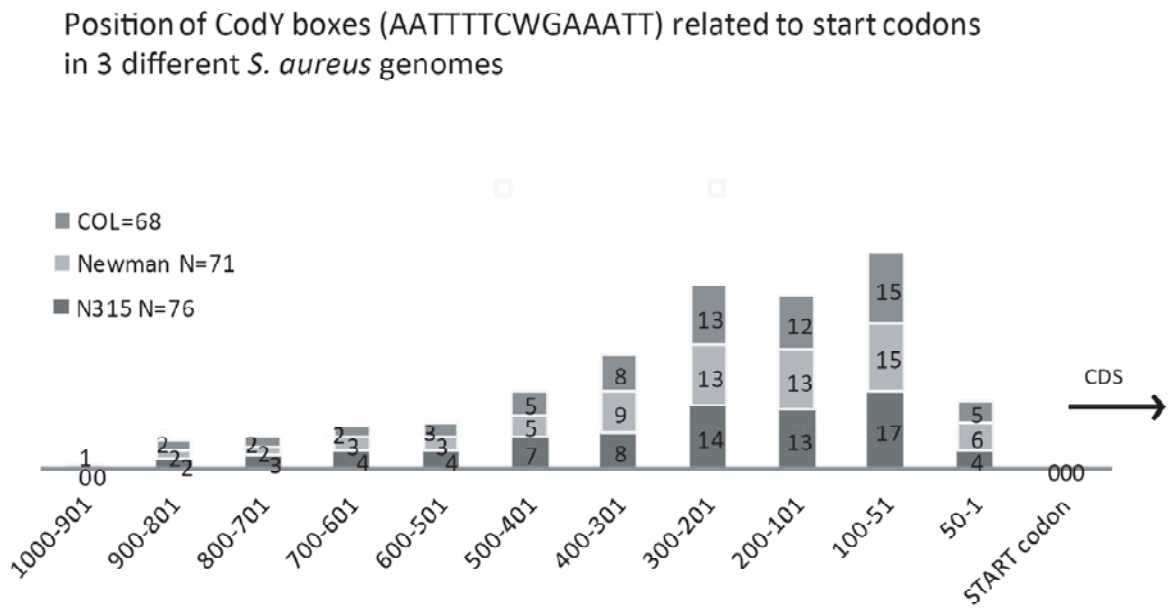

Supplementary table 2

List of genes containing putative *codY* box including a limit of 2 mismatches compared to the consensus sequence found differentially expressed in microarray analysis*.

ORF in Newman	*Description*	*Short name*	*COG*	*New vs New codY*	*Putative codY box*	*Position of cod-box*
NWMN_0667	hypothetical protein	-	KG	0.5*	aattttatataaaatt	-202
NWMN_1277	prephenate dehydrogenase	tyrA	E	0.50	attattctgaaaatt	-34
NWMN_0516	branched-chain amino acid aminotransferase	ilvE	EH	0.47	aatttttagaaaatt	-92
NWMN_0721	putative S30EA family ribosomal protein	-	J	0.45	aaatatcagaaaatt	-100
NWMN_0350	hypothetical protein	-	E	0.36	aattattagaaaatt	-229
NWMN_0130	Branched-chain amino acid transport system II carrier protein	brnQ1	E	0.33	aattttcaaaaaatt	-117
NWMN_2370	putative transport protein	-	H	0.19	tattttctgaaaatt	-75
NWMN_0855	oligopeptide transport system permease protein	oppB	-	0.15	aattttctgaaatct	-191
NWMN_1305	aspartate semialdehyde dehydrogenase	asd	E	0.12	aatattctaaaaatt	-45
NWMN_2500	hypothetical protein	-	E	0.10	aattgttagaaaatt	-227
NWMN_2222	hypothetical protein	-	R	0.10	aattttcagaaaagt	-78
NWMN_1616	putative soluble hydrogenase subunit	-	E	0.08	aattttcagaaaatt	-54
NWMN_2578	hypothetical protein	-	E	0.06	aattttctgaaaaag	-142
NWMN_0883	hypothetical protein	-	E	0.03	aatattctgaaaaat	-18
NWMN_1960	dihydroxy-acid dehydratase	ilvD	EG	0.03	aattgtaagaaaatt	-366
NWMN_0351	hypothetical protein	-	E	0.02	aattttctgaataat	-511

Values indicated in the table are average of two independent microarray experiments and appear statistically differentially regulated following our analytical strategy (see Material and Methods).

IV. A natural ochre mutation in IcaR reveals a CodY dominant effect on both PIA production and biofilm formation in *Staphylococcus aureus*

IV.A. Introduction

The present article focuses on the functions of CodY regarding biofilm formation in *S. aureus*, according to the principal goal of the thesis. Therefore, the ± *codY* ± *agrA* mutants created for the previous study were used again with the addition of a ± *codY* in the S30 background. The different strains were grown during 24 hours in TSB medium. In this experiment, their respective growth was automatically recorded in a Tecan apparatus, the biofilm quantities were measured with the crystal violet staining assay with and without digestion of putatively matrix-incorporated proteins using proteinase K, the production of PIA was recorded using antibodies, and finally, the biofilm phenotypes were analyzed using the Congo red agar test. The biofilm phenotypes of *codY* mutants were divergent when comparing the various backgrounds. Indeed, the *codY* mutant in S30 showed decreased production of biofilm as compared to the parent, whereas the *codY* mutant in UAMS-1 displayed increased production of biofilm compared to its parent. DNA sequencing showed that both strains were naturally affected in factors involved in an opposite manner in the regulation of *icaADBC* expression. Notably, sequencing the *ica* regulatory region in S30 showed that the natural repressor *ica*R was defective, probably explaining at least partially the S30 hyper-biofilm phenotype and high levels of PIA production, whereas genome sequencing of UAMS-1 allowed the identification of a mutation in a repressor of *ica*R called *rbf*, probably responsible for UAMS-1 lower biofilm production and the absence of PIA. I complemented *ica*R before analyzing the transcription of all the *ica* ORFs in function of both the over-expression or absence of both *codY* and *ica*R. Results suggested the implication of at least one additional factor involved in the CodY-mediated regulatory control of the *icaADBC* transcription, notably because both over-expression and disruption of *codY* resulted in a decrease of the *icaADBC* transcription, suggesting two different pathways. Another surprising result was that over-expressing *ica*R in the absence of CodY increased the level of the *icaADBC* operon, while *ica*R was described as the natural repressor of that operon and whereas over-expressing *ica*R when *codY* remains intact clearly resulted in decreased *icaADBC* transcription. PIA

and biofilm measurements in function of over-expression or alteration of both *icaR* and *codY* were well correlated together. When depleting the intracellular GTP by decoyinine treatment in *codY* over-expressing conditions, wild-type biofilms were restored, suggesting that CodY was needed for full biofilm production in S30 without activation by GTP. Finally, ChIP experiments were designed to study the *in vivo* binding of CodY to the *ica* region using CodY-antibodies for the test and ClfA-antibodies as negative control. The results suggested a binding of CodY within both the *icaR* and *icaB* ORFs. This is consistent with the localization of two CodY binding sequences in these ORFs. These results suggested that CodY dominated the control of *icaADBC* expression as compared to *icaR* by repressing both of them, probably by acting as a roadblock for the transcriptional elongation in *icaR* and *icaB*, again consistent with the previously mentioned transcriptional study. My contribution to this work was to perform the experiments and to write the manuscript.

This work showed that the integrity of other genes is an important factor for the study of the effects of CodY with respect to biofilm formation in *S. aureus*. Moreover, CodY may act oppositely when repressing the *icaADBC* operon, which affects negatively the biofilm, and when repressing the *agr* system, which affects positively the biofilm phenotype by increasing the expression of adhesins.

IV.B. Article:

A natural ochre mutation in IcaR reveals a CodY dominant effect on both PIA production and biofilm formation in *Staphylococcus aureus*

A natural ochre mutation in IcaR reveals a CodY dominant effect on both PIA production and biofilm formation in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus (*S. aureus*) biofilm related infections occurring notably onto prosthetic devices are characterized by bacterial persistence, antibiotics resistance, pain for patients, and increased costs for hospitals. In *S. aureus*, the formation of biofilm involved the expression of different genes among which the *icaADBC* locus was known for long time to play a central function, whereas the implications of the pleiotropic repressor CodY was reported more recently. A previous report showed that *S. aureus* CodY repressed both the *icaADBC* locus, additionally repressed by IcaR, and the *agr* system. The IcaADBC proteins are responsible for the production of the polymeric intercellular adhesin (PIA), the major extracellular polymer component encountered in Staphylococcal matrix whose production is needed for inter-bacterial adhesion and biofilm thickening. Additionally, in the post-exponential growth phase, the *agr* system down-regulates different adhesins and up-regulates secreted toxins such as hemolysins. Thus, both adhesins-mediated adhesion of *S. aureus* to abiotic surfaces and the production of extracellular matrix surrounding bacteria are probably affected in an opposite manner by CodY, whereas both adhesion of bacteria to surfaces and production of extracellular matrix define the biofilm life style.

In the present work, the *agr*-dependant and independent contributions of CodY to both biofilm formation and PIA production were tested in different *Staphylococcus aureus* strains, using Δ*codY*, Δ*agrA* and Δ*agrA* Δ*codY* mutants. The results were strain-dependant, putatively explainable by missense or nonsense mutations that could have occurred among factors implicated into CodY-regulated pathways needed for biofilm establishment. These additional partners were searched in the different *S. aureus* tested strains. Interestingly, an ochre mutation in *ica*R was identified in S30, whereas *rbf,* which represses *ica*R, was reported to be a pseudogene in UAMS-1. The *ica*R-deficient S30 strain producing thick biofilm not digestible by proteinase K treatment showed a drastic reduction of biofilm production in its isogenic Δ*codY* mutant, whereas the *rbf*-deficient UAMS-1 strain producing flat and low quantities of biofilm digestible by proteinase K showed an increased of biofilm production when *codY* was altered. Both mutations occurring in UAMS-1 (*rbf*) and S30 (*icaR*-) were then confirmed by sequencing. Our results suggest that the effect of CodY on biofilm formation

in *S. aureus* depends on the integrity of other genes notably implicated in the multi-factorial regulation of *icaADBC*.

In order to understand putative interactions occurring between IcaR and CodY on the regulation of *icaADBC*, the *ica*R and *codY* genes were complemented *in trans* in *ica*R-deficient S30 wild-type and Δ*codY* mutant. Then, *ica*R, each *icaADBC* ORF and *codY* mRNAs were then quantified by q-RT-PCR, revealing that CodY probably dominates IcaR for the repression of *icaADBC* by repressing also *ica*R transcription. Both over-expressions of *ica*R and *codY* resulted in a decrease of *icaADBC* mRNA levels, PIA production and biofilm formation in S30. As CodY is able to repress transcription by DNA-binding activity, immuno-precipitations of CodY-DNA cross-linked complexes were performed in *S. aureus* S30, and DNA recovery was quantified by q-PCR. Results revealed two *in vivo* binding sites for CodY, most probably located inside *icaB* and *ica*R. Moreover, CodY DNA binding consensus sequences were identified *in silico* in *icaB* and *ica*R ORFs. Thus, CodY potentially serves as a roadblock for transcription in both genes. Taken together, our results suggest that CodY, interacting both with *icaADBC* operon and RNAII/RNAIII, provides a mechanism to switch from planktonic to biofilm forming mode of growth in *S. aureus*, in function of the metabolic state of the bacteria and interfering with different factors implicated in stress response and quorum sensing.

Introduction

Bacterial biofilms are thought to derive from an ancestral adaptation to the accumulation of nutrient in the bottom of oceans [62]. In biofilm (prev. Slime), population(s) of micro-organisms adhere to surfaces and produce an extracellular matrix surrounding themselves [26]. Plasmid exchange increases in biofilm-incorporated bacteria as compared to planktonic bacteria probably related to promiscuity [18]. Different mechanisms of resistance against hostile environments, such as chemical compounds, antibiotics and host defenses appear to be probably due to slow-growing bacteria localized deeply inside biofilms [61]. In the case of *Staphylococcus aureus* biofilms, often associated to prosthetic devices, persistent infections remain usually unsolved even after adapted and prolonged antibiotic treatments [19,26]. The lengthening of lifespan in human and the development of prosthetic devices are probably responsible for increasing the number of implanted prostheses in developed countries [51,52]. The cost of *S. aureus* infections occurring in prosthetic devises containing patients is greater as compared to other patients [13,50]. Moreover, the impact of *S. aureus* biofilm-related infections on post-surgery was probably largely underestimated, probably because the concept of biofilm was introduced lately and the diagnostic remains difficult to performed routinely [22]. Therefore, the comprehension of mechanisms leading to biofilm formation in pathogenic bacteria remains a major health concern [18,19,61].

In our approach to investigate *S. aureus* biofilms, 153 different clinical strains collected from patients and preserved at the Geneva University Hospital were screened for biofilm production with a crystal violet staining assay, leading to the characterization and identification of *S. aureus* strain S30 as the strain associated with the maximal quantity of biofilm [65]. Mutagenesis with an Em-Mu transposon identified genes responsible for the establishment and the maturation of biofilm. As expected, many insertions occurred in the *icaADBC* operon, underlining its already known important contribution in *S. aureus* biofilms. In addition, genes not previously known to be involved in the production of biofilm in *S. aureus* were identified, notably CodY, a GTP-dependent pleiotropic repressor present in low G+C Gram positive bacteria and involved in the post-exponential adaptive response to starvation [59].

The gene *codY* was discovered in 1993 by Slack *et al* in *Bacillus subtilis* in a genetic screen performed to identify mutations that allow inappropriate expression of a dipeptide operon [56]. In *S. aureus*, the *codY* gene is the last of four open reading frames (ORF) in an operon [57]. The 3 other genes of the operon are *xerC/clpX*, *hslU/clpQ* and *hslV/clpY* [57]. HslU and HslV are both ATP-dependent proteases whereas XerC is a tyrosine recombinase. It is not understood why *codY* is co-transcribed with these structural genes.

In *Lactococcus lactis*, CodY was able to bind an AATTTTCWGAAAATT palindromic DNA sequence [17]. This CodY-binding consensus motif appeared to be conserved in *B. subtilis* as well [6], and CodY has homologues in many other Gram-positive bacteria [59]. In order to be able to bind DNA at its consensus sequence, CodY has to be activated by cofactors binding [55]. The binding of CodY to the *ilvB* promoter region appeared enhanced in the presence of GTP and/or the branched chain amino acids (BCAA) valine, leucine and isoleucine [55]. The crystal structure of CodY revealed a cofactor binding domain for BCAA that form a hydrophobic pocket at the N-terminal domain of CodY [33]. The binding of CodY to GTP is thought to occur inside putative GTP binding motifs but additional structural data remain necessary [32].

In the work of Tu Quoc *et al.*, the biofilm formation capacity of Ω6900 (S30 *codY::em-mu*) decreased to 20 % as compared to the parental S30 strain. Moreover, the production of **p**olysaccharide **i**ntercellular **a**dhesin (PIA), also called **p**oly-**N**-**a**cetyl **g**lucosamine (PNAG), or **P**oly**s**accharide/**a**dhesin (PS/A) decreased drastically [65]. The PIA remains the principal component of sugaric-based matrix in *S. aureus* and *S. epidermidis*, resulting from polymerization, export and partial de-acetylation of poly-N-acetyl-glucosamine residues, mediated by IcaADBC [24]. Therefore, a relationship between CodY and the *icaADBC* operon was suspected. Note that the transcription of the *icaADBC* operon was regulated negatively by IcaR [24]. Other factors were also implicated into the complex regulation of the *icaADBC* operon in *S. aureus*, such as TcaR, SarA, and likely numerous other factors [4 28].

Others have replaced the *codY* gene with an *ermC* cassette in UAMS-1 and SA564 [37]. The function of *codY* in these strains was tested for the expression of virulence factors; *hld*, RNAII & RNAIII, *icaA* and *icaR*, as well

as virulence phenotypes, hemolytic activity, biofilm formation and PIA production. Results show a de-repression of *icaA* and an increase of both PIA and biofilm in *codY* mutants.

Thus, *codY* was responsible for an opposite effect both on PIA and biofilm productions when comparing results obtained with UAMS-1 and S30. Interestingly, UAMS-1 *Staphylococcus aureus* strain presented a basal biofilm production compared to S30. According to the authors, the regulatory network of PIA-dependent biofilm formation in S30 may be different than in UAMS-1. In the present study, the goals were 1) to resolve the contradictory effect of CodY on PIA and biofilm formation between different *S. aureus* strains, notably UAMS-1 and S30, 2) to identify the genetic basis of the S30 hyper-biofilm phenotype, and finally 3) to investigate the relationship between IcaR and CodY.

Material and methods

Strains and growth conditions

Strains and plasmids are listed in table 1. For biofilm studies, strains were grown in trypticase soy broth (TSB) [60]. For induction of the $P_{xyl/tet}$ promoter carried by pCG30, a non toxic analogue of tetracycline, the anhydrotetracycline (AHT) was added to the medium at 250 ng/ml. That concentration corresponds to an optimal concentration for $P_{xyl/tet}$ activation, according to a previous study [2]. For pIcaR, pCN38 and pCG30 selection, strains were grown in the presence of 10 µg/ml chloramphenicol [11,43]. For *Em-Mu* selection, strains were grown in the presence of 10 µg/ml erythromycin (Sigma, cat. num. E-6376). For depleting the intracellular GTP level, decoyinine (dissolved in 1 N KOH) was added to a final concentration of 500 µg/mL to the appropriate cell cultures, with an equivalent volume of 1 N KOH added to control cell cultures, as previously described [45].

Table 1

Strains	Relevant genotype and (plasmid carrying)	antibiotic resistance	References
MW2	ATCC BAA-1707™, CA-MRSA, PVL$^+$, *mecA* , Type IVa SCCmec.	met^R, peni^R	Baba T *et al*., 2002, Lancet, 25;359(9320):1819-27
MW2 Δ*codY*	*ΔcodY::tetM*	tet^R, met^R, peni^R	Present study
SA113	ATCC 35556, (M+; R-) host strain	-	Iordanescu S *et al*., 1976, J. Gen. Microbiol. 96: 277-281
SA113 Δ*icaADBC::tet*	*ΔicaADBC::tet*	tet^R	Cramton SE *et al.*, 1999, Infect Immun, 67(10):5427-33
RN4220	NCTC 8325: 11-bp deletion in *rsbU* ; (M+; R-) host strain	-	Kreiswirth *et al.*, 1983, Nature 305, 709–712
RN4220 pCN38	(pCN38)	chloram^R	Present study
RN4220 pIcaR	(pIcaR)	chloram^R	Present study
RN4220 pT181	(pT181)	tet^R	Present study
S30	*icaR::G292A* (pT181)	tet^R, peni^R	Tu Quoc P *et al.,* 2007, Infect Immun, 75(3):1079-88
S30 pIcaR	(pT181 & pIcaR)	tet^R, peni^R, chloram^R	Present study
S30 pCN38	(pT181 & pCN38)	tet^R, peni^R, chloram^R	Present study
S30 pCG30	(pT181 & pCG30)	tet^R, peni^R, chloram^R	Present study
Ω6900	*codY::em-mu* , (pT181)	tet^R, peni^R	Tu Quoc P *et al.,* 2007, Infect Immun, 75(3):1079-88
Ω6900 pIcaR	*codY::em-mu* , (pT181 & pIcaR)	tet^R, peni^R, chloram^R	Present study
Ω6900 pCN38	*codY::em-mu* , (pT181 & pCN38)	tet^R, peni^R, chloram^R	Present study
Ω6900 pCG30	*codY::em-mu* , (pT181 & pCG30)	tet^R, peni^R, chloram^R	Present study
UAMS-1	MSSA, *rbf*$^-$ (+2 pb at 50th codon), *ΔfnbA, hla*$^-$ (nonsense after codon 112th)	-	Gillaspy *et al*., 1995, Infect Immun 63: 3373–3380. Cue *et al.*, 2009, J Bacteriol, 191(20): 6363–6373
UAMS-1 21	*ΔcodY::tetM*	tet^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
UAMS-1 21/30	*ΔcodY::tetM, (*pCG30)	tet^R, chloram^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
UAMS-1 21/*agr*	*ΔcodY::tetM, agrA::Tn551*	tet^R, ery^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
UAMS-1 *agr*	*agrA::Tn551*	ery^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
ISP479r	UV-mutagenized strain, *rsbU* restored	-	Pattee PA *et al*., 1981, J Bacteriol 145: 479–488; Toledo-Arana *et al.*, 2005
ISP479r 21	*ΔcodY::tetM*	tet^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
ISP479r 21/*agr*	*ΔcodY::tetM, agrA::Tn551*	tet^R, ery^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
ISP479r *agr*	*agrA::Tn551*	ery^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
ISP479r 21/30	*ΔcodY::tetM, (*pCG30)	tet^R, chloram^R	Present study
ISP479r pCG30	(pCG30)	chloram^R	Present study
ISP479r pT181	(pT181)	tet^R	Present study
Newman	NCTC 8178; ATCC 13420; *fnbAB*$^-$	-	Duthie ES *et al*., 1952, J. Gen.Microbiol., 6, 95-107
Newman 21	*ΔcodY::tetM*	tet^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
Newman 21/30	*ΔcodY::tetM,* (pCG30)	tet^R	Present study
Newman 21/-*agr*	*ΔcodY::tetM, agrA::Tn551*	tet^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
Newman *agr*	*agrA::Tn551*	*ery*^{R}	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
Newman pCG30	(pCG30)	chloram^R	Present study
Newman 21 Δ*icaR*	*ΔcodY::tetM, icaR::targetron*	tet^R	Present study
Newman Δ*icaR*	*icaR::targetron*		Present study
Newman pT181	(pT181)	tet^R	Present study
Newman *ΔspA::Tc*^{R}	*ΔspA::Tc*^{R}	tet^R	Massey *et al*., 2002, Infect Immun 70: 5339–5345
Other backgrounds			
S. epidermidis 1457	sugaric-based biofilm producing strain		Rohde H *et al*., 2005, Mol Microbiol 55: 1883–1895
S. epidermidis 5179-R	proteinaceous biofilm-forming strain, *aap* $_{5179,}$ *ΔicaA::IS257*	nd	Rohde H *et al*., 2005, Mol Microbiol 55: 1883–1895
E. coli JM109	*endA1 glnV44 thi-1 relA1 gyrA96 recA1 mcrB+ Δ(lac-proAB) e14- [F' traD36 proAB+ laclq lacZΔM15] hsdR17(rK-mK+)*	-	Sigma, cat. num. J 3895
E. coli JM109 pCN38	(pCN38)	amp^R	Present study
E. coli JM109 pIcaR	(pIcaR)	amp^R	Present study

Plasmids	Description and genotype	antibiotic resistance	References
pCN38	20-25 copies in *S. aureus* , 5.4 kb shuttle *E. coli* & *S. aureus*	chloram^R (*S. aueus*), amp^R (*E. coli*)	Charpentier E *et al.*, 2004, Applied and Environmental Microbiology, 70 (10), 6076-6085
pIcaR	20-25 copies in *S. aureus* , 6.7 kb pCN38 with *icaR* insert	chloram^R (*S. aueus*), amp^R (*E. coli*)	Present study
pCG30	20-25 copy plasmid, *codY* under $P_{xyl/tet}$	chloram^R (*S. aueus*), amp^R (*E. coli*)	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63
pT181	20 copies in *S. aureus* , 4.4 kb pT181-like plasmid	tet^R (*S. aureus*)	Present study
pCG29	pMAD with tetM between *codY* flanking regions	ery^R tet^R	Pohl K *et al*., 2009, J Bacteriol, 191(9):2953-63

Genetic constructions

All the different constructions are listed in table 1, with their relevant genotypes, plasmids and antibiotic resistances. Plasmids used for manipulations are first transformed into *E. coli* by heat shock prior to electroporation into competent *S. aureus* RN4220 and electroporation to the strain of interest, as previously described [14,31]. Therefore, plasmids were extracted with QIAGEN Plasmid Mini Kit according to manufacture instructions except that bacteria are incubated during 10 min at 37°C in presence of 10 µg lysostaphin (Sigma) in the P1 solution of the kit. The *codY* was replaced by a *tetM* cassette in UAMS-1, ISP479r and Newman as previously described [43]. The *agrA* was inactivated by insertion of Tn551 in RN6112

and this inactivation was transduced using Φ11 into UAMS-1, ISP479r and Newman wild-types and Δ*codY::tetM*, respectively [43]. Inactivation of *codY* in S30 was accomplished with the insertion of an *em-mu* transposon leading to Ω6900 [65]. To complement *codY*, pCG30 plasmid carrying the *codY* allele under the control of a $P_{xyl/tet}$ inducible promoter was electroporated into UAMS-1, ISP479r, S30, Newman, and their respective *codY* mutants. For complementation, the *icaR* gene with its native promoter was amplified by PCR from genomic DNA of the Newman strain using primers "FP_icaR_13.1.09" and "RP_icaR_BamH1_b_13.1.09", (table 2).

Table 2

Name	Sequence 5'->3'	Purpose	TM	References
Primer1_F_ica	TTGCATTTAATTTATAAAATTCATATACAT	sequencing *icaR*region	50.9	present study
Primer2_F_ica	CACTTTTAGCTATATCATCAAGTGTTGTACCGTC	sequencing *icaR*region	60.8	present study
Primer3_F_ica	ATCCTGTATTTATGTCTATTTACTGGATTGTCGG	sequencing *icaR*region	61.1	present study
Primer4_R_ica	CGACTATCAATAAAGAGTGCGACTGTAAATTGAA	sequencing *icaR*region	62.7	present study
FP_icaR_13.1.09	TTAGCGTTGGGTATTCCCTCTGTCTG	complementation of *icaR*	60.5	present study
RP_icaR_BamH1_b_13.1.09	TGTGTGGGATCCTATCGATTTGTATTGTCAAC	complementation of *icaR*	64.4	present study
pT181_vLx_1-20_F	CGCCAGGGTTTTCCCAGTCA	insert detection in pCN38	59.4	present study
ampcolori_vLx_3328-3347_R	TTTCCTGCGTTATCCCCTGA	insert detection in pCN38	54.5	present study
SdrCDE_2746_F	TTACGGATCATGATGATTTCA	MLVA	47.9	François P *et al.*, 2005
SdrCDE_3326_R	CAYTACCTGTTTCTGGTAATGCTT	MLVA	51.5	François P *et al.*, 2005
Spa_798_F	AGCACCAAAAGAGGAAGACAA	MLVA	50.8	François P *et al.*, 2005
Spa_1082_R	GTTTAACGACATGTACTCCGT	MLVA	46.1	François P *et al.*, 2005
SspA_841_F	ATCMATTTYGCMAAYGATGACCA	MLVA	57.9	François P *et al.*, 2005
SspA_969_R	TTGTCTGAATTATTGTTATCGCC	MLVA	50.8	François P *et al.*, 2005
ClfA_1684_F	GATTCTGACCCAGGTTCAGA	MLVA	48.6	François P *et al.*, 2005
ClfA_2867_R	CTGTATCTGGTAATGGTTCTTT	MLVA	45	François P *et al.*, 2005
ClfB_1607_F	ATGGTGATTCAGCAGTAAATCC	MLVA	50.1	François P *et al.*, 2005
ClfB_2435_R	CATTATTTGGTGGTGTAACTCTT	MLVA	47.5	François P *et al.*, 2005
FnBP-A_1903_F	GGTCAAGCRCAAGGACCART	MLVA	53.9	François P *et al.*, 2005
FnBP-A_2994_R	AATAATCCGCCGAACAACAT	MLVA	51.3	François P *et al.*, 2005
CNA_1616_F	AAAATGACAAAAATGGCAAG	MLVA	47.3	François P *et al.*, 2005
CNA_3474_R	CAGGTTTAGTTGGTGGTGTT	MLVA	46.5	François P *et al.*, 2005
mecA_321_F	CATTGATCGCAACGTTCAATTT	MLVA	54.3	François P *et al.*, 2005
mecA_419_R	TGGTCTTTCTGCATTCCTGGA	MLVA	54.5	François P *et al.*, 2005
sas_1457_F	TTGGAACATTCGAATATACAGAGT	MLVA	49.5	Koessler T *et al.*, 2006
sas_2749_R	TCGATGTACTGTCACTTAATGATG	MLVA	49	Koessler T *et al.*, 2006
plsR2_2400_F	AATTACAACGCCTCAAGCTG	MLVA	49.8	Koessler T *et al.*, 2006
plsR2_4346_R	GCACCATGGATGATTACTTC	MLVA	46.7	Koessler T *et al.*, 2006
F_Tet	TAGCAATTGTAGGAGGTTTA	sequencing pS30	42.2	present study
R_Tet	TAGTTTTCATCATATAAGGC	sequencing pS30	39.1	present study
F_tet_2	CATTTTAATACTACTCCTGGAATTACAAACTGGG	sequencing pS30	60.8	present study
R_tet_2	GCATAAACGGAATGTTTTTCCCTAGTTTAGGATT	sequencing pS30	63.9	present study
F_Tet_3	CAAAAGTTAAATCTGGAACAAATACAACGGG	sequencing pS30	61.9	present study
R_Tet_3	GCTCATTAAATCTATCTTGAAACGCTGTTAAAGC	sequencing pS30	62.6	present study
F_Tet_4	CAAAGCCTTTGGTTTTATAGGATCAATTGTAGC	sequencing pS30	62.9	present study
R_Tet_4	CCATGGCTACAAGAATATTACTATACACTCCAGAA	sequencing pS30	61.2	present study
F_Tet_5	CACGATTTAGACAATTTTTCTAAAACCGGCTACT	sequencing pS30	63.4	present study
R_Tet_5	CCTCTTTACTTGAGGTGACTAAAGTTTATAGGGGT	sequencing pS30	61.8	present study
F_Tet_6	GTGAGAGCCAAAAAACAGACCATATAAAGC	sequencing pS30	60.7	present study
R_Tet_6	CTGCTAAACCATTTAGTTTTGGTTCATCTTCTGT	sequencing pS30	62.8	present study
R_Tet_7	TGCCTTGTTTTTTTCTTGTAATATTTCTAGCTACA	sequencing pS30	61.1	present study
FP_codY_sequencing	AGATAGTCGTACAACTATCTTCCCAATTTTAGGTG	sequencing *codY*	61.5	present study
RP_codY_sequencing	CGTAAGATTTCCATACCAATAACTGTAGCAGC	sequencing *codY*	61.5	present study
icaR 345-346a-IBS	AAAAAAGCTTATAATTATCCTTAAAATCCAACTATGTGCGCCCAGATAGGGTG	disrupting *icaR*	74.1	present study
icaR 345-346a-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCAACTATTCTAACTTACCTTTCTTTGT	disrupting *icaR*	71.8	present study
icaR 345-346a-EBS2	TGAACGCAAGTTTCTAATTTCGATTAAACTTCGATAGAGGAAAGTGTCT	disrupting *icaR*	72.4	present study
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	disrupting *icaR*	57.2	present study
FP_icaR_13.1.09	TTAGCGTTGGGTATTCCCTCTGTCTG	verification of Δ*icaR*	60.5	present study
RP_icaR_BamH1_b_13.1.09	TGTGTGGGATCCTATCGATTTGTATTGTCAAC	verification of Δ*icaR*	64.4	present study
EBS Universal	CGAAATTAGAAACTTGCGTTCAGTAAAC	verification of Δ*icaR*	57.2	present study
REBS Universal	GTTTACTGAACGCAAGTTTCTAATTTCG	verification of Δ*icaR*	57.2	present study

The *icaR* containing insert and the pCN38 shuttle vector were double digested with *Bam*HI & *Eco*RI before ligation and transformation. Restriction enzymes were purchased from New England BioLabs. pIcaR was

verified by restriction and sequencing. Inactivation of *icaR* in Newman and its respective Δ*codY* mutant was performed with TargeTron® Gene Knockout System (Sigma, cat. num. TA0100) using icaR_345_346a_IBS, icaR_345_346a_EBS1d and icaR_345_346a_EBS2 primers listed on table 2 [69]. The Newman Δ*icaR*- simple and Δ*icaR* Δ*codY* double mutants were verified by PCR using primers EBS_universal, REBS_Universal, FP_icaR_13.1.09 and RP_icaR_BamH1_b_13.1.09, listed on table 2. Multi-locus variable number of tandem repeat analysis (MLVA) was performed to confirm the strain identity after transformation process [20,40].

Congo red agar and sheep blood agar

Congo red agar (CRA) medium was prepared as previously described [29]. Briefly, 0.08 % of Congo Red (CR, Serva, cat. num. 27215) is added to Brain Heart Infusion Broth (BHI, Fluka, cat. num. 70138) and poured in agar plate. Sheep blood agar (BA) plates were prepared with Tryptic Soy blood agar following recommendations of the manufacturer and supplemented with a 5% concentration of defibrinated sheep blood as commonly used to visualize hemolytic phenotypes of *S. aureus* [30]. Drops (10 µl) of bacteria in suspension (OD_{540} = 1,5) were spotted onto CRA and BA plates and the phenotypes were analyzed.

Crystal violet biofilm assay and bacterial growths

Biofilm staining assays were performed as previously described [41,60]. Briefly, after bacterial growth, polystyrene flat bottom sterile microtiter plates (Multiwell™ 96 wells, Becton Dickinson, cat. num. 353915 and 353071) were washed twice with PBS, fixed for 20 minutes at 80°C, and stained for 10 minutes with 1% (w/v) crystal violet solution freshly diluted 2-fold in 1% (v/v) ethanol/distilled water. Plates were then washed with water and photographed. The crystal violet was dissolved in DMSO during 1h before OD_{600} measurements [65]. Bacterial growth inside 96 wells microtiter plates was automatically recorded with a Tecan apparatus measuring Optical density (OD) at 540 nm every 15 minutes for 20 hours. Note that the optical path was shorter in the Tecan apparatus, corresponding to 0.5 cm length when 200 µl are deposited into a well of 96-wells containing plate, as compare to 1 cm length cuvette when using classical spectrophotometers. Proteinase K treatment of biofilm was performed by incubating formed biofilm during 1 hour with 4 milli-

Anson units (mAU) of proteinase K (coming from DNeasy Tissue Kit, QIAGEN, cat. num. 69504) diluted into PBS buffer to a 200 µl final volume per well of Multiwell™ 96 wells whereas biofilms without proteinase K treatment was inoculated with 200 µl of PBS only. *S. epidermidis* 5179-R1 was used as a control for proteinaceous biofilm related to inactivation of *icaA* and proteinaceous biofilm-positive revertant phenotype, whereas *S. epidermidis* 1457 was used as control for sugaric-based biofilm [47].

DNA Sequencing by Capillary Electrophoresis

The *codY* and *icaADBC* regulatory region from S30 as well as pT181 were sequenced with primers listed in table 2. Bacterial colonies were suspended in 200 µl 10 mM Tris 1 mM EDTA*Na_2 pH 8 (TE) containing 10 µg (20 U) lysostaphin (Sigma, cat. num. L4402) and incubated for 15 min at 37°C. Genomic DNA (gDNA) was extracted using the DNeasy kit (QIAGEN, Germany) following manufacturer's instructions. DNA concentration and purity were assessed by using the NanoDrop ND-1000 spectrophotometer. gDNA extracted was diluted to 1 ng/ul. 5 ng was used in PCR reactions with 0.3 µM forward and 0.3 µM reverse primers, 0.2 mM dNTPs, 1 mM $MgSO_4$, 1 U KOD Hot Start DNA polymerase and PCR buffer in 50 µl as final volume (Novagen, cat. num. 71086-4). 1 µl of the ended PCR reaction was analyzed with the Agilent 2100 BioAnalyzer using 1000 or 7500 DNA chip kit (Agilent). Products were either purified with MinElute PCR purification kit (Qiagen) or loaded onto 0.8 % agarose gel electrophoresis and extracted with QIAquick PCR Purification Kit (Qiagen) .The purified amplicon was then diluted according to its size (1-3 ng for 100-200 bp; 3-10 ng for 200-500 bp; 5 to 20 ng for 500-1000 bp; 20-50 ng for >2000 bp) in 1 µl for next step reaction. Diluted amplicons were labeled using Big Dye Terminator Cycle Sequencing v.1.1 (Applied Biosystems) with 0.25 µM forward or reverse, 1.5 µl Kit-Mix, into 10 µl final volume. Thermal cycle conditions are 96°C during 1 min, 25 cycles of i) 96° C during 10 sec, 50 °C during 5 sec, 60°C during 90 sec. 5 µl water was added to the sample before purification with AutoSeq G-50 Dye Terminatore Removal Kit (GE Healthcare) according to the manufacturer's instructions. Labeled samples were then sequenced using the capillary sequencer 3130xl Genetic Analyzer (Applied Biosystems). Sequences were visualized using

Bioedit software version 7.0.5.3, and assembled using contigExpress tool of Vector NTI software version 10.3.0.

mRNA quantification

Primers and probes used in that study for q-RT-PCR experiments are listed in table 3.

Table 3

Name	Sequence	Purpose	TM	References
icaA_F	GCCATGTGTTGGATGTTGGTT	q-RT-PCR	54.4	present study
icaA_R	CCCCTTGAGCCCATCTCA	q-RT-PCR	52.9	present study
icaA_P	[FAM]-CGTTGCTTCCAAAGACCTCCCAATGTTT-[BHQ1]	q-RT-PCR	65.6	present study
icaD_F	CAGAGGGAATACCCAACGCTAA	q-RT-PCR	54.9	present study
icaD_R	GTACCAATATAAACGAGTAGAACAACTAAACA	q-RT-PCR	55	present study
icaD_P	[VIC]-AGAAACAGCACTTATCGCT-[NFQ-MGB]	q-RT-PCR	44	present study
icaB_F	AGCACACTGGATGGTCATCATA	q-RT-PCR	51.9	present study
icaB_R	TGCCAGAGCACTATTTTCTTTATATT	q-RT-PCR	52.4	present study
icaB_P	[FAM]-TGCAAATGCAGATGACGATTCACCTAA-[BHQ1]	q-RT-PCR	62.4	present study
icaC_F	GGGTGGATCCTTAGTGTTACAATTTT	q-RT-PCR	55.1	present study
icaC_R	ACTTTTTGGTAATTCAAGGTTGTCAGT	q-RT-PCR	55	present study
icaC_P	[NED]-TATTGTGATTTTTGGTACACCTT-[NFQ-MGB]	q-RT-PCR	48	present study
icaR_F	TTCTTCCACTGCTCCAAA	q-RT-PCR	45.8	present study
icaR_R	GCTGTTTCTTGAAAGTTGGT	q-RT-PCR	46.1	present study
icaR_P	[6FAM]-TTTTGCGAAAAGGATGCTTTCA-[BHQ1]	q-RT-PCR	56.5	present study
rRNA16S-1024F	GATAGAGCCTTCCCCTTCGG	q-RT-PCR	54.3	present study
rRNA16S-1174R	CCGGCAGTCAAGTTAGAGTGC	q-RT-PCR	54	present study
rRNA16S-1071P-JOE	[JOE]-ACATCTCACGACACGAGCTGACGACA-[BHQ1]	q-RT-PCR	63.8	present study
CodY_F	GGTATTGCGGTTGATTTTAAAGATG	q-RT-PCR	55.2	present study
CodY_R	CGACGCGATACAATAAATACATTTG	q-RT-PCR	54.9	present study
CodY_P	[FAM]-ACAAACGATTAGTAGCGTAAC-[MGB]	q-RT-PCR	43.8	present study
codY_F_post_insertion	CGCGATAAAGCTGCTATTACAATG	q-RT-PCR	55.2	present study
codY_R_post_insertion	TCTGCAACTTTTGATGCGATTAA	q-RT-PCR	54.4	present study
codY_Rev_Prob	[FAM]-CCTTCCGTACCGCCAAGTTCTTCAAAGATA-[TAMRA]	q-RT-PCR	65.6	present study
rRNA_16s_1024F	GATAGAGCCTTCCCCTTCGG	q-RT-PCR	54.3	present study
rRNA_16s_1174R	CCGGCAGTCAAGTTAGAGTGC	q-RT-PCR	54	present study
rRNA_16s_1071_FAM	[FAM]-ACATCTCACGACACGAGCTGACGACA-[TAMRA]	q-RT-PCR	63.8	present study

For total RNA sample preparation, one colony of each strain was incubated overnight into TSB ± 10 µg/ml chloramphenicol (for pCN38, pCG30, pIcaR selection). Then, 120 µl of culture adjusted to OD_{540} = 1.5 was inoculated in 2 ml final volume containing TSB and 250 ng/ml of anhydrotetracycline hydrochloride (Sigma). After 1h30 growth at 37°C with 220 t/min agitation in 2 ml tubes, cells were centrifuged at 4000 rpm at 4°C during 5 min. Pellets were fixed with 1:1 acetone (Flucka-ethanol for 10 min at room temperature [23]. Cells were centrifuged at 4000 rpm at 4°C and washed in 1X Dulbecco's Phosphate Buffered Saline (D-PBS, Invitrogen, cat. num. 14040-083). Protoplasts were prepared by incubating cells for 5 min at 37 °C into 200 µl solution containing 10 mM Tris 1 mM EDTA pH 8 (TE), 1 M glucose (Merck), 10 µg (20 U) lysostaphin (Sigma). Samples were treated using QIAshredder (Qiagen) and RNeasy Micro Kit (Qiagen) including DNAse I treatment (Qiagen) for 40 minutes [23,46]. RNA samples were then immediately snap-freeze into liquid nitrogen. One aliquot was used to for RNA concentrations and quality assessments by using the NanoDrop

ND-1000 spectrophotometer (NanoDrop Technologies) and the BioAnalyzer using RNA 6000 Nano Kit (Agilent). Transcription levels of the *S. aureus codY*, *icaR*, *icaA*, *icaD*, *icaB*, *icaC* were monitored by quantitative real-time reverse-PCR (qRT-PCR), with normalization to the ribosomal 16S rRNA transcript levels [46,68] in a StepOne Real-Time PCR system (PE Biosystems). Probes for targeted genes were coupled either to Ned, Vic or Fam reporters whereas the Joe reporter was selected for the 16S rRNA transcript, allowing duplex quantification. Primers and probes are listed in table 3. Setting the primers and probes concentrations was performed on the genomic DNA template with ABsolute™ QPCRMix (Thermo Scientific) and varying primers concentration from 0.1 to 0.2 µM and probes concentrations from 0.05 µM to 0.2 µM. For qRT-PCR, ThermoScript Reaction mixture (Invitrogen) was used and coupled to a hot-start PCR provided by Platinum® Taq DNA Polymerase [12,54]. RT-PCR conditions were 40 cycles of 15' at 50°C, 2' at 95°C, 15" at 95°C, 30" at 30°C. Primers and probes were designed with Primer Express ® Software Version 3.0.

PIA Dot Blot

Antibodies against PIA [35] and commercial F_{ab} fragments of goat polyclonal anti-rabbit coupled respectively to phosphatase alkaline (PA) or Horseradish peroxidase (HP) conjugates were used to detect α-PIA after incubation with their respective substrates, 5-bromo-4-chloro-3-indyolylphosphate–nitroblue tetrazolium (BCIP/NBT) and SuperSignal® West Pico Trial Kit (Thermo Scientific). *S. aureus* strains SA113, its isogenic Δ*icaADBC::tet* mutant as well as Newman wild-type and its isogenic Δ*spa::Tc*^{R} mutant were used for genetic control of specific PIA and non-specific SpA detections [15,38]. Equilibrated overnights (OD_{450} nm = 1.5) diluted 20 folds in TSB were incubated for 2 hours before being filtrated (Φ= 0.5 µm, Sartorius sterile filters) and equilibrated to OD_{450} nm = 0.1. 1 ml final volume of equilibrated sample were then heated to 96 °C and incubated during 30 min at 37 °C in presence of 10 µg of proteinase K (Sigma, P2308). Then, 100 µl of each samples were loaded and fixed onto a nitrocellulose membrane (Amersham) by aspiration with a Dot Blot apparatus (BioRad). Membranes were dried at 80 °C during 10 minutes, blocked 1 hour at room temperature into 50 ml of 3% Skim Milk, 0.05% Tween 20, 1x TBS before overnight incubation with 1:3000 (v/v) α-PIA antibodies diluted in 2% BSA, 0.01% Tween 20, 1x TBS (TTBS). Membranes were washed twice for 10

minutes at room temperature before incubation with 1:5'000 (v/v) secondary antibodies diluted in TTBS for 1 hour. After tree washings steps with 10 minutes incubations in TTBS, the membranes were incubated with the appropriated substrates of the secondary antibodies and signals were recorded, normalized and quantified using a standard scanner and the imageJ open-source software.

CodY antibodies production

Rabbit antibodies able to detect the *S. aureus* CodY protein were produced by Eurogentec (Seraing, Belgium) following a 28 days procedure beginning with the injection of two peptide sequences of CodY (H_2N-VSRRGKILGSSLNELC-$CONH_2$ & H_2N-FPPENRELFIDSRTTI-$CONH_2$) into Rabbits. Both α-CodY-1 and α-CodY-2 antibodies populations were then purified on column by the two antigenic respective peptides.

Total protein sample preparations

Total protein extracts were prepared from 20 ml TSB overnights cultures of *S. aureus* with 250 ng/ml final concentration of AHT for the last two hours of incubation when the pCG30 was present. Bacterial pellets were dissolved in 1.5 ml lysis solution (10 mM Tris-HCl pH 7,4; 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTE, proteases inhibiting cocktail (Roche)) containing 10 µg of lysostaphin (Sigma) prior incubation at 37°C for 15 minutes. Samples were treated with 10 µl of DNAse I (Sigma) during 5 minutes at 37°C prior to 10 min of centrifugation at 10'000 rpm. Samples were then ultra-filtered for 1 hour at 4000 rpm using Amicon®Ultra column (Millipore) with 3 kDa cutoff. Protein concentration was assessed with Micro BCA method (Pierce).

Western Blot

100 µg of protein samples in Laemmli buffer were separated on 12 % polyacrylamid denaturing precast gels (BioRad, cat. num. 456-1046) before 2 hours of semi-dry transfer (48 mM Tris, 30 mM glycine, 0.037 % SDS, 20 % ethanol) with 32 mA current onto 8.3 cm * 5.2 cm nitrocellulose membranes. Membranes were blocked 2 hours at room temperature in 5% skim milk solution supplemented with 0.2 % SDS before overnight incubation of 1:3000 (v/v) diluted α-CodY-1 or α-CodY-2 antibodies (0.44 mg/ml) into 2% BSA, 0.01%

Tween 20, TBS (TTBS). After 4x five minutes washing (0.1 % Tween in PBS) with agitation, membranes were incubated for 1 hour with 1:10'000 (v/v) goat F_{ab} fragments anti-Rabbit coupled to alkaline Phosphatase (Sigma) into TTBS prior to four washing of 15 minutes. Then, membranes were exposed to alkaline phosphatase substrate BCIP/NBT according to manufacturer instructions (Sigma, cat. num. B-5655) and scanned.

Immuno-precipitation of CodY-DNA complexes

S30 and Ω6900 grown overnight in 40 ml starting volume of TSB medium were suspended for 10 minutes in a cross-linking solution containing 1 % of formaldehyde before stopping the reaction by re-suspending the bacteria into a gylcine containing solution coming from the ChIP-IT Express Kit of Active Motif. The protocol of the kit was carefully followed, with the exception that the steps corresponding to the lysis of cells and the centrifugation of nuclei, as described in the handbook, were replaced by breaking the bacteria with the FastDNA Spin Kit for soil (MP) and a Fast Prep instrument (Bio101). The sonication procedure of cross-linked DNA was determined empirically using a Branson 250 sonifier, working on ice with 15 successive cycles of sonication, avoiding contamination of sample by the tips of the instrument. DNA fragmentation was visualized on 1 % agarose gel. Immuno-precipitation of S30 and Ω6900 cross-linked DNAs were performed with a 1:1 ratio of both antibodies against the CodY protein, whereas antibodies against the clumping factor A (ClfA) were used as negative control [39]. Final DNA recovery was estimated by Q-PCR using ABsolute™ and primers and probes matching the various *ica* ORFs, listed on table 3, and used also for q-RT-PCR experiments, as previously described. Results were expressed in relative DNA quantity corresponding to $2^{-\Delta_{Ct}}$ between normalized inputs and IPs samples.

Statistical analysis

To assume a normal distribution of Q-RT-PCR & biofilm measurements, quantile-quantile plot (QQplot) comparing experimental values to theoretical values were generated in "R" software version 2.7.0. When QQplots resulted in linear fitting, the use of standard deviations and standard errors were accepted. For

statistical significance, T-tests were performed in Microsoft Office Excel 2007 with distribution containing 2 tails of 2 samples with unequal variances.

Results and discussion

Effects of CodY and AgrA on *S. aureus* biofilms and hemolysis

The Δ*agrA* and Δ*codY* double and single mutants were generated in strains UAMS-1, Newman and ISP479r, in order to distinguish *agr*-dependant from *agr*-independent effects of CodY towards *S. aureus* biofilm formation and hemolysis [43]. We did not succeed to disrupt *agrA* in S30 and Ω6900 because that background showed resistance against Φ-11 mediated transduction. The overall results of the bacterial growths (first column), the biofilms quantities (second column), the PIA productions (third column) and the phenotypes of bacterial spots onto Congo red agar (CRA) and blood agar (BA) (fourth column) are shown in figure 1. Results were standardized to S30 and set as one for CV assay and PIA production because this strain remains the strongest producer of PIA and biofilm between the different tested strains (Figure 1).

Growth curves automatically recorded in the TECAN apparatus were established in order to control whether a biofilm decrease is due to a growth defects among the different strains. Results showed that Δ*codY* mutations were responsible for a decrease of the planktonic stationary phase in UAMS-1 (Figure 1, black ovals and triangles) independently of disruption of *agrA* (Figure 1, white ovals and triangle). In Newman, mutations occurring in *agrA* or *codY* did not influence the growth, whereas in ISP479r *codY* mutation independently of *agrA* mutation increased the stationary phases as an inverse result compared to UAMS-1. In the S30 background, the *codY* mutation increased the rate of exponential growth (compare the slope of black ovals to the slope of white ovals). All together, these results suggest that mutations of *codY* and *agrA* could lead to slight changes of the growth of the bacterial population in a strain-dependent manner.

The Crystal violet assay (CV assay) for presence of biofilm was carried out with and without proteinase K treatment to distinguish sugaric from proteic nature of biofilms, (figure 1, CV assay, black bars correspond to proteinase K digested biofilms, white bars correspond to biofilm quantities without digestion). In Newman

and ISP479r backgrounds, no statistically significant changes of biofilm quantity occurred without proteinase K treatment whereas a *codY*-dependent and *agrA*-independent significant increase of biofilm formation was found for ISP479r with proteinase K treatment. These results suggested a CodY mediated repression of PIA production in ISP479r. In strain UAMS-1 (also in MW2, not shown), *codY* mutants produced more biofilm than their parents and the differences were statistically significant according to T-test without proteinase K treatment (Figure 1). In S30 background, a significant decrease of biofilm was found in Ω6900 with and without proteinase K treatment. Note that S30 wild type biofilm was unaffected by proteinase K, suggesting a sugaric-base composition of S30 biofilm, whereas wild-type biofilms in the other backgrounds were partially digested by the proteinase K. As the nature of biofilm differed, this observation could explain somehow the opposite effect of *codY* mutation between S30 and the other backgrounds. Thus, according to our crystal violet staining assay, the contribution of CodY in biofilm production was strain-dependent.

PIA productions were also measured by dot blot using specific antibodies recognizing the sugaric-based PIA molecule and performed on proteinase K digested samples to avoid cross-detection of protein A (Figure 1, PIA). PIA dot blot experiments did show a general tendency of PIA increase linked to absence of *codY* gene but independently of the presence of *agrA*. This tendency of PIA increase was statistically significant in ISP479r background but not in UAMS-1 and Newman. But in S30, the disruption of *codY* in Ω6900 resulted in a drastic reduction of PIA production, an opposite effect compared to the other backgrounds, also recorded for the biofilm productions. Note that opposite and conflicting effects of *codY* mutations on biofilm formation in the S30 and UAMS-1 strains were reported previously [37,65]. Therefore, the Congo red agar (CRA) was used as a second method to investigate biofilm formation in *S. aureus*.

To control CRA phenotypes, all the S30 biofilm defective mutants resulting from the mutagenesis project [65] were spotted onto CRA. Results showed that below 30 % of biofilm formation compared to S30 wild-type, as measured by crystal violet staining assay, the mutants from the mutagenesis project generated flat or smooth red spots on CRA, whereas above 30 % of biofilm formation, spots appeared rough or crystalline structured with a tendency to acquired a black color, (not shown). Observations of CRA phenotypes in UAMS-1 reveal

a slight crystalline structure of bacterial spots linked to the Δ*codY* mutation and independently of the Δ*agrA* mutation, suggesting that amyloid and/or PIA increased when CodY-mediated repression disappear in that background, as in ISP479r background but in a higher level, with a black color of *codY* deficient bacterial spots. Interestingly, CRA results showed that ISP479r Δ*codY*, Δ*codY* Δ*agrA* and S30 produced the highest quantity of biofilms according to CV assay, the highest quantities of PIA, and showed a correlation between the resistances of their respective biofilms against the proteinase K treatment and their respective dark phenotypes onto CRA plates (figure 1).

Observation of bacteria spotted onto sheep blood agar plates showed that the hemolytic activity toward sheep erythrocytes increased with Δ*codY* mutation and decreased with Δ*agrA* mutation, suggesting that CodY represses the *agr* system and that the *agr* system promotes the hemolytic activity, as previously observed (figure 1, BA) [37]. In UAMS-1, the alpha hemolysin (*hla*) encoding gene harbors a non-sense mutation, probably responsible for the lack of hemolytic activity of that strain toward the sheep blood. Nevertheless, a small hemolytic activity was observed when *codY* is deleted, which could correspond to the activity of the beta-hemolysin [7,37,37,43]. ISP479r wild-type is not hemolytic in BA for unknown reason, but its Δ*codY* mutant acquired full hemolytic activity

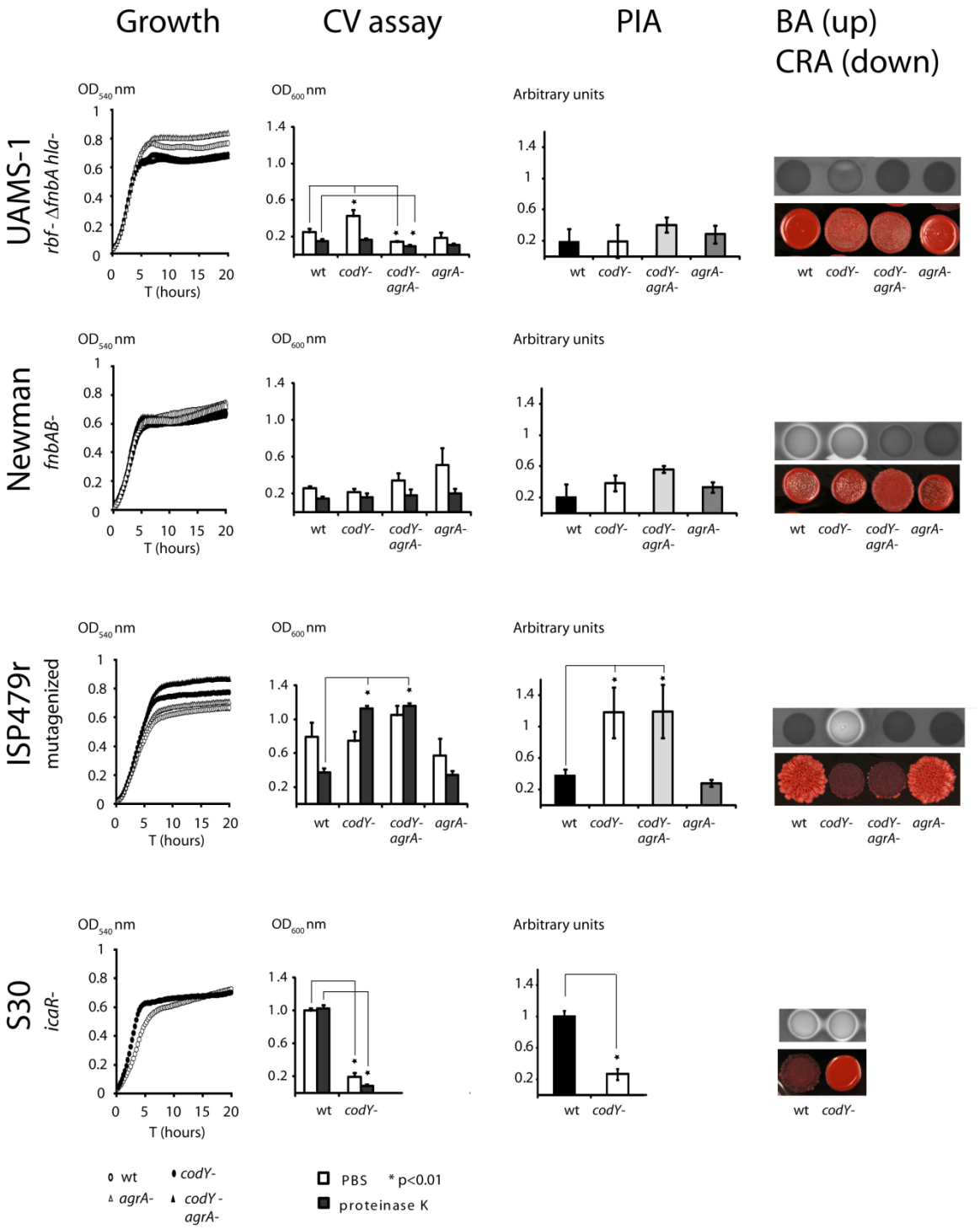


Figure 1 - The *agrA*-dependent and *agrA*-independent effects of Δ*codY* mutation onto different *S. aureus* biofilms.

Three independent triplicates were performed to obtain mean and standards errors (errors bars) shown in figure 1. **Growth**: bacterial growth curves according to OD_{540} nm determination automatically reported each

15 minutes into a Tecan apparatus during 20 hours of growth into TSB medium at 37 °C without agitation. (○) wild type strain, (●) *ΔcodY::tetM* mutant, (▲) *ΔcodY::tetM agrA::Tn551* mutant, (Δ) *agrA::Tn551* mutant of UAMS-1, Newman, ISP479r and S30. Note that we do not succeed to obtain *agrA::Tn551* mutation into S30 and Ω6900. **CV assay**: crystal violet staining assay quantifying biofilm produced after 20 hours of growth. (■) Biofilm was treated with 4 mAU proteinase K during 1 hour prior to wash, fixation and staining, (□) or with PBS only. **PIA**: measurements of PIA quantities according to standardized PIA dot blot results. (*) Statistical significant differences calculated with t-test leading to $p < 0.05$ both for CV assay and PIA. **CRA**: phenotype of bacterial spots grown onto Congo red agar medium. **BA**: phenotype of bacterial spots grown onto sheep blood agar medium. Strains are named in the left part of the figure. Genes known to be altered in these strains are mentioned below their name. ISP479r is mentioned as being mutagenized, but the altered genes are not identified.

Complementation of *codY* in S30

Interestingly, the *codY* mutation in the S30 background led to contradictory results compared to the other backgrounds. In order to exclude that the observed phenotypes were due to an indirect effect of the transposon or to a second mutation, the *codY* mutation was complemented. This was performed using pCG30 in Ω6900 in presence of 250 ng/ml AHT inducer before crystal violet staining assay. The biofilm of Ω6900 was partially restored in presence of the inducer and the pCG30 plasmid, strongly suggesting that the decreased biofilm reported in Ω6900 is due to alteration of *codY*, (complementary figure S1). Nevertheless, the complementation was not totally achieved. The partial phenotype could be explained either by the natural presence of pT181 in S30 background or by the over-expression of CodY. Indeed, pT181 plasmid carrying a *tet* gene is potentially able to export the inducer out of the bacteria, whereas over-expressions of *codY*/CodY at both the transcript and the protein levels were detected by q-RT-PCR and western blot, respectively, which is not comparable to the levels recorded in the parental strain. Nevertheless, the *codY* complementation results suggest strongly that *codY* is needed for biofilm production in S30.

An ochre mutation in *icaR* is probably responsible for the hyper-biofilm phenotype of S30

S30 is able to produce higher amounts of both biofilm and PIA compared to the other strains tested. Therefore, we decided to sequence the regulatory region of the *icaADBC* operon of S30. A point mutation (G292A) was identified in *icaR*, leading to a stop (TAA) at the 98th codon in place of glutamine "Q" (CAA). In order to evaluate the impact of the *icaR* mutation on biofilm production in S30, we decided to complement

the mutation. A fragment of DNA containing a non-mutated *ica*R gene under its natural promoter, amplified from Newman genomic DNA, was cloned in the *Eco*RI & *Bam*HI sites of pCN38, resulting in pIcaR. Results showed that the complementation of *ica*R in S30 decreased biofilm production significantly ($p<0.01$, t-test).

Transcriptional studies of *codY, icaR* and *icaADBC* operon in S30

To understand the involvement of *codY* and *ica*R in the regulation of the *icaADBC* operon, its transcription was quantified in function of the absence and the over-expression conditions of these transcriptional regulators (figure 2). The probed regions matched every ORF of the *icaADBC* operon (figure 2A). In the case of *codY*, the two probed regions are localized before and after the insertion site of the *em-mu* in Ω6900, (figure 2D).

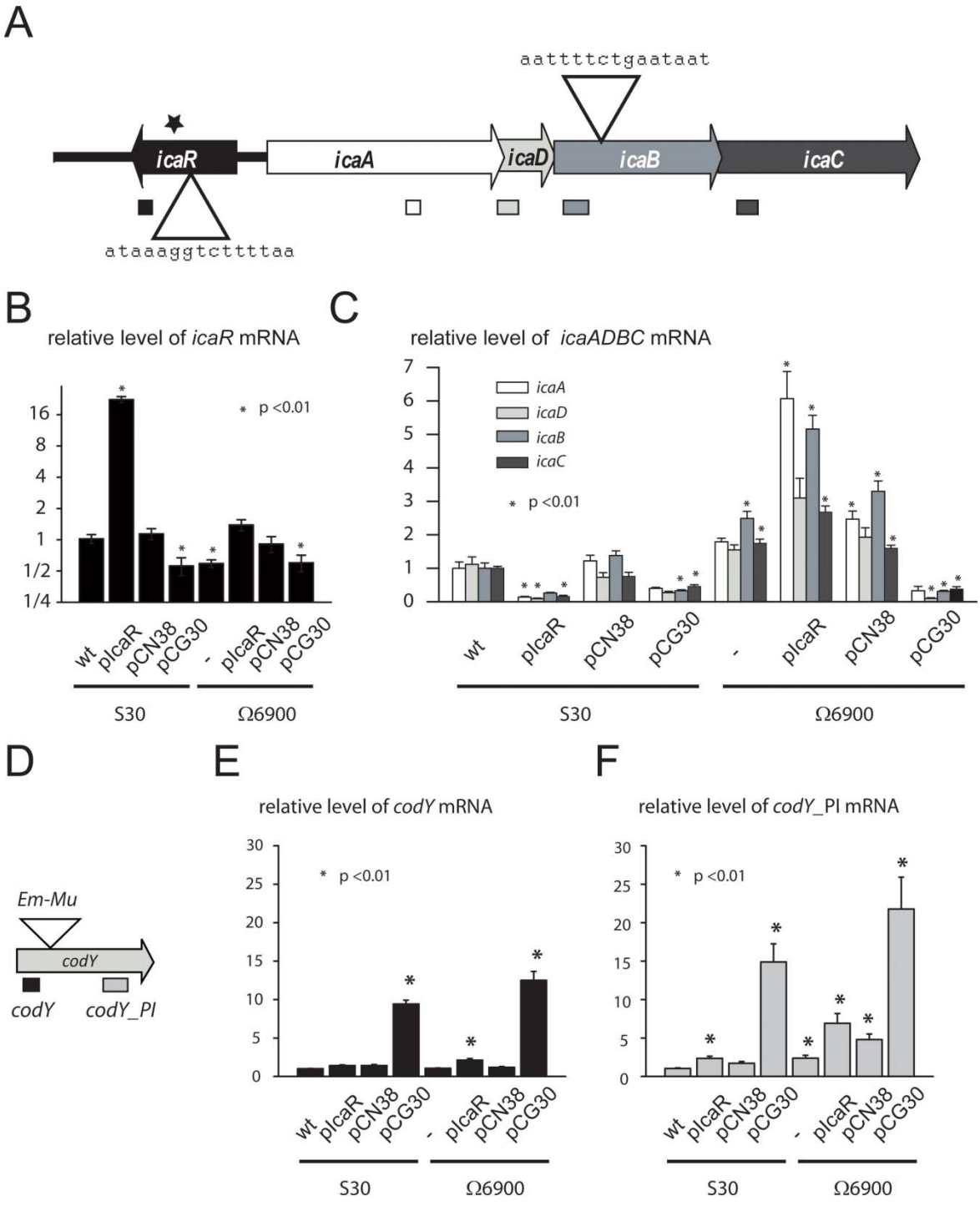
First, quantification of *ica*R mRNA levels showed that both over-expressions as well as disruption of *codY* decreased *ica*R expression two fold compared to the level reported in S30 wild-type, (figure 2B, bars 4,5,8). These results look contradictory. But they could be explained by the involvement of two different pathways for CodY-mediated repression of *ica*R mRNA level. At least one unknown additional factor seems to be involved. This factor should be repressed by CodY and able to repress *ica*R. Additionally, *ica*R mRNA level in Ω6900 containing pIcaR resulted is an increase of *ica*R expression by 2.4 fold leading to similar levels of *ica*R as reported in S30, (figure 2B, bar 6). This suggests that complementation of *ica*R in a *codY* disrupted condition restored a wild-type *ica*R mRNA relative level. All together, these results suggested that increasing or decreasing the transcriptional rate of *codY* in S30 background resulted in a reduction of the transcriptional rate of *ica*R.

Second, to decipher CodY and IcaR mediated networks involved in the regulation of the *icaADBC* operon, all ORFs of the operon were probed by Q-RT-PCR (figure 2C). Results in S30 showed a drastic decrease of *icaADBC* operon mRNA level for each *ica* ORF under *ica*R over-expressing conditions, consistent with previous reports of IcaR as a repressor of *icaADBC*, (figure 2C, bar group 2). Surprisingly, pIcaR in Ω6900 resulted in an increase of *icaADBC* mRNA relative levels, (figure 2C, bar group 6). These results suggest a

CodY mediated IcaR repression of *icaADBC* operon reversing IcaR positive effects when *codY* is inactivated. The mRNA relative levels from the different ORFs of the *icaADBC* operon were affected in a similar manner for the eight S30 derived genetic constructs presented in figure 2, even if differences in quantified mRNAs were observed between *icaA* and *icaB* on one side, and *icaD* and *icaC* on the other side (figure 2C). We think that these differences were likely due to probe specificity and do not reflect a real differential level of expression. CodY itself behaved clearly as an independent repressor of the *icaADBC* operon because over-expression of *codY* resulted in a drastic decrease in mRNA level of the *icaADBC* operon. Moreover, inactivation of *codY* by *em-mu* caused to an increase in *icaADBC* operon mRNA, coherent with results reported for *icaA* in UAMS-1 [37].

Third, Q-RT-PCR results showed over-expression of *codY* mRNA level in presence of pCG30 both in S30 and Ω6900 with values ranging from 9.4 to 21 fold (figure 2EF). The disruption of *codY* did not change *codY* mRNA level before the insertion site of *em-mu* (figure 2E, bar 5), but a significant 2.37 fold increase of *codY* mRNA level was observed downstream of the insertion (figure 2F, bar 5). This increase was probably due to the inserted promoter within the *em-mu* element. These results suggested that in S30, CodY did not influence the rate of its own transcription, whereas a strong auto-repressing activity of CodY was reported in *Lactococcus lactis* [17]. Finally, the presence of pIcaR in both S30 and Ω6900 resulted to increase slightly the *codY* transcriptional level (figure 2EF, bars 2, 6).

All together, these results demonstrate an effective over-expression of *codY* linked to pCG30 and an effective over-expression of *ica*R linked to pIcaR. The results suggested the probable existence of an additional factor involved in the negative regulation of *ica*R linked to CodY, demonstrate that CodY is a repressor of the *icaADBC* operon and finally suggest that the *ica*R-mediate repression of *icaADBC* needs CodY.



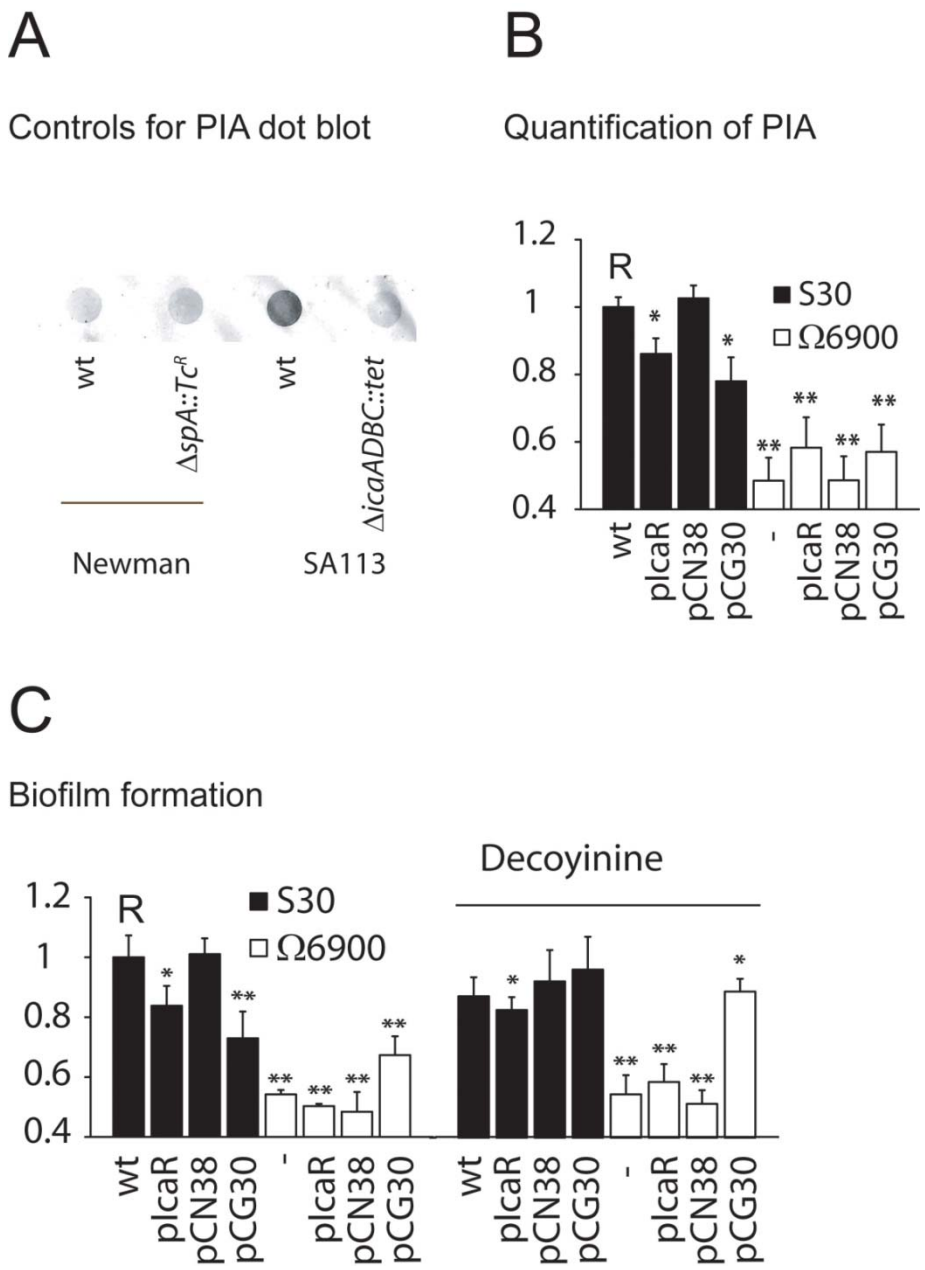
<u>Figure 2</u> - Transcriptional studies of *codY*, *icaR* and *icaADBC* operon between S30 deriving genetic constructs.

(**A**) Schematic representation by arrows of *icaADBC* operon in sense and its *ica*R repressor in antisense with the location of the G292A mutation in *ica*R shown as a black star (★). 2 putative CodY-binding sites were detected through an informatics query in the "*ica* region", in *icaB* ORF and in *ica*R ORF. (Δ) Their locations are shown by white triangles surrounded by a thin black line up and down the schematic representation of the *icaADBC* operon. Their DNA sequences are indicated. Regions probed for q-RT-PCR experiments are

shown as small rectangles below the operon, positions and colors compared to the schematic representation of the "*ica* region". Each q-RT-PCR experiment was performed at least in tree independent triplicates or two independent sextuplets producing the data presented in panel **B**, **C**, **E**, **F**. The housekeeping 16S rRNA was used as an endogenous control assuming that it remains expressed at similar levels in all samples. Note that the JOE reporter was exclusively used for the 16S rRNA probe and allowed duplex quantification of 16S rRNA and one of the other probes in the same well. Results from S30 total mRNA were used as reference allowing comparison of that condition to the other conditions. T-tests were performed comparing dataset of mRNA relative levels from S30 compared to datasets from the other conditions assuming a two tails distribution of values with two samples of unequal variances. (*) Small stars represent statistical significant differences with p-value smaller than 0.01 between the condition below the star and S30 wt. mRNA levels assessed in the present figure followed a normal distribution of values according to QQ plots. Standard errors were calculated to illustrate the variations of reported mRNA levels and are represented by error bars. (**B**) Relative levels of *ica*R mRNA assessed in the 8 S30 derived constructs. (**C**) Relative levels of *icaADBC* mRNA in the 8 S30 derived constructs assessed with probed regions matching each individual *ica* ORFs; (□)*icaA*, (■) *icaD*, (■)*icaB* and (■)*icaC*. (**D**) Schematic representation of *codY* gene as a light gray arrow. The two probed region for quantification of mRNA relative levels are shown as dark and light rectangles below the *codY* gene. (▽) The localization of insertion of the *em-mu* genetic element responsible for the production of Ω6900 mutant is shown with a white triangle. (**E**, **F**) Relative levels of *codY* mRNA probed (**E**) before and (**F**) after the insertion of the *em-mu* in the 8 S30 derived constructs.

PIA production in function of CodY and IcaR

As the level of *icaADBC* operon varies between the tested conditions, the PIA was quantified with a dot blot experiment using antibodies. To avoid protein A non-specific detection, we digested proteins present in samples with proteinase K. Controls showed no detection of protein A but specific detection of PIA, (figure 3A). PIA dot blot results showed a highly reproducible decrease of PIA when *ica*R and *codY* were over-expressed in S30, in accordance with their repressing effects on the transcription of *icaADBC* (figure 3B, bars 2, 4, 8). Interestingly, the disruption of *codY* resulted in the absence of PIA, consistent with drastic biofilm decrease (figure 3B, bar 5). Conditions of *codY* and *ica*R over-expressions associated to *codY* genomic disruption resulted in an intermediate PIA level, between strong decrease recorded in Ω6900 and partial decrease in S30-associated *codY* and *ica*R over-expressions (figure 3B, bars 6, 8). These results suggested that CodY was required for PIA production whereas *codY* over-expression resulted in PIA-repression in the S30 background. Moreover, in Ω6900, increased expression of the *icaADBC* operon was linked with abolition of PIA production, suggesting that CodY may affect other factors that are important for PIA formation.



<u>Figure 3</u> - PIA production and biofilm formation respectively assessed through dot blot experiments and crystal violet staining assay with and without decoyinine.

At least three independent triplicates were performed. Stars represent statistical significant differences (*) $p<0.05$ and (**) $p<0.01$ between each condition compared to S30 wild-type used as reference (R). Black bars represent S30 with different plasmids whereas white bars represent Ω6900 with different plasmids. Error bars correspond to standard errors. Note that Y scale starts from 0.4. (**A**) Scanned and cropped image of PIA-detected by immuno-chemical reaction (α-PIA antibody and phosphatase conjugated secondary antibody) leading to visible spots on the nitrocellulose membrane for the PIA and Spa control conditions. Newman wild-type and its deriving *ΔspA::Tc*^{R} are used to control eventual non specific detection of protein A whereas SA113 and its *ΔicaADBC::tet* are used to control specific PIA detection. (**B**) Histogram presenting PIA quantity assessed with 3 independent experiments produced by the different S30 derived constructs. (**C**) Histogram showing relative biofilm quantities produced with and without decoyinine treatment, by 8

equilibrated (starting OD_{540} nm = 1.5) and 10 fold diluted S30 deriving genetic constructs. Biofilm formation occurred after 2h of growth at 37 °C without agitation in 1 ml/well of inoculated TSB medium in 24 wells polystyrene plates. Biofilm-incorporated crystal violet was solubilized in DMSO before measurement of OD_{600} nm. Note that AHT is present in every condition at 250 ng/ml whereas decoyinine is present only when mentioned on the picture at 500 µg/ml.

GTP-dependant CodY dominant effect compared to IcaR towards S30 biofilm formation

To see if activation of CodY by GTP is implicated for biofilm formation in S30, bacterial samples were treated with decoyinine. Decoyinine is known to be responsible for intracellular GTP decrease in bacteria, by specifically inhibiting the GMP synthase [66]. A biofilm crystal violet staining assay was performed with the 8 S30 derived constructs in three independent triplicates, splitting each sample for decoyinine and TSB conditions (figure 3C).

First, in absence of decoyinine treatment, both over-expressions of *ica*R and *codY* significantly affected the formation of biofilm negatively in the S30 background, consistent with *icaADBC* mRNA and PIA levels (Figure 3C, left, bars 2, 4, 8). Despite the fact that the *codY* over-expression mediated a decrease of biofilm formation, disruption of *codY* also drastically decreased biofilm production, (figure 3C, bar 5). This suggests that two different pathways could involve CodY in biofilm formation in S30. Moreover, the results are consistent with PIA reported levels. Interestingly, biofilm decrease seemed to be independent of pIcaR in Ω6900, suggesting a dominant effect of CodY over IcaR, (figure 3C, bar 6). These results, contradictory with the observed *icaADBC* increase but consistent with the PIA levels, suggested that transcription of *icaADBC* is not always sufficient for the synthesis of PIA and for the formation of biofilm. Note that additional *ica*-independent pathways were involved in biofilm formation in *S. aureus* [64]. In Ω6900, de-repressed targets of CodY somehow should inactivate the synthesis of PIA despite the observed increased of *icaADBC* expression at the transcriptional level. This could be by acting on the level of N-acetyl-glucosamine substrates needed for PIA synthesis, or by a post-transcriptional repression of IcaADBC.

Second, under decoyinine treatment, similar results compared to TSB conditions were obtained except when *codY* was present. Indeed, S30 biofilm quantity decreased slightly whereas Ω6900 biofilm quantity remained

equivalent, (figure 3C, right, bars 1, 5). In *codY* over-expressing conditions (S30 pCG30 and Ω6900 pCG30), biofilm formation increased up to the level reported for decoyinine-treated S30, (figure 3C, bars 4, 8). Over-expression of *codY*, under condition of intracellular GTP decrease trough decoyinine treatment restored biofilm formation to the level of the wild-type, suggesting that CodY non activated by GTP was needed for full biofilm in S30.

In vivo binding of CodY on *icaB* and *icaR*

Two CodY binding sequences that contain two mismatches from the AATTTTCWGAAAATT consensus were identified inside *icaB* and *ica*R, (figure 4C). CodY binding to *ica* DNA was investigated by cross-linking and immuno-precipitation with CodY antibodies followed by Q-PCR. Indeed, if CodY blocks the transcription in *ica*R or in *icaB*, it could have opposite effects on the expression of the *icaADBC* operon. Detection of DNA recovered through CodY-immuno-precipitation was performed with primers and probes matching the different ORFs of the *icaADBC* operon. Relative DNA quantity calculated using the ΔCt method obtained from Q-PCR was maximal when the cross-linked DNA from S30 was immuno-precipitated with antibodies against CodY in the *ica*R and *icaB* regions, suggesting that CodY protein bound these regions *in vivo*, (figure 4C). The recovered DNA using S30 and antibodies against CodY decreased between *ica*R and *icaB* as well as after *icaB*, consistent with the position of the *in silico* detected CodY binding sites, (figure 4C). In Ω6900, recovered DNA stayed bellow as compared to S30 and stationary along the *ica* region, consistent with the absence of CodY protein that was verified with western blot, (figure 4A). Note that the obtained resolution corresponded to 1 kb length form the probed region according to the migration of sonicated DNA samples in agarose gels, (figure 4B). The entire procedure was also performed using antibodies against ClfA to control that CodY-DNA complexes were specifically immuno-precipitated by CodY antibodies, (figure 4C). The results were compatible with *in vitro* binding of CodY within *icaB* detected trough genome-wide analysis of NTCT 8325 DNA but not for the binding into *ica*R [36]. The difference concerning *ica*R was probably not directly due to the ochre mutation, because the DNA substitution is not located in the CodY box. We chose ChIP approach to study CodY binding in the *ica* region in S30 even if this technique is not

suitable for precise localization of binding sites as compared to footprinting experiments, because we expected that additional binding sites for other proteins should be also co-localized in the *ica* region. In the case another protein binds that region, it could prevent CodY binding *in vivo*, which should not be detected in footprinting experiments performed *in vitro*. Note that we showed here for the first time that CodY binds to the *icaADBC* operon and also its specific *ica*R repressor *in vivo*. The way CodY controls the transcriptional level of both *ica*R and *icaADBC* will probably depend on the respective affinities of CodY for each of the two boxes. The binding of CodY in *ica* DNA region could probably be responsible for repression of *icaADBC* expression, superseding IcaR mediate repression.

PIA detection in function of *codY* and *icaR* mutations in Newman

The *ica*R was disrupted by TargeTron® Gene Knockout System in Newman wt and *ΔcodY*, to investigate effects of *ica*R and *codY* on PIA production in another background. Results of PIA quantification did show an increase of PIA more related to *ica*R disruption than *codY* absence in Newman (not shown). Nevertheless, even the most PIA producing constructs (Newman *ΔicaR ΔcodY*) did not reach the PIA level recorded in S30 (not shown). These results suggested that the S30 genome probably contains additional particularities other than the ochre mutation in *ica*R responsible for PIA overproduction and the hyper-biofilm phenotype. These additional particularities are currently studied by comparison of genomes, (D. Hernandez, personal communication).

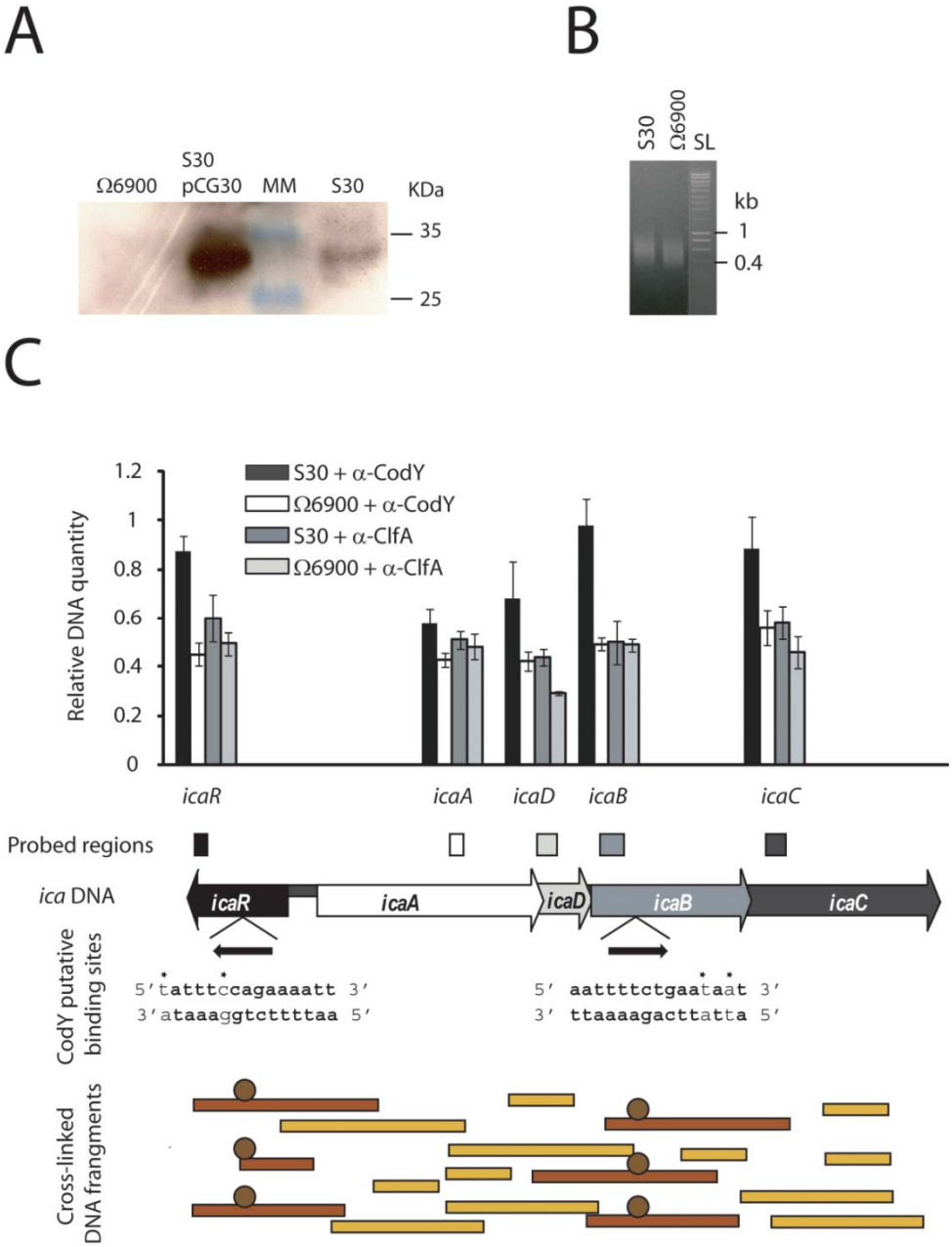


Figure 4 - CodY binds *in vivo* in *icaR* and *icaB*

(**A**) CodY detected by Western blot in Ω6900, S30 pCG30 and S30. MM, molecular marker PageRuler™. (**B**) Photography of agarose gel electrophoresis containing cross-linked and sheared DNA from both S30 and Ω6900. SL, smart ladder. (**C**) ChIP-QPCR results probing CodY binding along the *ica* region using ChIP and inputs samples prepared from S30 and Ω6900 that were immuno-precipitated with antibodies α-CodY and α-ClfA (clumping factor). Relative DNA quantities according to Q-PCR were calculated from 3 independent experiments. Error bars represent standard errors. The 5 probes regions are shown above the X axis legend of the graphic and respect the sizes and the positions according to *ica* region. The *ica* DNA positioned above

the probed regions is drawn with 5 arrows representing each *ica* ORF respecting their relative sizes and orientations. 2 CodY putative binding sites, located into the *ica* region, are shown with small arrows oppositely oriented above inverted V. Their respective sequence is shown for the two DNA strands and asterix represent mismatches compared to the palindromic consensus AATTTTCWGAAAATT. DNA fragments are drawn above the binding sites as rectangles with various sized between 0.4 and 1 KDa. Red fragments symbolized CodY-bound whereas yellow fragments symbolized probably not bound by CodY. Brown circles symbolized CodY.

Conclusions

CodY is a pleiotropic repressor, and therefore its biological roles are multiple. In *S. aureus*, the locations of CodY DNA binding sites were studied previously by microarray analysis of mRNA transcripts comparing *codY* mutant and parent strains, by bioinformatics detection of consensus boxes in *S. aureus* genomes, by *in vitro* pull down assays with purified CodY, and by using DNase I footprinting analysis [36,43]. 179 genes were overexpressed by more than 2-fold in the *codY* mutant from the study of Majerczyk *et al.* (2010), compared with 106 genes in the study of Pohl *et al.*, (2009). Depending on the strain, different numbers of CodY boxes were identified with bioinformatics approaches: 68 CodY boxes in COL, 76 in N315 and 71 in Newman [43]. 231 positions of high DNA enrichment were identified in a CodY pull down assay [36]. Note that 58% of DNA sequences that were bound CodY *in vitro* are intergenic in NTCC8325, whereas the intergenic regions represent only approximately 15 % of NTCC8325 *S. aureus* genome [36]. Inside intergenic regions, small transcripts were reported [3]. In *Bacillus subtilis*, CodY was reported to activate the transcription of a small RNA [44]. CodY regulating functions of small RNAs remain totally unknown in *S. aureus* except for RNAIII. In this particular case, CodY regulates a genetic element that is itself a regulator of other genes. The integrity of CodY targeted genes is important when studying CodY effects on phenotypes. In the present study, CodY has different effects on the biofilms formed by different *S. aureus* strains. An intensive study on the *icaR* deficient strain S30 was performed. Interestingly, some genes implicated in the adhesion process or biofilm maturation are naturally defective among the other tested strains. These gene defects can explain strain differences in term of biofilm formation.

First, the Newman strain carries an amber mutation inside the *fnbB* gene coding for fibronectin-binding protein B, which was probably transferred inside *fnbA* [25]. As a result of these mutations the LPXTG motif needed for sortase-mediated protein export was deleted and consequently FnbPA & FnbPB are secreted instead of being anchored to the cell wall, resulting in the loss of FnBPAB-dependent functions, such as strong adhesion to immobilized fibronectin, binding of fibrinogen, and host cell invasion [25]. CodY was able to bind *in vitro* to the promoter region of FnBPs [36]. *fnBP* genes are negatively regulated by Agr [53], whereas *agr* is negatively regulated by CodY [37]. The effects of *codY* and *agr* single and double mutations in Newman biofilm revealed no statistically significant differences. This absence of consequences of both CodY and Agr on Newman biofilms could be partially due to the absence of FnBP-dependent functions, although additional experiments are necessary to support this statement.

Second, in UAMS-1, a regulator of biofilm formation (*rbf*, ORF SA0622 in strain N315) was reported to be a pseudogene [34]. A 2-bp insertion in the 50th codon of the *rbf* affects the open reading frame in this strain, explaining why deleting *rbf* in UAMS-1 did not produce differences in term of biofilm formation [16], and explaining also probably why the biofilm of UAMS-1 contains only a monolayer of adhesive bacteria when observed with fluorescent microscopy [60]. Rbf possesses a consensus region signature of the AraC/XylS family of regulators [34]. This putative transcriptional regulator repressed *ica*R without direct binding, suggesting the implication of (an) additional factor(s) in this process [16]. Δ*rbf* mutants fail to produce a multi-cellular aggregation stage in biofilms [34]. PIA mediates the contact of the bacterial cells to each other and bacteria become embedded in this slimy matrix during the cumulative phase of biofilm formation in *S. epidermidis* [70]. Note that FnbPA is not present in UAMS-1 and the *hla* gene coding for hemolysin A contains a non-sense mutation [7,37,37,43]. UAMS-1 wild-type is not hemolytic for sheep blood, (figure 1, BA). Moreover, deletion of the *ica* locus in UAMS-1 had no impact on biofilm development, suggesting an *ica*-independent pathway for biofilm formation in UAMS-1 [5]. No CodY box was found in the regulatory region of Rbf by bio-informatics approaches and the *rbf* regulatory region was not detected in CodY pool down assay [36,43]. In UAMS-1, a strong biofilm increase in the Δ*codY* mutant of that *rbf* defective strain was observed in the present study and

also reported by others [37]. Complementation of the defective *rbf* gene in both UAMS-1 and its *codY* deriving mutant could unveil putative CodY-Rbf interactions involved in biofilm formation and maturation.

Third, derived from an original cutaneous infection isolate, ISP479r is a highly manipulated strain. Notably, UV mutagenesis has occurred in that strain [1,58]. Its genome probably contains a lot of mutations. In this strain, PIA increases related to *codY* mutation in an *agr*-independent manner was clearly recorded and a strong correlation was observed with proteinase K digested biofilm and CRA phenotypes. In ISP479r Δ*codY* and Δ*codY* Δ*agrA* mutants, as well as S30, the Congo red probably bound to the PIA in a highly structured manner. Congo red dye molecules remain randomly located in absence of organized polymers. That could be responsible for the dark phenotypes onto CRA, probably due to the polarization of each molecules of the dye located into the polymer, which change its absorption of the light, a property named birefringence and previously reported for Congo red stained amyloid or starch [49,63]. But the additional presence of amyloid compounds of protein nature cannot be excluded, because various proteases-resistant amyloid fibers specifically detectable by the Congo red dye were identified in various biofilm matrices, such as curli produced by *Escherichia coli*, or TasA fibers providing structural integrity to the biofilm in *Bacillus subtilis* [10,49]. Since CodY binds the *icaADBC* operon *in vivo* in S30 and *in vitro* in NTCC8325, the direct effect of CodY on *icaADBC* operon is likely to be more visible in ISP479r, because additional targets leading to complex networks have an increased probability to be mutated in this strain. ISP479r genome sequencing would lead to identify mutations in this strain. Finally, we identified an ochre mutation in *icaR* of S30. IcaR is the natural repressor of the *icaADBC* operon and its ORFs remain 100 % identical between 12 sequenced strains of *S. aureus* available on NCBI. In the S30 *icaR*-deficient and hyper-biofilm producing strain, *codY* mutation leads to an opposite phenotype compared to *codY* mutation in *rbf* defective UAMS-1. Note that UAMS-1 did not produce PIA or only at negligible quantity, whereas S30 is over-producing PIA. Rbf is able to repress *icaR*, and the interactions involving *icaR*, *codY* and *rbf* can partially explain the opposite phenotypes observed between UAMS-1 and S30. Further studies of *codY* mutants with both complementation of *rbf* in UAMS-1 and *icaR* in S30 would be of a great interest to validate the opposite phenotypes between the two *codY* mutants. Moreover, sequencing the genome of S30 will be an advantage for the identification of putative

additional genetic particularities leading to hyper-biofilm phenotype, which is an ongoing project in our laboratory.

We found that CodY binds *in vivo* inside *ica*R and *icaB* by ChIP-QPCR experiments. We found two binding sites in the corresponding regions (figure 4). Over-expression of CodY repressed *ica*R whereas disruption of *codY* increased *icaADBC* expression in S30. CodY probably blocks the transcription of *ica*R and *icaB*. By that mechanism, CodY seems to dominate the transcriptional control of the operon as compared to *ica*R. The influence of CodY binding on the expression of the *icaADBC* operon depends heavily on the availability of the two boxes and the respective affinity of CodY for these boxes.

Additional factors are also implicated in the regulation of *icaADBC*. The staphylococcal accessory regulator A (SarA) and the alternative sigma factor (σ^B) were previously identified as positive regulators of *icaADBC* expression and are also required for *ica*R expression whereas σ^B was able to activate *sarA* [4,5,8]. σ^B was shown to be involved in recovery from heat shock at 54°C and in acid and hydrogen peroxide resistance [9]. But according to a contradictory report, SarA and not σ^B is essential for biofilm development by *Staphylococcus aureus* [67]. Finally, Spx, an additional global regulator in *S. aureus*, appears to activate *ica*R transcription, leading to decreased *ica* operon expression [21] [42]. Inactivation of Spx in *Staphylococcus aureus* rendered the cells hypersensitive to a wide range of stress conditions including high and low temperature, high osmolarity, and hydrogen peroxide [42]. The complex network regulating the *icaADBC* operon and CodY pathways are summarized in a final schematic shown in figure 5.

Our main conclusion is that CodY is involved in a multi-factorial complex network of interacting elements in the context of biofilm formation. The strain specific nature of CodY impact on *S. aureus* biofilm is probably due to the fact that natural mutations occurred in the interacting genes of the different tested strains. Genetic integrity of the interacting genes is needed to unravel a clear CodY-dependent effect. Moreover, CodY could be seen as repressing system responding to intracellular level of GTP and BCAA able to repress a quorum sensing system responding to cell density. Increasing or decreasing bacterial population or changing the metabolic state of the bacteria would lead to CodY involvement and adapted behavior of the bacterial population. CodY seems to be the heart of a molecular network implicating interacting elements, allowing for

an entire community to make a switch in the mode of growth from planktonic to biofilm formation and vice versa, as a function of the metabolic state of the bacteria, in connections with stress response and quorum sensing.

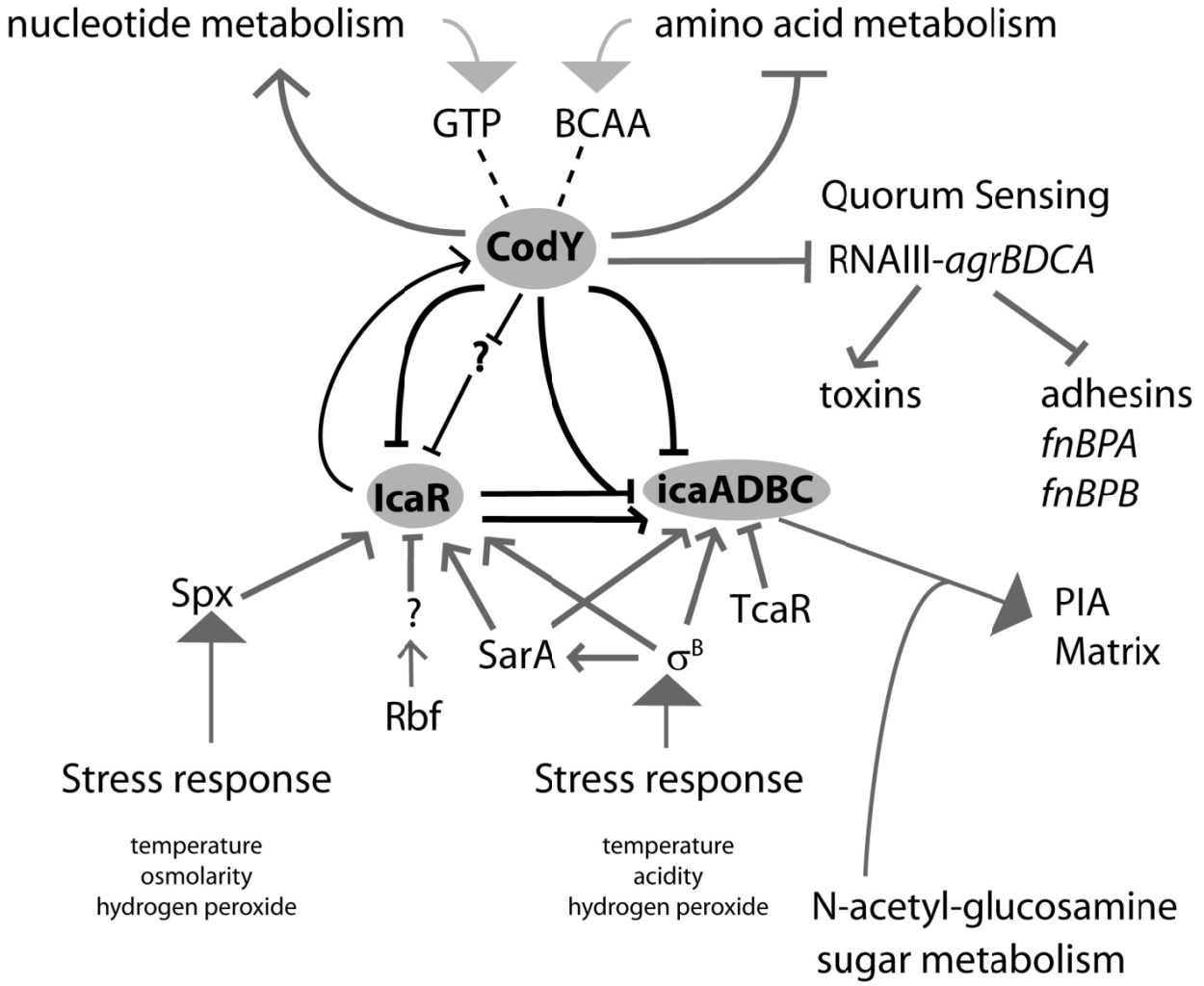


Figure 5 - *S. aureus* CodY interacting network towards biofilm formation
The picture presents CodY interacting network in *S. aureus* according to recent reports. Interactions observed in the present study are drawn in bold whereas interactions reported from other studies are drawn in gray. Blunted lines indicate repressing effects whereas arrows indicate activation. Interactions usually concern the transcriptional control of one gene product towards the transcription of another gene or a pool of genes with some exceptions explained bellow. For example, CodY represses a pool of genes implicated in the metabolism of amino acids. For commodities, amino acid metabolism is shown without the gene names. The arrow from the *icaADBC* operon to the PIA indicates that *icaADBC* is implicated in the synthesis of PIA. Toxins activated by RNAIII-*agrBDCA* are not named in the picture. Only *fnbA* and *fnbB* adhesins repressed by the *agr* system are indicated but other adhesins not mentioned in the picture remain also repressed by the *agr* system. The arrows from stress response to SpX or σ^B indicate that σ^B and SpX were reported to be activated in stress conditions. Question mark indicates that an additional factor is thought to be implicated

without being identified. Dashed arrows indicate physical interactions between GTP and/or BCAA (valine, isoleucine and leucine) with CodY.

The genomes of *S. aureus* show a well conserved core region corresponding to approximately 80% of the genome [27]. But *S. aureus* also displays a wide diversity of accessory genetic elements [27]. These observations confirm an important genetic diversity and high plasticity of the bacterium and suggest that these contribute to its adaptation to environmental changes, including antibiotic selection pressure [48]. Here, strains tested have also shown genetic diversity in the core genomes with mutations in conserved genes. Therefore, the core genome of *S. aureus* also could be seen as variable. These variations have lead to opposing results for the role of CodY in biofilm formation. The mutation of IcaR occurring in S30 leads to a strong biofilm phenotype, whereas bacterial biofilm formation could be seen as an adaptation of the bacteria against antibiotic pressure.

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Supplemental figures:

Figure 1S

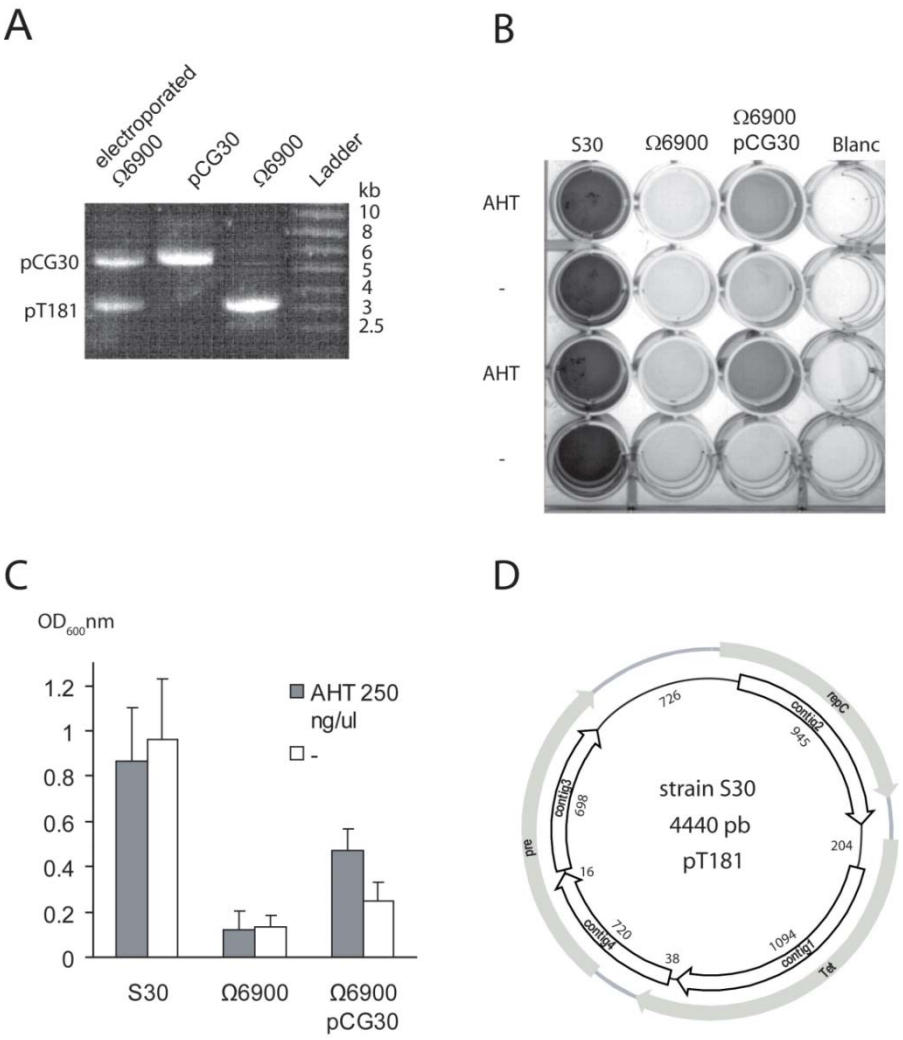

Figure 1S: Complementation of Ω6900 mutant with the pCG30.
A. 0.8 % agarose gel electrophoresis of 1 µg plasmids extracted from Ω6900 after electroporation of pCG30, first column. Second vertical migration column corresponds to 1 µg of pCG30 extracted from U21/30 [43]. Third vertical column corresponds to 1 µg of plasmid extracted from Ω6900. Fourth vertical column corresponds to the smart ladder used for evaluations of DNA sizes. Note that plasmids are not digested and that their migrations do not correspond to their real sizes. In the pCG30, the *codY* sequence was inserted between *Eco*RI sites and is located under the control of a $P_{xyl/tet}$ promoter [43]. The pT181 plasmid was detected by electrophoresis of plasmid extracts in S30 and its derivative, and was identified by sequencing using primers listed on table 2. **B**. Photography of a CV-stained microtiter plate performed to quantified biofilm in various S30 strains. S30 was inoculated onto wells located onto the first vertical column; Ω6900 was inoculated onto the second

column, Ω6900 pCG30 on the third vertical column, and blank in the fourth vertical column. 250 ng of AHT inducer was added for each ml of the medium for every strain on first and third horizontal rows (AHT), and is absent of the second and fourth horizontal rows (-). The anhydrotetracycline (AHT) is a non-lethal analogue of tetracycline used to induce transcription of *codY* in pCG30. The basal activity of the promoter without the inducer was estimated to be negligible and the optimal concentration of the inducer was estimated to be 250 ng/ml [2]. Results showed partial complementation for biofilm production in presence of induced pCG30 in Ω6900, whereas the biofilm produced by Ω6900 remains low and those produced by S30 remains high, independently of the presence of AHT. **C**. Quantification of biofilm production of the conditions shown in B, (■) in presence of AHT, (□) in absence of AHT, based on three triplicates. Errors bars represent standards deviations. **D**. Map of the contigs produced by PCR-based sequencing of pieces of the plasmid present in S30 and Ω6900, matching perfectly the pT181. The first pair of primers designed to sequence an unknown plasmid matches the *tet* gene, because tetracycline resistance was detected in S30, was reported to be mainly plasmid-encoded in *S. aureus*, and allowed successful acquisition of tetracycline resistance after transformation of various strains with that plasmid. The other set of primers were designed according to the previously acquired sequences, in a step-by-step manner. (⇨), contigs resulting from the assembly of sequences performed into vector NTI software. (➜) genes encoded into pT181. That plasmid contains amongst other the gene *tet*, coding for a tetracycline/H+ antiporter conferring tetracycline resistance. The presence of pT181 in S30 was confirmed by the sequencing of S30. We obtained a circular contigs of 4,44 kb that perfectly match the sequence of pT181 (accession number: CP000045.2).

GENERAL DISCUSSION

In developed countries, the expected live-time of human has increased during the last century, principally due to both the development of the modern medicine and prosperity [346]. Population ageing observed in developed countries is due to decreases of both the birth and the fertility rates, probably due to the education and emancipation of women, and the decrease of crude mortality and infant mortality rates, but all these changes are not at all comparable when considering all parts of the world [375]. In developed countries, the population will continue to get older during the next century, even if some parameters remain unknown, such as human migrations [63]. With ageing, deterioration of joints increases together with the need for orthopedic devices able to replace the function lost by damaged articulations, explaining also why the use of indwelling medical devices in ageing patients has increased during the past century [14,376]. As bacterial biofilm related infections can develop on abiotic surfaces of medical devices, it is very likely that such infections will continue to occur and will probably increase, underlining the need to further investigate pathogenic bacteria forming biofilms.

Another important aspect is that different genetic determinants of bacterial resistances against antibiotics were identified, such as the *van* operon conferring resistance against vancomycin, or the penicillinase conferring resistance against penicillin [180,272]. The biofilm lifestyle increases the survival of bacteria in the presence of antibiotics, while genetic exchanges are increased inside biofilms [325]. Therefore, it could be that bacterial multispecies biofilms provide an opportunity for the acquisition of genes conferring resistance against antibiotics. The worldwide surge in bacterial resistance rates against antibiotics and the decreased availability of new antibiotics highlight the fact that we need to develop alternatives to antibiotic treatments [50]. In gram positive bacteria with low GC content, CodY seems to control virulence and biofilm formation as a function of the metabolic status of

bacteria, as summarized in the review (part D2). Therefore, CodY is an interesting target for the development of such alternative approaches, where pathogenic bacteria could be kept in a non-pathogenic status and beeing susceptible to clearing by the host immune system.

During my thesis, different projects were performed to study the formation of biofilm in *S. aureus*. First, we published a case report of a biofilm-related infection occurring on a cochlear implant in a patient (part I.B.). This work illustrates general aspects of biofilm-related infections such as the persistence of the infection despite antibiotic treatment and the need to re-operate the patient in order to remove the medical device. Indeed, 17 months after the surgery the patient felt an increasing retro-auricular pain combined with the apparition of a hematoma that had developed inside the muscle pouch containing the cochlear implant processor (part I.B.). The symptoms were treated with corticoids and amoxicillin/clavulanate. First, corticoids are steroids elaborated by the adrenal cortex and were used as anti-inflammatory agents in the present case. Second, amoxicillin corresponds to semi-synthetic penicillin. As this drug is susceptible to degradation by β-lactamase-producing bacteria, it was combined with clavulanic acid, a β-lactamase inhibitor produced by fermentation of *Streptomyces clavuligerus* [8]. After that first treatment, the swelling and pain disappeared in 15 days, but reappeared 6 weeks later. Puncture removed 3 cc of a citrin liquid, and subsequent culture of the liquid showed the growth of *Staphylococcus aureus* sensitive to all tested antibiotics with the exception of penicillin G. Today, most *S. aureus* strains are resistant to Penicillin G, notably when carrying beta-lactamases and/or mutated forms of penicillin binding proteins. The patient received then a treatment of ciprofloxacin and rifampin for 8 weeks, but the swelling, redness, and pain persisted. Ciprofloxacin is a second-generation fluoroquinolone killing *Staphylococcus aureus* bacteria by interfering with the topoisomerase IV enzymes GrlA and GrlB. These enzymes are required for the decatenation of DNA inside the bacteria, a process needed for DNA and protein synthesis. Mutations occurring inside *grlA* conferred resistance of *S. aureus* against fluoroquinolones [255].

Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding to the beta-subunit, thus preventing transcription to RNA and subsequent translation to proteins [342]. The combination of these two different antibiotics did not resolve the infection. Then, surgical drainage and irrigating the processor with antibiotics also did not allowed to resolve the infection. Finally, the processor was removed. Importantly, the strain was resisting various antibiotics treatment when grown *in vivo* as a biofilm deposited onto the implant inside the patient, whereas this strain remained susceptible when growing *in vitro* according to antibiogram with the exception of penicillin G. This illustrates the fact that strains susceptible to antibiotics can become resistant when growing in a biofilm without the need of a genetic support for this resistance.

The main difficulty encountered in this project was the limitation of the starting material, the explanted device. Additionally, the material was contaminated with debris of the patient, rendering more difficult the observation of the bacterial biofilm. For example, we used the crystal violet to stain the bacterial biofilm, which was correctly criticized by reviewers, because crystal violet could have stained cellular debris of patient's origin. But we were able to immuno-detect PIA located on the implant, and PIA remained a rather specific material generated by staphylococcal matrices. Reports of bacterial infections associated with an explanted medical device remain quite rare in comparison with the occurrence of such infections, probably because the main goal for clinicians is to treat rapidly the infection, and not necessarily to understand the lifestyle of bacteria that are encountered onto such infected devices. But such case reports are important for the evaluation of the incidence and the prevalence of medical device-associated infections. We cannot extrapolate that each bacterial infection occurring on a device involves a biofilm without some experimental evidence. Our approach involving the use of immuno-microscopy to identify a specific compound of the matrix is not useful in case of infections involving other types of bacteria. A compound specific of the biofilm lifestyle but not specific of the bacterial species, that could be specifically

detected by antibodies would be a useful diagnostic method, even if such substances remain to be identified (part B.2.).

Another project was the study of oleic fatty acid effects on *S. aureus* biofilms (part II.B.). This was studied before, notably by Campbell *et al* [45], but in our work, oleic acid showed opposite effects on the formation of biofilm, depending on the primary adhesion of bacteria to the surface of wells containing the inoculated medium. Indeed, when oleic acid was emulsified (0.1 v/v) in the medium prior to the inoculation of various *S. aureus* strains, biofilm formation was inhibited. But when the bacteria could grow in absence of oleic acid during 2 hours before the replacement of the medium with oleic acid emulsion, biofilm formation increased. This opposite phenotypes could be explained by the difference between the interactions occurring during the primary adhesion and those occurring during the maturation of biofilm (see part B.3.a and B.3.b). During the primary adhesion, also called reversible attachment, interactions occurring between the surfaces of both the bacteria and the solid mainly involved electrostatic charges or van der Waals interactions. Interestingly, oleic acid molecules interact with the membrane of *S. aureus*. Indeed, bacterial membranes are the prime targets of free fatty acid [87]. As the oleic acid molecule is composed of a hydrophobic carbon chain, the molecule should probably be able to insert itself inside the bacterial membrane, whereas the carboxyl terminal group carrying a negative charge should be kept at the surface of the bacteria. Therefore, this molecule should change the net charge of the *S. aureus* surface, becoming more negative according to the carboxyl groups, changing also the van der Waals interactions occurring between both previously mentioned surfaces. This is in accordance with a report showing that "*S. aureus* mutant bearing a stronger negative surface charge due to the lack of D-alanine esters in its teichoic acids can no longer colonize polystyrene or glass" [126]. These anthors further state "The mutation abrogates primary adhesion to plastic while production of the glucosamine-based polymer involved in later steps of biofilm formation is not affected" [126]. Additionally, oleic acid has an

inducing effect on biofilm formation by *S. aureus*, which could result from an ionic interaction of the positively charged PIA with the negatively charged oleic acid [123]. Thus our results suggest that oleic acid has dual and opposite effects on biofilm formation depending on the developmental phases of the biofilm; inhibiting primary adhesion but promoting maturation, in addition with a killing effect. Analysis of substances able to prevent biofilm formation remains of high potential, because problems that are the consequence of the formation of biofilm are highly difficult to solve, not only in the medical field, but also in industry (part B). Our paper was cited in a review considering the effects of different free fatty acids on bacteria [87]. These molecules are implicated in algae, animals and plants host defenses against pathogenic and opportunistic microorganisms. They have a broad spectrum of activities and their mode of action is not specific [87]. In case of human skin, free fatty acids are mainly produced by lipolytic cleavage of lipids secreted from the sebaceous glands [94]. There is increasing evidence that free fatty acids could be useful for bacterial killing, and each alternative to antibiotics should be carefully considered today.

The fact that adjunction of isoleucine can stop the growth of bacteria containing CodY allowed an additional alternative to control infection. This observation remains valuable only in cases where isoleucine functions as a cofactor for CodY. An important achievement was the minimal medium for *S. aureus* growth, allowing the study of the effects of amino acid supplementations and its effect on *S. aureus* growth in function of the presence of CodY. In a "normal situation", the isoleucine should reflect the availability of the various essential amino acids for the bacteria, sensed by CodY. Indeed, the primary function of CodY seems to be the derepression of genes implicated in extracellular digestion of proteins, transport of dipeptide and biochemical pathways involved in the synthesis of various amino acids when the bacteria is starved. In cases where the bacteria are starved for amino acids except isoleucine, the CodY regulon remains repressed whereas its derepression is needed for the acquisition of other amino acids. Interestingly, Δ*codY* mutant were able to grow in

absence of threonine by opposition to wild-type. In this case, isoleucine is present and the CodY regulon is repressed, among which are genes involved in the synthesis of threonine. But in the Δ*codY* mutants, the threonine synthesis pathway is totally derepressed, allowing endogenous synthesis of that amino acid and subsequent growth. Note that all genes involved in the threonine synthesis pathway were derepressed in absence of CodY (see microarrays data, part III.B.). In the study of the CodY regulon by microarrays, as well as that addressing the impact of CodY on the metabolism and the expression of virulence factors in *S. aureus* (part III.B.), fundamental aspects of CodY regulation in *S. aureus* were solved. Notably, we showed that the majority of CodY-regulated genes was involved in amino acid metabolism and was probably directly repressed by CodY in an *agr*-independent-manner. A pool of six genes involved in nucleotide metabolism and transport (ORFs names in Newman are NWMN_0016, NWMN_0379, NWMN_1110 to NWMN_1112 and NWMN_1249) was up-regulated in presence of CodY, putatively in an *agr*-independent manner even if the statistical significance was limited and except for the two first mentioned genes, suggesting that CodY probably represses a repressor of these genes, (see table 2 on page 2960 of the article presented in part III.B.). As the nucleotide GTP is probably an active additional cofactor for CodY in *S. aureus*, it seems logical that nucleotides metabolism will be also affected by CodY. 6 genes (NWMN_0016, NWMN_0379, NWMN_0110 to NWMN_0112, NWMN_1249) implicated into nucleotide transport and metabolism were up-regulated in presence of CodY, mainly in an *agr*-independent manner. Interestingly, the *cap* operon, implicated into the synthesis of the capsule, was almost completely derepressed in absence of CodY and in an *agr*-independent manner. It would be interesting to test if this derepression was sufficient for the presence of the capsule in the Δ*codY* mutant, using india ink staining, allowing to see the capsule as a refractive zone surrounding bacteria [36]. Interestingly, the high-molecular-weight polysaccharides-based capsule was associated with virulence and biofilm formation [36]. Virulence factors were mainly down-regulated in the parent as

compared to the Δ*codY* mutant, in accordance with the repressing effects of CodY on the *agr* system and the positive regulation of virulence factors by the *agr* system. For example, the catalse (*kat*), which is used for *S. aureus* identification in classical microbiology, (see figure 1 on page 17), and which is considered as a virulence factor because allowing the bacteria to resist against peroxygen released by immune cells, was derepressed in absence of CodY independently of the *agr* presence. In contrast, the delta-hemolysin (*hld*), encoded by the RNAIII, was derepressed in absence of CodY in an *agr*-dependant manner, (see table 2 on page 2961 of the article presented on part III.B.). Virulence factors that were up-regulated in presence of CodY were the Staphylocoagulase precursor (NWMN_0166), the superantigen-like protein 7 (NWMN_0394), the cell wall-anchored protein (NWMN_2392) and surprisingly, the *fnBPA* (NWMN_2399). Finally, an important pool of hypothetical proteins with unknown functions was derepressed in absence of CodY and independently of the *agr* presence, notably the gene called *lpl2nm* supposed to be coding for a lipoprotein according to informatics prediction.

The main goal of this thesis was to determine the function of CodY in the formation of biofilm in *Staphylococcus aureus*. Therefore, we generated a model with *codY* mutants, parents as well as complemented strains, and measured the biofilm of these strains using a crystal violet staining assay (part IV.B.). It appeared that the results were difficult to interpret principally because strain-dependent variations did not allow drawing a general conclusion for all cases. Even opposite phenotypes were observed in the crystal violet (CV) assay used to quantify biofilm produced by wild-type and Δ*codY* mutant in both UAMS-1 and S30 backgrounds (figure 1 of the article presented on part IV.B). Because the opposite phenotypes were statistically significant in both backgrounds, we decided to compare corresponding genomes in order to find their respective specificities. We have recently obtained the sequences of both genomes and we compared them. This work was performed in collaboration with Fasteris for the sequencing process (http://www.fasteris.com), and

with Dr David Hernandez for the assembling and the genome comparison processes [140]. The minimal size of sequences differencing between the two strains was set as 500 pb. The preliminary results show a total of 185 kb composed of 66 different sequences greater than 500 pb present specifically in S30 strain, and 182 kb composed of 62 sequences present specifically in UAMS-1. We are currently searching for ORFs localized in these sequences. Note that the point mutation present in *ica*R of S30 would not be seen when using that approach. This approach should allow the detection of genes bigger than 500 pb present only in one of the two strains, whereas the mean of *S. aureus* genes sizes is around 800 and 900 pb. Smaller differences could be seen in a second and more stringent approach. It would be also interesting to compare the genome of S30 to the genome of Newman. Indeed, we successfully disrupted *ica*R in Newman wild-type and the *ΔcodY* mutant using the TargeTron® Gene Knockout System, work performed by Dr. Anna Corvaglia, and monitor the production of PIA among these different strains (part IV.B.). Results of PIA quantification showed an increase of PIA more related to *ica*R disruption than *codY* absence in Newman, whereas the disruption of *codY* in S30 *ica*R-deficient strain resulted in a drastic decrease of PIA. Therefore, the genome of S30 strain probably contains at least one additional difference other than the ochre mutation in *ica*R, implicated in the formation of PIA, compared to the genome of Newman.

As CodY is a pleiotropic repressor probably controlling the expression of up to 5 % of the genome, and perhaps even more, and as the biofilm formation involved the expression of different pathways, the effects of CodY on the formation of biofilm should be seen as multi-factorial. The *ica*-dependent production of PIA as well as the *agr*-dependent production of adhesins seem to be the two major CodY regulated pathways implicated in biofilm formation in *Staphylococcus aureus*. But additional pathways are expected to be involved in biofilm formation, such as the expression of proteases able to degrade proteinaceous matrices, pathways implicated in cell death leading to the release of extracellular DNA, stringent response putatively connected to the GTP-CodY, and so on.

Different questions remain to be considered regarding CodY and biofilm formation. Are both GTP and BCAA CodY co-factors differentially implicated in the function of the studied pathways? Indeed, CodY in *S. aureus* seems able to be activated by both the GTP and the isoleucine cofactors, when considering the growth inhibition observed when isoleucine is present in excess (part III.B.) and the changes of biofilm quantities occurring specifically when *codY* is present and observed in presence of decoyinine (part IV.B.). CodY-DNA binding studies using both isoleucine and decoyinine could address this question. Does each CodY monomer recognize half of the consensus palindrome? Structural studies on CodY-DNA crystallized complexes could address this question, but the crystallization of that particular complex has not yet been achieved today. How is the stress responding regulator SigB connected with CodY and how in general are the different main regulators connected together? Complementation of naturally affected regulators among *S. aureus* strains combined to gene expressions studies seems a good approach for analyzing the interactions between the different identified regulators. But the results will probably be difficult to analyze, as the function of one or two regulators is already highly complex and redundant. As the PIA can also be implicated in the adhesion process, does the CodY regulated PIA production compensates the hypothetical CodY-related decrease of adhesins? One way to study that question could be adhesion studies between PIA producing and adhesins-defective mutants compared to PIA-defective and adhesins-expressing strains.

In addition, the formation of biofilm occurring on implanted material in clinical context is probably highly more complex when compared to the simplified situation of the *in vitro* growth of strains in culture media. Notably, interactions between the immune system and the bacterial biofilm were not studied in the present work. The impact of the immune system on the CodY regulated pathways and CodY regulated biofilms remains unknown according to my knowledge. Moreover, it appears that

some genes are naturally affected across the different strains of *S. aureus* probably rendering the interpretation of the data highly difficult even in the relative simple *in vitro* context.

During the work, a particular impressive observation was the *ica*R deficiency in S30. Only one bp mutation in approximately 2.8 millions of bp is supposed to have occurred naturally leading to a hyper-biofilm phenotype. This aspect was highly important in my study and remains highly essential in general because false conclusions can be extrapolated from results obtained with a strain, when that strain is affected in factors belonging to the studied pathways. The literature reported such cases of conclusions that are explained later by the identification of strain-specific genetic alterations. This time, it was highly difficult to identify such alterations because sequencing an entire genome of *S. aureus* was challenging financially, timely, and technically. The development of the new generation of sequencers now allows to control the genetic integrity of a studied bacterium. Finally, the general conclusion of the present work is that CodY probably represses the formation of PIA when the bacterium is well fed by repressing the *icaADBC* operon and activates the expression of *agr*-regulated adhesins by repressing the *agr* system again when the bacterium is well fed.

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