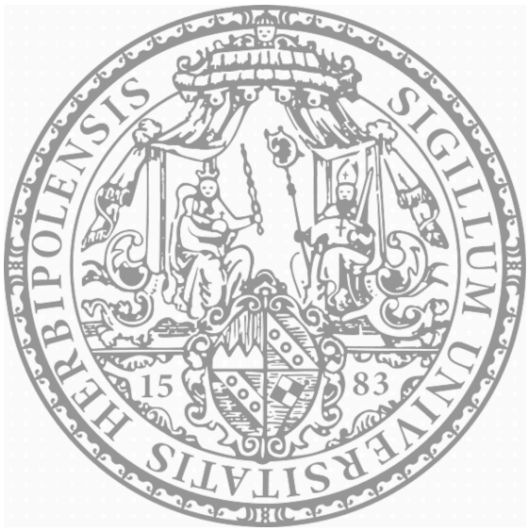

Divergence of cell-fates in multicellular aggregates of *Staphylococcus aureus* defines acute and chronic infection cell types

Divergenz von Zelldifferenzierung in multizellulären Aggregaten von *Staphylococcus aureus* grenzt Zelllinien für akute und chronische Infektionen voneinander ab

Doctoral thesis for the degree of Doctor of Philosophy (Ph.D.) in Science at Graduate School of Life Sciences, Julius Maximilians University of Würzburg

Section Infection and Immunity

Submitted by

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From

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AFFIDAVIT

Here, I declare that the doctoral dissertation entitled, "Divergence of cell-fates in multicellular aggregates of *Staphylococcus aureus* defines acute and chronic infection cell types" is the result of my own work, has been written independently and with no other sources and aids than quoted. I did not receive any help or support from commercial consultants. All sources and/or materials applied are listed and specified in the thesis. Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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EIDESSTATTLICHE ERKLÄRUNG

Hiermit erkläre ich an Eides statt, die Dissertation "Divergenz von Zelldifferenzierung in multizellulären Aggregaten von *Staphylococcus aureus* grenzt Zelllinien für akute und chronische Infektionen voneinander ab" eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben. Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

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Juan Carlos García Betancur

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TABLE OF CONTENTS

SUMMARY

Staphylococcus aureus is a versatile human pathogen that normally develops acute or chronic infections. The broad range of diseases caused by this bacterium facilitates the escape from the host's immune response as well as from target-specific antimicrobial therapies. Nevertheless, the underlying cellular and molecular mechanisms that enable *S. aureus* to cause these disparate types of infections are largely unknown.

In this work, we depicted a novel genetic program involved in the development of cell-fate decision, which promotes the differentiation of the staphylococcal cells into two genetically identical but differently heritable cell lines capable of defining the course of an infection, by simultaneously progressing to (i) a biofilm-associated chronic infection or (ii) a disperse acute bacteremia. Here, *S. aureus* growing in architecturally complex multicellular communities harbored different cell types that followed an exclusive developmental plan, resulting in a clonal heterogeneous population. We found that these cell types are physiologically specialized and that, this specialization impacts the collective behavior within the multicellular aggregates. Whereas one cell line that we named *BRcells*, promotes biofilm formation that engenders chronic infections, the second cell line, which we termed *DRcells* is planktonic and synthetizes virulence factors, such as toxins that can drive acute bacteremia.

We identified that the positive feedback loop present in Agr *quorum* sensing system of *S. aureus* acts a bimodal switch able to antagonistically control the divergence of these two physiologically distinct, heritable cell lines. Also, we found that this bimodal switch was triggered in response to environmental signals particularly extracellular Mg^{2+}, affecting the size of the subpopulations in specific colonization environments. Specifically, Mg^{2+}-enriched environments enhanced the binding of this cation to the staphylococcal teichoic acids, increasing the rigidity of the cell wall and triggering a genetic program involving the alternative sigma factor σ^B that downregulated the Agr bimodal switch, favoring the enrichment of the *BRcells* type. Therefore, colonization environments with different Mg^{2+} content favored different outcomes in the bimodal system, defining distinct ratio in the *BRcells/DRcells* subpopulations and the *S. aureus* outcome in our *in vitro* model of development of multicellular aggregates and, the infection outcome in an *in vivo* mice infection model.

In this prime human pathogen cell-fate decision-making generates a conserved pattern of heritable, physiological heterogeneity that actively contributes to determine the course of an infection through the emergence and spatio-temporal dynamics of distinct and specialized cell types. In conclusion, this work demonstrates that cell differentiation in pathogenic bacteria is a fundamental phenomenon and its understanding, is central to understand nosocomial infections and to designing new anti-infective strategies.

ZUSAMMENFASSUNG

Staphylococcus aureus ist ein wandlungsfähiges humanes Pathogen, das im Allgemeinen akute oder chronische Infektionen entwickelt. Das breite Spektrum von Krankheiten, die von diesem Bakterium verursacht werden, erleichtert es, sowohl der Immunantwort des Wirts als auch gezielten antimikrobiellen Therapien zu entgehen. Dennoch sind die zellulären und molekularen Mechanismen, die *S. aureus* die Entwicklung dieser verschiedenartigen Infektionsarten ermöglichen, weitgehend unbekannt.

In dieser Arbeit beschreiben wir ein neues genetisches Programm, das bei der Entwicklung der Zelldifferenzierung beteiligt ist und die Differenzierung der Staphylokokken-Zellen in zwei genetisch identische, aber unterschiedliche, erbliche Zelllinien fördert. Diese können den Verlauf einer Infektion bestimmen, indem sie sich gleichzeitig entwickeln zu (i) einer Biofilm-assoziierten chronischen Infektion oder (ii) einer sich ausbreitenden akuten Bakteriämie.

Hier verbirgt *S. aureus*, der in architektonisch komplexen multizellulären Bakteriengemeinschaften wächst, verschiedene Zelltypen, die einem einzigartigen Entwicklungsplan folgen, resultierend in einer klonal heterogenen Population. Wir haben festgestellt, dass diese Zellzypen physiologisch spezialisiert sind, und dass diese Spezialisierung das kollektive Verhalten innerhalb der multizellulären Aggregate beeinflusst.

Während eine Zelllinie, die wir als *BRcells* benennen, Biofilm-Bildung fördert, was chronische Infektionen erzeugt, ist die zweite Zelllinie, als *DRcells* bezeichnet, planktonisch und synthetisiert Virulenzfaktoren wie Toxine, die eine akute Bakteriämie verursachen können. Wir haben identifiziert, dass die im Agr *Quorum sensing* System von *S. aureus* vorhandene positive Rückkopplung als bimodaler Schalter agiert, der antagonistisch die Divergenz dieser beiden physiologisch unterschiedlichen, vererbbaren Zelllinien kontrolliert. Wir haben auch gefunden, dass dieser bimodale Schalter durch Signale aus der Umgebung ausgelöst wird, insbesondere durch extrazelluläres Mg^{2+}, wodurch die Größe der Subpopulationen in spezifischen Kolonisierungsumgebungen beeinflusst wird. Besonders Mg^{2+}-angereicherte Umgebungen fördern die Bindung dieses Kations mit den Teichonsäuren von Staphylokokken, welche die Steifigkeit der Zellwand erhöhen und ein genetisches Programm initialisieren, welches den alternativen Sigmafaktor σ^B beinhaltet. Dieser regelt den bimodalen Agr Schalter herunter und begünstigt die Anreicherung des *Brcells* Zelltyps. Daher begünstigen verschiedene Kolonisierungsumgebungen mit verschiedenem Mg^{2+} Gehalt unterschiedliche Ergebnisse im bimodalen System, welche sich in individuellen Verhältnissen der *Brcells/Drcells* Subpopulationen und dem Ergebnis für *S. aureus* – sowohl in unserem *in vitro* Modell der Entwicklung multizellulärer Aggregate als auch der Entzündungsentwicklung in einem *in vivo* Maus-Infektionsmodell.

In diesem primären Humanpathogen generiert die Zelldifferenzierung ein bleibendes Muster von vererbbarer, physiologischer Heterogenität, die aktiv dazu beiträgt, den Infektionsverlauf durch das

Auftreten und die räumlich-zeitliche Dynamik verschiedener spezialisierter Zelltypen zu bestimmen. Zusammenfassend zeigt diese Arbeit, dass Zelldifferenzierung in pathogenen Bakterien ein grundlegendes Phänomen ist. Diese zu erfassen ist zentral für das Verständnis nosokomialer Infektionen und die Konzeption neuer Strategien gegen Infektionen.

PREFACE

Despite the discovery of antibiotics, bacterial infections are still the most prevalent cause of mortality, due to the ability of microbes to evade conventional antimicrobial therapies (Cantón and Morosini 2011). Bacteria have refined multiple evolutionary traits to survive, persist and proliferate within the host. These features range from the acquisition of antibiotic resistance genes (Kos et al. 2012; Edwards et al. 2013) and the development of physiological and metabolic changes to scape immune response (Rooijakkers, van Kessel, and van Strijp 2005), to sophisticated mechanisms that involve phenotypic diversification and the arise of multicellular-level traits within clonal populations (Vlamakis et al. 2013; Saint-Ruf et al. 2014; Ackermann 2015).

Successful pathogens cause a broad range of diseases using diverse virulence factors, which enable them to escape from target-specific antimicrobial therapies (Bush et al. 2011). *Staphylococcus aureus* is one such pathogen, it is able to cause different types of life-threatening infections in hospital settings, from acute bacteremia to endocarditis, pneumonia and chronic biofilm-associated infections in prosthetic devices (Otto 2012b). The underlying cellular processes that enable *S. aureus* to cause these disparate types of infections are largely unknown, although specific yet to be described, extracellular signals are likely to play a role in the adaptation of *S. aureus* to generate distinct and locally define types of infections (Veening, Smits, and Kuipers 2008; Veening et al. 2008; López and Kolter 2010). Hence, determining the cellular processes and the nature of the extracellular signals that define the different outcomes of the infections is crucial to understand the development of difficult-to-treat bacterial infections and improve the strategies to overcome antimicrobial resistance.

In *S. aureus* the outcome of and infection is controlled to a large extent by the accessory gene regulator (Agr) *quorum* sensing (QS) program, which is activated in response to the self-produced extracellular signal AIP (autoinducing peptidic pheromone) (Recsei et al. 1986), which binds extracellular the histidine kinase AgrC and activates its cognate regulator AgrA via phosphorylation. AgrA~P induces changes in cellular gene expression programs resulting in the bacteria dispersing rapidly in the host and causing acute bacteremia (Thoendel et al. 2011). Bacterial dispersion requires, among others, the upregulation of surfactant phenol-soluble modulins (PSMα and PSMβ) that allow detachment of bacteria (M. Li et al. 2009; Peschel and Otto 2013), membrane-disrupting toxins (Hlα, Hlβ and Hlg) and other antigens that facilitate the disruption of tissues during septicemia (Recsei et al. 1986). In addition, the activation of Agr indirectly downregulates the *icaADBC* operon, necessary to produce the extracellular polysaccharidic matrix (PNAG or PIA) and many adhesion proteins (e.g. SpA) responsible for cell attachment and aggregation during biofilm formation (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a). Biofilms, which are associated with untreatable chronic infections, protect bacteria from antibiotics and host defenses. Thus, the Agr system antagonistically regulates the activation of the planktonic and the biofilm-associated lifestyles (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a), contributing to the development of acute and chronic infections. The Agr is inhibited by the σ^B stress-induced alternative sigma factor and is activated in response to different

types of cellular stresses and triggers the expression of a general-stress response regulon that includes the formation of biofilm, via Agr repression (Bischoff, Entenza, and Giachino 2001; Kullik, Giachino, and Fuchs 1998; Shaw et al. 2006; Lauderdale et al. 2009).

Since the Agr system antagonistically controls the process of biofilm formation and virulence, we explored the possibility that this constitutes an autonomous genetic program that contributes to the development of acute and chronic infections through the generation of cell heterogeneity and cellular differentiation within multicellular communities of *S. aureus*. The Agr system provided a natural model to study how *S. aureus* switches between a chronic and a acute infection and in addition, allowed us to identify the environmental cues promoting the activation of the program of cell heterogeneity and cell differentiation that makes one type of infection to prevail.

In this work, we report that clonal communities of the pathogenic bacteria *S. aureus* diversify into physiologically distinct cell types during the process of biofilm formation *in vitro* as *in vivo*. While, one cell type lineage specializes in developing biofilm-associated chronic infections, the other cell lineage specializes in dispersion-associated processes related to acute bacteremia. We found that this diversification is achieved through the activation of the QS system Agr (Novick et al. 1995) as a bimodal switch that antagonistically promotes the emergence of one cell lineage while inhibiting the other. The molecular mechanism underlying the bimodal behavior of the Agr involves the presence of extracellular Mg^{2+}, which serves as the environmental signal to downregulate Agr. This cation specifically binds to the cell wall teichoic acids (TAs) (Heptinstall, Archibald, and Baddiley 1970a; Lambert, Hancock, and Baddiley 1975b; Heckels, Archibald, and Baddiley 1975; Lambert, Hancock, and Baddiley 1975a; Heckels, Lambert, and Baddiley 1977) promoting an increase in cell wall rigidity, which serves a cue to activate a σ^B stress-response that represses Agr activity. The differential level of Agr activity within the bacterial community guides the bimodal switch responsible for the differentiation of the two exclusive cell lineages.

In Chapter 1, general and relevant aspects of *S. aureus* will be presented. We will review in detail strain diversity within *S. aureus* species, staphylococcal virulence factors, the process of biofilm formation, and structural characteristics of this bacterial species, such as the cell wall TAs.

In Chapter 2, important aspects about the genetic and physiological nature of the staphylococcal Agr QS mechanism will be presented, emphasizing on signal transduction, two-component systems (TCS) and the role of Agr in the development of staphylococcal multicellular communities. This section will lead to the description of Agr as a bistable switch in the context of a positive-feedback loops (PFL) and their role in the process heterogeneity and during staphylococcal pathogenesis.

In Chapter 3, we will present general notions on cell heterogeneity and its relevance in the process of cell differentiation, bacterial multicellularity and bacterial pathogenesis. This chapter will include specific examples of how this concept has been proven to exist in *S. aureus*.

INTRODUCTION

Chapter 1

1. *Staphylococcus aureus*, a prime human pathogen

Staphylococcus aureus is a Gram-positive facultative anaerobe cocci with a diameter of 1 μm, present as a commensal species in the anterior nares of about 1 of every 3 people in the general population (Plata, Rosato, and Wegrzyn 2009) but also, is considered as one of the most frequent human pathogens nowadays. It was originally isolated from surgical wounds by the Scottish surgeon Alexander Ogston in 1881 (Thomer, Schneewind, and Missiakas 2016) and, has been catalogued as a prime human pathogen due to its particular and versatile pathogenicity potential that causes diverse life-threatening infections (Costerton et al. 1987; A. L. Cheung and Projan 1994; Costerton 1999; P. S. Stewart and William Costerton 2001; Otto 2012b; Joo and Otto 2012; Otto 2012c; Sanchez et al. 2013).

S. aureus is the most common causes of intrahospitalary infections, particularly in intensive care units (ICU) (van Hal et al. 2012; Haar and Amato-Gauci 2015) and the leading cause of prosthetic and medical devices bacterial infection (Götz 2002; Barrett and Atkins 2014). In the preantibiotic era, mortality rates due to *S. aureus* bacteremia (SAB) reached 83% while nowadays; the overall rates worldwide are still high and estimated in a 30% (van Hal et al. 2012). This mortality rate is caused by the inherent arsenal of virulence factors, the rapid evolution of resistance mechanisms against last resort antibiotics in the methicillin and vancomycin resistant *S. aureus* strains (MRSA and VRSA) and the lack of an efficient and effective vaccination therapy (Köck et al. 2010; Thomer, Schneewind, and Missiakas 2016).
The annual mortality reaches more than 15000 cases in the European union (EU) and more than 20000 in the United States (Köck et al. 2010; Haar and Amato-Gauci 2015; Thomer, Schneewind, and Missiakas 2016). Recurrent staphylococcal infections are now a major health-care system concern, accounting for annual extra in-hospital costs estimated in nearly EUR 500 millions only in the EU (Haar and Amato-Gauci 2015). It has been observed that the probability of developing a recurrent staphylococcal infection after complete antibiotic treatment is 25% (Thomer, Schneewind, and Missiakas 2016), which supposes a major challenge for clinicians and for the health system.

Patients with indwelling catheters, endotracheal intubation, medical implantation of foreign bodies and hemodialysis, have shown increased rates of recurrent and chronic *S. aureus* infections, as well as higher mortality rates (Thomer, Schneewind, and Missiakas 2016). In addition, host factors such as age, gender, ethnicity, socioeconomic status, immune status and the presence of comorbidities and baseline conditions, have positively shown to have a great impact in the probability of dying of SAB (van Hal et al. 2012; Thomer, Schneewind, and Missiakas 2016). Diverse epidemiological studies have shown raises in mortality rates that range from 5% up to 60% as direct consequence of specific host factors (Köck et al. 2010; van Hal et al. 2012).

Mortality outcomes showed to be highly dependent on the source of infection, showing higher mortalities in patients with infective endocarditis and bacteremic pulmonary infections (20.5%), compared to the patients with venous catheter-related and orthopedic implant infections (6.8%) (van Hal et al. 2012).

Mortality risk	Source of infection	Mortality rate
Low	Intravenous catheter. Urinary tract. Ear, nose, throat. Gynecological.	5%
Intermediate	Bone and Joint. Soft tissue. Unknown foci.	13%
High	Endovascular. Lower respiratory tract. Intra-abdominal. Central nervous system.	30%

Table 1: Mortality stratification due to *S. aureus* infections depending on the source of infection. Adapted from (van Hal et al. 2012).

Nevertheless, the main factor associated with the acquisition of a *S. aureus* invasive infection is the presence of prosthetic devices (Thomer, Schneewind, and Missiakas 2016). Central venous catheters, implanted materials and orthopedic prostheses constitute the ideal niches for the proliferation of *S. aureus* through biofilm formation but also, are considered the most direct entry point to the intravascular space (Götz 2002).

In addition to the host factors already mentioned, the vast repertoire of molecular strategies employed by *S. aureus* to colonize and to evade immune response and antimicrobial therapy, represent a major challenge. The recurrence in *S. aureus* infections supports the hypothesis that this bacterium has evolved sophisticated genetic mechanisms to coordinately switch between chronic and acute infections, depending on the environmental conditions.

1.1. Genetic background in staphylococcal strains and its role in disease

The emergence of the MRSA and VRSA strains is the classical example of how the bacterial genetic background determines the development and course of a staphylococcal infection (Gordon and Lowy 2008; Köck et al. 2010). In MRSA strains, the presence of the mobile genetic element (MGE) known as the staphylococcal cassette chromosome mec (SSCmec) (Katayama, Ito, and Hiramatsu 2000), which carries the antibiotic resistance among other virulence factors, determines the course of the infection and increase mortality from nosocomial MRSA bacteremia when compared with methicillin susceptible *S. aureus* (MSSA) strains (Gordon and Lowy 2008).

In a similar manner, most clinical strains of *S. aureus* possess particular genetic characteristics associated with the foci of infection, making them suitable for the study of a wide range of pathogenic traits.

In particular, *S. aureus* strain Newman is a clinical strain originally isolated from a typical biofilm-associated infection of human tubercular osteomyelitis (DUTHIE and LORENZ 1952). This antibiotic susceptible isolate is recognized for the variety of virulence factors and infective phenotypes. Interestingly, the reduced ability of this strain to form biofilms has been linked with nonsense mutations in the *fnbA* and *fnbB* genes, which generate truncated fibronectin-binding proteins FnbA and FnbB that cannot bind the cell surface (Grundmeier et al. 2004; O'Neill et al. 2008; Houston et al. 2011). The introduction of an intact copy of the *fnbA* gene increased biofilm formation in strain Newman to similar levels of the strain UAMS-1 (Mainiero et al. 2010; Mrak et al. 2012). But most importantly, the poor biofilm forming phenotype *in vitro* of the strain Newman has been strongly associated with a naturally acquired mutation in the *saeS* gene, which substitutes a Proline for a Leucine at position 18 (L18P or $saeS^{L}$ allele) (Baba et al. 2007; Adhikari and Novick 2008). This mutation generates a constitutively active histidine kinase, which provokes a hyperactive SaeRS TCS (Giraudo et al. 1999; Steinhuber et al. 2003; Baba et al. 2007; Adhikari and Novick 2008; Cue et al. 2015). Nevertheless, under specific culture conditions, this clinical isolate has shown higher expression of attachment proteins (Harraghy 2005; Schafer et al. 2009) and significantly higher adhesion to Fibronectin matrixes (Blickwede 2005), suggesting that the reduced biofilm phenotype results from the standard laboratory culture conditions.

In particular, the SaeRS locus is involved in the regulation of the gene expression of several exoproteins, including biofilm-related proteins (Spa, Emp, Eap, FnbA, Efb and Fib, among others) a DNase nuclease, hemolysins and coagulases (Steinhuber et al. 2003; Giraudo et al. 2003; Adhikari and Novick 2008; Mainiero et al. 2010). In addition, the SaeRS acts synergistically with the QS system Agr, coordinating the effects of environmental signals with QS (Novick 2003a). Those environmental signals seem to be related to changes in bacterial cell wall since, it was demonstrated that SaeS kinase is preferentially activated by alterations in the bacterial membrane, allowing the pathogen to cope with stressful external conditions (Geiger et al. 2008).

The discrepancy between the biofilm phenotypes *in vitro* and *in vivo* might reflect the utility of an altered SaeRS functionality in nature, where *S. aureus* is usually exposed to rapidly fluctuating environmental conditions (la Fuente-Núñez et al. 2013). In fact, a comparative study of inter-strains infectivity showed that Newman was in general, the most virulent strain in a sepsis model when compared to other clinical isolates (Herbert et al. 2010). In addition, it was recently demonstrated that the SaeRS is fundamental for appropriate biofilm development, by controlling the concerted migration of bacterial cells within organized multicellular aggregates (Moormeier et al. 2014).

2. Staphylococcal toxins, major virulence factors driving acute infections

Multiple host and bacterial factors define the causes and course of chronic and acute infections. In *S. aureus*, the characterization of virulence factors have allowed to catalogue them in large families composed of diverse members that share functional, genetic and structural characteristics.

S. aureus possesses an important arsenal of essential virulence factors (Otto 2012c; Zecconi and Scali 2013), which are specifically active during chronic and acute presentations of the disease. The

prevalence of a clonal lineage of *S. aureus* highly depends on the presence of a particular toxin gene or, on the expression level of a subset of them (GOERKE 2004; Henry 2012). In particular, extracellular toxins (exotoxins) constitute essential virulence factors associated with acute presentations of the *S. aureus* as well as with the evasion of the immune response during the early stages of the staphylococcal disease.

Many virulence factors of *S. aureus* belong to the group of secreted cytotoxic factors or toxins, which are generally highly expressed during process of acute infection (Powers and Wardenburg 2014; Dal Peraro and van der Goot 2016). Specifically, these toxins promote a generalized inflammatory state, impair immune response, alter coagulation and compromise the vascular integrity at organ and cellular level (Powers and Wardenburg 2014; Dal Peraro and van der Goot 2016).

Some of the most extensively studied toxins in *S. aureus* are the polypeptides belonging to the leukocidin family (Henry 2012). This family includes the Panton-Valentine (PVL), the AB/GH (LukAB/GH), the ED (LukED) and the α- and γ-hemolysin (Hla and Hlg) (Henry 2012). These bi-component exotoxins targets a broad range of immune cells like neutrophils (Löffler et al. 2010), T lymphocytes, polymorphonuclear cells, macrophages (Pédelacq et al. 1999), mononuclear phagocytes (Powers and Wardenburg 2014) and even erythrocytes (Pédelacq et al. 1999; DuMont and Torres 2013). Their mode of action relies on the selective targeting of specific immune cells and the formation of lethal transmembrane hetero-octamer β-barrel pores (Pédelacq et al. 1999; DuMont and Torres 2013) through the sequential binding of the slow- and fast-fractions (WOODIN 1960; Henry 2012). Although, these toxins are mainly secreted, are present also attached to the staphylococcal surface, suggesting an alternative but mechanistically similar mode of action during phagocytosis and attachment to the host matrix (de Bentzmann et al. 2004; Tristan et al. 2009; Ventura et al. 2010; Dal Peraro and van der Goot 2016). The cellular receptors for leukocidins include the Toll-Like Receptors 2 and 4 (Inden et al. 2009; Zivkovic et al. 2011; Henry 2012) and some membrane lipids, although in general, are largely uncharacterized.

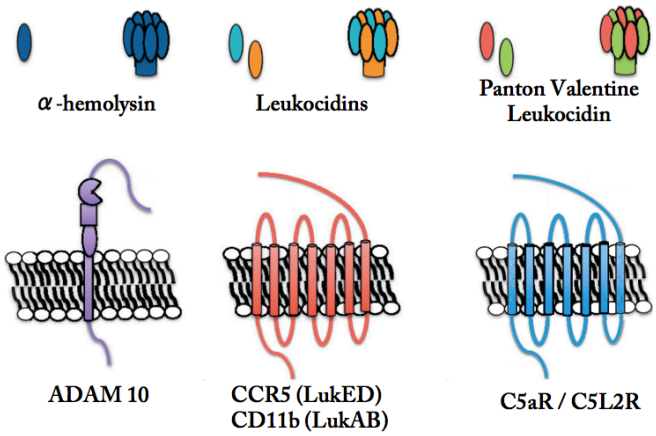

Figure 1. Mode of action and proposed cellular receptors for the major exotoxins secreted by *S. aureus*. These cytotoxins mediate cell disruption through a pore-forming strategy. Adapted from (DuMont and Torres 2013).

Hemolysins such as the α- and β-hemolysins are another class of membrane-damaging staphylococcal virulence factors. The mechanism of action of the α-hemolysin resembles the leukocidins (Bhakdi and Tranum-Jensen 1991) where an aqueous pore is formed to transport of K^+ and Ca^+, creating a misbalance in ion concentrations that leads to the necrotic death of the target cell (Henry 2012). These hemolysins constitute a particular class of toxins since they act upon binding to specific host receptors. The α-hemolysin binds phosphocholine lipids (Galdiero and Gouaux 2004), A-disintengrin and the Metalloproteinase Domain-containing protein 10 (ADAM10) (Powers, Kim, and Wang 2012). The β-hemolysin acts through an enzymatic reaction that involves the hydrolysis of the sphingomyelin in red blood cells, altering the membrane fluidity and destabilizing the bilayer structure (Ira and Johnston 2008). An alternative mechanism for β-hemolysin action resides the formation of ceramide-rich signaling platforms (Henry 2012). In particular, this toxin has proven to be specific targeting monocytes, but no activity against inactive lymphocytes, granulocytes or fibroblasts has been observed, where no specific receptor for β-hemolysin has been characterized yet (Walev et al. 1996).

Another well-recognized *S. aureus* virulence factor is the immune evasion cluster (IEC) (van Wamel et al. 2006; Rooijakkers et al. 2006). In nature, the β-hemolysin-converting bacteriophages βC-ϕs (ϕ13 and ϕ42) incorporates IEC into the chromosome using a specific 14-bp sequence via the attB-attP mechanism of phage-related *S. aureus* pathogenicity islands (SaPI) (Coleman, Knights, and Russell 1991; Novick, Christie, and PenadEs 2010). IEC insertion inactivates the β-hemolysin but incorporates several immune-modulating genes such as the staphylokinase (*sak*), the staphylococcal complement inhibitor SCIN or (*scn*), the staphylococcal enterotoxin A (SEA) and P (SEP) (*sea* and *sep*) and the chemotaxis inhibitory protein CHIP (*chp*) genes (van Wamel et al. 2006; Henry 2012; Verkaik et al. 2014). There are 7 different IEC types characterized, all carrying the *scn* gene and a different combination of the *sea*, *sak* and *chp* genes (van Wamel et al. 2006; Verkaik et al. 2014).

The staphylokinase is a 15.5 KDa thrombolytic protein that activates the plasminogen and promotes plasmin formation (Bokarewa, Jin, and Tarkowski 2006), preventing phagocytosis (Verkaik et al. 2014) and facilitating the bacterial penetration into deeper tissues (Bokarewa, Jin, and Tarkowski 2006). In addition, this protein destroys directly antimicrobial peptides such as α-defensins as well as pro-opsonic immunoglobulin IgG (Rooijakkers et al. 2005; van Wamel et al. 2006; Verkaik et al. 2014).

Figure 2. Genomic organization of a typical IEC element or *S. aureus*. The different IEC types depend on the presence of the different loci, except the *scn* gene, common to all. Adapted from (van Wamel et al. 2006).

The SCIN family is composed of four 9.8 KDa proteins structurally similar to the IgG-binding modules of the SpA protein (Ricklin et al. 2009). Once secreted, SCIN binds to C3b and blocks the formation of the complement complexes on the surface of *S. aureus*, abolishing phagocytosis, neutrophil chemotaxis and opsonophagocytosis (van Wamel et al. 2006; Ricklin et al. 2009; Verkaik et al. 2014).

The staphylococcal enterotoxins SEA and SEP are serologically classified extracellular small peptides from 26 to 29 KDa responsible for the toxic shock-like syndrome during food poisoning (Mehrotra, Wang, and Johnson 2000; Ortega et al. 2010). Together with the toxic shock syndrome toxin 1 (TSST-1), enterotoxins belong to the group of staphylococcal superantigens (Mehrotra, Wang, and Johnson 2000; Ortega et al. 2010), which promote a massive and non-specific lymphocyte proliferation (Rahimpour et al. 1999).

Finally, the CHIP protein blocks neutrophil chemotaxis by binding the formylated peptide receptor and attenuates the complement C5a receptor on neutrophils (de Haas et al. 2004; Postma et al. 2004; van Wamel et al. 2006).

3. Cell wall-anchored proteins, virulence factors involved in adhesion, immune evasion and biofilm formation

The cell wall-anchored proteins (CWAs) are cell surface adhesins specifically involved in establishment and proliferation of the staphylococcal disease, through the adhesion of the *S. aureus* cells to the host tissue and the impairing of the innate immune response (Zecconi and Scali 2013; T. J. Foster et al. 2014). CWAs facilitate the dissemination of *S. aureus* from the bloodstream to the different organs (Thomer, Schneewind, and Missiakas 2016) attaching to different plasma molecules and endothelial cells (Sinha et al. 1999; T. J. Foster et al. 2014).

Within CWAs, MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Patti et al. 1994) are CWAs covalently anchored to the bacterial surface, ranging from 50 up to 240 KDa, which play a central role in the facilitation of microbial adhesion to the host extracellular matrix (ECM) and in the internalization of the pathogen (Isberg and Leong 1990). Apart of their complementary function in immune evasion (Zecconi and Scali 2013), many of these MSCRAMMs adhesins have been also proved to actively cooperate during the process of biofilm formation (Cucarella et al. 2001; Cucarella et al. 2002; Walsh et al. 2008; Merino et al. 2009; Gruszka et al. 2012; Speziale et al. 2014).

Unlike toxins, MSCRAMMs usually recognize a specific ligand of the host ECM (Patti et al. 1994) such as collagen, fibrinogen or fibronectin. Depending on the *S. aureus* strain, the repertoire and pattern of MSCRAMMs expressed on the surface can vary greatly, which supposes a high degree of genetic regulation (McCarthy and Lindsay 2010). Indeed, models of *S. aureus* hematogenous infection have demonstrated the association of specific MSCRAMMs with particular ECM components such as collagen fibers, bone and cartilage during the initial stages of biofilm formation (Voytek et al. 1988).

Staphylococcal MSCRAMMs are structurally diverse and they are classified based on its modular structure. Most CWAs possess a N-terminal sequence (S) that serves as a secretory signal, followed by a unique and non-repetitive sequence of diverse size (U), a cluster of repetitive domains (R), a cell wall-spanning domain (W), a hydrophobic membrane-spanning domain that contains the classical MSCRAMMs motif LPXTGX (Leucine-Proline-X-Threonine-Glycine-X) (M) and a characteristic

sorting signal positively charged at the carboxy terminus (C), which facilitates a covalent anchorage to the peptidoglycan cell wall (Patti et al. 1994; T. J. Foster et al. 2014). The M motif is used by the enzyme Sortase A to anchor covalently these proteins to the peptidoglycan (Navarre and Schneewind 1999).

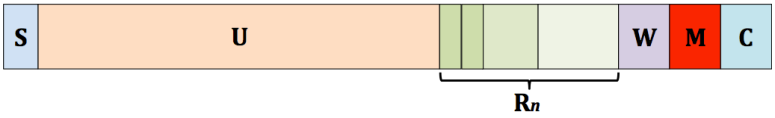

Figure 3. Basic structure of a typical staphylococcal MSCRAMM protein. MSCRAMM size in Gram-positive bacteria range between 16 and 110 KDa. Adapted from (Patti et al. 1994).

The staphylococcal protein A (SpA) is the best-studied CWAs due to its role in attachment during the process of biofilm formation, as well as its strong immune-modulatory effect (Forsgren and Nordström 1974; Klaesson et al. 1993; Palmqvist et al. 2002; Merino et al. 2009; Thammavongsa et al. 2015). SpA possesses five subdomains that bind the Fc region of the IgG and impair opsonization during phagocytosis. In addition, SpA binds the von Willebrand factor, the complement protein C3 and the TNFR1 receptor, increasing adhesion to platelets, interfering with the complement activation and reducing the inflammatory signaling (Zecconi and Scali 2013).

Importantly, *in vitro* and *in vivo* studies have also shown that SpA is an essential component of the staphylococcal biofilm, acting independently or synergistically with the PNAG exopolysaccharide, another major component of these biofilms (Toledo-Arana et al. 2005; Merino et al. 2009). These findings confirm that SpA, as many other MSCRAMMs, is critical during the initial steps of biofilm formation (Merino et al. 2009). Biofilm formation in many *S. aureus* strains depends mainly on CWAs rather than on the PNAG exopolysaccharide, highlighting the relevance of the *ica*-independent pathway of biofilm formation (T. J. Foster et al. 2014).

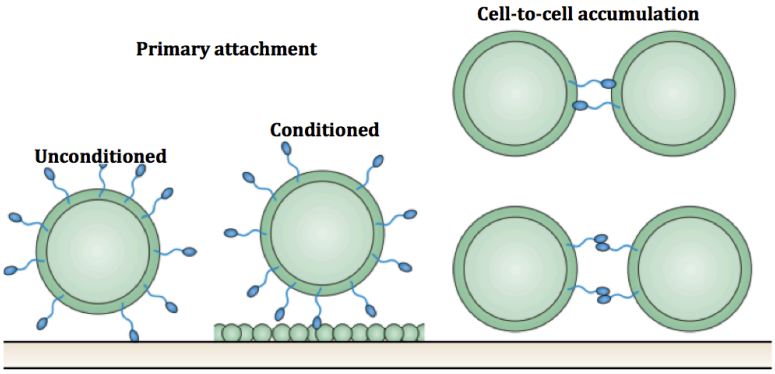

Figure 4. Modes of interaction of different MSCRAMMs during the process of biofilm formation. Primary attachment can be unconditioned or conditioned by plasma proteins. Accumulation can be mediated by the interaction of these adhesins with the cell wall or by protein-protein interactions. Adapted from (T. J. Foster et al. 2014).

Other CWAs involved in biofilm formation are the Bap family (Cucarella et al. 2001), the Zn-dependent SasG (Corrigan et al. 2007; Geoghegan et al. 2010), the immunomodulatory extracellular adherence protein (Eap) also known as Map (Palma, Haggar, and Flock 1999), the ClfA and the ClfB adhesins and, the Fibronectin-binding proteins FnBPA and FnBPB (Walsh et al. 2008). All these adhesins showed to be fundamental promoting the attachment of the staphylococci over the surface and creating a multilayer bacterial cluster via cell-to-cell interactions.

CWAs are crucial for cell attachment during the initial stages of biofilm formation but also, are an important part of the staphylococcal extracellular matrix that protects the biofilm. Proteins such as SpA, SasC, SasG, Embp, Bap, FnbpA and FnbpA are present alone or in association with the PNAG exopolysaccharide in different staphylococcal species (Joo and Otto 2012).

Adhesin	**Gene name**	**Putative ligand**	**Function in infection**
Fibronectin binding protein A, B	*fnbA* *fnbB*	Fibronectin, fibrinogen and elastin.	Adhesion to ECM. Endocarditis. Mastitis. Foreign body infection.
Clumping factor A, B	*clfA* *clfB*	Fibrinogen	Adhesion to ECM. Nasal and skin colonization. Endocarditis. Kidney abscesses. Septic arthritis.
Collagen adhesin	*cna*	Collagen	Immune evasion. Prevention of complement activation. Septic arthritis.
Staphylococcal protein A	*spa*	IgG, IgM, TNFR1, von Willebrand factor.	Biofilm formation and immune evasion. Prevents opsonization and complement activation. Septic arthritis.
Iron-regulated surface determinant A, B, C, H	*isdA* *isdB* *isdC* *isdH*	Fibrinogen, fibronectin, transferrin, hemoglobin, hemin, fetuin, haptoglobulin.	Adhesion to ECM of epithelial cells. Immune evasion. Survival in neutrophils. Invasion of non-phagocytic cells. Degradation of C3b. Iron acquisition in iron-restricted environments. Resistance to bactericidal lipids, antimicrobial peptides and lactoferrin. Kidney abscesses.
Extracellular matrix protein-binding protein	*emp*	ECM.	Biofilm formation in iron-depleted conditions.
Extracellular adherence protein (also known as Map).	*eap* (*map*)	Laminin, Collagen, Fibronectin, ICAM-1, prothrombin.	Biofilm formation in iron-depleted conditions. Immune evasion. Interferes with T cell-mediated responses.
Biofilm-associated protein	*bap*	Endoplasmin (gp96 / HSP90B1)	Biofilm formation. Prevents entry of S. aureus into epithelial cells. Only found in bovine strains.
Serine-aspartate repeat protein C, D, E (SdrE, also known as Bone sialoprotein-binding protein).	*sdrC* *sdrD* *sdrE* (*bbp*)	Fibrinogen, β-Neurexin, complement factor H.	Immune evasion. Degradation of the complement factor C3b. Endocarditis infection.
S. aureus surface protein A, B, C, D, F, G, J, K, L X	*sasA* (*sraP*) *sasB*	Human platelets. Human epithelial	Biofilm formation in the presence of Zn^{2+}. Immune

	sasC *sasD* *sasF* *sasG* *sasJ* *sasK* *sasL* *sasX*	cells.	evasion. Binding to platelets. Nasal and skin colonization. Endovascular infection.
Plasmin-sensitive protein	*pls*	Fibronectin, IgG.	Cell-to-cell interactions. Binding to host's lipids and glycolipids. Septic arthritis.
Elastin-binding protein	*ebpS*	Elastin.	Biofilm formation in Zn^{2+}-dependent manner.
Enolase	*eno*	Laminin.	Abundant in biofilm conditions. Mastitis.

Table 2. Some of the well-characterized CWAs of *S. aureus*, their putative eukaryotic ligand (if reported) and role in infection and biofilm formation. Adapted from (Zecconi and Scali 2013; T. J. Foster et al. 2014).

4. Phenol soluble modulins, reconciling virulence with biofilm formation

Phenol-soluble modulins (PSMs) are a class of amphipathic membrane-damaging small peptides (< 5 KDa) with diverse roles in staphylococcal pathogenesis that include lysis of red and white blood cells, phagosomal scape and immune evasion, stimulation and modulation of the inflammatory response, antibacterial activity, intracellular persistence, biofilm formation and structuring, biofilm maturation and dissemination (Mehlin, Headley, and Klebanoff 1999; Otto et al. 2004; Yao, Sturdevant, and Otto 2005; R. Wang et al. 2007; Verdon et al. 2009; Schwartz, Syed, Stephenson, Rickard, and Boles 2012a; Periasamy et al. 2012; G. Y. C. Cheung, Joo, et al. 2013; Otto 2014).

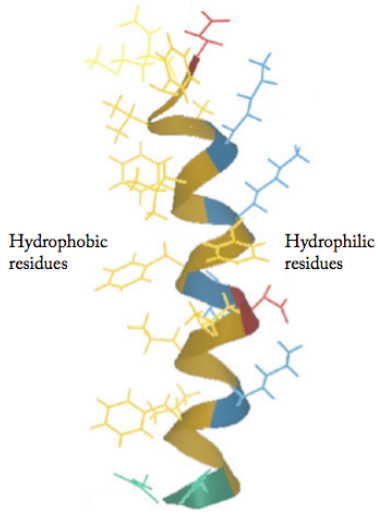

Figure 5. Typical structure of the staphylococcal PSMs. Here, a representative diagram of the PSMα3. Adapted from (G. Y. C. Cheung, Joo, et al. 2013; G. Y. C. Cheung, Kretschmer, et al. 2013).

PSMs include, the neutrally charged δ-hemolysin or PSMγ (26 aa and embedded in the RNAIII regulatory RNA), four positively charged PSMα (22 aa), two negatively charged PSMβ (44 aa) and the

PSMmec, which is only found in HA-MRSA strains and is encoded within 3 of the 11 described MGE SSCmec genomic islands responsible of the methicillin resistance (S. Li et al. 2013).

The lytic ability of PSMs resides in their capacity to aggregate and form transmembrane pores that destabilize the plasma membrane via lipid binding. Also, at high concentrations PSMs solubilize cell membranes through a detergent-like mechanism due to their strong surfactant-like properties (Otto 2009; Peschel and Otto 2013; S. Li et al. 2013).

PSMs possess a pronounced immunomodulatory effect over dendritic cells (DC), acting upon binding to the immune receptor N-formyl-peptide receptor 2 (FPR2) and contributing to the immune modulation and evasion that characterizes highly virulent *S. aureus* strains (Peschel and Otto 2013; Schreiner et al. 2013).

Recent experimental evidence has shown that PSMs are important virulence factors involved in the initial evasion of the immune response, facilitating the survival of *S. aureus* in intracellular compartments of non-professional phagocytes for prolonged periods of time and then, promoting the reactivation of the pathogen after periods of persistence within these cells (Giese et al. 2011). In addition, at low concentrations PSMs are sensed by the immune system and elicit neutrophil activation (Kretschmer et al. 2010), promoting *S. aureus* uptake and intracellular persistent survival (G. Y. C. Cheung, Duong, and Otto 2012).

PSMs are potent antibacterial and bacteriostatic peptides, due to their enhanced and differential binding to prokaryotic lipids and lipoproteins (Rautenberg et al. 2011; Chatterjee et al. 2013). This mechanism mediates staphylococcal colonization in the competitive environment created by the human microflora and apparently, constitutes the original role of these peptides, from where it is believed the virulent characteristics evolved (Saravanan Periasamy 2012). The lack of any secretion signal peptide and their short size, led to the discovery of a dedicated PSMs secretion system, based on a four-component ABC transporter, which selectively exports all the PSMs and at the same time provides immunity to their lytic action (Chatterjee et al. 2013). No homologous systems have been found in other bacteria, supporting the hypothesis that PSMs originally evolved as a cluster of antibacterial peptides to compete against other bacteria found in the colonization niche selected by this human commensal (Chatterjee et al. 2013).

In addition, PSMs are fundamental structural components of the staphylococcal biofilm, particularly involved in the maturation of the multicellular structure and the dispersal of the staphylococcal cells (R. Wang et al. 2007; Queck et al. 2009; R. Wang et al. 2011; G. Y. C. Cheung et al. 2011; Periasamy et al. 2012). In particular, PSMβ are responsible for channel formation within mature biofilms of *S. epidermidis* and *S. aureus* and also contribute to the subsequent cell dispersion process (R. Wang et al. 2011; Periasamy et al. 2012).

The pronounced amphipathy and surfactant properties of PSMs contributes to PSM aggregation helps cell spreading in solid surfaces and emulsifies nutrients for uptake during biofilm expansion (Peschel and Otto 2013). In particular, PSMα and the PSMγ lower the surface tension of the liquid-to-air interface during biofilm development (Kaito and Sekimizu 2007; Tsompanidou et al. 2011; Tsompanidou et al. 2012) similar to the mechanism employed by other organisms that secrete

biosurfactants to develop multicellular structures (Branda et al. 2001; Boles, Thoendel, and Singh 2005; Angelini, Roper, and Kolter 2009; Schwartz, Syed, Stephenson, Rickard, and Boles 2012a).

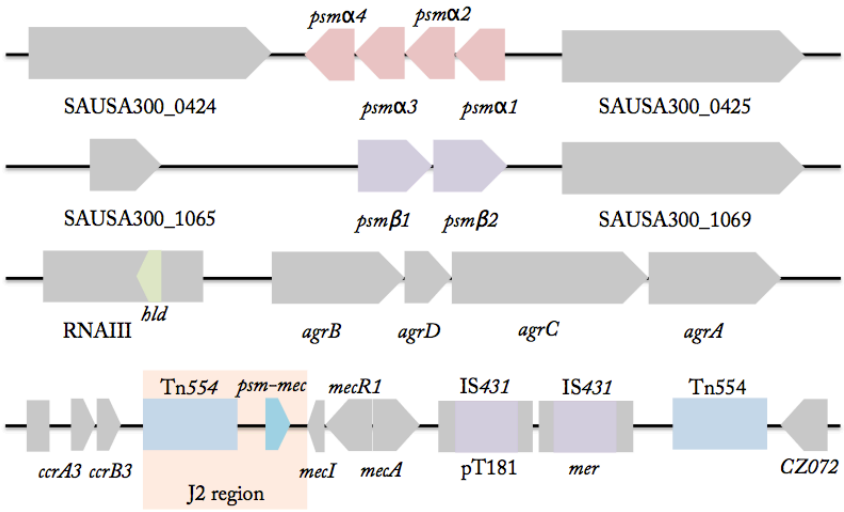

Figure 6. Genomic organization of the different PSMs operons and genes in *S. aureus*, based on the annotation for *S. aureus* strain USA300 FPR3757. The PSM*mec* is located within the SSC*mec* elements (types II, III and VIII) found in most MRSA strains. Adapted from (Peschel and Otto 2013).

Deformylated PSMs can polymerize and form fibrillar structures with amyloid-like properties that significantly increase the biofilm structure *in vitro* (Schwartz, Syed, Stephenson, Rickard, and Boles 2012b). Finally, the cell-cell disruptive capacities exhibited by these peptides explain how these peptides contribute to the detachment of the staphylococcal cells from the mature biofilm (Otto 2012a). Although, all *S. aureus* strains carry the PSMs genes (Peschel and Otto 2013; Schreiner et al. 2013), important differences in their expression have been observed among different *S. aureus* strains (M. Li et al. 2009), suggesting a strain-specific genetic regulation for these peptides. Gene regulation of PSMs might be related to their dual and antagonistic role during biofilm formation (R. Wang et al. 2011): while amyloidal PSMs promote biofilm structuring and maturation (S. Li et al. 2013), soluble monomeric PSMs contribute to biofilm disassembly and cell dispersal, suggesting a synergistic function of all PSMs for the concerted development of these multicellular aggregates.

PSMs are transcriptionally regulated by the AgrA (Pang et al. 2010; Periasamy et al. 2012) and, Agr-deficient strains display robust and unstructured biofilms *in vitro* unable to disseminate efficiently, due to the absence of these multifunctional peptides (Vuong et al. 2000; Vuong et al. 2003; R. Wang et al. 2011; Periasamy et al. 2012). In the particular case of the HA-MRSA strains, the *psm-mec* mRNA has the ability to downregulate the PSMα3 production by direct binding to the *agrA* mRNA, specifically suppressing colony spreading and promoting biofilm formation, not only by repressing *psmα3* transcription but, also repressing the expression of many other virulence factors controlled by the Agr QS system (Kaito and Sekimizu 2007; Kaito et al. 2011; Kaito et al. 2013).

5. Biofilm formation in *S. aureus*, determinant mechanism for chronic infections

Simply stated, biofilms can be defined as groups of microbes attached to a surface using a self-produced matrix (L. Shapiro, Agabian-Keshishian, and Bendis 1971; Costerton et al. 1987; Costerton 1999; Yarwood et al. 2004; J. A. Shapiro 2008; Zielinska et al. 2011; Hobley et al. 2013). Nonetheless, this definition is broad and neither describes the physiological attributes of the attached microorganisms. Virtually every microbe investigated thus far has the ability to colonize surfaces under at least some conditions, suggesting that surface-associated bacterial growth is important in microbial physiology (Costerton 1999; J. A. Shapiro 2008). As a consequence, research in the last two decades has unraveled that spatiotemporal organization characterizes biofilm-associated microbial communities (J. A. Shapiro 2008; Ackermann 2015) and thus, different from the physiologies discovered through bacterial suspensions in agitated cultures. Based on these investigations, biofilm formation can be defined as a developmental process in which bacteria undergo a regulated lifestyle switch from a nomadic unicellular state to a sedentary multicellular state where different cell types co-exist and are spatially and temporally organized as a consequence of extracellular matrix production (López and Kolter 2010). Cell arrangements in microbial biofilms facilitate the stable association of physiological cooperative individuals, generating organization patterns that suggest a programmed and orchestrated mechanism of genetic regulation. Now, is generally accepted that biofilms represent the predominant mode of microbial growth in the natural environments and indeed, the ability to adhere, proliferate and persist over solid surfaces is a general attribute to almost all bacteria (L. Shapiro, Agabian-Keshishian, and Bendis 1971; Costerton et al. 1987; Costerton 1999; Yarwood et al. 2004; J. A. Shapiro 2008; Zielinska et al. 2011; Hobley et al. 2013).

It has been estimated that between the 65% and the 80% of all human infections involve biofilms (Joo and Otto 2012). Among bacterial species that form biofilm in the human host, staphylococci are the leading cause of nosocomial infections on indwelling medical devices (Otto 2008) and in addition, biofilm-associated persistent *S. aureus* was determined as a surrogate for complications in the outcome and for an increase in mortality rates (van Hal et al. 2012). Indeed, close to 40% of all *S. aureus* bacteremia episodes upon appropriate antibiotic therapy were related to a persistent case of *S. aureus* infection (van Hal et al. 2012). In general, mortality rates associated with biofilm infections in implanted medical devices range from low rates of 1% to 3% in mammary implants up to significantly high 25% to 50% in cardiac assist devices (Lynch and Robertson 2008).

Infection or disease	**Common biofilm bacterial species**
Musculoskeletal infections.	Gram-positive cocci.
Osteomyelitis.	*S. aureus* and other bacterial and fungal species often mixed.
Sutures.	*S. aureus* and *S. epidermidis*.
Exit sites.	*S. aureus* and *S. epidermidis*.
Arteriovenous shunts.	*S. aureus* and *S. epidermidis*.
Schleral buckles.	Gram-positive cocci.
Contact lens.	Gram-positive cocci and *Pseudomonas aeruginosa*.
Hickman catheters.	*S. epidermidis* and *C. albicans*.
Central venous catheters.	*S. epidermidis* and others.
Ventricular assist devices.	*S. aureus* and *P. aeruginosa*.

Coronary stents.	*S. aureus*.
Breast implants.	Staphylococci.
Neurosurgical ventricular shunts.	Staphylococci.
Fracture-fixation devices.	*S. aureus* and *S. epidermidis*.
Endotracheal tubes.	Staphylococci and enteric Gram-negative bacteria.
Cochlear implants.	*S. aureus*.
Mechanical heart valves.	*S. aureus* and *S. epidermidis*.
Vascular grafts.	Gram-positive cocci.
Orthopedic devices.	*S. aureus* and *S. epidermidis*.
Penile prostheses	*S. aureus* and *S. epidermidis*.
Endocarditis.	*S. aureus.*

Table 3. Common persistent infections caused by staphylococcal biofilms. Adapted from (Costerton 1999; Lynch and Robertson 2008).

For *S. aureus*, biofilm formation constitutes a fundamental virulence mechanism and, is responsible for the establishment of the chronic, recurrent and difficult-to-treat presentation of this bacterial disease (Archer et al. 2014). The ability of *S. aureus* to attach to a surface and to assembly an organized multicellular community depends primarily on four different structural components: MSCRAMMs, the PNAG exopolysaccharide, PSMs and extracellular DNA. Many studies have demonstrated that each of these structural components undergoes precise regulatory mechanisms with many genetic systems involved (Fitzpatrick, Humphreys, and O'Gara 2005; Toledo-Arana et al. 2005; Mann et al. 2009; Joo and Otto 2012; Otto 2012b; Periasamy et al. 2012; Archer et al. 2014).

5.1. Extracellular matrix exopolysaccharide

Biofilm formation in *S. aureus* varies depending on the bacterial strain, the matrix composition and the environmental conditions (Schwartz, Syed, Stephenson, Rickard, and Boles 2012a; Lister and Horswill 2014; Schwartz et al. 2015; Payne and Boles 2015). Nevertheless, the extracellular matrix of the staphylococcal biofilm is generally structured by the same structural components: CWAs, PNAG, PSMs and eDNA, which greatly differ in ratios among the different models evaluated (Fitzpatrick, Humphreys, and O'Gara 2005; Toledo-Arana et al. 2005; Mann et al. 2009; Otto 2012b; Periasamy et al. 2012; Joo and Otto 2012; Archer et al. 2014).

The staphylococcal polysaccharide intracellular adhesin (PIA) is a polymer of the monosaccharide derivative of D-glucose, N-acetylglucosamine (GlcNAc or NAG) (MACK, Fischer, Krokotsch, and Leopold 1996). Initially described as *slime*, the PIA or PNAG has been proposed as the major component of *S. epidermidis* and *S. aureus* biofilms associated with prosthetic devices and, the main responsible for the intercellular aggregation leading to the accumulation of multilayers of cell clusters within those biofilms (Otto 2009). Importantly, not all staphylococcal strains synthetize PNAG but nevertheless, these PNAG-negative strains are still able to successfully adhere to surfaces and develop functional biofilms, confirming that the staphylococcal biofilm is highly diverse, extremely versatile and, that not only the *slime* is responsible for the biofilm-forming phenotype (MACK, Haeder, et al. 1996; MACK, Fischer, Krokotsch, and Leopold 1996; Götz 2002; Otto 2009).

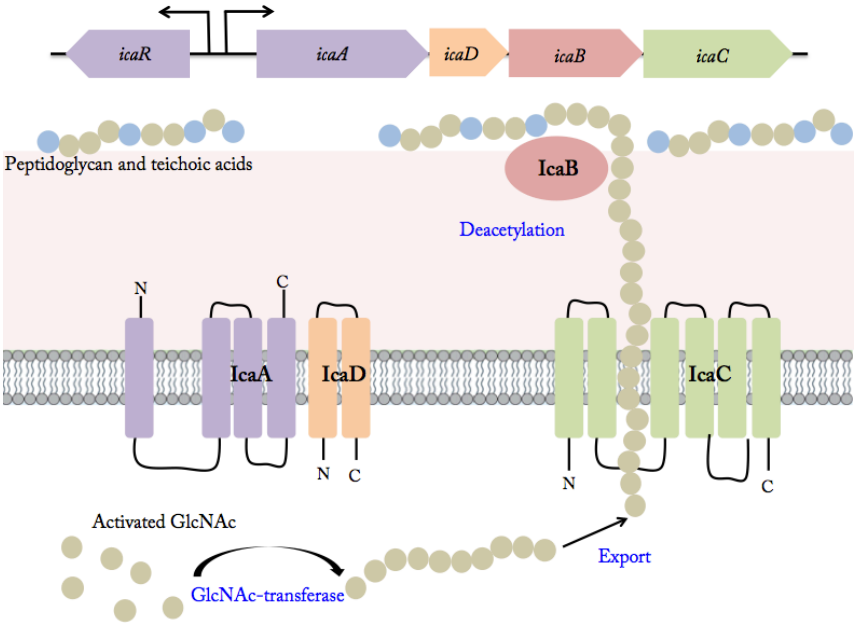

Figure 7. Upper part, genomic organization of the *icaADBC* operon and the *icaR* regulatory gene in staphylococci species. Lower part, structure and function of the PNAG-producing machinery in *S. epidermidis* and *S. aureus*. Adapted from (Otto 2009).

The genetic basis of PNAG production (Heilmann et al. 1996) resides in an operon termed the intercellular adhesion (ica), composed of four biosynthetic (*icaADBC*) and one regulatory (*icaR*) genes (Heilmann et al. 1996; Ziebuhr et al. 1997; K. M. Conlon, Humphreys, and O'Gara 2002; Jeng et al. 2008).

The protein product of *icaA* is a 4-helices transmembrane protein of 38 KDa with N-glycosyl-transferase activity responsible for the initial step, where NAG monomers (UDP-N-acetylglucosamine) are polymerized intracellularly into a polysaccharide of 10 to 20 residues of β-1,6-linked-N-acetylglucosamine (Heilmann et al. 1996; Gerke et al. 1998). The polymerization activity of IcaA is only reached in the presence of the gene product of *icaD* (Gerke et al. 1998). The *icaD* gene is located between and overlaps with, *icaA* and *icaB*. IcaD is a transmembrane protein of 12 KDa with no glycosyl-transferase activity by its own but that significantly enhances the IcaA transferase activity, presumably acting as a chaperone for IcaA (Gerke et al. 1998).

The IcaB is a secreted protein of 34 KDa with a typical AXA-motif for signal peptidases (Heilmann et al. 1996), responsible for the cation-dependent N-deacetylation of the secreted PNAG (Vuong, Kocianova, et al. 2004; Pokrovskaya et al. 2013). IcaB introduces positive charges in the neutral-charge polymer PNAG allowing electrostatic interactions that facilitate cell-to-cell adhesion, immune evasion and adhesion to epithelial cells (Vuong, Kocianova, et al. 2004).

IcaC is a transmembrane protein of 42 KDa involved in the translocation of the PNAG polymer through the membrane (Heilmann et al. 1996). The *icaC* start codon overlaps with *icaB* and, the only rho-independent termination sequence found in the operon is downstream of *icaC*, suggesting the translational coupling and co-transcription of this operon (Heilmann et al. 1996).

In several studies, *ica*-deficient strains showed reduction in virulence (Kropec et al. 2005; Arciola et al. 2015) while, naturally occurring PNAG-negative isolates are often associated with a commensal and non virulent lifestyle (Ziebuhr et al. 1997; Peacock et al. 2002). Natural occurrence of PNAG-negative strains in *S. epidermidis* is associated with the insertion sequence element IS256, which reversible integrates at the *icaA* and *icaC* locus in a process called phase variation (Ziebuhr et al. 1999). In *S. aureus* exists a similar process that does not involve the IS256 but instead, the expansion and contraction of a tetranucleotide tandem repeat (TTTA) that inactivates the *icaC* gene (Brooks and Jefferson 2014; Arciola et al. 2015).

In a model of bacteremia and renal infection, the total loss of the *ica* operon significantly reduced the amount of *S. aureus* in kidneys (Kropec et al. 2005), demonstrating the essential role of the PNAG in immune evasion as well as in the establishment of a persistent infection in renal abscesses (Vuong, Kocianova, et al. 2004; Vuong, Voyich, et al. 2004; Kropec et al. 2005).

In a matter that is still under debate, it has been proposed that a putative and alternative staphylococcal QS system LuxS, is directly involved in the repression of PNAG synthesis via *icaR* activation (Yu et al. 2012). Nevertheless, given that LuxS is poorly active in *S. aureus*, in addition to the facts that no AI-2 receptor has been found in *S. aureus* and that the LuxS protein has been associated with general metabolism, it is unlikely that a true second-QS system is functional in *S. aureus* and that downregulates PNAG production as a result of increasing cell densities.

6. Cell wall teichoic acids

Found as the major component of the cell surface (L. Glaser 1973; Joo and Otto 2012) in most Gram-positive species, TAs are carbohydrate-based polymers attached to the bacterial cell membrane or to the peptidoglycan that constitutes the cell wall, implicated in diverse roles in bacterial physiology that range from cell division, protection against antimicrobial peptides, activation of the immune response and inflammation, to the host-cell adhesion and biofilm formation (Weidenmaier and Peschel 2008).

The primary role assigned to the TAs was the assimilation of bivalent cations for enzymatic reactions, particularly Mg^{2+}, for the maintenance of high concentrations in the region of the cytoplasmatic membrane (Archibald, Baddiley, and Heptinstall 1973; Heckels, Lambert, and Baddiley 1977). Specifically, it was shown that bacterial membrane enzymes preferred Mg^{2+} bound to TAs rather than Mg^{2+} supplied in solution to the suspending medium (Heckels, Lambert, and Baddiley 1977). It was demonstrated that, although not essential fur survival *in vitro*, *S. aureus* TAs had the ability of Mg^{2+} preferential binding over other cations of similar characteristics (Heptinstall, Archibald, and Baddiley 1970b; Lambert, Hancock, and Baddiley 1975a; Bhavsar et al. 2004). Therefore, TAs together with the peptidoglycan and CWAs have been considered as a polyelectrolyte matrix with ion-exchange properties required for the maintenance of cation homeostasis and for the traffic of ions, nutrients, proteins and antibiotics (Neuhaus and Baddiley 2003).

Many additional roles have been found for TAs including the control of different envelope proteins like autolysins and the cell division machinery (Campbell et al. 2012), the protection of the cell

integrity against muralytic enzymes, antimicrobial peptides and the innate immune response (Thomer, Schneewind, and Missiakas 2016; Weidenmaier and Peschel 2008), phage adsorption (Neuhaus and Baddiley 2003; Koprivnjak et al. 2008) and as an colonization factor *in vivo*. Using a colonization model in cotton rats, it was demonstrated that TAs were an essential virulence factor in *S. aureus* for the colonization of anterior nares, where *S. aureus* resides normally as a commensal (Weidenmaier et al. 2004).

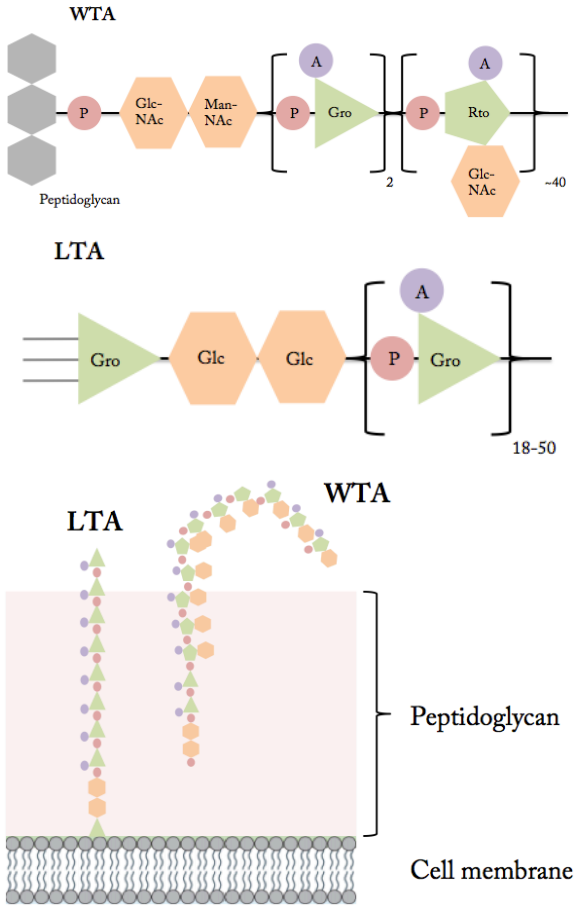

Figure 9. Staphylococcal WTAs and LTAs. WTAs are peptidoglycan-anchored cell wall glycopolymers while, LTAs are membrane-anchored glycopolymers. Composition, organization, decoration and length vary among WTAs and LTAs in *S. aureus*. Adapted from (Weidenmaier and Peschel 2008; Swoboda, Meredith, et al. 2009).

WTAs were found to contribute with the nonproteinaceous adhesion to endothelial cells *in vitro*, leading to the development of infective endocarditis and preventing dissemination into renal tissues (Weidenmaier, Peschel, Kempf, et al. 2005). LTAs were shown to contribute to invasion rather than adhesion to endothelial cells and correlated with effective dissemination into brain tissue (Sheen et al. 2010). Based on these findings, it is possible that changes in the ratio WTAs/LTAs could facilitate invasion after adhesion, and promote the survival of invading *S. aureus* that induce host inflammatory response (Neuhaus and Baddiley 2003; Koprivnjak et al. 2008).

Depending on their localization, type of constituent sugar, net charge and decoration of the repeating units, TAs are divided in two major classes: WTAs and LTAs (Percy and Gründling 2014; Santa Maria et al. 2014). Wall teichoic acids or WTAs, are the TAs covalently attached to the peptidoglycan of the bacterial cell wall by invariant phosphodiester bonds between N-acetylmuramic acids and the disaccharide N-acetylglucosamine-N-acetylmannosamine (Araki and Ito 1989). WTAs are the major TAs present in staphylococcal strains (Swoboda, Campbell, et al. 2009). The linkage unit of WTAs to peptidoglycan is invariant but the chain lengths of these polymers are highly variable among species and strains and range from 5 to 50 units (Neuhaus and Baddiley 2003), suggesting that WTAs have evolved in function of a specific tropism for determined hosts or tissues (Weidenmaier et al. 2004). Lipoteichoic acids (LTAs) are the TAs embedded through and attached to the cell membrane and are only constituted by a backbone of poly glycerolphosphate (Gro-P) tailored with D-alanine esters.

Different genetic pathways assemble Rbo-P-WTAs, Gro-P-WTAs and LTAs. In *S. aureus* and *B. subtilis* strain W23, Rbo-P-WTAs are synthetized for the 10-genes tar operons (*tarADBJFIL*, *tarO* and *tarGH*) while, the LTAs proteins in *S. aureus* are encoded by the *ypfP*, *ltaA* and *ltaS* genes (Percy and Gründling 2014). Particularly, in *B. subtilis* strain 168 the Gro-P-WTAs biosynthetic machinery is encoded by an alternative 5-genes *tag* operon (teichoic acid glycerol) *tagABDEF* (Qian et al. 2006). In *S. aureus*, the Rbo-P-WTAs *tar* genes are the major TAs biogenesis pathway and are organized in several clusters and with several duplications in many staphylococcal genomes, particularly for the *tarI*, *tarJ* and *tarL* genes (Qian et al. 2006). Annotation is frequently confusing in *S. aureus* and in most databases all the genes responsible of Rbo-P-WTAs synthesis are repeatedly found under the *tag* annotation. From now, Rbo-P-WTAs will be simply referred as WTAs and the tar annotation used.
The *tagO* and *tagA* genes are the only WTAs genes that can be knocked-out without abolishing staphylococcal viability, in a phenomenon known as conditional essentially (Weidenmaier et al. 2004; D'Elia et al. 2006; Falugi et al. 2013). Nevertheless, *tagB*, *tagD* and *tagI* mutants retaining some minor level of residual activity have been isolated in *S. aureus* strain COL. These mutants showed dramatic alterations in their WTAs abundance and polymer length, as well as stress-sensitive phenotypes (H. Wang et al. 2013).
The biosynthesis of LTAs is mechanistically simpler than WTAs. In *S. aureus*, anchorage of the LTAs to the cell membrane is achieved by a glycolipid produced in the cytoplasm by the YpfP glycosyltransferase, which adds two glucosyl residues to diacylglycerol (DAG) giving raise to the anchoring molecule, β-diglucosyl-DAG. This lipid is then translocated to the outer part of the membrane by the flippase LtaA (Percy and Gründling 2014) and once anchored to the membrane, the transmembrane LTA synthase LtaS repeatedly adds the glycerolphosphate units to the nascent LTA polymer (Gründling and Schneewind 2007).

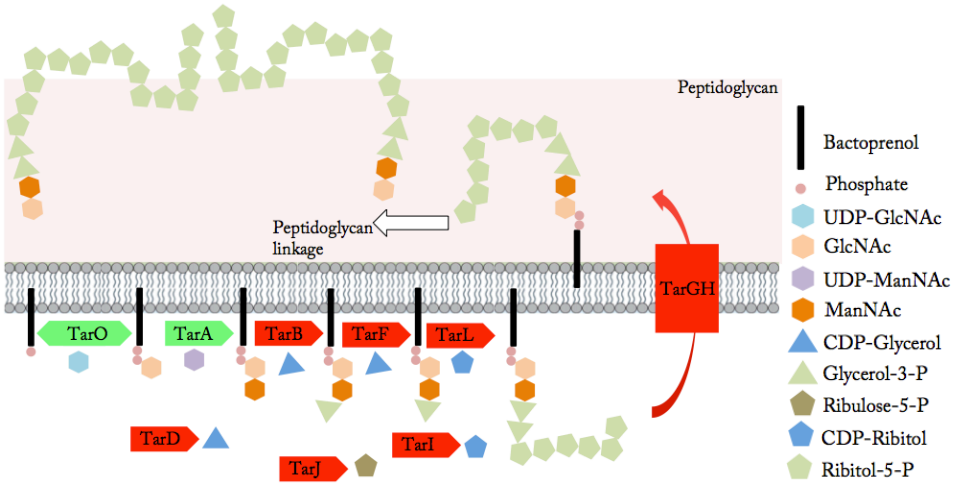

Figure 10. Simplified biosynthetic pathway for *S. aureus* WTAs. The *tarO* and *tarA* are not essential enzymes (colored in green) while; conditionally essential proteins are colored in red. Their deletion is lethal and only permitted in strains *tarO*- or *tarA*-deficient. Adapted from (Swoboda, Meredith, et al. 2009).

LTAs and WTAs seem to have similar but complementary functions, since cannot be simultaneously deleted without losing viability, both use the same D-alanylation machinery to add D-alanine esters to their sugar-based backbones (Neuhaus and Baddiley 2003) and their individual deletion results in cell division defects (Oku et al. 2009).

The D-alanylation of WTAs and LTAs play a crucial role in the staphylococcal physiology. The genetic mechanism of D-alanylation involves the activity of the *dltABCD* operon (Peschel et al. 1999; Weidenmaier, Peschel, Kempf, et al. 2005) and at final steps, activated D-alanine is enzymatically transferred by the DltAC complex to the glycerol phosphate backbone of the LTAs. There, esterified LTAs serve as the precursor for the D-alanylation of WTA, establishing that the D-alanylation of WTAs occurs via LTAs (Neuhaus and Baddiley 2003).

Deletion of any *dlt* genes results in a reduced *in vitro* growth, increased susceptibility to cationic antimicrobial peptides and reduced biofilm formation (Peschel et al. 1999; Peschel et al. 2000). D-alanine modulates the net charge of the cell envelope regulating electrostatic interactions and, regulates the activity of cell surface enzymes providing them with particular cofactors, particularly Mg^{2+}. WTAs lacking D-alanine esters bind up to 60% more Mg^{2+} than D-alanylated WTAs (Heptinstall, Archibald, and Baddiley 1970a) and indeed, the D-alanylation level modulates the divalent cation binding properties of TAs, providing a regulatory mechanism of cation scavenging for intracellular metabolic pathways (Koprivnjak et al. 2006). The absence of D-alanine in WTAs alters bacterial cell size from 5.1 to 10.1 ml/g and cell charge, enhancing autolysis and antibiotic susceptibility (Ou and Marquis 1970). Strikingly, all these effects were suppressed by the simultaneous addition of Mg^{2+} (Wecke, Madela, and Fischer 1997). The electrostatic characteristics of D-alanylated TAs are thought to play a fundamental role in the initial steps of biofilm formation and in fact, mutations in the *dlt* operon render dramatically biofilm formation on abiotic surfaces due to the augmentation of a net negative charge of the envelope, which repulses bacteria from the colonization surface (Joo and Otto 2012).

Indeed, fully functional TAs are interacting partners of surface polymers and scaffolds for protein attachment during the initial steps of biofilm formation (Joo and Otto 2012).

Disruption of the WTAs synthesis by targocil, an antibiotic that selectively inhibits the TAs transporter TarG (TagG), led the appearance of cluster of swollen cells displaying evidence of osmotic stress (Campbell et al. 2012) and also activated the cell wall stress stimulon (CWSS) (Utaida 2003; Muthaiyan et al. 2008), accompanied with the reduction in the expression of virulence genes and the *dltABCD* operon (Campbell et al. 2012). In addition, the inability of a *S. epidermidis tagO*-deficient mutant to grow under high salinity conditions, indicates the important role of WTAs in stress tolerance (Holland, Conlon, and O'Gara 2011). Interestingly, surface changes associated with loss of WTAs in a *tagO*-deficient *S. aureus* activated the alternative sigma factor σ^B, resulting in an increased *icaADBC* transcription and higher biofilm formation (Knobloch et al. 2004; Holland, Conlon, and O'Gara 2011).

Chapter 2

1. The Agr *quorum* sensing system

The Agr is the archetypical global regulator in *S. aureus* and, it is defined as a TCS that senses bacterial density in a QS manner and antagonistically controls the expression of extracellular toxins, lipases, proteases and surface proteins (Morfeldt, Tegmark, and ARVIDSON 1996; Bronner, Monteil, and PrÃ vost 2004). During biofilm development, the Agr system exerts a negative regulation during early stages of biofilm formation, through the derepression in the expression of some surface proteins involved in attachment (Queck et al. 2009; Otto 2012c) while positively regulates the dissemination phase in late stages, through the expression of proteases, PSMs and some exotoxins (Arciola et al. 2012; Periasamy et al. 2012). In addition, Agr is required for endosome escape, antimicrobial susceptibility and intracellular survival, persistence and replication (Qazi et al. 2001). From mid-exponential to post-exponential phase, the Agr system represses the transcription of cell wall proteins involved in adhesion, colonization or biofilm formation such as SpA and FnBPA (Novick et al. 1993; Dunman et al. 2001; Novick 2003b) and activates toxins production (α-toxin, β-hemolysin and the PSMs) (Dunman et al. 2001; Periasamy et al. 2012; Peschel and Otto 2013).

Within the relatively small staphylococcal genome (2.9 Mb), there are more than 50 accessory genes directly involved in *S. aureus* pathogenesis (the *virulon*), which are regulated by Agr (Dunman et al. 2001; Novick 2003b). In addition to the *virulon*, Agr regulates and is regulated, by many other staphylococcal global regulators, such as the pleiotropic regulator SarA and the Sar family of transcriptional regulators (Beenken et al. 2010).

The Agr is an autoregulatory system that senses bacterial density during the transition from exponential to stationary growth phase (Vuong et al. 2000). The Agr can be defined as a four-component system (*agrACDB*) with an embedded traditional TCS (Novick et al. 1995). AgrC is a transmembrane sensor histidine kinase of 46 KDa that binds a specific extracellular ligand. Binding of the ligand changes AgrC conformation and promotes its homodimerisation, subsequently followed by a *trans*-autophosphorylation by the transfer of a phosphate from ATP to a histidine residue located in one of the cytoplasmatic domain (Novick and Geisinger 2008). Then, this phosphate is transferred to an Aspartate domain in the response regulator AgrA, a 34 KDa protein member of the LytTR family of response regulators (Novick and Geisinger 2008). Once activated, AgrA~P binds to specific DNA promoter sequences, leading to the control of its own gene expression, as well as the gene expression of several other genes (C. W. Tseng, Zhang, and Stewart 2004; Cafiso et al. 2007; Matsumoto et al. 2007; Beenken et al. 2010; Rutherford and Bassler 2012).

Within the *agr* operon, the AgrA-binding DNA sequence is a heptanucleotide repeat which is found in the 120 bp Agr intergenic region (IGR) (Morfeldt, Tegmark, and ARVIDSON 1996). This IGR possesses the DNA-binding sequences for its own gene, as well as for the expression of the regulatory small RNA (sRNA) called RNAIII (Novick et al. 1993; Boisset, Geissmann, and Huntzinger 2007). Therefore, the Agr system is composed of two adjacent transcripts, the P2 (RNAII) that controls the

expression of the *agrACDB* operon and the P3, which promotes the expression of a 514 nt RNAIII but also, of the *hld* gene (δ-hemolysin or PSMγ) that is located within the RNAIII molecule transcript. The RNAIII that has the ability to modulate several virulence factors at the transcriptional and translational level (Janzon and Arvidson 1990; Morfeldt, Tegmark, and ARVIDSON 1996). RNAIII is the pleiotropic effector of Agr and is characterized by 14 β-hairpins and 3 long-range interactions that presumably forms stable secondary structure with its target-DNA sequences creating appropriate protein-binding sites for the activation of gene expression (Novick et al. 1993) or by an anti-SD (Shine-Dalgarno) action that resides in the unpaired regions of the stem-loops 7 and 14, which are complementary to the canonical SD sequence and therefore, interfere with the translation of other regulatory proteins (Novick 2003b). RNAIII targets include most of the extracellular protein genes, some adhesion surface proteins (Saravia-Otten, Müller, and ARVIDSON 1997), the SarT and SarS regulator genes (McNamara et al. 2000; K. A. Schmidt, Manna, and Gill 2001), among others.

Apart of the four-heptanucleotide repeats that are located at -45 bp from P2 and -66 bp from P3, there is a strong 17 bp inverted repeat (IR) overlapping with the second and third heptanucleotide repeats, which has been proposed as a bidirectional regulatory protein binding site, involved in the Agr-independent regulation of P2 and P3 gene expression (Bayer, Heinrichs, and Cheung 1996). In addition to the two most proximal heptanucleotide repeats are required for P3 activation, a 19 nucleotides spacing between the -10 and -35 elements of the P3 promoter has shown to be important for RNAIII regulation (Morfeldt, Tegmark, and ARVIDSON 1996).

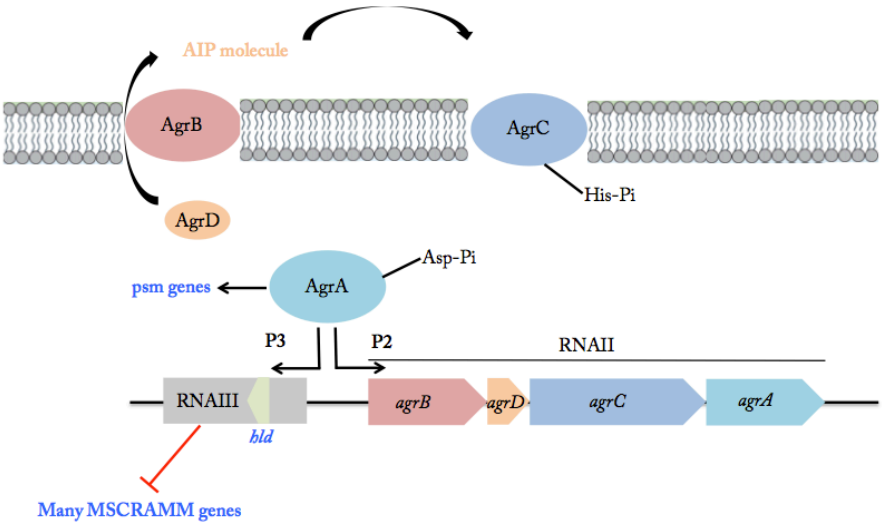

Figure 11. The *agr* locus exerts QS in staphylococci. The AgrCA constitutes a typical TCS. The AgrB processes the QS molecule AgrD, also known as AIP. Together, the Agr system (RNAII) comprises an autoregulatory system that responds to increased AIP concentration, which normally correlates with increased bacterial density. AgrA~P is a transcriptional factor that controls the expression of the Agr operon, the effector regulatory sRNA called RNAIII as well as many genes, such as the PSMs. The RNAIII molecule controls the expression of many other genes, including the expression of exotoxins and the repression of adhesion molecules such as MSCRAMMs. Adapted from (Joo and Otto 2012).

All these data points to a regulation of P2 and P3 to respond differentially to specific regulatory signals and systems. Some studies have demonstrated that, apart of AgrA~P the SarA also binds to the Agr IGR and that, differential ratios AgrA~P/SarA are associated with this IGR depending on the bacterial growth phase (A. L. Cheung and Projan 1994; Heinrichs, Bayer, and Cheung 1996; Morfeldt, Tegmark, and ARVIDSON 1996). Although, the precise mechanism governing the preferential binding of AgrA to the P2 or P3 promoter has not been characterized yet, it might rely on the local intracellular concentration of AgrA, its phosphorylation level, differential polymerization states, the strength of the binding to the promoter DNA sequence and the presence of other regulatory proteins like SarA and SarR (Reyes et al. 2011).

AgrA directly upregulates the expression of the *psm* genes (R. Wang et al. 2007; Periasamy et al. 2012) but also, downregulates the expression of many genes involved mainly in carbohydrate metabolism, amino acid metabolism and the genes involved in the synthesis of staphyloxanthin (Queck et al. 2008). These results suggest that in parallel with the upregulation in the production of exoproteins and toxins via RNAIII, the Agr system promotes a general reduction of the general metabolism, coping with both, the increase in cell densities (QS) and the diversion of the energetic resources for the RNAIII-dependent gene upregulation. This work revealed an interesting hypothesis about the staphylococcal physiology and the role of the Agr system: that the RNAIII regulation of virulence-related genes emerged evolutionary later than the primary function of the Agr in the control of QS. In there, the AgrA and the P2 transcript specifically evolved to downregulate genes involved in metabolism in response to QS and nutrient limitation. Then, and later in evolution, the addition of the regulatory RNAIII to the P3 transcript that originally could had been for the embedded *hld* gene, created a new regulatory function for the Agr system in a host-pathogen scenario (Queck et al. 2008), optimizing the properties of *S. aureus* as a pathogen (Sittka and Vogel 2008).

The environmental signal sensed by AgrC is the auto inducing peptide (AIP), encoded by the *agrD* gene, which increases its extracellular concentration with the increase in the bacterial cell density. The AgrD is a semi-cyclic peptide of 7 to 9 aa with an functionally important thiolactone group located between a Cysteine residue and the C-terminal carboxyl group (Ji, Beavis, and Novick 1997; Mayville et al. 1999). The AgrD peptide is modified and subsequently secreted trough the transmembrane protein AgrB (26 KDa) (Ji, Beavis, and Novick 1997; L. Zhang et al. 2002; L. Zhang and Ji 2004). When extracellular levels of AIP reach certain high activation threshold, the Agr system is autoactivated through a positive feedback loop and also, activates a plethora of genes including the adjacent RNAIII regulatory RNA (Ji, Beavis, and Novick 1995).

The sequence of the *agrD* gene, as well as of certain regions in the *agrB* and *agrC* genes, display certain level of sequence variability that defines 4 allelic AIP groups with cross-inhibition properties in a phenomenon called *bacterial interference* (Ji, Beavis, and Novick 1997). Although, no specific allelic group is responsible for a specific human disease presentation or tropism, particular *S. aureus* allelic groups have shown major differences in the temporal control of the Agr autoinduction, as well as in the production of specific exotoxins (Ji, Beavis, and Novick 1997; Jarraud, Lyon, and Figueiredo 2000;

Jarraud et al. 2002), suggesting an evolutionary role for these variances and might underlie correlations between the allelic group and the site or severity of the infection.

2. Staphylococcal *QS* in biofilm formation

In *S. aureus*, cell-cell communication via QS modulates in time and space the expression of hundreds of virulence factors (Otto 2004). Between the large repertoires of regulatory genes characterized in *S. aureus*, the only true QS system described in this species is the Agr (Peng et al. 1988; Kornblum et al. 1990). Although, the presence of additional staphylococcal QS systems such as the RAP-TRAP and RIP systems has been proposed, their existence and functionality is controversial (Balaban and Novick 1995; Novick et al. 2000; Lyon and Novick 2004).

The role of the Agr QS system in biofilm formation was demonstrated showing that the expression of the *agr* correlated with a decreased ability to adhere to polystyrene surfaces (Vuong et al. 2000). In addition, it was proved that the *agr* phenotype was autolysin- and PNAG-independent, suggesting additional factors involved in *S. aureus* biofilm formation. The transition from late exponential to stationary phase is accompanied by the activation of the autoregulatory mechanism of Agr QS, resulting in the downregulation of many cell surface proteins involved in biofilm formation and in parallel, upregulating the expression of several virulence factors involved in biofilm disassembly and biofilm dispersion (Yarwood and Schlievert 2003).

Experiments *in vivo* showed that *agr* expression contributed to staphylococcal pathogenesis in abscesses, arthritis and endocarditis models (Bunce et al. 1992; Abdelnour et al. 1993; A. L. Cheung and Projan 1994). *S. aureus agr*-defective strains facilitated biofilm formation in prosthetic and indwelling devices (Boles and Horswill 2008a) while; *agr* mutant strains were attenuated in models of endocarditis (A. L. Cheung et al. 1994), osteomyelitis (Gillaspy et al. 1995), skin abscesses (Mayville et al. 1999; Wright, Jin, and Novick 2005) (Mayville et al. 1999; Wright, Jin, and Novick 2005) and septic arthritis (Abdelnour et al. 1993).

A revealing study proposed the basic model of QS-mediated biofilm formation, where the temporal and spatial confined expression of *agr* was determining the cycle of biofilm formation and dispersal in *S. aureus* (Yarwood et al. 2004). The model suggested that *"…in most areas of a biofilm at most times,* agr, *and likely* agr-*dependent virulence genes, is not expressed. However, cells that do express* agr *appear to be released from the biofilm, cells that may be also expressing* agr-*dependent virulence factors… crowding within the localized infection activates the* agr-*dependent* quorum *response and synthesis of extracellular factors, enabling the staphylococci to scape the access and spread to new sites."* (Yarwood et al. 2004).

Therefore, Agr arises not only as the prime regulatory system modulating *virulon* expression but also, as a key determinant in biofilm formation. In this context, QS signals the multicellular community that a population burden has been reached through the activation of both the P2 the P3 promoter at a high RNAIII/AgrA ratio. Increased AgrA drastically downregulates the carbohydrate and amino acids metabolism (Queck et al. 2008) and biofilm-related genes are repressed. In addition, high expression

levels of AgrA and the RNAIII molecule promote the expression of virulence genes that facilitate the detachment and the colonization of a new niche.

The Agr QS system exerts a fine control over the process of biofilm formation in *S. aureus* and PSMs regulation constitutes an interesting case. PSMs are proinflammatory peptides involved in the modulation of immune response and phagocytic scape but also, strongly influence biofilm development and dispersal. A complete analysis of staphylococcal biofilms demonstrated that PSMs significantly impact biofilm volume, thickness, roughness and channel formation (Periasamy et al. 2012). In addition, PSMs have intrinsic amyloid-like properties and can polymerize into aggregates that actively contribute to biofilm development (Schwartz, Syed, Stephenson, Rickard, and Boles 2012b). Together, these results indicate that PSMs are Agr-dependent key players in biofilm development and biofilm disassembly. It might be feasible that depending on the aggregation state, these peptides perform antagonistic functions within the biofilm: as monomers they might promote biofilm disassembly while, as polymers they might favor biofilm formation (Solano, Echeverz, and Lasa 2014).

The main regulatory system of the Agr system is itself. Nevertheless, additional staphylococcal regulatory systems such as many staphylococcal TCS, as well as the global regulators σ^B and SarA have shown to regulate Agr expression and, significantly influence biofilm formation and virulence in *S. aureus* in a Agr-dependent manner.

3. σ^B stress response, alternative sigma factor

S. aureus is a highly adaptable organism with a large genetic plasticity that allows this pathogen to successfully cope with the environmental changes. Diverse structural and regulatory components protect the cell against specific stresses (Clements and Foster 1999). Among those regulatory components, the alternative sigma factor σ^B is a key player in the rapid regulation of gene expression, virulence and biofilm formation in staphylococcal species. Contrary to σ^A, the primary staphylococcal initiation factor that promotes de attachment of the RNA polymerase to specific initiation sites, σ^B is mainly expressed during the stationary phase of growth and is activated upon stress signals such as starvation and energy depletion (ATP/ADP ratio) (Novick 2003b), oxidative stress, heat shock, environmental acidification and strong osmolarity changes (Chan et al. 1998; Rachid et al. 2000). Interestingly, different to many other bacteria that have several σ factors that range between 6 (*E. coli*) to 17 (*B. subtilis*) (Nyström 2003; Paget 2015; Helmann 2016) additional sigma factors, in *S. aureus* only two sigma factors have shown to be consistently active and a total of 4 have been reported: the housekeeping sigma factor σ^A (Deora and Misra 1996), the alternative σ^B (Wu, de Lencastre, and Tomasz 1996; Deora, Tseng, and Misra 1997), σ^H (Morikawa et al. 2003) and σ^S (Shaw et al. 2008). While σ^H is not expressed in laboratory conditions and seems to be important for DNA transformation (Morikawa et al. 2012), the function of σ^S remains elusive (Shaw et al. 2008).

Undoubtedly, σ^B is the most studied alternative sigma factor in staphylococci due to its relevance in stress response. Proteomic and transcriptomic studies have identified about 200 genes under direct and indirect positive control of σ^B, many of them involved in cell envelope turnover, membrane transport

processes and adhesion through CWAs, among many other diverse physiological functions (Gertz et al. 2000; Ziebandt et al. 2004; Hecker, Pané-Farré, and Uwe 2007).

Sharing high levels of identity with the *sigB* operon of *B. subtilis*, the σ^B chromosomal gene cluster is composed of four genes: *rsbU*, *rsbV*, *rsbW* and *sigB* (Wu, de Lencastre, and Tomasz 1996). σ^B recognizes the unique promoter sequence in the form GTTT(N_{14-17})GGGTAT, which has been identified for at least 23 *S. aureus* genes (Gertz et al. 2000). Since the σ^B stimulon overcome this number, it is feasible that σ^B also regulates gene expression indirectly through other global regulators and other transcription factors like the Sar family and the Agr QS system (Novick 2003b). In this context, σ^B is known to be an Agr antagonist (Novick 2003b).

Although the molecular mechanism for σ^B regulation differs depending on the bacterial species and the origin of the metabolic or environmental signals (Chan et al. 1998), the general notion in *S. aureus* is that stress signals release the anti-σ^B factor RsbW from SigB inducing its activation. This process is guided of RsbV, an antagonist of RsbW (also known as an anti-anti-σ^B factor) (Haldenwang 1995), which once dephosphorylated becomes active and has the ability to dephosphorylate RsbW, promoting the release of SigB (Pané-Farré et al. 2009). RsbU is the phosphatase protein that carries out the dephosphorylation of RsbV. Interestingly; RsbU is a protein of the PP2C-type and requires Mg2+ for its correct function.

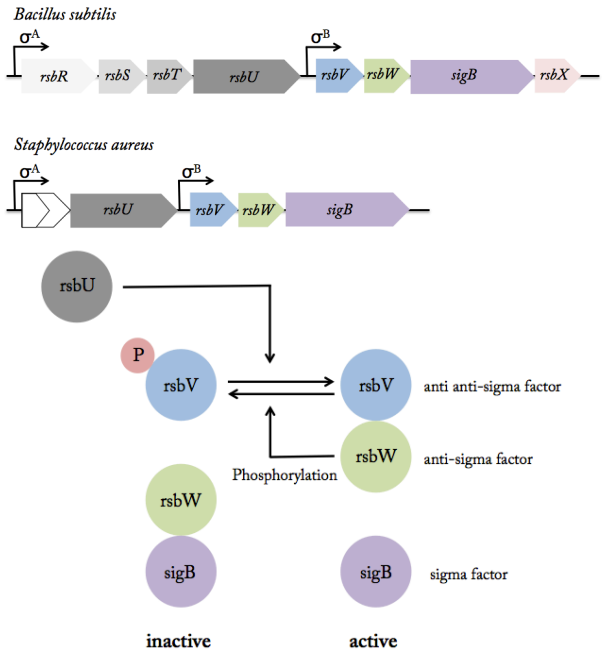

Figure 11. In the upper part, comparison of the *sigB* operons in *B. subtilis* and *S. aureus*. At the amino acid level, SigB proteins share between 37% and 56%. In *S. aureus*, the stress-induced pathway is incomplete and includes only the RsbU phosphatase, which dephosphorylates the anti anti-sigma factor RsbV (lower part). Adapted from (Pané-Farré et al. 2009).

In normal, non-stressed conditions, the kinase activity of RsbW constantly phosphorylates RsbV (Dufour and Haldenwang 1994; Delumeau, Lewis, and Yudkin 2002). The mechanism by which

RsbU is activated to dephosphorylate RsbV is not known since, different to *B. subtilis*, which possesses the *stressosome* proteins RsbR, RsbS and RsbT that control RsbU activity, *S. aureus* does not have them (Pané-Farré et al. 2009).

Biofilms offer bacteria protection against unfavorable environmental conditions and is already known that, the transition between planktonic and sessile life styles can be triggered by external stress conditions (Deretic et al. 1994; Lapaglia and Hartzell 1997; Mathee et al. 1999; Rachid et al. 2000). Particularly, σ^B plays an important role in the *ica*-dependent process of biofilm formation in staphylococcal species. In *S. aureus*, biofilm formation is triggered by osmotic stress conditions and, the deletion of the *sigB* gene resulted in the abolition of biofilm formation under these conditions, due to drastic reduction in the transcription of the *ica* operon (Rachid et al. 2000). Since there is not σ^B consensus sequence within the promoter region of the *ica* genes, it was suggested that the regulation should be indirect by additional regulatory factors.

In *S. epidermidis* was proved that this stress-related sigma factor promotes biofilm formation by repressing the *icaR* transcription but also, by promoting *sarA* expression (Knobloch, Bartscht, and Sabottke 2001; Knobloch et al. 2004). Nevertheless, further investigations established that SarA and not σ^B was the essential factor for the *ica*-dependent pathway of biofilm formation in some *S. aureus* strains (Valle et al. 2003). Although, *sigB* mutations decreased the *ica* operon transcription, PNAG production was not affected, opposite to what happened in *sarA* mutations. It was then proposed that both, SarA and σ^B might contribute to biofilm formation under stress conditions by promoting *ica* expression via SarA, while repressing the transcription of the *ica* repressor *icaR,* via σ^B (Valle et al. 2003).

Importantly, in *S. aureus* was proved that active σ^B inhibited *agr* expression, favoring the reduction of exotoxins and secreted proteases and hence, promoting a *ica*-independent process of biofilm formation and biofilm maturation (Shaw et al. 2006; Lauderdale et al. 2009; Arciola et al. 2012). These results demonstrated that σ^B is a direct and an essential regulator of the Agr and hence, of the *agr*-dependent process of biofilm formation.

Interestingly, σ^B-overexpressing *S. aureus* showed a significantly higher attachment to fibrinogen and fibronectin coated surfaces, together with a positive induction in the expression of the biofilm-related CWAs genes *clfA*, *fnbA* and *spa*. In addition, in an *in vivo* model of experimental endocarditis, σ^B-overexpressing *S. aureus* showed enhanced bacterial densities in aortic vegetations during early stages of infection, suggesting the importance of this regulatory system in early stages of biofilm development (Entenza et al. 2005). It has been proposed that σ^B plays a critical role in adhesion, allowing the bacteria to persist at the attachment site without damaging the host through the repression of toxin production. This antagonist behavior would allow *S. aureus* to colonize and increase bacterial cell densities in early stages (Entenza et al. 2005).

The σ^B regulon proved to influence strongly the adhesion and internalization of *S. aureus* to osteoblasts through the expression of CWAs such as FnbA and ClfA (Nair et al. 2003), suggesting that σ^B is an active contributor to the process of biofilm formation *in vivo*. In a σ^B-defective strain, the RNAIII molecule increased (Lauderdale et al. 2009). Surprisingly, no consensus sequence for σ^B binding has

been characterized in the P2 or P3 promoters of the Agr system, suggesting that σ^B might be repressing AgrA using an intermediate regulator (Lauderdale et al. 2009). In this context, σ^B repression of the Agr, might ensure that *S. aureus* does not undergo QS under conditions when the bacteria must dedicate resources to alleviate stress (Beveridge and Murray 1976) or perhaps, that *S. aureus* undergoes biofilm formation and collective behaviors under stress conditions, as a mechanism to protect from environmental conditions.

Together, these results strongly suggest that σ^B is a crucial regulatory factor involved in the switch between persistent and invasive states in response to the stress host conditions (Ziebandt et al. 2004; Hecker, Pané-Farré, and Uwe 2007). Indeed, an extensive proteomic and transcriptomic analysis of σ^B-defective, *agr*-defective and σ^B-repaired strains, unequivocally demonstrated that Agr and σ^B have an opposite effect on the regulation of several extracellular toxins and CWAs. In this study, the Agr system showed to positively influence the expression of toxins, proteases, autolysins and lipases while repressing CWAs (Ziebandt et al. 2004). Additionally, it was found the *sarA* transcription was positively regulated by σ^B and, that most of the proteins up-regulated by *agr* and repressed by σ^B were also repressed by SarA (Ziebandt et al. 2004).

Chapter 3

1. The Agr system is a positive feedback loop

One of the most remarkable characteristics of the Agr system is its autoregulatory nature. Autoregulatory systems, also known as positive feedback loops (PFL) and negative feedback loops (NFL), have been identified in basically all living forms and have emerged as the fundamental biochemical control of multitude biological processes. At the most fundamental level, the control exerted by PFL is based on the amplification of the response to an external signal (Saini et al. 2010).

The amplification achieved by PFL is a key determinant to stabilize steady expression states by extending the lifetime of transcriptional pulses in a gene expression system, slowing downs and therefore overcoming the intrinsic stochastic fluctuations of these systems (Fraser and Kaern 2009).

In theory, external signals such as environmental stress are the strength modulators of feedback loops and recreate a simple sense-and-response circuit. In this model, in the absence of stress, *noise* is crucial because it causes above-threshold gene expression in a small subset of cells, seeding the feedback-based switch (Becskei, Séraphin, and Serrano 2001; Williams, Savageau, and Blumenthal 2013). Nevertheless, when the signal (stress) is positively sensed, the stochastically preactivated cells would be able to upregulate their gene expression rapidly through regulatory PFL and, stably pass this better-fit expression state to their progeny, facilitating the process of cell fate decision-making (Fraser and Kaern 2009).

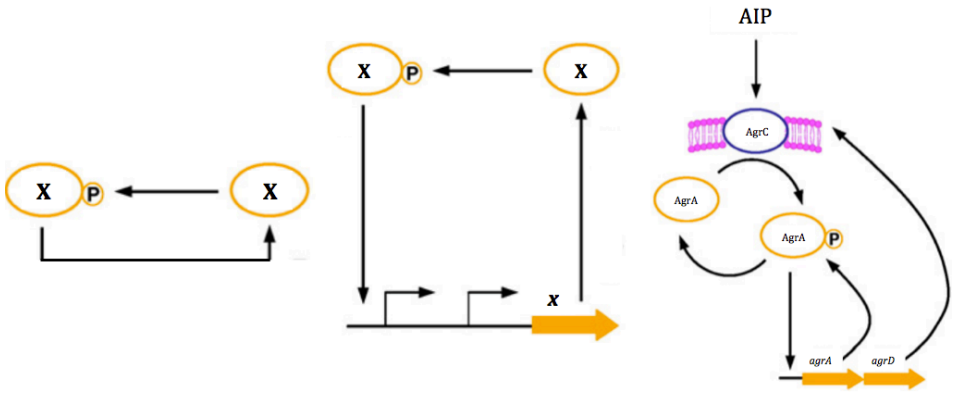

Figure 12. The Agr QS system is a genetic regulatory positive feedback. PFL are architecturally simple where a regulator X activates its own expression. In biological systems, PFL are normally associated with the phosphorylation of these regulators X~P, which bind the promoter region of its own gene *x* to activate the PFL. Finally, the architecture of the staphylococcal Agr system as a TCS, shares all the characteristics of a PFL. Adapted from (Mitrophanov, Hadley, and Groisman 2010).

The switching between stable states requires a perturbation that exceeds the noise threshold, which in this context can be provided by the autoregulatory and signal amplification characteristics that define PFL. A graded gene expression pattern can be converted to a binary pattern by the concerted action of PFL, which has the ability to increase the input noise of any biochemical system (Avery 2006).

PFL involve the group of biochemical interactions where the activation or accumulation of certain component is influenced by its own and leads to its activation or accumulation. On the other hand,

NFL involves the group of biochemical interactions where the activation or accumulation of certain component is influenced by its own and leads to its deactivation or depletion (Mitrophanov and Groisman 2008b; Mitrophanov, Hadley, and Groisman 2010).

In general, PFL and NFL are considered switching regulatory mechanisms involved in the efficient response of living cells to changes in the intracellular and extracellular environment and, define basic physiological functions from enzyme production to more sophisticated phenomena like bistability, hysteresis and cell heterogeneity (Mitrophanov and Groisman 2008b).

Bacterial PFL incorporate nonlinear response elements that amplify the polarization of stable cellular phenotypes. These regulatory factors require activation mechanisms such as multimerization or cooperativity in order to activate or repress themselves as well as their targets (Dhar and McKinney 2007). In bacteria, PFL contribute to the efficiency of a transcriptional regulatory system and relies mainly, in the activation mechanism of the autogenous regulator by phosphorylation (Mitrophanov and Groisman 2008b). Of special interest, the increase in the amount of the regulator protein does not necessarily mean an increase in the ability of a certain cell to activate genes since, it is the phosphorylation state and not the amount of the protein what defines its activity as a transcriptional regulator (Laub and Goulian 2007). These autoregulatory systems seem to be highly efficient since, limiting the production of regulator and grounding the functionality of the whole system on the rates of phosphorylation, avoid unnecessary waste of resources and circumvents the possibility of having phosphorylation crosstalk by a non-physiological partner in response to a non-physiological signal (Mitrophanov and Groisman 2008b). Therefore, PFL activated by phosphorylation states create the perfect equilibrium between protein production and protein activity where the total protein level never will exceed the phosphorylation level, avoiding any unnecessary waste of energy and resources. In addition to the activation of response regulators, autophosphorylating kinases have the potential to store memory (Veening, Smits, and Kuipers 2008), a phenomenon known as hysteresis. Kinases have the ability to remain activated even when the signal is absent or stimulus and as a result, when activated cells divide, they have the ability to pass the *on* state to the offspring, functioning as memory devices and therefore, dictating the future life-history of the new cell (Veening, Smits, and Kuipers 2008).

Although, this behavior means a delay time in activation when compared to activator proteins constitutively produced, it guarantees to instruct and amplify in the right time the action of cellular response mechanisms. Cells have evolved to accelerate this process by interlinking feedback loops to enhance switching performance (Brandman et al. 2005; Alexander Y Mitrophanov 2008; Mitrophanov, Hadley, and Groisman 2010; Brandman and Meyer 2008). However, so far there is no evidence that some loops are fast whereas others are slow (Saini et al. 2010), suggesting that biochemical circuits could evolve a fast and robust response by simply linking together multiple regulators that alone lack the requisite properties or, when all the activating signals are present (Saini et al. 2010; Chang et al. 2010). Indeed, in nature simple bistable systems with a single PFL are rarely encountered and normally, biological systems interlink several feedback loops with or without additional regulatory mechanisms. The combination of different loops, even as simple as two interacting PFL, overcomes

the practical lack of low- and high- activity systems and at the same time, provides the different timescales necessary to offer resistance to noise, to produce an ultrasensitive response, to provide fine-tuning of the system and, to increase bistability robustness over a wide range of conditions (Ferrell and Xiong 2001; Ferrell 2008; Chang et al. 2010).

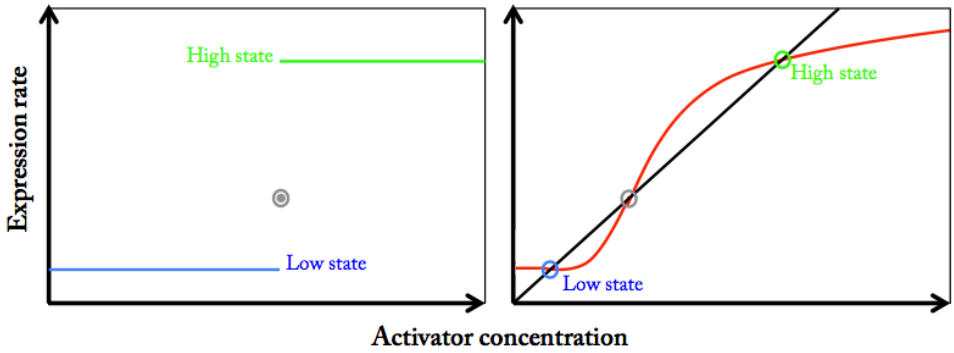

Figure 13. PFL and bistable systems. As observed in the left panel, PFL promote an abrupt transition from a low to a high steady state after the activating signal has reached certain threshold level, which can be represented as a sigmoid function. PFL are required to be hypersensitive and generate three equilibria, of which only the high- and low- expression states are stable. Then, relatively small fluctuations in the concentration of the autoactivator do not affect the system, which always will fall into one of the two stable states. On the other hand and, as observed in the right panel, systems without a PFL show a lineal distribution of expression (red curve) and degradation (black line) states where, low and high expression rates are observed for low and high concentration of the activator, respectively. Adapted from (Siebring et al. 2012) and (Smits, Kuipers, and Veening 2006).

2. PFL are the key mechanistic determinant of bacterial bistability

A simple definition of noise in gene expression can be how "c*lonal populations of bacteria exhibit unimodal variation in the expression of a given gene, due to random fluctuations in the rates of synthesis and degradation of the cognate gene product*" (Dubnau and Losick 2006). It is clear that noise does not imply bistability by its own, either the mere existence of a PFL. Indeed, both are strictly necessary for the appearance of bistability and associate in how the noise displayed by a biological/biochemical system can be amplified by the existence of an autoregulatory PFL, giving rise to a bifurcation of the population in two stable subpopulations, leaving the intermediate states relatively unoccupied.

The stochasticity relies on the fact that, at least in experimental systems where cells are randomly distributed in a defined space, the choice of which individual cells exhibit altered gene expression is random (Dubnau and Losick 2006).

Experimental approaches have proved the hypothesis that noise in gene expression is an important biological variable subject to natural selection (Kaern et al. 2005). Mechanisms like PFL and redundancy of regulatory pathways, which operate at the level of gene and cell-regulatory control systems, have evolved specifically to minimize the impact of intra- and extracellular fluctuations and noisy signal transduction by assembling high transcription rates and low translational efficiency as an operational selective pressure for noise reduction (Kaern et al. 2005).

The activation of PFL normally results in the dynamic control and the modulation of several biological processes. In most living organisms including bacteria, one of the most remarkable features displayed by these genetic circuits is their ability to generate bistability, bimodality and hysteresis. Bistability is defined as the property of a system to have two stable steady states where, one of the steady states corresponds to the low activity of the system while the other corresponds to its high activity. Frequently mistaken with bistability but closely associated with it, bimodality is the property of a system to display a probability distribution with two distinct maxima. Bimodality is then, the property of bistability displayed by a biochemical system related to the distribution of its components (Mitrophanov and Groisman 2008b).

Normally, the definition of the system's activity can be observed as the bimodal activity of a defined reporter gene (component), tracked by the fluorescence emission of a transcriptional fusion at the single cell level (Maamar and Dubnau 2005; Sprinzak and Elowitz 2005; Dubnau and Losick 2006; Eldar and Elowitz 2010; Levine and Elowitz 2014). Now it is recognized that bacterial heterogeneity and the process of cell differentiation in bacterial populations are ruled by the activation of several-combined PFL, which directly and indirectly cooperate to activate their own expression and the expression of several genes in complex regulatory cascades that conclude with the emergence of cell heterogeneity that is able to be inherited and to endure in time, in what is known as hysteresis. Hysteresis is formally defined as "*the ability to produce different steady-state signal-response curves for the cases of increasing and decreasing stimulus intensity*" (Mitrophanov and Groisman 2008b). In other words and in the context of biochemical/biological systems, hysteresis implies that the threshold level to activate the system is higher than the corresponding threshold to deactivate the system. That is, that the expression state of the system appears to display a memory of previous expression states (Basset et al. 2012) and, its most intense manifestation is the irreversibility of a system. Bistability is, in fact, a prerequisite for hysteresis (or epigenetic memory): *"the ability of genetically identical cells to differ in phenotypes and to maintain their distinct phenotypes from one generation to the next*" (Sneppen, Krishna, and Semsey 2010).

In developmental biology, has been largely believed that irreversibility of a differentiated state can be maintained by feedback loops (W. Xiong and Ferrell 2003). There, hysteresis is considered as the principal mechanism able to generate a self-sustained pattern of gene expression, producing a systems-level memory of transient stimuli. Indeed, experimental data on bistable systems have shown that they can function as memory modules relevant in the physiological process of cell fate induction. A remarkable study based on the *Xenopus* oocyte maturation, showed that a bistable signalling system converts a transient stimulus into a reliable, self-sustaining and irreversible pattern of protein activity (W. Xiong and Ferrell 2003).

The activation dynamics observed in a bistable system denotes the fundamental importance of autoregulatory mechanisms such as PFL as a necessary condition to amplify the intrinsic and extrinsic noise (stochasticity) nature of biological systems. PFL guarantee signal amplification, the rapid activation of the system and finally, the emergence of two well-differentiated bimodal probabilities maxima (Alexander Y Mitrophanov 2008; Raj and van Oudenaarden 2008; Locke et al. 2011).

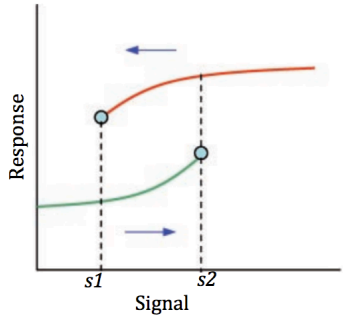

Figure 14. Hysteresis in a bistable system. The transition from the low to the high steady state is achieved when the activating signal reaches the *s2* threshold. Hysteresis is achieved within the interval *s1-s2* where, even if the activating signal is removed (less than *s2* but greater than *s1*) the high state coexists with the low state. In bacteria bistable systems are reversible, since the increase in the signal from *s1* to *s2* or *vise versa*, can reconstitute the coexisting high and low steady states. Adapted from (Mitrophanov and Groisman 2008b).

The bistability property of bacterial TCS has been defined as "learning behaviors" in gene regulation processes (Mitrophanov and Groisman 2008b). This interesting definition is based on the fact that, activation of the TCS normally results in increased levels of both, the sensor kinase and the response regulator, even after signal removal. Then, subsequent reactivation of the autoregulatory system will be characterized by a shorter activation time. Different activation times and different activation thresholds, which are known characteristics of different bistable TCS in bacteria, define distinctive gene expression patterns for a particular system under specific conditions and/or after sensing specific environmental changes.

Bistability is then, a steady-state property of regulatory systems like the bacterial TCS and, guarantee rapid, sensitive and efficient switching behaviors, robustness in the presence of noise and tunability in response to changing environmental and intracellular conditions. Even the simplest PFL system can display transient surge-like behavior that is critical for many transient bacterial physiological responses such as virulence (Mitrophanov and Groisman 2008b) or biofilm formation and, the elimination of PFL has shown to affect the regulatory and temporal precision of many developmental processes (Gao and Stock 2013).

Bacterial TCS and the PFL embedded in them have shown to be the driving force for heterogeneity in bacterial populations (toepel 2005). The fact that in the presence of the activation signal, a proportion of the cell population remained uninduced suggests that TCS are based on activation thresholds, instead of graded activation responses (Lamarche et al. 2008). The PFL that autoregulates this TCS is likely to contribute to the emergence of multiple stable phenotypes within the bacterial population not only by controlling its own expression, but also by contributing to the propagation and autoamplification of the response to downstream genes (Pedraza and van Oudenaarden 2005). Indeed, this kind of mechanisms have been largely observed in several biological systems and are thought to be a driving force in the determination of cell fate and development in complex organisms by promoting

gene expression in a small subpopulation, which would be always ready for diverse changes in a fluctuating environment (Elowitz 2002; toepel 2005).

The function of single and/or coordinated PFL under particular circumstances, might contribute to stabilize the process of cell heterogeneity that finally might maximize the fitness of the whole bacterial community in fluctuating environments (Balaban et al. 2004; Kaern et al. 2005). As result, environmental conditions able to trigger collective behavior and induce a specific developmental program, together with the resulting microenvironments found within a bacterial multicellular community, lead to the expression of properties never anticipated for individual bacterial cells, enhancing genetic diversity and adding further levels of complexity to bacterial adaptability (Steinert 2014). The complexity elicited by the PFL found in the bacterial TCS, offers some clues about the fundamental shift between unicellular and multicellular life style in the evolutionary history of all living forms.

3. Bistability, heterogeneity and bacterial cell differentiation

Bacterial populations are not longer believed to be uniform with respect to the physiology and morphology of the individual cells. Heterogeneity is now considered a general attribute of bacterial communities and, PFL are key genetic determinants in the emergence of different states (or subpopulations) within a bacterial population. Heterogeneity was first observed during the process of spore formation and during the appearance of genetically competent cells in *B. subtilis* (L. Shapiro, Agabian-Keshishian, and Bendis 1971; Dubnau 1991; Branda et al. 2001; J. A. Shapiro 2008; López, Vlamakis, and Kolter 2009). Bacteria use interlinked networks of sensing and regulation pathways, such as the TCS, to conduct several different adaptive processes simultaneously (Hamoen 2003).

Phenotypic heterogeneity found in bacterial multicellular communities is both, the cause and the consequence of the diversity of chemical states displayed in these multicellular communities. The mechanisms that contribute to the physiological heterogeneity include mostly, microscale chemical gradients of oxygen, nutrient, intermediate metabolites and metabolic products and, the genetic adaptation of bacteria to those local environmental conditions (P. S. Stewart and Franklin 2008). However, the process that generates chemical gradients in biofilms reciprocally is the result of the diversification of metabolic activities of the cells within the community as they adapt to those gradients, which creates a constant cycle of challenges and responses between the environment and the cells. Therefore, the diversification of metabolic activities together with the merely physical diffusional processes, result in concentration gradients of nutrients, signalling molecules and bacterial metabolic products (waste) to which bacteria respond and adapt through well defined genetic circuits of stress response, generating a considerable structural, chemical and phenotypic heterogeneity (P. S. Stewart and Franklin 2008). Microscale heterogeneity in solute chemistry, exemplified by the microscale gradients of oxygen, is one of the clearest mechanisms involved in the physiological diversification found in biofilms (P. S. Stewart and Franklin 2008). The presence of oxygen-concentration gradients determines the response of the cells and the emergence of different physiological states as the result of a phenotypic differentiation. As a result, bacteria in the differentially exposed to oxygen adapt a

physiological response to this element and adapt a particular phenotypic state where growth and activity are slowed or arrested owing to substrate limitation. In a similar way, gradients of nutrients, QS molecules, metabolic products and even mechanical forces, determine the fate of the individuals within organized bacterial communities. Although chemical heterogeneity and physiological adaptation to the local environment explain much of the biological heterogeneity in biofilms, it is likely that other mechanisms also contribute to the phenotypic heterogeneity (P. S. Stewart and Franklin 2008).

4. Cell differentiation, bacterial multicellularity and division of labor

Now it is accepted that heterogeneity is a general attribute of bacterial populations. Indeed, in highly organized multicellular communities known as biofilms, heterogeneity is accompanied by an organized process of cell differentiation and division of labor (Ackermann 2015).

The ability of bacterial cells to individually respond to stochastic environments is an evolutionary solution adopted by almost every bacterial system (C. J. Davidson and Surette 2008) and therefore, biofilm formation might be understood as a natural evolutionary consequence of individuals cells delimited in space and time to reduce the stochastic influence of the environment over whole the clonal population.

From a simple perspective, when bacterial cells associate in a biofilm, they acquire population-level properties (Furusawa and Kaneko 2002): (I) recursive production of cells at a cooperative level, where cells ensemble as a unit and set their own boundaries as a multicellular organism; (II) cell differentiation, where all cells in the community are not identical and coexist with different characteristics and playing a different functional role, although they proceed from the same ancestor cell; (III) a robust developmental process, which means that the multicellular community responds and develops in a similar way to molecular and environmental fluctuations and implies the existence of finely tuned genetic control mechanisms that enable cells to respond and associate and; (IV) the emergence of a germ-line, where only one or few types of cells can produce the next generation (Furusawa and Kaneko 2002).

Importantly, multicellularity is attributed to the ability of cells to communicate and in bacteria this ability resides in the QS systems. Gene expression of most of the master regulators of QS in bacteria has shown to have autoregulatory characteristics, several nested feedback loops along the signaling cascade, relatively low stochasticity and autoinducer-dependent noise (Balázsi, van Oudenaarden, and Collins 2011). These characteristics imply that several QS circuits have evolved as network structures capable of noise reduction and modulation of the bacterial individuality during the transition to a population-level behavior (Becskei, Séraphin, and Serrano 2001; Nevozhay, Adams, and Murphy 2009; Balázsi, van Oudenaarden, and Collins 2011). The genetic architecture of most QS, together with their fundamental characteristic of being the driving force of bacterial communication, makes them the perfect mechanism to generate cell differentiation in multicellular communities of bacteria.

A mathematical approach for the emergence of multicellularity revealed that increases in high cell density make cell systems unstable promoting differentiation and, the emergence of another cell type stabilizes the dynamics of each cell again (Furusawa and Kaneko 2002). These authors suggest that

differentiation is a general feature of a system of interacting units, where each of them possesses an individual and nonlinear internal dynamics.

As bacteria cannot store genetically all the information required to develop multicellular attributes under all possible environmental conditions, it has developed cooperative features for the colonial organization to proceed by using the informatics capabilities of each individual cell as an autonomous system capable of sensing, storing, processing, assessing and amplifying the environmental information (Ben-Jacob 2008). By exploiting the inherent noise of each biological system, bacterial communities can afford self-alteration and promote cell-to-cell communication that in consequence might initiate alterations of the surrounding individuals. As a result, collectively bacteria store information, perform *decision-make* decisions and learn from past experience (epigenetic) in a very efficient way, features associated with rudimentary forms of intelligence (Ben-Jacob 2008) as well as with bacterial biofilms (Balázsi, van Oudenaarden, and Collins 2011; Ackermann 2015).

4.1. Population-level behavior: bacterial multicellular communities and division of labor

The fact that multicellular behavior in bacteria is now recognized as the preferred life style for these organisms (Costerton et al. 1987; Donlan and Costerton 2002), suggest that stochastic cellular decisions in clonal bacterial populations may undoubtedly contribute to the course of how bacteria join forces to orchestrate population-level behaviors such as biofilm formation, virulence or luminescence. The complexities in the architecture of bacterial biofilms suggest that this process is not stochastic (O'Toole, Kaplan, and Kolter 2000).

Multicellularity in bacteria appear early in the fossil record (~3.25 billion years ago) and coincides with the first evidence of an evolutionary transition from unicellular to multicellular organization, suggesting that bacteria that transitioned into a biofilm life style might have been the first multicellular life forms (la Fuente-Núñez et al. 2013).

One of the most remarkable features displayed by bacterial biofilms is the highly organized population structure. At the microscopic and macroscopic level, this characteristic reflects the high degree of spatial heterogeneity within bacterial multicellular communities, which has shown to promote the evolution of cooperative traits in the environment by increasing the frequency of interactions and communication between cells and favoring cooperation (Celiker and Gore 2013; Serra et al. 2013).

The spatial constraint found in biofilms is a consequence but also a requisite for the emergence of efficient cell differentiation and division of labor. Although stochasticity and bet hedging have been considered the major benefits of phenotypic heterogeneity in bacteria, one fundamental adaptive benefit of an organized multicellular aggregate is based on the interactions and the division of labor between phenotypically different individuals in a clonal group (Ackermann 2015). In the case of biofilms, the characteristic feature is that the benefits for a given individual depend on the phenotypes of the other cells in the same microenvironment and, this diversity of phenotypes is promoted by the spatial limitation that supports communication, interactions and the division of labor between individual cells (Ackermann 2015). It is generally accepted that division of labor is coordinately regulated within multicellular communities through intercellular communication (JEFFERSON 2004).

QS is a density-dependent phenomenon and, if activated in a highly organized population structure, guides the emergence of sophisticated mechanisms of cooperativity and cell differentiation that finally leads the survival and the growing of the clonal population as a whole. In bacterial biofilms, division of labor leads to collective functionality, where social interactions within phenotypically different individuals improve the fitness of the multicellular community, affecting growth and/or survival for every individual in the same environment (Ackermann 2015).

In biofilm formation, decisions depend on the coordination among progeny and this requires some degree of memory that enables multicellular cooperation. By coordinating memory behavior across many generations, the timing provided by interlinked feedback loops enforces cooperation among the progeny of a cell that initiates a process of cell differentiation in a multicellular community (Norman et al. 2013). Long-term commitment to a particular cell type during an early process of biofilm formation, relies on coupled feedback mechanisms that lock cells into the multicellular state, where at least of the feedback loops is activated in response to particular environmental signals (a desirable niche, starvation or hypoxia for example) that can be periodically re-evaluated by the same feedback mechanisms (Norman et al. 2013). Subsequently, the process of cell fate decision-making leading to multicellularity and the early steps of biofilm formation in *B. subtilis* was defined as a *"...type of excitable dynamics, in which the system is randomly kicked out of a stable state but returns after a well-defined excursion...explained in terms of linked feedback loops...that could have a role in narrowing the probability distribution of time spent producing matrix proteins...and implicated in other* B. subtilis *decision networks."* (Norman et al. 2013).
Although noise is inherent to any biological system and cannot be eliminated, gene networks comprising, for example, feedback loops are capable of controlling and adjusting gene expression noise, the rate of random phenotypic switching and the rate and outcome of phenotypic differentiation (Balázsi, van Oudenaarden, and Collins 2011).

4.2. QS, heterogeneity and community cooperativity

Bacterial heterogeneity is the fundamental biological phenomenon behind biofilm formation and dissemination in bacteria and at the same time, QS genetic networks drive most of the cell heterogeneity in bacteria (Stoodley et al. 2002). Bacterial QS promotes cooperativity by controlling the production of public goods for the benefit of any member of the community. Interestingly, in multicellular communities, not all individuals respond to QS signals and hence, not all individuals produce public goods (Dandekar, Chugani, and Greenberg 2012). The communication system enabled by QS provides a system for the effective coordination of cooperative behaviors, co-regulating the functions required for utilization of public and private good and providing a genetic mechanism against cheating. In natural habitats, QS could serve to promote multicellular behaviors and ultimately the dispersal of individuals to new niches where cooperative behavior is needed, selecting phenotypes against social cheats (Dandekar, Chugani, and Greenberg 2012). The QS control of multicellular behaviors might be observed from a collective perspective: QS is an energetically expensive process that enables bacteria to (spatially synchronize and) survive as a community only when the impact of such a

expense is beneficial on certain environment or host and, the survival probabilities can be maximized (Rutherford and Bassler 2012).

Growing evidence suggest that QS-dependent responses, as many other bacterial bistable responses, are not always homogenous (Grote, Krysciak, and Streit 2015). The QS system of *L. monocytogenes* represents an interesting example. In this human pathogen, the QS system is produced by the *agrBDCA* operon. Fusing the *agr* promoter region to GFP, it was shown that the agr system is in general highly heterogeneous, concluding that the *L. monocytogenes* agr system is in general subject to heterogeneous regulation. Of importance, these authors found that growing conditions, for example liquid cultures or biofilm growth in flow cells, had a significant impact on the degree of heterogeneity. In fact, biofilm lifestyle appeared to increase the observed heterogeneity of *L. monocytogenes* cells (Garmyn et al. 2011; Grote, Krysciak, and Streit 2015).

In bacteria, is not possible to separate QS from biofilm formation. Although the mechanism and timing of production differ among bacterial species, QS plays a role in dictating when this process occurs and particularly, high concentration of QS molecules has been correlated with the ability of cells to detach from the multicellular communities in many species, such as *Rhodobacter sphaeroides* and *P. fluorescens* (Stoodley et al. 2002; Rutherford and Bassler 2012). Within biofilms, bacterial communication is more robust because the cells are in intimate associations and, if the QS signaling displays an heterogeneous (bistable) behavior, members of biofilm communities most certainly respond more effective but at the same time divergently, ultimately profiting from the ability to differentially communicate and respond, creating a multicellular community with a variety of phenotypes and therefore, a diversification of their physiology. In this respect, a link between heterogeneity in the QS-response and subsequently, downstream in all the QS-dependent structural and regulatory-network genes, might indicate that similarly, in other bacterial species such as *S. aureus, E. coli*, *P. aeruginosa* and *V. cholerae,* where biofilm formation is strongly controlled by QS systems, cell heterogeneity might be the driving force in the process of cell differentiation and division of labor.

Given the fact that QS regulates global gene expression patterns, other regulatory systems might have co-opted QS heterogeneity to control additional products, probably also in a bistable manner, in response to particular environmental conditions (Rutherford and Bassler 2012). In a similar manner, heterogeneity in the several gene regulatory systems that feed information into (upstream) the QS systems and fine-tune the networks that control biofilm formation and/or virulence might determine the heterogeneous fate of the QS systems and call for an structured multicellular community where different cell types with different phenotypes coexist and cooperate.

5. Staphylococcal heterogeneity. Bringing all together: environmental stress, Agr QS and biofilm formation

The versatility of *S. aureus* manifestations within the host, which easily switches between a harmless commensal to a dangerous pathogen or, from a chronic biofilm to a aggressive septicemia, strongly suggests that cell heterogeneity is actively operating across the entire staphylococcal population and the

host environment to cause such a *switching probability* (Dubnau and Losick 2006). Therefore, understanding the physiological and molecular basis of *S. aureus* heterogeneity is fundamental to fight this live-threatening infectious organism.

S. aureus was the first species where bacterial heterogeneity was observed. J.W. Bigger discovered that a subpopulation of staphylococcal cells was always persisting after penicillin and then, might be responsible for the failure in treatment. Importantly, those subpopulations were not resistant mutants (Korch, Henderson, and Hill 2003; Balaban 2011). Now, it is recognized that persistence is a stochastic process for the generation of heterogeneity, which seems to reside in the toxin-antitoxin (TA) modules (Korch, Henderson, and Hill 2003; Korch and Hill 2006; K. Lewis 2007; K. Lewis 2010). The fine signaling and genetic regulation of TA modules and its influence on the emergence of a subpopulation of persisters has not been fully elucidated but, it has been established that in *E. coli* the alarmone ppGpp, which regulates bacterial stringent response that reprograms cellular metabolism (Maisonneuve, Castro-Camargo, and Gerdes 2013). The fact that most of the persister subpopulation appears at late exponential phase, suggest that persister heterogeneity and QS follow a similar dynamics. Indeed, P. *aeruginosa* and *S. mutants* modulate persister frequency in response to the QS-signaling molecules. In *E. coli* and *Salmonella* Typhimurium, indole, which is produced under nutrient-limited growth conditions, was suggested to induce persister cell formation (Maisonneuve and Gerdes 2014).

In *S. aureus*, the MazF toxin of the *mazEF* locus is the most studied. MazF is a sequence-specific RNase that cleaves a number of transcripts, including mRNA of pathogenicity factors but, no direct involvement in persistence formation as been reported. The transcription of the MazEF system is coupled to the σ^B locus and interestingly, the *mazEF* promoter is needed for full activity of σ^B system (C. Schuster and Bertram 2016), suggesting a direct role of MazEF in stress response and maybe, in the emergence of persisters during particular environmental conditions.

Persistence and biofilm formation are both reversible lifestyles and, PFL-mediated bistability and threshold amplification of intracellular regulatory noise have been identified as critical for bacterial persistence as well as in biofilm development (Balaban 2011), suggesting that the mechanisms involved or presumed to be involved are also similar.

An essential study of *S. aureus* biofilms showed that the expression of the *agr*-dependent RNAIII gene was restricted to patches within cell clusters and oscillated in time, with the loss of fluorescence coinciding with the detachment of cells from the biofilm. These results indicated a role for *agr* expression in biofilm development and in particular that the behavior of the Agr system was highly dependent on the environmental conditions (Yarwood et al. 2004). The expression of the *agr* reporter was not unimodal along the mature biofilm and, not all cells in a cluster expressed the gene, demonstrating a heterogeneous pattern of gene expression of the Agr system in staphylococcal biofilms (Yarwood et al. 2004). In addition, there was an oscillation in expression in these patches appearing in periodic waves, starting at late stages of biofilm development at 16 h, 20 h and 32 h. Surprisingly; the loss of fluorescence was accompanied of the detachment only of the *agr*-expressing cells from the

clusters but, most of the region under observation was covered by the biofilm, strongly suggesting an specific physiology for the dispersed cells.

A technically challenging study showed the confinement-induced QS of individual *S. aureus* bacteria, extending the QS concept and postulating that the Agr QS is a genetic system that can be also beneficial not only at the community level but also, at the single-cell level. Interestingly, this work demonstrated that the Agr QS system is able to promote genetic reprogramming in a single isolated organism, suggesting that the agr might act as an autonomous mechanism to generate cell heterogeneity, independently of high-cell densities. Remarkably, it was shown that QS allows *S. aureus* to sense confinement and to activate virulence and metabolic pathways needed for survival at the single-cell level and, that under this particular conditions, the agr QS system does provide a significantly greater viability over non-QS bacteria. Here, it is emphasized that for medically important pathogens such as *S. aureus*, QS might act as an autonomous autoregulatory system that can contribute significantly to the survival of the isolated individuals in natural systems during infections such as within an endosome or a phagosome and, maintain cell viability under conditions of complete chemical and physical isolation (Carnes et al. 2009).

An interesting mathematical model analyzed the emergence of cell heterogeneity in bacterial biofilms as a consequence of the spatial heterogeneity of autoinducer regulation systems, which enable coordinated behavior of bacterial populations. It was found that in bacterial aggregates, gradients of signals and environmental substances emerge to create a regulation network based on the autoinducer molecule, generating a spatially heterogeneous behavior that resembles multicellular division of work, especially under nutrient-controlled conditions (Hense et al. 2012).

Later on, a more general approach was used to define the spatial patterns of DNA replication, protein synthesis, and oxygen concentration within staphylococcal biofilms, demonstrating that these communities are constituted of subpopulation of cells with diverse physiological states (Rani et al. 2007). This study found stratified patterns of DNA and protein synthesis activity in three different biofilm systems where, two bands of DNA synthetic activity were evident: one along the air interface of the biofilm and a thinner region along the membrane interface while; protein synthesis progressed inward into the biofilm over time, demonstrating large variation along the structure with a single band of bright green fluorescence at the air interface of the biofilm. These patterns permitted to define a first zone, characterized by an anabolic activity at the air interface that corresponded with the depth of oxygen penetration and, a second zone of activity along the nutrient interface. Interestingly, it was found that much of the biofilm (around the 70% of the total biomass) was constituted by cells anabolically inactive but viable and that, only the 10% of the biofilm population was populated by dead cells (Rani et al. 2007). These results suggested that staphylococcal biofilms contain cells in at least 4 distinct states: growing aerobically, growing fermentatively, dormant and dead; all of them contributing to the special ecology and tolerance to antimicrobial agents that characterizes these multicellular structures. In addition, oxygen measurements defined that this element is diffused passively into biofilms and hence, is locally depleted. Actually, the lower two-thirds of these biofilms are anoxic and

respiring bacteria found on the surface consume it before it reaches the lower parts of the community (Rani et al. 2007).

MATERIALS AND METHODS

1. Bacterial strains, growing media and culture conditions

We used the clinical isolate *S. aureus* strain Newman (DUTHIE and LORENZ 1952; Lipiński, Hawiger, and Jeljaszewicz 1967) during the development of this work. In addition, we used the laboratory *S. aureus* strain RN4220 (Kornblum et al. 1990) for cloning purposes. *Bacillus subtilis* strain 168 was used as genetic background to generate a staphylococcal *agr* synthetic strain. The strain *Escherichia coli* DH5α was used for propagating plasmids and genetic constructs in laboratory conditions. *B. subtilis* and *E. coli* strains were regularly grown in LB medium. A complete list of strains is shown in Table S1 (Appendix A).

When required, selective media were prepared in LB agar using antibiotics at the following final concentrations: ampicillin 100 μg/ml, kanamycin 10 μg/ml, chloramphenicol 5 μg/ml, tetracycline 10 μg/ml, and erythromycin 2 μg/ml.

S. aureus strains were routinely propagated in liquid TSB (Becton Dickinson) medium incubated with shaking (220 rpm) at 37 °C for 16 h. When required, selective media were prepared in TSB using antibiotics at the following final concentrations: kanamycin 10 μg/ml, chloramphenicol 10 μg/ml, tetracycline 10 μg/ml, erythromycin 2 μg/ml, neomycin at 75 μg/ml, Spectinomycin at 300 μg/ml and Lincomycin at 25 μg/ml.

To generate multicellular aggregates, *S. aureus* was grown overnight (ON) on TSB (Becton Dickinson) plates at 37 ºC and then incubated in TSB medium (BD) supplemented with $MgCl_2$ at a final concentration of 100 mM (TSBMg) at 37 °C with shaking. Two (2) μl of the overnight culture were pelleted and resuspended in 250 μl of TSBMg and then, 4 μl were spotted on the surface of TSBMg agar plates and incubated at 37°C for up to 5 days.

2. Generation of fluorescence-labeled and mutant strains

To generate chromosomally integrated transcriptional fusions of *S. aureus*, we followed the protocol proposed by Yepes et al. (Yepes et al. 2014). To generate mutant strains, we followed the two-step double recombination process proposed by Arnaud et al. (Arnaud, Chastanet, and Débarbouillé 2004). Briefly, to generate the different *S. aureus* mutants; we amplified 500 bp upstream and 500 bp downstream of the target gene and the respective antibiotic cassettes. The primers used for these PCR contained homology fragments in the 5'- and 3'- ends to generate the joining partners for a long-flanking homology PCR (LFH-PCR). The joining fragment was digested using *EcoRI* and *BamHI* (NEB), following the manufacturer's instructions. The resulting fragments were cloned into pMAD plasmid (Arnaud, Chastanet, and Débarbouillé 2004) and transformed by electroporation into *E. coli* DH5α by electroporation. The plasmidic DNA (pDNA) was isolated from *E. coli* and purified using MiniPrep columns kit (Macherey Nagel) and, 1 μg of purified pDNA, quantified by spectrophotometry, was transformed by electroporation into the laboratory strain *S. aureus* RN4220. To transfer the mutations from RN4220 to Newman and to generate the double mutant strains, φ11 phage lysates

were generated from RN4220 mutants to infect Newman (Rudin et al. 1974). Clones resistant to the respective antibiotic were further verified to carry the mutation using PCR. Positive clones were validated to carry the mutation using Sanger sequencing using the flanking-regions and the antibiotic cassettes primers in all possible combinations.

To generate the *S. aureus* strains single-labeled with P_{ica}-*yfp*, P_{spa}-*yfp*, $P_{psm\alpha}$-*yfp*, $P_{psm\beta}$-*yfp*, P_{dnaA}-*yfp* and double-labeled with P_{spa}-*yfp* P_{ica}-*mars*, $P_{psm\beta}$-*yfp* $P_{psm\alpha}$-*mars*, $P_{psm\alpha}$-*yfp* P_{ica}-*mars*, P_{spa}-*yfp* $P_{psm\alpha}$-*mars*, P_{ica}-*yfp* P_{clfA}-*mars*, P_{ica}-*yfp* P_{isdA}-*mars*, P_{spa}-*yfp* P_{clfA}-*mars* and P_{spa}-*yfp* P_{isdA}-*mars* transcriptional fusions, the respective promoter region comprising 200 to 500 bp upstream of the start codon were PCR amplified from purified genomic DNA from Newman using primers with additional *EcoRI* and *HindIII* restriction sites at the 5'-end, digested and fused to the PCR amplified *yfp* reporter-gene using the plasmids pKM003 or to the *rfp* (mars) reporter-gene using the plasmid pKMmars. The amplified promoter region and the respective pKM plasmids were digested with *EcoRI* and *HindIII* (NEB), purified by osmosis on nitrocellulose filters on double distilled water for 15 min and then, ligated ON at 16 ºC using T4 ligase (NEB) following the manufacturer's instructions and adding 1 µl of ATP 1 µM to improve the ligation reaction. Next day, the ligation was purified again using nitrocellulose filters and transformed into *E. coli* DH5α by electroporation. The pDNA was isolated from *E. coli* and purified using MiniPrep columns kit (Macherey Nagel) and then, digested with *EcoRI* and *BamHI* to release the transcriptional fusion. The digested plasmid was run in a 0.8 % agarose gel and the fusion was cut and purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel). These fusions were subcloned into the plasmids pAmy and pLac (Yepes et al. 2014) and transformed by electroporation into *E. coli* DH5α, propagated, purified and then, 1 µg of pDNA transformed into *S. aureus* RN4220. In *S. aureus* RN4220 the plasmids were integrated into the neutral *amy* and *lac* loci of *S. aureus* chromosome to ensure a uniform and chromosome-equivalent copy number of the reporters in all the cells within the microbial community. The integration of reporters in *amy* and *lac* neutral loci occurs in a two-step recombination process, as described in (Yepes et al. 2014). Briefly, integration of the plasmid into the chromosome of *S. aureus* RN4220 occurs via a first single recombination event. This first recombination occurs by growing the plasmid-carrying strain overnight at 30 ºC, plating serial dilutions onto selective media (erythromycin 2 µg/ml and X-Gal 100 µg/ml) and incubating the plates at 44 ˚C. This is a temperature-sensitive plasmid, which does not allow plasmid replication at higher temperatures (Arnaud, Chastanet, and Débarbouillé 2004). Therefore, incubation at 44 ˚C allows only the strains that incorporate the plasmid into the chromosome to grow. The genetic constructs obtained from the first recombination process in the strain RN4220 were transferred to strain Newman by φ11 phage transduction (Rudin et al. 1974). Once the constructs were transferred to the strain Newman, we forced a second recombination process to leave only the reporter in the integration locus. To do this, a culture of a light-blue colony was incubated overnight at 30 ˚C in the absence of erythromycin, plating serial dilutions onto selective (erythromycin and X-Gal) media and then incubating the plates at 44 ˚C. After 48 h of incubation at 44 ˚C, the light-blue colonies still carrying the plasmid in the chromosome were discarded. A four-step process of screening, including

fluorescence, antibiotic susceptibility, PCR and Sanger sequencing, was designed to validate whether the white colonies carried the corresponding insertion in the neutral loci.

The *S. aureus* strains lower-*tagB* (NWMN_0187), lower-*tagG* (NWMN_1763) and lower-*tagH* (NWMN_1763) were obtained by φ11 phage transduction using as donor strain the respective mutants deposited in the transposon-mapped mutant collection (Bae et al. 2004). Clones were verified using PCR and Sanger sequencing. The *S. aureus* strain that overexpresses the *tagB* gene (NWMN_0187) was obtained by cloning the complete ORF into the replicative plasmid pJL74 (Klijn et al. 2006). The plasmid pJL74 has the *sarA* promoter sequence and in addition, we added the ribosomal binding site (RBS) of the *sodA* gene to guarantee high expression levels in *S. aureus*.

A complete list of plasmids and primers are shown in Table S2 and Table S3 (Appendix A).

3. Generation of the synthetic Agr orthologous model in *B. subtilis*

The two genes *agrC* and *agrA*, which are adjacent in the operon *agrBDCA*, were cloned as a chimeric version *agrCA*. The gene *agrC* encodes the histidine kinase and the gene *agrA* encodes the cognate regulator. Briefly, as the *agrA* gene is immediately downstream the staphylococcal *agrC* gene, we amplified by PCR the complete region, adding the restriction sites *HindIII* and *SphI* to the 5' and 3'-ends of the forward and reverse primers, respectively. The resultant construct was integrated into the *amyE* neutral locus of *B. subtilis* strain 168 chromosome.

In a similar way, the P_{ica}-*yfp*, P_{spa}-*yfp*, $P_{psm\alpha}$-*yfp*, $P_{psm\beta}$-*yfp*, P_{RNAII}-*yfp* and P_{RNAIII}-*yfp* transcriptional fusions were cloned into plasmid pDR183 and integrated into the neutral locus *lacA*. For transformation via double heterologous recombination, all plasmids were linearized and added to competence-induced liquid cultures of *B. subtilis* strain 168 as proposed in (Vlamakis et al. 2008; Garcia-Betancur et al. 2012). Resultant colonies were verified that contained the reporter using Sanger sequencing.

The synthetic model that recreates the divergent P2 and P3 promoters of *S. aureus* contains a DNA fragment of the construct of RNAII and RNAIII joined to the *cfp* and *yfp* genes divergently transcribed by the P2 (RNAII) and P3 (RNAIII) promoter, respectively. This fragment was cloned into the plasmid pDR183 and integrated into the neutral locus *lacA*. The integration of the fragment occurs by double heterologous recombination. The plasmids were linearized and added to competence-induced liquid cultures of *B. subtilis* strain 168. The resultant colonies were verified to contain the construct using Sanger sequencing.

4. Microtiter plate-based biofilm assay

For *S. aureus* traditional biofilms, we followed the protocol proposed by (O'toole and Kolter 1998) with some modifications. Briefly, an overnight liquid culture was inoculated 1:100 in fresh TSB media and after 6 hours at 37 ºC with shaking at 220 rpm, the OD_{600} nm was measured and normalized to a final OD_{600} 0.05. From the normalized cultures, 5 µl was inoculated in 995 µl of TSB or TSBMg in a 24-well microtiter plate (Thermo) and incubated up to 48 h at 37 ºC without shaking inside the original

plastic bags closed with adhesive tape to avoid dehydration and to guarantee a more homogeneous environment. Biofilm formation was measured as follows: media was discarded by mild aspiration; wells were washed twice with PBS and allowed to dry for 45 min at 65 ºC. Then, 500 µl of a solution of Crystal Violet at 0.1% in water was added and to stain the organic material associated with the well for 5 minutes. After staining, wells were washed three times with deionized water. For quantitative analyses, Crystal Violet was solubilized using 500 µl of acetic acid at 33%. The solubilized dye was diluted 1:100 in deionized water and transferred to 96-well microtiter plates (Thermo). The absorbance was determined at 595 nm using an InfiniteF200 Pro microtiter plate reader (Tecan). Background was corrected by subtracting the absorbance values of non-bacteria inoculated wells.

5. Analyses of the *B. subtilis* synthetic Agr orthologous system

For the experiments using the synthetic *agr* model generated in *B. subtilis*, cells were incubated in MSgg medium at 220 rpm at 37 ºC until cultures reached an OD_{600} nm = 0.5. After incubation, 50 µl of the culture was added to 50 ml of MSgg and allowed to grow for 4 hours at 37 ºC at 220 rpm. After incubation, 500 µl of culture was used to inoculate 50 ml of fresh MSgg and allowed to grow for 24 h at 37 ºC with constant shaking. Addition of AIP to the culture defined the initiation of the experiment (time = 0 h). Samples were taken at 0 h, 2 h, 4 h, 6 h, 8 h, 10 h, 12 h and 24 h.

6. Staphyloxanthin extraction and quantification

After 72 h of growth, cells from liquid TSB, TSBMg culture or from the multicellular communities of *S. aureus* wild type and the different mutant strains analyzed (Δ*dltA* and Δ*sigB*) were harvested, washed once and resuspended in PBS buffer. The cell densities at OD_{600} nm were measured and the samples normalized. Following the protocol proposed by (Pelz 2005; Morikawa et al. 2001), 1 ml of cells were centrifuged and the pellet resuspended in 200 µl of methanol and heated at 55 ºC for 3 min. Samples were centrifuged to eliminate debris. Then, 200 µl of the supernatant was taken and the methanol extraction repeated. A volume of 180 µl was recovered and added to 820 µl of methanol. Absorption spectra of the methanol extracts were measured using a spectrophotometer at a peak of 465 nm, normalized and reported as relative absorbance to express the total amount of staphyloxanthin pigment.

7. Atomic Force Microscopy

Mechanical indentation via atomic force microscopy (AFM) was applied according to previous publications (Dufrêne 2014; Formosa-Dague et al. 2016). Overnight cultures of *S. aureus* wild type or Δ*dltA* were diluted into fresh TSB or TSBMg liquid media to a final OD_{600} of 0.05. Cells were grown overnight again at 37 ºC and 220 rpm. One (1) ml of the culture was pelleted by centrifugation (5000 rpm for 1 min at room temperature) and washed with sterile PBS or PBS supplemented with $MgCl_2$ final concentration 100 mM (PBSMg) depending on culture conditions and, were normalized to a final OD_{600} of 0.5 in PBS or PBSMg. Cells were fixed with 4% p-formaldehyde for 6 min and washed twice with 500 µl of PBS or PBSMg. One series of 25 pulses of mild sonication, defined as a power output

of 70% and cycle of 0.7 sec, was applied to produce a homogenous sample of single cells. Finally, 40 µl of a dilution of 1:5 in deionized water was immobilized on poly-lysine coated microscopy slides. Samples were washed twice with Milli-Q water and allowed to dry. Samples were processed immediately after immobilization, in air and at room temperature, using a CFM conical probe AFM (Nanotec, Spain) with nominal spring constant 3 N/m and resonant frequency 75 kHz. Optical lever calibration and sensitivity were obtained by tapping the probe cantilever onto the glass surface of the slide and measuring the force response to z-piezo extension (z is vertical to the glass surface). For cell indentation, the AFM probe was placed above a cell and repeatedly pressed down onto the surface (and retracted) at 50 nm/s over distances of 100 nm, several times at several positions of the cell surface. Z position and speed of the AFM probe were controlled by a piezoelectric translator. The force response of the cell membrane was measured at three different positions for three individual cells. Young's modulus was obtained by fitting the resulting force-indentation curves for forces <10 nN, resulting in indentations < ~20 nm. Best fits were produced with a modified Hertz model assuming a conical punch probe geometry.

8. Purification of AIP

To obtain an enriched fraction of AIP, we used a protocol adapted from (MDowell et al. 2001). Briefly, 500 ml culture of the wild type *S. aureus* strain Newman was grown for 24 h in TSB and, after the removal of bacterial cells by centrifugation at 5000 x g for 20 min at 4 °C, the supernatant was filtered through a 0.22 µm membrane filter. The filtered supernatant was loaded into a C18 Sep-Pak cartridge (Millipore/Waters) previously stabilized with aqueous 20% CH_3CN and 0.1% trifluoroacetic acid. Elution of AIP was achieved with a 20-45% concentration range of CH_3CN. Fresh AIP fractions were used in each experiment due to the instability of the preparation.

9. Peptidoglycan extraction and quantification

Peptidoglycan (PG) from *S. aureus* was purified using a protocol adapted from (Bera et al. 2005; Peterson et al. 1978). Cells were grown in 2 L of TSB medium and incubated overnight at 37 °C with vigorous shaking. Bacteria were harvested by centrifugation (5,000 × g, 4 °C, 10 min), washed with cold Buffer 1 (20 mM ammonium acetate, pH 4.8) and resuspended in 30 ml of Buffer 1. Cell suspension was transferred to a falcon tube, centrifuged and determined the weight of the pellet. Cell pellet was resuspended in 2 ml of Buffer 1 and cells were disrupted with a bead beater (Genogrinder, SPEXsamplePrep, USA). After centrifugation, the interphase between glass beads and the foam at the top of the tube was collected and treated with 40 U of DNase, 80 U of RNase and 5 mM of $MgSO_4$ and were incubated 5 h at 37 °C. Cell walls were resuspended in 2% SDS in Buffer 1 and incubated 1 h at 65 °C. The material was washed twice with distilled water and resuspended in 30 ml of Buffer 1. Then, 5% trichloroacetic acid was added to remove WTAs from peptidoglycan and incubated 4 h at 60 °C. PG was then washed four to six times with cold Milli-Q water, lyophilized, and weighed. Before use, PG was resuspended in PBS buffer and sonicated on ice.

Quantification of peptidoglycan was performed using a protocol adapted (R. Zhou, Chen, and Recsei 1988; Nocadello et al. 2016). PG pellets were resuspended in 5 ml of cold Buffer 1 and diluted 1:50 in a final volume of 2 ml. PG was labeled with Remazol Brilliant Blue (RBB) (Sigma) by incubating the samples with 20 mM RBB in 0.25 M NaOH at 37 ºC with constant shaking ON. The labeled samples were neutralized with HCl and pelleted by centrifugation at 14000 rpm for 20 min at room temperature. We performed intense washing using distilled water to eliminate the remaining RBB. After washing, the RBB-PG complexes were diluted 1:50 and the OD 595 nm was determined. OD 595 nm values were normalized to wet weight of each PG-isolated sample.

10. Stereomicroscopy and fluorescence microscopy

Digital images of the development of *S. aureus* multicellular aggregates were captured with an AxioCAm-HR digital camera (Carl Zeiss) using AxioVision AC Release 4.3 software (Carl Zeiss).

To assay fluorescence microscopy, cells from the multicellular communities or from the liquid cultures were washed in PBS and resuspended for fixation in 0.5 ml of 4% paraformaldehyde solution and incubated at room temperature for 6 min. After two washing steps with PBS buffer, samples were resuspended in 0.5 ml of PBS buffer. Mild sonication was applied to the samples prior to single-cell analysis. Microscopy images were taken on a Leica DMI6000B microscope equipped with a Leica CRT6000 illumination system (Leica). The microscope was equipped with a HCX PL APO oil immersion objective with 100x1.47 magnification and a color camera Leica DFC630FX. The YFP fluorescence signal was detected using an excitation filter 489 nm and an emission filter 508 nm (excitation filter BP 470/40 and suppression filter BP 525/20). The RFP-mars fluorescence signal was detected using an excitation filter 558 nm and an emission filter 582 nm (excitation filter BP 546/12 and suppression filter BP 605/75). Excitation times were 567 and 875 msec, respectively. Transmitted light images were taken with 21 msec of excitation time. Linear image processing was done using Leica Application Suite Advance Fluorescence Software V3.7 (Leica).

11. Thin sectioning of mature *S. aureus* multicellular aggregates

For thin cryosectioning of *S. aureus* multicellular communities, we used previously published protocols (Vlamakis et al. 2008; Serra et al. 2013). Mature multicellular communities of *S. aureus* growing on TSBMg agar surface were cut and extracted from the agar and immediately immersed a solution paraformaldehyde 4% and let to fix at room temperature during 30 minutes. The fixing solution was carefully removed and the multicellular aggregates were immediately embedded in Tissue-Tek® O.C.T and let to penetrate for 30 minutes at room temperature. Blocks were frozen using liquid Nitrogen and immediately transferred to -20 ºC until processing. 4 µm-thick sections were obtained using a CM 3050 S cryostat set to -20 ºC (Leica). These sections were placed on SuperFrost® plus poly lysine-coated slides (Thermo) and immediately carefully rinsed twice with PBS buffer precooled at 4 ºC. To determine the structural features of these thin sections and localize the fluorescence, a series of horizontal optical sections were collected using a Leica TCS SP5 II confocal microscopy with a z-step

size of 0.3 µm. Width and height format in X and Y was set to 1024 x 1024 pixels at a scan speed of 200 Hz. Air 1 pinhole was set to automatic detection. A HCX PL APO CS 40.0X1.30 OIL UV objective was used for image acquisition. Digital images were captured using the Leica AF 6000 system software that is provided with the confocal microscope. All parameters were kept identical for the unlabeled control and the different labeled samples.

12. Histological analyses of infected mice organs

The infected mice organs were aseptically extracted and immersed in a solution 1:1 of PBS and paraformaldehyde 4% and left at 4 ºC overnight. 4 µm-thick sections were obtained using a CM 3050 S cryostat set to -20 ºC (Leica). These histological sections were placed on SuperFrost® plus poly lysine-coated slides (Thermo) and immediately rinsed twice with PBS buffer precooled at 4 ºC. Then, fixed-samples where stained with Giemsa staining solution (Sigma) including a dehydration step before the staining and a rehydration step after staining using Xylol and ethanol at 96%, 70% and 50%. Slides were immediately mounted with coverslips and processed by confocal microscopy.

To assess bright field microscopy of fixed infected mice organs, the infected organs were examined using a Leica DM4000B microscope. Digital images were obtained using the Diskus software (Hilgers). To assess fluorescent images of fixed and infected organs, a Leica TCS SP5 II confocal microscope equipped with A HCX PL APO CS 100X1.47 OIL objective was used (Leica). The hardware settings included: Argon laser power at 25% and 496 nm laser intensity at 10-15%. Bright field images were collected using the PMT-1 Trans scan channel at 512 V with a gain offset of -0.15%. Fluorescent images were collected using the HyD-2 channel with a gain of 10 and an emission bandwidth of 500 nm for excitation and 550 nm for emission (excitation filter BP 470/40 and suppression filter BP 525/20). The acquisition mode included a xyz scan mode, with z-stacks in the z-wide mode from 4 to 8 µm. To localize fluorescence, a series of horizontal optical sections were collected using a z-step size of 0.2 µm and with an optimized system. Width and height format in X and Y was set to 1024 x 1024 pixels at a scan speed of 200 Hz. Air 1 pinhole was set to automatic detection. Digital images were captured using the Leica AF 6000 system software provided with the confocal microscope. All parameters remained constant during the examination of the different labeled samples.

13. Live microscopy and cell-lineage tracking

For hysteresis determination using live fluorescence microscopy, 5 day-old multicellular communities of *S. aureus* were removed from the agar surface, resuspended in 500 µl of TSBMg medium and disrupted by mild sonication. Then, 5 µl of the cell suspension was diluted in 495 µl of pre-warmed TSBMg, and placed in microscopy pads containing TSBMg and 1.5% Low Melting Point Agarose. This microscopy pad was placed upside-down over the coverslip of a 35 mm glass-bottom microwell culture dish (MatTek) with wet Whatman paper to avoid dehydration of the thin layer. The MatTek dish was sealed with parafilm and images were taken every 30 min up to 6 hours using a Leica

DMI6000 B inverted microscope equipped with a temperature setting chamber that was set to 37 ºC. The time of image acquisition was 17 msec and 445 msec for bright field and fluorescence signals, respectively, to reduce phototoxicity. Digital images were captured using the Leica AF 6000 system release 2.6.0.7266 software.

The bright field and fluorescence images were used for parsimony mapping of the fluorescence inheritance to create the cell-lineage trees. Data analysis was performed using a semi-automatic protocol that involved manual measuring of the cell division in individual frames and determination of the fluorescence intensity using the software Image J. The cell lineage information included parental and fluorescent (fate) state. The fluorescence state was reported as a binary code (fluorescence *on* and *off*) based on a cutoff value of > 20 arbitrary fluorescence units above the background of a non-labeled strain. This information was used to generate a parsimony reconstruction using an *in house* script to generate the Newick file for the visualization software FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Within the tress, every node of the tree represents a cell division event in a time-lapse of 5 hours, of a five individual frames analyzed for each experiment.

14. Fluorescence dilution experiment

S. aureus cells carrying pMAD plasmid that contained the P_{psma}-*yfp* were grown in liquid TSB medium at 30 ºC for 16 h in the presence of erythromycin 2.5 µg/ml to propagate the plasmid. To generate a culture of low-fluorescent cells, a 1:10000 dilution was grown for 16 h at 30 ºC in TSB + erythromycin 2.5 µg/ml and after 16 hours of incubation, we performed several passage to fresh media (TSB + erythromycin 2.5 µg/ml) at 1:5000, 1:500 and 1:10 dilutions. Each one of the passages were allowed to grow for 4 h at 30 ºC in TSB + erythromycin 2.5 µg/ml. Cells were washed with PBS to eliminate traces of erythromycin and grown in a filtered-sterilized (0.22 µm pore-size filter) TSB and TSBMg. Last passage was monitored for the occurrence of fluorescence using flow cytometry and fluorescence microscopy at every hour until high-fluorescence appeared (time point t = 0 h). The sample was split in two samples that were treated differently (t = 0), one sample was reinoculated at 30 ºC + erythromycin 2.5 µg/ml. The second sample was reinoculated at 44 ºC in absence of erythromycin and in the presence of chloramphenicol. Chloramphenicol inhibits *de novo* protein proteins thus in the presence of chloramphenicol, detection of fluorescence signal correlates to the amount of YFP produced before chloramphenicol was added to the sample. A sublethal dose of chloramphenicol 1 µg/ml was added to the cultures. We took samples of 150 µl every hour of incubation and centrifuged at 5000 rpm for 2 min, resuspended in 300 µl of PBS and analyzed using fluorescent microscopy and flow cytometry. This procedure was performed every hour until fluorescence disappeared (time point t = +2 h). We took additional samples for RNA isolation and cDNA to quantify *yfp* gene expression.

15. Flow Cytometry

For flow cytometry analysis, cells from the multicellular communities were fixed with a treatment of 4% paraformaldehyde as mentioned above, washed and resuspended in PBS buffer. After fixation, a

sonication treatment was required to separate single cells in the sample. In this case, samples were subjected to three consecutive series of 25 pulses (power output 70% and cycle 0.7 sec) and kept on ice. Dilution of samples 1:500 was necessary prior flow cytometry analyses. For YFP fluorescence, a laser excitation of 488 nm coupled with 530/30 and 505LP sequential filter was used. The photomultiplier voltage was set at 777 V.

16. Fluorescence-Activated Cell Sorting for physical separation of *S. aureus* cell types

Multicellular communities at day 5 of development and labeled for cells expressing the extracellular matrix-production reporter (P_{ica}-*yfp*) or the detachment/virulence reporter ($P_{psm\alpha}$-*yfp*) were scraped from the TSBMg plates and immediately resuspended in *RNAlater* (Qiagen) in 1.5 ml RNAse-free Eppendorf tubes, in order to fix the cell fluorescence and at the same time preserve the RNA within the cells. Previous reports (Rosenberg et al. 2003) and fluorescence microscopy experiments performed in our laboratory showed that the fixing procedure of these multicellular communities in *RNAlater* had no effect in the conservation of the fluorescence when compared to cells fixed using 4% paraformaldehyde. Multicellular communities were disrupted in the *RNAlater* by extensive pipetting, followed by one series of mild sonication as mentioned above, and previously treating the sonicator with RNaseZap® RNase Decontamination Solution (Life Technologies). All procedures were performed on ice.

After mild sonication, samples were immediately processed using FACS. Cell fixation and subsequent mild sonication allowed cell separation at the single-cell level without affecting cell integrity. For the sorting procedure, 50 µl of the cell suspension was resuspended in 10 ml of filtered and autoclaved PBS buffer prepared in DEPC-treated water. This cell suspension was sonicated, changing cycles from 70% to continuous (100%) and performing 1 round of 20 sec. Immediately, cells were FACS-sorted based on their fluorescence intensity in a FACS Aria III (Becton Dickinson) using the following parameters: Nozzle size of 70 microns, FITC/Alexa Fluor® 488 nm laser, a 530/30 nm filter for data collection and a 502 LP mirror. Flow cytometry parameters were set as: SSC 341 V with a threshold of 500, FSC 308 V with a threshold of 500, FITC 769 V and a variable Flow Rate to guarantee that the number of events per second never exceeded 1500; hence, the Sorting Efficiency never dropped from 97%. These data were analyzed using the BDIS FACS Diva software version 7.0 provided with the FACS Aria III. Sorting was performed in the Precision Mode set to *Single Cell* in a first round, followed by a second sorting round set to *Purity*. Using the sorting Precision Mode, we recovered approx. 25 million cells of each subpopulation and their respective non-fluorescent counterparts, based on manually established Target Gates P1 for highly fluorescent cells and P2 for non-fluorescence cells. Once sorted (approximately 5 million cells per 15 ml tube), cells were immediately quick-frozen by immersing the tubes in liquid nitrogen prior to ultra-freezing until sorting was completed.

17. Mouse infection studies

All animal studies were approved by the local government of Lower Franconia, Germany (license number 55.2- DMS-2532-2-57 and were performed in strict accordance with the guidelines for animal care and animal experimentation of the German animal protection law and directive 2010/63/EU of the European parliament on the protection of animals used for scientific purposes. Female BALB/c mice (16 to 19 g) were purchased from Charles River (Charles River Laboratories, Erkrath, Germany), housed in polypropylene cages and supplied with food and water *ad libitum*. The different *S. aureus* strains used were cultured for 18 h at 37 °C on BHI medium. Subsequently, cells were collected and washed three times with PBS and diluted to reach an $OD_{600\ nm}$ = 0.05. Viable cell counts were determined by plating dilutions of the inoculum on TSB agar plates.

To compare the representation of each cell type of *S. aureus* in the bacterial communities that colonize the distinct organs, three cohorts of 3 mice for the unlabeled strain and 6 mice for each labeled strains (P_{ica}-*yfp* and $P_{psm\alpha}$-*yfp*) were infected with 150 µl of cultures of *S. aureus*, containing $1x10^7$ cells via tail vein injection. Each strain was used to infect one cohort of mice. The infections were allowed to progress until severe infection signs occurred or to an endpoint of 4 days. Animals were sacrificed when they met the following criteria: 1) loss of at least 20% of body weight; 2) loss of at least 15% of body weight and ruffled fur; 3) loss of at least 10% of body weight and hunched posture; or 4) 4 days of infection. Infected kidneys and hearts were aseptically harvested and processed for thin sectioning, histology, confocal microscopy and RNA isolation, as described above and below.

Organs were also used to calculate bacterial burden calculated as CFU/g of organ, as proposed by (Marincola et al. 2012). For this purpose, kidneys and hearts were homogenized in 2 ml of sterile PBS using GentleMACS™ M Tubes (Miltenyi Biotec) and serial dilutions from 10^{-2} to 10^{-8} of the organ homogenates were immediately plated on TSB plates and incubated at 37 °C for 24 hours.

To compare the representation of the *S. aureus* lower-*tagB* and higher-*tagB* strains colonizing the kidneys, heart, femora, liver, lungs and spleen, four cohorts of 5 mice each, were infected with 150 µl of cultures of *S. aureus* and organs were processed as described above. Three days after bacterial challenge all mice were euthanized, organs were aseptically harvested and processed as described above, CFU determined and RNA isolated.

18. RNA isolation

For the FACS-sorted bacterial cells, the ultra-frozen samples were thawed using a 37 °C water bath. The volume was poured in a vacuum filter system provided with a 47 mm filter diameter and 0.45 µm pore-size. The equipment was previously sterilized using 75% ethanol in DEPC-treated water, followed by two DEPC-treated water rinses and finally UV light for 180 seconds and then precooled at -20 °C. Filters containing each of the sorted samples were individually ground using liquid Nitrogen in an RNAse-free, sterile precooled mortar. The powder was carefully scraped out from the mortar and placed in a 2 ml RNAse-free Eppendorf tube and the RNA isolated as described below.

To isolate RNA from *S. aureus* infected organs, 450 µl of the homogenized organs were incubated for 15 min on ice with 50 µl of *RNAlater* and 5 µl of Triton X100 briefly vortexing every 5 minutes to lyse the murine cells. After lysis, RNA was isolated as described below, following the hot-phenol protocol proposed by (Blomberg, Wagner, and Nordström 1990; Dugar et al. 2013) with some modifications. To isolate RNA from *S. aureus* infected organs, 450 µl of the homogenized organs were incubated for 15 min on ice with 50 µl of RNAlater and 5 µl of Triton X100 briefly vortexing every 5 minutes to lyse the murine cells. After lysis, RNA was isolated as described below.

One volume of TE lysis buffer (Tris 20 mM pH 7.5, EDTA 10 mM) prepared in DEPC-prepared water and 25 µl of Lysostaphin (1 mg/ml) were added and taken to a hybridization oven (with rotation), pre-warmed at 37 ºC. Samples were incubated for 30 min at 37 ºC prior to transferring the whole material to a new tube for mechanical lysis. This was performed using FastPrep™ Lysing Matrix glass beads in a Fast Prep Shaker (MP Biomedicals) set at 6500 rpm for 50 sec. Samples were lysed using 2 cycles of 50 sec. The lysate was transferred to a new 2 ml RNAse-free Eppendorf tube and centrifuged for 10 min at 14000 rpm and 4 ºC to remove the filter and beads. The supernatant (700 µl) was recovered and used for RNA isolation using the standard hot phenol methodology with some modifications. The sample recovered from was mixed with 60 µl of 10% SDS and incubated at 64 ºC for 2 min. 66 µl of sodium acetate 3 M pH 5.2 and 750 µl of phenol Roti-Aqua® (Carl Roth) were added and the mixture was incubated at 64 ºC for 6 min with mixing every 30 sec. The sample was centrifuged for 10 min at 13000 rpm and 4 ºC and the upper aqueous layer was transferred to a 2 ml Phase Lock Gel Heavy (PLGH) tube (5Prime). Then, 750 µl of Chloroform was added and the sample was centrifuged for 12 min at 13000 and 15 ºC. The aqueous layer was transferred to a new tube and mixed with 2 volumes of a 30:1 ethanol and sodium acetate 3 M pH 6.5 mix. Additionally, 0.5 µl of GlycoBlue™ co-precipitant (15 mg/ml) (Life Technologies) was added. This mix was left at -20 ºC overnight. The following day, the sample was removed from storage at -20 ºC, centrifuged for 30 min at 13000 rpm and 4 ºC and the pellet was washed with 300 µl of precooled 75% ethanol. After washing, the total RNA was resuspended in 42 µl of RNAse-free water (Qiagen) were added and the sample was incubated at 65 ºC at 1000 rpm for 5 min prior storage in ice. To remove any DNA traces, the isolated RNA was treated (one to three times, depending on the sample) with 4 Units of RNase-free DNase I (Thermo), 10 Units of SUPERase In™ RNase Inhibitor (Life Technologies) and incubated for 45 min at 37 ºC. To remove the DNase I, 50 µl of RNAse-free water and 100 µl of Roti®-Aqua-P/C/I (Phenol, Chloroform, Isoamyl alcohol 25:24:1 pH 4.5 – 5) (Carl Roth) were added to the reaction tube, mixed, transferred to a 2 mL PLGH tube and centrifuged for 12 min at 13000 rpm and 15 ºC. The sample was mixed with 2.5 volumes of the 30:1 ethanol and sodium acetate with 0.5 µl of GlycoBlueTM, which led to RNA precipitation when stored at -20 ºC overnight. On the following day, samples were centrifuged for 30 min at 13000 rpm and 4 ºC, washed with 200 µl of 75% ethanol and the pellets were dried and resuspended in 50 µl of DEPC-water. To remove phenol residues, 50 µl of RNAse-free water and 100 µl of Chloroform (Carl Roth) were added to the reaction tube, mixed by inversion for 1 min, transferred to a 2 mL PLGH tube and centrifuged for 12 min at 13000 rpm and 15 ºC. The sample was mixed with 2.5 volumes of the 30:1 ethanol and sodium acetate

with 0.5 µl of GlycoBlueTM, which led to RNA precipitation when stored at -20 °C overnight. On the following day, samples were centrifuged for 1 h at 13000 rpm and 4 °C, washed with 200 µl of 75% ethanol and the pellets were dried. This RNA was resuspended in 22 to 44 µl of RNAse-free water and then incubated at 65 °C at 1000 rpm for 5 min. To assess the concentration and purity of the total RNA, OD_{260} was measured using a Nanodrop (Thermo) and the OD_{260}/OD_{280} ratio and the OD_{260}/OD_{230} ratio determined.

19. RNA-Seq library construction, sequencing and quantitative-PCR analysis

The cDNA prepared was strictly strand-specific, allowing transcriptome sequencing and expression profiling in both the forward and reverse strands. The combined-length of the flanking sequences was 100 bases. The cDNA is generated and size fractionated by preparative gel electrophoresis or by using the LabChip XT fractionation system from Caliper/PerkinElmer in order to obtain cDNA fractions, optimally suited for the different NGS systems. For this, the RNA samples were poly (A)-tailed using a poly(A) polymerase. The 5'-PPP were removed using tobacco acid pyrophosphatase (TAP) followed by the ligation of the RNA adapter to the 5'-monophosphate of the RNA. First-strand cDNA synthesis was performed with an oligo (dT)-adapter primer and the M-MLV reverse transcriptase. The resulting cDNA was PCR-amplified to reach a concentration of 20-30 ng/µl using a high fidelity DNA polymerase. The cDNA was purified using the Agencourt AMPure XP kit (Beckman) and was analyzed by capillary electrophoresis. The primers used for PCR amplification were designed for TruSeq sequencing according to the instructions of Illumina. The following adapter sequences flank the cDNA inserts: TruSeq_Sense: 5'-AATGATACGGCGACCACC-GAGATCTACACTCTTTCCCTACACGACGCTCTTCCGAT-T-3' TruSeq-Antisense NNNNNN (NNNNNN = Barcode) 5'-CAAGCAGAAGACGGCATAC-GAGATNNNNNNGTGACTGG-AGTTCAGACGTGTGCTCTTCC-GATC(dT25)-3'.

20. RNA-Seq and bioinformatics analysis

The cDNA libraries were generated by Vertis Biotechnology (Vertis Biotechnologie, Freising, Germany) as described previously for eukaryotic microRNAs but omitting the RNA size-fractionation step prior to cDNA synthesis (Dugar et al. 2013). The libraries were sequenced with an Illumina HiSeq 2500 machine with 100 cycles in single end mode. The pooled sequence reads were processed using fastq_quality_trimmer (from the FastX suite version 0.0.13). The reads were processed using the RNA-analysis pipeline READemption version 0.3.3 using default parameters. The de-multiplexed FASTQ files and coverage files in wiggle format have been deposited in NCBI Gene Expression Omnibus (Edgar, Domrachev, and Lash 2002) and are accessible through GEO Series accession number GSE69835 (Appendix A SI4).

The pooled sequence reads were de-multiplexed and the adapter sequences were removed. After that, the reads in Fastq format were quality trimmed using fastq_quality_trimmer (from the FastX suite version 0.0.13) with a cut-off Phred score of 20 and converted to Fasta format using Fastq_to_Fasta

(also from the FastX suite). The reads were processed, which included poly(A) removal, size filtering (minimum read length of 12 nucleotides after clipping), statistics generation, coverage calculation and normalization, which was performed with the RNA-analysis pipeline READemption version 0.3.3. READemption uses segemehl version 0.1.7 for the read alignment to the reference and DESeq 1.18.0 (Anders and Huber 2010) for the differential gene expression analysis. The reference genome NC_009641 was taken from the NCBI database for the purpose of alignment and gene-quantification. DESeq calculates statistically significant expression fold change and their log2 values by computing the ratio of normalized read counts of each gene in two libraries. The genes with log2fold value higher than 1.5 and lower than -1.5 were selected as up- and down-regulated gene-sets, respectively. Scatter plots for the visualization of sample correlation were generated using matplotlib 1.4.2 and the coverage data were visualized into circular layout using software package Circos (Krzywinski et al. 2009).

21. Statistical analysis

All statistical analyses were performed using the software Prism® 6 (version 6.0f, GraphPad). Graphs represent data from independent experiments with at least three independent technical replicates for experiment. Error bars represent the standard error of the mean. For the analysis of experiments with two groups, the parametric unpaired two-tailed Student's t-test with Welch's correction was done and, the non-parametric unpaired Mann-Whitney test were done. For the analysis of experiments with three or more groups, the parametric one-way ANOVA or the non-parametric Kruskal-Wallis tests were done. *Post hoc* analysis included multiple comparison Dunnett's test or Dunn's tests. Differences were considered significant when p value was smaller than 0.05. Statistical significance: ns = not statistically significant, * p<0.05, ** p<0.01, *** p<0.001.

22. Mathematical modeling of the bimodal behavior of the orthologous Agr system

To study the dynamic processes of the synthetic network, we mathematically described the reactions of the genetic circuitry of the orthologous system. In this circuit, the phosphorylation state is only reached inside the cell after the concentration of AIP is above the threshold. The complex AgrA~P is the transcription factor that upregulates the expression of P2 promoter (also called P_{RNAII}) responsible for activation of the positive feedback loop. Moreover, AgrA~P upregulates the expression of the rest of the promoters that were used in this work; P3 (Also called P_{RNAIII}), $P_{psm\alpha}$, $P_{psm\beta}$. P_{ica} and P_{spa} promoters are not directly regulated by AgrA~P and were therefore used as negative controls. The reactions that define the former genetic network are the following:

Activation/deactivation: $$AgrA^{P} + P_{agrA} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P^{a}_{agrA} \quad (1)$$

Fast transcription: $$P^{a}_{agrA} \xrightarrow{k_2} P^{a}_{agrA} + mAgrA \quad (2)$$

Slow transcription: $$P_{agrA} \xrightarrow{k_3} P_{agrA} + mAgrA \quad (3)$$

Translation: $$mAgrA \xrightarrow{k_4} mAgrA + AgrA \quad (4)$$

Complex formation: $AgrA + P \xrightarrow{k_5} AgrA^P$ (5)

Creation (appearance): $\phi \xrightarrow{k_6} P$ (6)

Activation/deactivation: $AgrA^P + P_x \underset{k_{-7}}{\overset{k_7}{\rightleftharpoons}} P_x^a$ (7)

Fast transcription: $P_x^a \xrightarrow{k_8} P_x^a + mYFP$ (8)

Slow transcription: $P_x \xrightarrow{k_9} P_x + mYFP$ (9)

Translation: $mYFP \xrightarrow{k_{10}} mYFP + YFP$ (10)

Degradation (disappearance): $mAgrA \xrightarrow{k_{11}} \phi$ (11)

Degradation (disappearance): $AgrA \xrightarrow{k_{12}} \phi$ (12)

Degradation (disappearance): $AgrA^P \xrightarrow{k_{13}} \phi$ (13)

Degradation (disappearance): $P \xrightarrow{k_{14}} \phi$ (14)

Degradation (disappearance): $mYFP \xrightarrow{k_{15}} \phi$ (15)

Degradation (disappearance): $YFP \xrightarrow{k_{16}} \phi$ (16)

where k_1 and k_{-1} are the binding and unbinding rates of AgrA~P to P2, k_2 is the transcription rate of P2 once AgrA~P binds the promoter, k_3 is the basal transcription rate of P2, k_2 and k_3 produce the mRNA of *agrA*, k_4 is the translation rate of AgrA protein, k_5 is the phosphorylation rate of AgrA, k_6 is the availability rate of phosphate in the system, k_7 and k_{-7} are the binding and unbinding rates of AgrA~P to the different promoters (P_x), k_8 is the transcription rate of P_x once AgrA~P binds to the promoter (P^a_x), k_9 is the basal transcription rate of P_x, k_{10} is the translation rate of the YFP protein and k_{11} to k_{16} are the degradation rates of mRNAs and proteins involved in this system.

Deterministic modeling using differential equations pointed to a quasi-steady state assumption (Murray, 2002), which we used to identify the elements responsible for the behavior of the system. The resulting equations are:

$$dAgrA^P/dt = \frac{k_6 \cdot AgrA}{AgrA + \delta} - k_{13} \cdot AgrA^P \quad (17)$$

$$dAgrA/dt = \frac{\alpha_1 + \beta_1 \cdot AgrA^P}{\gamma_1 + AgrA^P} - \frac{k_6 \cdot AgrA}{AgrA + \delta} - k_{12} \cdot AgrA \quad (18)$$

$$dYFP/dt = \frac{\alpha_2 + \beta_2 \cdot AgrA^P}{\gamma_2 + AgrA^P} - k_{16} \cdot YFP \quad (19)$$

where $\alpha_1 = k_3 k_{-1} k_4 Pt_{agrA}/k_1 k_{11}$, $\beta_1 = k_2 k_4 Pt_{agrA}/k_{11}$, $\gamma_1 = k_{-1}/k_1$, $\delta = k_{14}/k_5$, $\alpha_2 = k_9 k_{-7} k_{10} Pt_x/k_7 k_{15}$, $\beta_2 = k_8 k_{10} Pt_x/k_{15}$, $\gamma_2 = k_{-7}/k_7$ and $Pt_i = P_i + P^a_i$ with i = [*agrA*, *x*].

The following values are used to run the Gillespie algorithm of the full model (reactions 1-16). We focused our first set of simulations on the dynamics that affect YFP directly, to further study the behavior of the system when considering variations in AgrA and AgrA~P. The first set of simulations

allowed us to find parameters that show a fixed value among all of the experiments independently on the promoter that is under consideration. These parameters are shown here:

Parameter	Meaning	Value
k_1	Binding rate	0.01 molecules/hour
k_{-1}	Unbinding rate	2/hour
k_2	Transcription rates	500/hour
k_3	Basal transcription rate	50/hour
k_9	Basal transcription rate	90/hour
k_4, k_{10}	Translation rates	50/min
k_5	Phosphorylation rate	0.05 molecules/hour
k_6	Entry rate	40/molecules hour
k_{11}, k_{15}	Degradation rates	10/hour
k_{12}	Degradation rate	0.05/hour
k_{13}	Degradation rate	0.1/hour
k_{14}, k_{16}	Degradation rates	1/hour

Consequently, the values of these parameters did not change through the rest of the simulations that aimed to characterize the kinetics of each particular promoter. Initial simulations assumed the high stability of AgrA~P to test the response of the system to saturated levels of AgrA~P. The following simulations assumed unstable AgrA~P. The specific rates to simulate each promoter were defined as follows:

Parameter	Meaning (rate)	P_{RNAIII}	$P_{psm\alpha}$	P_{RNAII}	$P_{psm\beta}$	P_{RNAIII^-dual}	P_{RNAII^-dual}
k_8	Transcription	300	300	450	470	300	450/hour
k_7	Binding	3	6.5	10	20	12	40×10^{-4}*
k_{-7}	Unbinding	0.08	0.08	0.1	0.1	0.08	0.1/hour

*/molecules hour

The simulations that involved an unstable AgrA~P established values of $k_5 = 0.2 \times 10^{-4}$ and $k_6 = 5$. Using these values, we resolved a 3-mode decision-making model. Further simulations that involved changeable values of k_6 (phosphate availability) revealed that the activation rate of P2 and P3 occurs within a range of 0 and a maximum value of saturation of 40. The values for the rest of the parameters were selected from standard numbers obtained from previously reported studies (Andersen et al. 1998; Balagaddé et al. 2008; Ben-Tabou de-Leon and Davidson 2009; Dublanche et al. 2006; Goñi-Moreno and Amos 2012).

23. Mathematical modeling of the development of the multicellular communities

We used a so-called reaction diffusion system, which not only describes the changes of concentrations and density in time to any type of reaction but also their spread in space. In our case, we used a model with two spatial dimensions. Our system consisted of four equations, describing how the concentrations of nutrients, AIP and the density of replicative and non-replicative bacteria evolved in time. In the following, $n(x,t)$ denotes the nutrient concentration, $b(x,t)$ the density of replicative bacteria, $s(x,t)$ the density of non-replicative bacteria and $q(x,t)$ the concentration of AIP. We assumed that nutrients and AIP underlie diffusion. Diffusion parameters are denoted by d_n and d_q, respectively. In the case of the replicative bacteria, the diffusion coefficient depends on nutrient

concentration and the density of replicative bacteria. It is of the form $d_b = \sigma nb$ where σ also contains a stochastic fluctuation of random movement. This form for the diffusion of active bacteria cells was chosen according to the model developed by (Kawasaki et al. 1997). We assumed that non-replicative bacteria are not able to diffuse on their own and their diffusion is driven by movement of the replicative cells. The diffusion of non-replicative bacteria depends on the density of replicative bacteria. If there are more replicative bacteria, the non-replicative cells will be pushed in a type of diffusion. This effect is limited. The diffusion coefficient for the non-replicative bacteria is assumed to be of the form:

$$d_s = \tau \frac{b}{b_s + b}$$

where b_s and τ are constant. The replicative bacteria proliferate by consuming nutrients. In our system of equations, the consumption rate of the nutrients was given by $G_1 f(n, b)$ and the bacterial growth was described by the term $G_2\, f(n, b)$ where G_2/G_1 was the conversion rate of consumed nutrients to bacterial growth. We assumed $f\ (n, b)$ to be of the form:

$$f(n, b) = \frac{bn}{1+\gamma n}\left(1 + \frac{1}{q_m + \delta q}\right)$$

Here, we chose a Monod growth term (Monod 1949) to describe the increase in the concentration of replicative bacteria in relation to nutrient consumption. It reproduces the fact that a high nutrient concentration will cause a faster increase in the concentration of replicative bacteria but that these bacteria cannot reproduce infinitely fast. An additional factor accounts for the effect of the *quorum* sensing signal. If the concentration of active bacteria is already high, a high concentration of AIP slows down the conversion process. We furthermore assumed that there is only a transition from replicative to non-replicative cells. This process is described by a term of the form:

$$\varepsilon a(b, n) = \varepsilon \frac{\mathrm{b}}{\left(1 + \frac{\mathrm{b}}{a_1}\right)\left(1 + \frac{\mathrm{n}}{a_2}\right)}$$

This choice is in agreement with (Matsushita et al. 1999). The equation number four described the concentration of AIP. We considered that the diffusion and the increase in the concentration of AIP are related with the concentration of replicative bacteria but we also considered that there is degradation process of AIP. The degradation of AIP is described by μ_q, whereas the production of AIP typically has two levels in a Hill type function (Gustafsson et al. 2004) with Hill coefficient 2 to reflect bistability. This is caused by a positive feedback including nonlinearity in the underlying regulation system. The low production rate of AIP is denoted by $p_1$while the increased production rate is denoted by p_2. The threshold between the low and the increased production is denoted by q_{thr}. For more details see (J. Müller et al. 2006). With the above-mentioned terms we achieved the following system of equations, which are able to represent the growth dynamics of *S. aureus* multicellular aggregates:

$$\frac{\partial n}{\partial t} = d_n \nabla^2 d - G_1 \frac{bn}{1+\gamma n}\left(1 + \frac{1}{q_m+\delta q}\right) \quad (20)$$

$$\frac{\partial b}{\partial t} = \nabla(\sigma \mathrm{nb} \nabla \mathrm{b}) + G_2 \frac{bn}{1+\gamma n}\left(1 + \frac{1}{q_m+\delta q}\right) - \varepsilon \frac{b}{\left(1+\frac{b}{a_1}\right)\left(1+\frac{n}{a_2}\right)} \quad (21)$$

$$\frac{\partial s}{\partial t} = \nabla\left(\tau \frac{b}{b_s+b} \nabla s\right) + \varepsilon \frac{b}{\left(1+\frac{b}{a_1}\right)\left(1+\frac{n}{a_2}\right)} \quad (22)$$

$$\frac{\partial q}{\partial t} = d_q \nabla^2 q + \left(p_1 + p_2 \frac{q^2}{q_{thr}^2+q^2}\right) b - \mu_q q \quad (23)$$

It remains necessary to define the initial and boundary conditions. Since the bacteria grow on an agar plate, we chose no-flux boundary conditions, e.g. $\frac{\partial n}{\partial x}|_{\partial \Omega} = 0$, where Ω denotes the area of the agar plate. The agar has the same concentration of nutrients throughout and therefore we defined the initial condition for the nutrients as constant over the entire domain, e.g. $n(x,0) = n_0$ for $x \in \Omega$. The replicative bacteria are set on the agar as a drop in the center. At this stage, neither non-replicative bacteria nor AIP exist and thus the initial conditions read $b(x,0) = b_M \exp\left(-\frac{x^2+y^2}{6.25}\right)$ with a compact support, $s(x,0) = 0$ and $q(x,0) = 0$. The (non-dimensionalized) parameters chosen for this simulation are given by:

$\sigma = 0.5; G_1 = G_2 = G = 7; \varepsilon = 5; \tau = 0.25; q_m = 0.3; d_n = d_q = 1; z = 1; \rho = 1;$
$\gamma = 1; b_s = 2;\ \delta = 1; q_{thr} = 1; \mu = 1; n_0 = 1.11; p_1 = \ p_2 = 1;$
$a_1 = 2400;\ a_2 = 120.$

Our model was able to capture differences in texture on the surfaces of different *S. aureus* mutants. For the simulation of mutants, we only changed the parameter value, which corresponds to the modified gene and phenotype for the mutant, the other parameter values were maintained from the wild type, as follows:

Background	σ	G	p_1	p_2
Wild type	0.5	7	1	1
spa	0.5	3	1	1
ica	0.75	2	1	1
psmα	1	8	1	1
psmβ	1	4	1	1
agr	*1*	5	0	0
spa ica	1	2	1	1

The non-mentioned parameters were maintained at the same value as the wild type strain. In the case of the Δspa mutant, as influencing the biofilm, we modified the biofilm production rate constant G from 7 to 3. The mutants $\Delta psm\alpha$ and $\Delta psm\beta$ differ from the wild type also by their ability to move on; therefore apart from G also the parameter σ was modified. To obtain the agr mutant not only a simple change in the parameters is needed, but also a combination of different mutant behaviors since the agr system influences several components of biofilm production and properties. Especially the ability of the model to produce AIP is lost, expressed by setting p_1 and p_2 to 0.

RESULTS

1. Integrative plasmid collection for the study of *S. aureus* heterogeneity at the single cell level

The main focus of this project involves the analyses of multicellular aggregates of *S. aureus* at the single cell level. Therefore, we created a new molecular toolbox to facilitate the insertion of fluorescent single-copy transcriptional reporters into the *S. aureus* chromosome using the neutral loci *amyE* (*NWMN_2354*) and *lacA* (*NWMN_2098*). The AmyE is a glutamyl-aminopeptidase highly similar to a *B. subtilis* extracellular α-amylase and, the LacA is a β-galactosidase. Both are nonessential proteins for the propagation of *S. aureus* under laboratory conditions and the disruption of this genes (Δ*amy* and Δ*lac)* showed no differences with the wild type in all the phenotypic properties tested (Yepes et al. 2014).

In addition, this plasmid toolbox was also created to generate the gene deletions, in an improved 1-step recombination process (Yepes et al. 2014). The chromosomal insertion of these fluorescent reporters does not add any antibiotic resistance gene since the screening is based on the emission of fluorescence, making them amenable for further genetic manipulations.

This system is based on the existent vector pMAD (Arnaud, Chastanet, and Débarbouillé 2004), which carry the ampicillin resistance cassette for its propagation in *E. coli*, the erythromycin resistance cassette for its propagation in *S. aureus* and in addition, the *bgaB* gene for blue-white X-gal screening. We modified this vector to carry different fluorescent reporter genes an/or a multiple cloning site (MCS). To construct the pAmy and pLac plasmid, the respective 1 Kb upstream and downstream DNA regions of the *amy* and *lac* loci were PCR amplified. The upstream and downstream PCR products generated for each locus were joined in a second PCR, introducing the MCS between these two fragments. This cassette was cloned into the pMAD vector. The *yfp* gene was amplified from pKM003 and cloned into pAmy using the restriction enzymes SalI/BamHI or into pLac using SalI/NheI. The *rfp-mars* gene was amplified from pmRFPmars (Paprotka, Giese, and Fraunholz 2010) and cloned into pAmy using SalI/BamHI and pLac using SalI/NheI.

To create a particular transcriptional fusion, it is necessary to amplify by PCR the promoter region (between 200 and 500 bp upstream the start codon) of the *gene of interest* adding the XhoI and SalI restriction sites in the 5' and 3' ends, respectively. Then, this DNA fragment can be integrated into the respective cargo using XhoI/SalI, which is already inserted into the pAmy or pLac plasmid. Alternatively, the promoter region can be cloned in pKM003 or pKMmars using XhoI/SalI, extracted using EcoRI/BamHI and then subcloned in the pAmy or pLac vectors. Additional experimental procedures are described in the section Materials and Methods.

2. *Staphylococcus aureus* multicellular aggregates

In *S. aureus*, the global regulator, TCS and *quorum* sensing system Agr (accessory gene regulator), modulates the outcome of infections in response to bacterial cell density and extracellular signals such as the self-produced autoinducing peptide (AIP) (Recsei et al. 1986). This regulatory has a central role in staphylococci pathogenesis controlling virulence (Novick et al. 1993; Novick et al. 1995) and biofilm

formation (Yarwood et al. 2004) in an antagonistic manner and, contributing to the chronic nature of biofilm-associated staphylococcal infections (Yarwood and Schlievert 2003).

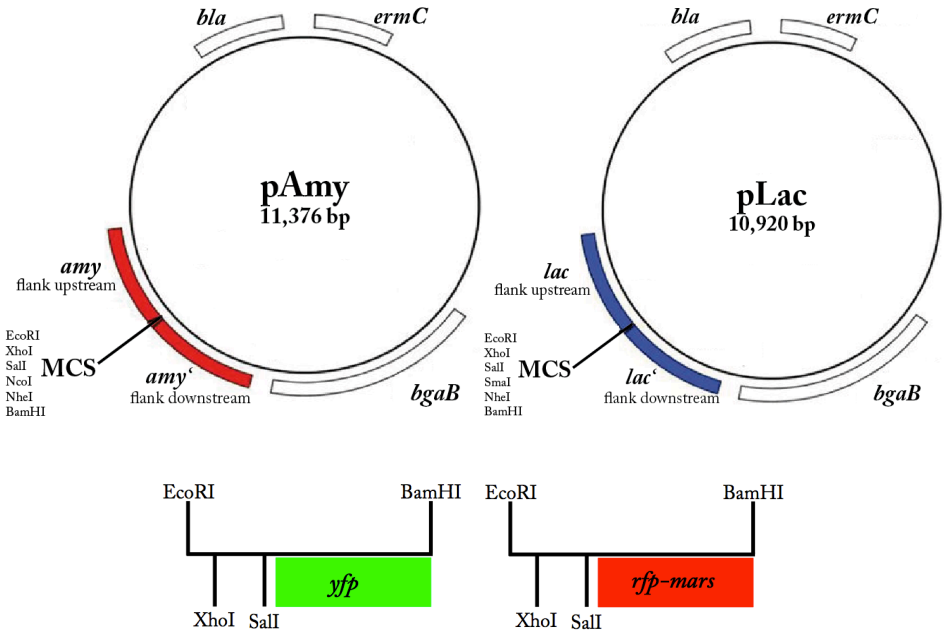

Figure 15. In the upper panels, plasmids pAmy and pLac for genomic integration of single-copy fluorescent transcriptional fusion in *S. aureus*. The plasmids pAmy and pLac carry the *bla* gene that encodes a β-lactamase, the *ermC* gene that encodes an rRNA methylase and, the *bgaB* gene that encodes a β-galactosidase. In the lower panel, representation of the transcriptional fusions to the fluorescent proteins YFP and RFP-Mars. Graph adapted from (Yepes et al. 2014).

Briefly, at high bacterial cell densities extracellular AIP binds to the membrane histidine kinase AgrC and activates its cognate response regulator AgrA via phosphorylation. Once phosphorylated, AgrA~P induces massive changes in gene expression resulting in the bacteria dispersing rapidly in the host and causing acute bacteremia (Thoendel et al. 2011). On the other hand, the activation of agr is inversely correlated with the expression of the *ica* operon necessary to synthetize the PNAG exopolysaccharide that constitute the extracellular matrix of the staphylococcal biofilms, as well as of many adhesion proteins such as the Spa and other adhesins responsible for cell aggregation and attachment during biofilm formation (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a). Biofilms, which are associated with untreatable chronic infections, protect bacteria from antibiotics and host defenses (Parsek and Singh 2003; Otto 2008; Nadell, Xavier, and Foster 2009; Lopez, Vlamakis, and Kolter 2010). Therefore, the Agr system antagonistically modulates biofilm formation and dissemination during *S. aureus* infections, which respectively contribute to the development of chronic and acute infection outcomes.

In addition, the Agr system is inhibited by the alternative σ^B sigma factor, which is activated in response to different types of bacterial stress signals and hence, triggers a general-stress response that includes the formation of biofilm via agr repression (Kullik, Giachino, and Fuchs 1998; Bischoff, Entenza, and Giachino 2001). In addition to the signaling cascade that is triggered by AIP, *S. aureus*

cells are exposed to a variety of other external environmental signals during the course of an infection that have the potential to influence bacterial gene expression and thus, the infective potential of this pathogen. These signals include, but are not limited to, changes in nutrient availability, concentration of oxygen, pH gradients, osmolarity or temperature. Then, *S. aureus* may be able to respond collectively to these extracellular cues to adapt their behavior in a fluctuating environment and thus allowing staphylococcal communities to generate distinct and locally define types of infections (Veening et al. 2008; López and Kolter 2010). Indeed, it has been hypothesized that changes in bacterial virulence potential occurs in response to local concentration of tissue-specific cues, which ultimately play an important role in determining the outcome of infection (A. L. Cheung et al. 2004). Nevertheless, such microenvironments do fluctuate rapidly and, how bacteria prepare for such unpredictable environmental changes is a question that still remains unanswered but, extensive research has highlighted a fundamental feature of microbial cells: their ability to adapt to diverse environmental conditions by physiologically differentiating into specialized cell types and developing collective division-of-labor (López, Vlamakis, and Kolter 2009; López and Kolter 2010). In most cases, extracellular signals are responsible for defining coexisting cell fates in bacterial populations. The classical example of this behavior in *S. aureus* is persistence. Antibiotics usually kill most bacterial cells but it is frequent to observe a small subpopulation of genetically identical but physiologically different, antibiotic-resistant persister cells that can develop recurrent infections after the antibiotic exposure (Bigger 1944; K. Lewis 2006).

The Agr represents a rather straightforward model controlling the switch between biofilm-associated and planktonic lifestyles in *S. aureus* and then, provides us with a natural model to study how do *S. aureus* cells collectively choose between establishing chronic or acute infection lifestyles, as well as to identify the extracellular cues that influence the activation of the cellular program that promotes the emergence of one type of infection to prevail.

2.1. *S. aureus* develops an architecturally complex multicellular community

To understand the processes that define the development of acute or chronic infections during *S. aureus* colonization, we explored the role of Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles in this human pathogen.

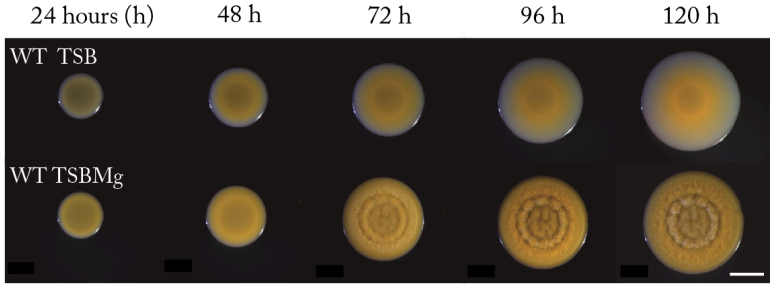

Figure 16. *S. aureus* wild type development in TSB and TSBMg. Top-view pictures of the development of microbial communities of *S. aureus* growing during 5 days (120 h) at 37 ºC. In Mg^{2+}-supplemented TSB medium agar plates, *S. aureus* develops architecturally robust and complex multicellular aggregates. In standard TSB agar

plates, *S. aureus* does not develop complex multicellular aggregates and grows as a flat macrocolony. Scale bar is 5 mm.

We found that under specific growing conditions on agar surface, *S. aureus* is able to develop a distinctive, heterogeneous and architecturally complex multicellular community, characterized by a macroscopic structure with a particular temporal and spatial developmental plan. When *S. aureus* is grown on Mg^{2+}-enriched TSB agar at a final concentration of 100 mM (TSBMg) at 37 ºC in laboratory conditions during 5 days (120 hours), develops these multicellular communities (**Figure 15**).

In a previous study performed in our laboratory, we found that these multicellular aggregates did occur in Mg^{2+}-enriched TSB agar and not in the presence of other cation (Koch et al. 2014), suggesting that Mg^{2+} is an important environmental cue to trigger biofilm formation and also consistent with the fact that biofilm-associated *S. aureus* colonization generally occurs in in Mg^{2+}-enriched niches, such as bones and kidneys, in which chronic staphylococcal infections usually develop (Elin 2010; Günther 2011; Jahnen-Dechent and Ketteler 2012). By contrast, organs and tissues depleted in Mg^{2+} are prone to an acute *S. aureus* infection such as the particular case where, sequestration of Mg^{2+} from vaginal tissues due to the use of tampons led to an outbreak of *S. aureus* in the USA (Schlievert 1985).

Interestingly, we observed that most *S. aureus* clinical isolates can develop these robust and complex multicellular communities in solid TSBMg, in contrast with laboratory strains such as *S. aureus* RN 4220, which lacks the concentric wrinkles, considered as the main macroscopic readout for extracellular matrix production (Branda et al. 2006) (**Figure 17**).

Importantly, the ability of the different staphylococcal strains to develop these complex multicellular communities directly correlates with their ability to form biofilm in the traditional pellicle microtiter assay using crystal violet, which is used as gold-standard for biofilm formation in bacteria (O'toole and Kolter 1998); establishing a correlation between the developmental architecture of these aggregates and their ability of biofilm formation (**Figure 18**).

These multicellular aggregates are macrocolonies of approximately 12-15 mm in diameter with three architecturally different areas: i) a central or origin area (*O*) characterized by a flat central zone surrounded by a ring of bulk sections, ii) a wrinkled area (*W*) that radiates extensively and constitutes a conserved pattern of the typical wrinkles found in other microbial multicellular biofilms growing on agar surface (Branda et al. 2001; Serra, Richter, and Hengge 2013) and finally, iii) a peripheral area (*P*) characterized for a accumulation of actively dividing cells of smooth appearance and absence of the extracellular matrix found in the *O* and *W* sections (**Figure 19**).

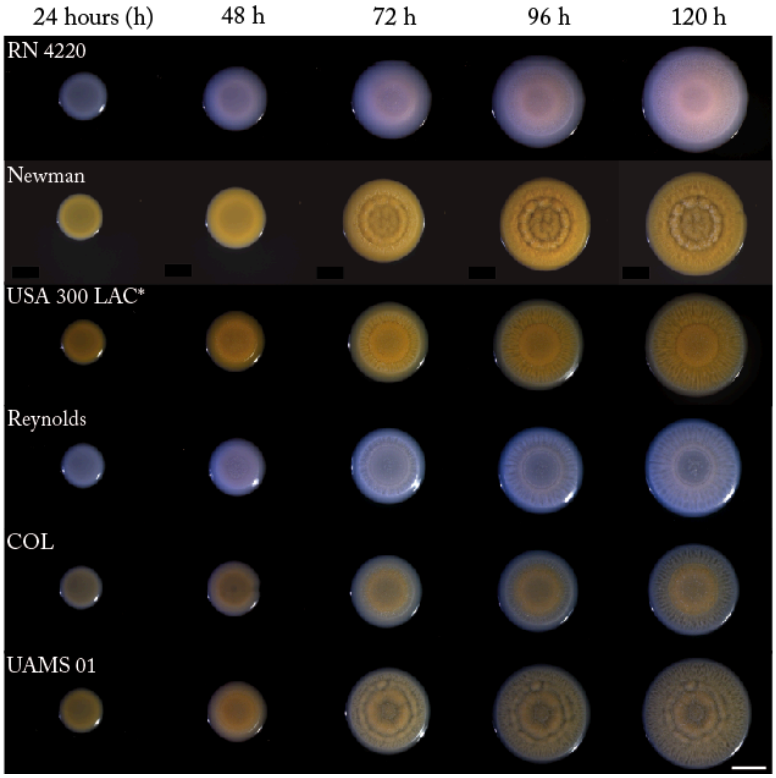

Figure 17. Development of multicellular communities in TSBMg of the laboratory strain RN4220 and, of different clinical isolates of *S. aureus*. Top-view pictures of the development of microbial communities of different strains of *S. aureus* growing during 5 days (120 h) at 37 ºC in TSBMg. Laboratory-adapted strains such as RN4220 are deficient in biofilm formation and appear as flat macrocolonies lacking wrinkles. On the other hand, clinical isolates, which are recognized as strong biofilm formers, display a large range of architectural features characterized by the presence of a wrinkled surface as well as well defined morphological areas. Scale bar is 5 mm.

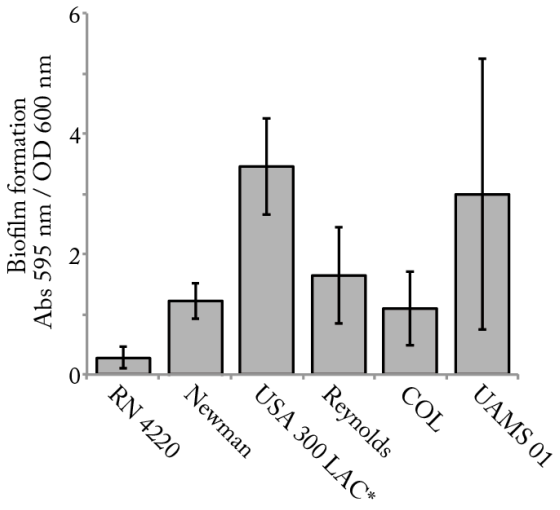

Figure 18. Traditional quantification of biofilm formation of different laboratory strains (RN4220) and clinical isolates of *S. aureus* growing in TSBMg liquid medium in 24-well titer plates. Using this approach, biofilm formation directly correlates with the phenotypes observed in **Figure 17**. The crystal violet assay is highly variable between replicates, which can be observed by the large error bars, contrary to our model of biofilm formation that is highly reproducible. Biofilm formation quantification using the traditional crystal violet assay (O'toole and Kolter 1998) of *S. aureus* growing in TSBMg in 24-well microtiter wells during 48 h at 37 ºC.

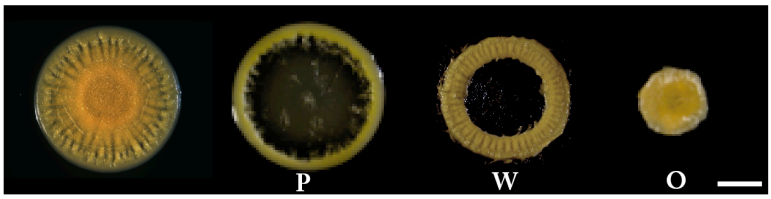

Figure 19. Multicellular communities of *S. aureus* growing in TSBMg develop architecturally different areas. Dissection of a 5 days old multicellular community in three distinct morphological regions: a *peripheral* region (*P*), a surrounding *wrinkled* area (*W*) and a central older region or *origin* (*O*). From the TSBMg agar surface, these regions can be physically separated from the aggregate and further processed independently. Scale bar is 5 mm.

It has been proposed that the accumulation of cells in the borders of the community during the initial stages of development, might lead to the generation of the conserved *P/W/O* patterns (J. A. Shapiro 1987). A process of swimming of bacteria during the drying process of the bacterial seeding spot would generate the preferential accumulation of cells at the edges of the inoculation zones creating what it was called as "crater-like" structure (J. A. Shapiro 1987). This structure guides the development of the multicellular community through a process of internal division and senescence within the core (or *O* in our model), a process of waves of division and expansion that could give rise to the concentric zones and the wrinkled structures (or *W* in our model) and, the outer ring (or *P* in our model) of actively and newly dividing cells (J. A. Shapiro 1987).

2.2. *S. aureus* multicellular communities in different genetic backgrounds

To determine the role of the different regulatory and structural components involved in biofilm formation in *S. aureus*, we performed experiments to evaluate the contribution of those gene products in the development of the *S. aureus* multicellular aggregates. In TSBMg medium, the formation of microbial aggregates was more prominent in the *agr*-defective strain, while flat dispersed communities were observed in strains lacking the biofilm associated *ica* and *spa* genes (**Figure 20**), consistent with previous publications demonstrating the correlation between *agr* repressed genes and biofilm aggregation (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a).

Using the traditional microtiter biofilm assay the Δ*sigB* mutant was unable to form biofilm in TSBMg and the biofilm formation phenotype was partially recovered in a Δ*sigB*Δ*agr* double mutant (**Figure 21**). Interestingly, in the absence of Mg^{2+} biofilm formation in the Δ*sigB* mutant strain was significantly higher than in the WT and, the double mutant Δ*sigB*Δ*agr* displayed no partial recovery as it happened in TSBMg, suggesting that in the absence of this particular cation biofilm formation follows an *sigB*- and *agr*-independent pathway, controlled perhaps by some other global regulators such as SarA (A. L. Cheung and Projan 1994; Trotonda et al. 2005; A. L. Cheung et al. 2008; Beenken et al. 2010; Atwood et al. 2016).

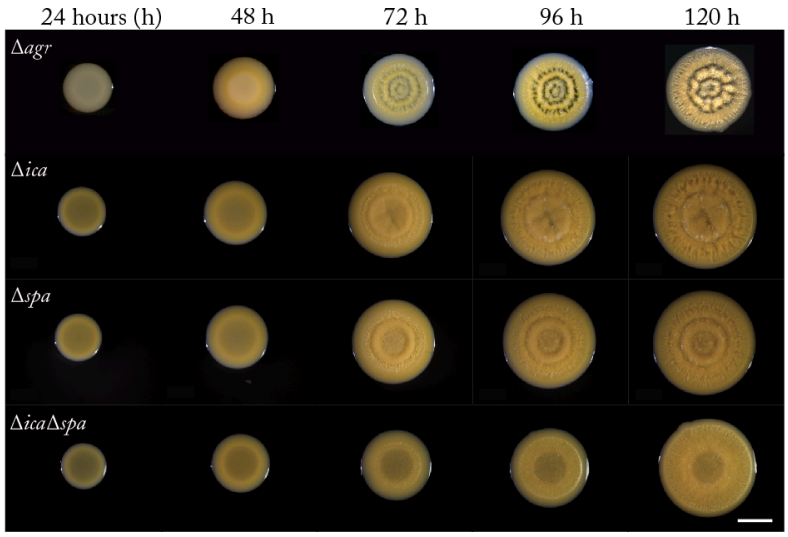

Figure 20. Development of multicellular communities in TSBMg of different genetic backgrounds of *S. aureus* strain Newman. Deletion of the *agr* operon produces significant changes in the development of the staphylococcal multicellular aggregates, generating more robust communities, with a reduced diameter and a thicker and more wrinkled extracellular matrix. On the other hand, deletion of the genes responsible of the structural components of the staphylococcal biofilm, the PNAG exopolysaccharide and the MSCRAMM adhesion SpA, generates flatter aggregates with a reduced wrinkled surface, confirming the antagonistic involvement of the Agr regulator and the *ica* operon and the *spa* gene in the development of these multicellular communities. Top-view pictures of the development of these microbial communities during 5 days (120 h) at 37 ºC in TSBMg. Scale bar is 5 mm.

Stress response and the balance between growing and maintenance are highly dependent on alternative σ factors (Nyström 2002) and, the stability of the genome and the correct inheritance depends on the correct function of the alternative σ factor and its interaction with the whole regulatory genetic network. Therefore, it is not surprising that the SigB- and Agr-deficient strains display strong phenotypic changes in the development of the multicellular communities.

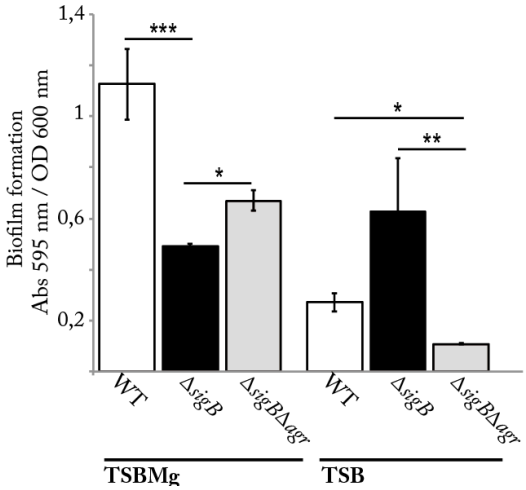

Figure 21. Quantification of biofilm formation in liquid TSBMg and TSB of *S. aureus* Newman strains Δ*sigB* and Δ*sigB*Δ*agr*. The deletion of the *sigB* gene has a significant effect in biofilm formation in the presence of extracellular Mg^{2+} but not, in standard TSB liquid medium. The deletion of the *agr* operon recovers partially the

ability to form biofilm when growing in TSBMg; while in standard TSB the double mutant Δ*sigB*Δ*agr* shows a more pronounced reduction. *S. aureus* growing in TSBMg in 24-well microtiter wells during 48 h at 37 ºC. Biofilm formation quantified using the crystal violet assay. Statistical significance was measured by one-way ANOVA. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Changes in development of the different structural (**Figure 20**) and regulatory (**Figure 21**) genetic backgrounds correlated with the ability to form biofilm using the traditional microtiter biofilm assay. In general, more robust and thicker aggregates correlated with increased biofilm formation in the microtiter biofilm assay while, more relaxed, flatter and thinner multicellular communities correlated with decreased biofilm formation in microtiter wells (**Figure 22**).

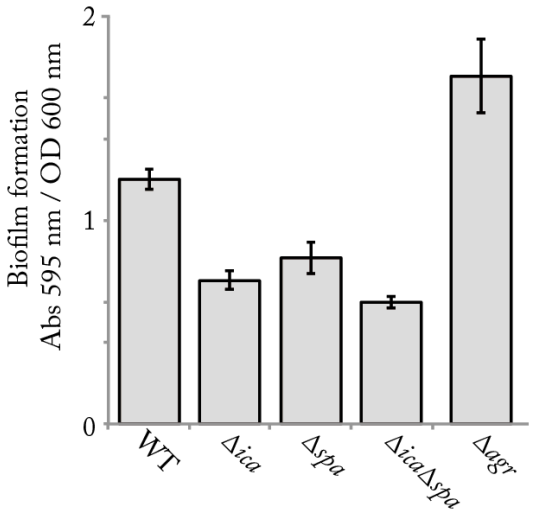

Figure 22. Quantification of biofilm formation in liquid TSBMg of *S. aureus* Newman strain of different structural and regulatory genetic backgrounds. There is a strong correlation between the architectural complexity of the multicellular aggregate (**Figure 20**) and the ability of the strain to form biofilm in the classical biofilm formation assay that entitles forming submerged pellicles in the bottom of a well that contains liquid medium. *S. aureus* growing in TSBMg during 48 h at 37 ºC and biofilm formation was quantified using the crystal violet assay.

We tested how different deletion mutant strains influenced the development of the staphylococcal multicellular aggregates. Simple- and double- deficient strains displayed a miscellaneous repertoire of developmental features that qualitatively and quantitatively correlated with the measurements of the biofilm formation ability using the microtiter assay. In particular, multicellular aggregates of *S. aureus* deficient for the production of exopolysaccharide PNAG (Δ*icaADBC*), the extracellular adhesion protein A (Δ*spa*), or the phenol soluble modulins type β (Δ*psmβ1-2*) (**Figure 23**) showed a reduced pattern of wrinkle formation when compared to the WT strain. The extracellular matrix of biofilms is mainly composed of exopolysaccharides, extracellular proteins, amyloid-like peptides and eDNA (Joo and Otto 2012). In *B. subtilis*, multicellular communities, reduction in wrinkles correlates with a reduction in the structural components of the extracellular matrix, like such as the exopolysaccharides (Nagorska et al. 2010) or extracellular adhesins (Branda et al. 2006; Vlamakis et al. 2008). Comparing the developmental phenotypes found for the PNAG, SpA and the PSMβ mutant strains, we suggest a

role for the *psmβ1-2* gene products in the structural development of the extracellular matrix of *S. aureus* multicellular communities, as has been demonstrated for *S. aureus* and *S. epidermidis* traditional biofilms (R. Wang et al. 2011; Periasamy et al. 2012). On the other hand, the Δ*psmα1-4* mutant strain showed a more robust architecture, characterized by a reduced diameter, a thicker extracellular matrix and a dense O region with well-defined central clumps (**Figure 23**), which resembled at some extent the development of the *agr*-deficient strain. It is known that the PSMs are directly regulated by AgrA (Otto 2011; Periasamy et al. 2012; Chatterjee et al. 2013), which explains how the absence of the *agr* operon or the *psmα1-4* developed a robust architecture and an increased biofilm formation in the traditional biofilm microtiter assay. Nevertheless, the *agr*-dependent PSMs expression cannot explain the flatter and thinner Δ*psmβ1-2* phenotype, suggesting a structural connection between the PSMβ1 (highly hydrophilic 44 amino-acids long) and the PSMα (highly hydrophilic and formylated 22 amino-acids long) peptides during the structural development of the staphylococcal biofilm, promoting the amyloid-like polymerization of these short peptides and the enlargement, dissemination and detachment of the multicellular staphylococcal biofilms (Mehlin, Headley, and Klebanoff 1999; R. Wang et al. 2007; Schwartz, Syed, Stephenson, Rickard, and Boles 2012a; Periasamy et al. 2012; Peschel and Otto 2013). While the Δ*psmα1-4* multicellular aggregates are thicker and reduced in diameter, the Δ*psmβ1-2* multicellular communities are thinner and spread more when compared to the wild type isogenic strain. These contrasting phenotypes suggest that these peptides might have complementary functions during the development, maturation and dissemination of the staphylococcal biofilm defined by a particular and *agr*-dependent PSMα/PSMβ ratio.

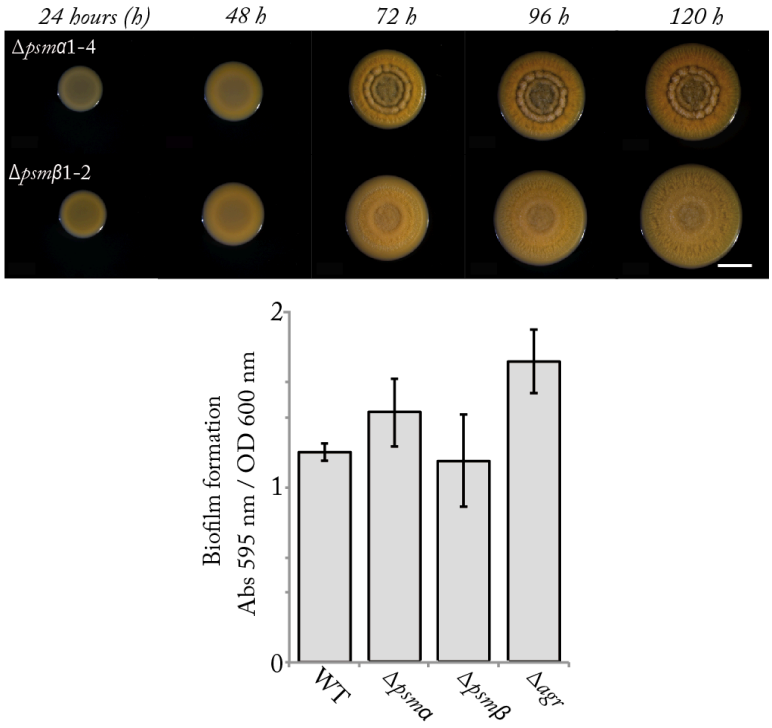

Figure 23. Upper part, development of multicellular communities in TSBMg of *S. aureus* strain Newman Δ*psmα1-4* and Δ*psmβ1-2*. These PSMs are both directly regulated by AgrA~P. Deletion of the *psmα1-4* operon generates a more thick and robust phenotype that resembles the Δ*agr* strain. Intriguingly, deletion of the *psmβ1-2* operon give

rise to the opposite phenotype, which resembles more the Δ*ica* and Δ*spa* strains. Top-view pictures of the development of these microbial communities during 5 days (120 h) at 37 ºC in TSBMg. Scale bar is 5 mm.
Lower part, quantification of biofilm formation in liquid TSBMg of *S. aureus* Newman strain of different structural and regulatory genetic backgrounds. There is a strong correlation between the architectural complexity of the multicellular aggregates of Δ*psmα1-4* and Δ*psmβ1-2* and their ability to form biofilm in the classical biofilm formation. *S. aureus* growing in TSBMg during 48 h at 37 ºC and biofilm formation was quantified using the crystal violet assay.

In general, the phenotypic characteristics displayed by the different genetic backgrounds during the different stages of development are informative about the structural role of the different components for biofilm formation in *S. aureus*. The examination of the fully developed staphylococcal multicellular communities at the macroscopic level revealed a high degree of morphological organization with the display of large-scale and well-conserved elements in structure, space and time that could be traced in their individual features by the generation of the different mutants.

2.3. Multicellular communities in *S. aureus* Newman derivative strains

Among all the clinical isolates initially tested, we deliberately selected the classical model strain Newman to perform further experiments despite the fact this strain has been catalogued as poor biofilm former using the classical pellicle formation assay in liquid TSB (Cue et al. 2015). Nevertheless its behavior in standard laboratory conditions, the Newman strain was originally isolated from a long-term bone-associated infection that usually involves biofilm formation (DUTHIE and LORENZ 1952) and bones are important reservoir of Mg^{2+} in the body (Günther 2011; Jahnen-Dechent and Ketteler 2012), possibly Newman strain naturally develops strong biofilms in Mg^{2+}-enriched growing conditions that could resemble the colonizing niches in which Newman develops biofilm-associated infections. It is important to remark that the features of organisms are the result of environmental conditions as well as genes (Darwin 1859). Therefore, it is perhaps most accurate to describe a phenotype as a product of the interaction between a set of genes and an environment (Moxon et al. 1994). The reduced ability of the Newman strain to form biofilms in TSB medium has been attributed to a point mutation in the SaeS histidine kinase, which generates a constitutively active SaeS that activates the *sae* regulon, which includes inhibition of biofilm formation (Cue et al. 2015). Thus replacement of the Newman SaeS with a wild type copy of SaeS (NewHG strain) or deletion of saeRS (Δ*saeRS*) restored biofilm formation (Cue et al. 2015). However, it is possible that the point mutation in *saeS* in Newman strain occurred to naturally develop strong biofilms in Mg^{2+}-enriched growing conditions because Newman strain is a robust biofilm former in TSBMg. Therefore, we tested the capacity of the NewHG and Δ*sae* strains to form multicellular aggregates in TSBMg medium and, in contrast to the Newman wild type, the NewHG strain showed limited capacity to form aggregates in TSBMg (**Figure 24**). Moreover, the Δ*sae* strain showed almost totally impaired to develop aggregates in TSBMg. These results are consistent with the hypothesis that a constitutively active SaeS facilitates biofilm formation of Newman strain in Mg^{2+}-enriched colonization niches. In addition, the traditional biofilm assay using liquid TSB

and TSBMg and 24-microtiter titer plates (**Figure 25**) showed similar results: in TSBMg the *S. aureus* Newman wild type showed increased biofilm formation when compared to the strain NewHG and Δ*sae* deletion mutant. Interestingly, when growing in standard TSB non-supplemented with Mg^{2+}, the Newman wild type showed reduced biofilm formation when compared to the two derivative strains, suggesting that natural selection provided *S. aureus* stain Newman the mutation in the *saeS* gene to cope efficiently with environmental conditions with elevated levels of extracellular Mg^{2+}.

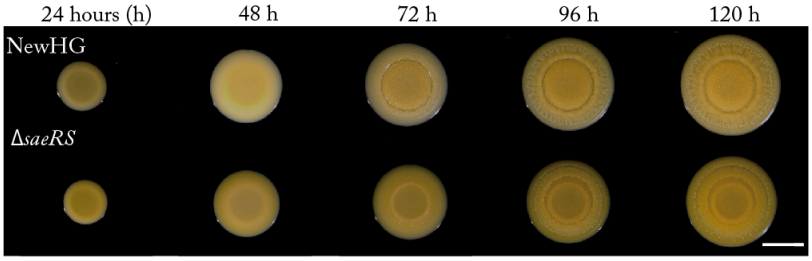

Figure 24. Development of multicellular communities in TSBMg of different *S. aureus* strain Newman derivative strains. Restoration of the *saeS* allele in NewHG from the hyperactivated constitutively active version in the wild type strain Newman reduces the ability of this corrected strain to develop architecturally complex and robust multicellular communities in TSBMg. Similarly, deletion of the *saeRS* TCS produces significant changes in the development of the staphylococcal multicellular aggregates, generating a flatter and thinner multicellular community, suggesting that the ability to develop highly structured multicellular communities in TSBMg medium directly correlates to *sae* expression levels. Top-view pictures of the development of these microbial communities during 5 days (120 h) at 37 ºC in TSBMg. Scale bar is 5 mm.

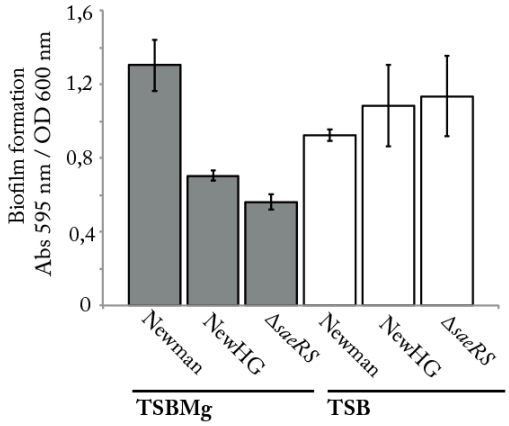

Figure 25. Quantification of biofilm formation of Newman and derivative NewHG and Δ*saeRS* strains when they grow in liquid TSBMg and TSB in 24-well titer plates for 48 hours at 37 ºC. The strains showed different biofilm behavior in TSBMg and TSB media. The capacity to form biofilm in TSB medium directly correlates to *sae* expression levels. However, in TSBMg, biofilm formation indirectly correlates to *sae* expression.

3. Increase in cell wall rigidity favors multicellular aggregation in *S. aureus*

We explored the role of Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles in *S. aureus* in order to understand the processes that define the development of acute and chronic infections during staphylococcal colonization. We found that the ability of most *S. aureus*

clinical isolates to form stronger biofilms in the traditional liquid microtiter assay, as well as to develop highly structured multicellular aggregates on the surface of agar TSB relied on the presence of Mg^{2+} (Koch et al. 2014). Moreover, we also found that biofilms occurred only in Mg^{2+}-enriched TSB but not in the presence of other cation (Koch et al. 2014), suggesting that this ion in particularly important as an environmental cue to trigger biofilm formation. Our findings are also consistent with the fact that biofilm-associated *S. aureus* colonization generally occurs in in Mg^{2+}-enriched niches, such as bones and kidneys, in which chronic staphylococcal infections usually develop (Elin 2010; Günther 2011; Jahnen-Dechent and Ketteler 2012) while; tissues naturally depleted of this cation are prone to acute staphylococcal infections. A classical example is that sequestration of Mg^{2+} from vaginal tissues due to the use of tampons led to an outbreak of *S. aureus* in women in the USA (Schlievert 1985).

Together, with the increased biofilm formation in liquid and agar TSBMg we also found that in the presence of Mg^{2+}, staphyloxanthin was significantly overproduced in *S. aureus* strain Newman wild type (**Figure 26**).

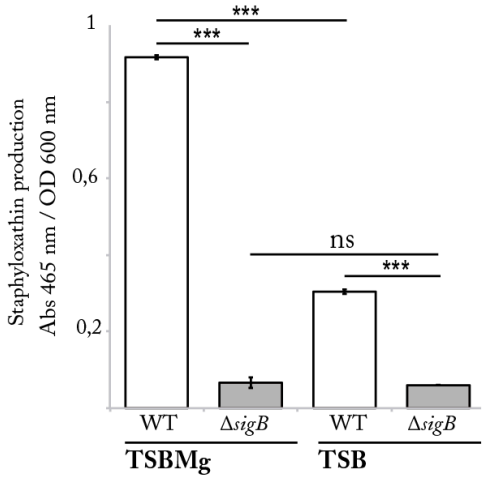

Figure 26. Staphyloxanthin production in *S. aureus* wild type and Δ*sigB* growing in the TSBMg and standard TSB. When growing in the presence of extracellular Mg^{2+}, the production of staphyloxanthin is significantly increased in *S. aureus*. As σ^B directly regulates staphyloxanthin production, the *sigB*-deficient strain does not produce this pigment. TSBMg activates σ^B stress regulon in *S. aureus*. Staphyloxantin production was used as a proxy to monitor activation of σ^B, since the expression of the operon responsible for staphyloxanthin production is directly regulated by σ^B (Giachino, Engelmann, and Bischoff 2001). Statistical significance was measured by unpaired two-tailed test. *** $p<0.001$, ns no significant differences.

The pigment staphyloxanthin, which gives the typical yellow coloration to *S. aureus* is directly regulated by the alternative Sigma factor σ^B (Giachino, Engelmann, and Bischoff 2001). These results suggest that the signaling cascade acts upon *agr* downregulation via activation of σ^B, consistent with previous publications showing that σ^B is a stress sigma factor that represses Agr activity (Kullik, Giachino, and Fuchs 1998; Giachino, Engelmann, and Bischoff 2001) and Agr downregulates biofilm-related genes.

Since σ^B is the staphylococcal stress-related Sigma factor, we reasoned that the stress mechanism that activates σ^B should be related to the specific role of extracellular Mg^{2+} in TSBMg. In previous studies it

was demonstrated that Mg^{2+} plays a specific role in stabilizing the cell wall of Gram-positive bacteria, which are decorated with phosphate-rich teichoic acids (TAs) that contribute to membrane integrity (Heptinstall, Archibald, and Baddiley 1970a). Cell wall TAs preferentially bind free Mg^{2+} cations and form a consolidated network that strengthens the rigidity of the cell envelope and hence, alleviates the electrostatic repulsive interactions between neighboring phosphates (Lambert, Hancock, and Baddiley 1975b; Heckels, Lambert, and Baddiley 1977; Swoboda, Campbell, et al. 2009). Therefore, we hypothesized that the Mg^{2+} present in the TSBMg increases the cell wall rigidity of *S. aureus* and in turn, serves as an external cue to activate σ^B. These results, together with the fact that a SigB-defective is a poor biofilm former and, that the biofilm formation phenotype is partially recovered in *S. aureus* strain Newman Δ*sigB* Δ*agr* (**Figure 21**), suggest that biofilm formation in *S. aureus* in the presence of Mg^{2+} is an stress-related process triggered by the sensing of an increased cell wall rigidity in this bacterium.

To follow this hypothesis, we performed experiments using atomic force microscopy (AFM) to monitor, at the single cell level, the structural rigidity of *S. aureus* cell wall when grown in TSB and TSBMg media (**Figure 27**). We found that when growing in the presence of Mg^{2+}, cell wall rigidity in *S. aureus* wild type significantly increases, demonstrating that this cation might act as an environmental cue to trigger biofilm formation through σ^B repression of the Agr functionality.

AFM is the gold-standard technique to quantitatively measure cell wall rigidity in bacteria and works by quantifying the forces acting between a sharp tip and the bacterial cell wall after pressure (Dufrene 2002; Neumann 2008; Formosa-Dague et al. 2016). It is known that binding of Mg^{2+} to the cell wall depends on the presence of TAs (Heptinstall, Archibald, and Baddiley 1970a) and the D-alanine ester content of TAs (Archibald, Baddiley, and Heptinstall 1973). In particular, the D-alanylation of TAs introduces positively charged amines and prevents repulsive interactions between neighboring TAs, stabilizing the staphylococcal cell wall. As the membrane-bound protein complex DltA-E is responsible for D-alanine esterification of TAs (Perego et al. 1995), the cell wall rigidity in a Δ*dltA* in standard TSB is significantly reduced due to a increased destabilization of the bacterial surface (**Figure 27**). As expected, in non-supplemented TSB the Δ*dltA* displayed significantly low cell wall rigidity due to the absence of positive charges. Nevertheless, when this DltA-deficient strain was grown in TSBMg, AFM results showed that cell wall rigidity recovered to values similar to the wild type strain growing also in TSBMg and even higher that the wild type strain grown in non-supplemented TSB media.

We also demonstrated the direct correlation between the cell wall rigidity and the activation of the σ^B stress-related response by measuring staphyloxanthin production and biofilm formation in these conditions (**Figure 28**). As seen in **Figure 28**, the wild type and the Δ*dltA* strains produced significantly elevated levels of staphyloxanthin when growing in the presence of Mg^{2+}, which completely correlated with the AFM results (**Figure 27**). In the same manner, the presence of Mg^{2+} also increased the ability of this mutant strain to form biofilm to similar levels of the isogenic wild type strain. In addition, the ability of the Δ*dltA* strain to develop robust multicellular aggregates when growing on the solid surface

of TSBMg plates was strongly affected by the presence of Mg^{2+} (**Figure 29**). Together, these results demonstrated that the *S. aureus* senses Mg^{2+} as an extracellular stress cue that activates σ^B and promotes biofilm formation downregulating *agr* expression.

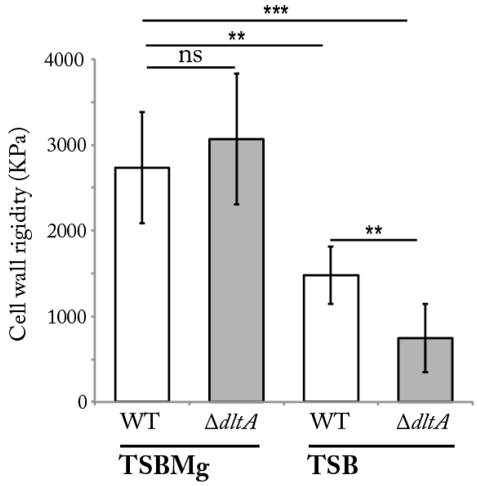

Figure 27. Quantification of the cell wall rigidity of *S. aureus* cells in TSB and TSB. *S. aureus* wild type and the Δ*dltA* strain were grown in TSBMg or TSB and the rigidity of the cell surface was quantified at the single cell level using AFM. In the presence of extracellular Mg^{2+}, the cell wall rigidity significantly increases. The absence of D-alanine decorating the TAs in the Δ*dltA* strains generates a defective cell wall with a significantly reduced surface rigidity. The addition of Mg^{2+} to Δ*dltA* cultures recovers the rigidity of this mutant strain to the wild type levels. Statistical significance was measured by unpaired two-tailed test. ** p<0.005, *** p<0.001, ns no significant differences.

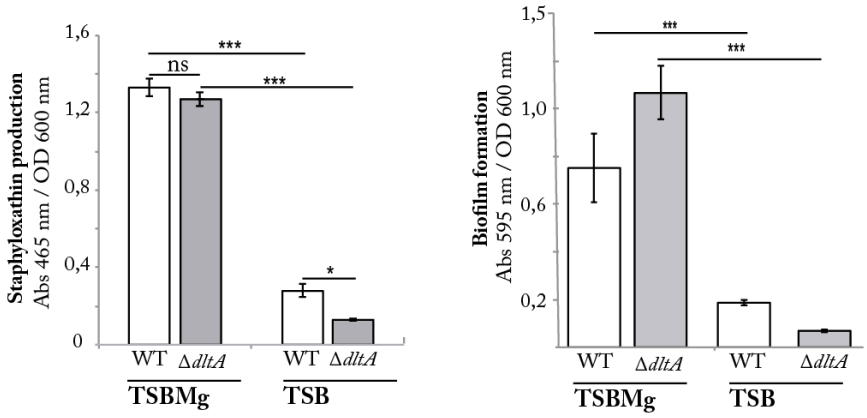

Figure 28. Quantification of staphyloxanthin production and biofilm formation in WT and *dltA*-defective strain in TSBMg and TSB media. The DltA-E machinery is responsible for D-alanylation of TA, which introduces positively charged amines and prevents repulsive interactions between neighboring TA, stabilizing the bacterial cell wall. This D-alanylation increases cell wall rigidity (Perego et al. 1995), similar to the effect of Mg^{2+} incorporation to the cell wall. In standard TSB medium, cell wall rigidity is severely compromised in the Δ*dlt* mutant, whereas in TSBMg, extracellular Mg^{2+} binding complemented the cell wall rigidity defect in this mutant, as TA-coordinated Mg^{2+} can provide cell wall rigidity in the absence of the Dlt machinery. *S. aureus* grew in TSBMg in 24-well microtiter wells during 48 h at 37 ºC. Biofilm formation quantified using the crystal violet assay. Statistical significance was measured by unpaired, two-tailed t-test. * p<0.05, *** p<0.001, ns no significant differences.

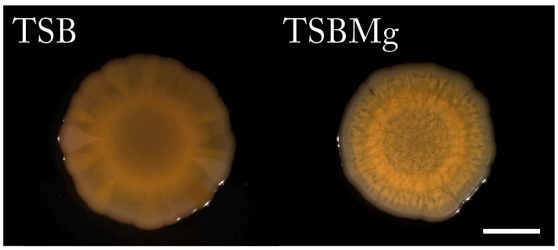

Figure 29. Development of multicellular communities *S. aureus* Δ*dltA* in TSB and TSBMg. In the absence of extracellular Mg^{2+}, the DltA-deficient strain does not develop the robust multicellular communities. The addition of Mg^{2+} to TSB, strongly recovers the complex architecture of the staphylococcal multicellular communities. In TSBMg, *S. aureus* Δ*dltA* display similar features as the wild type strain: reduced diameter, thick extracellular matrix, a wrinkled surface and well defined concentric areas. Top-view pictures of fully developed microbial communities during 5 days (120 h) at 37 ºC in TSBMg. Scale bar is 5 mm.

Furthermore, to correlate biofilm formation in TSBMg medium and the specific capacity of TAs to bind Mg^{2+} and thus to increase cell wall rigidity, we performed additional biofilm formation experiments using the selective inhibitor of TAs biosynthesis tunicamycin (Campbell et al. 2011) (**Figure 30**).

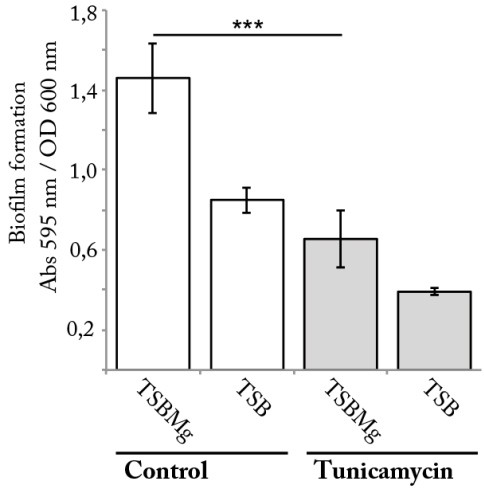

Figure 30. Quantification of biofilm formation in TSBMg and TSB alone or with sublethal tunicamycin concentration (0.8 mg/ml). Tunicamycin selectively inhibits the synthesis of the TAs, which correlates with a decreased ability of *S. aureus* cells to bind extracellular Mg^{2+} and therefore, a significant reduction in biofilm formation. Therefore, cells treated with this antibiotic are not able to respond to extracellular Mg^{2+}. Biofilm formation quantified using the crystal violet assay. Statistical significance was measured by unpaired, two-tailed t-test. *** $p<0.001$.

Cells treated with a sublethal concentration of the antibiotic tunicamycin were not able to respond to Mg^{2+} and, pellicle formation was abrogated even in TSBMg. Based on these results, we used *S. aureus* strains that downregulate and upregulate important genes related to TAs biosynthesis and tested their capability to form biofilms (**Figure 31**) in TSB supplemented with Mg^{2+}. Strains exhibiting downregulation of diverse *tag* genes were unable to respond to Mg^{2+} and failed to develop biofilms in

TSBMg (henceforth *tag*-lower strains) comparable with the wild type strain. This behavior was similarly found in different clinical isolates of *S. aureus* (USA300 LAC* and Newman strains) and interestingly, for all the *tag*-lower strains and, in both clinical isolates, when Mg^{2+} was not present, not statistical differences in biofilm formation were observed between the wild type and the *tag*-deficient strains.

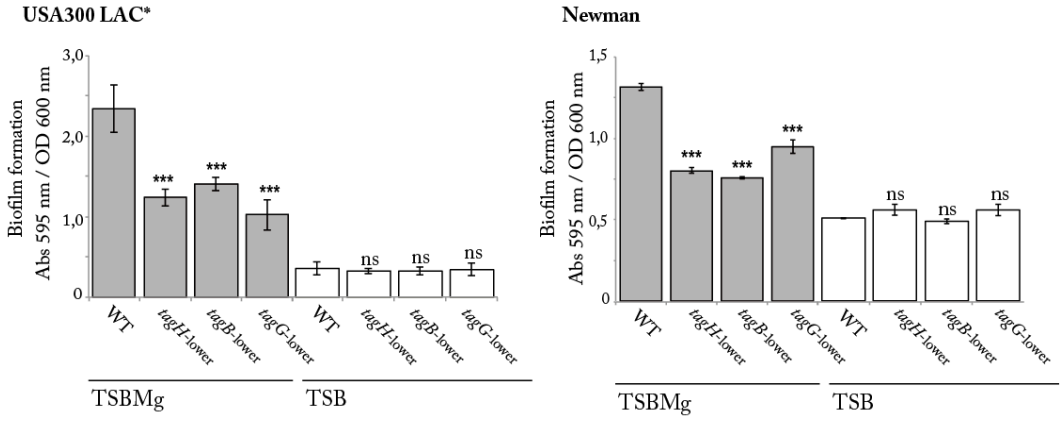

Figure 31. Quantification of biofilm formation of different genetic backgrounds in TSBMg and TSB media. Biofilm formation was monitored in wild type and different genetically engineered strains that produced different levels of TAs in USA300 LAC* and Newman. In the presence of Mg^{2+}, there are significant differences in biofilm formation between the wild type strain and the strains defective in TAs biosynthesis. Interestingly, no differences in biofilm formation between the wild type and the genetically engineered strains were observed when growing in standard TSB. *S. aureus* grew in TSBMg in 24-well microtiter wells during 48 h at 37 ºC. Biofilm formation quantified using the crystal violet assay. Statistical significance was measured by unpaired, two-tailed t-test. *** $p<0.001$, ns no significant differences.

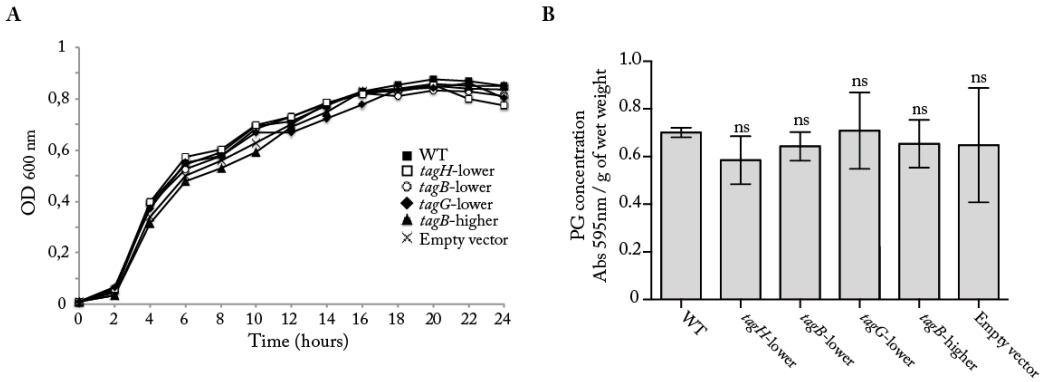

Figure 32. Growth curves and peptidoglycan (PG) quantification in *S. aureus* strain Newman wild type and different *tag*-lower and *tagB*-higher strains. Genetically engineered strains that produced different levels of TAs showed no differences in growth or peptidoglycan production. **(A)** Growth curves of WT and several genetically engineered strains that produced different levels of TAs in TSB medium. **(B)** Peptidoglycan quantification of WT and several genetically engineered strains that produced different levels of TAs.

The differences in biofilm formation observed between the wild type and the different *tag*-lower strains were associated with the ability of these strains to bind extracellular Mg^{2+} since no differences were

observed in standard TSB and their growth rate and peptidoglycan synthesis were similar (**Figure 32**), demonstrating that the biofilm phenotype was not due to a deleterious fitness disadvantage of the modified *S. aureus* strain when growing in standard laboratory conditions.

In contrast, strains showing upregulation of *tagB* using a high-copy number plasmid system became hypersensitive to extracellular Mg^{2+} and produced more robust biofilms (*tagB*-higher strains) (**Figure 33**). Interestingly, even in the absence of Mg^{2+}, the *tagB*-higher strain developed more biofilms. This behavior might be the result of an increased amount of TAs on the surface of bacterial cell, which might render *per se* elevated cell wall rigidity. Also, increased TAs levels expressed on the staphylococcal cell wall directly correlate with an increased D-alanylation, generating a more rigid bacterial cell wall. In a similar manner to the different *tag*-lower strains, the *tagB*-higher strain showed no differences in growth or in PG synthesis when compared to the isogenic wild type strain (**Figure 32**).

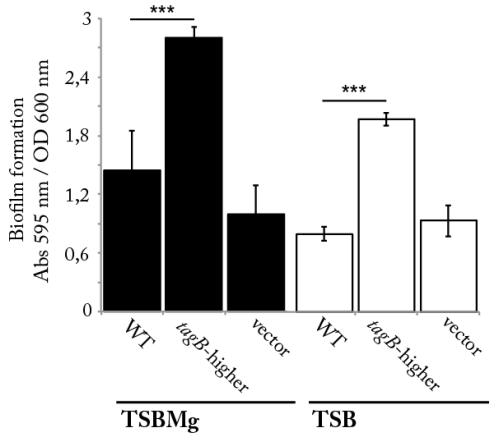

Figure 33. Quantification of biofilm formation of different genetic backgrounds in TSBMg and TSB media. Biofilm formation was monitored in wild type and different genetically engineered strains that produced different levels of TAs in USA300 LAC* and Newman. In the presence of Mg^{2+}, there are significant differences in biofilm formation between the wild type strain and the strains defective in TAs biosynthesis. Interestingly, no differences in biofilm formation between the wild type and the genetically engineered strains were observed when growing in standard TSB. *S. aureus* grew in TSBMg in 24-well microtiter wells during 48 h at 37 ºC. Biofilm formation quantified using the crystal violet assay. Statistical significance was measured by unpaired, two-tailed t-test. *** $p<0.001$, ns no significant differences

The biofilm phenotypes observed for the different *tag*-lower and *tag*-higher strains showed to be directly correlated with the activity and expression of the alternative sigma factor σ^B (**Figure 34**). We measured staphyloxanthin production (**Figure 34A**) in these genetically engineered strains in TSB and TSBMg and we found a significant changes in σ^B activity among the wild type and the modified strains. In particular, the *tagB*-lower strain showed a significant decrease in staphyloxanthin production, in agreement with the hypothesis that a reduced expression of TAs decreases cell wall rigidity, due to the inability of *S. aureus* to bind extracellular Mg^{2+} and hence, there is inefficient σ^B activation. On the contrary, the increased TAs concentration in the *tagB*-higher strain elevates σ^B

activity due to an increased Mg^{2+} binding and therefore, an augmentation in cell wall rigidity. Interestingly, σ^B activity was unaffected by the genetic background in the absence of extracellular Mg^{2+}, confirming the direct role of the TAs in Mg^{2+} and in the activation of the σ^B. In addition, we also measured the expression of the σ^B protein by immunodetection (**Figure 34B**) to confirm not only σ^B activity but also σ^B overproduction in response to the cell wall stress caused by the binding of Mg^{2+} to the cell wall TAs. As expected, σ^B expression was altered in response to extracellular Mg^{2+} in the different strains that can sense this cation.

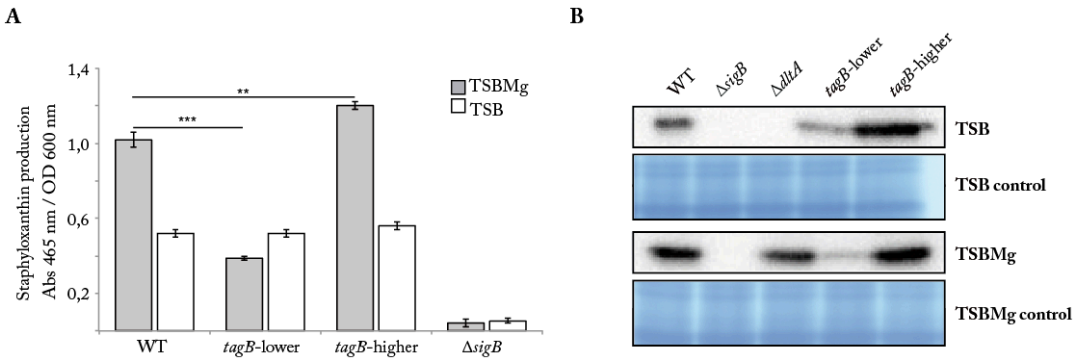

Figure 34. (A) Quantification of staphyloxanthin production in *S. aureus* Newman wild type and genetically engineered strains that produce different levels of TAs. **(B)** Immunodetection of σ^B protein in *S. aureus* wild type and different strains with altered TAs expression and composition. In response to extracellular levels of Mg^{2+} found in the TSBMg, σ^B activity **(A)** and expression **(B)** is significantly altered. Statistical significance was measured by unpaired, two-tailed t-test. ** $p<0.01$, *** $p<0.001$.

All together, these results are consistent with the hypothesis that Mg^{2+} of TSBMg stabilizes TAs, causes an increase in cell wall rigidity and triggers the stress sigma factor σ^B, which activates biofilm formation via downregulation of *agr*.

4. Multicellular aggregates of *S. aureus* specialize *BR* and *DR* heritable cell fates

The results from above sections above determined the nature of the signal and the mechanism that triggers biofilm formation of *S. aureus* in TSBMg. Next, we further explored the role of Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles at the single-cell level, in the presence of this particular environmental cue that is promoted in TSBMg medium.

To identify cells associated with the different stages of biofilm formation, we used transcriptional fusions of biofilm associated *ica* and *spa* genes and planktonic *psmα/β* agr-related genes. These transcriptional fusions were constructed by introducing them in neutral loci into the S. *aureus* chromosome to ensure the expression of a single copy of the reporter in each one of the cells (Yepes et al. 2014) and their expression in S. *aureus* aggregates was monitored using fluorescence microscopy and flow cytometry.

Based on the literature available, we chose reporter gene markers that might constitute some of the different cell types relevant during the different stages that define a chronic or an acute infection:

matrix exopolysaccharide-producing cells (*ica* operon), adhesion and attachment cells (*spa*), and virulent/disseminating cells (*psmα1-4* and *psmβ1-2*) (Forsgren and Nordström 1974; Klaesson et al. 1993; MACK, Fischer, Krokotsch, and Leopold 1996; MACK, Haeder, et al. 1996; MACK, Fischer, Krokotsch, Leopold, et al. 1996; Heilmann et al. 1996; Ziebuhr et al. 1997; Mehlin, Headley, and Klebanoff 1999; Götz 2002; Palmqvist et al. 2002; K. M. Conlon, Humphreys, and O'Gara 2002; Otto et al. 2004; Yao, Sturdevant, and Otto 2005; R. Wang et al. 2007; Jeng et al. 2008; Otto 2009; Merino et al. 2009; Verdon et al. 2009; Periasamy et al. 2012; Schwartz, Syed, Stephenson, Rickard, and Boles 2012a; G. Y. C. Cheung, Joo, et al. 2013; Otto 2014; Thammavongsa et al. 2015).

During the development of the multicellular aggregates in TSBMg agar surface, all the reporters displayed bimodal expression and revealed the bifurcation of two subpopulations of cells in *S. aureus* multicellular communities, one subpopulation with low fluorescence and another subpopulation exhibiting higher fluorescence (**Figure 35**). This bimodal expression pattern was different from the unimodal expression behavior of the Agr-independent gene *dnaA* that we used as control reporter (**Figure 36**). We used the P*dnaA-yfp* promoter as the control reporter since the chromosomal replication initiator protein DnaA is an essential housekeeping protein involved in the initiation of chromosomal replication during cell division, regulates its own expression (Skarstad and Boye 1994; Moriya et al. 1999; Murai et al. 2006; Kurokawa et al. 2009) and is not part of the Agr regulon (GOERKE et al. 2000).

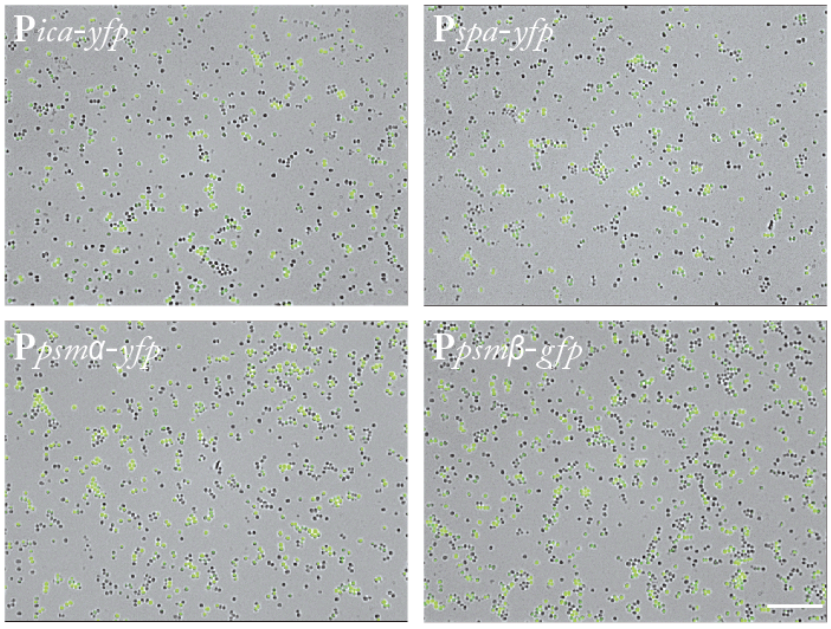

Figure 35. Gene expression in *S. aureus* multicellular aggregates displays a bimodal behavior. Fluorescence microscopy pictures of fields of single-labeled cells harboring distinct transcriptional fusions for different reporter genes involved in biofilm formation (P*ica* and P*spa*) and virulence and dissemination (P*psmα1-4* and P*psmβ1-2*). The fluorescence signal is false-colored in green. Scale bar is 10 μm.

We monitored the temporal dynamics of the different subpopulations that bifurcates during the development of the staphylococcal microbial community to understand this previously unknown

bifurcation of coexisting cells types in *S. aureus* multicellular communities. As observed in **Figure 36**, *S. aureus* growing on TSBMg agar surface revealed a larger subpopulation of *ica* and *spa* expressing cells during the early stages of development (24 h to 48 h of development), while concomitantly the number of cells expressing *psmα* and *psmβ* increased over time (72 h to 120 h of development), consistent with the previously reported antagonistic regulation of *ica/spa* and *psmα/psmβ* by *agr* during biofilm formation and biofilm maturation and dispersal (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a).

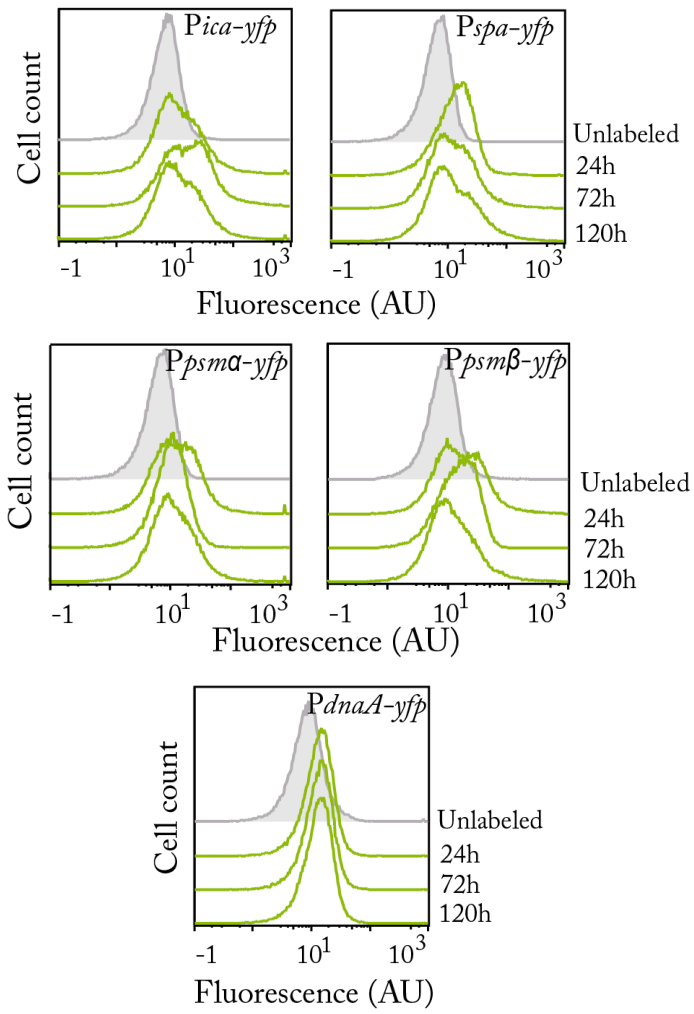

Figure 36. *Staphylococcus aureus* multicellular aggregates contain specialized cell types. Flow cytometry analyses shows that the temporal expression pattern of *ica* is similar to that of *spa* and the temporal expression pattern of *psmα* is similar to that of *psmβ*. Cell count analyses of single-labeled cells at different times during aggregate formation.

The results of microscopy cell count from **Figure 35** and the flow cytometry quantification from **Figure 36** showed a similar pattern for bimodal behavior found for the *ica/spa* and *psmα/psmβ* cell types. Basically, *ica/spa* displayed a higher number of expressing cells at early stages while the *psmα/psmβ* cell types displayed higher number of expressing cells at late stages, suggesting that the cell types involved in biofilm formation (*ica/spa*) and the cell types involved in virulence and biofilm dispersion

(*psmα/psmβ*) could be following the same developmental plan. Therefore, in order to define if the expression of the *ica/spa* and *psmα/psmβ* cell types is generated in discrete cell types, we generated strains labeled with pairwise combinations of these reporters, to examine their simultaneous expression during the development of *S. aureus* microbial communities (**Figure 37**).

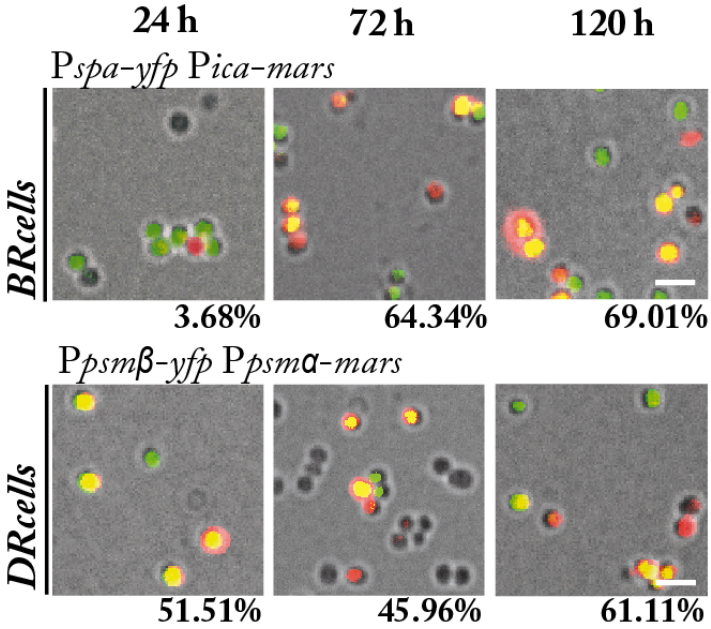

Figure 37. Fluorescence microscopy pictures of double-labeled cells at different times during aggregate formation (24, 72 and 120 h) harboring distinct transcriptional fusions. The total percentage of cells in which the two signals overlap is shown at the bottom left in every panel. A positive overlap of the fluorescence signal was attributed to cells that simultaneously expressed the two reporters with comparable fluorescence intensity within the 2:1–1:2 expression rates. Cells expressing the *ica* operon and cells expressing the *spa* gene positively overlap, particularly at mid and late stages during aggregate formation. Similarly, *psmα* and *psmβ* expressing cells also overlap during all the stages of aggregate formation tested. This coexpression supports the hypothesis that the *ica/spa* and the *psmα/psmβ* constitute two discrete and exclusive cell lines involved in biofilm formation and biofilm dispersal, respectively. Fluorescence signal related to the expression of YFP is false-colored in green. Fluorescence signal related to the expression of Mars is false-colored in red. Cells in which the two signals overlap appeared yellow. Percentages of cells quantified in the fields with positive overlapping signal are shown. Scale bar is 2 μm.

Using this approach, we detected coexpression of *ica* with *spa* and *psmα* with *psmβ* in two distinct subpopulations of cells, which allowed us to define that the bimodal bifurcation generated during the development of these multicellular communities generates two distinct subpopulation of cells: one of them specialized in expressing the biofilm related genes *ica/spa* and the other, specialized in expressing the dispersion related genes *psmα/psmβ* (**Figure 37**). Therefore, we named these cell types as *BRcells* (biofilm-related cells) and *DRcells* (dispersion-related cells), respectively. Additionally, we generated *S. aureus* strains comprising different *BRcells* reporters together with *DRcells* reporters and analyzed its coexpression during the development of the multicellular aggregate (**Figure 38**).

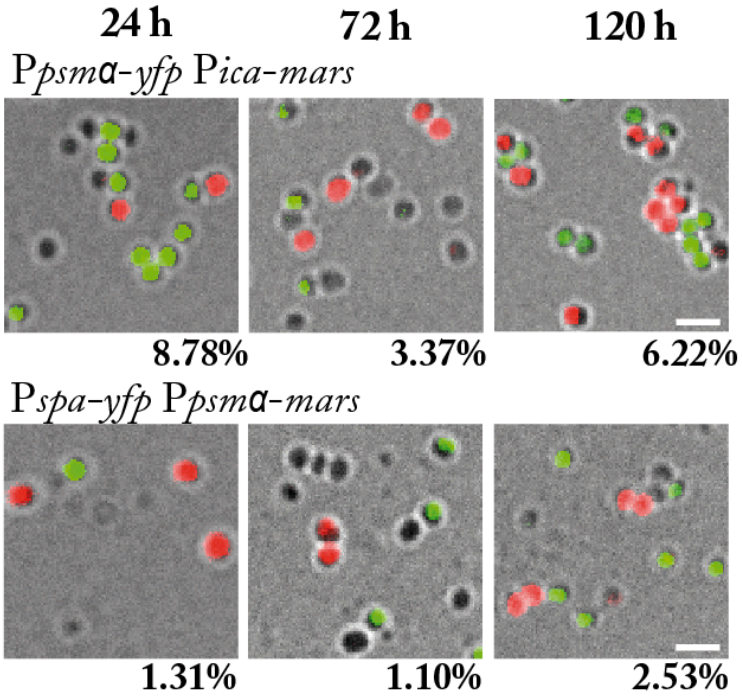

Figure 38. Fluorescence microscopy pictures of double-labeled cells at different times during aggregate formation (24, 72 and 120 h) harboring distinct transcriptional fusions. The *ica* expressing cells and *psmα* expressing cells do not significantly overlap. Similarly, *spa* expressing cells and *psmα* expressing cells do not overlap higher that 2.53 %. The exclusive expression of the *ica* and *spa* cell types respective to *psmα* expression supports the findings presented in **Figure 37**, where we defined the *BRcells* and *DRcells* types. Fluorescence signal related to the expression of YFP is false-colored in green. Fluorescence signal related to the expression of Mars is false-colored in red. Cells in which the two signals overlap appeared yellow. Percentages of cells quantified in the fields with positive overlapping signal are shown. Scale bar is 2 μm.

These results demonstrated that during the development of these staphylococcal biofilms the *BRcells* reporters, *ica* and *spa* do not coexpress, or do it in a significantly low proportion with the *DRcells* reporters, *psmα* or *psmβ*, confirming that biofilm formation and dispersal in *S. aureus* multicellular communities growing in TSB supplemented with Mg^{2+}, is driven by particular and exclusive subpopulations of cells and moreover, highlighting the antagonistic role of the *agr* system in the regulation of planktonic and biofilm-associated lifestyles, based on these results, at the single-cell level. To confirm that the BRcells indeed express additional genes involved in attachment and biofilm formation, we performed additional experiments with additional pairwise combinations of the *ica* and the *spa* reporters with reporters for adhesins that play important role in biofilm formation, specifically the *clfA* and the *isdA* adhesin genes (**Figure 39**).

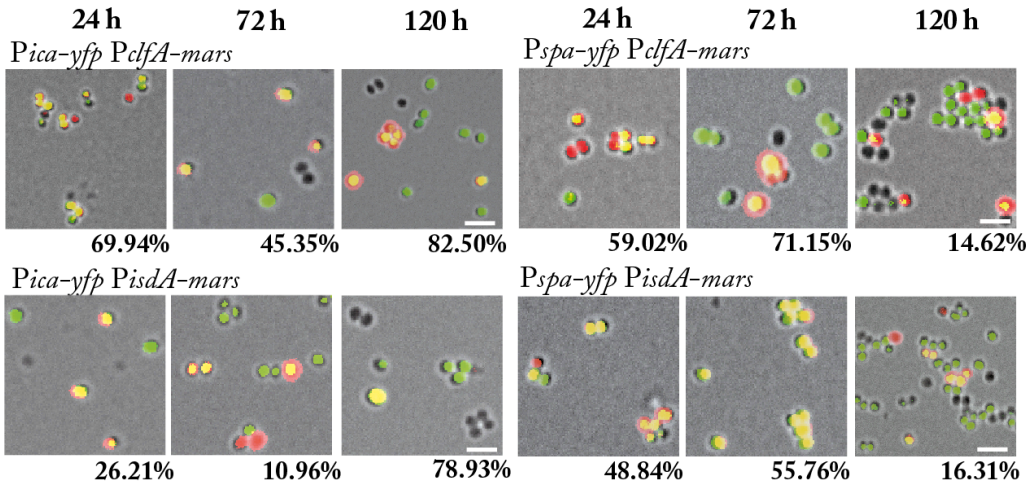

Figure 39. Fluorescence microscopy pictures of double-labeled cells at different times during aggregate formation (24, 72 and 120 h) harboring distinct transcriptional fusions. Fluorescence microscopy pictures of fields of double-labeled cells harboring the *BRcells* transcriptional fusions (P*ica* and P*spa*) and the P*clfA-mars* and P*isdA-mars* transcriptional fusions. *BRcells* simultaneously expresses *ica*, *spa* and other genes coding for adhesion proteins. The *BRcells* reporter genes positively coexpress with additional MSCRAMMs involved in adhesion and biofilm formation. Fluorescence signal related to the expression of YFP is false-colored in green. Fluorescence signal related to the expression of Mars is false-colored in red. Cells in which the two signals overlap appeared yellow. Percentages of cells quantified in the fields with positive overlapping signal are shown. Scale bar is 2 μm.

As expected, the coexpression of these adhesins with the *ica* and *spa* revealed that these reporters are positively coexpressed in the same subpopulation of cells. The ClfA, or clumping factor A, is a recognized cell wall protein implicated adhesion and in early phases of biofilm formation (McDevitt et al. 1994). IsdA, a member of the heme scavenging factors family, is a CWAs MSCRAMM surface protein (Clarke, Wiltshire, and Foster 2004). The iron concentration in TSB medium is ~10 μM, enabling *isd* expression, although is nonetheless possible to achieve higher expression using iron chelators. Conversely, higher iron concentration inhibits *isd* expression, as shown by many publications when TSB medium is supplemented with 50 μM $FeSO_4$ (Mazmanian et al. 2003; Clarke, Wiltshire, and Foster 2004). Moderate *isd* reporter expression is sufficient to recognize this subpopulation through this approach. These results reinforce our hypothesis of dedicated *BR* and *DR* cell linages during the development of staphylococcal communities in the presence of Mg^{2+} as an environmental cue that triggers *agr* modulation via σ^B activation.

In order to determine if the *BRcells* and *DRcells* constitute indeed tractable cells types and display a heritable line-dependent expression of the biofilm- or dissemination-related cell fate, we performed live time-lapse fluorescence microscopy to track the fate of hundreds of *BRcells* and *DRcells* through multiple generations. We investigated the stability of the different biofilm- and dissemination-related cell fates in which the bacterial population bifurcates during the biofilm development using this real-time microscopy technique and to perform this, cells were dispersed from the biofilm, washed to remove the AIP signal and grown in fresh TSBMg solid agars as it was detailed in Materials and

Methods. The cell fate from separate microcolonies were analyzed using fluorescence microscopy and a parsimony reconstruction was generated (**Figure 40**).

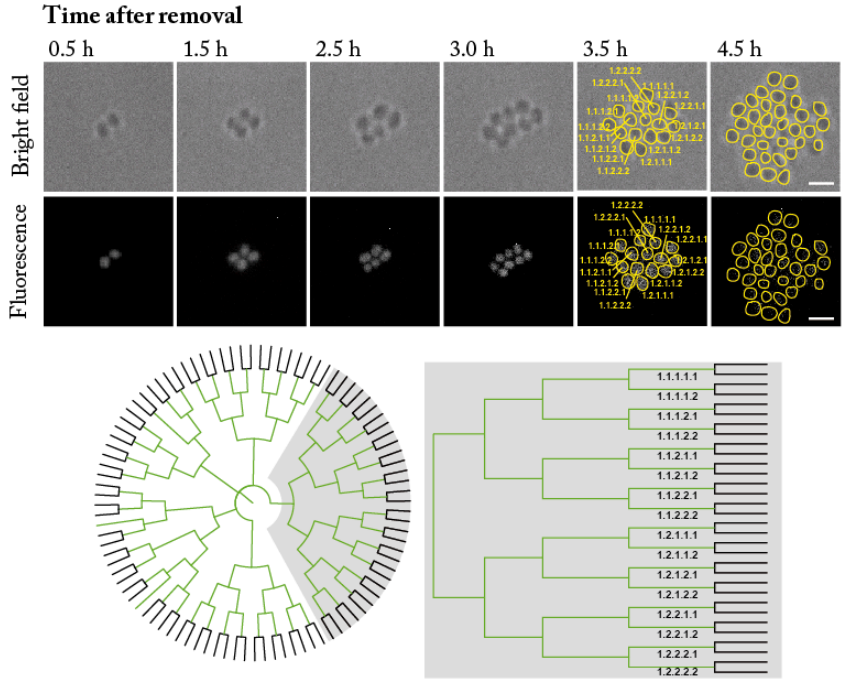

Figure 40. In the upper panel, representative fluorescence time-lapse microscopy assay of the outgrowth of *S. aureus* cells into a microcolony and labeled with the P*ica-yfp* (top) or P*psmα-yfp* transcriptional fusions, as proxies of the *BRcells* and *DRcells* types, respectively. Scale bar is 2 μm. In the lower panel, representative phylogenetic clustering of the expression of the *BRcells* and *DRcells* reporters in microcolonies during cell division in time-lapse fluorescence experiments. Parsimony clustering of the expression of the respective reporter is generated on the right side of the panel. Green lines represent inheritance in the expression of the fluorescent reporter to the progeny. Black lines denote the loss of the fluorescence signal to the cell progeny. The grey frame shows the clustering of the inheritance that is represented in the time-lapse experiment. A subpopulation of *BRcells* or *DRcells* was traced during colony growth by following the expression of respective transcriptional reporter. Cells were removed from a mature aggregate, washed them to eliminate any remaining AIP QS signal from the medium and inoculated in fresh TSBMg. Cells proliferated in the fresh media, generated a microcolony and the expression of the reporter is transmitted to the progeny, reflecting the temporally heritable character in the cell fate decision making of the *BRcells* and *DRcells* types.

In **Figure 40**, green lines represent inheritance in the expression of the fluorescent reporter to the progeny and black lines denote the loss of the fluorescence signal to the cell progeny. Using this technique, we were able to trace the gene expression in *BRcells* and *DRcells* up five cell divisions events, even in the absence of the AIP signal, showing that the fluorescence expression pattern is transmitted to the progeny during several generations, in a process that is known as hysteresis (Lacasta et al. 1999; Ozbudak et al. 2004; Smits, Kuipers, and Veening 2006; Alexander Y Mitrophanov 2008; Mitrophanov and Groisman 2008a; Guttal and Couzin 2010) and, that the phylogenetic clustering is line-dependent and attributable to a heritable expression of the reporter. We confirmed the heritable

nature of this behavior by impairing new mRNA transcription and protein synthesis of the yellow fluorescent protein (YFP) (**Figure 41**). Here, we found that our *yfp* gene mRNA transcript and the respective fluorescent protein were unstable and the cell fluorescence was lost in approximately 1.5 h under similar growing conditions as in **Figure 40**.

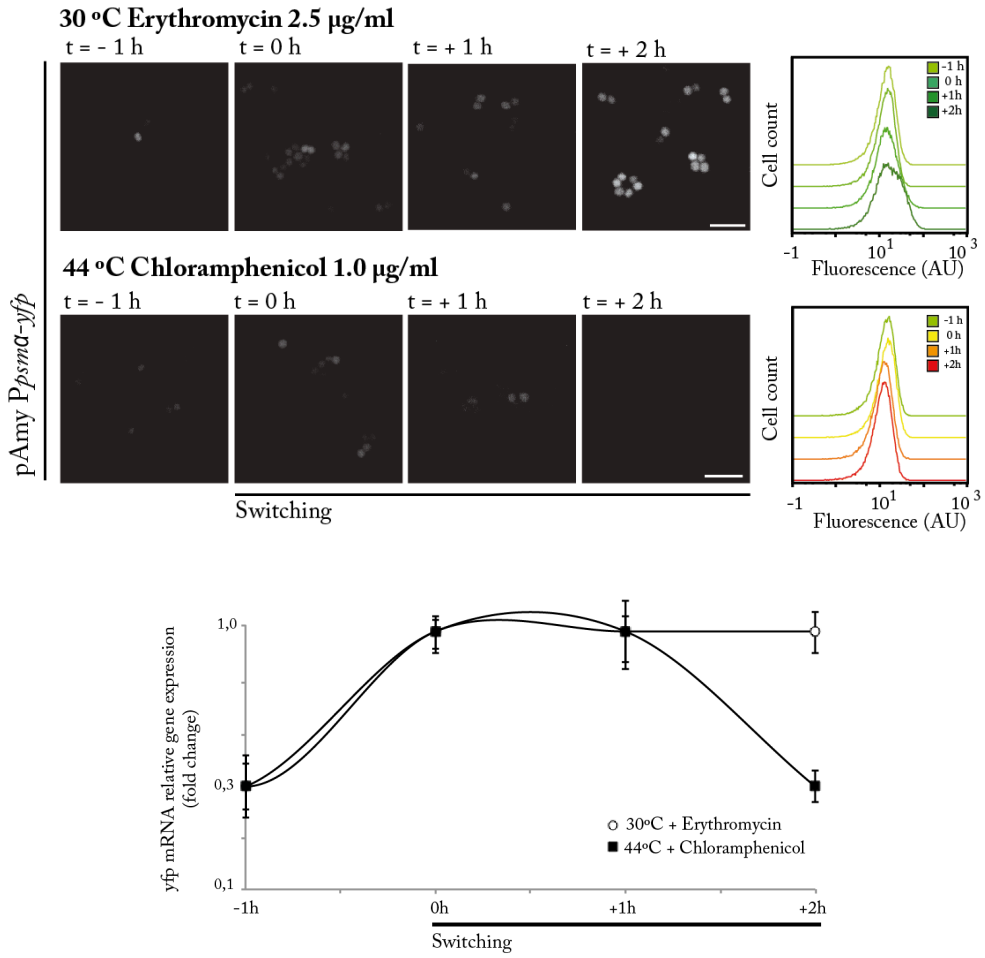

Figure 41. Fluorescence stability of the YFP protein. Cells harboring a pMAD temperature-sensitive vector carrying the P*psma-yfp* reporter were propagated in TSB medium at 30 ºC. The control experiment in the upper part shows the time-lapse experiment at 30 ºC in the presence of erythromycin. These conditions ensured the replication of the plasmid during cell growth and thus fluorescence signal of the reporter was detected in the cells. Samples that were incubated at 44 ºC in the presence of chloramphenicol (1 µg/ml) lost the fluorescence signal after 120 min. High temperature prevents plasmid replication and the presence of chloramphenicol prevents new protein synthesis. Scale bar is 2 µm. The flow cytometry profiles of the samples show the continuous increase in the fluorescence intensity of the culture maintained at 30 ºC, in contrast to the decrease in the fluorescence observed in cells at 44 ºC with chloramphenicol, shown in the lower right panel. In the lower graph, quantification of *yfp* gene expression by RT-qPCR. Total RNA was isolated from cells harboring the pMAD temperature-sensitive vector carrying the P*psma-yfp* reporter. The *yfp* transcripts decreased after inhibiting plasmid replication by incubating cultures to 44 ˚C.

In this experiment, cells harboring the low-copy number and temperature-sensitive plasmid pMAD vector containing the P*psma-yfp* reporter were growing normally at 30 ºC in the presence of the

antibiotic erythromycin to guarantee vector replication and synthesis of the fluorescence protein. Microscopy and the flow cytometry profiles of the samples showed the continuous increase in the fluorescence intensity of the culture maintained at 30 ºC and erythromycin and, contrasted with the loss of fluorescence before 120 min observed in cells where plasmid replication and *yfp* transcription was impaired by switching to 44 ºC with chloramphenicol. These results suggest that *BRcells* and *DRcells* may retain certain kind of *temporal memory* of exposure environmental signals like the extracellular Mg^{2+} and morphogens such as the autoinducing QS molecule AIP, which might determine the activation of specific genetic programs leading to the emergence of these particular cell types during the development of the staphylococcal multicellular aggregates growing on the agar surface of TSBMg medium.

In developmental biology, spatial and temporal organization of different cell types normally respond to a threshold activation of a programmed genetic circuit that generates a bistable behavior based on concentration of diverse molecules called morphogens, which include environmental stimuli like nutrients availability and signaling molecules such as QS autoinducing peptides (Kopfová 2006; Lek et al. 2010). This bistable switch mechanisms induce the expression of transcription factors that modulate global gene expression in the particular subpopulation of cells that reached that threshold and as result, promote the process of cell differentiation. In these systems, differentiated cells have the ability to retain and transmit to the progeny their pattern of gene expression, what is known as memory or hysteresis (Harfe et al. 2004). Together, these results suggest that the staphylococcal biofilm contains a least two different and heritable cell fates expressing genes that are relevant for biofilm formation or cell dissemination and virulence, which are normally associated with the chronic and acute presentation of the *S. aureus* infections.

4.1. Bifurcation of the staphylococcal subpopulations is affected by the extracellular Mg^{2+} sensing

Having determined the nature of the signal that triggers biofilm formation in TSAMg, we explored the role of Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles in response to extracellular Mg^{2+}. Expression of the different transcriptional fusions of the *BRcells* and *DRcells* types were monitored in the presence and absence of extracellular Mg^{2+} (**Figure 42**). In TSBMg, these reporters showed bimodal expression and indicated the bifurcation of two cell subpopulations in *S. aureus* aggregates, one with low and another with high fluorescence levels. Both subpopulations were detected in TSB and TSBMg cultures, although the size of the different subpopulations was strongly influenced by the presence of Mg^{2+} in the medium, suggesting that cell differentiation is induced by environmental conditions rather than occurrence of any mutations, for example. In addition, the absence of extracellular Mg^{2+} abolished the bimodal character of all *BRcells* and *DRcells* genes analyzed, suggesting that in the absence of Mg^{2+}, the nature of the Agr as a PFL that coordinates the bifurcation of *S. aureus* cells into different subpopulations is completely lost.

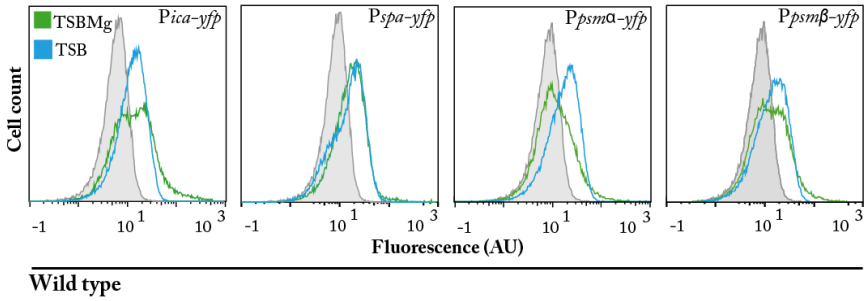

Figure 42. Flow cytometry profiles of the different *BRcells* and *DRcells* reporter genes in the presence (green peaks) and absence (blue peaks) of extracellular Mg^{2+}. The absence of Mg^{2+} abolishes the bimodal character of the different *BRcells* and *DRcells*

The heritable differential expression of Agr-related genes in the distinct cell types led us to analyze the molecular basis of Agr-mediated cell fate decision-making. The Agr system is activated if the extracellular AIP concentration reached a given threshold and if the Agr-repressor σ^B is not induced by extracellular Mg^{2+}. Since Agr activation upregulates *psmα/β* expression directly (Queck et al. 2008), we used flow cytometry to monitor *DRcells* expression to determine the influence of these two input signals on Agr activity and thus on cell differentiation in *S. aureus*. In contrast to the wild type, a *S. aureus* strain unable to sense AIP (Δ*agr*) differentiated a small subpopulation of *DRcells* independently of growth conditions (TSB or TSBMg) (**Figure 43**). In contrast, *S. aureus* Δ*sigB* mutant differentiated a larger *DRcells* subpopulation than that of the wild type strain in the presence and absence of extracellular Mg^{2+}. These results indicate that cell differentiation in *S. aureus* relies on extracellular Mg^{2+} and σ^B activation, which effectively control Agr activity can thus modulate the sizes of specialized cell type subpopulations.

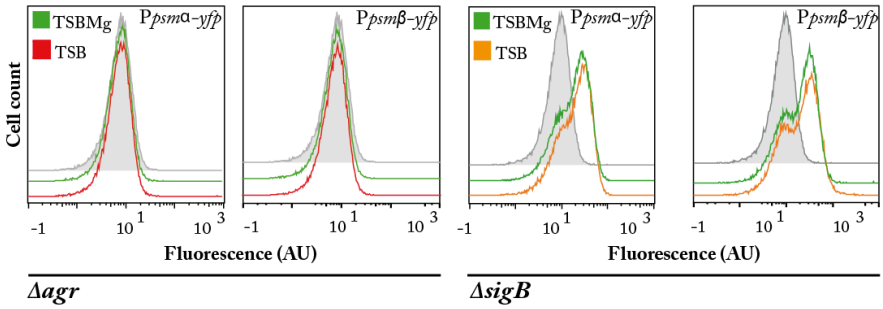

Figure 43. The *agr QS* system is an autonomous program for cell fate decision-making. Flow cytometry profiles of Agr-related promoters in TSBMg and TSB of different *S. aureus* genetic backgrounds representing the two inputs necessary for the activation of the Agr bimodality circuit: extracellular Mg^{2+} and σ^B.

5. The bimodal behavior of the Agr system generates the *BRcells* and *DRcells* heritable cell fates

The heritable and differential expression of Agr-related genes in the distinct cell types led us to investigate the molecular basis for the Agr-mediated cell-fate decision-making. Following the dynamics of a typical PFL, the Agr system autoactivates if the extracellular concentration of AIP has

reached a threshold concentration and also, if the Agr repressor σ^B is not induced by extracellular signals, such as Mg^{2+}. Upon activation, phosphorylated AgrA (AgrA~P) directly upregulates the expression of *psmα/β* (Queck et al. 2008), among others, but also binds to two adjacent, divergent promoters P2 and P3, which trigger the expression of RNAII and RNAIII transcripts, respectively (Koenig et al. 2004). RNAII upregulates the *agrBDCA* operon, which encodes the agr signal transduction cascade, including the AIP signal, the AgrC sensor kinase and its AgrA cognate regulator. The genetic architecture of the agr system, based on the binding of AgrA~P to P2 promoter constitute a PFL in which the product of the gene induces its own expression (Novick and Geisinger 2008; Thoendel et al. 2011). If AgrA~P is slightly activated in a cell after certain threshold level of AgrA~P is reached, the AgrA itself and the repertoire of genes controlled by this transcriptional factor will be activated in that particular cell. This includes indeed, upregulation of the RNAIII transcript that in turn modulates a pool of *agr*-dependent genes coding for the cytotoxic toxins and virulence factors that are responsible for an acute infection (Koenig et al. 2004). In contrast, cells that do not reach the threshold of expression to induce the PFL will remain inactive for the response (Losick and Desplan 2008). However, the presence of a PFL is necessary but not sufficient to generate bimodality in a bacterial population (Angeli, Ferrell, and Sontag 2004) and, additional parameters need to be evaluated to define that a PFL is bimodal, such as the activation threshold of the PFL, the time to respond to the input signal and the self-perpetuation of the PFL in the absence of the input signals (Angeli, Ferrell, and Sontag 2004).

To investigate the bimodal behavior of the *S. aureus* Agr system and, to determine whether is responsible for cell differentiation in staphylococcal multicellular communities as an autonomous program for cell fate decision-making, we genetically engineered a synthetic orthogonal Agr system in the model organism *B. subtilis*, that is exempt from interference from additional staphylococcal regulatory inputs. We used this system to identify the minimal components required for bimodal expression of Agr-related genes using the expression the *psmα* and *psmβ* genes as direct readout for agr activity (**Figure 44**) (F. Zhang et al. 2015). The orthogonal *S. aureus* Agr system in *B. subtilis* is particularly simple and can be converted as a AND logic gate, which represents the two input signals that control Agr activity are thus the activating signal AIP and the inhibiting signal σ^B.

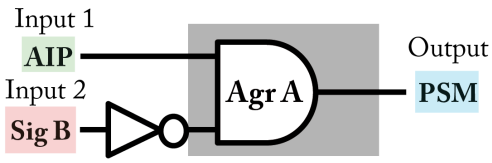

Figure 44. Conversion of the Agr signal transduction pathway into a Boolean circuit using binary logic gates. The *agr* system is represented by an AND gate to indicate that the simultaneous action of two inputs is necessary to activate the circuit. Input 1 is the presence of a threshold concentration of AIP and input 2 is the absence of σ^B activity. The output is the expression of the P*psmα-yfp* and P*psmβ-yfp* as proxies of the AgrA autoactivation.

In this orthogonal system, *B. subtilis* harbored a P*psmα-yfp* and P*psmβ-yfp* reporters and expressed the membrane kinase AgrC and its cognate regulator AgrA under the control of P2 promoter as a chimeric version AgrCA. In particular, this orthogonal system does not contain the staphylococcal σ^B, which ensures no inhibition of the Agr system. The addition of purified AIP (1 μM) to these *B. subtilis* cultures activates the Agr system and resulted in the bimodal expression of chromosomally integrated P*psmα-yfp* and P*psmβ-yfp* reporters (**Figure 45**), showing a subpopulation of cell expressing the reporters increasingly over time, similar to the situation observed in *S. aureus*.

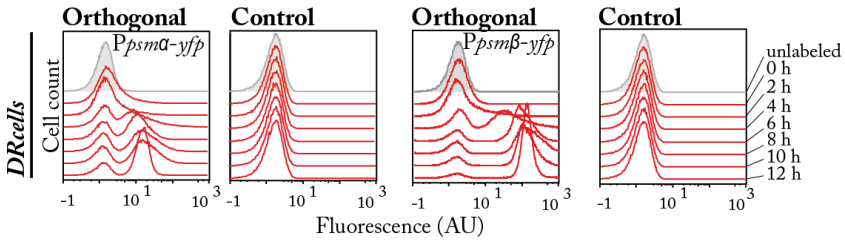

Figure 45. Flow cytometry profiles of the orthogonal system showed bimodal expression of *psmα* and *psmβ* reporters at different times after AIP induction. Control panels show the expression of reporters in *B. subtilis* without the AgrCA chimera. The addition of purified staphylococcal AIP (1 μM) and the intrinsic absence of the σ^B allow the amplificatory autoactivation of the AgrA as a PFL, generating two stable states (subpopulations) in the expression of the PSMα and PSMβ.

Similar experiments performed with transcriptional fusions of the biofilm-related reporter genes *ica* and spa did not show variation (**Figure 46**), consistent with previous publications that demonstrate that the expression of *ica* operon and *spa* gene is not directly regulated by AgrA (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a).

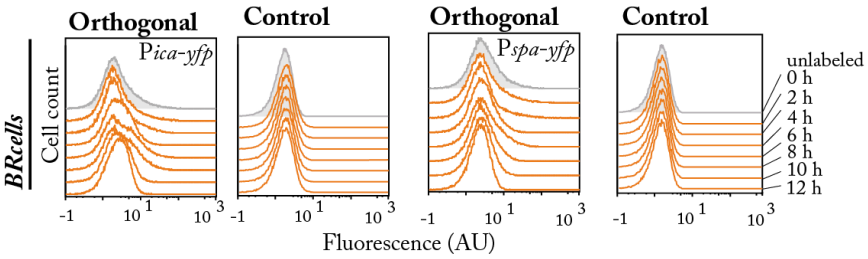

Figure 46. Flow cytometry profiles of the orthogonal system showed bimodal expression of *ica* and *spa* reporters at different times after AIP induction. Control panels show the expression of reporters in *B. subtilis* without the AgrCA chimera. There is no bimodality in the *ica* and *spa* promoters expression since they are not directly regulated by AgrA~P.

In a similar way as we showed it in **Figure 40**, we determined the heritable nature of this PFL-mediated behavior and to do so, activated *B. subtilis* cells from the orthogonal system were removed from the AIP-containing exhausted LB medium and were grown in LB medium with no AIP to investigate the stability of the activated cell types over cell division using time-lapse fluorescence microscopy (**Figure 47**). Using this approach, we observed that the bifurcated subpopulations

maintained reporter expression over more than seven cell divisions events, suggesting that the minimal Agr genetic program harbored in the orthogonal system acts as an autonomous program for differentiation and is sufficient to define heritable cell types in *S. aureus*.

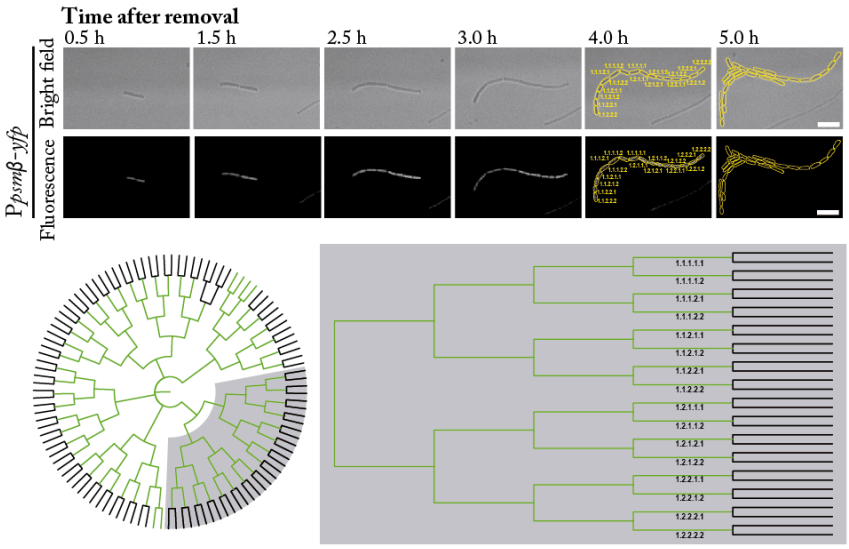

Figure 47. Expression of P*psmβ-yfp* in the orthogonal system was followed during several generations in the absence of AIP. The upper panel shows a fluorescence time-lapse microscopy assay of the outgrowth of cells expressing the P*psmβ-yfp* reporter. Lower panel shows the parsimony clustering of the expression of the reporter. Scale bar is 3 μm.

In this case, we followed the expression of P*psmβ-yfp* in the orthogonal system and, we found that the activated character of the bifurcated subpopulations was maintained during several generations, even in the absence of AIP, confirming that the PFL mechanism harbored in the Agr system is sufficient to provide activated cells with certain kind of *memory* that may lead to cell differentiation and, suggesting that the minimal Agr genetic program harbored in the orthogonal system acts as an autonomous program for differentiation and is sufficient to define heritable cell types in *S. aureus*.

To gain more insight into the molecular mechanisms that leads Agr to act as autonomous program to define cell fate in *S. aureus*, two additional orthogonal systems were created to investigate the activation of P2 (PRNAII-*yfp*) and P3 (PRNAIII-*yfp*) independently (**Figure 48**). In response to exogenous AIP (1 μM), the subpopulation of P2-expressing cells differentiated earlier and showed a more intense fluorescence signal in a larger subpopulation of cells in comparison to the orthogonal system that differentiated P3-expressing cells. These results suggest that P2 responds more sensitively than P3 to the activation of Agr, which is one of the intrinsic characteristics of PFL (Mitrophanov and Groisman 2008b; Mitrophanov and Groisman 2008a).

This system showed an 8-h transition period for cells to activate, after which subpopulation sizes remained constant. In response to exogenous AIP, however, the subpopulation of P2-expressing cells differentiated earlier and showed a more intense fluorescence signal in a larger cell subpopulation over time compared to the orthogonal system that differentiated P3-expressing cells, which suggested that

that P2 is more sensitive than P3 to Agr activation and hence, is the promoter region involved in the PFL hypersensitive autoactivation.

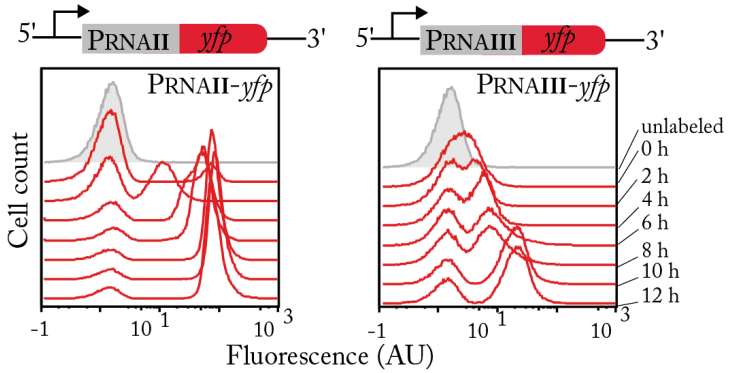

Figure 48. Flow cytometry profiles of the orthogonal system showed bimodal expression of the P2 (left panel) and P3 (right panel) promoters of the Agr system at different times after AIP induction. The addition of purified staphylococcal AIP (1 μM) generates two stable subpopulations that differ in activation time and intensity, demonstrating the hypersensitivity of the P2 (RNAII) promoter, which controls the autoactivation of the Agr PFL.

We hypothesized that the differential sensitivity of P2 and P3 promoters (**Figure 48**) to AgrA~P is the driving force that generates the Agr PFL and thus cell differentiation in *S. aureus*. Earlier P2 activation could lead to higher AIP production and the Agr regulatory cascade, which triggers the positive feedback loop (PFL) only in the cell subpopulation that activates P2 above a certain threshold. This subpopulation produces higher AgrA~P levels, which licenses them to trigger the P3 promoter and induce the Agr regulon responsible for dispersion and virulence, whereas cells that express P2 below the threshold cannot activate the Agr PFL and are thus, unable to induce P3 promoter expression. In this subpopulation, Agr-repressed genes are upregulated, which licenses them to differentiate as biofilm-producing cells.

We tested this hypothesis using a dual orthogonal system that harbored both P2 (PRNAII-*cfp*) and P3 (PRNAIII-*yfp*) reporters expressed as two adjacent transcriptional units transcribed in opposite directions, similar to the chromosomal organization that show in *S. aureus* genome (**Figure 49**). We performed flow cytometry analysis with simultaneous detection of CFP and YFP signals to quantitatively determine whether the subpopulation of P2-expressing cells becomes P3-expressing cells over time.

At 4 h after AIP induction, a subpopulation of cells activating P2 was detected. A fraction of this subpopulation activated P3 at later time points (6 h). Moreover, the subpopulation of P2- and P3-expressing cells increased over time (8 h and 10 h) to finally differentiate into a uniform subpopulation of P2- and P3-expressing cells (12 h) with a significantly small fraction of P2- and P3-inactive cells (non CFP, non YFP cells) and also, a subpopulation of cells only expressing the P2 promoter (CFP-expression non YFP cells). These results demonstrated that higher sensitivity of P2 leads to activate the PFL in a subpopulation of cells, which activate later the expression of the lower affinity promoter P3 that differentiate the subpopulation of *DRcells*.

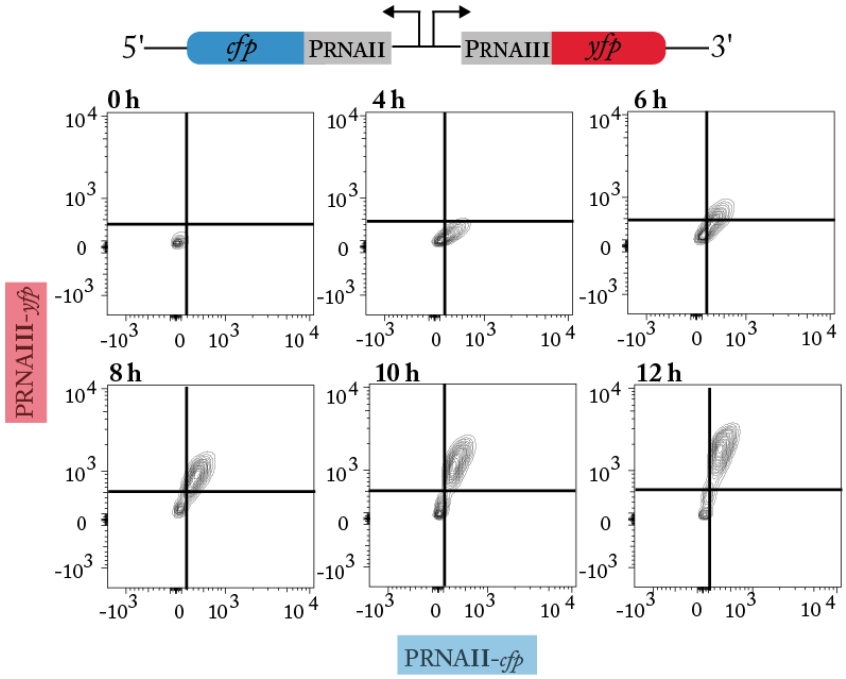

Figure 49. Flow cytometry monitoring simultaneous expression of P2 (y-axis) and P3 (x-axis) in the dual orthogonal system at different times after AIP induction. In this dual system, the P2 (CFP) promoter is activated earlier and is subsequently followed by the activation of the P3 (YFP) promoter. This dual system recreates the appearance of two subpopulation of cells: one subpopulation that activates P2 in early stages and then, P2 and P3 in later stages and, one subpopulation that remains inactivated.

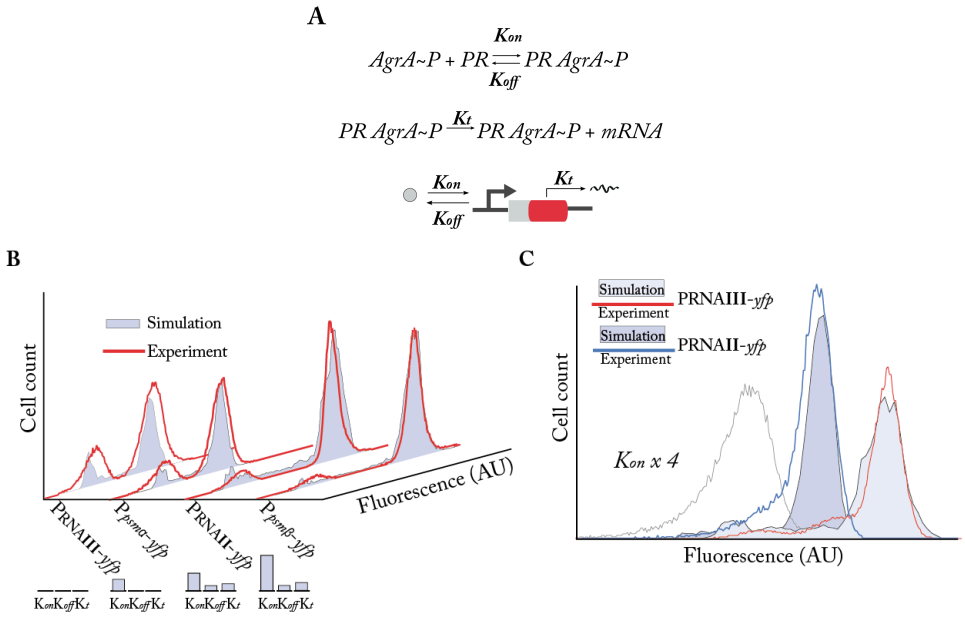

Figure 50. (A) Description of the reactions in the genetic circuitry of the orthologous system after AIP concentration has reached the activation threshold. **(B)** Computational simulation analysis of promoter expression (blue colored) in reference to experimental data (red lined) in the individual orthologous systems. Comparison of their expression profiles allowed the definition of the relative expression parameters: K_{on} (AgrA~P-promoter binding rate), K_{off} (release rate) and K_t (transcription rate). **(C)** Computational simulation analysis of promoter expression (colored) in reference to experimental data (lined) in the dual P2-P3 system showed that K_{on}, which

displayed a 4-fold increase in comparison to the expression of the same RNAII and RNAIII promoters analyzed individually.

To analyze the dynamics of the Agr PFL as an autonomous genetic program for cell fate decision-making, mathematical modeling coupled to computational simulations was performed (Golding et al. 2005; Chong et al. 2014). Mathematical simulations of the individual and dual orthogonal systems revealed the existence of a P2-activated PFL that induces P3-driven bimodal expression in response to AIP fluctuations (**Figure 50-51**) and time (**Figure 52**).

The simulations showed in **Figure 50** defined that the binding rate in the promoter region of the RNAII regulon is particularly high, defining a hypersensitive circuit that leads to the early activation of the P2 (*agr*) operon, followed by the late activation of the RNAIII, revealing that a P2-activated bimodal system induces a P3-driven bimodal expression in response to AIP fluctuations, suggesting the existence of a P2 PFL that promotes bimodality in the P2 and P3 systems. Interestingly, the dual system showed a higher intensity for the RNAIII promoter following an early activation of the P2 promoter (**Figure 51**). In addition, the mathematical analyses of the dual system showed a 4-fold increase in the binding rates for the P2 and P3 promoters, suggesting that this divergent genetic organization of the RNAII (the *agr* operon) and RNAIII might have evolved to increase the sensitivity of the P2 PFL to generate bimodality and heterogeneity in the AgrA~P response in *S. aureus*.

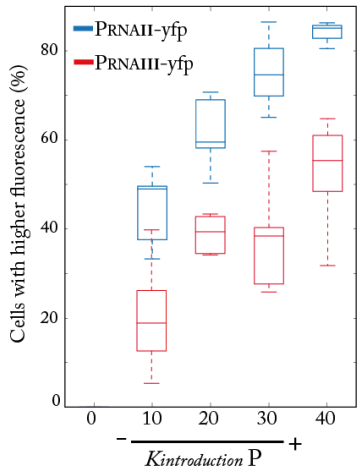

Figure 51. Mathematical simulation of the effect of AIP in the orthogonal system to induce the activation of RNAII (P2) expression (blue bars) and RNAIII (P3) expression (red bars) promoters. The maximum value of 40 represents the saturation level of AIP. Expression of the promoters is activated in response to an increase of AIP concentration (right panel) or in response to an increase in the exposure time to a defined AIP concentration (left panel).

Figure 51 shows the mathematical simulation analysis of the effect of AIP entry into the system to activate the expression of P2 (blue bars) and P3 (red bars) promoters where, the maximum value of 40 represents the saturation level of AIP. Here, the mathematical model responded to an increase in AIP concentration similarly to as an experimental increase in the exposure time to a defined extracellular

concentration of AIP, as observed in the left panel. In the simulation presented in the right panel, the expression of P_{RNAII}-*yfp* (reporter used as a proxy to monitor P2 expression) and P_{RNAIII}-*yfp* (reporter used as a proxy to monitor P3 expression) in the orthogonal system responded to an increase of the AIP entry rate into the system. The X-axis represents the different rates for AIP entry of AIP ($K_{introduction}$ P) while the Y-axis represents the activation of the Agr system via quantification of the size of the subpopulation that expresses the reporter.

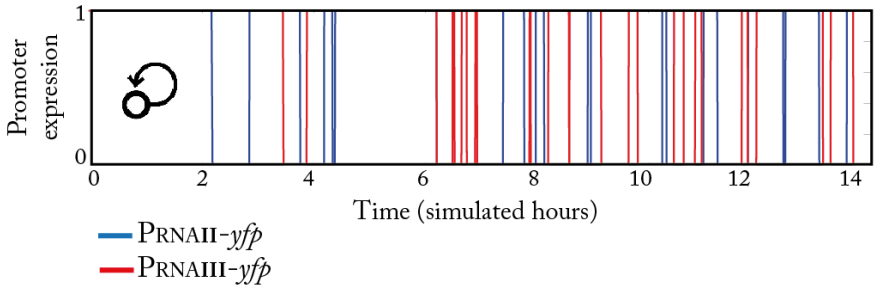

Figure 52. Temporal *bursts* of promoter expression for the P2 and P3 promoters. This mathematical modeling of P2 and P3 expression simulates the behavior of the AgrA PFL in relation to the binding efficiency of the AgrA~P. Blue lines, which represent RNAII (P2) activation appear first, followed by the appearance of red lines (RNAIII), demonstrating that the AgrA PFL acts as a retardant that controls activation of the system.

Quantitative changes in the activation state of P2 and P3 can be explained by just altering the entry rate of AIP into the system and demonstrated that, the level of AIP directly affects the concentration of AgrA~P inside the cell, which in turn, plays a key role in activation of the program for cell-fate decision-making. This particular behavior is similar to what observed experimentally in the orthogonal system when we increased the exposure time to a defined concentration of AIP, as simulated in **Figure 50**.

The temporal *bursts* of promoter expression for the P2 and P3 promoters (**Figure 53**) shows the mathematical modeling of P2 and P3 expression simulating the behavior of the PFL (as the time to become functional), in relation to the binding efficiency of the AgrA~P. This simulation shows that early activation (*bursts*) of P2 is followed by later and more frequent *bursts* of P3. The PFL acts as a retardant that controls activation of the system. Interestingly, activation of P2 occurred after approximately three hours of exposure to AIP and the number of bursts increased significantly after three hours of exposure (i.e. four to six hours), which strongly suggests the existence of a PFL. Activation of P3 relies on activation of the PFL and occurred after seven hours of exposure to AIP, once the PFL is active.

Moreover, the mathematical model was performed simultaneously in a simulated community of 20,000 cells to define the response of each cell to above-threshold AIP concentrations (saturation of AgrA~P) at a given time-point (**Figure 53** first panel). Under this condition, cells were distributed in discrete subpopulations of specialized cells with a defined abundance of P2- and P3-expressing cells. In contrast, below-threshold concentrations of AIP (i.e. null or limited AgrA~P) (**Figure 53** second and third panel, respectively) drastically reduced the probability that cells simultaneously activated P2 and

P3 and therefore, to differentiate. Importantly, these simulations revealed that most of the cells could be assigned to one of the subpopulations and also defined the flow of the decision process, suggesting that the subpopulation of P3-expressing cells resulted of the sequential activation of both promoters rather than from a non-expressing state.

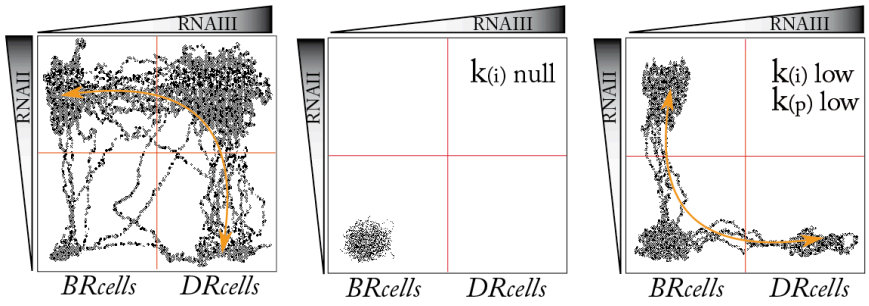

Figure 53. Mathematical simulation of P2 (y-axis) and P3 (x-axis) expression in 20,000 cells of the dual system. Grey dots represent cells. Arrows indicate the transition between states. Left panel shows saturation of AgrA~P; center panel shows absence of ~P; right panel shows low concentration of AgrA~P ($K_{(i)}$ P introduction rate, $K_{(p)}$ phosphorylation rate). The saturation of AgrA~P as the result of an autoamplificatory PFL, generates the bifurcation of the cell population in discrete subpopulations of specialized cells that express both the P2 and P3 promoters. In contrast, null or low (below-threshold) levels of AgrA~P prevents cell differentiation.

Together, these biological and mathematical-modeling experiments demonstrated by the Agr system shares all the characteristics of a PFL and, works as an autonomous bistable switch mechanism able to trigger the bifurcation of *S. aureus* cells into specialized *BRcells* and *DRcells* based on the expression level of the transcriptional regulator AgrA~P.

6. *BRcells* and *DRcells* spatially organize in the staphylococcal multicellular aggregates

Cell differentiation in *S. aureus* multicellular aggregates growing in TSBMg is heritable during cell division and, cells form groups of specialized cells within these macrocolonies. Since the development of these multicellular aggregates is completely reproducible, characterized by macroscopic and specific phenotypic features (the *origin*, the *wrinkles* and the *periphery*) and in particular, the different genetic backgrounds produce significant changes in the morphology of the community, we reasoned that cell types may display a defined spatial organization during the development of the biofilm multicellular community (Yarwood et al. 2007).

We examined whether microbial development and the heritable activity of the Agr autonomous system is influenced by the external factors we considered prominent and inherent to a microbial community, such as local concentration of AIP, activation of σ^B in response to extracellular TA-bound Mg^{2+}, nutrients and bacterial growth rates. Taking all these factors into consideration together with the phenotypic features displayed by the different genetic backgrounds, we developed a mathematical model to delineate the growth of multicellular aggregates in the form of a non-linear reaction diffusion equation system (**Figure 54**).

To understand the underlying mechanisms driving the spatial localization of specialized cell types within these multicellular communities and how, this might explain the pattern formation, we used partial differential equations in space and time (PDEs) to formulate a mathematical model for the growth dynamics of the bacterial communities. To be more precise, we use the so-called reaction diffusion system, which does not only describe the changes of concentrations and densities in time by any kind of reaction but also, spreading out in space. In our case we used a 2D model. Our system consists of four equations, describing how the concentrations of nutrients, replicative as well as non-replicative bacteria and the QS molecule AIP evolve in time. We assumed that all substances underlie diffusion and, in the case of nutrients and AIP, the process of diffusion depended on the constant diffusion parameters. In the case of the replicative bacteria, the diffusion coefficient is chosen to depend on the nutrient concentration as well as the concentration of replicative bacteria and contains a stochastic fluctuation of random movement, chosen according to the model developed by Kawasaki (Kawasaki et al. 1997). Also, we assumed that non-replicative bacteria cells are not able to diffuse by themselves and, in these bacteria diffusion might be induced by the movement of the replicative cells, establishing that the diffusion of non-replicative bacteria cells to depend on the concentration of replicative cells. Therefore, if there are more replicative bacteria around, the non-replicative cells will be more pushed forward leading also to a kind of limited diffusion and is chosen to be constant. As replicative bacteria cells proliferate by consuming the nutrients, our system of equations comprises a relation between the consumption rate of nutrients and bacterial growth, setting a conversion rate of consumed nutrients to bacterial growth based on Monod's growth term (Monod 1949), to describe the increase of the replicative bacteria concentration due to nutrient consumption: as a fact, high nutrient concentration cause a fast increase of the concentration of replicative bacteria but, that the bacteria cannot reproduce infinitely fast. Then, we multiplied this term by a factor that accounts for the effects of QS where, a high concentration/density of active bacteria is accompanied with a high concentration of the QS molecule AIP and, slows down the conversion process. We furthermore assumed that there is only a transition from active to inactive cells, according to Matsushita (Matsushita et al. 1999). Finally, the fourth equation of our system describes the concentration of AIP as an indicator of QS. Apart from the process of diffusion, we took into account that the production of AIP increased with the concentration of replicative bacteria and also, that degradation/consumption processes take place. The degradation/consumption of AIP is described by the constant, whereas the production of AIP typically has two levels (high and low) in a Hill-type function (Gustafsson et al. 2004), caused by a PFL including dimers in the underlying regulation system. There, the threshold between the low and the increased production can be denoted (J. Müller et al. 2006). In conclusion, with the terms described above and, the system of non-linear reaction diffusion equations that we developed, we achieved a mathematical representation of the growth dynamics of the *S. aureus* multicellular communities.

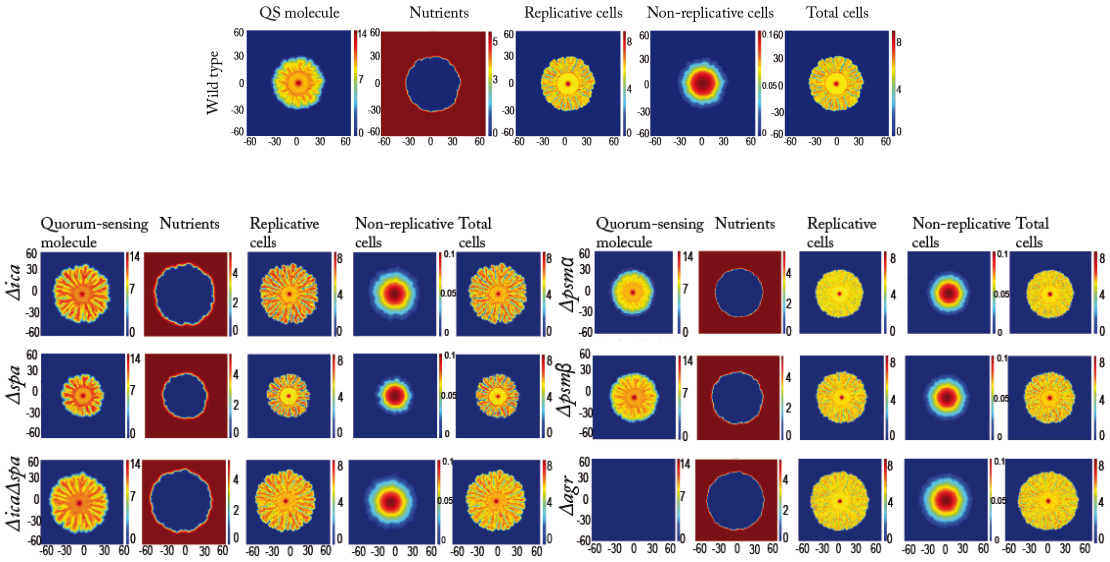

Figure 54. Spatial distribution of AIP, nutrients, replicative and non-replicative cells *in silico* multicellular communities of *S. aureus* wild type (upper panel) and different *S. aureus* genetic backgrounds (lower panels). Local concentration of AIP and nutrients control the spatial distribution of replicative and the non-replicative cells in multicellular aggregates of *S. aureus*. Mathematical modeling of *S. aureus* growth in different genetic backgrounds, in 5 day-old multicellular aggregates.

The results of the mathematical model of the 5-days development of a wild type strain, as well as of the different genetic backgrounds showed that the local concentration of AIP and nutrients control the spatial distribution of replicative and the non-replicative cells in multicellular aggregates of *S. aureus*. Here, the different properties of the microbial community that are quantified were represented in columns and their concentration shown in an arbitrary color scale. In **Figure 54,** each one of the rows represents the mathematical modeling of a different genetic background. The different properties of the microbial community that are quantified are represented in columns and their concentration is shown in a color scale. In general, the mathematical model predicted that the extracellular matrix of the multicellular aggregates controls the diffusion of the AIP molecule, generating higher concentrations of AIP in the centered and older regions of the aggregate. Therefore this mathematical model showed that the diffusion of the AIP is more efficient in matrix-deficient mutants, such as Δ*ica*, Δ*spa* and Δ*ica*Δ*spa* mutants (first column, left panels). Moreover, the mathematical model predicted that, as an aggregate grows and expands through the surface of the agar, the nutrients and also extracellular signal molecules such as Mg^{2+} exhaust in the centered older region and therefore, all center regions of the aggregates contained higher concentration of non-actively dividing cells. In addition, as the centered and older region is particularly enriched in AIP concentration (and presumably depleted of nutrients and hence, free extracellular Mg^{2+}), it also might contain a higher representation of differentiated *DRcells*. On the other hand, as the aggregate expands and moves towards the edges, nutrient (and Mg^{2+}) availability increases and cells divide actively, generating newer regions with lower concentrations of AIP and higher concentrations of Mg^{2+}, which might promote *BRcells* enrichment of specialized cells involved in the process of biofilm formation. Interestingly, PSM-deficient strains

(right panel) showed a clear decrease in the number of dividing cells (*BRcells*) compared to the matrix-deficient and wild type strains, suggesting that these small molecules might have an important structural role in the expansion and dispersion of the multicellular aggregate, as has been proposed by some authors (R. Wang et al. 2007; Periasamy et al. 2012; Schwartz, Syed, Stephenson, Rickard, and Boles 2012b; Kizaki et al. 2016; Qi et al. 2016) but even more, demonstrates the division of labor and cooperativity needed between different cell types for the correct development of the multicellular aggregate. On the other hand, matrix-deficient strains showed an increase in the numbers of dividing cells, suggesting that the physical constrain provided by the extracellular matrix is also necessary for an equilibrium within the multicellular community.

Using this mathematical modeling, we found that matrix-deficient mutants might display a more efficient diffusion of the AIP and, experimental data confirmed these predictions (**Figure 55**). We corroborated this mathematical predictions showing that the size of the subpopulation of *DRcells* were more abundant in the Δ*ica*Δ*spa* matrix-deficient strain than in WT aggregates, suggesting that indeed the presence of extracellular matrix prevented AIP diffusion and therefore, the differentiation of *DRcells*. These findings demonstrate experimentally the cooperativity that exists within this multicellular aggregates where; some biosynthetic products generated by the specialized *BRcells* have a large influence in the emergence of the antagonistic cell type, the *DRcells*.

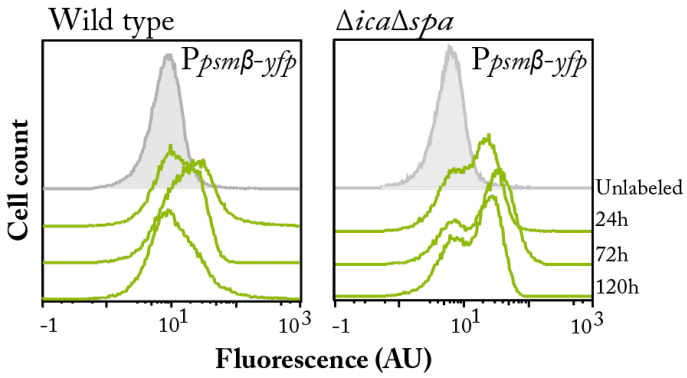

Figure 55. Flow cytometry profile of $P_{psm\beta}$-*yfp* labeled cells of *S. aureus* wild type (left panel) and Δ*ica*Δ*spa* (right panel) strains. As the mathematical model predicted it, in the absence of extracellular matrix AIP diffuses more freely, affecting a higher number of staphylococcal cells within the multicellular aggregate and therefore, increasing the number of *DRcells* within the community.

To further test this hypothesis, mature aggregates were sectioned into concentric morphologically distinct regions (**Figure 19**) and the size of the distinct *DRcells* and *BRcells* subpopulations were analyzed using flow cytometry (**Figure 56**). One particular characteristic of our model of *S. aureus* biofilm formation on the surface of TSBMg agar is that, these multicellular communities show discrete morphological areas can be isolated and analyzed independently using microscopy and flow cytometry (**Figure 19**). In **Figure 19** we showed the dissection of a 5-days old multicellular aggregate in three distinct morphological regions and separate a peripheral region (*P*), a surrounding wrinkled area (*W*) and a central older region or origin (*O*).

In agreement with our mathematical predictions, the spatial localization of the distinct cell types within the morphologically different regions of the multicellular aggregates showed that, the most peripheral region contained a higher proportion of *BRcells* representing the 40% of the total, compared to a small proportion of *DRcells* representing only the 20% of the total. On the other hand, *DRcells* were enriched in the center (*O*) of the aggregate with a 40% of the total, compared to a lower proportion of *BRcells* (**Figure 56**).

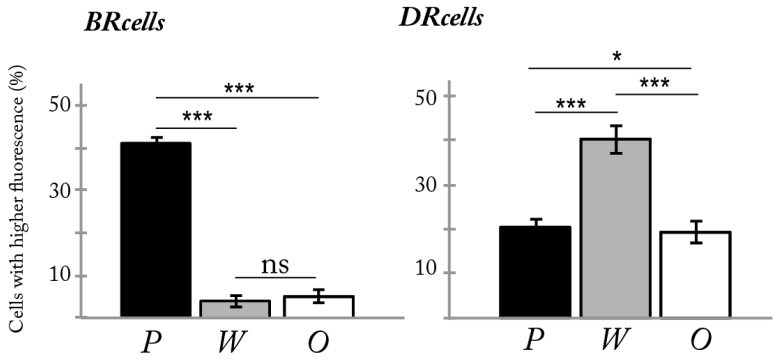

Figure 56. Quantitative analysis of flow cytometry data of *BRcells* and *DRcells* from the dissection of a 5-day multicellular aggregate in the three distinct morphological regions *peripheral* (*P*), *wrinkled* (*W*) and *origin* (*O*) independently processed. *BRcells* are preferentially enriched at the edge of the multicellular community (*P*) while; *DRcells* are predominant at the *W* wrinkled area. At the *P* and *O* areas, *DRcells* are equally misrepresented. Statistical significance was measured by unpaired, two-tailed t-test. * p<0.05, *** p<0.001, ns No significant differences.

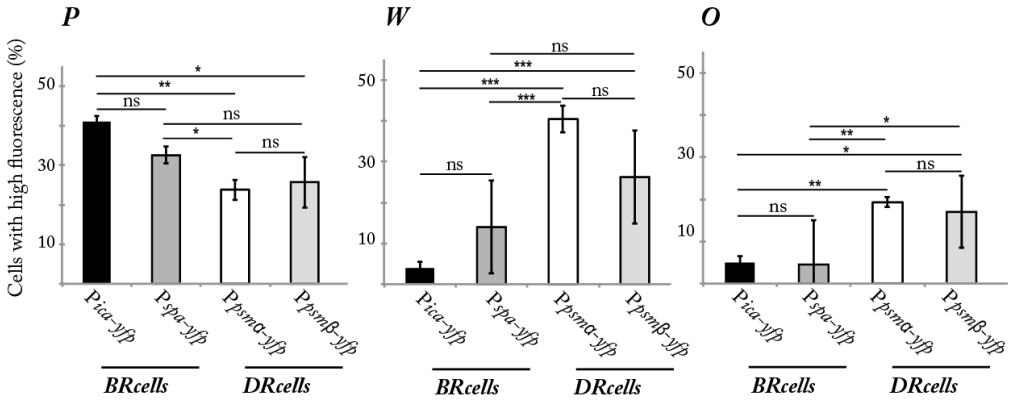

Figure 57. Flow cytometry quantification of the size of each cell type of *BRcells* and *DRcells* in the three dissected morphological regions (*P, W* and *O* areas). Fluorescence signal is represented by the percentage of fluorescent cells over the total community using an unlabeled strain as control for no fluorescence. In general, *BRcells* types (*ica* and *spa* expressing cells) are equally similarly represented in each concentric area. In a similar manner, *DRcells* types (*psmα* and *psmβ* expressing cells) do not show significant differences within the *P, W* and *O* areas. On the other hand, *BRcells* and *DRcells* were distinctly represented among areas, showing a preferential enrichment of *BRcells* in the *periphery* (*P*) and *DRcells* preferentially found in the inner regions *W* and *O*. Statistical significance was measured by unpaired, two-tailed t-test. * p<0.05, ** p<0.01, *** p<0.001, ns No significant differences.

The data presented in **Figure 56** represents consolidated data from the all the respective *BRcells* and *DRcells* reporter genes. Detailed flow cytometry quantification of the size of each cell type representing both, *BRcells* and *DRcells* type in the three dissected morphological regions, showed that the *ica* and *spa* cell types (*BRcells*) are preferentially localized in the outer *P* region while, the *psmα* and *psmβ* (*DRcells*) are predominant in the *W* and *O* regions (**Figure 57**).

These results showed that the actively growing area of the aggregate (the *P* region), is significantly enriched in both, *ica* and *spa BRcells* cell types while, the wrinkled area (the *W* region) presents a significantly higher proportion of the QS Agr-regulated *DRcells psmα* and *psmβ* cell types. Of particular interest, comparing the proportions of the different cell types found in the *P* and *W* regions with those found within the *O* region, it can be observed that this inner region is less active, confirming the predictions obtained with our mathematical model. In addition, these results suggest that the *W* region is a semi-active area characterized by a subpopulation of staphylococcal cells preferentially expressing Agr-regulated *psmα*, presumably involved in dissemination, dispersion and virulence. Also, that the *O* region is an inactive area in general but, especially regarding biofilm formation (*BRcells*). Nevertheless, the *O* region is significantly enriched in *DRcells*, as the mathematical modeling showed it. Finally, that the *P* region is the most active area for all cell types evaluated, with a significantly higher representation of *BRcells*, which are directly involved in the structural development of the multicellular community and are not directly controlled by the Agr system.

Moreover, we used a combination of cryosectioning and confocal microscopy to determine the localization and size of each subpopulation within the inner zones of thick biofilms. Remarkably, we obtained 4 μm-thick vertical cryosections of a fully developed multicellular community and, we found an extraordinary spatial organization characterized by at least the same 3 discrete areas (**Figure 58**).

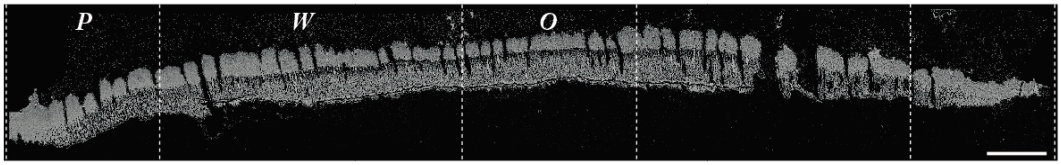

Figure 58. Longitudinal-transverse 4 μm-thick cryosection of a mature *S. aureus* aggregate. Similarly to the sectioning presented in **Figure 19**, the cryosectioning of a *S. aureus* multicellular aggregates shows at least three morphologically different concentric areas. In addition, this cryosectioning approach revealed the architectural complexity of these bacterial multicellular communities. Bar = 500 mm.

The outer *P* region starts with no evident organization of the cells but transitory, develops a more structured architecture where a lower- and mid- matrix becomes evident. In the *W* region, the lower matrix becomes more evident and acquires more density and start organizing in a second dense matrix in the upper part of the multicellular community that holds an abundant group of cells that display a characteristic *mushroom-like* shape. The *O* region is characterized by a fully developed architecture where up to 4 different regions can be observed: the lower matrix, which gives rise to a low-density area that is found along the complete structure; a high-density matrix that holds the *mushroom-like*

structures; the *mushroom-like* structures localized in the upper part of the multicellular aggregate and finally, a membrane-like structure that cover the abovementioned *mushroom-like* structures and that resemble somehow, the complex microanatomy of bacterial biofilms and the dispersion structures found in other bacterial multicellular communities (Branda et al. 2001; B. Lee et al. 2005; Goldman et al. 2006; Serra et al. 2013; Claessen et al. 2014).

Using cryosection coupled with confocal microscopy in 5-days old labeled *S. aureus* multicellular aggregates to obtain longitudinal transversal cryosections, we localized BRcells in the outer P region and, the inner regions closer to nutrient source that is found on the agar surface (Cramton et al. 2001) (**Figure 59**). Semi quantitative quantification of the fluorescent area found in the 3 different areas confirmed that the *P* region was significantly enriched in *BRcells* types, *ica* and *spa* subpopulations.

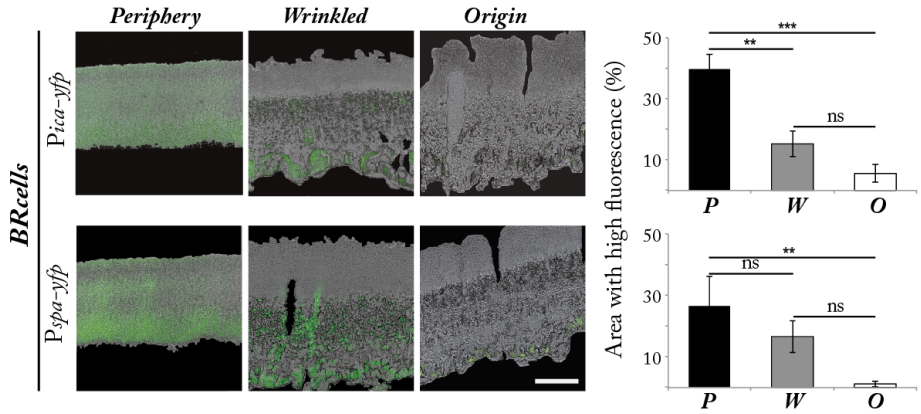

Figure 59. Spatial localization of the distinct *BRcells* types within the longitudinal plane of the microbial aggregate. Fluorescence micrographs of selected *P, W* and *O* regions. Fluorescence signal is false-colored in green. Scale bar is 100 μm. Percentages denote the proportion of fluorescent area over the total aggregate area. Graphs represent data analyses from three independent areas. Localization of the fluorescence signal is spatially confined to regions of the multicellular aggregate and preferentially localized in the *P* area. Statistical significance was measured by unpaired, two-tailed t-test. ** p<0.01, *** p<0.001, ns No significant differences.

On the contrary, *DRcell*s were localized preferentially away from the nutrient source (**Figure 60**) and particularly enriched in the W and O regions, consistent with the results obtained for the concentric regions analyzed independently (**Figure 56-57**).

These experiments showed that *BRcells* are enriched in newer regions of the biofilms while *DRcells* show enrichment in older regions of the multicellular community, suggesting that the cells respond differently to distinct local conditions since they represent two physiologically distinct cell types.

7. Global analysis of gene expression shows specialization of *BRcells* and *DRcells* cell fates

To understand the potential physiological specialization of *BRcells* and *DRcells*, their transcriptional profiles were determined using Illumina RNA-sequencing after enrichment by fluorescence-activated cell sorting. Briefly, we grew mature aggregates of cells labeled with the P*psmα-yfp* or P*ica-yfp* reporter fusions as proxy to identify the subpopulation of *DRcells* or *BRcells*, respectively. Between the 4th and

the 5th day of growing, multicellular aggregates were resuspended and disaggregated in *RNAlater* for cell fixation and, a homogenous suspension of single cells was obtained using mild sonication to proceed with the FACS sorting of the different subpopulations: cells expressing the reporter in a tube and the subpopulation of cells that did not express the reporter in a different tube. We used filtration to collect and concentrate the cells and isolated total RNA using hot phenol extraction protocol (Blomberg, Wagner, and Nordström 1990; Dugar et al. 2013) with some modification. The fluorescent and non-fluorescent cells from the mature aggregates were physically sorted/separated and simultaneously collected in individual samples.

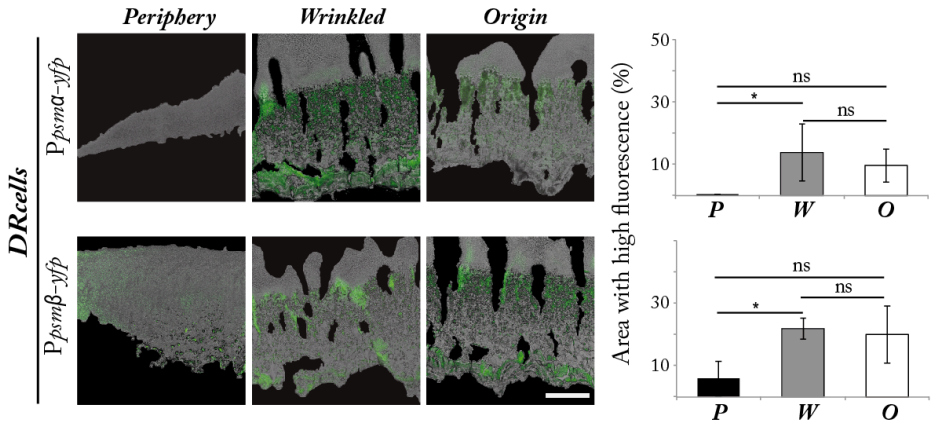

Figure 60. Spatial localization of the distinct *DRcells* types within the longitudinal plane of the microbial aggregate. Fluorescence micrographs of selected *P, W* and *O* regions. Fluorescence signal is false-colored in green. Scale bar is 100 μm. Percentages denote the proportion of fluorescent area over the total aggregate area. Graphs represent data analyses from three independent areas. Localization of the fluorescence signal is spatially confined to regions of the multicellular aggregate and preferentially localized in the *W* and *O* areas. Statistical significance was measured by unpaired, two-tailed t-test. * $p<0.05$, ns No significant differences.

In an initial approach and as a proof-of-principle, we wanted to determine if the FACS was sensitive enough to separate individual bacterial cells based on their fluorescence emission. To test this, we performed a control experiment of bacterial cell separation/sorting where, *S. aureus* wild type and non-labeled living cells from liquid cultures were mixed with fluorescence P*ica-yfp* labeled cells from liquid cultures in OD_{600} nm relation 3:1 (equivalent to a 75%-25% ratio). Indeed, FACS profiling showed the enrichment of a non-fluorescent subpopulation in the mix (**Figure 61**). Following the procedure detailed in Materials and Methods, we separated only the fluorescent cells using FACS and we analyzed this sorted sample using flow cytometry and growing the sorted cells in TSB plates, confirming that our separation protocol works and significantly enriches, reaching up to 97% of efficient enrichment, a particular cell population based on its fluorescence profile. In addition, as we used living cells for this control experiment, the FACS-sorted bacterial population was diluted (using a 10^{-6} dilution) and plated on TSB and, the resulting colonies were examined for viability and for the emission of fluorescence using a fluorescence stereoscope.

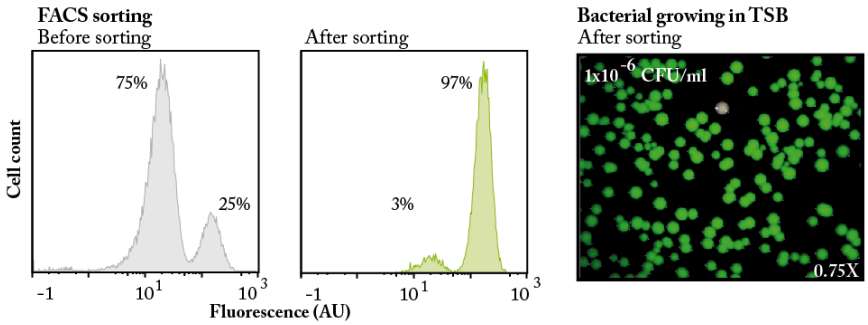

Figure 61. Experimental workflow to sort *BRcell*s and *DRcells* using Fluorescence Activated Cell Sorting (FACS) to analyze and compare transcriptomic profile. Control experiment of cell sorting. In the first panel, *S. aureus* wild type (non-fluorescence) living cells from liquid cultures were mixed with P*ica-yfp* labeled cells from liquid cultures in ratio 3:1 and analyzed using FACS. In the second panel, cytometry results after the FACS sorting. Here, the FACS-sorted bacterial population reached 97% enrichment of fluorescent cells. In the third panel, this sample was highly diluted and plated on TSB and the resulting colonies were examined for viability (growing) and for emission of fluorescence using a fluorescence stereoscope. Consistent with our flow cytometry enrichment data, around the 97% of the colonies grew, carried the transcriptional fusion and were fluorescent.

Consistent with our flow cytometry enrichment data, more than 95% of the colonies carried the transcriptional fusion and were fluorescent and also, viability of the sorted population was significantly high, demonstrating that FACS sorting might not compromise cell stability and hence, does not influence the quality of the bacterial RNA in further analysis.

Next, we evaluated if the success in bacterial cell sorting could be achieved using multicellular aggregates of *S. aureus*. A 5 day-old multicellular aggregate of P*ica-yfp* labeled strain was fixed using RNAlater, cells were dispersed and their fluorescence signal was analyzed using flow cytometry (**Figure 65**). Strikingly, cytometry analysis showed higher representation of the fluorescent reporter in the subpopulation of cells (subpopulation P1) after FACS sorting, as evidenced by the shoulder observed in the fluorescence expression profile of these cells. The FACS sorting applied to this sample led to an efficient separation of the subpopulation of fluorescence cells (P1) and the subpopulation of non-fluorescent cells (P2) in independent tubes/samples.

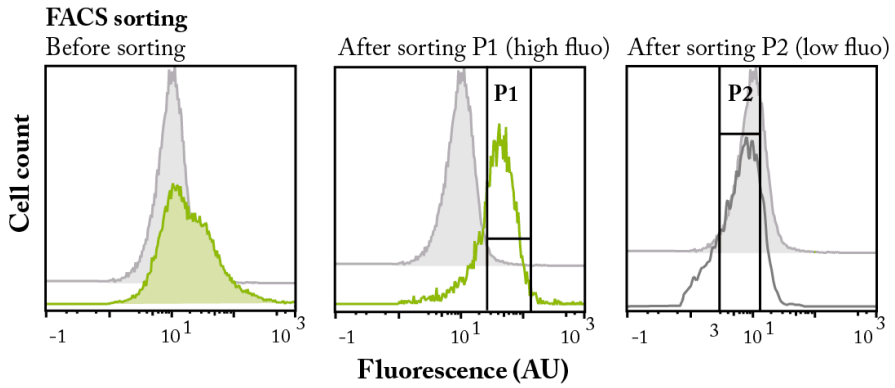

Figure 62. Experimental workflow to sort *BRcell*s and *DRcells* using Fluorescence Activated Cell Sorting (FACS) to analyze and compare transcriptomic profile. Control experiment of cell sorting. Cell sorting of multicellular

aggregates of *S. aureus*. A 5-days-old multicellular aggregate of P*ica-yfp* labeled strain was fixed using RNA*later*. Cells were dispersed and their fluorescence signal was analyzed using flow cytometry (first panel). FACS of this sample led to an efficient separation of the subpopulation of fluorescence cells (P1) and the subpopulation of non-fluorescent cells (P2) in two different tubes. Flow cytometry analyses of P1 and P2 fluorescence signal showed that most of the cells from the P1 sample were fluorescent (second panel), whereas most of the cells from the P2 sample were non-fluorescent (third panel).

Flow cytometry analyses of the fluorescence signal of P1 and P2 subpopulations showed that most of the cells that contained the P1 sample were fluorescent, whereas most of the cells that contained the P2 sample were non-fluorescent. In addition, fluorescence microscopy examination of P1 and P2 samples also showed that most of the cells that contained the P1 sample were fluorescent, whereas most of the cells that contained the P2 sample were non-fluorescent.

Together, these control experiments demonstrated that the implementation of physical separation of bacterial cell subpopulations using FACS was possible and, allowed us to perform the decisive experiments using *BRcells*-labeled and *DRcells*-labeled multicellular aggregates to physically separate *BRcells*+, *DRcells*+ and their counterparts *BRcells*- and *DRcells*- in order to isolate total RNA, perform RNAseq sequencing and analyze all their transcriptional profiles to dissect their global physiological profile.

We used a P*ica-yfp* labeled strain to separate a P1 subpopulation of fluorescent cells (*BR*+ sample) and a P2 subpopulation of non-fluorescent cells (*BR*- sample). Likewise, we used a P*psmα-yfp* labeled strain to separate a P1 subpopulation of fluorescent cells (*DR*+ sample) and a P2 subpopulation of non-fluorescent cells (*DR*- sample). We sorted approximately 25 million cells per sample prior to RNA isolation. To test the efficiency in RNA isolation and the complete DNA depletion, we performed a series of additional measurements that included PCR amplification of the *rrna*16s coding gene over the total RNA samples, which showed that samples were free of DNA contamination after treatment with DNaseI (**Figure 63**).

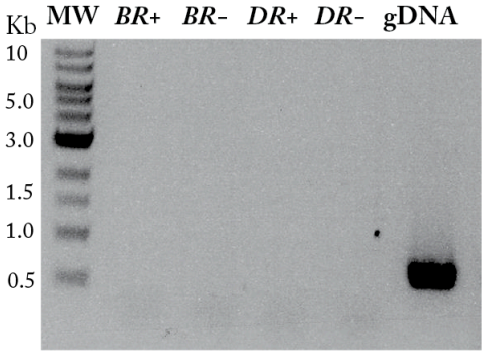

Figure 63. Experimental workflow to sort *BRcell*s and *DRcells* using Fluorescence Activated Cell Sorting (FACS) to analyze and compare transcriptomic profile. PCR analysis of total RNA samples showed that the definitive samples were free of DNA contamination after DNaseI treatment. Amplification of *rrna*16s control gene was detected only in the positive control (genomic DNA from *S. aureus*). Molecular weight = 500 bp ladder.

Quantification of total RNA concentration using spectrophotometry, which measured the RNA concentration and absorption ratios for each sample (**Figure 64A**) and, a quality check of the RNA samples using a MultiNA microchip electrophoresis to define the total RNA concentration and the quality of the RNA (**Figure 64B**).

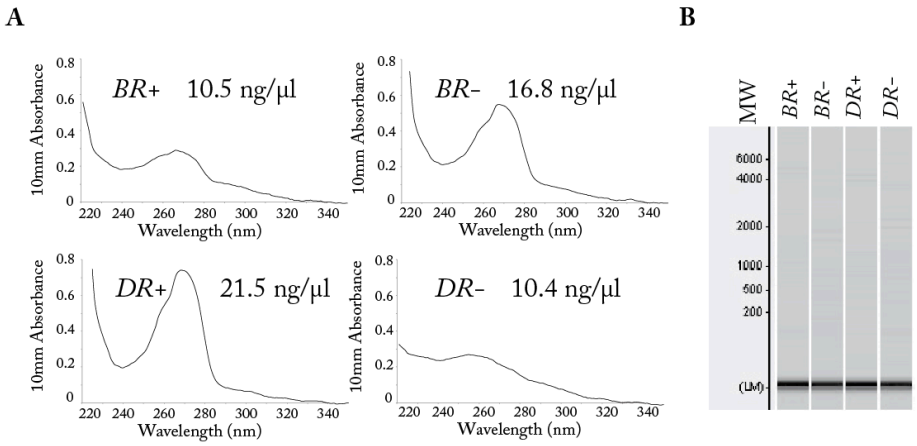

Figure 64. (A) Quantification of RNA concentration using spectrophotometry. The RNA concentration and absorption ratios for each sample were determined using the Nanodrop. Concentration is shown in the top right section of each panel. **(B)** Quality check of the RNA samples. RNA samples were examined using MultiNA microchip electrophoresis (Shimadzu) to determine quality and concentration of the RNA. After analysis, cDNA was synthesized following the protocol described in Materials and Methods. Molecular weight = 200 bp ladder.

We determined that, although in low concentration, our total RNA was enough to be used as template for cDNA synthesis. Total RNA was used to construct cDNA libraries that were sequenced using the Illumina HiSeq 2500 and the results were analyzed using diverse bioinformatics tools. The cDNA was synthesized following the protocol described in Materials and Methods and, the analysis of the PCR-amplified cDNA samples on a Shimadzu MultiNA microchip electrophoresis system showed that it was suitable for RNAseq (**Figure 65A**).

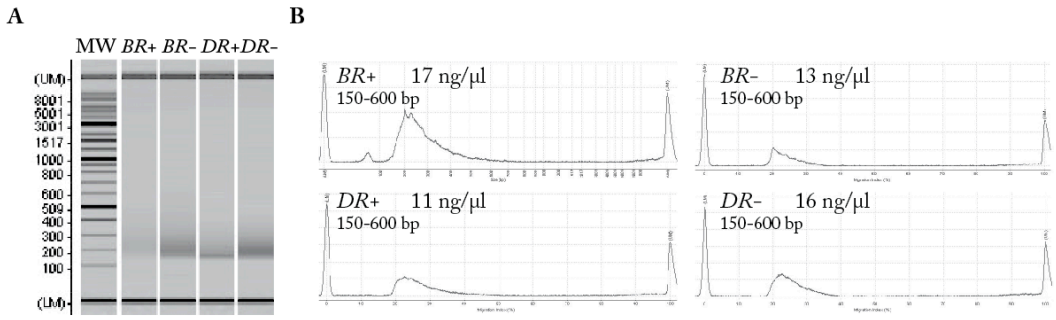

Figure 65. (A) Analysis of the PCR-amplified cDNA samples on a Shimadzu MultiNA microchip electrophoresis system. For Illumina sequencing, the cDNA was size-fractionated in the size range of 150–600 bp using a differential cleanup system. **(B)** An aliquot of each cDNA was analyzed by capillary electrophoresis. Each double-stranded cDNA sample was flanked with different adapter sequences to generate a cDNA with a combined length of 100 bases. Length range and concentration are shown at the top left section of each panel.

Next, for Illumina sequencing the cDNA was size-fractionated in the size range of 150–600 bp using a differential cleanup system and, an aliquot of each cDNA was analyzed by capillary electrophoresis (**Figure 65B**). Each double-stranded cDNA sample was flanked with different adapter sequences to generate a cDNA with a combined length of 100 bases and, the length range and concentration shown at the top left section of each panel in **Figure 65B**, confirmed that was suitable for the sequencing procedure.

After sequencing and the initial set of sequence-cleaning standard protocols, the total number of reads allowed us to map approximately 96% to the *S. aureus* total genome (**Figure 66**). The data presented in **Figure 66**, show the basic statistics of the samples showing the number of reads obtained from the sequencing procedure, the number of reads that passed the bioinformatics standard quality check and were aligned against the reference genome, the number of aligned and unaligned reads and the percentage of aligned reads. On average, we obtained 7 million reads per sample and approximately 97% of reads could were aligned to the reference genome. In addition, the distribution of the aligned reads in each library into different RNA classes based on the annotation obtained from NCBI database showed that all the different libraries shared similar distributions characterized, as expected, by a high representation of ribosomal RNA, followed by tRNA and coding genes that were subsequently used for the bioinformatic analyses.

	BRcells +	*BRcells -*	*DRcells +*	*DRcells -*
Number of input reads	5.991.727	10.158.224	5.877.599	6.031.017
Number of reads used for alignment	5.853.600	10.043.706	5.323.032	5.893.904
Total number of aligned reads	5.565.398	9.827.149	4.491.223	5.741.161
Total number of unaligned reads	288.202	216.557	831.809	152.743
Aligned reads (%)	95.08	97.84	84.37	97.41

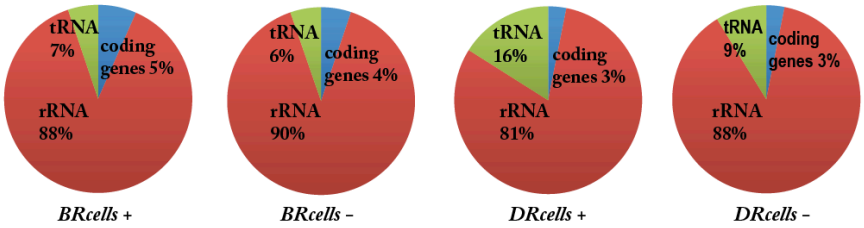

Figure 66. Read alignment statistics and distinctive transcriptomic profile of *BRcells* and *DRcells*. In the upper panel, basic statistics of the sequenced and aligned samples. Number of reads obtained from sequencing; number of reads that passed the bioinformatics standard quality check and thus were aligned against the reference genome, number of aligned and unaligned reads and the percentage of aligned reads are shown. In the lower panel, distribution of the aligned reads in each library into different RNA classes based on the annotation obtained from NCBI database.

To initially determine if the subset of sorted subpopulations displayed a different transcriptomic profile, we performed a Pearson's correlation index of the relative gene expression within different samples (**Figure 67**). The first panel showed correlation within the *BR*+ and *BR*- populations with and *r* index of 0.88. The second panel, comprising the correlation within the *DR*+ and *DR*- populations showed an r = 0.81, suggesting that the *DR*+ cell type differs more to its counterparts that the *BR*+ cell type does and finally, the third panel showed that the correlation within the *BR*+ and *DR*+ populations reached

an r = 0.76. These correlation indexes are low and hence, confirm that these cells types posses a divergent transcriptional that might reflect a different and exclusive physiological state.

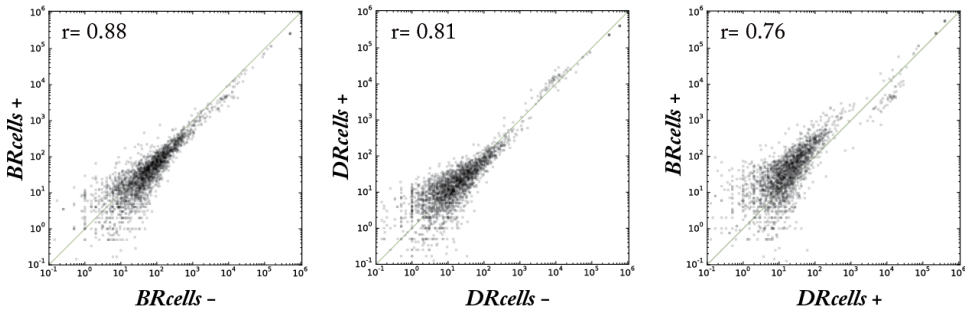

Figure 67. Pearson correlation index of the relative gene expression within *BRcells* and *DRcells*. First panel shows correlation within the *BR*+ and *BR*- populations (r = 0.88). Second panel shows the correlation within the *DR*+ and *DR*- populations (r = 0.81). The third panel shows the correlation within the *BR*+ and *DR*+ populations (r = 0.76). These results denote a different expression pattern between *BR*+ and *BR*- cells as well as between *DR*+ and *DR*- cells. These *r indexes* demonstrate a significant different expression pattern detected between *BRcells* and *DRcells*.

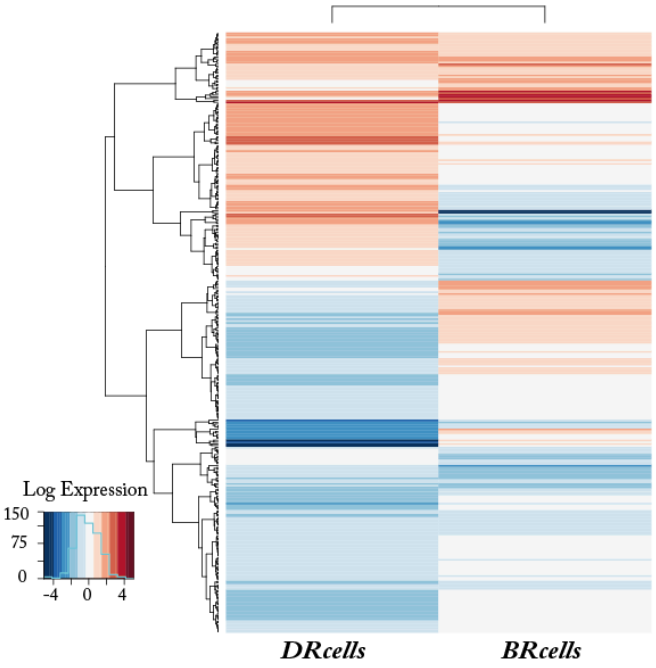

Figure 68. *BRcells* and *DRcells* subpopulations have different gene expression profiles. Unsupervised hierarchical clustering of commonly expressed genes differentially regulated (>3- or <-3-fold change) in at least one of the libraries. Clustering of commonly expressed genes differentially regulated show a divergent expression pattern between *DRcells* and *BRcells* confirming that these cell types represent a particular and exclusive physiological state. The color scales represent $\log_2$ fold-changes for differential expression.

These results suggest that the relative gene expression patterns differ within *BRcells* (comprising *BR*+ and *BR*-), within *DRcells* (comprising *DR*+ and *DR*-) and even more within *BRcells* and *DRcells*. To further explore this hypothesis, we created an unsupervised hierarchical clustering of commonly

expressed genes differentially regulated (≥3 fold or ≤-3 fold change) in at least one of the libraries (**Figure 68**).

The results obtained from this hypergeometric analysis revealed that *BRcells* have significant differences in the expression profile when we compared the set of all genes that were commonly expressed in both cell types, confirming that *BRcells* and *DRcell*s indeed have a different expression profile what signifies most likely, a particular and specific physiological state. The classification of the differentially expressed genes into functional categories (**Figure 69**) using TIGRfam, SEED and Gene Ontology (GO) functional categories, followed by manual curation, showed that each particular cell type possesses an enriched profile of functional categories, suggesting once again a particular physiological state.

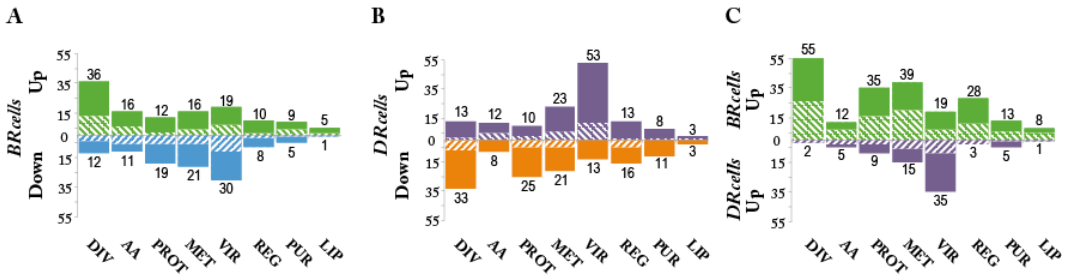

Figure 69. Functional classification of the differentially expressed genes in the four analyzed cDNA libraries, using TIGRfam, SEED and Gene Ontology (GO) functional categories, followed by manual curation. For each category, solid and dashed columns represent the number of regulated genes from DESeq analysis by using the raw read threshold of >8 and >25, respectively. **(A)** Functional categories upregulated (Up) and downregulated (Down) in the *BRcells* (*BR*+) when comparing its transcriptomic profile against the *BR*-. **(B)** Functional categories upregulated (Up) and downregulated (Down) in the *DRcells* (*CR*+) when comparing its transcriptomic profile against the *DR*-. **(C)** Functional categories upregulated (Up) in the *BRcells* (*BR*+) and *DRcells* (*DR*+) when comparing it with each other. *DIV*, DNA replication, cell envelope and cell division; *AA*, amino acid synthesis; *PROT*, protein synthesis and processing; *MET*, energy and intermediary metabolism; *VIR*, virulence, binding and transport; *REG*, regulation, transcription and signal transduction; *PUR*, purines, pyrimidines, nucleotides and nucleosides; *LIP*, lipid metabolism.

Of interest, these findings show that *BR*+ cells are preferentially enriched in cell division, protein, energy and intermediate metabolism, as well as in regulation and signal transduction, while *DR*+ cells preferentially match the categories of virulence, in agreement with their antagonistic role in chronic and acute infection outcomes. Further analyses of the transcriptomic profile of the four different subpopulations revealed that they posses a particular and distinctive gene expression profile. Genome-wide identification of the genes that are differentially expressed in the different *BRcells* and *DRcells* subpopulations demonstrated that the normalized gene expression profile of *BR*+, *DR*+, *BR*- and *DR*- subpopulations differed greatly. Gene expression analyses demonstrated a significant number of genes differentially expressed in a particular subpopulation, suggesting a specific physiological state for these cell types. In addition to the comparison of their normalized gene expression profiles at the genome-wide level, we further validated this data using qRT-PCR (**Figure 70**) revealed important differences

between the two subpopulations, confirming a specific physiological state can be attributed to each of the cell type.

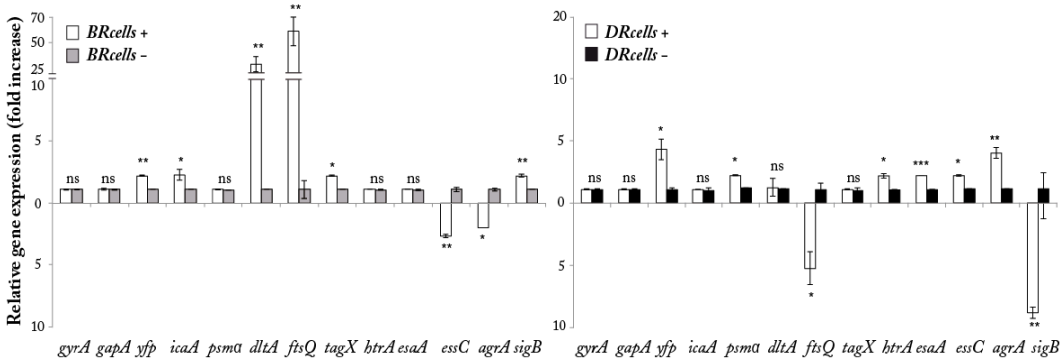

Figure 70. Quantitative qRT-PCR analysis of selected genes that showed different expression pattern in the transcriptomic data of *BRcells* and *DRcells*. Left graph shows the qRT-PCR analysis of the selected genes expressed in *BRcells*. The right graph shows the qRT-PCR analysis of selected genes expressed in *DRcells*. Expression of the *yfp* gene, which is not part of the *S. aureus* reference genome, was used as gene expression control experiment. qRT-PCR experiments follow the standard MIQE guidelines for publication of qRT-PCR experiments (Bustin et al. 2009). Statistical significance was measured by unpaired, two-tailed t-test. * $p<0.05$, ** $p<0.01$, ns No significant differences.

In particular, *BRcells* contained a high number of upregulated genes, including *sigB* sigma factor that is a repressor of *agr* expression but also, of many biofilm-related genes and genes related to peptidoglycan turnover, cell division and DNA replication, in addition to the upregulation of 49 tRNAs, which indicates a higher metabolic activity of this cell type and their physiological predisposition to cell proliferation. In contrast to *BRcells, DRcells* showed a reduced number of upregulated genes, which we attributed to a lower gene expression activity potentially due to the lower physiological activity of this cell line. Among the few upregulated genes we detected, we found a significant number of genes related to toxin secretion and host invasion, such as the type-VII secretion system (Burts et al. 2005), as well as genes related to coping with the immune system, such as *hssRS-htrAB* haemin detoxification system (Stauff, Torres, and Skaar 2007). We also detected upregulation of multi-drug efflux pumps that confer resistance to diverse antimicrobials, as well as regulators such as *graR* and *arsR*, which positively control gene related cell-wall antibiotic resistance and metal ion stress. Our results suggest that the subpopulation of *DRcells* showed lower metabolic activity and is predisposed to resist the presence of different types of antimicrobials.

8. *BRcells* and *DRcells* cell fates arise during *in vivo* infections

An important question arising from the physiological differences detected between *BRcells* and *DRcells in vitro* concerns the role and impact of these particular cell types during the progression of *S. aureus* infections. To address this question, we developed a mouse-infection model (Marincola et al. 2012; Koch et al. 2014) in which, mice were intravenously infected with 10^7 CFU of *S. aureus* strain Newman

cells wild type (unlabeled) and labeled with P*ica-yfp* or P*psmα-yfp* reporters as proxies to track *BRcells* or *DRcells*, respectively. Infections were allowed to progress during four days and mice were sacrificed and the infected organs collected. Bacterial proliferation occurred actively in kidneys, as the infected kidneys that we collected from mice showed a bacterial load of 10^8 CFU/g of tissue while, infected hearts reached only 10^4 CFU/g of tissue (**Figure 71**), demonstrating that *BRcells* and *DRcells* distribute differently in the distinct infected organs.

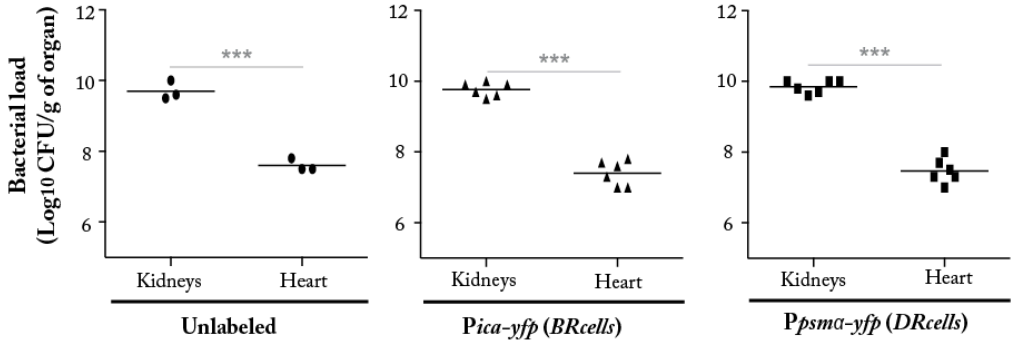

Figure 71. *BRcells* and *DRcells* distribute differently in the distinct infected organs. Bacterial loads of unlabeled, P*ica-yfp*- and P*psmα-yfp*-labeled *S. aureus* strain Newman in kidneys and hearts of infected mice. Statistical significance was measured by Mann-Whitney test. *** $p<0.001$.

Histological preparations of infected mice kidneys revealed bacterial compact aggregates surrounded by immune cells infiltrates (**Figure 72**), which are indicative of biofilm formation during septicemia (Prabhakara et al. 2011). In this histological preparation of infected kidneys stained with Giemsa solution and visualized using light microscopy, the analyzed area corresponded to a 40X and 100X magnified area of the infected kidney. Confocal microscopy analyses of these histological preparations and, a semi quantitative cell fluorescence estimation showed approximately a three-fold higher representation of *BRcells* than *DRcells* in kidneys aggregates, in agreement to what we detected in our *in vitro* experiments where *BRcells* are involved in biofilm formation, are not directly regulated by the Agr system, are preferentially found in actively growing areas of the multicellular community and, have a particular physiological profile associated with cell division and cell proliferation and also, consistent with previous reports showing that kidneys are reservoirs of Mg^{2+} of the body (Günther 2011; Jahnen-Dechent and Ketteler 2012) and 82% of the patients with urinary catheterization develop long-term *S. aureus* infections (Muder et al. 2006).

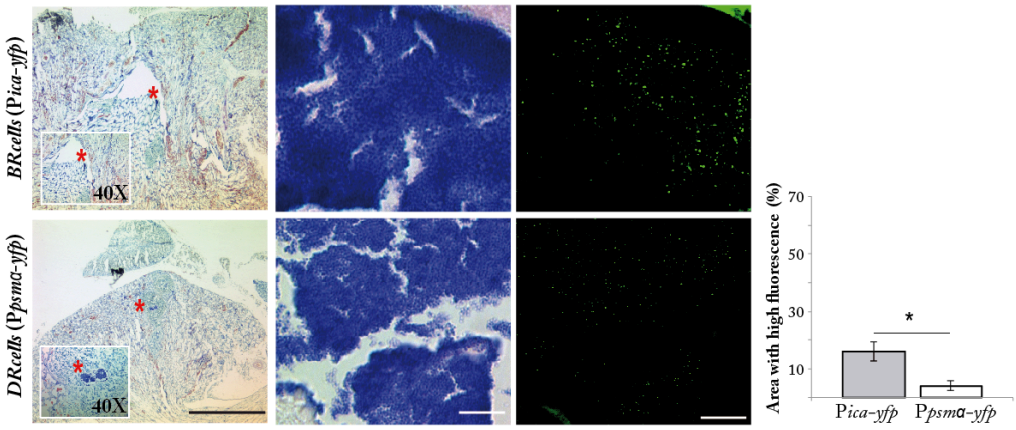

Figure 72. *BRcells* and *DRcells* distribute differently in the distinct infected organs. Histological preparations of infected kidneys stained with Giemsa solution and visualized using light microscopy. Left column, 5X magnification of the preparation. Asterisk, 40X magnification. Bar = 500 mm. Center column, 100X magnification of the area highlighted with a red asterisk. Compact aggregates of *S. aureus* cells can be seen in dark blue. Bar = 20 mm. Right column, confocal fluorescence microscopy images of the bacterial populations that colonized infected organs. Magnification 100X. Fluorescence signal in green. Bars = 20 mm. Semi-quantitative estimation of the signal in reference to bacterial population is shown as a percentage of the fluorescent area over the total bacterial area. Statistical significance was measured by unpaired, two-tailed t-test. * $p < 0.05$.

In contrast, infected hearts showed a bacterial load of 10^4 CFU/g of tissue suggesting that *S. aureus* cells that colonize heart tissues did not proliferate as actively as the cells located at the kidneys (**Figure 73**). In this histological preparation of an infected heart stained with Giemsa solution and visualized using light microscopy, the analyzed area corresponded to a 40X and 100X magnified area of the infected organ. It is likely that infected hearts promoted differentiation of *DRcells*, consistent with the lower metabolic activity and lower proliferation rate of *DRcells* that we observed *in vitro* but also and particularly important, due to the lower concentration of Mg^{2+} typically found in heart tissues (Günther 2011; Jahnen-Dechent and Ketteler 2012). Histological preparations of infected hearts revealed abundant deposits of disperse cells without immune cells infiltrates, which might be indicative of tissue invasion during acute bacteremia (McAdow et al. 2011). Further confocal microscopy and, a semi quantitative cell fluorescence estimation analysis showed that more than 60% of the total bacterial population identified in the heart tissues belonged to the *DRcells* cell type.

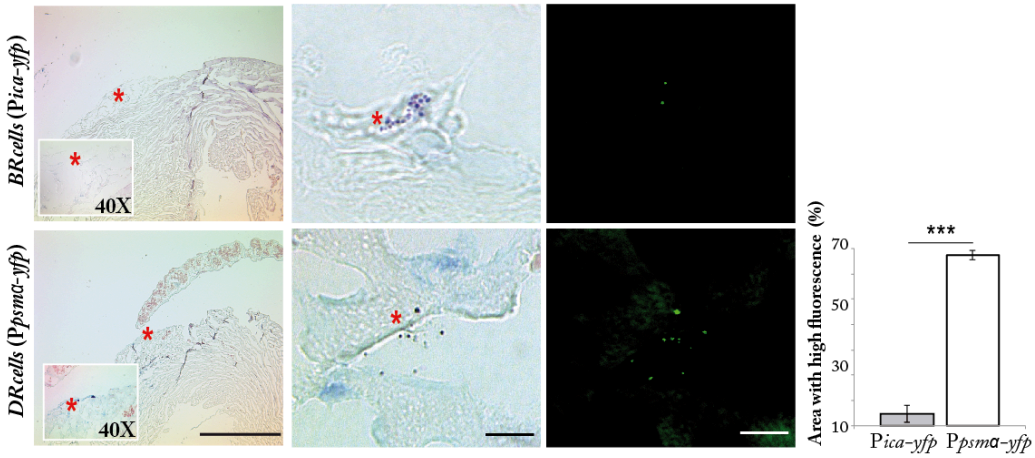

Figure 73. *BRcells* and *DRcells* distribute differently in the distinct infected organs. Histological preparations of infected hearts stained with Giemsa solution and visualized using light microscopy. Left column, 5X magnification of the preparation. Asterisk, 40X magnification. Bar = 500 mm. Center column, 100X magnification of the area highlighted with a red asterisk. In infected hearts staphylococcal cells do not aggregates and are dispersed through the infected organ. Bar = 20 mm. Right column, confocal fluorescence microscopy images of the bacterial populations that colonized infected organs. Magnification 100X. Fluorescence signal in green. Bars = 20 mm. Semi-quantitative estimation of the signal in reference to bacterial population is shown as a percentage of the fluorescent area over the total bacterial area. Statistical significance was measured by unpaired, two-tailed t-test. *** p <0.001.

To further investigate the influence of extracellular Mg^{2+} on the infection outcome, we performed additional infection studies using the suite of lower-*tagB* and higher-*tagB* strains that respectively, display a lower and higher sensitivity to extracellular Mg^{2+} as function to the concentration of TAs in their cell wall. Lower-*tagB* and higher-*tagB* strains displayed a distinctive infection pattern in different infected organs. In these experiments, infected kidneys showed a reduction in the bacterial load when infected with the lower-*tagB* strain, which is less sensitive to extracellular Mg^{2+} (**Figure 74**). However, this strain colonized heart tissues more efficiently that wild-type cells. In contrast, the higher-*tagB* strain, which is more sensitive to extracellular Mg^{2+} given the augmented amount of cell wall TAs that can actually bind more of this cation and induce a more prominent σ^B stress-related response repressing *agr* activity, showed a significantly lower colonization of heart tissue but, an increased infection of kidneys, in agreement with our hypothesis. In addition, the deletion of the *sigB* gene in the higher-*tagB* strain reverted this effect and indeed, this strain showed similar colonization patterns of the lower-*tagB* strain, confirming the role of this alternative sigma factor in the regulatory cascade that leads to cell differentiation in *S. aureus* via *agr* and drives the progression and the outcome of chronic and acute staphylococcal infections. This derivative locked-up strain that also lacks σ^B has a higher TAs content that may cause an increase in cell wall rigidity in response to Mg^{2+} but the absence of σ^B prevents the activation of biofilm formation via downregulation of Agr.

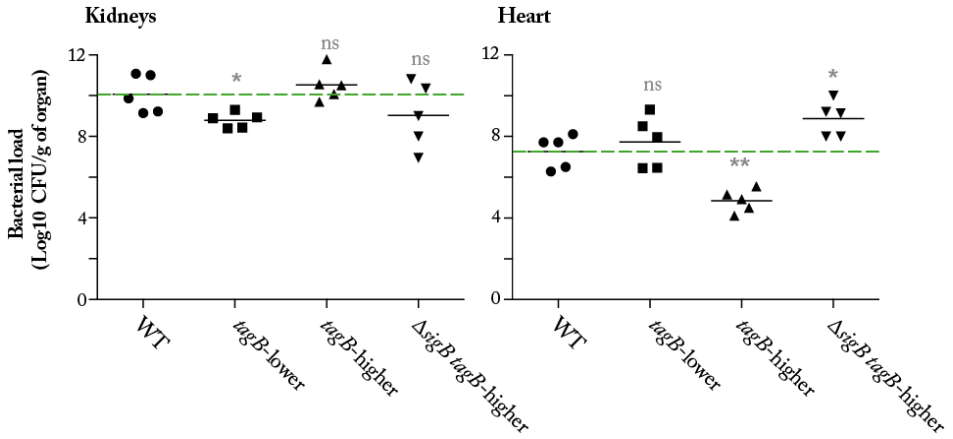

Figure 74. Lower- and higher-*tagB* strains display different infection patterns. Bacterial loads of different genetic backgrounds in kidneys and hearts of infected mice. *S. aureus* strains with a reduced synthesis of cell wall TAs displayed a significantly reduced ability to infect Mg^{2+}-enriched organs such as the kidneys while; strains with an increased synthesis of TAs displayed a significantly reduced ability to infect Mg^{2+}-depleted organs such as the hearts. The absence of σ^B decreases infectivity in Mg^{2+}-enriched organs but, increases it in Mg^{2+}-depleted organs. Statistical significance was measured by multiple comparison analysis testing using One-way ANOVA. * $p<0.05$, ** $p<0.01$, ns Not significant differences.

Additional qPCR experiments verified that these differences were associated with upregulation of key genes. In infected kidneys, we found that the lower colonization load of the lower-*tagB* and the σ^B-depleted higher-*tagB* strains was correlated with a significant increase in the expression of *DRcells*-related genes (e.g. *agrA*, *agrB* and *psmα/β*) (**Figure 75**), suggesting that infections with these two strains contained a higher representation of *DRcells*, impairing the multicellular community to establish a biofilm-related infection in this Mg^{2+}-enriched organ. By contrast, a higher-*tagB* strain that is hypersensitive to extracellular Mg^{2+}, showed an increment in the expression of *BRcells*-related genes (e.g. *icaA*, *icaB* and *spa*), which may account for its increased ability to infect kidneys more efficiently than the wild type but, its impaired colonization of heart tissue (**Figure 75**).

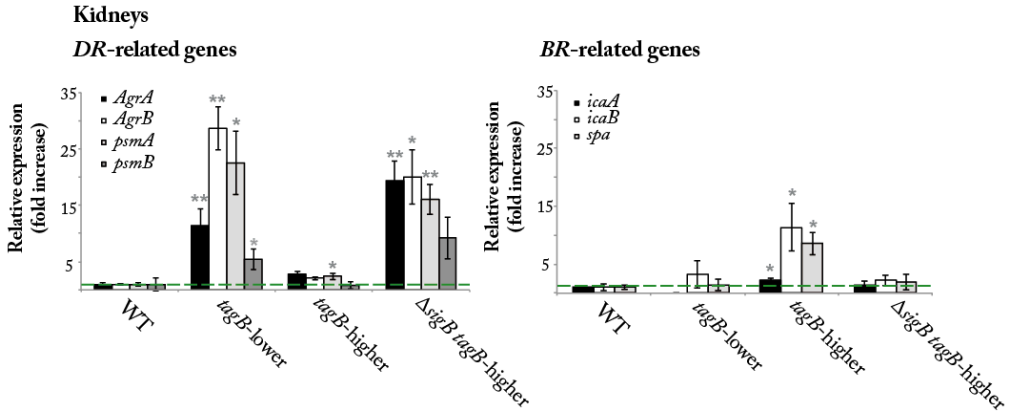

Figure 75. qRT-PCR analysis of *BRcell*-related genes and *DRcell*-related genes in kidneys of mice infected with different *S. aureus* strains that differentially synthetize cell wall TAs. The *tagB*-lower strain and the σ^B mutant, which are insensitive to extracellular Mg^{2+}, have a more pronounced expression of *DRcells*-related genes. On the contrary, the *tagB*-higher strain, which is hypersensitive to extracellular Mg^{2+}, shows a higher expression of

BRcells-related genes. Significance was measured by unpaired, two-tailed t-test. ns = not significant, * p<0.05, ** p<0.01, *** p<0.001.

Similarly, qPCR analyses experiments performed in infected hearts showed that the higher colonization load of the lower-*tagB* and the σ^B-depleted higher-*tagB* strains was correlated with a significant increase in the expression of some *DRcells*-related genes (**Figure 76**), in parallel with a downregulation of these genes in the higher-*tagB* strain, which did not colonize heart tissue as efficiently as the wild type, the lower-*tagB* or the σ^B-depleted higher-*tagB* strains. These results suggest that the higher infection load of these strains might be the result of a higher representation of virulent *DRcells*, which do not undergo the regulatory network that inactivates Agr functionality. The fact that the higher-*tagB* strain, which is hypersensitive to extracellular Mg^{2+} cannot colonize this organ efficiently, highlights the role of this cation absent in the heart tissue, as an extracellular signal driving the establishment of a biofilm-related staphylococcal infection.

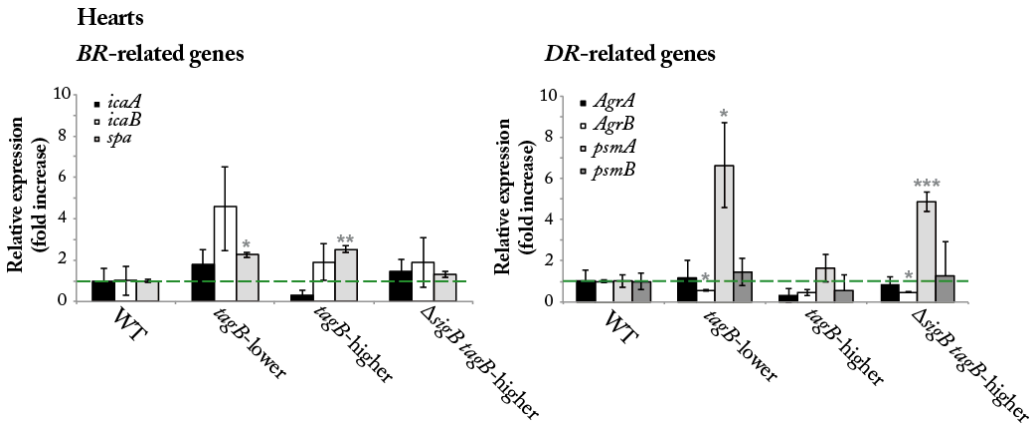

Figure 76. qRT-PCR analysis of *DRcells*-related genes and *BRcells*-related genes in hearts of mice infected with different *S. aureus* strains that differentially synthetize cell wall TAs. The *tagB*-lower strain and the σ^B mutant, which are insensitive to extracellular Mg^{2+}, have a more pronounced expression of *DRcells*-related genes, which might favor heart colonization (**Figure 74**). On the contrary, the *tagB*-higher strain, which is hypersensitive to extracellular Mg^{2+}, shows no differential expression of *DRcells*-related genes, suggesting that in Mg^{2+}-depleted organs, cell wall TAs are not particularly relevant for efficient colonization. Significance was measured by unpaired, two-tailed t-test. ns = not significant, * p<0.05, ** p<0.01, *** p<0.001, ns Not significant differences.

Infected livers, which have a moderate concentration of Mg^{2+} in their tissues (Günther 2011; Jahnen-Dechent and Ketteler 2012), also showed more comparable bacterial loads between lower-*tagB*, higher-*tagB* and the σ^B-depleted higher-*tagB* strains (**Figure 77**), demonstrating the role of this cation in the bifurcation of specialized cell lines in *S. aureus* and finally, in the outcome of a chronic or an acute infection.

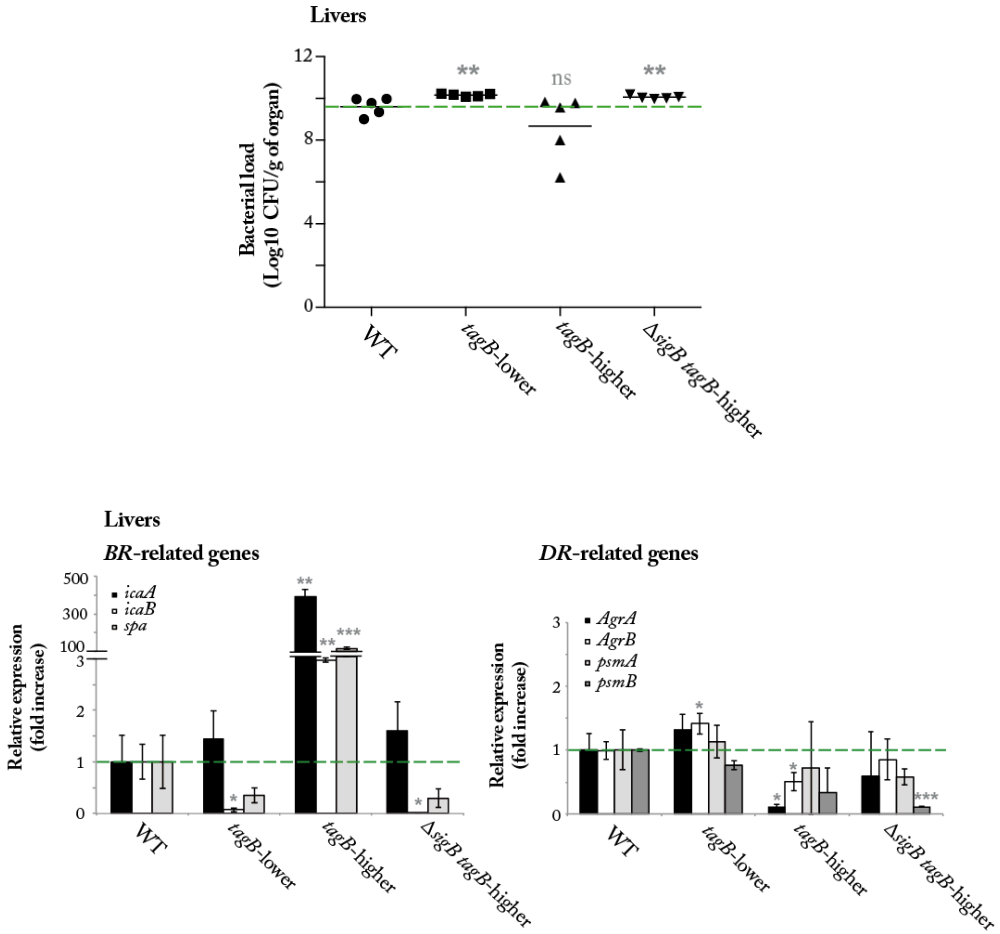

Figure 77. Upper panel, bacterial loads of different genetic backgrounds in livers of infected mice. *S. aureus* strains with a reduced synthesis of cell wall TAs or, insensitive to extracellular Mg^{2+} displayed a significantly increased ability to infect this organ while; strains with an increased synthesis of TAs displayed reduced infectivity, resembling the infection pattern for a Mg^{2+}-depleted organ such as the heart. Statistical significance was measured by multiple comparison analysis testing using One-way ANOVA. * $p<0.05$, ** $p<0.01$, ns Not significant differences. Lower panels, qRT-PCR analysis of *BRcells*-related genes and *DRcells*-related genes in livers of mice infected with different *S. aureus* strains that differentially synthetize cell wall TAs. Infected livers show the significant overexpression of biofilm-related genes for the *tagB*-higher strain together with a general decrease in the expression of *DR-related* genes, which somehow resembles the gene expression profile found in infected kidneys. Significance was measured by unpaired, two-tailed t-test. ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ns Not significant differences.

Interestingly, qPCR results of infected livers revealed a significantly increased expression of *BRcells*-related genes together with a downregulation of *DRcells*-related gene in the higher-*tagB* strain, which might account for an increased liver colonization. Surprisingly, compared to the wild type infection loads, this strain did not show any significant differences. These results seem contradictory but on the other hand, highlight the importance of extracellular signals such as the Mg^{2+} in the modulation of the gene expression and the subsequent bifurcation of specialized cell type to establish a successful infection outcome.

DISCUSSION

This work demonstrates that the human prime-pathogen *S. aureus* shows an Agr-mediated bimodal switch that results in the existence of at least two exclusive and heritable cell fates that we named *BRcells* and *DRcells,* which physiologically specialize in biofilm- (sessile) and disperse-associated (planktonic) lifestyles during the course of an infection. We studied the this phenomenon of cell differentiation at the single-cell level and in addition, we dissected the molecular mechanism by which *S. aureus* activates this bimodal switch in response to high, nevertheless physiologically relevant, concentrations of extracellular Mg^{2+}. In pathogenic bacteria, extracellular signals play a key role in the adaptation of the microorganism to diverse and fluctuating host environments and hence, are determinants in the generation of distinct and locally defined types of infections (Veening et al. 2008; López and Kolter 2010; Balaban 2011).

We found that under specific, Mg^{2+}-enriched growing conditions, *S. aureus* was able to develop a complex and robust architecture resembling *B. subtilis* biofilms (Mielich-Süss and López 2014). This complexity was directly associated with the ability to form biofilms in the traditional 24-well microtiter crystal violet assay (Peng et al. 1988; A. L. Cheung et al. 1994; O'toole and Kolter 1998; Vuong et al. 2000; Boles and Horswill 2008a; Coelho et al. 2008; Periasamy et al. 2012). In particular, the striking phenotypic changes observed in the Δ*agr* strain, as well as in the wild type strain growing in ordinary TSB, led us to hypothesize that in response to extracellular Mg^{2+}, this QS system might be involved in the arise of a developmental plan, which might involve the arising of cell heterogeneity and multicellular-level characteristics such as division of labor, as has been already reported for other bacterial species such as *B. subtilis* and *E. coli* (Vlamakis et al. 2008; López, Vlamakis, and Kolter 2009; Serra et al. 2013). Indeed, the macroscopic features shared by these species and our multicellular aggregates as well as the remarkable morphological similarities, strongly suggested that bacterial cell differentiation was the driving force behind this developmental plan. Intimately related, the Agr system has shown to play a fundamental and antagonistic role in the control of virulence and biofilm formation through QS (Recsei et al. 1986; Novick et al. 1993; Balaban and Novick 1995; Novick et al. 1995; Ji, Beavis, and Novick 1995; Yarwood and Schlievert 2003; Yarwood et al. 2004; R. Wang et al. 2007; Thoendel et al. 2011; Periasamy et al. 2012).

We considered that during the course of an infection Mg^{2+} content in an specific colonization niche might be modulating the differential activation of the Agr system, promoting the prevail of one or another cellular behavior through a concerted mechanism of cell differentiation. We used the staphylococcal Agr QS system as a rather straightforward model able to control the switch between biofilm-associated and planktonic lifestyles in *S. aureus*. In our model, Agr acts as a natural model of a genetic circuit triggered by environmental cues that allows *S. aureus* cells to collectively choose between establishing a sessile or a planktonic lifestyle and hence, permitting us to identify the cellular process underlying the emergence of one type of infection, chronic or acute, to prevail.

1. Extracellular Mg^{2+} promotes the development of architecturally complex multicellular aggregates in *S. aureus*

We based our analyses to the particular case of the *S. aureus* strain Newman (DUTHIE and LORENZ 1952), a clinical isolate derived from a long-term bone-associated infection that usually involves biofilm formation. Newman has been traditionally catalogued as poor biofilm former using the classical biofilm formation assay in TSB medium (Cue et al. 2015), which emphasizes the importance of Mg^{2+}-enriched niches, such as the bone tissue, in the development of biofilm-associated infections. The inability of Newman to form biofilm in standard laboratory conditions has been attributed to a point mutation in the SaeS histidine kinase, which generates a constitutively active SaeS (Cue et al. 2015). Replacement of the *saeS* gene in Newman with a repaired copy, generating the NewHG strain, resulted in a biofilm-proficient phenotype (Cue et al. 2015). Nevertheless, our results in TSBMg diverge from these findings, since we found that among Newman, NewHG and a Δ*saeRS* strains, the Newman strain developed the most robust aggregates and the stronger biofilms only in the presence of extracellular Mg^{2+}. These results might imply that the point mutation in *saeS* has been naturally acquired by this strain to effectively react to extracellular levels of Mg^{2+} and to develop strong biofilms in Mg^{2+}-enriched colonizing niches *in vivo*, where Newman was found to develop biofilm-associated infections (DUTHIE and LORENZ 1952). In general, environmental conditions, genes and the acquisition of mutations in these genes influence the phenotypes of organisms (Darwin 1859). Therefore, a phenotype is the result of the interaction between a set of genes and an environment (Moxon et al. 1994) and as consequence Newman, which is a poor biofilm former in the traditional TSB assays, is a robust biofilm former in solid TSBMg that might resemble a particular colonization niche. Therefore, our experimental observations in Newman, NewHG and Δ*saeRS* were consistent with the hypothesis that, a constitutively active SaeS histidine kinase facilitates *S. aureus* Newman to develop biofilms specifically, in the presence of extracellular Mg^{2+}. As the result of an altered SaeS activation, Newman would have an enhanced capacity to form a biofilms in the presence of Mg^{2+} by a mechanism that might involve, for example, the reduction in the synthesis of extracellular proteases (Beenken, Blevins, and Smeltzer 2003) but also, indirectly augmenting the presence of TAs on the cell wall, diminishing their D-alanylation or altering the expression and/or activation of the global regulators σ^B and Agr. Nevertheless, since most *S. aureus* clinical isolates retained the ability to develop robust multicellular aggregates in TSBMg, biofilm formation in Mg^{2+}-rich environments in staphylococcal species do not rely exclusively in the activation of the SaeRS and could be considered as a general attribute of *S. aureus* isolates. For the case of Newman, is might the feasible that this mutation in the *saeS* gene was fixed to provide an evolutionary advantage in its natural habitat, where it is more likely that access to Mg^{2+} is limited (Cassat and Skaar 2011; Takeda et al. 2014). Together with the intrinsic characteristics of the SaeRS TCS in signal transduction, the *downstream* regulatory pathways altered by the *saeS* mutation in Newman and the multicomponent character of the staphylococcal biofilm; the presence of a strong environmental signal such as Mg^{2+} provides an evolutionary advantage to this strain and offers a

solution to fully exploit the process of biofilm formation during the colonization of the host, through the occurrence of cell heterogeneity, cell differentiation and division of labor, as we will discuss below.

We proposed that the bimodal gene expression observe in *S. aureus* growing on solid TSBMg might be the result of the activation of the Agr PFL and its response via σ^B sigma factor to the TAs-bound extracellular Mg^{2+}. In consequence, the deletion of the Agr system in the Δ*agr* strain abolished completely the bimodality in gene expression, placing the Agr system as a fundamental genetic program involved in the emergence of cell heterogeneity in *S. aureus*. Therefore, the PFL and bimodal switch contained within the Agr QS and how this PFL is modulated in response to the presence of extracellular signals, provides this pathogen with a fundamental mechanism for the generation and maintenance of cell heterogeneity, which is of particular importance for cell differentiation and the concerted process of division of labor during the development of these multicellular-level bacterial aggregates.

The Agr bimodal switch seems to be modulated via σ^B and, it is known that in staphylococcal species σ^B represses *agr* expression (Giachino, Engelmann, and Bischoff 2001; Kullik, Giachino, and Fuchs 1998; Baker et al. 2009). In all bacteria, the big repertoire of alternative σ factors have shown to influence greatly the cellular response to basically all kinds of internal and environmental conditions by radically changing the patterns of gene expression (Haldenwang 1995; Kazmierczak, Wiedmann, and Boor 2005; Paget 2015; Helmann 2016). Interestingly, while most bacterial species have several alternative σ factors (Nyström 2003; Paget 2015; Helmann 2016) that specifically and differentially respond to different sources of stress conditions such as nutrient availability, ATP fluxes, oxygen and/or ROS gradients, temperature, physical or mechanical insults and antibiotics; in *S. aureus* only two sigma factors have shown to be consistently active and only 4 have been reported: the housekeeping sigma factor σ^A (Deora and Misra 1996), the alternative σ^B (Wu, de Lencastre, and Tomasz 1996; Deora, Tseng, and Misra 1997), σ^H (Morikawa et al. 2003) and σ^S (Shaw et al. 2008). While σ^H is a cryptic sigma factor expressed under very particular conditions and seems to be important for DNA transformation (Morikawa et al. 2012), the function of σ^S remains elusive (Shaw et al. 2008). Therefore, σ^B can be considered the only alternative sigma factor in *S. aureus* and in consequence, it has demonstrated to be activated upon a variety of sources of cellular and environmental conditions (Bischoff, Entenza, and Giachino 2001; Horsburgh et al. 2002; Valle et al. 2007; Stauff, Torres, and Skaar 2007; Mitchell et al. 2010; Mitchell et al. 2013), triggering a general stress response that includes, among others, biofilm formation (Rachid et al. 2000). In particular, it has been shown that during the induction of the staphylococcal stress regulon, the Agr system is inhibited by this alternative σ^B factor leading to a physiological switch that increases biofilm formation via Agr repression (Kullik, Giachino, and Fuchs 1998; Giachino, Engelmann, and Bischoff 2001). Then, it was feasible to postulate that extracellular Mg^{2+} was sensed as an environmental cue, activating σ^B that in turn would modulate Agr activity by repressing *agr* expression, enhancing the ability of *S. aureus* to develop these multicellular aggregates. Multicellular aggregate of the *sigB* mutant was characterized by an enlarged dissemination on the agar surface, the expected absence of staphyloxanthin pigmentation and a flatter and significantly less-wrinkled architecture (Koch et al. 2014). Surprisingly, biofilm formation of a

Δ*sigB* in ordinary TSB cultures showed a significantly increase in biofilm formation and even more, there was a no recovery in the double mutant Δ*agr*Δ*sigB* strain, suggesting a negative effect in biofilm formation of the Agr system in standard TSB cultures. These results might be contradictory, nevertheless it is known that *S. aureus* Δ*sigB* strain display increased biofilm formation under specific nutritional conditions, specifically by activating an Agr-independent pathway of biofilm formation that involves the preferential synthesis of PNAG (Valle et al. 2003; Archer et al. 2014). These findings indicate that *S. aureus* is able to respond collectively to particular extracellular signals to adapt its behavior, allowing staphylococcal communities to generate a complex and conserved pattern of multicellular development (Veening et al. 2008; López and Kolter 2010), as well as distinct and locally-defined outcome of infection when evaluated *in vivo*. Consequently, *S. aureus* responds to local concentrations of external signals such as Mg^{2+} by altering phenotypic features found in the multicellular aggregates, in a mechanism intimately related with the expression and activity of two of the main staphylococcal regulatory systems: the Agr QS system and σ^B. We conjecture that such genetic pathway evolved as an adaptive strategy in *S. aureus* to generate a multicellular-level developmental plan involving the physiological differentiation of the staphylococcal cells into specialized cell types, with the underlying collective division-of-labor process behind (Ackermann 2015). Indeed, it has been demonstrated that in most cases, extracellular signals are responsible for defining coexisting cell fates in bacterial populations (López, Vlamakis, and Kolter 2009; López et al. 2009; López and Kolter 2010).

We also evaluated the contribution of different structural gene products in the development of these multicellular aggregates and, results were consistent with previous studies that demonstrate the correlation between Agr repressed genes such as *spa*, and biofilm aggregation (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008a). We also analyzed the developmental features displayed by isogenic mutants of the Agr-controlled *psmα1-4* and *psmβ1-2* gene operons (G. Y. C. Cheung et al. 2011; R. Wang et al. 2011). PSMs were initially identified as cytolytic peptides in MRSA *S. aureus* (R. Wang et al. 2007) but also, demonstrated to be critical for biofilm formation and maturation in staphylococcal species (R. Wang et al. 2011; Periasamy et al. 2012; Schwartz, Syed, Stephenson, Rickard, and Boles 2012b). Our results suggest that during the process of biofilm formation, PSMα and PSMβ might have opposite and/or complementary roles, cooperating to regulate the process of biofilm formation and maturation. PSMs have amyloidogenic properties important during biofilm formation (R. Wang et al. 2011; Schwartz, Syed, Stephenson, Rickard, and Boles 2012b; Periasamy et al. 2012) and might be feasible that extracellular cooperativity exists between PSMα and PSMβ to modulate amyloid polymerization during the process of biofilm formation and maturation, similar to what it happens during the process of colony spreading (Kizaki et al. 2016) or during curli formation in *E. coli* where, the nucleator protein CsgB cooperates to promote the polymerization into amyloid fibers of CsgA (Hammar, Bian, and Normark 1996; Chapman et al. 2002; Barnhart and Chapman 2006; Hammer, Schmidt, and Chapman 2007; Shu et al. 2012). The contrasting phenotypes observed for the Δ*psmα* and Δ*psmβ1-2* aggregates, as well as the differences in biofilm formation in liquid TSBMg, support our idea of an active interaction of these amphipathic peptides between them and, with

additional components of the staphylococcal biofilm such as eDNA (Schwartz et al. 2015) or even parts or the cleaved *agrD* gene product, the AIP QS molecule (Schwartz et al. 2014). In addition, during the analysis of the synthetic Agr system in *B. subtilis* we found that the dynamics of expression for the *psmβ* promoter greatly differed from that for the *psmα*, supporting the idea that these two sets of peptides have different roles during biofilm formation in *S. aureus* and hence, might have a different temporal regulation independently of their direct regulation by AgrA (Queck et al. 2008). Based in our results and the existing data, we propose that PSMs might work as surfactants to promote colony spreading (Kizaki et al. 2016) during early stages of biofilm formation and in the most peripheral areas of the multicellular aggregates and later in time, facilitate the the process of biofilm maturation and disassembly (R. Wang et al. 2011; Schwartz, Syed, Stephenson, Rickard, and Boles 2012b; Periasamy et al. 2012), polymerizing into amyloid fibers composed of PSMα, PSMβ, δ-toxin, eDNA and even MSCRAMM adhesins, which all together might contribute to the structuring, maturation and dissemination of the multicellular aggregate. There, switch in roles of these PSMs based on the PSMα/PSMβ ratio might be possible at different stages of the development of the bacterial multicellular community. In the absence of the PSMα, PSMβ might not be nucleated and could not polymerize properly, giving the thicker and more robust appearance of the Δ*psmα1-4* while; in the absence of the PSMβ, the PSMα would polymerize deficiently, resulting in a more expanded, thinner and flatter Δ*psmβ1-2* multicellular aggregate.

Our model of multicellular aggregates of *S. aureus* and the phenotypic characteristics displayed by the different genetic backgrounds during the different stages of development are informative about the role of the different components for biofilm formation in *S. aureus*, possibility that is almost null in the traditional biofilm assay (O'toole and Kolter 1998). The examination of the fully developed staphylococcal multicellular communities at the macroscopic level revealed a high degree of morphological organization with the display of large-scale and well-conserved elements in structure, space and time that could be traced in their individual features by the generation of the different mutants.

2. Multicellular aggregates of *S. aureus* contain specialized cell lines

To explore the role of the Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles, we proceeded to evaluate the expression of these genes at the single-cell level within the multicellular aggregates of *S. aureus* growing in TSBMg. For every gene studied, we observed a particular temporal dynamics, which correlated with the nature of the Agr system antagonistically controlling the process of biofilm formation and virulence, and supporting our hypothesis of a QS-based switch that promotes the divergence of the *BRcells* state to a *DRcells* state by 1) means of a repression in the synthesis of biofilm structural components such as PNAG and SpA and by, 2) overcoming the biofilm-formation signal (Mg^{2+}) with the overexpression of the Agr system (AIP) and hence, of the whole staphylococcal Agr virulon. The SpA adhesion is an important component of the staphylococcal biofilm (Palmqvist et al. 2002; Procopio Evagrio George 2006; Merino et al. 2009) and, the observed abundance during the early stages of development of our

multicellular aggregates suggest a role of this adhesion protein in the initial attachment to the TSBMg surface. In addition and opposite to the expression of the *icaADBC* operon, it is known that *spa* expression is controlled by the *agr*-dependent molecule RNAIII (Huntzinger et al. 2005). Therefore, the differences found in the temporal dynamics between the *ica*- and the *spa*- expressing cells might be related to the different regulatory mechanisms that drive their expression, as well as to their role in the process of biofilm formation in *S. aureus*. The expression of the *ica* operon is not directly modulated by the Agr system but includes additional regulatory elements mainly, SarA-mediated regulation (K. M. Conlon, Humphreys, and O'Gara 2002; Cue, Lei, and Lee 2012; Tormo et al. 2005; Valle et al. 2003; Periasamy et al. 2012). Gene expression monitoring of the *psmα1-4* and *psmβ1-2* genes at the single-cell level revealed that the temporal dynamics of these types responded to cellular densities in a QS manner and therefore, to the local concentrations of AgrA~P and hence AIP as proposed by (Queck et al. 2008), incrementing their cellular representation within the multicellular aggregate as it grows and expands, reflecting the role of these versatile molecules in promoting biofilm maturation, dissemination and detachment from mature multicellular structures (N. Wang et al. 2011; Periasamy et al. 2012; Schwartz, Syed, Stephenson, Rickard, and Boles 2012b). We propose that the high initial abundance of *DRcells* might be associated to the residual activation of this cell lineage when growing in ordinary TSB to obtain the initial inoculum for the development of the multicellular aggregates. In addition, the differences found between the temporal dynamics of the *psmα*- and the *psmβ*-cell types imply that these operons are differentially regulated by the Agr system and support the results of the mathematical modeling of the orthogonal Agr system synthetically reproduced in *B. subtilis*. As we discussed above, soluble monomeric PSMα and PSMβ might contribute to biofilm disassembly and cell dissemination while, insoluble, polymeric and amyloid aggregated PSMs might contribute to biofilm formation and maturation (S. Li et al. 2013). Our results indicate that specialized cell types coexist within multicellular communities of *S. aureus* and display a bimodal expression profile. Based on deliberately-selected specific reporter genes that to our knowledge represent two antagonistic lifestyles of *S. aureus* (sessile and planktonic), all cell types analyzed behaved independently and possessed their own temporal dynamics, dictating the development and maturation of these architecturally complex aggregates This implies that, during the process of biofilm formation, gene expression in clonal communities of *S. aureus* is highly heterogeneous and *logically* regulated in a time-dependent manner. Our results allowed us to conclude that, during the development of the multicellular aggregates in TSBMg *S. aureus* cells bifurcate into two subpopulations of cells involved in biofilm-formation and in dispersion. When we further analyzed double-labeled strains with pairwise combinations of the different cell types, we found a well-conserved coexpression of these promoters in divergent lineages or cell lines that we named *BRcells* or *DRcells*. For the *BRcells* cell line, coexpression rates augment as the aggregate grows and expands. On the other hand, as *psmα* and *psmβ* expression is directly controlled by AgrA~P, their coexpression rates within the *DRcells* line is stable during the development of the aggregate. Pairwise combinations of a *BRcells* reporter with a *DRcells* reporter gene demonstrated the exclusive character of these two cell lines. Interestingly, we were able to determine that the *BRcells phenotype* comprises more than the *spa*- and *icaADBC*- expressing cells and includes additional genes

involved in adhesion and biofilm formation: particularly the adhesion protein ClfA (McDevitt et al. 1994) and the iron-regulated IsdA membrane protein (Clarke, Wiltshire, and Foster 2004) that showed to be particularly important for the development of kidney abscesses during *S. aureus* infection (Cheng et al. 2009). We selected the *icaADBC* operon and *spa* gene as reporters for biofilm formation based on the literature available, which repeatedly reported that these two structural components are predominant and common in different models of biofilm formation in *S. aureus* (Patti et al. 1994; Ziebuhr et al. 1997; Götz 2002; Merino et al. 2009) but also because these two labeled transcriptional fusions displayed the best fluorescence signal for all the experiments we planned to perform. Moreover, the coexpression pattern for *ica* and *isdA* might reflect the possibility that, as the IsdA protein is an iron-regulated protein that is preferentially expressed in iron-depleted conditions (Clarke, Wiltshire, and Foster 2004), as the aggregates grows and nutrient are consumed iron becomes even more scarce in the TSBMg medium, promoting an overrepresentation of the *isdA-expressing* cells.

The morphological development of these multicellular aggregates of *S. aureus* is the result of a particular cell fate that governs an temporally-organized process of cell differentiation, as it occurs in other bacterial species (Branda et al. 2006; Vlamakis et al. 2008; López, Vlamakis, and Kolter 2009; López and Kolter 2010). In multicellular organisms, it is well known that spatial and temporal organization of the different cell types normally respond to a threshold activation of a programmed genetic circuit, generating a heritable and stable bimodal behavior in gene expression based on gradients of diverse molecules, including environmental stimuli, autocrine and paracrine signaling molecules (Kopfová 2006; Lek et al. 2010).

We hypothesize that, as the multicellular aggregate grows and expands, differentiated staphylococcal cells localized in areas differentially exposed to the presence of extracellular Mg^{2+} and gradients AIP, the latter which drives the autoactivation of the Agr bimodal switch. In response to these signalling molecules, the Agr system modulates the bifurcation of the bacterial population into biofilm- and dissemination-related cell lines. Specifically, the response of the Agr system to Mg^{2+} is its repression (analog to an *agr* system *off*) and leads to the emergence of the *BRcells* through an indirect process where *DRcells* are not licensed to appear. On the other hand, as the aggregate grows encased in the extracellular matrix, the bacterial *quorum* increases concomitant with the concentration of AIP, leading to the emergence of the *DRcells* line (analog to an *agr* system *on*) through a phenomenon we believe, involves the overcoming of the Mg^{2+} signaling by the AIP molecule and the activation of a hypersensitive and autoamplificatory Agr system, which is indeed a typical PFL. Therefore, the temporal and spatial equilibrium reached by this bimodal mechanism results in the bifurcation of the different cell fates within the bacterial population during biofilm development.

Taking into account that Mg^{2+} and AIP are both extracellular signals that promote the bifurcation of the staphylococcal population and, that the autoactivation of the Agr bimodal switch is an autoamplificatory signaling-loop process, the gene expression pattern for *BRcells* and *DRcells* might be maintained during several generations and be transmitted (Mitrophanov and Groisman 2008b) to the progeny, even in the absence of the extracellular bifurcation-signals. To maintain a determined cell fate indefinitely, the Agr switch might be constantly *fed back* with extracellular Mg^{2+} or with above-

threshold concentrations of AIP. Our hysteresis experiments proved that, the intentional removal of the staphylococcal cells from the multicellular community that drastically disturbs those conditions, the inheritance in the expression of *BRcells*- or *DRcells*-genes was depending only on the internal genetic program determined by the Agr bimodal switch. The persistence of the specific *BRcells* or *DRcells* expression pattern was suggestive of an internal genetic heritance program responsible of transmitting the *BRcells* or *DRcells* fate to the new daughter cells.

Current techniques to track cell division in bacterial cocci species have not been fully developed thus we developed a semi-automated method using the two-dimensional data obtained from the live time-lapse fluorescence microscopy. As microscopy images offered us only two-axes of the staphylococci cell division plane, we consider that division events in these microcolonies might have been higher and therefore, the 3-D division plane of *S. aureus* might imply a bigger parsimony reconstruction superior to the observed 5 division events. This remains speculative, but it has been highlighted that imaging analysis of coccid bacteria is particularly difficult and some groups are actively working in the development of imagining techniques and automatic algorithms to define the heritability of specific traits during the division process in this kind of microorganisms that divide in three planes (Ackermann 2015). The heritability of this cell fate might account for a genetically programmed bifurcation of the staphylococcal population and in consequence, the physiological specialization of the particular cell types expressing a particular subset of genes relevant for biofilm formation or virulence, which are normally associated with the chronic and acute presentation of the *S. aureus* infections.

3. Extracellular Mg^{2+} increases cell wall rigidity in *S. aureus*

To understand the cellular process that define the bifurcation of the staphylococcal cells into *BRcells* and *DRcells*, which ultimately facilitate the development of chronic or acute infections, we explored the specific role of extracellular Mg^{2+} in the Agr-mediated antagonistic regulation of planktonic and biofilm-associated lifestyles in *S. aureus*. Mg^{2+}, although scarce and limited in the extracellular environment, is the most abundant cation within living cells and, is particularly important for several enzymatic reactions such as RNA formation, protein structuring, enzymatic catalysis and genome stability (Takeda et al. 2014). Therefore, Mg^{2+} scavenging is fundamental for bacterial physiology and, it has been largely known that in Gram-positive bacteria environmental Mg^{2+} sequestration resides mainly in the preference of the cell wall TAs to bind this metal (Archibald, Baddiley, and Heptinstall 1973; Lambert, Hancock, and Baddiley 1975b; Heckels, Archibald, and Baddiley 1975; Heckels, Lambert, and Baddiley 1977; Peschel et al. 1999; Weidenmaier, Peschel, Kempf, et al. 2005; Koprivnjak et al. 2006; Biswas et al. 2012). The fact that *B. subtilis* uses TAs-bound Mg^{2+} rather than free Mg^{2+} available in the growing media (Lambert, Hancock, and Baddiley 1975b; Lambert, Hancock, and Baddiley 1975a).

To dissect the molecular mechanism behind it, we explored the hypothesis that Mg^{2+} might be sensed by the σ^B factor, which has shown to repress Agr function (Bischoff, Entenza, and Giachino 2001) and also, to be the only sigma factor identified in *S. aureus* to respond to several stress-related environmental signals (Deora and Misra 1996; Wu, de Lencastre, and Tomasz 1996; Kullik, Giachino,

and Fuchs 1998; Gertz et al. 2000; Rachid et al. 2000; Cao et al. 2002; Horsburgh et al. 2002; Valle et al. 2007; Pané-Farré et al. 2009). The nature of the Mg^{2+}-induced stress signal was unknown nevertheless, important clues were obtained from studies performed in the 1970s that demonstrated that the cell wall of Gram-positive bacteria had the ability to preferentially bind Mg^{2+} ions via cell wall TAs (Archibald, Baddiley, and Heptinstall 1973; Heckels, Archibald, and Baddiley 1975; Heckels, Lambert, and Baddiley 1977; Neuhaus and Baddiley 2003). It is known that free Mg^{2+} preferentially binds the phosphate-rich TAs that decorate the cell wall, forming a consolidated network that alleviates the electrostatic repulsive interactions between neighboring phosphates, strengthens the cell envelope and contributes to the bacterial membrane integrity (Heptinstall, Archibald, and Baddiley 1970a; Lambert, Hancock, and Baddiley 1975b; Heckels, Lambert, and Baddiley 1977; Swoboda, Campbell, et al. 2009). Found as the major component of the cell surface in Gram-positive species (Fielder and Glaser 1973; Joo and Otto 2012; Heptinstall, Archibald, and Baddiley 1970a), TAs have been implicated in diverse roles in bacterial physiology that include, physical protection against antimicrobial peptides, host-cell adhesion and biofilm formation (Weidenmaier and Peschel 2008). In *B. subtilis*, it was found that TAs had the ability to assimilate preferentially Mg^{2+} over other divalent cations, suggesting a role for TA in the maintenance of high concentrations of Mg^{2+} near to the cytoplasmatic membrane as a source of this cofactor for enzymatic reactions (Heptinstall, Archibald, and Baddiley 1970a; Wright and Heckels 1975; Heckels, Lambert, and Baddiley 1977; Bhavsar et al. 2004). It was reported that bacterial membrane enzymes preferred Mg^{2+} cations bound to TA rather than to Mg^{2+} supplied in solution (Heckels, Lambert, and Baddiley 1977), which may help to understand why high Mg^{2+} concentrations are needed to generate multicellular aggregates *in vitro* and to develop chronic biofilm-related infection *in vivo* in organs as bones and kidneys, which have high Mg^{2+} concentrations (40 and 60 mM, respectively) (Elin 2010; Günther 2011; Jahnen-Dechent and Ketteler 2012); the concentration of free Mg^{2+} might be not equivalent to the concentration of $MgCl_2$ normally hyperhydrated and hence, significantly lower than the original 100 mM found in TSBMg. Apart of binding Mg^{2+}, many additional roles have been found for TAs (Neuhaus and Baddiley 2003; Koprivnjak et al. 2008; Weidenmaier and Peschel 2008; Campbell et al. 2012) and of special interest, it was found that TAs were essential for the establishment of *S. aureus* in the anterior nares using a cotton rat model of *in vivo* colonization (Weidenmaier et al. 2004; Weidenmaier, Peschel, Kempf, et al. 2005). Not only TAs are important by their selves, the D-alanylation of these polymers are also crucial for staphylococcal physiology (Neuhaus and Baddiley 2003). Deletion of any of the *dlt* operon genes results in a reduced growing *in vitro*, increased susceptibility to antimicrobial peptides and reduced biofilm formation (Peschel et al. 1999; Peschel et al. 2000; Gross et al. 2001; Santa Maria et al. 2014). The absence of D-alanine in the TAs significantly altered the bacterial cell wall charge, enhancing autolysis and increasing susceptibility to antibiotics. Interestingly, all these effects were suppressed by the simultaneous addition of Mg^{2+} (Wecke, Madela, and Fischer 1997) and indeed, TAs lacking D-alanine esters bound up to 60% more Mg^{2+} (Heptinstall, Archibald, and Baddiley 1970a; Swoboda, Campbell, et al. 2009), which strongly suggest that in our case, the absence of D-alanine esters in the *S. aureus* Δ*dltA* strain might promote augmented Mg^{2+} binding and hence, the clear recovery of the wild

type phenotypes. Some authors have proposed that in *S. aureus*, regulated D-alanylation might provide a mechanism of cation scavenging for intracellular metabolic pathways, regulating the expression of the *dlt* operon in function of the extracellular concentration of this cation (Koprivnjak et al. 2006). In addition, D-alanylation is fundamental in the stabilization of the cell wall TAs and thus, deeply influences the physiology of Gram-positive bacteria (Perego et al. 1995; Wecke, Perego, and Fischer 1996; Peschel et al. 1999; Neuhaus and Baddiley 2003; Koprivnjak et al. 2006; Kovacs et al. 2006; Walter et al. 2007; Sonenshein and McBride 2011; Saar-Dover et al. 2012).

Therefore, we hypothesized that extracellular Mg^{2+} was bound by the staphylococcal TAs, changing the cell wall properties of *S. aureus* cells that in turn might be sensed by σ^B to be activated and finally, repressing the activation of the *agr* system to promote biofilm formation. Our results demonstrated that the presence of Mg^{2+} in the TSBMg increased the expression and activity of σ^B activity as the result of its binding to the cell wall TAs, showing a direct correlation between Mg^{2+}-binding to the TAs, σ^B expression and activity and biofilm formation. Together, these results strongly suggested that the signaling cascade acts upon *agr* downregulation via activation of σ^B, consistent with previous publications showing that σ^B is a stress sigma factor that downregulates *agr* (Kullik, Giachino, and Fuchs 1998; Bischoff, Entenza, and Giachino 2001) and *agr* downregulates biofilm-related genes (Recsei et al. 1986; Boles and Horswill 2008a). Using different genetic backgrounds and experimental approaches, we demonstrated that an increased amount of TAs decorating the cell wall of *S. aureus* changes its rigidity and, similar to the effect of extracellular Mg^{2+} binding these TAs activates σ^B, promoting biofilm formation. Binding of extracellular Mg^{2+} to the TA, presumably reduces the repulsive net charge of the highly negatively charged TA as the D-alanylation does (Lambert, Hancock, and Baddiley 1975b; Heckels, Lambert, and Baddiley 1977; Peschel et al. 1999; Peschel et al. 2000; Weidenmaier, Peschel, Xiong, et al. 2005; Kovacs et al. 2006) and as a result, the physical characteristics of the staphylococcal cell wall change, serving as an extracellular signal for the activation of σ^B. It is known that the Dlt protein machinery decorates TA with D-alanine esters to reduce repulsive interactions between TA negative charges, which also increase cell wall rigidity (Perego et al. 1995). Supplementation of ordinary TSB with Mg^{2+} had an extraordinary effect in the Δ*dltA* strain. The simple addition of Mg^{2+} to the medium recovered to the wild type levels the growing, σ^B expression, staphyloxanthin production and more importantly, the ability to form strong biofilms and to develop a robust and architecturally complex multicellular aggregate. These results undoubtedly and strongly demonstrated that the effect of extracellular Mg^{2+} was specifically associated with its binding to the TAs, supplying a similar role of the D-alanine esters.

It has been proposed that, depending on the bacterial species and the nature of the cue, biofilm formation is induced as a response to different sources of intracellular or environmental stress (Rachid et al. 2000; Knobloch, Bartscht, and Sabottke 2001; Knobloch et al. 2004; Frees et al. 2004; Pamp et al. 2006; Rode et al. 2007; Dubrac et al. 2007; Qamar and Golemi-Kotra 2012; Islam et al. 2014). According to our hypothesis, in *S. aureus* Mg^{2+} stabilizes the cell wall and provoking an increase in the rigidity, which is sensed by σ^B to activate the stress response that downregulates *agr* and hence, induces the development of the multicellular aggregates. The absence of D-alanine esters in the TAs causes

significant alterations of the net charge of the cell envelope, leading to several physical defects on the cell wall by the deregulation of electrostatic interactions and, the downstream deregulation in the activity of cell surface enzymes. On the other hand, in the presence of elevated levels of Mg^{2+} the staphylococcal cell wall acquires an unusual increase in rigidity that serves as an extracellular cue to promote *sigB* expression and the activation of the stress regulon that includes the downregulation of the *agr*. Indeed, it has been shown that the removal of D-alanyl esters from TAs caused a significant increase in cell size (Ou and Marquis 1970), providing evidence that TAs content as well as D-alanylation of them determines the rigidity of the cell wall of *S. aureus*, allowing cells to contract or expand more or less freely (Neuhaus and Baddiley 2003). Accordingly, it has been proposed that TAs provide to the cell wall of Gram-positive bacteria the inherent ability to swell or shrink in response to environmental factors and thus, activate stress-response mechanisms (R. E. Marquis, Mayzel, and Carstensen 1976; Thwaites and Surana 1991; Doyle and Marquis 1994; Neuhaus and Baddiley 2003). In addition, *S. aureus* functional mutants in the *tagB*, *tagD* and *tagI* genes, which retained some minor level of residual activity, showed dramatic alterations in their TA abundance and polymer length, together with stress-sensitive phenotypes (H. Wang et al. 2013), suggesting a feasible connection between the ability of TAs to modulate cell wall rigidity by means of their Mg^{2+} binding ability, the activation of σ^B and the activation of the stress regulon or *stressome*. Complementing our findings, it was demonstrated that destabilization of the cell wall in *S. aureus* activated the stress response regulon (Utaida 2003; Muthaiyan et al. 2008; Mlynek et al. 2016) and in particular, disruption of the TAs synthesis leaded to the activation of the stress-response in parallel with a significant reduction in the expression of virulence genes (Campbell et al. 2012). Indeed, surface changes associated with loss of TA in a *tagO*-deficient *S. aureus* strain activated the alternative factor σ^B, which results in an increased *icaADBC* transcription and higher biofilm formation (Knobloch et al. 2004). Consequently, colonization niches with higher Mg^{2+}, such as bones and kidneys, might show higher propensity to develop biofilm-associated infections while colonization niches with lower concentration of Mg^{2+} may promote faster colonization and acute sepsis. Consistently, it is known that *S. aureus* cells that are deficient in TAs also exhibit poor adhesion and poor biofilm formation capabilities (Vergara-Irigaray et al. 2008) and consequently, might explain why these mutants fail to colonize nasal tissues (Weidenmaier et al. 2004) or endothelial tissues derived from kidneys (Weidenmaier, Peschel, Xiong, et al. 2005). Although, it is most likely that different extracellular cues are responsible to define the outcome of *S. aureus* infections, the mere presence of extracellular Mg^{2+} and hence, the increase in cell wall rigidity represents an important cue to trigger biofilm formation and possibly develop staphylococcal biofilm-associated infections.

4. The staphylococcal Agr QS system is an autonomous program for cell fate decision-making

The inactivation of the Agr promotes the emergence of the *BRcells* line, specialized in the expression of biofilm-related genes. Then, during the development of the multicellular community the

bimodal, autoregulatory and autoamplificatory nature of the Agr system license it to self activate when the extracellular concentration of AIP autoinducing molecule overcome certain threshold, generating the emergence the *DRcells* line, specialized in the expression of dissemination-related genes, which contribute to the maturation and disassembly of the staphylococcal biofilm. The temporal and spatial balance reached by the Agr system in response to extracellular Mg^{2+} and AIP gradients determines its bimodal-switch behavior and hence, the bifurcation of the staphylococcal population into *BRcells* and *DRcells*, which display particular and exclusive temporal and spatial dynamics within the architecturally complex multicellular aggregate.

We explored the genetic architecture of the Agr system, based on the binding of AgrA~P to the divergent P2 and P3 promoters that constitute a PFL in which, if the P2 is slightly activated in a particular cell after certain threshold level of AgrA~P is reached, the Agr system itself activates an autoamplificatory loop as well as the expression of a repertoire of genes directly controlled by this transcriptional factor, provoking the emergence of the *DRcells* phenotype. Once a cell differentiates into *DRcells*, undergoes the upregulation of the RNAIII transcript that in turn modulates a pool of *agr*-dependent genes coding for the cytotoxic toxins and virulence factors responsible for dissemination, dispersion of biofilm and *in vivo,* for an acute infection (Koenig et al. 2004). In contrast, any *BRcells* that cannot reach the AIP threshold level, will remain inactive for that specific response (Losick and Desplan 2008). Based on this proposed switch that generates *BRcells* and *DRcells in vitro*, we analyzed some of the principal characteristics of the *agr* signal transduction pathway to define its bimodal nature and, to determine whether is responsible for cell differentiation in staphylococcal multicellular communities, acting as an autonomous program for cell-fate decision-making.

Our isolated system is depleted of the staphylococcal σ^B, ensuring no inhibition of the Agr system. Although, the known σ^B-dependent promoters in *S. aureus* are similar to the consensus sequence in *B. subtilis*: GTTT(A/T)(A/T)-$_{NNNNNNNNNNN(NNN)}$-GGG(A/T)A(A/T) for the -35 and -10 regions, respectively (Bischoff et al. 2004); no consensus sequence is found within the promoter region of the *agr* and indeed, it has been proposed that the repression of *agr* by σ^B could be indirect (Bischoff, Entenza, and Giachino 2001; Horsburgh et al. 2002; Bischoff et al. 2004). As σ^B is upstream the *agr* regulatory, its exemption guarantees no influence over the engineered artificial *agr* system. Our experiments demonstrated that, when no orthogonal *agr* system (the AgrCA chimera) was present in *B. subtilis*, the different strains labeled with P*psmα-yfp* and P*psmβ-yfp* fluorescent reporters showed no bimodality even after adding AIP, confirming that the stochastic expression of the Agr system does not account for the emergence of bimodality and in consequence, that is the triggering of the PFL upon AIP priming, the responsible for the bimodal bifurcation of the staphylococcal population. Similar experiments, where we analyzed the expression of chromosomally integrated biofilm-related reporter genes P*ica-yfp* and P*spa-yfp* did not show bimodality, consistent with previous publications that demonstrate that the expression of genes such as the *ica* operon and *spa* gene are not directly regulated by AgrA (Recsei et al. 1986; Peng et al. 1988; Boles and Horswill 2008b) and hence, are not influenced by the activation of the Agr amplificatory loop. In this context, in the natural host the Agr system might determine the appearance of the *DRcells* once those cells reach the threshold levels given by the

gradients of AIP while, cells with an Agr system repressed, might respond primarily to the high extracellular concentrations of Mg^{2+}.

One particular advantage derived from the construction of this orthogonal system was the possibility to recreate the complete genetic circuit to determine and quantify the basic dynamic characteristics of this molecular system and, to define quantitatively how the Agr can successfully generate cell differentiation in *S. aureus*. The comparison between the orthogonal systems that resemble the activation of the P2 (PRNAII-*yfp*) and P3 (PRNAIII-*yfp*) promoters in response to exogenous AIP, permitted us to demonstrate that the dynamics of the Agr-system activation is guided by a early and strong activation of the a subpopulation of cells that express the *agr* operon via P2, generating an stable autoamplificatory loop of AgrA expression. The generation of bistability by PFL is characterized by an abrupt transition from a non-active to an active steady state (Raj and van Oudenaarden 2008), as we observed for the emergence of clear bimodality in our synthetic orthologous system. Activation dynamics of P2 and P3 suggest that the staphylococcal Agr system might have evolved to generate a sensitive genetic circuit (the P2 *agr* operon) prone to react rapidly to environmental cues such as increased AIP levels as a autoamplificatory PFL. The bimodality observed for the P2 and P3 systems is in agreement with our hypothesis that the Agr system constitutes a bimodal switch: the activation of the P2 never affects the whole bacterial population. Additionally, only the subpopulation of P2-activated cells, which produce higher levels of AgrA~P, would be licensed to subsequently trigger P3 (RNAIII) expression. The analysis of the P3 expression revealed that in comparison to P2, the expression guided by this promoter is weaker and appears at later time points. The differences in the expression rates found between the P2 and P3 promoters reinforce our hypothesis that the P2 operon constitutes indeed a PFL but also, allow us to conjecture that the P3 might have evolved adjacent to the P2 PFL in order to generate a transitional state where a sequential activation of P2 and P3 is necessary for the generation of an stable process of cell differentiation, setting a temporal switch control in the production of all the gene products responsible for the phenotypic and physiological characterization of the *DRcells* cell lineage. In agreement, it has been proposed that the primary and unique function of the P3 gene product is the upregulation of exotoxins and other virulence factors, the downregulation of MSCRAMM adhesion surface proteins, as well as the regulation other pleiotropic regulators such as Rot, SarT and SarS (Novick et al. 1993; Saravia-Otten, Müller, and ARVIDSON 1997; Novick 2003b; Novick and Geisinger 2008), most of them intimately associated with the regulation of the process of biofilm development.

The P2-regulated gene product AgrA has been found to modulate the expression of several genes, including those involved in carbohydrate and amino acids metabolism as well as genes involved in the synthesis of staphyloxanthin (R. Wang et al. 2007; Queck et al. 2008; Periasamy et al. 2012). Together, the temporally concerted gene regulation exerted by the P2 and P3 systems might guarantee the correct spatial and temporal emergence of the *DRcells* lineage: first, the activation of the P2-PFL might promote a reduction of the general metabolism and the switch to P3 expression, switching the *BRcells* lineage off, allowing the multicellular community to cope with ascending cell densities and then, generating the subsequent emergence of the physiologically differentiated *DRcells* lineage. In this

context, our results are in agreement with the statement that "*the RNAIII regulation of virulence-related genes emerged evolutionary later than the primary function of the Agr in the control of QS. Then and later in evolution, the addition of the regulatory RNAIII to the P3 transcript (that originally could had been for the embedded hld gene) created a new regulatory function for the Agr system in a host-pathogen scenario, optimizing the properties of S. aureus as a pathogen"* (Queck et al. 2008; Sittka and Vogel 2008), making feasible that the P2-P3 genetic architecture emerged as an efficient temporal switch to guarantee that only P2 activated cells are licensed to activate the P3 expression and become stable *DRcells*. The precise mechanism governing the preferential binding of AgrA~P to the P2 or P3 promoters might rely on the external level of AIP QS molecule, local intracellular concentration of AgrA~P, its phosphorylation level, differential polymerization, the strength of the binding to the promoter DNA sequence and the presence of other regulatory proteins like SarA and SarR (Novick et al. 1995; Reyes et al. 2011). It has been largely known that apart of AgrA~P, the transcriptional regulator SarA also binds to the Agr IGR, with differential ratios of AgrA~P/SarA depending on the bacterial growth phase (A. L. Cheung and Projan 1994; Heinrichs, Bayer, and Cheung 1996; Morfeldt, Tegmark, and ARVIDSON 1996).

Interestingly, in the dual system the activation of P2 occurs first but not with the intensity reported in the individual system, suggesting that embedded within the *agr* IGR, the RNAIII has a more sensitive *reaction switch* that can cooperate to clearly differentiates the *DRcells* subpopulation. In addition, the quantitative analysis of the dual orthologous system revealed that the binding rate (K_{on}) of the AgrA~P to augments 4-fold in comparison to the single systems, suggesting that the architecture of this IGR might play a role in the establishment of a PFL sensitive enough to specific environmental cues to generate an specialized cell type by sequentially activating the P2 and then the P3 regulons. The dissection of the Agr system allowed us to define quantitative characteristics of this autoregulatory system (Golding et al. 2005; Zeng et al. 2010; Chong et al. 2014). Comparison between the experimental data and the mathematical simulations showed clear differences in the K_{on} of AgrA~P between the *psmα* and the *psmβ* and, between the P2 and P3 promoters. As discussed above, the K_{on} for the P3 promoter was low and indeed, was the lowest among all the promoters analyzed individually. Nevertheless, the mere introduction of this promoter in the Agr P2-P3 IGR radically changed this scenario, highlighting the importance of the genetic architecture in the transcriptional efficiency of the effector RNA molecule RNAIII. Although all the promoters studied here are directly controlled by the effective binding of AgrA~P, only P3 could be considered *out* of the P2/AgrA regulon. Together, quantitative data supports our hypothesis about the role of the P2 PFL in the *preparation* of a subset of cells to become fully and physiological active *DRcells*: only upon the activation of the sensitive PFL (P2) and other genes directly controlled by Agr (such as *psmα* and the *psmβ*) cells may activate the P3 expression and the subsequent gene regulation exerted via the RNAIII molecule. On the other hand, the differences found between P*psmα* and the P*psmβ*, might be related to their functional role during the process of biofilm formation. As we proposed above, the PSMβ molecules might be involved in the maturation of the multicellular aggregate more than in its disruption or dissemination, which might explain why this promoter showed an increased and stronger K_{on}. It has been proposed that PSM not only are proinflammatory peptides involved in the modulation of immune response and phagocytic

scape but also, that strongly influence biofilm development and dispersal (Periasamy et al. 2012). Taking into consideration that the K_{on} differs between the *psmα* and the *psmβ* promoters it could be suggested that during the process of biofilm formation, maturation and dispersal in response to cell densities via QS and to the autoactivation of the Agr PFL; the ratio between PSMα and PSMβ might vary controlling antagonistic or cooperative functions depending on such a ratio. The *psmβ* genes might react faster to reduced amounts of AgrA~P while on the other hand, the PSMα molecules might have evolved a weaker promoter in order to react only to particularly high cell densities (QS) and strictly when a full activation of the P2-PFL and an *irreversible* RNAIII activation guarantee the emergence of the *DRcells*. Here, each particular K_{on} sets a response to the AIP, implying a temporal but also a spatial regulation of the Agr-related transcription. Specifically, higher K_{on} guarantee a faster response to lower concentrations of AgrA~P, setting a subset of genes that respond earlier and then, delineate a temporal-related physiological state that defines the temporal and spatial development of the staphylococcal multicellular aggregate, when considering a multicellular community constraint by the extracellular matrix. It is known that variations in the different components of the system, such as K_{on}, activation rates, as well as posttranslational processes, might cooperate to modulate the velocity at which certain PFL can react to a particular environmental cue (Saini et al. 2010). Then, using mathematical simulations of the effect of AIP entry in the orthogonal system we addressed the abovementioned suggestion that the process of cell differentiation in staphylococcal multicellular aggregates modulated by the Agr system, is ruled by temporal and spatial features related to the local concentration of AgrA~P (AIP) and the specific K_{on} of all *agr*-controlled genes. These analyses demonstrated that the expression of the promoters is activated in response to an increase of AIP concentration (spatial) or in response to an increase in the exposure time to a defined AIP concentration (temporal). These mathematical simulations recreated the experimental results simply by increasing the exposure time to a defined concentration of the QS molecule. Moreover, further analysis on the expression of the P2 and P3 promoters over the time, showed the appearance of clear temporal bursts (Chong et al. 2014), corroborating our findings and demonstrating that the activation of the Agr in function of the binding efficiency of the AgrA~P is guided by an early burst, fast and sensitive (a PFL) of P2 activation followed by later and more frequent burst of P3.

All together, our results strongly suggest that the Agr system represents a PFL, which acts as a *temporal timer*, as a retardant that controls the sequential activation of the P2 and P3 systems embedded in the chromosomal architecture of the Agr IGR. PFL guarantee signal amplification, the rapid activation of the system and finally, the emergence of two well-differentiated bimodal probabilities maxima (Mitrophanov and Groisman 2008b; Locke et al. 2011). The level of extracellular AIP directly affects the concentration of AgrA~P inside the cell, which in turn, plays a key role in activation of the program for cell-fate decision-making, contributing to the concerted emergence of the *DRcells* lineage that overcome the effect of the extracellular Mg^{2+}.

The simulation of a multicellular community of 20.000 staphylococcal cells demonstrated that above-threshold concentrations of AIP at a given time-point generate cells distributed in discrete subpopulations of *BRcells* and *DRcells*, depending on the specific activation level of the P2 (RNAII)

and P3 (RNAIII). Specifically, an activated Agr-PFL (RNAII high) accompanied by high levels of RNAIII would lead the emergence of a discrete subpopulation of *DRcells*. Below-threshold concentrations of AIP (limited AgrA~P) drastically reduced the probability that cells activate P2 and subsequently P3, revealing that most of the cells could be assigned to one of the subpopulations on the basis of the sequential activation of both promoters rather than from a non-expressing state, following a particular path for the decision process, where the subpopulation of P3-expressing cells (*DRcells*) results from a former subpopulation of P2-expressing cells (*BRcells*). The absence of AIP in the system abolishes the activation of the PFL and cell differentiation, reflecting our experimental observations in the *S. aureus* Δ*agr* strain, which does not differentiate as well as the results obtained in our orthologous system where the AIP was not present. When concentrations of the components in the Agr system were low, the system may experience stochastic fluctuations due to its intrinsic noise nature as well as due to the external variations that affect the system (Raj and van Oudenaarden 2008). But, when a perturbation occurs, those levels overcome certain activation threshold, the noise is exponentially increased and the system displays bimodality.

It has been largely known that PFL are the key mechanistic determinant of bacterial bistability but they do not generate bistability by its own. Indeed, they need intrinsic noise in gene expression, for which these PFL serve as amplifiers, minimizing the impact of changing external fluctuations by assembling high transcription rates and low translational efficiencies (Kaern et al. 2005; Dubnau and Losick 2006). Like many other PFL system, the assembly found between AgrA~P binding and their respective transcription rates might serve as an operational selective pressure for noise reduction after AIP sensing, resulting in the modulation and the *perpetuation* of the biological process of cell-fate decision-making. The presence of the Agr system determines the bimodality in the gene expression in *S. aureus* while; the absence of extracellular Mg^{2+} or the deletion of *sigB* do not abolish the bimodality but instead, modify the bimodal ratio between *on* and *off* cells. These results confirm that among all the components of the genetic circuit, the Agr system is the autonomous PFL program determining the process of cell differentiation. In our system, the concentration of AIP to activate the system needs to be above-threshold and higher that the corresponding threshold to deactivate it, strengthening our idea that this system indeed displays heritability. The fact that AgrA~P levels are subject to the amplificatory role of the PFL and also, need to be above-threshold levels to guarantee gene expression and to overcome the intrinsic noise expression level of the Agr system explained as constitutive levels the AgrC transmembrane histidine kinase and the AgrA transcriptional factor, to generate bimodality in gene expression and finally, to lead to the emergence of a physiologically different staphylococcal cell types, guarantees that once a cell has made the decision to become *DRcells* it will remain in such a state and will not easily deactivate, unless the threshold level of deactivation is reached again. Bacterial heterogeneity is commonly ruled by the activation of combined PFL, which cooperate to generate cell heterogeneity and to transmit and preserve this phenotypic and physiologic diversity in time, what is known as epigenetic memory or hysteresis (Mitrophanov and Groisman 2008b; Basset et al. 2012). Also, the irreversibility of a differentiated state can be maintained by feedback loops (W. Xiong and Ferrell 2003). The staphylococcal Agr system seems to share both characteristics.

Many studies have attributed a *stochastic nature* to the bistability exhibited by bacterial TCS, due to the spontaneous, unknown and promiscuous nature of the triggering signals (Elowitz 2002; L. Zhou et al. 2005; Pedraza and van Oudenaarden 2005). Noise-triggered excitable systems allow cells to probabilistically enter a state using a combination of positive and negative feedback loops (Eldar and Elowitz 2010). Nevertheless, many authors have recently questioned the essentiality of intrinsic noise in gene expression as the driving force for bistability, proposing that stochasticity is not the determinant force in cellular decision-making. Instead, they have found that that pre-existing differences may explain to a significant degree cell fate decisions in biological systems (St-Pierre and Endy 2008; Weitz et al. 2008; Balázsi, van Oudenaarden, and Collins 2011). In particular, the kinetics of how the Agr is autoactivated is determined mainly by *pre-existing* gene activation parameters such as the AIP concentration and the promoter strength (K_{on}). How cells adopt a particular fate is usually thought of as being deterministic by virtue of an acquired lineage that has been inherited from the previous state (hysteresis) and/or by virtue of their proximity to the trigger inductive signal (Losick and Desplan 2008), Mg^{2+} or AIP in our particular case. Although the stochastic nature of the autoactivation in PFL is still a matter of debate, particularly in bacterial growing in standard flask conditions, it might be considered that predetermined (evolutionary acquired) response to external signals are strength-modulators of feedback loops and, recreate a simple sense-and-response circuit. In the absence of environmental stress, noise is crucial because it causes above-threshold gene expression in a small subset of cells, seeding the feedback-based switch (Becskei, Séraphin, and Serrano 2001) or because it guarantees basal level of expression. Nevertheless, when the signal is positively sensed, the stochastically preactivated cells would be able to upregulate their gene expression rapidly through regulatory PFL and, stably pass this better-fit expression state to their progeny, facilitating the process of cell fate decision-making (Fraser and Kaern 2009). Stochasticity has important mechanistic requirements that might be summarized as the pure existence of noise in gene expression, which in turn is strictly necessary for any deterministic process ruled by a PFL. Fluctuations due to noise are generally small and transient and then, are the PFL the mechanistic partner involved in the amplification and stabilization of these fluctuations once they have been decanted to one choice or another, making each decision-state resistant to small perturbations and then, as consequence of this locked-state, to persist for extended periods of time and be transmitted over a certain number of generations, exhibiting the typical memory that characterizes hysteresis (Losick and Desplan 2008). Considering that the Agr activates only in late exponential or stationary growth phase, the metabolic potential of QS system might rely in the fact that is a dedicated genetic circuit that rapidly amplifies a signal. It has been hypothesized that at low bacterial densities, AIP is produced linearly and might bind individually to the AgrC dimer, generating a partial, constitutive or basal activation of the system (noisy expression). When QS is reached, the amount of AIP increases to a threshold level, where two AIP molecules can bind the AgrC dimer promoting full activity and triggering the exponential production of AIP and hence, the full activation of the Agr system (Novick and Geisinger 2008). The Agr is then, an autoregulatory mechanism that senses and signals the state of cell density that is activated during different physiological transitions (Vuong et al. 2000).

Together, our *in vitro* and *in silico* experiments demonstrated that the autoregulatory nature of the Agr strongly relies on the presence of a PFL systems that permits the Agr QS system of *S. aureus* to work as an autonomous bistable switch able to trigger the bifurcation of *S. aureus* cells, based on the temporal control of the expression level of the transcriptional regulator AgrA~P and the existence of the adjacent P2 and P3 regulons, which define a intricate downstream genetic network that cooperate to establish a physiological divergent state. In *S. aureus*, the activation of the Agr PFL and the subsequent bifurcation of the bacterial population into *BRcells* and *DRcells* is determinant for the outcome of the infection and as we have demonstrated, it is influenced by extracellular signals specific to the colonization niche. The existence and activation of a PFL in the Agr system helps to understand how nosocomial pathogens such as *S. aureus* cause dissimilar types of infections in distinct organs, and show that cell differentiation in nosocomial pathogens is particularly relevant for adaptation to different host tissues. Therefore, the local concentration of the different environmental signals determine the sustainability of the *BRcells* and *DRcells* states as the most intense manifestation is the irreversibility of a Agr system (Sneppen, Krishna, and Semsey 2010; Basset et al. 2012). PFL are likely to contribute to the emergence of multiple stable phenotypes within the bacterial population not only by controlling its own expression, but also by contributing to the propagation and autoamplification of the response to downstream genes (Pedraza and van Oudenaarden 2005). Community-level decisions mainly depend on certain degree of coordination among progeny and hence, require a memory mechanism to boost and preserve this multicellular cooperation. The generational timing provided by interlinked feedback loops enforces cooperation among the progeny of a cell that decides to initiate a process of cell differentiation in a multicellular community (Norman et al. 2013).

5. Multicellular aggregates promote collective behavior and cell heterogeneity in *S. aureus*

Phenotypic heterogeneity can be understood as the cause and consequence of a primary diversity of chemical states that are innately found in any environment and how, the genetic adaptation of bacteria react to those local environmental conditions (P. S. Stewart and Franklin 2008). The activity of the Agr PFL is necessary but not strictly sufficient to generate a clear bimodal pattern that can be inherited to be stable in time. We have demonstrated that the multicellular level observed in these aggregates is facilitated by the direct influence of the QS system, determining how each cell make its own decision based on the local concentration of extracellular Mg^{2+} and the diffusible molecule AIP. In the context of structured multicellular communities such as biofilms, phenotypic heterogeneity leads to the emergence of a more elaborated pattern of division of labor between individuals, increasing the rate at which populations grow and the functions that they can perform, providing benefits to the whole clonal population, once it has reached the multicellular organization level (Ackermann 2015). In *B. subtilis* biofilms, the emergence of physiologic and phenotypically different cells is the result of interlinked genetic networks that involve intrinsic noise in gene expression, complex feedback loops and a variety of sensing and regulatory systems (Hamoen 2003; Smits et al. 2005; Maamar and Dubnau 2005; Veening et al. 2008; Balázsi, van Oudenaarden, and Collins 2011; Gamba, Jonker, and Hamoen 2015). In a similar manner, our model of multicellular development in *S. aureus* showed that cell

differentiation was heritable during cell division, suggesting that within these multicellular aggregates cell types might become spatially organized during colony development (Yarwood et al. 2007). We tested this hypothesis using a mathematical model of the development using the observed features displayed by the wild type and mutant aggregates. We represented the growth dynamics of these aggregates and captured the differences in the morphology of the different mutants by modifying specific parameters within the four-equations system. By changing values such as the dynamics of AIP diffusion, we successfully recreated it the phenotypes observed *in vitro*, demonstrating the key influence of these variables in the development of these bacterial multicellular communities.

Mathematical approaches that address the emergence of multicellularity in bacteria have revealed that once communities reach high cell densities, they undergo a process of destabilization that can be only solved by the emergence of a particular cell type able to stabilize again the dynamics of the whole system (Furusawa and Kaneko 2002). These findings suggest that differentiation is a general feature of a system of interacting units and that multicellularity and its innate heterogeneity, is a necessity for evolution. In agreement with our experimental data, it has been established that the positional information of the different cell types plays a fundamental role for the development of the multicellular organism and is maintained by the interplay between intra- and intercellular dynamics (Furusawa and Kaneko 2002). We demonstrated that the local concentration (gradients) of AIP and nutrients control the spatial distribution of replicative and the non-replicative cells in multicellular aggregates of *S. aureus*. Importantly, this mathematical model allowed us to predict that the extracellular matrix of the multicellular aggregate is a fundamental component for the diffusion of the QS molecule AIP. According to our model, higher concentrations of AIP will be accumulated in this centered and older region and as we established, above-threshold levels of AIP have the potential to activate the Agr PFL, generating the emergence of a stable *DRcells* subpopulation. On the other hand, outer areas of the aggregate would be active with replicative cells, metabolically active and depleted of AIP, generating suitable conditions for the stabilization of a *BRcells* line. As a multicellular community grows and expands, nutrients become scarce in the centered regions, generating the transition from the replicative to the non-replicative state. The consequence of this transition is the concentration of non-actively dividing cells with a presumably low metabolic activity. Then, centered older regions of the aggregate would be metabolically less active and would contain a higher representation of specialized *DRcells* due to the high AIP levels. Therefore, this area might represent a preferable zone for biofilm disassembly, hypothesis in agreement with the morphological features observed in this area, as well as with previous studies of *B. subtilis* multicellular communities where it was demonstrated that dissemination structures such as the fruiting bodies and the spores were localized on top of the extracellular matrix in the older regions of the biofilm (Branda et al. 2001).

Previous mathematical modeling analyzed the emergence of cell heterogeneity in bacterial biofilms as a consequence of the spatial heterogeneity of autoinducer regulation (Hense et al. 2012). It was found that in bacterial aggregates gradients of signals and environmental substances emerge, creating a regulation network that generates spatially heterogeneous behavior that resembles a multicellular-like division of work, especially under nutrient-controlled conditions. There, spatial gradients of the

autoinducer resulted in a spatial organization of its own induction. In addition to the these gradients, potential nutrient gradients in these aggregates seemed to affect further the spatio-temporal heterogeneity of its own regulation, concluding that the relation between nutrients and the autoinducer systems is critical for the potential of heterogeneity development (Hense et al. 2012). Together, our *in vitro* and *in silico* corroborated that local concentrations of the two input signals that control Agr, determine cell differentiation and the development of the multicellular aggregate. In particular, an altered extracellular matrix composition affects the bifurcation pattern of the *S. aureus* cells, suggesting that not only the absence of a particular structural component of a bacterial biofilm affect its morphology but also, has a profound influence over the process of cell differentiation, supposing a constant feedback between cell differentiation and biofilm structure that determines the correct development of the multicellular community. A concerted and regulated program of cell differentiation that relies in the fine interconnection between structural components and the activation of complex genetic networks has been already demonstrated in *B. subtilis* multicellular aggregates (Vlamakis et al. 2008). The extracellular matrix constitutes an active participant of the process of cell differentiation in ***B. subtilis* biofilm**. Biofilms developed by matrix-deficient mutants showed a significant decrease in the emergence of spores (Vlamakis et al. 2008). Interestingly, this cell differentiation defects was restricted to biofilms growing on solid surfaces. In liquid biofilms, the process of spores cell differentiation was not affected by the absence of these genes (Branda et al. 2006), supporting our mathematical modeling, where the spatial distribution within these multicellular aggregates is a fundamental need for the emergence of cooperative cell types and, demonstrating that the process of cell differentiation within multicellular communities of bacteria occurs with an extraordinary degree of spatiotemporal organization. In agreement and as predicted by the mathematical models, we demonstrated experimentally the specific localization of the *BRcells* and *DRcells* in the morphological distinct regions. Specifically, *BRcells* were preferentially found in the regions closer to the nutrients source and to Mg^{2+}, in agreement with previous findings in other models of bacterial biofilms (Cramton et al. 2001). Interestingly, transversal cryosections revealed a fascinating internal architecture with well defined architectural areas and in particular, the emergence of *mushroom-like* structures localized on top of the most centered regions of the aggregate, which resemble dispersion structures found in other bacterial multicellular communities (Branda et al. 2001; B. Lee et al. 2005; Goldman et al. 2006; Claessen et al. 2014) and provide clues of how staphylococcal multicellular communities could develop dispersion structures, as the result of the concerted production and assembly of diverse *agr*-related gene products such as the PSMs (Romero et al. 2010; Ostrowski et al. 2011; R. Wang et al. 2011; Periasamy et al. 2012; Schwartz, Syed, Stephenson, Rickard, and Boles 2012a; Taglialegna, Lasa, and Valle 2016). In *B. subtilis* biofilms, the emergence of spores as a particular cell type is an active process and, has been found that sporulation takes place preferentially in upper part of solid-matrix colony biofilms, with the emergence of the aerial structures or fruiting bodies (Branda et al. 2001; Vlamakis et al. 2008). The emergence of these fruiting bodies is facilitated by the presence of the wrinkles formed by the extracellular matrix (Veening et al. 2008; Romero et al. 2010; Romero 2013; Vlamakis et al. 2013; Cairns, Hobley, and Stanley-Wall 2014; Mielich-Süss and López 2014) and in addition, these wrinkles

provide the multicellular community with an increased surface-to-volume ratio that facilitates the access of cells to oxygen (Dietrich et al. 2008; Kolodkin-Gal et al. 2012; Dietrich et al. 2013; Mielich-Süss and López 2014) as well as the formation of a intricate network of channels to facilitates the free circulation of molecules (Wilking et al. 2013; Mielich-Süss and López 2014). Similarly, in *E. coli* multicellular aggregates are characterized by a process of cell morphologic differentiation where, actively growing cells are found in the bottom and nutrient-enriched layer while; the top and nutrient-depleted layers are inhabited by non-growing starving cells (Serra et al. 2013; Serra, Richter, and Hengge 2013; Serra and Hengge 2014). In these macrocolonies, the formations of wrinkled, ring structures is associated with surface breaks and are believed to be generated by propagating cells located below, together with adjacent curliated cell layers. These large breaks generate *dome*-like shapes through the ring pattern found within mature macrocolonies. It might be feasible that, in our *S. aureus* multicellular communities, the emergence of the *mushroom*-like structures observed during the cryosectioning of mature aggregates are also the result of the pressure generated by actively growing cells within the matrix, giving rise to specialized structures with the potential to serve as dissemination structures.

Biofilm formation can be interpreted as an evolutionary solution of individual cells embedded in a finite space and time to cooperate among them and minimize the stochastic influence of the environment for the benefit of the whole clonal population. Cells associated in multicellular aggregates acquire population-level properties such as recursive production of cells at a cooperative level, cell differentiation, a robust developmental process and the emergence of a germ-line (Furusawa and Kaneko 2002). The recursive production of cells at the cooperative level can be easily understood by observing the *BRcells* line while, cell differentiation is a direct consequence of the activation of the Agr as an autonomous program of cell fate decision making in the *DRcells*, based on QS cell communication. The reproducibility of our developmental program points out the robustness of this model and, the heritability of the cell fate strongly suggest the emergence of an stable germ-line within these aggregates. It has been proposed that cell differentiation process that leads to the rise of metabolically and physiologically heterogeneous zones within bacterial multicellular communities, is a key determinant of biofilm formation and its resistance features (Serra and Hengge 2014). The study of multicellular aggregates growing in solid surfaces has offered a highly valuable model to study physiological differentiation and architectural development. Interestingly, biofilms growing on other matrixes do not exhibit the high level of organization at the microscale or the elaboration of macroscopic and architectural complex phenotypes (Serra and Hengge 2014). In addition, the development of these multicellular aggregates has provided a suitable model for the identification and quantitative analyses of distinct cell types, as well as for the identification of the different environmental signals and conditions that modulate the emergence of this multicellular-level structures (Vlamakis et al. 2008; López, Vlamakis, and Kolter 2009; Serra and Hengge 2014; Mielich-Süss and López 2014). In particular, it was demonstrated that the emergence of spatial and temporal phenotypic diversity in multicellular communities of *E. coli* (Serra et al. 2013; Serra, Richter, and Hengge 2013)

was driven by a high level of heterogeneity in gene expression patterns, in growth, metabolic activity, morphology, stress response induction and death, suggesting that bacterial multicellular colonies provide an ecological opportunity for the generation and maintenance of phenotypic diversity, increasing the probability of population survival in unpredictable environments (Saint-Ruf et al. 2014). The heterogeneity displayed by these multicellular macrocolonies was driven by the nutritional status that influenced the stress response and the switching to a stationary-phase physiological state as a prerequisite matrix formation (Serra and Hengge 2014). The main regulator of this process is the stationary-phase sigma factor σ^S (*rpoS* gene), which controls the expression of a vast amount of genes (Lange and Hengge-Aronis 1991; Serra et al. 2013; Pesavento et al. 2008) responsible for the synthesis of the exopolysaccharide cellulose, amyloid curli fibers (Prigent-Combaret et al. 2000; Kikuchi et al. 2005; Brombacher 2003), unusual ovoid cells (Lange and Hengge-Aronis 1991) and flagella (Serra et al. 2013). The response of σ^S to environmental changes showed to be specific to envelop stress, as well as of signalling molecules that modulate gene expression for the emergence of bacterial heterogeneity in biofilm formation. Similarly to our proposed model in *S. aureus*, in addition to the environmental stress conditions that promote the activation of σ^S, the increase of signaling molecules such as the second messenger cAMP, the alarmone (p)ppGpp and the QS signaling molecule c-di-GMP in post-exponential growth phase, also played fundamental roles for the development of the *E. coli* macrocolonies (Botsford and Harman 1992; Magnusson, Farewell, and Nyström 2005; Potrykus and Cashel 2008; Pesavento and Hengge 2012).

Stratified growth of multicellular communities has been also reported in *P. aeruginosa*. One of the characteristic features of the pseudomonad multicellular communities is the *mushroom*-like structure, whose developmental process involves the emergence of heterogeneous subpopulations within the multicellular community (Klausen, Heydorn, et al. 2003; Klausen, Aaes-Jørgensen, et al. 2003). In particular, this structures are composed of a subpopulation of non-motile cells that form the mushroom stalk and, a migrating subpopulation that climbs the stalk using type IV pili and aggregates at the top (Klausen, Aaes-Jørgensen, et al. 2003; Häussler 2004; B. Lee et al. 2005). In this process, an eDNA scaffold binds type IV pili to mediate cell attachment and the migration of a particular group of leader cells through interconnected channels. As a *leader* cell type coordinately moves, it releases aligned eDNA strands using an specific secretion mechanism involving holin-antiholin systems similarly to the system found in *S. aureus*, strands which are followed by other cell types (Gloag, Turnbull, and Huang 2013; Steinberg and Kolodkin-Gal 2015; Rice et al. 2007; Montanaro et al. 2011). In *P. aeruginosa* multicellular communities it has been observed that regions close to the surface of the macrocolonies, oxygen is consumed and is not accessible for the rest of the multicellular community, defining the two-layer pattern found in these aggregates and determining how the top layer exhibits a subpopulation of metabolically active and elongated cells while, the middle an bottom layers are constituted of a subpopulation of metabolically inactive and ovoid cells that allow nutrients to diffuse from the agar surface to the top layer (Werner et al. 2004). Interestingly, in *P. aeruginosa* biofilm exopolysaccharides such as alginate, Psl or Pel are synthetized in response to different and specific environmental factors, displaying an interesting level of physiological heterogeneity in response to particular niches. Alginate

is preferentially produced by isolates from lungs of CF patients resulting in the typical mucoid colony phenotype, but is not essential for biofilm formation in the traditional microtiter plate assay (Steinberg and Kolodkin-Gal 2015). Psl is fundamental for biofilm development by producing trails as it moves by twitching motility along the surface, creating routes that are followed by other cells (Zhao et al. 2013). Also, Psl acts as a signal that activates the production of c-di-GMP, which in turns promotes the expression of Psl during biofilm formation. This autocrine signaling mechanism constitutes indeed a PFL that deeply influences biofilm formation in multicellular communities of *P. aeruginosa* (Irie et al. 2012) and together with the Psl trails, demonstrates that bacterial microcolony initiation is a self-organized process highly dependent on cell-to-cell communication and physiological heterogeneity. The local concentration of Psl is used to induce PFL, where cells then tend to reside in areas with significant Psl accumulation (Zhao et al. 2013). A specialized subpopulation of *pioneer* cells locally increases the production of Psl to attract more cells that later cooperate to increase the size of the extracellular matrix and serve as the founding population of the multicellular microcolony (Zhao et al. 2013; Steinberg and Kolodkin-Gal 2015).

Bacterial communication and QS also play an important role in the development of *P. aeruginosa* multicellular communities. The PQS molecule induces the rhl QS system, involved in biofilm formation *in vitro* and *in vivo* using programmed cell death (PCD) in areas of high cell density that generates the particular morphology of the microbial aggregate (Singh et al. 2000; D'Argenio et al. 2002). Also, QS activation in *P. aeruginosa* is associated with biofilm detachment and the appearance of the *central hollowing*, which involves the formation of internal cavities in determined areas of the multicellular community that fracture to release the motile bacteria (Sauer, Camper, and Ehrlich 2002; Hunt et al. 2004; Boles, Thoendel, and Singh 2005). *Central hollowing* is facilitated by the presence of rhamnolipids, a biosurfactant with structural roles motility and in the formation of the fluid channels that surround biofilm structures (Davey, Caiazza, and O'toole 2003). In particular, rhamnolipids synthesis is modulated by the QS molecule acylhomoserine-lactone, which accumulates in the center of the biofilm structures (Boles, Thoendel, and Singh 2005), demonstrating that cellular communication guarantees the concerted development of the community as a whole. Gene expression of most bacterial master regulators of QS has autoregulatory properties, characterized by the presence of different feedback loops, signaling cascades, relatively low stochasticity and autoinducer-dependent noise, which together modulate bacterial individuality during the transition to a population-level behavior (Becskei, Séraphin, and Serrano 2001; Nevozhay, Adams, and Murphy 2009; Balázsi, van Oudenaarden, and Collins 2011).

In *S. aureus* cell differentiation has not been extensively addressed although, heterogeneity in gene expression in staphylococcal biofilms has been observed (Yarwood et al. 2004). In particular, *S. aureus* biofilms actively express *agr*-dependent genes in a heterogeneous manner. Under specific culture conditions, the expression of the regulatory RNAIII was restricted to patches within cell clusters and oscillated in time, with the loss of fluorescence coinciding with the detachment of cells from the biofilm at late stages (Yarwood et al. 2004), suggesting a specific physiology for the dispersed cells. This work provided important evidence about the heterogeneity displayed in staphylococcal biofilms.

In an additional study, confined *S. aureus* single-cells demonstrated that the Agr QS system might constitute a genetic system beneficial at the multicellular-level, by means of its fundamental role in the activation of QS, but also that it had the ability to alter gene expression at the single-cell level (Carnes et al. 2009). Single *S. aureus* cells were confined in space, which allowed the AIP molecule to reside undiffused and exert its function as activator of an autoregulatory system, in an autonomous manner. This work provided important evidence that spatial constraints are fundamental players in the emergence of heterogeneity. Specifically, under traditional batch cultures, the emergence of heterogeneity as a result of the activation of the Agr system could be unnoticed. Nevertheless, in the confinement that the multicellular aggregate imposes, AIP diffusion follows specific patterns, generating chemical gradients that together with other environmental gradients such as oxygen, nutrients, extracellular Mg^{2+} and other bacterial metabolites, determine the specific activation of the Agr PFL in a particular subpopulation of cells, promoting heterogeneity within the bacterial community.

To define the role of chemical gradients in the emergence of heterogeneity within *S. aureus* biofilms, spatial patterns of DNA replication, protein synthesis, and oxygen concentration were measured and it was demonstrated that these staphylococcal biofilms were constituted of heterogeneous areas that display diverse physiological states (Rani et al. 2007). In this study, two bands of DNA synthetic activity were observed along the staphylococcal biofilm: one localized in the upper region of the community, in contact with the air interface and a second, localized in the bottom region. Protein synthesis progressed inward into the biofilm, with large variations along the structure and, with a characteristic band in the upper region, colocalizing with the DNA synthetic activity. In agreement, our experimental data demonstrated that cells close to the nutrient source were metabolically active, reflecting the physiological state of the *BRcells*, involve in the expansion of the multicellular community following nutrient and oxygen gradients while; cells embedded within the extracellular matrix were metabolically less active but viable, which might represent the *DRcells* physiological state. The fact that most of the cells within biofilms are viable with a reduced metabolic activity (Rani et al. 2007), agrees with our observations, where inner regions of our multicellular communities where particularly enriched in *DRcells*. The localization of the *DRcells* lineage along the biofilm, together with the determination of its physiological state by transcriptomic analyses, strongly suggest that *DRcells* embedded in inner regions of the biofilm display complementary role in biofilm maturation and dissemination, in response to high AIP levels. The presence dead cells within *S. aureus* biofilms as an additional *cell type* (Rani et al. 2007) results particularly interesting and closely related to the emergence of the *DRcells* lineage since, PCD and eDNA release were found to be fundamentally important during biofilm maturation. Specifically, it was demonstrated that eDNA facilitates the formation of functional PSM amyloids in *S. aureus* biofilms (Schwartz et al. 2015) and, that PSMs mixed with eDNA were less cytotoxic than soluble PSMs peptides, suggesting that at late stages during the maturation of a staphylococcal multicellular community, eDNA might sequester these toxins by favoring aggregation of free peptides (Schwartz et al. 2015), which in turn are known to be fundamental structural components involved in the formation of biofilm channels and the process of biofilm detachment (Periasamy et al.

2012) in areas of the biofilm where PSMs are actively produced and *DRcells* are predominately localized. Genes involved in the secretion of eDNA were steadily increased in late stages of biofilm formation, suggesting that eDNA was an important component of biofilm maturation while; carbohydrates in the matrix appeared to play an important role in structural stability (Grande et al. 2014). Interestingly, this work also demonstrated that the expression of the Agr system is temporally coupled to the expression of biofilm components involved in late stages of biofilm formation such as maturation and detachment.

It was recently found that under specific culture conditions that resemble a persistent human carrier, the regulation of the *cap* operon and CP production displays and heterogeneous pattern (George et al. 2015). The capsular polysaccharide (CP) protects against phagocytosis and also impedes adherence to endothelial cells and/or matrix proteins. In this study, it was demonstrated that CP heterogeneity was a growth-dependent and QS-dependent phenomenon driven by the Agr system, particularly by the effector molecule RNAIII. Using this model, the pattern of CP production and *cap* expression demonstrated to be heterogeneous but interestingly, the activity of the Agr system (RNAIII) was homogeneous. The heterogeneous pattern *cap* expression, represents another example of evolved heterogeneity playing a role in pathogenesis, possibly by providing better fitness of the bacterial population during infection and colonization (George et al. 2015). For the case of CP production, an active Agr seems to be necessary but, its bimodality does not define the bimodality in CP production, making feasible that the heterogeneity found in response to AIP works on top of the regulatory network and determines the activation of additional downstream bimodal switches involved in the emergence of additional cell types within staphylococcal communities. In this study, authors suggest that *in vivo* or in biofilms, small changes in the microenvironment are likely to modulate the Agr QS response and hence, could have an additional effect on the emergence of heterogeneity in CP production and explain why the temporal expression of *cap* is severely repressed in bacteria in the exponential growth phase, when *agr* activity is normally low (George et al. 2015).

Other studies have shown heterogeneity in *S. aureus* biofilms and in particular, one study showed that the expression of the *S. aureus* thermonuclease Nuc, displayed a heterogeneous pattern of expression in a subpopulation of cells during the process of biofilm formation (Moormeier et al. 2014). The heterogeneity found in the expression of this gene emerged in late stages of biofilm formation, after changes in matrix composition and the reorganization of the cells within the mature biofilm. Interestingly, the appearance of this specialized cell line showed to be associated to the secretion of eDNA for biofilm maturation and, the deletion of the *nuc* gene abolished the *exodus* of cells, causing hyperproliferation of the community and disrupting the normal development of *tower*-like structures, characteristic feature of these biofilms. The *nuc*-type cell differentiation process was independent of the Agr activity but instead, was governed by the presence of the TCS SaeRS (Moormeier et al. 2014), which has been proposed to act upstream of the Agr system regulatory network (Novick 2003a). Nevertheless, a fascinating connection emerged from these results, showing that P3 (RNAIII) activity was particularly elevated in and was restricted to the *tower* structures (Moormeier et al. 2014).

The benefits for a given individual would depend on the phenotypes of the other cells in the same microenvironment, which in parallel imposes the spatial limitation that supports effective communication, physical and chemical interactions, emergence of different phenotypes and finally, the coordinated division of labor between individual cells (JEFFERSON 2004; Ackermann 2015). In bacterial biofilms, division of labor leads to collective functionality, where social interactions within phenotypically different individuals improve the fitness of the multicellular community, affecting growth and/or survival for every individual in the same environment.

6. Staphylococcal cell lines found within multicellular aggregates posses a specialized physiological state

The environment strongly influence the differential expression of genes involved in the different stages of biofilm formation in bacteria and, define the emergence of particular cell types. As a result of the process of cell differentiation, biofilm formation alters the microenvironment of its own, leading to additional changes in gene expression and in the dynamics of its own regulatory networks, repeatedly changing the physiological status of a subset of the cells present in this heterogeneous multicellular community (JEFFERSON 2004). This supposes an extremely complex cycle of interactions and responses as well as an enormous physiological challenge for the individuals comprising the multicellular community. Although, extensive work has allowed identifying the fundamental changes in gene expression during the process biofilm formation in *S. aureus* in diverse conditions and diverse genetic backgrounds (Dunman et al. 2001; Bischoff et al. 2004; Beenken et al. 2004; Resch et al. 2005; Nagarajan and Elasri 2007; Eiff et al. 2006; Loughman et al. 2009; Tan et al. 2015), the physiological profile of a specific cell type has not been addressed, due mainly to the technical limitations imposed at isolating a particular cell type. Nevertheless, given the complexity of the developmental process in *S. aureus* aggregates growing on TSBMg, dissecting the physiological state of our particular cell lines resulted particularly interesting and constituted an important advance in the understanding of how bacteria acquire, evolve, interlink and modulate this multicellular-level behavior.

The reproducible, well-defined and architecturally complex developmental pattern of the *S. aureus* aggregates growing on TSBMg, as well as the temporal and spatial dynamics of the *BRcells* and *DRcells* lines indicate that the staphylococcal community embedded in this extracellular matrix might display certain level of physiological heterogeneity to accomplish with particular roles during the development of the multicellular community. We addressed this hypothesis by physically isolating the different cell types using FACS and to the best of our knowledge; this was the first time that physical separation of a defined bacterial cell type has been used in order to dissect the specific transcriptomic patterns. At the transcriptomic and at the single-gene level, both approaches showed marked differences between the *BRcells* and *DRcells* subpopulations, suggesting that indeed these sorted cells are particular cell types within the multicellular aggregate with a specific physiological state. Global gene analysis of the *BRcells* lineage showed a large number of upregulated genes, including many genes directly involved in biofilm

formation but most importantly, involved in peptidoglycan turnover, DNA replication and cell division, as well as the presence of 49 upregulated tRNAs, indicating that the *BRcells* are undergoing an active process of cell division and are metabolically active, as proposed during the mathematical modeling. Interestingly, the expression of *sigB* was upregulated in the *BRcells* and was accompanied by a downregulation in the expression of the *agrA* gene, in agreement with the molecular mechanism we propose where, the presence of extracellular Mg^{2+} increases the rigidity of the cell envelope by binding the cell wall TAs, inducing σ^B expression and activation. These results indicate that the *BRcells* constitute a particular cellular phenotype within the multicellular communities, characterized by a higher metabolic activity and a physiological predisposition to cell proliferation. In particular, the *sigB* and *agrA* expression pattern found for this cell lineage evidence that the molecular mechanism employed by *S. aureus* to modulate gene expression in response to extracellular Mg^{2+} involves the activation of the stress response and the repression of the Agr system. Therefore, the high metabolic activity evidenced by the transcriptomic profile and the repression of the *agr* operon, might account for a direct link between the Agr PFL activation via AIP, the bifurcation of the staphylococcal community into *BRcells* and *DRcells* and the spatial localization of theses cell types within the multicellular aggregate.

In contrast, *DRcells* showed a lesser number of upregulated genes reflecting a lower general metabolic activity, which might be related to a reduced physiological activity of this cell type, in accordance to the mathematical predictions and the observed localization of these cell type in older areas of the aggregate with lower concentration of nutrients. In agreement with the developmental model that we propose and, supported by our mathematical approach, older regions of the biofilm would promote the concentration of high levels of AIP and the subsequent reach of the Agr PFL activation threshold that generates the bifurcation of the bacterial community into the formerly described *BRcells* lineage and the *DRcells* cell line. Interestingly, among the few upregulated genes detected we found a notable number of genes related to virulence: toxin secretion and host invasion, such as the type-VII secretion system (Burts et al. 2005), as well as genes related to immune evasion and protection of *S. aureus* from the host immune system such as the *hssRS-htrAB* hemin detoxification system (Stauff, Torres, and Skaar 2007). Also, the global transcriptomic analyses of the *DRcells* subpopulation allowed us to detect the upregulation of multi-drug efflux pumps that confer resistance to diverse antimicrobials, and of regulators such as *graR* and *arsR*, which positively control gene-related cell-wall antibiotic resistance and metal ion stress (M. Li et al. 2007; Sass and Bierbaum 2009; Falord et al. 2011; Falord et al. 2012; Kashyap et al. 2014; Fang et al. 2016). *DRcells* also showed a significantly higher expression of the *agrA* gene concomitant with a downregulation of the *sigB* expression, displaying an antagonistic expression pattern to the *BRcells*. These results suggest that the *DRcell* subpopulation has lower metabolic activity than *BRcells* and is predisposed to resist different types of antimicrobials. In particular, the reduced expression of *sigB* found in these cells might be the result of a possible reduction in the Mg^{2+}-induced stress response since these cells are localized apart of the nutrients/Mg^{2+} source, which might account for the derepression of the *agr* operon and the activation of the QS signaling cascade that promotes the emergence of the *DRcells* phenotype. Also, the *agrA* upregulation might be also related to the

amplificatory effect of the Agr PFL, which overcomes SigB repression and autoactivates the regulatory network involved in the appearance of the *DRcells* lineage.

Using RNAseq sequencing we dissected the whole transcriptomic profile of *BRcells* and *DRcells* lineages, which allowed us to identify the particular pattern of gene expression for each cell type, comprising a considerable number of genes differentially expressed with defined roles in the emergence of a particular physiological state with the multicellular aggregate. At the individual gene level, this approach demonstrated to be highly valuable finding specific genetic signatures associated with a particular lineage and in addition, to corroborate our hypothesis about the genetic and molecular mechanism involved in the cell differentiation process. Nevertheless, the acquisition of these large volumes of data for an individual cell type also allowed us to extend our analysis to the complete set of differentially expressed genes, in order to fully determine the correlations found between the expression profile of *BRcells* and *DRcells*. Hierarchical clustering groups genes into particular collections based on similar features, for example their fold-change expression, producing a dendogram that shows the hierarchy of these clusters. Interestingly, unsupervised clustering does not take any of the experimental variables into account, creating an unbiased hierarchical classification of genes (D'haeseleer 2005). One major advantage of this analysis is it ability to generate an overview of the gene expression profile and to identify major relationships between the samples compared, for example in cells lines (Seol et al. 2011; R. Andersson et al. 2014). The comparison of these unsupervised hierarchical clusters in *BRcells* and *DRcells*, resulted to be a valuable tool to delineate a global and unbiased comparison of the physiological state of these cell lineages, based on their gene expression patterns and most importantly, to extract important clues about the transcriptional networks that determine the process of cell differentiation and the emergence of a particular cell type within these multicellular aggregates: the application of this unsupervised clustering permits to establish the connection or the correlation between a particular cell lineage and the patterns of gene expression. Therefore, when we compared the pattern of expression in the clusters of genes differentially expressed in the *BRcells* or the *DRcells* libraries, our analyses revealed a major line of variation between these cell lineages. The commonly differentially expressed genes between *BRcells* and *DRcells* displayed dramatic changes in their patterns, represented by evident variations in the fold expression for most clusters. The unsupervised analyses demonstrated that most clusters of genes differentially expressed between lineages strongly diverge. When we extended these analyses to the functional classification of the differentially expressed genes using TIGRfam (Haft, Selengut, and White 2003), SEED (Overbeek et al. 2005) and Gene Ontology (GO) (Ashburner et al. 2000), we confirmed an exclusive expression pattern belonging to each particular cell lineage. Our findings in gene expression patterns at this scale are in agreement with the hypothesis that *BRcells* and *DRcells* are physiological divergent cell types involved in particular roles within the multicellular community. This type of global approach circumvents the limitation of the analysis of particular genes where differences can be modest, statistically not significant and with analytic noise masking underlying informative changes in gene expression with biological significance (Ptitsyn et al. 2006). Unsupervised hierarchical clustering is based on the observation of similarities within the data and avoids speculative assumptions about gene function. Using a *natural classification*

strategy the only assumption being made is that the most common gene expression patterns associated with a particular characteristic, in our case a cell type, are expected to be found many times, providing enough information about the particular gene expression patterns that delineate the emergence of a particular physiological state, in our case a particular cell type (Ptitsyn et al. 2006). Gene ontology (GO) classification adds more informative physiological data and in addition, categorizes genes in subgroups based on biological function, narrowing the hierarchical clustering into biologically relevant information to define, in the physiological context, what makes a *BRcells* or a *DRcells*. Based on these functional classifications, *BRcells* were characterized by a high division rate result of a robust metabolism of energy, proteins and intermediate metabolites. The active metabolism that characterizes this cell type was accompanied by a dynamic gene regulation and positive signal transduction activation. On the contrary, *DRcells* displayed a low metabolic activity, with their energetic efforts directed to the activation of QS, synthesis of virulence factors and evasion of the host's immune response.

Transcriptomic patterns found in multicellular communities of some bacterial species have demonstrated a remarkable physiological heterogeneity in function of the spatial localization of the cells that comprise them. Transcriptome characterization of *P. aeruginosa* biofilms demonstrated that bacteria localized at the top of the biofilms were actively dividing and displayed a high expression of genes involved in general metabolic functions while, the expression of housekeeping genes was low in cells at the bottom of the biofilms. In addition, it was shown that the dividing cells were more susceptible to killing by antibiotics, suggesting that cells deep in the biofilm are viable but in an antibiotic-tolerant slow-growth state (Williamson et al. 2012). In our approach, the physical separation and the transcriptomic analysis of two of the cell types that compose the staphylococcal multicellular communities, allowed us to confirm our hypothesis and the previous results but in addition, provided large amounts of additional data on the specific and particular physiological state of these cell types, which undoubtedly contributes to the understanding about the biofilm physiology and about the implications that these heterogeneity have on the development, the course and the outcome of *S. aureus* infections. These results indicate that *S. aureus* uses cell differentiation as an evolutionary strategy to protect and persist from the action of external insults by means of the *DRcells* physiology and to adapt to and exploit new colonization niches, by means of the *BRcells* phenotype. Specifically, cells expressing the bimodal switch actively become *DRcells*, which are inclined to detach from the mature biofilm, disperse to new niches with most favorable nutritional conditions and, in the context of bacterial infections, to spread rapidly in the host and upregulate genes responsible for toxin production responsible of tissue destruction and acute bacteremia. In addition, analyzing the global gene expression profile of this cell type, *DRcells* do not divide actively, which is a beneficial strategy as they show a reduced number of targets against the action of traditional antimicrobials (D. I. Andersson 2003; Balaban et al. 2004). Also, this physiological feature is unique for dispersed cells that are highly expose to the action of the immune system. In contrast, cells responding to a high nutritional input and to elevated levels of extracellular Mg^{2+} and low QS AIP concentrations, present an inactive Agr bimodal switch, physiologically representing biofilm-encased *BRcells*. Given the transcriptional profile

of these cell type, *BRcells* are metabolically active and show higher predisposition to cell division, which affects its sensitivity to the action of antibiotics and the immune system. However, the matrix of the biofilm can protect cells from the action of antimicrobials (Egan and Vollmer 2013), which could enable *BRcells* to proliferate and develop long-term infections (Costerton 1999; Parsek and Singh 2003).

Taken together, the accumulative data on the complete transcriptomic profile of these cell lineages implies a major role of specific host micro-environmental signals in the activation of key *S. aureus* biofilm and virulence genes in an heterogeneous manner that involves the emergence of particular cell types that display division of roles and tasks during the development of a multicellular bacterial community. The expression profile of these cell lineages might be interpreted as the result of a specific mechanism of gene regulation that involves particular changes in gene expression in response to external signals and not, a global deregulation of the expression pattern in response to a generic environmental cue. We tested if the extracellular Mg^{2+} was causing a general deregulation in gene expression independently of the σ^B- and Agr- mediated mechanism. The expression of these selected housekeeping genes (Sihto, Tasara, and Stephan 2014) showed no significant differences between cells growing in TSB and in TSBMg, confirming that the observed transcriptomic profile for the *BRcells* and *DRcells* is ruled by a concerted genetic mechanism that involves the response to extracellular Mg^{2+}, the activation of the σ^B stress response and the bifurcation of the bacterial community as a consequence of the activation of the Agr PFL. Bacteria uses signal transduction pathways to sense environmental stress signals and promote physiological heterogeneity through the ability of PFL to generate bistability and bypass stochasticity in gene expression. Heterogeneity is then, a prerequisite for cell differentiation and, cell differentiation is mandatory for the emergence of division of labor, phenomenon that becomes evident in highly organized bacterial multicellular communities known as biofilms (Ackermann 2015).

7. Staphylococcal cell fates arise during *in vivo* infections

A central question in infectious biology concerns about the role of bacterial QS-mediated cooperative behavior during the progression of an infection. It is fundamental to understand if, during the invasion of host tissues, *S. aureus* has the ability to generate the bifurcation of the clonal population into physiologically different cell types and if so, to dissect the role and impact of these cell types during the progression of the staphylococcal infection. We followed the progression of the infection in different organs and furthermore, we tracked the appearance of *BRcells* and *DRcells* in these organs. Our results suggest that kidneys might possess specific conditions to promote the efficient colonization of this pathogen. Consistent with our *in vitro* results, organs characterized for being reservoirs of high levels of Mg^{2+}, such as kidneys, displayed significantly high bacterial proliferation when compared with organs depleted of this cation, such as the heart. Under normal physiological conditions in the body, Mg^{2+} concentrations vary from 1 mM in blood to several fold higher in the rectum while; the urinary tract, the amniotic fluid, the vagina, the gastrointestinal tract, and the neonatal lung have concentrations of Mg^{2+} that vary widely from 1 up to 45 mM (Wrong and Metcalfegibson 1965).

Tissues such as bones and organs such as kidneys have Mg^{2+} concentrations around the 40 mM and 60 mM, respectively (Elin 2010; Günther 2011; Jahnen-Dechent and Ketteler 2012).

Histological preparations of infected kidneys showed abundant proliferation of bacterial aggregates surrounded by immune cell infiltrates, indicative of long-term colonization during septicemia (Prabhakara et al. 2011) and, frequently associated with the renal model of staphylococcal biofilm formation (Günther 2011; Jahnen-Dechent and Ketteler 2012). On the other hand, no traces of microbial aggregates were found in heart and on the contrary, *S. aureus* cells in this organ were frequently found as individual and dispersed cells along the cardiac tissue. Confocal microscopy analyses showed a higher representation of the *BRcells* lineage within the kidney aggregates, with approximately three-fold more *BRcells* than *DRcells* in this organ. These results were consistent with our *in vitro* results, where *BRcells* is the lineage actively involved in biofilm formation due to its particular physiological state associated with high metabolic activity and active cell division and proliferation and hence, are preferentially found in actively growing areas of the multicellular community; as well as with reports that establish that kidneys are important reservoirs of Mg^{2+} in the body (Günther 2011; Jahnen-Dechent and Ketteler 2012). Interestingly, it has been reported that up to 82% of patients with urinary catheterization develop long-term *S. aureus* infections (Muder et al. 2006), which might reflect the ability of this bacteria to develop biofilm-encased multicellular communities in this Mg^{2+}-enriched organ; hindering the effectiveness of the antibiotic treatment and therefore, the complete clearance of this pathogen. Oppositely, infected hearts displayed an evident lower bacterial proliferation with no signs of active bacterial aggregation, suggesting that *S. aureus* cells that colonize heart tissues proliferate less actively and cannot establish a multicellular community. It has been largely observed that cardiac catheters promote biofilm formation in the inner cardiac tissue (the endocardium) (Götz 2002; Weidenmaier, Peschel, Xiong, et al. 2005; Gibson et al. 2007; Steger:2012ji Hanses et al. 2014). Nevertheless, in our systemic model of staphylococcal infection, endocarditis and biofilm formation within the heart valves were not evident, suggesting that the particular conditions where a prosthetic implant disturbs the cardiac tissue is the fundamental alteration promoting biofilm formation in heart but not, that this tissue is a target during hematogenous dissemination of a *S. aureus* infection. The dispersed cells found along the heart tissue showed to actively express the fluorescent marker that defines the *DRcell* subpopulation, which in agreement with our *in vitro* results indicate the lower metabolic activity and lower proliferation rate of these cells in relation with the reduced Mg^{2+} concentrations typically found in heart tissue (Günther 2011; Jahnen-Dechent and Ketteler 2012). Together, these results indicate that *DRcells* found in infected hearts might be dispersed cells coming from a different site of infection or, cells residing the heart but expressing a virulent physiological profile intended to rapidly disseminate through this organ expressing the set of *agr*-related genes such as cytolytic PSMs, RNAIII, proteases and other virulence factors, without establishing a multicellular aggregate such as the one found in kidneys, in a process of acute septicemia (McAdow et al. 2011). These results demonstrate that different organs display dissimilar infection patterns after an intravenous model of systemic infection, highlighting the importance of particular environmental conditions in the development of the staphylococcal infection.

We further determined the influence of extracellular Mg^{2+}-binding to the TAs on the infection outcome in these organs. Since it is not possible to deliberately deplete a specific organ of Mg^{2+}, we performed infection studies using the suite of *tagB*-lower and *tagB*-higher strains. In agreement with our *in vitro* results, in kidneys bacterial loads significantly decreased when infected with the *tagB*-lower strain. Interestingly, the pattern of infection among the different strains significantly differed in the different organs analyzed, suggesting that Mg^{2+} concentration in the colonization niche *in vivo* strongly contributes to the development of a chronic or acute *S. aureus* infection. The *tagB*-lower strain displayed a reduced ability to colonize renal tissue, probably due to the reduced amount of cell wall TA. Similarly, a *sigB*-deficient strain, which cannot display any level of repression of the Agr system, should behave similarly *in vivo*. We generated a new strain derived from the *tagB*-higher strain that also lacks σ^B. Higher TA content in this strain could increase cell wall rigidity in response to Mg^{2+}, but the lack of σ^B prevented activation of biofilm formation via downregulation of *agr*. As expected, we found reduced bacterial loads of this strain in infected kidneys. Although a *tagB*-higher strain might display enhanced colonization levels due to its increased ability to bind extracellular Mg^{2+}, the depletion of σ^B abolished any level of Agr repression and as consequence, reduced the emergence of the *BRcells* phenotype. On the other hand, a *tagB*-higher strain (with a functional σ^B activity) showed relatively higher bacterial loads in kidney tissues, in agreement with the proposed mechanism of Mg^{2+} sensing, σ^B activation and Agr repression. Expression of specific genes related to *BRcells* and *DRcells*, demonstrated that strains unable to sense efficiently extracellular Mg^{2+} such as the *tagB*-lower and Δ*sigB tagB*-higher, had relatively low expression of genes involved in biofilm formation, concomitant with a significant increase in the expression of QS and Agr-related genes, such as *agr* and *psm* genes. On the other hand, the *tagB*-higher strain, which is characterized by an increased ability to form biofilms *in vitro*, showed significantly high expression levels of the biofilm-related genes and a strong reduction in the expression of characteristic *DRcells* genes. Together, these results confirm that the reduction in colonization is associated with a marked representation of *DRcells* and an underrepresentation of the *BRcells* subpopulation. In contrast, the increased bacterial loads and the gene expression profile found in the *tagB*-higher strain, suggest that this strain differentiated a larger subpopulation of *BRcells*, which efficiently might promote the establishment and development of a long-term infection characterized by an efficient multicellular aggregation or, biofilm formation.

In cardiac tissue, we found the opposite behavior. In this tissue, *tagB*-lower and Δ*sigB tagB*-higher strains, which showed higher representation of *DRcells* based on their patterns of gene expression, produced higher colonization rates when compared to the wild type strain. In contrast, the *tagB*-higher strain, characterized for its increased representation of *BRcells* based on the overexpression of biofilm-related genes, produced significantly lower bacterial loads, corroborating the role of the cell wall TA in the sensing of extracellular Mg^{2+} as an effective mechanism to generate cell differentiation and the bifurcation of the staphylococcal population in biofilm-producers (*BRcells*) and virulent and planktonic (*DRcells*) cell types. In agreement with their infection patterns, gene expression of these strains showed moderate changes pointing to a significant decrease of the *BRcells* phenotype in this tissue, together

with an augmentation of *DRcells* representation, particularly associated with the strain that better colonized this organ: the *tagB*-lower and Δ*sigB tagB*-higher strains. Together, these results suggest that organs with low concentration of Mg^{2+} are not targets for biofilm formation and on the contrary, are prone to the dissemination of the staphylococcal infection through a process of acute bacteremia (McAdow et al. 2011) , characterized by the presence of a highly virulent *S. aureus* cell type, which expresses a whole repertoire of QS and Agr-related virulence factors, as it is the *DRcells* lineage. Interestingly, when comparing the relative gene expression levels between kidneys and hearts, it was evident a relatively lower expression of all genes in the heart tissue, suggesting the lower bacterial loads found in this organs but also, a feasible lower metabolic activity of the bacterial cells when residing on this kind of tissue.

We explored the infection pattern and *S. aureus* gene expression levels in organs such as livers, which had a moderate Mg^{2+} concentration (Günther 2011; Jahnen-Dechent and Ketteler 2012). In this organ, bacterial loads were high and comparable to those found in kidney but, the representation of the different *tagB*-lower, *tagB*-higher and Δ*sigB tagB*-higher *S. aureus* strains adopted a similar pattern to the one observed in hearts: increase in the colonization for the *tagB*-lower and Δ*sigB tagB*-higher, strains which cannot sense extracellular Mg^{2+} properly and, a relatively decrease for the *tagB*-higher strain. In principle, organs with low and moderate levels of Mg^{2+} might promote the overrepresentation of the *DRcells* subpopulation and the dissemination of an acute infection, which might partially correlate with the high bacterial loads found in the liver while; only organs with elevated levels of Mg^{2+} such as the kidneys could host biofilm-associated infections with the subsequent overrepresentation of the *BRcells* cell type. Nevertheless, the gene expression pattern for the *tagB*-lower and Δ*sigB tagB*-higher did not support this hypothesis. Although gene expression of the *BRcells*-related genes was moderately reduced in these strains when infecting the liver, the expression pattern for the *DRcells*-related genes did not account for an increased proportion of *DRcells*, opposite to what do happens in the heart and indeed, a general downregulation of the *DRcells* marker genes is observed in the Δ*sigB tagB*-higher strain.

Of interest, the high bacterial loads found for the strains unable to sense properly extracellular Mg^{2+} (*tagB*-lower and Δ*sigB tagB*-higher) was not associated with a significant augmentation in the expression of *DRcells* marker genes but curiously, the *tagB*-strain that is hypersensitive to Mg^{2+}, showed a strong expression of *BRcells*-related genes accompanied by a general downregulation of *DRcells*-relate genes but, did not display an enhanced colonization pattern as we should expect based on the role of *BRcells* in developing multicellular aggregates, as it happens in the kidneys. Based on these results, it could be plausible that this hypersensitive strain senses moderate levels of extracellular Mg^{2+} and indeed represses Agr activity via σ^B; in principle this might account for the emergence of the *BRcells* lineage. Nevertheless, additional and specific environmental conditions found in the liver may account for the premature disruption of the multicellular community, preventing this organ from high bacterial loads and/or a long-term biofilm-associated infection. On the other hand, those strains that displayed higher bacterial loads did not achieve it by means of and increased Mg^{2+} sensing or by an active process of

DRcells differentiation. Otherwise, *DRcells*-related genes would be significantly overexpressed for these strains in this organ. These results, instead of contradictory, highlight the importance of extracellular Mg^{2+} in the modulation of the gene expression and the subsequent bifurcation of the bacterial population into specialized cell types to establish a successful infection outcome. Also, is evident that within the host there are additional environmental cues such as gradients of oxygen, reactive Nitrogen and Oxygen species and other nutrients, among others, that coordinately act together with the Mg^{2+} to promote the emergence of cooperative traits in multicellular communities of *S. aureus*.

The analyses of the bacterial infection patterns in femora, spleen and lungs showed variable patterns related with the levels of Mg^{2+} found in these organs. Bones are also important reservoirs of Mg^{2+} in the body and although, our mouse-infection model does not facilitate the occurrence of osteomyelitis, we found a pattern of bacterial colonization similar to the infected kidneys. We observed that in bone tissue, the *tagB*-lower and Δ*sigB tagB*-higher strains could not establish an efficient colonization and, no bacterial infection was found for some of the infected mice processed. In contrast, the bacterial loads for the *tagB*-higher strain, which can sense extracellular Mg^{2+} efficiently, displayed slightly higher bacterial loads than the wild type strain and actually, all the mice infected reported bacterial loads over 10^8 CFU/g of tissue. Finally, organs such as the spleen and the lungs showed no particular patterns of bacterial infection associated with the ability of a particular strain to sense extracellular Mg^{2+}, suggesting, as in the liver, the key role of the Mg^{2+} in the development of a biofilm-associated staphylococcal infection.

In synthesis, we found that *in vivo* staphylococcal infection using the hematogenous spread route occurs in function of specific environmental cues found in specific organs, such as the presence of Mg^{2+}. In particular, we found the *S. aureus* infections developed a larger *DRcell* subpopulation in tissues with low Mg^{2+} levels (e.g., cardiac tissue), in which the microbial population did not develop multicellular aggregates and became instead, highly dispersed. Oppositely, the *BRcell* phenotype was more prevalent in tissues with high Mg^{2+} concentrations (e.g. bone, kidney), where bacteria organized in microbial aggregates characteristic of biofilm-associated infections. Interestingly, our results recapitulate clinical studies in which, remarkable number of biofilm-associated persistent infections develop in urinary tract and bone usually following surgery or catheterization (Muder et al. 2006; Brady et al. 2008; Flores-Mireles et al. 2015; Idelevich et al. 2016).

CONCLUSIONS

Nosocomial pathogens have evolved the ability to cause a broad range of infections within the same host, partly due to the presence of extracellular signals specific to particular colonization niches. In *S. aureus*, we have found that the presence of high, nevertheless physiologically relevant, concentrations of extracellular Mg^{2+} serve as a specific extracellular cue that promotes cell differentiation in *S. aureus*. In this work, we demonstrated that infections generated by clonal populations of this pathogen bifurcate into distinct specialized cell types that physically localize in different colonization tissues during the course of an infection. The prevalence of physiologically distinct bacterial cells in different infection niches provide bacteria with either an adaptive strategy to increase the chances to evade the immune system during bacterial infection or, with a *bet-hedging* strategy to increase the possibilities of survival if facing antimicrobial therapy (Beaumont et al. 2009).

We showed that Mg^{2+} acts as a specific environmental cue that promotes cell differentiation, division of labor and biofilm formation in *S. aureus*. Precisely, available Mg^{2+} cations specifically bind the phosphate-rich teichoic acids found in these bacteria in order the alleviate the repulsive interaction between the neighboring phosphate, forming a consolidate network which strength the cell wall, resembling the mechanism exerted by the D-alanylation machinery of *S. aureus*. The binding of free Mg^{2+} to the teichoic acids stabilizes the cell wall and furthermore, increases the rigidity of the staphylococcal cell wall, event that is intracellularly sensed by the alternative sigma factor σ^B and activates a stress response mechanism characterized by the repression of the Agr QS system. Previous studies showed that biofilm formation in *S. aureus* is highly dependent on the activity of the *dlt* operon gene product (Gross et al. 2001), as well as for developing biofilm-associated infections *in vivo* (Weidenmaier, Peschel, Kempf, et al. 2005). Our results are consistent with these findings suggesting that, although many different extracellular signals are probably responsible for defining the outcome of *S. aureus* infections, it is likely that the mere presence of extracellular Mg^{2+} and hence, the augmentation in cell wall rigidity are an important cue to triggers biofilm formation and the development of biofilm-associated infections. We demonstrated the importance of Mg^{2+} as a specific extracellular signal that promotes the development of staphylococcal multicellular aggregates, which display an organized pattern of temporal and spatial distribution of the divergent cell types, supposing the emergence of a concerted phenomenon of division of labor. As is already known, bacterial communities encased within biofilms are the result of a genetic-programmed process of cell differentiation that raises the emergence distinct subpopulations of specialized cells responsible of specific task in the different stages of the biofilm formation (Aguilar et al. 2007; J. A. Shapiro 1998; J. J. Schuster and Markx 2013; Vlamakis et al. 2008).

Our results have demonstrated that in response to the presence of extracellular Mg^{2+}, the repression of the Agr system upregulates the expression of a repertoire of genes involved in biofilm formation,

promoting the emergence of a particular cell line (*BRcells*) involved in biofilm formation and characterized to simultaneously express the genes responsible for the synthesis of the attachment MSCRAMM protein SpA, the *icaADBC* operon involved in the synthesis of the major matrix exopolysaccharide of staphylococcal species and some other adhesins such as ClfA and IsdA, associated with initial attachment during the process of biofilm formation. Deep-sequencing transcriptomic analyses of this cell type showed that *BRcells* display a particular physiological state characterized by a high expression of *sigB*, the downregulation of the *agr* operon and a metabolically active state prone to DNA replication, cell proliferation and cell division. Therefore, during early stages of niche colonization, the repression of Agr allows the synthesis of structural components involved in the first steps of biofilm formation. As the multicellular aggregate grows and expands, high cell densities are reached and the Agr QS system is derepressed by the activation of the PFL that resides within the *agr* operon, leading to the spatially localized accumulation of the QS molecule AIP, which once reaches a determined threshold concentration stimulates the downregulation of several genes associated with the *BRcells* cell type and, promotes the bifurcation of the staphylococcal population in a second exclusive cell line (*DRcells*), characterized by the simultaneous expression of the Agr-dependent operons *psmα* and *psmβ* genes, involved in biofilm maturation, cell detachment, virulence and toxicity. As *BRcells*, the *DRcells* cell line possesses a particular physiological state characterized by a reduced metabolic activity and low *sigB* expression, the specific expression of genes associated with QS, virulence and immune evasion.

Using *in vitro* techniques and *in silico* mathematical simulations, we found that the concerted temporal and spatial localization of these two cells types determines the structural and physiological development of these multicellular aggregates and drives the dynamics of biofilm formation and dispersal that characterizes in many cases the long-term infectious pattern found during the staphylococcal disease. Therefore, we propose that the Agr system, composed of a particular genetic architecture of the adjacent P2 and P3 promoters, is an autonomous system based on a positive feedback loop that generates bimodality in gene expression and hence, cell heterogeneity within the staphylococcal community; controlling temporally and spatially the process of cell differentiation. Using a repertoire of conditions and genetic backgrounds we demonstrated that the absence Mg^{2+} and, the *sigB* or *agr* genes influenced the emergence of cell heterogeneity and profoundly altered the architectural features of the multicellular aggregates. The adaptational behavior achieved by bacterial biofilms is characterized by a sophisticated and intricate genetic developmental process triggered by external stress signals that has been selected through evolution to assure bacterial survival and involves regulatory circuits that cause temporary rather than permanent genetic alterations (la Fuente-Núñez et al. 2013).

When bacteria decides to establish a multicellular community, cooperation becomes a norm among the different individuals and division of labor arises in order to provide the population with signal molecules that coordinate and synchronize communication between cells, extracellular enzymes that extract nutrients from the environment and process structural components, antibiotics that outcompete

other species, extracellular polymers that facilitate the establishment and maturation of the biofilm and surfactants that enable cooperative movement and dissemination (Dogsa, Oslizlo, and Stefanic 2014). Evolution has granted division of labor within bacteria biofilms as an effective mechanism to enhance the total fitness of the entire clonal bacterial community (Veening, Smits, and Kuipers 2008). Prime examples have been provided by the studies on the cellular differentiation in *B. subtilis* biofilms, which have demonstrated that these multicellular communities follow a precise spatiotemporal ordering dictated by a *nutritional stated-dependent logic* (Branda et al. 2001; Smits, Kuipers, and Veening 2006; Vlamakis et al. 2008; López, Vlamakis, and Kolter 2009; Serra and Hengge 2014).

Bacterial heterogeneity is the fundamental biological phenomenon behind biofilm formation and dissemination in bacteria and at the same time, QS genetic networks drive most of the cell heterogeneity in bacteria (Stoodley et al. 2002). Bacterial QS promotes cooperativity by controlling the production of public goods for the benefit of any member of the community. The communication system enabled by QS provides a system for the effective coordination of cooperative behaviors, co-regulating the functions required for utilization of public and private good and providing a genetic mechanism against cheating. In natural habitats, QS could serve to promote multicellular behaviors and ultimately the dispersal of individuals to new niches where cooperative behavior is needed, selecting phenotypes against social cheats (Dandekar, Chugani, and Greenberg 2012). Given the fact that QS regulates global gene expression patterns, other regulatory systems might have co-opted QS heterogeneity to control additional products, probably also in a bistable manner, in response to particular environmental conditions (Rutherford and Bassler 2012).

The large amount of *in vitro*, *in silico* and *in vivo* data, undoubtedly will help to understand how nosocomial pathogens such as *S. aureus* can simultaneously cause dissimilar types of infections in distinct organs, and show that cell differentiation in nosocomial pathogens is particularly relevant for adaptation to different host tissues. Understanding cell differentiation in pathogenic bacteria is fundamental in order to design new anti-infective strategies that target specific cell subpopulations, which in the case of *S. aureus* is considered endemic in hospitals and, with a mortality rate of about 20%, is currently one of the leading causes of death by a single infectious agent (Klevens et al. 2007).

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APPENDIX A

Table S1. List of bacterial strains.

Species	Strain	Genotype	Strain number	Reference
Escherichia coli	DH5α	Wild type	JCGB-0065	(Reusch, Hiske, and Sadoff 1986)
Bacillus subtilis	168	Wild type	IW 112	(Moszer, Glaser, and Danchin 1995; Moszer et al. 2002)
		amyE::P_{hp}-*agrAC*	JCGB-0558	This work
		amyE::P_{hp}-*agrAC* *lacA*::$P_{agrA(RNAII)}$-*yfp*	JCGB-0560	This work
		amyE::P_{hp}-*agrAC* *lacA*::$P_{psm\alpha 1-4}$-*yfp*	JCGB-0443	This work
		amyE::P_{hp}-*agrAC* *lacA*::$P_{psm\beta 1-2}$-*yfp*	JCGB-0442	This work
		amyE::P_{hp}-*agrAC* *lacA*::$P_{icaADBC}$-*yfp*	JCGB-0445	This work
		amyE::P_{hp}-*agrAC* *lacA*::P_{spa}-*yfp*	JCGB-0444	This work
		amyE::P_{hp}-*agrAC* *lacA*::P_{RNAIII}-*yfp*	JCGB-0568	This work
		amyE::P_{hp}-*agrAC* *lacA*::*yfp*-$_{IGRp3p2}$-*cfp*	JCGB-0655	This work
		lacA::$P_{psm\alpha 1-4}$-*yfp*	IW 145	This work
		lacA::$P_{psm\beta 1-2}$-*yfp*	IW 142	This work
		lacA::$P_{icaADBC}$-*yfp*	IW 146	This work
		lacA::P_{spa}-*yfp*	IW 144	This work
Staphylococcus aureus	RN 4220	Wild type	JCGB-0068	(Kornblum et al. 1990)
	USA 300 LAC*	Wild type	JCGB-0577	(McDougal et al. 2003)
		amy::$P_{icaADBC}$-*yfp*	JCGB-0724	This work
		amy::P_{spa}-*yfp*	JCGB-0725	This work
		amy::$P_{psm\alpha 1-4}$-*yfp*	JCGB-0726	This work
		amy::$P_{psm\beta 1-2}$-*yfp*	JCGB-0727	This work
		tagB::ϕNΣ (erm)	JCGB-0669	This work
		tagH::ϕNΣ (erm)	JCGB-0665	This work
		tagG::ϕNΣ (erm)	JCGB-0670	This work
	Reynolds	Wild type	JCGB-0224	(Albus, Arbeit, and Lee 1991)
	UAMS-1	Wild type	JCGB-0226	(Gillaspy et al. 1995)
	COL	Wild type	JCGB-0225	(Dyke 1969)
	Newman	Wild type	JCGB-0223	(DUTHIE and LORENZ 1952; Lipiński, Hawiger, and Jeljaszewicz 1967)
		NewHG	JCGB-0069	(Markus Mainiero 2010)
		NewHG *amy*::$P_{icaADBC}$-*yfp*	JCGB-0177	This work
		NewHG *amy*::P_{spa}-*yfp*	JCGB-0714	This work
		NewHG *amy*::$P_{psm\alpha 1-4}$-*yfp*	JCGB-0070	This work
		NewHG *amy*::$P_{psm\beta 1-2}$-*yfp*	JCGB-0715	This work
		pAmy$P_{psm\alpha 1-4}$-*yfp*	JCGB-0662	This work
		amy::P_{dnaA}-*yfp*	JCGB-0205	This work
		amy::$P_{icaADBC}$-*yfp*	JCGB-0151N	This work
		amy::P_{spa}-*yfp*	JCGB-0203	This work
		amy::$P_{psm\alpha 1-4}$-*yfp*	JCGB-0148N	This work
		amy::$P_{psm\beta 1-2}$-*yfp*	JCGB-0356	This work

		amy::P$_{spa}$-*yfp* *lac*::P$_{icaADBC}$-*mars*	JCGB-0490	This work
		amy::P$_{psm\alpha 1-4}$-*yfp* *lac*::P$_{icaADBC}$-*mars*	JCGB-0470	This work
		amy::P$_{spa}$-*yfp* *lac*::*Ppsmα1-4-mars*	JCGB-0464	This work
		amy::P$_{psm\beta 1-2}$-*yfp* *lac*::P$_{psm\alpha 1-4}$-*mars*	JCGB-0487	This work
		amy::P$_{icaADBC}$-*yfp* *lac*::P$_{clfA}$-*mars*	JCGB-0689	This work
		amy::P$_{icaADBC}$-*yfp* *lac*::P$_{isdA}$-*mars*	JCGB-0699	This work
		amy::P$_{spa}$-*yfp* *lac*::P$_{clfA}$-*mars*	JCGB-0701	This work
		amy::P$_{spa}$-*yfp* *lac*::P$_{isdA}$-*mars*	JCGB-0702	This work
		agrBDCA::tet	JCGB-0149N	(Novick 1990)
		agrBDCA::tet *amy*::P*psmα1-4*-yfp	JCGB-0346	This work
		agrBDCA::tet *amy*::P*psmβ1-2*-yfp	JCGB-0394	This work
		icaADBC::cat	JCGB-0299	This work
		spa::spc	JCGB-0150N	(Oesterreich et al. 2014)
		icaADBC::cat *spa*::spc	JCGB-0301	This work
		psmα1-4::kan	JCGB-0236	This work
		psmβ1-2::tet	JCGB-0620	This work
		sigB::erm	JCGB-0229	(Koch et al. 2014)
		sigB::erm amy::P*psmα1-4*-yfp	JCGB-0547	This work
		sigB::erm amy::P*psmβ1-2*-yfp	JCGB-0549	This work
		dltA::spc	JCGB-0663	(Peschel et al. 1999)
		saeRS::kan	JCGB-0212	(Markus Mainiero 2010)
		saeRS::kan amy::P*icaADBC*-yfp	JCGB-0104	This work
		saeRS::kan amy::P*spa*-yfp	JCGB-0251	This work
		saeRS::kan amy::P*psmα1-4*-yfp	JCGB-0080	This work
		saeRS::kan amy::P*psmβ1-2*-yfp	JCGB-0395	This work
		tagB::ϕNΣ (erm)	JCGB-0676	This work
		tagH::ϕNΣ (erm)	JCGB-0672	This work
		tagG::ϕNΣ (erm)	JCGB-0677	This work
		icaADBC::cat *spa*::spc *amy*::P$_{psm\beta 1-2}$-*yfp*	JCGB-0449	This work
		pJL74$_{tagB}$	JCGB-0711	This work
		pJL74	JCGB-0697	This work
		sigB::erm pJL74$_{tagB}$	JCGB-0713	This work
		sigB::erm agrBDCA::tet	JCGB-0715	This work

Table S2. List of plasmids.

Name	Description	Reference
pKM003	Harbors the *yfp* gene and an improved RBS for Gram-positive bacteria.	Prof. David Rudner, Harvard Medical School, Boston, USA.
pKM008	Harbors the *cfp* gene and an improved RBS for Gram-positive bacteria.	Prof. David Rudner, Harvard Medical School, Boston, USA.
pKMmars	Harbors the *rfp*-mars gene and an improved RBS for Gram-positive bacteria.	This work
pMAD	Shuttle vector for gene inactivation in naturally nontransformable *S. aureus*. *bla*, Ori pBR325, *ermC, bgaB, pclpB*.	(Arnaud, Chastanet, and Débarbouillé 2004)
pAmy	pMAD variant that harbors a MCS flanked by homologous regions to the Amylase gene of *S. aureus* strain Newman.	(Yepes et al. 2014)
pAmyP$_{spa}$-*yfp*	pAmy harboring the promoter region and 100 bp of the *spa* gene of *S. aureus* fused to the *yfp* gene.	This work
pAmyP$_{ica}$-*yfp*	pAmy harboring the promoter region of *ica* operon of *S. aureus* fused to the *yfp* gene.	This work
pAmyP$_{psm\alpha 1-4}$-*yfp*	pAmy harboring the promoter region *psmα* operon of *S. aureus* fused to the *yfp* gene.	This work
pAmyP$_{psm\beta 1-2}$-*yfp*	pAmy harboring the promoter region *psmβ* operon of *S. aureus* fused to the *yfp* gene.	This work
pLac	pMAD variant that harbors a MCS flanked by homologous regions to the Beta galatosidase gene of *S. aureus* strain Newman.	(Yepes et al. 2014)
pLacP$_{spa}$-*rfp*	pLac harboring the promoter region and 100 bp of the *spa* gene of *S. aureus* fused to the *rfp*-mars gene.	This work
pLacP$_{ica}$-*rfp*	pLac harboring the promoter region of *ica* operon of *S. aureus* fused to the *rfp*-mars gene.	This work
pLacP$_{psm\alpha 1-4}$-*rfp*	pLac harboring the promoter region *psmα* operon of *S. aureus* fused to the *rfp*-mars gene.	This work
pLacP$_{psm\beta 1-2}$-*rfp*	pLac harboring the promoter region *psmβ* operon of *S. aureus* fused to the *rfp*-mars gene.	This work
pDR111	Vector for integration into the *amyE* gene. It carries a polylinker downstream of the Phy-spank promoter and the gene for the *LacI* repressor. Spectinomycin resistance.	(Kearns 2005)
pDR183	Vector for ectopic integration for double crossover insertions into the nonessential *lacA* locus, Chloramphenicol resistant.	(Doan, Marquis, and Rudner 2005)
pDG1663	Vector for ectopic integration at the *thrC* locus in *B. subtilis*, MLS resistant.	(Guérout-Fleury, Frandsen, and Stragier 1996)
pIW001	Vector for ectopic integration at the *thrC* locus in *B. subtilis*, Chloramphenicol resistant.	This work
pJL74	Replicative plasmid that harbors the *sarA* P1 promoter and the *sodA* RBS for enhanced expression of proteins of *S. aureus*. Ampicillin and Erythromycin resistance.	(Klijn et al. 2006)
pJL74$_{tagB}$	pJL74 harboring the complete ORF of the *tagB* gene of *S. aureus* strain Newman (NWMN_0187).	This work

Table S3. List of DNA oligonucleotides.

Name	Sequence (5' - 3')	Reference
Gene promoter amplification		
pICA-fwd	TgA ATT CTC CgT AAA TAT TTC CAg AAA ATT C	This study
pICA-rev	TAA gCT TTT CTT TAC CTA CCT TTC gTT Ag	
pSpA-fwd	TAA gCT TCA ATA CCT ACA CCT AgT TTA C	This study
pSpA-rev	TgA ATT CAC ATA ATg AAC AAC TTT CTA T	
pPSMa-fwd	TgA ATT CAA TCT CTC gCA TAA TTg CTT ATg	This study
pPSMa-rev	TAA gCT TAA gAT TAC CTC CTT TgC TTA Tg	
pPSMb-fwd	TgA ATT CAg gCA ACT TAA TTg TgT TAA A	This study
pPSMb-rev	TAA gCT TTg AAA ACA CTC CTT AAA ATT T	
pClfA-fwd	TgA ATT CAA gCg ATT gAT TCA AgC ATT A	This study
pClfA-rev	TAA gCT TTA TTC CCT CTT TTT AAA AAg T	
pIsdA-fwd	TgA ATT CTT ggC AgT gTT TTT ACT AAT A	This study
pIsdA-rev	TAA gCT TTg TTT TCC TCC TAA ggA TAC A	
Gene flanking region amplification		
Amy-flank-pMAD-fwd	AAg AAg CTg AAg AAg CTg gCg	This study
Amy-flank-pMAD-rev	TTg TAT TCg CTg CCA CTT TCg	
Lac-flank-fwd	gTT ggC gAA CAA gTT gTT AgC	This study
Lac-flank-rev	gCg CAA gTA CTC TTg TTA CAT	
ica-R-flank-fwd	ATC TTA ATA TTC TCA ATA TCg	This study
ica-flank-rev	CCg CgT gTT TTT AAC ATA gCT	
SpA-flank-fwd	gTA TTg CAA TAC ATA ATT CgT	This study
SpA-flank-rev	AAT gAT ATC TAT CgT TgT gTA	
PSMa-flank-fwd	gCC CTA Tgg ATA AAA TgC AAA	This study
PSMa-flank-rev	ATT gCA TTg ATA AgT ggC TTg	
PSMb-flank-fwd	TTT gTT ATC TTC AAA TTT AAA	This study
PSMb-flank-rev	TgC ATA ACA TTA AAg TAT gCT	
agr-flank-fwd	ATA ATg ACA gTg Agg AgA gTg	This study
agr-flank-rev	TgA ATA CgC CgT TAA CTg ACT	
ica-upstream-fwd	gTC gAC TAT TCT TTA TAT TTg TgA ATg	This study
ica-upstream-rev	CTT gAT AAT AAg ggT AAC TAT TgC CAT TCT CTA ACA TTA TgT ATA A	
ica-downstream-fwd	ggg TAA CTA gCC TCg CCg gTC CAC ggC TAT gTT AAA AAC ACg Cgg T	This study
ica-downstream-rev	ggA TCC ACg CCT ACA AAA CAT gAT Tgg	
PSMa-upstream-fwd	gTC gAC TgT ggC gTg TTT TAT gTT TgA	This study
PSMa-upstream-rev	CCT ATC ACC TCA AAT ggT TCg CTg TTT gAA gAT CCA TAT CAA Tgg	
PSMa-downstream-fwd	CgA gCg CCT ACg Agg AAT TTg TAT CgA TgT TgA CCA TgA ATA CCg AA	This study
PSMa-downstream-rev	ggA TCC ATA gAg gTT AAg CTC ATg AAT	
PSMb-upstream-fwd	TgT CgA CCC gTA ATC ACg gAA CTC TTT T	This study
PSMb-upstream-rev	gAg AAC AAC CTg CAC CAT TgC AAg ATg AAA ACA CTC CTT AAA ATT T	
PSMb-downstream-fwd	TGG ATC CAT TAG TAA CAA TAT ATA AAT	This study
PSMb-downstream-rev	GGG ATC AAC TTT GGG AGA GAG TTC TAT AAT AAC TAA TAT TCT TTA	
Gene amplification		
YFP-pkm003-fwd	CAA TTg gAg TAT TTT gTT gAT	This study
YFP-pkm003-rev	TCA TAT gAA ACA gCA TgA CTT	
Mars-RFP-RBS-fwd	TAA gCT TAC AAA ggg AgA TAC TAC TAT ggC ATC ATC AgA AgA TgT T	This study
Mars-RFP-RBS-rev	Tgg ATC CTT ATC CTg CAC CTg TTg AAT g	
agrC-Fwd-Hind-RBS	TAA gCT TAC ATA Agg Agg AAC TAC TgT ggA ATT ATT AAA TAg TTA T	This study

agrA-Rev-SphI	TgC ATg CTT ATA TTT TTT TAA CgT TTC T	
pRNAIII-P3-Fwd-EcoRI	TgA ATT CTA ATA CTT TCT ACA TAA CAC C	This study
pRNAIII-P3-Rev-HindIII	TAA gCT TTA TTA AAA CAT gCT AAA AgC A	
YFP-fwd-SynBio	gAA TTC TTA TTT gTA Tag TTC ATC CAT	This study
YFP-rev-SynBio	gCT TTT AgC Atg TTT TAA TAT ACA TAA ggA ggA ACT ACT ATg	
P2-P3-IGR-fwd	CAT AgT AgT TCC TCC TTA TgT ATA TTA AAA CAT gCT AAA AgC	This study
P2-P3-IGR-rev	CAT AgT AgT TCC TCC TTA TgT AAT TTT ACA CCA CTC TCC TCA	
mCherry/cfp-fwd-SynBio	TgA ggA gAg Tgg TgT AAA ATT ACA TAA ggA ggA ACT ACT ATg	This study
mCherry/cfp-rev-SynBio	ggA TCC TTA CTT gTA Cag CTC gTC CAT	
tagB-RBS-fwd	TgA ATT CTT Agg Agg ATg ATT ATT TAT gAC AAA AAC gAA ACA AgC A	This study
tagB-rev	TCC ATg GTT AgC gTC TAA ACA AAT CTT T	
Gene amplification for RT qPCR		
qGyrA-fwd	TAT CCg CTT gTT gAT ggC CAA gg	This study
qGyrA-rev	CgC gCT TCA gTA TAA CgC ATT gC	
qGapA-fwd	TAC ACA AgA CgC ACC TCA CAg A	(Pang et al. 2010)
qGapA-rev	ACC TgT TgA gTT Agg gAT gAT gTT T	
qFtsQ-fwd	TgT AAT TTC AgT CgA CAA CgA	This study
qFtsQ-rev	TTg TCg CTT ACg TCT TAA CTT	
qTagX-fwd	CCT ACA TgT AAT AAT gAg gCA	This study
qTagX-rev	gTA ACC gTT CAA TCA TAg gAA	
qEssC-fwd	Agg CgA AgT CAT TCA TTT AgA	This study
qEssC-rev	ATg ATg CAT AAT CAg CTT CTg	
qEsaA-fwd	TCg CAA TTg TTA ACg Agg ATC	This study
qEsaA-rev	AAA CCA gAC TCA gCA ACg TTT	
qPSMa-fwd	TAT CAA AAg CTT AAT CgA ACA ATT C	(Koch et al. 2014)
qPSMa-rev	CCC CTT CAA ATA AgA TgT TCA TAT C	
qSigB-fwd	TgC CgT TCT CTg AAg TCg TgA	(Koch et al. 2014)
qSigB-rev	ATg ggg CAA CAA gAT gAC CA	
qYFP-fwd	ATT AgA Tgg TgA TgT TAA Tgg g	This study
qYFP-rev	TAA CCT TCg ggC ATg gCA CTC TTg	
qDltA-fwd	TgC CAA CgA CTg AAg TTA Cg	This study
qDltA-rev	ATg gCA ATT gTT CCA TCC AT	
qAgrA-fwd	gTg AAA TTC gTA AgC ATg ACC CAg TTg	This study
qAgrA-rev	TgT AAg CgT gTA TgT gCA gTT TCT AAA C	
qHtrA-fwd	CAT ACA gAT CAT TCT ACA ACA	This study
qHtrA-rev	gTA CTA gAA gTg CAC CAA TAA	
qIcaA-fwd	gTT gTC gAC gTT ggC TAC Tg	This study
qIcaA-rev	AgA CCT CCC AAT gTT TCT gg	
qIcaB-fwd	TTA TCA CAg gTC ATg TTg gg	(Koch et al. 2014)
qIcaB-rev	ATC gTC ATT CAT CAA gCC AT	
qSpa-fwd	TgA ATC TCA AgC ACC gAA Ag	(Koch et al. 2014)
qSpa-rev	TTg CTC ACT gAA ggA TCg TC	
qPsmb-fwd	TTA ACg CAA TTA AAg ATA CCg	This study
qPsmb-rev	ACC TAA TAA ACC TAC gCC ATT	
qAgrB-fwd	CCC ATT CCT gTg CgA CTT AT	This study
qAgrB-rev	gAA TTg ggC AAA Tgg CTC TT	
qFtsZ-fwd	TTA CTg gTg gCg AgT CAT Tg	This study

qFtsZ-rev	TTT ACg CTT gTT CCg AAT CC	
qRecA-fwd	CTT TCg gTA AAg gTg CCg TA	This study
qRecA-rev	CCA CCT ACA CCT AgC gCA TT	
q16srRNA-fwd	gCg gTA ATA CgT Agg Tgg Cag	This study
q16srRNA-rev	TTT CCA ATg ACC CTC CAC gg	
qSaeRS-fwd	CgA AgA TgC TAA TAC CgT gAA	This study
qSaeRS-rev	CCT AAT CCC CAT ACA gTT gTg A	
qSarR-fwd	gCT AAg TgC TCA gAg TTC AAA CC	This study
qSarR-rev	TgT TCT TTC gTC TTg TAA ACT TCT TT	

Supplemental Information (SI) 1. Gene quantification and differential expression analysis of BRcells and DRcells.

Available in digital format as *DESeq_Gene_quant.xlsx*.

Microsoft Excel® datasheet containing the tables for gene-wise quantification calculated for different cDNA libraries used in this study and the DESeq analysis data for the library comparison of *BRcells+* against *BRcells–*, *DRcells+* against *DRcells–* and *BRcells+* against *DRcells+*.

SI 2. Functional classification and Gene Ontology.

Available in digital format as *GO_Total_annot_count.xlsx*.

Microsoft Excel® datasheet containing the tables of functional classification and Gene Ontology classification of annotated genes. This data table contains the tables for the functional classification of the annotated genes that showed significant log2fold change value for differential expression generated by DESeq analysis. The sets of genes were selected by using thresholds of >8- and >25-reads and were annotated by TIGRFAM and SEED databases derived annotations. Additionally, this table contains the functional classification of up- and down-regulated genes for all the different comparison between the four cDNAs libraries. The Gene Ontology (GO) enrichment analysis was carried out using in house scripts for which the GO annotation was taken from UniProt database and the p-values were adjusted by Bonferroni and Benjamini-Hochberg correction method.

SI 3. Hypergeometric analysis for library comparison.

Available in digital format as *Hypergeometric.xlsx*.

Microsoft Excel® datasheet containing the tables comprising the log2fold values for differentially expressed genes that were shown to be expressed by DESeq analysis in both the sample sets, *BRcells+* against *BRcells-* and *DRcells+* against *DRcells–* and the hypergeometric probability was calculated for the regulated genes.

SI 4. Hypergeometric analysis for library comparison.

Available in digital format in: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69835

This web site address contains the RNAseq libraries de-multiplexed FASTQ files and coverage files in wiggle format deposited in NCBI Gene Expression Omnibus (Edgar, Domrachev, and Lash 2002) and accessible through GEO Series accession number GSE69835.

APPENDIX B

List of collaborators

Dr. Ángel Goñi-Moreno, School of Computing science, Newcastle University (United Kingdom), performed the mathematical modeling and the computational simulations of the synthetic Agr orthologous system created in *B. subtilis*.

Dr. Christina Kuttler and Thomas Horger from the group of Prof. Dr. Barbara Wohlmuth, Department of Mathematics of the Technical University of Munich, performed the mathematical modeling and computational simulations of the development of the multicellular communities of *S. aureus*.

Melanie Schott, from the group of Prof. Dr. Alma Zernecke, Institute of Clinical Biochemistry and Pathobiochemistry of the University of Würzburg, performed the thin cryosectioning of the multicellular aggregates of *S. aureus* and the histological thin sectioning of the infected mice organs.

Dr. Knut Ohlsen, Institute for Molecular Infection Biology, University of Würzburg, provided the animals for the *S. aureus* infections, performed the intravenous infections and the sacrifice of the infected mice. In addition, we kindly provided laboratory facilities for the completion of this project.

Malvika Sharan, Institute for Molecular Infection Biology, University of Würzburg, processed the files derived from the cDNA libraries sequenced by Illumina's HiSeq RNAseq and performed the DESeq analysis for differential gene expression. She wrote the script to generate the Newick file for the visualization of the parsimony mapping of the fluorescence inheritance in *S. aureus* cells.

Julian Eikmeier, Institute for Molecular Infection Biology, University of Würzburg, standardized the protocol for the FACS-physical separation of the different cell types present in multicellular aggregates of *S. aureus*.

LIST OF PUBLICATIONS

2016

Juan C Garcia-Betancur, A Goñi-Moreno, T Horger, M Schott, M Sharan, J Eikmeier, B Wohlmuth, A Zernecke, K Ohlsen, C Kuttler, D Lopez.
Cell-fate decision defines acute and chronic infection cell types in *Staphylococcus aureus.*
PLoS Biology (Submitted, under revision).

2014

Yepes Ana, Koch Gudrun, Waldvogel Andrea, **Garcia-Betancur Juan Carlos**, Lopez Daniel.
Reconstruction of mreB expression in *Staphylococcus aureus* via a collection of new integrative plasmids.
Appl Environ Microbiol.
2014 Jul; 80(13): 3868-78.

2013

Flórez-Zapata N, **García JC**, Del Portillo P, Restrepo S. Uribe-Vélez D.
Composition And Function Of The Microbial Community Related With The Nitrogen Cycling On The Potato Rhizosphere.
Acta Biol. Colomb.
18(3):449-464.

2012

Yepes A, Schneider J, Mielich B, Koch G, **García-Betancur JC**, Ramamurthi KS, Vlamakis H, Lopez D.
The Biofilm Formation Defect of a *Bacillus subtilis* Flotillin-defective Mutant involves the Protease FtsH.
Mol Microbiol.
2012 Oct; 86(2): 457-71.

Garcia-Betancur, J. C., Yepes, A., Schneider, J., Lopez, D.
Single-cell Analysis of *Bacillus subtilis* Biofilms using Fluorescence Microscopy and Flow Cytometry.
J. Vis. Exp.
2012 Feb; 15 (60)

Schneider J, Yepes A, **Garcia-Betancur JC**, Westedt I, Mielich B, López D.
Streptomycin-Induced Expression in *Bacillus subtilis* of YtnP, a Lactonase-Homologous Protein That Inhibits Development and Streptomycin Production in *Streptomyces griseus*.
Appl Environ Microbiol.
2012; 78(2):599-603.

J.C. Garcia-Betancur, M. Carmen Menendez, P. Del Portillo, M.J. Garcia
Alignment of multiple complete genomes suggests that gene rearrangements may contribute towards the speciation of Mycobacteria.
Infection, Genetics and Evolution.
2012 Jun; 12(4): 819-26

Curriculum vitae

Juan Carlos García Betancur
Doctoral Fellow at the Center for Infectious Diseases Research (ZINF-IMIB)
University of Würzburg, Germany.

juan.garcia_betancur@uni-wuerzburg.de

EDUCATION

PhD in Infection and Immunity University of Würzburg, Würzburg, Germany	May 2011 - present
MSc in Biological Sciences (Molecular Microbiology) Los Andes University, Bogotá, Colombia	2006 - 2008
BSc in Microbiology with a Minor in Chemistry Los Andes University, Bogotá, Colombia	2001 - 2005

RESEARCH EXPERIENCE

2011-present	PhD student	Bacterial cell differentiation in multicellular communities of *Staphylococcus aureus* Dr. Daniel López University of Würzburg, Germany
2009-2010	Research assistant	Metagenomics of *Solanum phureja* agricultural soils Patricia del Portillo Corpogen. Bogotá, Colombia
2008-2009	Research assistant	Comparative genomics of *M. tuberculosis* Dr. María Mercedes Zambrano Corpogen. Bogotá, Colombia
2006-2008	MSc student	Comparative transcriptomics in WSSV infected *Litopenaeus vannamei* Dr. Clarissa Granja MD. Corpogen. Bogotá, Colombia
2005-2006	BSc student	Neuro-immune virology of RABV Dr. Jaime Castellanos Institute of virology. El Bosque University. Bogotá, Colombia

AWARDS AND FELLOWSHIPS

2014
Fellowship from the Wilhelm and Else Heraeus Foundation.
566. WE-Heraus-Seminar on Mechanisms, Strategies, and Evolution of Microbial Systems at the Physikzentrum Bad Honnef.
June 15th to 19th.
Bad Honnef, Germany.

2013
Fellowship from the ERASynBio (European Research Area), the KBBE Synthetic Biology ST-flow FP7-project and the CSIC.
Summer School, Synthetic Biology in action! National Biotechnology Center CSIC.
June 23rd to July 6th.
Madrid, Spain.

2011
Fellowship from the Graduate School of Life Sciences (GSLS) of the University of Würzburg, funded by the Excellence Initiative of the German Federal and State Governments.
Doctoral position at the ZINF-IMIB.
Würzbug, Germany

2009
Fellowship from the International Centre for Genetic Engineering and Biotechnology (ICGEB).
Practical Course in Bioinformatics: Computer Methods in Molecular Biology. Co-sponsored by CEI -Central European Initiative.
Area Science Park, Trieste, Italy

2007
Mobility Fellowship from the Colombian Scientific Administrative Department – COLCIENCIAS.
Aquaculture Europe.
Istanbul, Turkey.

PUBLICATIONS

2016
Juan C Garcia-Betancur, A Goñi-Moreno, T Horger, M Schott, M Sharan, J Eikmeier, B Wohlmuth, A Zernecke, K Ohlsen, C Kuttler, D Lopez.
Cell-fate decision defines acute and chronic infection cell types in *Staphylococcus aureus.*
PLoS Biology (Submitted, under revision).

2014
Yepes Ana, Koch Gudrun, Waldvogel Andrea, **Garcia-Betancur Juan Carlos**, Lopez Daniel.
Reconstruction of mreB expression in *Staphylococcus aureus* via a collection of new integrative plasmids.
Appl Environ Microbiol.
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2013
Flórez-Zapata N, **García JC**, Del Portillo P, Restrepo S. Uribe-Vélez D.
Composition And Function Of The Microbial Community Related With The Nitrogen Cycling On The Potato Rhizosphere.
Acta Biol. Colomb.
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2012

Yepes A, Schneider J, Mielich B, Koch G, **García-Betancur JC**, Ramamurthi KS, Vlamakis H, Lopez D.
The Biofilm Formation Defect of a *Bacillus subtilis* Flotillin-defective Mutant involves the Protease FtsH.
Mol Microbiol.
2012 Oct; 86(2): 457-71.

Garcia-Betancur, J. C., Yepes, A., Schneider, J., Lopez, D.
Single-cell Analysis of *Bacillus subtilis* Biofilms using Fluorescence Microscopy and Flow Cytometry.
J. Vis. Exp.
2012 Feb; 15 (60).

Schneider J, Yepes A, **Garcia-Betancur JC**, Westedt I, Mielich B, López D.
Streptomycin-Induced Expression in *Bacillus subtilis* of YtnP, a Lactonase-Homologous Protein That Inhibits Development and Streptomycin Production in *Streptomyces griseus*.
Appl Environ Microbiol.
2012; 78(2):599-603.

J.C. Garcia-Betancur, M. Carmen Menendez, P. Del Portillo, M.J. Garcia
Alignment of multiple complete genomes suggests that gene rearrangements may contribute towards the speciation of Mycobacteria.
Infection, Genetics and Evolution.
2012 Jun; 12(4): 819-26

2009
Cháves D, Sandoval A, Rodríguez L, **Garcia JC**, Restrepo S, Zambrano MM.
Comparative analysis of six *Mycobacterium tuberculosis* complex genomes
Biomedica.
2010; 30(1)23:31.

Andrea Sandoval, Andrés Cubillos, Alejandro Reyes, Diego Chaves, **Juan Carlos García**, Silvia Restrepo, Patricia Del Portillo, María Mercedes Zambrano.
Genomic and Bioinformatic tools applied to the tuberculosis study
Biomedica.
2009;29 (Supl.):70-7.

Juan C Garcia, Alejandro Reyes, Marcela Salazar, Clarissa de Brito Granja.
Differential gene expression in White Spot Syndrome Virus (WSSV) infected naïve and previously challenged Pacific white shrimp *Penaeus (Litopenaeus) vannamei*
Aquaculture.
289 (2009) 253-258.

2005
Alejandro Rojas, Alberto Aparicio, **Juan C García.**
DNA Vaccines (Review).
Hypotesis
Vol 6. No 1
ISSN 1692-729X

ORAL PRESENTATIONS AT SCIENTIFIC MEETINGS

2014
Diversification of cell fate in staphylococcal multicellular aggregates.
J.C. García-Betancur
566. WE-Heraus-Seminar on Mechanisms, Strategies, and Evolution of Microbial Systems at the

Physikzentrum Bad Honnef.
June 15th.
Bad Honnef, Germany

2013
Coexistence of distinct cell types within multicellular aggregates of *Staphylococcus aureus*
Juan Carlos Garcia-Betancur, Julian Eikmeier and Daniel Lopez
SPP1617 meeting on phenotypic heterogeneity and sociobiology of bacterial populations.
November 28th.
Kloster Banz, Germany.

2012
Cell Differentiation in Biofilms Communities of *Staphylococcus aureus*
J.C. Garcia-Betancur, A. Yepes-Garcia, D. Lopez
Annual Conference of the Association for General and Applied Microbiology (VAAM)
March 21st
Tübingen, Germany

2010
Microbial Diversity in Neotropical Andean soils under contrasting agricultural managements assessed by 16S rDNA pyrosequencing
J.C. Garcia-Betancur, A. Cubillos, C. Osorio, S. Resptrepo, D. Uribe, P. Del Portillo
Soil Metagenomics 2010, Implications Of Next- Generation DNA Sequencing On Microbial Diversity Research And Soil Ecology

December 9th
Braunschweig, Germany

2007
Aquaculture Europe
Differential gene expression in L.vanammei with different degrees of resistance to WSSV infection
Juan C Garcia, Alejandro Reyes, Marcela Salazar, Clarissa de Brito Granja. Gustavo Parra
25th-28th October
Istanbul, Turkey

PUBLISHED CONFERENCE POSTERS AND ABSTRACTS

2014
Diversification of cell fate in multicellular *Staphylococcus aureus* communities.
Juan Carlos Garcia-Betancur, Ángel Goñi-Moreno, Thomas Horger, Melanie Schott, Julian Eikmeier, Victor de Lorenzo, Christina Kuttler and Daniel Lopez
SPP1617 International Conference on phenotypic heterogeneity and sociobiology of bacterial populations. October 15th to 17th. Kloster Irsee, Germany.

Towards understanding bacterial pattern formation by reaction-diffusion equations.
Thomas Horger, **Juan Carlos Garcia-Betancur**, Barbara Wohlmuth, Daniel López and Christina Kuttler.
SPP1617 International Conference on phenotypic heterogeneity and sociobiology of bacterial populations. October 15th to 17th. Kloster Irsee, Germany.

Distinct cell types coexist within multicellular aggregates of *Staphylococcus aureus*
Juan Carlos Garcia-Betancur, Julian Eikmeier, Ángel Goñi Moreno, Melanie Schott and Daniel Lopez
3rd Mol Micro Meeting May 7th to 9th. Würzburg, Germany.

2012

Cell differentiation in multicellular communities of *Staphylococcus aureus.*
Juan Carlos Garcia-Betancur, Ana Yepes Garcia, Daniel Lopez
7th International Symposium organized by the Students of the Graduate School of Life Sciences. October 16th and 17th. Würzburg, Germany

Cell Differentiation in Multicellular communities of *Staphylococcus aureus*
Juan Carlos Garcia-Betancur, Ana Yepes Garcia, Daniel Lopez
2nd Mol Micro Meeting. April 25th. Würzburg, Germany

2011
Functional Prediction of *Mycobacterium tuberculosis* Hypothetical Proteins Using Genomic and Post-Genomic Data
G. E. Hernandez-Neuta, D. Chaves, **J. C. Garcia-Betancur**, L. Lopez-Kleine, M. M. Zambrano
111th General Meeting. American society for Microbiology (ASM). May 24th. New Orleans, Louisiana, USA

Functional prediction of hypotetical proteins exclusive to the *Mycobacterium tuberculosis* Complex
Ginna Hernández-Neuta, Diego Chaves, **Juan García Betancur**, Luis Rodríguez Rojas, Silvia Restrepo, Liliana López Kleine, María Zambrano.
First Colombian Congress of Computational Biology. March 25th. Bogotá, Colombia

2010
Metagenomic analyses of soil from *Solanum phureja* landraces in Andean Neotropics
Suarez Silva Yudith Angélica, **García Betancur Juan Carlos**, Del Portillo Patricia
XX Latin America Meeting on Microbiology. Latin-American Association for Microbiology (ALAM). September 29th. Montevideo, Uruguay.

Comparative Genomics in Mycobacterium genus, a way to select genes as a potential drug targets
J. C. Garcia, D. Chaves, A. Sandoval, G. Hernandez-Neuta, L. M. Rodriguez, S. Restrepo, M. M. Zambrano. 110th General Meeting. American Society for Microbiology (ASM). May 24th. San Diego, California, USA.

2009
Genomic comparison of 17 Mycobacterial genomes
Diego Chaves M, Ginna E. Hernandez-Neuta, Andrea
Sandoval, Luis Rodríguez R, **Juan C. Garcia**, Adriana Bernal, Silvia, Restrepo, María Mercedes Zambrano
Mykobak09 German-Colombian Innovation Forum. November 23rd-26th. University of Antioquia. Medellin, Colombia.

Genomic and Bioinformatic tools applied to the tuberculosis study
Andrea Sandoval, Andrés Cubillos, Alejandro Reyes, Diego Chaves, **Juan Carlos García**, Silvia Restrepo, Patricia Del Portillo, María Mercedes Zambrano.
XIV Colombian Congress of Parasitology and Tropical Medicine "One hundred years of the Chaga's Disease". October 8th-11th. Medellin, Colombia.

TEACHING EXPERIENCE

2012
Practical course: *Die Prokaryotische Zelle, Die Zelle - Teil Mikrobiologie.*
Für 1. Semester Bachelor Biologie, Lehramt G, H, R und Gym und Nebenfach Biologie. Biozentrum.
November 5th and November 8th. University of Würzburg, Germany.

2010
Theorical course: Molecular Biotechnology and Genomic Sciences: Fundamentals and Applications

Conference in *transcriptomics.*
June 9th – 10th. Corpogen. Bogotá, Colombia.

2008
First Course in Molecular Biology and applications to Biotechnology.
Conference in *transcriptomics.*
May 24th-27th. Corpogen. Bogotá, Colombia.

2007
First *on line* course of Real Time PCR.
Topic: Relative quantification of gene expression.
Roche Colombia
http://www.virtualeduroche.com/
October 19th – November 19th. Bogotá, Colombia

INVITED REVIEWER FOR PEER-REVIEWED JOURNALS

2013
Evolutionary Bioinformatics
Libertas Academica Ltd.

2012
Infection, Genetics and Evolution.
Elsevier Editorial Group.

LANGUAGES

Spanish.	Native speaker.
English.	Very Good.
German.	Basic.
Portuguese.	Basic.

SIGNATURE

Juan Carlos García Betancur