Cell Heterogeneity in Staphylococcal Communities

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Abstract

The human pathogen *Staphylococcus aureus* is a gram-positive bacterium that causes difficult-to-treat infections. One of the reasons why *S. aureus* is such as successful pathogen is due to the cell-to-cell physiological variability that exists within microbial communities. Many laboratories around the world study the genetic mechanisms involved in *S. aureus* cell heterogeneity to better understand infection mechanism of this bacterium. It was recently shown that the *Agr* quorum-sensing system, which antagonistically regulates biofilm-associated or acute bacteremia infections, is expressed in a subpopulation of specialized cells. In this review, we discuss the different genetic mechanism for bacterial cell differentiation and the physiological properties of the distinct cell types that are already described in *S. aureus* communities, as well as the role that these cell types play during an infection process.

Staphylococcus aureus: A Versatile Pathogen

Staphylococcus aureus is a gram-positive facultative anaerobic bacterium and one of the most threatening human pathogens. It is able to infect practically every host organ [1–6], causing a great variety of infection patterns, such as acute bacteremia, pneumonia, endocarditis or chronic biofilm-associated infections in bone tissue and prosthetic devices [7–17]. *S. aureus* causes a wide variety of infections due to a large number of cytolytic toxins and additional virulence factors produced during infection [3,5,15,18–27]. These toxins and virulence factors damage the local tissue where the infection localizes to release the nutrients that bacteria need to grow and to block the host immune response. In addition, these toxins play an important role to the dissemination of the infection both within and between hosts [16,23,28–31].

S. aureus broad toxin arsenal provides this bacterium with a remarkable capacity to establish acute or chronic, biofilm-associated infection types in distinct infection niches. The adaptation of this pathogen to define distinct and locally defined infection types occurs in response to extracellular local signals and specific host–microbe interactions [32–37]. In particular, *S. aureus* can adapt to the different colonization niches by sensing extracellular signals or host–microbe interactions by modulating their metabolism or gene expression [26]. These signals include, but are not limited to, changes in nutrient availability, temperature, pH, osmolality or oxygen concentration. Extracellular signals that may cause cell differentiation of cells can also come from interaction with the host. Molecular oxygen, glucose or iron concentration is important for *S. aureus* growth, and the concentration of these molecules may vary enormously in the different tissues [38]. *S. aureus* can respond collectively to the presence of these cues to adapt its behavior in a fluctuating environment [39], allowing staphylococcal communities to generate distinct, locally defined types of infections [37,40].

The adaptation of microbial communities to the presence of specific extracellular signals leads to a

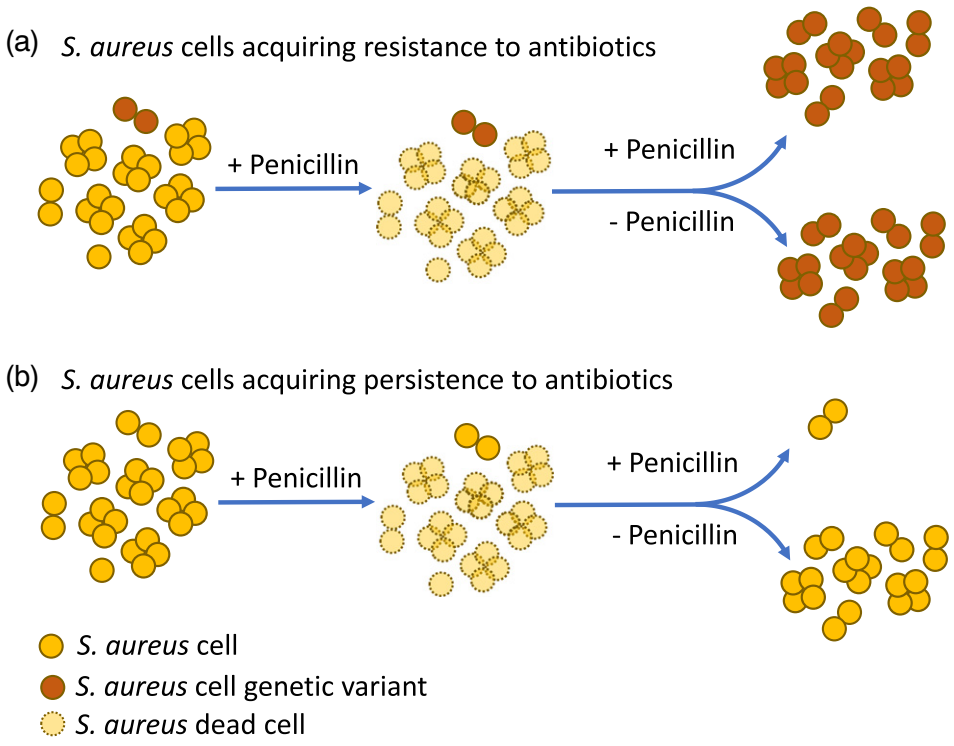

Fig. 1. Antibiotic resistance *versus* persistence. (1) Antibiotic resistance in microbial communities. A bacterial population exhibiting allelic variation can respond heterogeneously to the presence of an antibiotic. If any of the genetic variants gives a bacterium the advantage to grow in the presence of the antibiotic, this subpopulation is selected for growth and will increase in numbers to recapitulate a new bacterial population. This new bacterial population is genetically different from the original population. (2) Antibiotic persistence in microbial communities. An isogenic bacterial population contains a heterogeneous community with extensive phenotypic variation between individuals. Persister cells are usually the members of the community showing lower metabolic activity, considered to be in a dormant or inactive state, and thus can sustain antibiotic treatments. Persister cells do not divide as long as the stress conditions endure. As unfavorable conditions end, persister cells recapitulate growth and generate a new microbial community showing the same antibiotic susceptibility as the original population.

heterogeneous gene expression pattern within the microbial community [37,41]. This causes the differentiation and coexistence of distinct, specialized cell types [42–44] in the bacterial community. Specialized cell types are genetically identical bacterial subpopulations that express heterogeneously different sets of genes and different cell physiology and play different biological roles within the microbial community [32–34,37,45]. It is worth clarifying at this point that cell heterogeneity can exist in a microbial community at the genetic level. In this case, allelic variation occurs within the population, which may affect bacterial fitness and provide a selective advantage to thrive with harsh environmental conditions. Genetic diversity in microbial communities has important implications in clinic with acquisition of antibiotic resistance, for instance. This review, however, focuses on the mechanisms for bacterial cell differentiation that are not driven by allelic variation but phenotypic differences due to stochastic fluctuations that lead to differential gene expression within the same microbial community.

A classic example of cell differentiation of genetically identical cells is the emergence of persister cells in cultures of *S. aureus* that are treated with antibiotics [46] (see the following paragraph). In the presence of penicillins, the antibiotics kill most *S. aureus* cells of the cultures, but a small subpopulation of genetically identical but antibiotic persister cells can survive. This subpopulation of surviving cells can proliferate and generate a culture that is sensitive to the presence of the antibiotic, similar to the previous culture that originates the persister cells. Thus, persister cells can cause a recurrent infection in a post-antibiotic period [46,47]. In addition, there are numerous examples of heterogeneous cell differentiation within microbial communities that contribute to the spread of infections. Particularly to *S. aureus*, the formation of multicellular aggregates or biofilms is often associated with the coexistence of distinct *S. aureus* specialized cell subpopulations [33,48–51]. It is known as microbial biofilms that constitute an important virulence mechanism to generate physiological variability in the microbial community. Thus,

simple antibiotic treatments fail to eliminate all cells from the biofilm, as not all specialized subpopulations express the antibiotic target, and thus, they survive the treatment [52–56].

Cell Differentiation in *S. aureus* Communities

Bacterial persistence

The discovery of bacterial persistence is possibly the most relevant example of cell heterogeneity in microbial communities of *S. aureus* [46] (Fig. 1A and B). The pioneering work of Bigger in 1944 tested the capacity of the beta-lactam antibiotic penicillin to kill all bacteria in *S. aureus* cultures. The author monitored bacterial survival in cultures with different total number of cells using different concentration of antibiotics or different exposure time to antibiotics. In all cases, he detected a small population of surviving bacteria that did not show antibiotic resistance and thus were referred to as persisters. This subpopulation of surviving cells has not genetically acquired antibiotic resistance because they switched back to a normal phenotype in the absence of the antibiotic pressure; thus, they regrow as a new antibiotic-sensitive population. Thus, persister cells can grow in the absence of the drug and recapitulate a regular culture that is sensitive to antibiotics. As Bigger stated in his work: "If cultures of these organisms are tested, they will usually be found just as sensitive to the action of penicillin and just as easily killed by it as it was the strain isolated before treatment was commenced. Such organisms are persisters and not resisters" [46].

In this work, Bigger proposed that penicillin does not injure the staphylococcal cells that are not dividing. Thus, the subpopulation of non-dividing cells that is present in the culture can evade the action of the penicillin. Persister cells may survive a penicillin treatment because these cells are in a dormant, non-dividing state. They tested this hypothesis by resuspending bacteria in 1:800 diluted broth. In these growing conditions, the number of staphylococci remains practically constant, indicating that cell division does not occur in these conditions. When the cell resuspension is supplemented with penicillin, only a slight reduction in the number of living staphylococci is detected. This suggests that penicillin shows no effect on non-dividing bacteria. Altogether, this work describing persister cells provided the first evidence that cultures of *S. aureus* are heterogeneous, showing a small fraction of cells that do not divide and thus become protected to the bactericidal action of penicillin.

Since the work of Bigger [46], persistence has been described in many bacterial species with many classes of antibiotics [57–59]. Although bacterial persistence has been observed more than 70 years ago, it is still unknown what triggers this phenotypic differentiation or what is the mechanism underlying the phenomenon of persistence [60]. As it was proposed by Bigger [46], it is possible that persister cells are in a dormant, non-dividing state. Other theories are being considered as well. For instance, it is possible that bacteria are in some protected part of the cell cycle at the time of antibiotic exposure [61,62] or can adapt rapidly to the antibiotic stress [63]. It is also unclear how important persister subpopulations are to antibiotic treatment in human patients. There is, however, clinical evidence for persistence delaying and possibly preventing infection clearance to antibiotics during infection treatment [64,65].

DNA replication pattern in biofilms

A more general approach was more recently used to define spatial patterns of DNA replication, protein synthesis and oxygen concentration as a proxy for cell differentiation within staphylococcal biofilms [66]. These locally defined expression patterns demonstrate that *S. aureus* communities are constituted by subpopulations of genetically identical but physiologically different cells [66]. Stratified patterns of activity in DNA and protein synthesis are found in reduced areas of the biofilms, whereas ~70% of the total biomass of the biofilm is constituted by anabolically inactive but viable cells. In addition, 10% of the biofilm population is considered dead cells.

Capsule production

The laboratory of Christiane Wolz has reported that, at specific culture conditions that resemble a persistent human carrier, the regulation of the *cap* operon responsible for the production of the capsular polysaccharide (CP) in an *S. aureus* community displays a heterogeneous expression pattern [45]. The CP protects *S. aureus* cells against phagocytosis and also prevents adherence to endothelial cells and/or matrix proteins. The *cap* expression is mostly driven by the activity of the *Agr* system and most concretely to the induction of P3 promoter and the expression of RNAIII, but it does not account for the heterogeneous expression of *cap* within the microbial community over time [45]. The heterogeneous *cap* expression pattern possibly provides an adapted fitness to the bacterial population during infection and colonization [45]. The activation of the *Agr* system is necessary to detect *cap* expression in *S. aureus* cells. In the study, the authors suggest that *in vivo* or in biofilms, subtle changes in the microenvironment or variations in the extracellular signals modulate the *Agr* activity, which may have a tremendous effect on the differentiation of a subpopulation of cells specialized in CP production. This

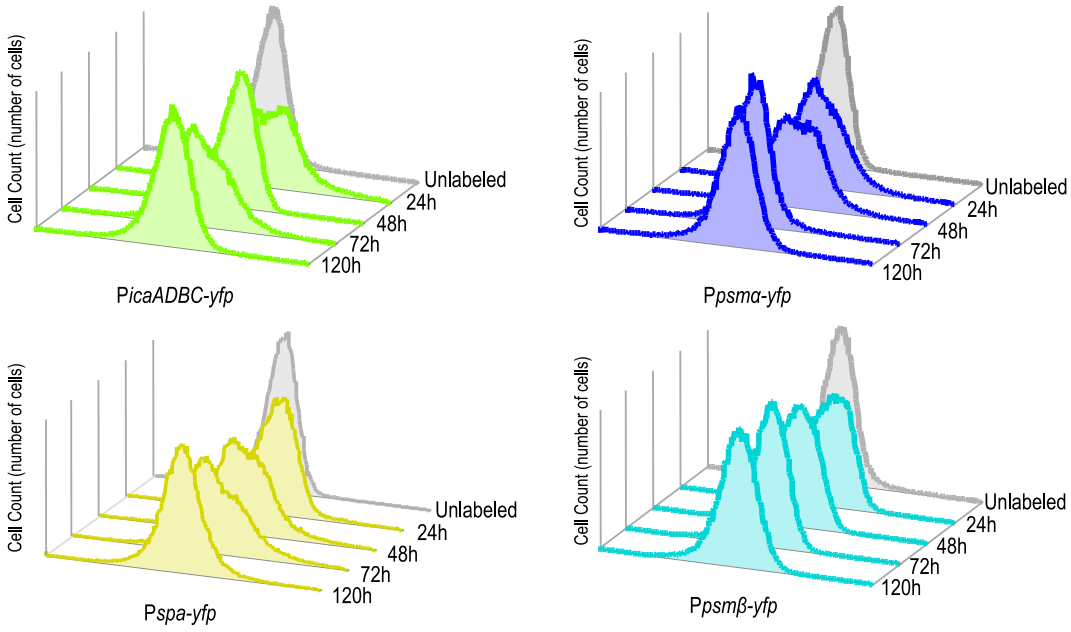

Fig. 2. Flow cytometry facilitates gene expression analyses in a bacterial population. Flow cytometry profiles of *S. aureus* cultures expressing different *Agr*-related promoters [48]. $P_{PSM\alpha}$ and $P_{PSM\beta}$ are positively regulated by Agr, whereas P_{ica} and P_{spa} are indirectly repressed by *Agr*. The expression of these promoters is restricted to a subpopulation of specialized cells, showing a bimodal distribution of the fluorescence peaks in the flow cytometry profiles. Cells were grown in TSB $MgCl_2$ 100 mM (TSBMg). Construction of the strains and growth conditions are published in Ref. [48]. Samples are collected at different time points during multicellular community development.

could explain why the temporal expression of *cap* is severely repressed at exponential growth phase, when *Agr* activity in *S. aureus* is normally low [45].

Nuclease expression

In *S. aureus* biofilms, a spatial localization of the thermonuclease *nuc* expression is detected [67]. The nuclease expression pattern is restricted to a subpopulation of cells, and this subpopulation emerges in late growth stages during biofilm formation, when cells reorganize to form a mature biofilm [67]. The differentiation of this specialized subpopulation is associated with the secretion of extracellular DNA. Extracellular DNA is a structural component of the extracellular matrix that contributes to biofilm organization in staphylococcal communities [68]. Consistently, an *S. aureus nuc* mutant is unable to generate mature biofilms and causes an amorphous proliferation of the microbial community. The differentiation of specialized cells expressing *nuc* gene is positively controlled by the SaeRS two-component system and does not depend on *Agr* activity directly [67].

Natural competence

Natural competence is a physiological state in which bacteria become capable of uptaking external DNA and incorporate it into their genome [69–71]. Induction of natural competence in microbial communities promotes genetic variability. Many bacterial species possess the genetic cascade to induce competence [72–74], and in most of the cases, the process is initiated in a specialized subpopulation of cells [75]. The natural competence protein machinery [76–80] is present in *S. aureus* [81,82]. Thus, it is possible to detect the differentiation of a small subpopulation of competent cells in *S. aureus* cultures. The differentiation of this subpopulation is positively regulated by the *sigH* alternative sigma factor (σH) and ComK-like regulator [76–80]. SigH production occurs in a small subpopulation of cells, which induces the expression of competent-related genes and become naturally competent. In addition, ComK is a mayor competence transcription factor in closely relative species, such as *Bacillus subtilis* and *Streptococcus pneumoniae* [77,83]. The role of ComK in inducing *S. aureus* natural competence has been studied using an engineered *comK*-overexpressing strain, which shows upregulation of the competence-related genes and thus higher capability to incorporate extracellular DNA [84,85].

Agr expression pattern in biofilms

A temporal and spatially defined expression of the *Agr* quorum-sensing system was described in *S. aureus* biofilms by Yarwood and colleagues [50]. They show clustered expression of *agr* in

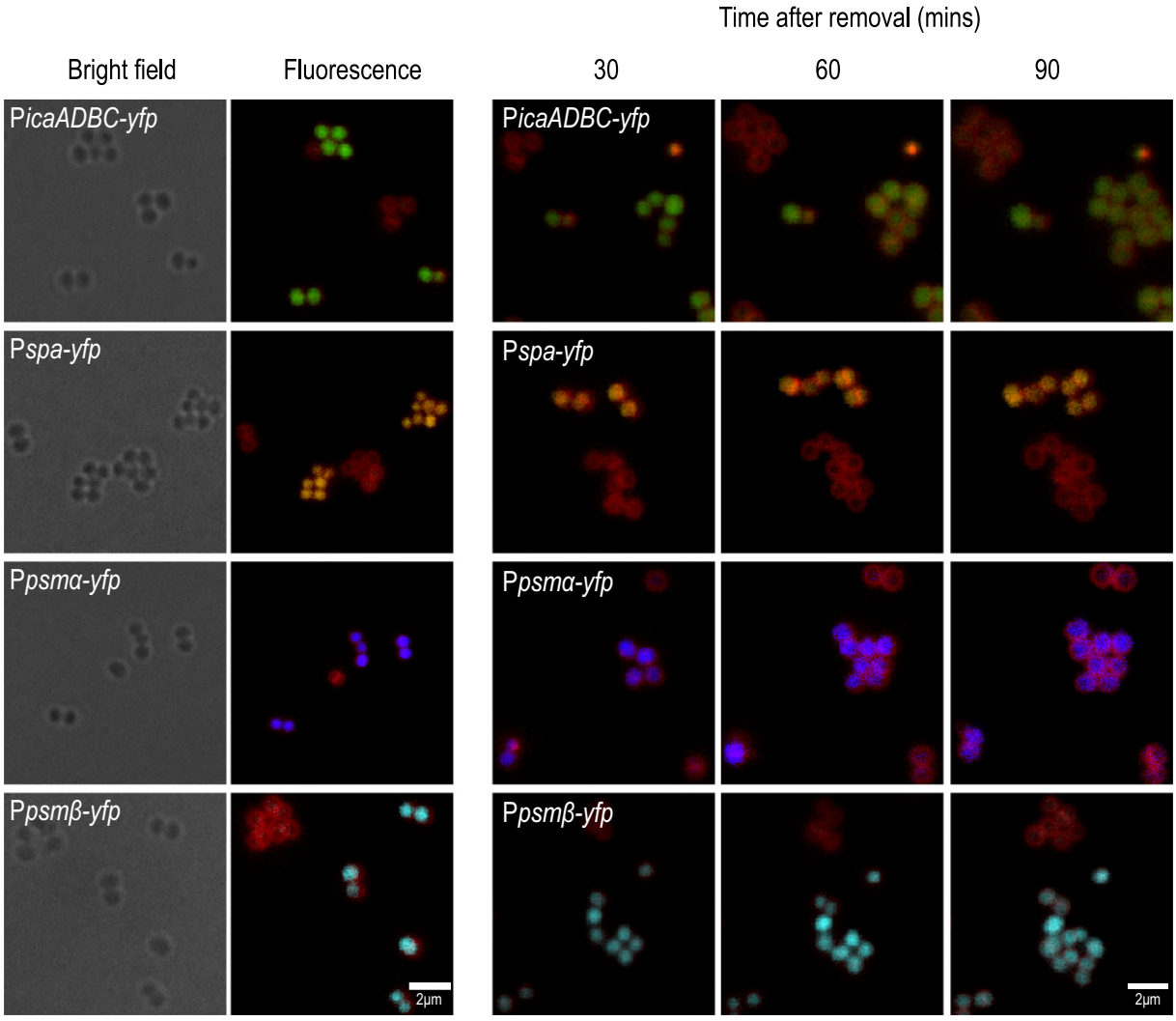

Fig. 3. Fluorescence microscopy to monitor gene expression at single-cell level over time. Fluorescence microscopy images of *S. aureus* cells labeled with different *Agr*-related promoters. $P_{PSM\alpha}$ and $P_{PSM\beta}$ are positively regulated by Agr, whereas P_{ica} and P_{spa} are indirectly repressed by Agr. The expression of these promoters is restricted to a subpopulation of specialized cells (right). Fluorescence microscopy images of a time-lapse experiments, in which cultures of labeled *S. aureus* (strain Newman) were grown in TSBMg for 48 h. Cells were resuspended in fresh TSBMg medium depleted from the AIP signal and monitored by fluorescence microscopy over time. The differentiation of subpopulations of specialized cells is observed during several generations and for more than 2 h, even in the absence of the AIP signal. Scale bar is 2 μm.

staphylococcal biofilms and that this *agr* spatio-temporal expression pattern is important for biofilm formation and for cell dispersal. Whereas most of the biofilm areas do not express *agr*, the staphylococcal cells that are released and dispersed from the biofilm show active *agr* expression. The locally restricted *agr* expression to patches oscillates in its expression intensity with time and coincides with the loss of fluorescence with the detachment of cells. This result suggested to Yarwood and colleagues [50] that the *agr* expression plays a role in cells detachment from the biofilm, indicating that cells detaching from the biofilm express the *agr* virulence regulon and thus are able to cause severe infections and disseminate to new colonization sites.

Positive Feedback Loops Induce Cell Differentiation in Microbial Communities

Many tools are nowadays available to facilitate gene expression studies at the single-cell level to explore a heterogeneous expression of genes in a bacterial population [86,87]. For instance, cells expressing a specific fluorescent reporter can be monitored using flow cytometry (Fig. 2) or

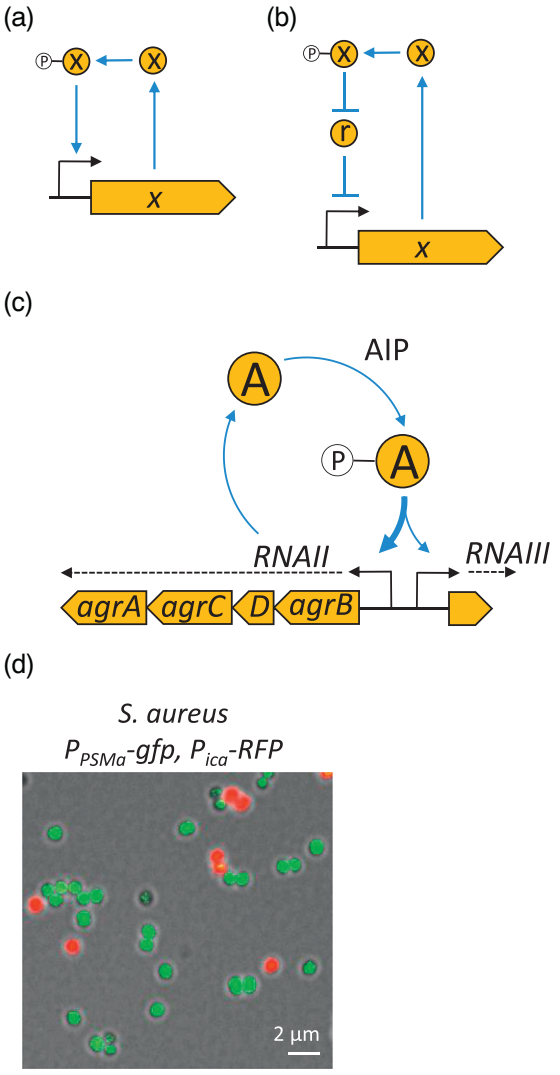

Fig. 4. PFLs determine bacterial cell differentiation. Schematic overview of genetic circuits that lead to the activation of a PFL. (A) Mechanism of bistable switch with a positive autoregulation, in which the regulator induces its own expression. (B) Double-repression system in which the regulator inhibits a repressor of the regulator. (C) Schematic overview of the PFL in the *Agr* quorum-sensing system of *S. aureus*. The *agr* operon (RNAII) comprises an autoregulatory system that responds to increased concentration of the AIP quorum-sensing signal. AgrA ~ P is a transcriptional factor that controls the expression of the *agr* operon. Thus, AgrA ~ P positively controls its own expression as well as the expression of the effector regulatory small RNA called RNAIII. The RNAIII molecule controls the expression of many other genes, including the expression of exotoxins. (D) Fluorescence microscopy images of *S. aureus* cells harboring the $P_{PSM\alpha}$ and P_{ica} *agr*-related promoters. $P_{PSM\alpha}$ expression is restricted to the subpopulation of DR-cells, whereas the expression of P_{ica} is restricted to the subpopulation of BR-cells [48]. The expression of both promoters is restricted to a subpopulation of specialized cells showing no overlap in the microbial community. Scale bar is 2 µm.

fluorescence microscopy (Fig. 3) to evaluate the distribution of fluorescence within the population. Using these two approaches, it is likely that the gene expression shows a uniform Gaussian distribution when expression is unimodal [88,89]. In other cases, the distribution of gene expression is bimodal as it clearly bifurcates into a subpopulation of activated cells expressing the reporter, which coexists with another subpopulation of non-activated cells [89–92] (Figs. 2 and 3). This phenomenon is referred to as bistability or bimodality, as it generates a dual stable pattern of gene expression, indicating the coexistence of two physiologically different subpopulations of bacteria within a single population of genetically identical cells [34,35,75,93,94].

The induction and coexistence of a bistable gene expression response in a microbial community is usually due to the activation of a positive autoregulation of the gene, because the gene product induces its own expression after reaching a certain threshold [95–97] (Fig. 4A and B). The production of a regulatory protein above a threshold results in high levels of theregulator, and the induction of the genes whose expression is controlled by that regulator, including its own gene expression. The induction of its own gene will produce more regulatory protein that induce the expression of the gene to higher levels, resulting in a positive feedback loop (PFL) that causes hyperactivation of the gene (Fig. 4A and B). In contrast, induction of the regulatory protein below the threshold will not produce the concentration of the regulatory protein necessary to induce the expression of the genes. As consequence, this does not activate the positive feedback mechanism; thus, hyperactivation of the gene does not occur, with the remaining cells being inactive for the response [32,34].

Another characteristic feature of PFL is that self-regulated proteins remain induced for a certain time even in the absence of the inducing signal. Activation of the PFL is sufficient to induce the expression of the PFL without the presence of the activating signal (Fig. 3B). As a result, when activated cells divide, they have the ability to pass this activated state to the next generation of cells, thus leading the fate of the future generations of bacteria [32,34] (Fig. 3B). This phenomenon is known as hysteresis; it is usually detected in bistable regulatory mechanisms and is particularly relevant for bacterial heterogeneity [98]. Hysteresis is thus inferred in bistable systems and is defined as the ability of genetically identical cells to differ in their phenotypes and to maintain the phenotype for more than a generation [99,100].

There are numerous examples of regulatory mechanisms that involve a PFL in nature, including prokaryotes. A classic example for positive autoregulation of gene expression is the case for activation of natural competence in *B. subtilis* [69,70,75,101–104]. The activation of the competence state in *B. subtilis*

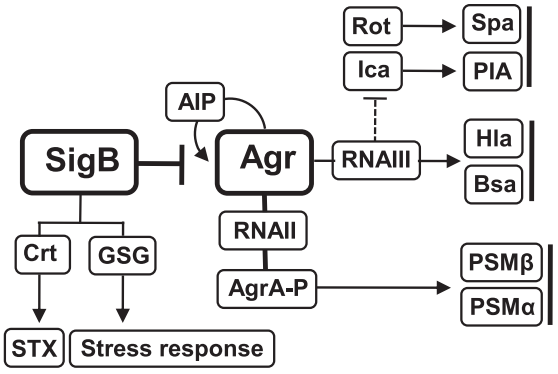

Fig. 5. The *Agr* autoregulatory network controls toxin production and biofilm formation. Schematic overview of the genetic circuitry that antagonistically regulates toxin secretion and biofilm formation in *S. aureus*. Arrows represent positive regulation and T-bars represent repression. Dashed lines represent indirect regulation. STX stands for staphyloxantin production and GSG for general stress genes. The Agr induces the expression of a PFL trough the AIP signal molecule. Following *Agr* activation, AgrA ~ P triggers the expression of the RNAII and RNAIII transcripts. The RNAII upregulates the *agrBDCA* operon that encodes the entire *Agr* signal transduction cascade, inducing its own expression and representing a PFL. The RNAIII positively regulates the expression of the *Agr* regulon, which includes many genes coding for cytotoxic toxins and virulence factors such as the Hla and the Bsa and simultaneously, and inhibits the expression of genes such as the *icaADBC* operon and *rot*, which are involved in cell aggregation during biofilm formation or in the regulation of the production of attachment proteins such as Spa.

cells is induced by the ComK master regulator; thus, induction of *comK* expression induces the competence pathway in *B. subtilis*. ComK regulator positively induces its own expression by upregulating the expression of its own gene *comK.* When levels of ComK reach a certain threshold, the expression of *comK* increases nonlinearly [77,83,92,105–110], leading to the activation of natural competence only in the subpopulation of *B. subtilis* cells that induced ComK expression above the threshold.

The *Agr* Quorum Sensing Is a PFL that Promotes Cell Differentiation in Multicellular Communities of *S. aureus*

S. aureus is a versatile pathogen that can develop different types of infections. In addition to the development of chronic, biofilm-associated infections, *S. aureus* causes acute bacteremia or general sepsis. Both infection outcomes are controlled by the *Agr* quorum-sensing system (Fig. 5). Agr is the most relevant genetic cascade controlling the expression of virulent factors in *S. aureus* and plays a pivotal role in the development of acute and chronic infections. Activation of Agr occurs in response to the self-produced extracellular *S. aureus* signal AIP (*auto*inducing *p*eptide) [111]. This small secreted-peptide AIP binds to the membrane-bound histidine kinase AgrC to phosphorylate and activate the regulator AgrA. AgrA ~ P induces the *agr* gene regulon responsible for bacterial dispersion in the host and the production of toxins and virulence factors that are necessary to develop an acute bacteremia [112]. For instance, bacterial dispersion requires upregulation of surfactant phenol-soluble modulins (*psm*α and *psm*β) whose expression is induced as part of the *Agr* regulon (Fig. 5). *psm*α and *psm*β are amphipathic small peptides that contribute to bacteria detachment and dispersion [20,113]. Moreover, acute bacteremia requires upregulation of the hemolytic toxins genes *hla*, *hlb* and *hlg* that facilitate tissue disruption during septicemia [111] (Fig. 5). In contrast, *Agr* activation indirectly downregulates the *icaADBC* operon genes needed to synthesize the extracellular polysaccharide matrix that is required for biofilm formation and protects cells within a biofilm (PNAG or PIA) (Fig. 5), as well as several adhesion proteins (SpA and other MSCRAMM proteins) responsible for cell aggregation/attachment during the process of biofilm formation [49,111,114]. Overall, the *S. aureus Agr* quorum-sensing system antagonistically regulates the activation of planktonic and biofilm-associated bacterial lifestyles [49,111,114], which contribute to the development of acute and chronic infection outcomes, respectively.

The *Agr* quorum-sensing system in *S. aureus* is also an autoregulatory system that induces the expression of a PFL [48] (Fig. 4C). Following *Agr* activation, AgrA ~ P directly binds to the two adjacent and divergently located promoters P2 and P3, which trigger expression of RNAII and RNAIII transcripts, respectively [115,116]. The RNAII transcript upregulates the *agrBDCA* operon that encodes the entire *Agr* signal transduction cascade that includes the *agrB* and *agrD*, both genes essential for the production of the AIP signal; the *agrC* gene, which codes for the AgrC membrane-bound sensor kinase and the *agrA* gene that codes for its AgrA cognate regulator. Therefore, AgrA ~ P binding to the P2 promoter represents a PFL [112,117] (Fig. 4C). In addition, AgrA-controlled genes include the upregulation of RNAIII *via* activation of the P3 promoter [115,118,119], which positively regulates the expression of the *Agr* regulon [111,118,120,121] that causes bacterial dispersion and acute bacteremia, which includes many genes coding for cytotoxic toxins and virulence factors that are responsible for the development of acute infections [115,117,122] such as the surfactant phenol-soluble modulins (*psm*α and *psm*β) [20,113] and the hemolytic toxins genes *hla*, *hlb* and *hlg* that facilitate tissue disruption

during septicemia [111] (Fig. 5). Simultaneously, the upregulation of RNAIII indirectly inhibits the expression of the *icaADBC* operon that synthesizes the extracellular polysaccharide matrix of the biofilm and adhesion proteins (SpA) involved in cell aggregation/attachment during biofilm formation [49,111,114] (Fig. 5). Thus, the *S. aureus Agr* quorum-sensing system antagonistically regulates the activation of planktonic and biofilm-associated colonization or infection patterns in *S. aureus* communities.

As published recently by our group in Garcia-Betancur *et al*. [48], the *Agr* system of *S. aureus* shows bimodal behavior; thus, it generates a subpopulation of *Agr* ON cells and a subpopulation of *Agr* OFF cells, which coexist in *S. aureus* microbial populations. This suggests that two different subpopulations of cells types, specialized in biofilm- (*BR-cells, b*iofilm-*r*elated cells*)* or dispersal-(*DR-cells, d*ispersal-*r*elated cells) associated lifestyles coexist in *S. aureus* communities (Fig. 4D). Differentiation of DR- and BR-cell types in *S. aureus* communities was explored using transcriptional fusions of *psmα* and *psmβ* genes, whose expression depends directly on *Agr* and code for cytolytic toxins that contribute to bacterial dispersion and acute staphylococcal infections [20,113]. These reporters showed bimodal expression in *S. aureus* cultures, with the bifurcation of one subpopulation with lower and another with higher fluorescence levels. The expression of transcriptional fusions of biofilm-associated *ica*/*spa* genes was also restricted to a specialized subpopulation, whereas pairwise combinations of these reporters showed the bifurcation of two distinct subpopulations of cells specialized in expressing *ica*, *spa* biofilm-related genes (BRcells) and another subpopulation of cells expressing *psmα* and *psmβ* genes dispersion-related genes (DR-cells) (Fig. 4D).

The *Agr* bimodal behavior is based on a sequential activation of the two adjacent divergent promoters P2 and P3. AgrA ~ P binds P2 with greater affinity than the P3 promoter [115]. The P2 promoter triggers the *Agr* PFL at lower AgrA ~ P concentrations and only in a given subpopulation of *Agr*-ON cells. Activation of the Agr PFL in this subpopulation produces high AgrA ~ P levels that trigger the less-sensitive P3 promoter to induce the *Agr* regulon [48]. This causes the differentiation of the *Agr*-ON cells, to specialize in dispersion and virulence and thus to become DR-cells. In contrast, the subpopulation of cells that expresses P2 below the threshold cannot activate the *Agr* PFL and become a subpopulation of *Agr*-OFF cells. In this subpopulation, there is not sufficient AgrA ~ P to induce P3 promoter expression. As consequence, this subpopulation shows upregulation of genes that are typically inhibited by AgrA ~ P, including biofilm-related genes. This causes the differentiation of Agr-OFF cells to specialize as biofilm-producing cells thus, to become BR-cells.

Any alteration that inhibits *Agr* activity compromises the bistable expression of the reporters. This demonstrates that *Agr* PFL is responsible for the differentiation of BR-cells and DR-cells. However, once the *Agr* bimodal switch is activated and BR-cells and DR-cells differentiate in microbial communities, the fluctuations in the concentration of extracellular cues that regulates the activation of the *Agr* bimodal switch only cause variations in the size of the two subpopulations. Their ratio differed in the overall bacterial community and that causes a variation in the infection outcome in *in vitro* and *in vivo* infection assays. For instance, the *Agr* system is inhibited by the σ^B sigma factor [123], which is induced at early stationary phase in response to cellular stresses [124] (Fig. 5). In response to stress, σ^B downregulates the *Agr* PFL (Fig. 4). *Agr* activation above the threshold becomes more difficult, which leads to differentiation of a smaller DR-cell and a larger BR-cell subpopulation, facilitating biofilm formation and the development of biofilm-associated chronic infections. Conversely, increasing the concentration of AIP above the threshold facilitates the activation of the *Agr* PFL. This causes the differentiation of a larger subpopulation of DR-cells and thus a smaller subpopulation of BR-cells, which in turn facilitates the dispersion of *S. aureus* and thus the development of an acute infection.

Overview and Perspectives

Microbial communities exposed to a homogenously distributed signal do not respond to the signal uniformly but differentiate distinct subpopulations of specialized cells. The nature of the signal can be quite diverse, such as quorum-sensing signals, environmental cues or signals that result from the interaction with the host [38], in the case of infectious bacteria. The description of persister cells in cultures of the human pathogen *S. aureus* is one of the most relevant examples of bacterial cell differentiation with important implications in the treatment of infections with antibiotics. In addition, more recent reports have described a number of specialized subpopulations of cells that contribute to determine the different infection lifestyles of this pathogen, which could explain the capacity of *S. aureus* to colonize distinct organs and cause different types of infections. For instance, *S. aureus* can live as dispersed community or embedded in the matrix of a biofilm, defining two different lifestyles and infection types, acute bacteremia or chronic biofilm-associated infection, respectively. Communities of *S. aureus* differentiate two subpopulations of cells that specialize into disperse cells (enable to cause bactermia) or biofilm-associated cells (enable to cause chronic infections) [48]. Extracellular signals that are derived from the host influence the numbers

of cells that specialize into one subpopulation or the other, which collectively influence the choice of the entire population. This defines the distinct infection lifestyles depending on which extracellular signals are sensed during the infection process [48]. It is yet unclear in the field what is the precise contribution of the presence of extracellular signals in activating the process of cell differentiation. Thus, an important challenge to address is determining the proportion of differentiating cells that results from stochasticity, from the presence of external signals or from the autonomous activation of the phenotypic programs.

Cell sorting methods are now used to separate a given subpopulation of specialized cells from the entire community, in order to characterize the physiology of a specific subpopulation in isolation, using molecular biology methods. Sorting of bacterial subpopulations in combination with transcriptomic analysis offers interesting possibilities for the physiological characterization of a given cell type by studying its overall global gene expression architecture as well as possible variations in gene expression in comparison to the entire bacterial community [125]. The recent advances in RNA-seq approaches also allow the analysis of genome-wide bacterial gene expression at the single-cell level [126–128] to obtain information about the physiological state of an individual cell. Using this technique, it is possible to explore the variability in gene expression that exists between all individuals of a bacterial community to define differences in their gene expression profiles and cluster expression patterns into different, specialized subpopulations represented within the community. Single-cell or single-subpopulation RNA-seq can certainly help to predict the evolution of the infections and thus to design better and more effective antimicrobial therapies. In this line of work, we anticipate the importance that dual RNA-Seq will have in determining the contribution of each subpopulation to the infection outcome [129,130]. The study and characterization of the different subpopulations of specialized cells relies on their physical separation followed by their gene expression analyses. Dual RNA-seq does not require physical separation of host and pathogen cells; thus, transcripts of the two organisms are pooled together and analyzed separately *in silico*, by assigning sequencing reads to their respective reference genomes. This approach will determine better characterization of microbial heterogeneity during infections, without any interference of the technical procedures for bacterial cell separation.

Experimental Details

All experimental details presented in this work are published in Garcia-Betancur *et al*. [48].

Strains and growth conditions

For *S. aureus* aggregates in TSBMg, 4 µl of an overnight liquid culture was spotted in TSBMg and dried in a sterile culture cabin. Plates were allowed to grow for 5 days at 37 °C [131].

Fluorescence microscopy

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 100 × 1.47 magnification and a color camera Leica DFC630FX. Linear image processing was done using Leica Application Suite Advance Fluorescence Software. For hysteresis determination, 5-day-old multicellular communities of *S. aureus* were disrupted and cells were washed twice with PBS to eliminate extracellular AIP. Here, 5 µl of washed cell suspension was diluted in 495 µl of pre-warmed in TSB or TSBMg and placed in microscopy pads containing TSB 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 h using a Leica DMI6000 B inverted microscope equipped with a temperature setting chamber that was set to 37 °C.

Flow cytometry

Cells from the multicellular communities were fixed with a treatment of 4% paraformaldehyde, 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 series of 25 pulses (power output 70% and cycle 0.7 s) and kept on ice. Dilution of samples 1:500 was necessary prior to flow cytometry analyses.

Strain list

Species	Characteristics	Strain name	Reference
S. aureus st. Newman	*amy*::$P_{icaADBC}$-*yfp*	JCGB-0151 N	[48]
S. aureus st. Newman	amy::P_{spa}-*yfp*	JCGB-0203	[48]
S. aureus st. Newman	amy::$P_{psm\alpha 1-4}$-*yfp*	JCGB-0148 N	[48]
S. aureus st. Newman	amy::$P_{psm\beta 1-2}$-*yfp*	JCGB-0356	[48]
S. aureus st. Newman	amy::$P_{psm\alpha 1-4}$-*yfp* lac::$P_{icaADBC}$-*mars*	JCGB-0470	[48]

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Keywords:
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Abbreviations used:
CP, capsular polysaccharide; PFL, positive feedback loop; AIP, autoinducing peptide.

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