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Role of Phenol-Soluble Modulins in Formation of *Staphylococcus aureus* Biofilms in Synovial Fluid

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**Staphylococcus aureus** is a leading cause of prosthetic joint infections, which, as we recently showed, proceed with the involvement of biofilm-like clusters that cause recalcitrance to antibiotic treatment. Here we analyzed why these clusters grow extraordinarily large, reaching macroscopically visible extensions (>1 mm). We found that while specific *S. aureus* surface proteins are a prerequisite for agglomeration in synovial fluid, low activity of the Agr regulatory system and subsequent low production of the phenol-soluble modulin (PSM) surfactant peptides cause agglomerates to grow to exceptional dimensions. Our results indicate that PSMs function by disrupting interactions of biofilm matrix molecules, such as the polysaccharide intercellular adhesin (PIA), with the bacterial cell surface. Together, our findings support a two-step model of staphylococcal prosthetic joint infection: As we previously reported, interaction of *S. aureus* surface proteins with host matrix proteins such as fibrin initiates agglomeration; our present results show that, thereafter, the bacterial agglomerates grow to extremely large sizes owing to the lack of PSM expression under the specific conditions present in joints. Our findings provide a mechanistic explanation for the reported extreme resistance of joint infection to antibiotic treatment, lend support to the notions that Agr functionality and PSM production play a major role in defining different forms of *S. aureus* infection, and have important implications for antistaphylococcal therapeutic strategies.

*S. aureus* is a major cause of septic arthritis and orthopedic infections, in particular those developing on prosthetic joints after arthroplasty (1). In the presence of a prosthetic device, joint infection rates are at about 1 to 2% (2). Joint infections can cause prolonged disability and increased health care costs, due to prolonged antibiotic treatment, multiple surgeries, and, in difficult cases, joint fusion. If recalcitrant to treatment, these infections can cause significant morbidity, including loss of limb, systemic infection, and even death (3).

Antibiotic treatment alone is usually insufficient to eradicate joint infections. We recently showed that the pronounced recalcitrance of *S. aureus* joint infections to antibiotic treatment is due to exceptionally strong bacterial aggregation and biofilm formation, which renders even the high concentrations of antibiotics given exceptionally strong bacterial aggregation and biofilm formation, as well as the polysaccharide intercellular adhesin (PIA), with the bacterial cell surface. Together, our findings support a two-step model of staphylococcal prosthetic joint infection: As we previously reported, interaction of *S. aureus* surface proteins with host matrix proteins such as fibrin initiates agglomeration; our present results show that, thereafter, the bacterial agglomerates grow to extremely large sizes owing to the lack of PSM expression under the specific conditions present in joints. Our findings provide a mechanistic explanation for the reported extreme resistance of joint infection to antibiotic treatment, lend support to the notions that Agr functionality and PSM production play a major role in defining different forms of *S. aureus* infection, and have important implications for antistaphylococcal therapeutic strategies.

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control of Agr by direct binding of the AgrA response regulator to the psm operon promoters (16). There are three loci in S. aureus that encode PSMs: the psma locus encodes the peptides PSMα1 through PSMα4; the psmb locus encodes PSMβ1 and PSMβ2; and RNAIII includes the gene encoding the PSM β-toxin (17, 18). In vitro studies using TSB growth medium showed that all S. aureus PSMs impact biofilm formation in a similar way (13), i.e., they structure biofilms and lead to biofilm dispersal, which is likely due to their surfactant properties. While PSMs are important for biofilm structuring and thus biofilm development, the overall impact of PSMs on the extent of biofilm formation is negative because PSMs disperse biofilms (13, 15). Thus, these factors could not be identified in our recent work, which sought to identify factors with a generally positive impact on biofilm-like aggregation (4). Notably, the role of PSMs during S. aureus in vivo biofilm infection is not well understood. While the PSM-mediated dispersal effect has been shown to promote systemic dissemination of biofilm-associated infection, it is not known if lack of PSM production also leads to more extensive in vivo biofilm formation on indwelling devices (13).

In the present study, we show that low activity of Agr and low PSM production in SF are a major cause for the exceptionally strong aggregation behavior of S. aureus in SF. We demonstrate that PSM production has a key role in defining biofilm extension under conditions that exemplify an in vivo situation and provide evidence that contributes to our understanding of how PSMs affect biofilm dispersal.

MATERIALS AND METHODS

Ethics statement. Human SF was drained from the joint during total knee arthroplasty in the operating room and collected with permission of the Thomas Jefferson University Institutional Review Board (IRB). A waiver for the requirement of informed written consent was obtained, because the samples were deidentified, obtained during routine procedures, and would normally have been discarded. Human serum was obtained from the blood bank of the NIH with an existing IRB protocol.

Preparation and storage of SF. SF that was obtained as mentioned above was stored at 4°C for a period not exceeding 6 months before use. Cellularity was not recorded. All samples containing blood upon visual examination were discarded. Of note, due to the conditions of storage, white blood cells were inactive (dead) at the time of use of SF for our experiments.

Strains and growth conditions. All experiments were performed using strain S. aureus LAC (pulsed-field type USA300), a community-associated methicillin-resistant strain, or its derivatives. The USA300 lineage is the main source of skin and prosthetic-joint infections (PJs) in the United States (19, 20). The total Δpsm mutant (with the psma and psmb operons deleted and translation of β-toxin abolished by mutation of the hld gene start codon) and the Δagr mutant of LAC were produced by sequential allelic replacement and by phage transduction from strain RN6911, respectively, and were previously described (18, 21). Of note, the LAC wild-type strain and its Δagr and Δpsm mutants do not show significant differences in growth rates and yield in SF when grown at 37°C in shaking incubators (data not shown). Strain LAC was transformed by electroporation with plasmids based on the pTXα plasmid background, constitutively expressing the psma (15), psmb (this study), hld (22), or agrA (16) gene/operon under the control of the xyl promoter. The xyl repressor gene has been removed in that plasmid series to produce a constitutive mode of expression (18). The control strain harbors the corresponding pTXα16 plasmid (18). Strains were grown in SF, serum, or tryptic soy broth (TSB), as indicated. Oligonucleotides used are shown in Table 1, and strains used are shown in Table 2.

Macroscopic determination of bacterial aggregation. Aggregation formation was monitored by UV-fluorescent imaging of ethidium bromide (EtBr)-labeled bacteria (10 μg/ml EtBr, 10 min in the dark).

Measurement of aggregate size distribution. Aggregate size distribution was measured using a T3 Cellometer (Nexelcom) as described previously (4). Briefly, overnight cultures were centrifuged and resuspended to a concentration of ~10^10 CFU/ml in sterile phosphate-buffered saline (PBS). One milliliter of SF, TSB, or serum was warmed to 37°C, and 5 μl of the bacterial suspension and 20 μl of trypan blue were added, and the sample was incubated for 20 min at 37°C with agitation. The Cellometer can detect aggregates between 3 and 100 μm in size. The parameters were set to detect oddly shaped aggregates (roundness was not set as a determining factor).

qRT-PCR. Strain LAC (initial inoculum 10^6 CFU/ml) was grown for 8 h in TSB, SF, or serum, in a shaking incubator at 180 rpm and 37°C. miRNA was collected using the RNeasy miikit (Qiagen), and quantitative real-time PCR (qRT-PCR) was performed as described previously (23). Oligonucleotides used are described in Table 1. All probes were labeled with 5′-carboxyfluorescein. All experiments were performed in triplicate, and data were normalized against the housekeeping gyrB gene.

Creation of luciferase reporter gene fusion constructs and luminescence measurement. The lux operon (luxABCDE; 5.7 kb) was amplified from plasmid pXen5 (kindly given by K. Francis) (24) using the primers luxA-f (5′ CCCCCGGGCG CGGCTCAAGC 3′) and luxB-r (5′ CAGCGGCCGG CACGGTCCTGC 3′). The P2, P3, psma, and psmb promoters were amplified from LAC genomic DNA and cloned into the resulting plasmid, pL29 (a gift from C. Lee) (25). The P2, P3, psma, and psmb promoters were amplified from LAC genomic DNA and cloned into the resulting plasmid, pL29lux (26) using SacI/BamHI (psmb promoter) or EcoRI/BamHI (all other promoters) restriction sites. Then, the integration procedure protocol was performed as described previously (25). Briefly, the pL29lux reporter fusion plasmids were electroporated into strain RN4220 carrying plasmid pL2787, which contains the di1 int gene. After selecting for clones in which the integration of the respective pL29 plasmid into the host attB sites had occurred, the chromosome-integrated pL29 plasmids carrying the respective lux reporter fusion were phage transduced into strain LAC. Correct integration was verified by analytical PCR. Light emitted by the luciferase reporter gene fusion constructs was measured using a Victor (Perkin-Elmer) instrument.

Immunoblotting test for PI. Strain LAC was inoculated at 10^6 CFU from a preculture grown in TSB and grown for 24 h at 37°C and 180 rpm in 1 ml of either TSB, serum, or SF. Immunodetection of polysaccharide intercellular adhesive (PIA) was then performed as described by Cramton et al. (26) to minimize cross-reactivity to protein A. In brief, all cultures were resuspended in 1 ml TSB to an optical density at 600 mm (OD600) of 10.0. Each sample was centrifuged and resuspended in 0.5 M EDTA (pH 8.0) and incubated for 5 min at 100°C. Samples were centrifuged and 40 μl of supernatant were incubated with 10 μl proteinase K (20 mg/ml) for 30 min at 37°C. A 3.5-μl portion of each sample was spotted onto nitrocellulose, dried, and blocked with 3% bovine serum albumin (BSA) for 1 h prior to the addition of anti-PIA serum (1:1,000; from rabbit). The membrane was then incubated at 4°C for 18 h. Afterwards, it was washed five times with Tris-buffered saline (TBS)–TWEEN (0.1%) and incubated for 2 h with Cy5-labeled goat anti-rabbit IgG (Life Technologies), followed by washing five times with TBS-TWEEN (0.1%). Signals were detected using a Typhoon Trio variable-mode imager (GE Healthcare).

SEM. To prepare for scanning electron microscopy (SEM), samples were fixed with 2% paraformaldehyde in 0.1 M sodium phosphate buffer. Samples were then sent to the SEM facility at Rocky Mountain Laboratories, NIAID, where they were sputter coated using a South Bay Technologies IBS/e instrument and imaged on a Hitachi SU8000 electron microscope.

Biofilm assays. For biofilm analysis by confocal laser scanning microscopy (CLSM), strains were incubated in TSB under static conditions in an 8-well borosilicate plate for 8 h. TSB was aspirated, replaced with 200 μl of SF, and incubated for a further 24 h. Samples were then washed gently three times with sterile PBS, stained with 4 μM propidium iodide for 30
min at 37°C, and imaged using a Zeiss LSM700 confocal microscope. Total biovolume was calculated using Imaris software from three randomly chosen fields. This protocol was used for all biofilm CLSM analyses with the exception of the protocol used for the image shown in Fig. 1B, for which *S. aureus* (10^9 CFU/ml) was incubated in SF for 20 min, stained with 4′,6-diamidino-2-phenylindole, and immediately imaged.

**Measurement of PSMs.** After butanol extraction of PSMs, PSM production was analyzed by reversed-phase high-pressure liquid chromatography/electrospray mass spectrometry as described previously (27).

**Statistics.** Statistical evaluation was performed using GraphPad Prism version 6.02, with *t* tests for the comparison of two and one-way ANOVA for the comparison of more than two groups. Multiple comparisons with one-way ANOVA were performed using Tukey's posttests.

**RESULTS**

Biofilms and free-floating aggregates form in synovial fluid isolated from patients. In the present study, we used an *ex vivo* ap-
TABLE 2 Strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Description</th>
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<td>Strains</td>
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<td>LAC Δagr</td>
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<tr>
<td>LAC P3-lux</td>
<td>Strain LAC containing genome-integrated luxABCDE genes under the control of the S. aureus Agr P3 promoter</td>
<td>This study</td>
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<tr>
<td>LAC psma-lux</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pTX-psma</td>
<td>Tet', psma operon under constitutive control of the xylose promoter</td>
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<tr>
<td>pTX_agrA</td>
<td>Tet', agrA gene under constitutive control of the xylose promoter</td>
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proach to study the mechanistic underpinnings of the formation of free-floating and surface-attached S. aureus agglomerates (biofilms) during prosthetic joint infection (PJI). We isolated synovial fluid (SF) from noninfected patients undergoing surgery, thus reproducing the environmental conditions the bacteria encounter when mounting a periprosthetic joint infection. All experiments were performed using strain LAC, representing pulsed-field type USA300, the predominant cause of PJI in the United States (19).

Even when SF was isolated from patients who had received high doses of prophylactic antibiotic, which leads to high concentrations of antibiotic in SF at high doses of prophylactic antibiotic, which leads to high concentrations of antibiotic in SF (28), biofilms formed on titanium disks over the course of 48 h (Fig. 1A), underscoring the notion that S. aureus PJs are recalcitrant to antibiotic treatment due to the formation of biofilms (4, 28). Notably, when SF was isolated from patients who had received high doses of prophylactic antibiotic, which leads to high concentrations of antibiotic in SF and serum, with a small increase over expression in TSB, indicating that these are also unlikely to be responsible for the strong aggregation phenotype (Fig. 2). Thus, while fibrinogen- and fibronectin-binding proteins are required as a basis for aggregate formation in SF, our results indicate that, taken together, differences in their expression do not appear to contribute considerably to the strength of aggregation behavior in SF.

Expression levels of the regulatory factors RsbU and TRAP also previously identified in our screen (4) were roughly equivalent in SF and serum, with a small increase over expression in TSB, indicating that these are also unlikely to be responsible for the strong aggregation phenotype (Fig. 2). Thus, while fibrinogen- and fibronectin-binding proteins are required as a basis for aggregate formation in SF, our results indicate that, taken together, differences in their expression do not appear to contribute considerably to the strength of aggregation behavior in SF.

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S. aureus shows low activity of Agr and low production of PSMs in SF. As an alternative explanation, we evaluated whether low activity of the biofilm regulator Agr is responsible for the excessive formation of aggregates and biofilms in SF. To investigate this hypothesis, we constructed reporter gene fusion constructs. In these constructs, the agr P3 or P2 promoters were fused to the lux genes driving production of light-emitting luciferase. P3 drives expression of RNAIII, the intracellular regulator of most Agr targets (29), and P2 drives expression of the Agr proteins making up the Agr autoregulatory quorum-sensing circuit (30). Notably, all luciferase promoter fusions were cloned in the genome of strain LAC. In contrast to plasmid-based systems, which are often used for this purpose, genome insertion ensures that the obtained results accurately reflect natural expression and are not due to multiple, plasmid-introduced copies of the respective genes. During all stages of in vitro growth, Agr (P2 and P3) activity in human SF was extremely low, lower than in human serum and much lower than in TSB (Fig. 3A and B). This was verified using qRT-PCR with the agrA (controlled by the P2 promoter) and RNAIII (controlled by the P3 promoter) genes (Fig. 3C and D).

To analyze expression of the Agr-controlled PSMs in SF, we again used genome-integrated luciferase reporter gene constructs in strain LAC in addition to qRT-PCR measurements. The results confirmed our expectations, inasmuch as expression of the psma and psmb loci was extremely low (Fig. 3E to G), as shown above for RNAIII (Fig. 3C), which also contains the hld gene encoding the δ-toxin. Furthermore, we measured PSM production on the...
protein level (Fig. 3H), which confirmed the results achieved on the transcriptional level. These results demonstrate extremely low production of all PSMs in SF and suggest that low PSM production is a major reason for the marked formation of aggregates in SF.

To analyze whether the observed low activity of Agr in SF and low production of the biofilm-dispersing PSMs are responsible for the aggregative phenotype in SF, we determined the extent of bacterial agglomeration of wild-type and isogenic \( \text{H9004} \) \( \text{agr} \) and \( \text{H9004} \) \( \text{psm} \) mutants as visualized microscopically and using a Cellometer. In the \( \text{H9004} \) \( \text{psm} \) mutant, all \( \text{psm} \) genes have been removed or translation has been abolished (21). We found that all strains, notably including the wild-type strain, showed extensive agglomeration in SF, while in TSB, only \( \text{H9004} \) \( \text{agr} \) and \( \text{H9004} \) \( \text{psm} \) mutants produced agglomerates (Fig. 4). Furthermore, the extent of agglomeration in SF was not statistically different in the wild type and the \( \text{Δagr} \) and \( \text{Δpsm} \) mutants. These results showed that low activity of Agr and suppression of the Agr-controlled PSMs cause exceptionally strong aggregate production in SF.

Low production of the Agr-controlled phenol-soluble modulins is a major factor promoting bacterial aggregation and formation of biofilms in SF. To analyze whether the observed low activity of Agr in SF and low production of the biofilm-dispersing PSMs are responsible for the aggregative phenotype in SF, we determined the extent of bacterial agglomeration of wild-type and isogenic \( \text{Δagr} \) and \( \text{Δpsm} \) mutants as visualized microscopically and using a Cellometer. In the \( \text{Δpsm} \) mutant, all \( \text{psm} \) genes have been removed or translation has been abolished (21). We found that all strains, notably including the wild-type strain, showed extensive agglomeration in SF, while in TSB, only \( \text{Δagr} \) and \( \text{Δpsm} \) mutants produced agglomerates (Fig. 4). Furthermore, the extent of agglomeration in SF was not statistically different in the wild type and the \( \text{Δagr} \) and \( \text{Δpsm} \) mutants. These results showed that low activity of Agr and suppression of the Agr-controlled PSMs cause exceptionally strong aggregate production in SF.

To provide further evidence for the role of PSMs and Agr in aggregation in SF and delineate the specific role of different PSMs...
types, we transformed the LAC wild-type strain with plasmids carrying the psmα, psmβ, hld (encoding δ-toxin), or agrA genes/operons under the control of a constitutively active promoter. In these constructs, low expression of those genes in SF is thus overcome by constitutive expression. We first analyzed the formation of macroscopic clusters during 24-h growth in microtiter plates. In SF, the LAC wild-type strain formed a large cluster, in accordance with our previous results (4); this was not the case in serum or TSB (Fig. 5). Importantly, no cluster formation was visible when the strains expressing psmα, psmβ, or hld genes/operons were incubated in SF. Similarly, no cluster formation was detected when agrA, which controls expression of all of these genes, was expressed (16). Furthermore, the Δpsm mutant, devoid of PSM production, showed large cluster formation in SF, similar to that observed with the wild-type and Δagr strain.

We next analyzed biofilm formation of the constitutive-expression constructs by confocal laser scanning microscopy (CLSM) with analysis of total biovolume. Similar to the results obtained for macroscopic cluster formation, biofilm formation by the constructs expressing psmα, psmβ, hld, or agrA was significantly less pronounced than that by the wild-type strain (Fig. 6). Together, these results identify the lack of PSM production as the main cause for the extensive biofilm and aggregate formation in SF and show that all PSM types have the capacity to disrupt aggregates.

PSMs work by dispersing biofilm matrix molecules such as PIA. The surfactant characteristics of PSMs suggest that these molecules function during biofilm development by disrupting the interaction of biofilm matrix molecules with each other and the bacterial cell surface (31). However, this has not yet been examined experimentally. PIA is considered a major biofilm matrix
component in staphylococci (6). We showed previously that strain LAC produces a large amount of surface-located PIA when grown in SF (4). Transcription of the ica PIA biosynthesis operon (as determined by qRT-PCR of the icaA gene) was not increased in SF (Fig. 7A). However, immunological assessment using PIA-specific antibodies revealed that (i) PIA retention on the bacterial surface was significantly higher in SF than TSB and serum (Fig. 7B) and (ii) PIA was released from the bacterial surface in a PSM-dependent manner (Fig. 7C). The latter was demonstrated by the fact that surface PIA levels were similarly high in the isogenic Δpsm mutant when it was grown in either TSB or SF and in the wild-type strain grown in SF (conditions without or with very low PSM production) but significantly higher than PIA surface levels in the wild-type strain grown in TSB (under which condition PSMs are produced) (Fig. 7C). These results suggest that PIA matrix molecules are abundant on the bacterial surface in the absence of PSMs, indicating that PSMs cause separation of the PIA matrix molecules from the bacterial surface.

To provide further evidence supporting that mechanism, we compared cluster formation of the wild-type and Δpsm mutant strains using scanning electron microscopy (SEM). This was done in TSB, because (i) there is no host-derived fibrous material overshadowing bacterial exopolymers during growth in TSB and (ii) we have shown here that there is virtually no PSM production in SF. SEM showed only single cells and no cluster formation of the wild-type strain (in accordance with the results shown in Fig. 4). In contrast, the Δpsm mutant formed clusters, which had fibrous material on their surface (Fig. 7D), which—in the absence of host material such as fibrin—are strongly indicative of the deposition of bacterial biofilm matrix molecules, such as PIA, but also likely includes other biofilm matrix components, such as teichoic acids, extracellular DNA, and biofilm matrix proteins. Thus, these findings further confirmed that PSMs interfere with the deposition of matrix molecules on the bacterial surface.

**DISCUSSION**

In our previous study, we discovered that S. aureus proteins that connect the bacteria to the human matrix proteins fibrin and fibronectin are prerequisites for the formation of biofilms and biofilm-like aggregates during joint infections (4). In the present study, we asked which factors are responsible for the excessively strong degree of aggregate and biofilm formation in SF, which is the basis for the notorious recalcitrance of such infections to antibiotic treatment (28). We identified the low activity of Agr and consequentially low production of PSMs as major factors contributing to that phenotype. Our results support a two-step model of aggregate formation during joint infection which includes (i) bacterial attachment to fibrin and fibronectin via ClfA, ClfB, FnbA, and FnbB and (ii) extensive agglomeration of cells and bacterial matrix molecules, owing to the absence of the surfactant-like, separating effect of the Agr-controlled PSMs. It appears surprising at first glance that there is low activity of the quorum-sensing regulator Agr in cellular aggregates, despite such aggregates representing a high-cell-density situation. However, we and others have observed overall low activity of Agr activity in *in vitro* staphylococcal biofilms (11, 12, 32). Furthermore, there has been considerable doubt about a direct correlation of cell density and the activity of quorum-sensing systems (33). Whether the low activity of Agr in SF is due to specific factors that are present in SF, such as hyaluronic acid (34, 35), or the overall chemical composition of SF (35) awaits further investigation. One specific possibility that remains to be explored is whether the increased concentration of serum proteins in traumatized SF (36) contributes to a quorum-quenching effect due to sequestration of the Agr pheromone, as described for apolipoprotein B (37).

**FIG 5** PSM expression abolishes the formation of macroscopic cell clusters in SF. The LAC wild-type strain (WT) was inoculated into 200 μl SF, human serum, or TSB at 10⁶ CFU and incubated under static conditions for 18 h. In addition, the LAC Δagr and Δpsm strains and derivatives of the LAC wild-type strain containing either plasmids (pTX₃) for constitutive expression of agrA, psmA, psmB, or hld genes or a control plasmid (pTX₃Δ) were inoculated and grown under the same conditions in SF. Plasmid-containing strains received 12.5 μg/ml tetracycline for plasmid maintenance. Afterwards, cell clusters were visualized by staining with ethidium bromide. Note that large clusters formed only in the WT (when grown in SF) and SF-grown Δagr, Δpsm, and plasmid control samples (arrows), whereas expression of any of the *psm* loci or the PSM regulator agrA resulted in abolishment of cluster formation.

**FIG 6** PSM expression abolishes biofilm formation in SF. (A) Derivatives of the LAC wild-type strain containing plasmids (pTX₃) for constitutive expression of agrA, psmA, psmB, or hld genes or a control plasmid (pTX₃Δ) were assayed for biofilm formation in SF under static conditions (24-h growth). Plasmid-containing strains received 12.5 μg/ml tetracycline for plasmid maintenance. Biofilms were stained with propidium iodide for CLSM. (B) The total biovolume was calculated using Imaris software using 3 randomly chosen image fields. Error bars show standard deviations (SD). ****, P < 0.0001.
The significance of our results extends beyond joint infection. For the first time, we provide evidence in a system closely resembling in vivo conditions, and this evidence underscores a key role of PSM production in defining the extent of *S. aureus* biofilms. Specifically, we demonstrate that low PSM production causes strongly increased biofilm formation. Furthermore, we show that absence of PSMs leads to increased formation of floating aggregates, which was shown previously only for surface-attached biofilms (13,15). Moreover, our results provide previously unavailable evidence for the mechanism by which PSMs disperse biofilms, inasmuch as we demonstrate PSM-dependent release of PIA from the bacterial surface.

Our results are of particular interest given that in vitro studies have led to two different models of how PSM production impacts *S. aureus* biofilm development. Our previous studies, performed using TSB, indicated that absence of PSMs leads to more extensive and compact biofilm formation, owing to a lack of PSM-mediated biofilm structuring and dispersal (13). In contrast, using a different growth medium, Schwartz et al. observed that PSMs form amyloid-like fibrils that promote (rather than decrease) biofilm formation in vitro (38). In our present study, we demonstrate that under conditions emulating the in vivo situation present during a biofilm-associated infection, absence of PSMs leads to extensive formation of biofilms, while the amyloid model of Schwartz et al. would have predicted that in the absence of PSMs, biofilms would be less pronounced. In accordance with our previous in vivo results (13), our present findings further suggest that the role of PSM amyloid fibrils in biofilm development applies only to a very specific in vitro setup.

Our results further support the notion that differences in Agr activity are associated with different types of staphylococcal infection and demonstrate the crucial role that PSMs play in that association. Mutants that are dysfunctional in Agr have been found more frequently in chronic, biofilm-associated infections (14, 39) and in cases of *S. aureus* bacteremia (40). In contrast, a functional Agr system and high production of Agr-regulated toxins, such as PSMs and alpha-toxin, are associated with acute forms of *S. aureus* infection, such as acute skin and lung infections (18,41, 42), and osteomyelitis (43). Since there is continual developing of drugs targeting the Agr system (44), we caution that the use of Agr-blocking therapeutics should be limited to certain infection types and would be counterproductive in others.

In conclusion, the findings from our study indicate that the exceptional recalcitrance of staphylococcal PJI to antibiotic treatment (28) is due to the specific environment in joints that suppresses Agr and production of biofilm-dispersing PSMs, which together with the interaction with host-derived fibrin leads to the formation of extensive bacterial agglomerates. These results further our understanding about the role Agr and PSMs play in defining biofilm-associated *S. aureus* disease, which could lead to the development of antibiotic therapy strategies against PJI. Our findings suggest that therapeutic strategies against staphylococcal...
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