**Staphylococcus epidermidis** surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice

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Biofilms are surface-attached agglomerations of microorganisms embedded in an extracellular matrix. Biofilm-associated infections are difficult to eradicate and represent a significant reservoir for disseminating and recurring serious infections. Infections involving biofilms frequently develop on indwelling medical devices in hospitalized patients, and *Staphylococcus epidermidis* is the leading cause of infection in this setting. However, the molecular determinants of biofilm dissemination are unknown. Here we have demonstrated that specific secreted, surfactant-like *S. epidermidis* peptides — the β subclass of phenol-soluble modulins (PSMs) — promote *S. epidermidis* biofilm structuring and detachment in vitro and dissemination from colonized catheters in a mouse model of device-related infection. Our study establishes in vivo significance of biofilm detachment mechanisms for the systemic spread of biofilm-associated infection and identifies the effectors of biofilm maturation and detachment in a premier biofilm-forming pathogen. Furthermore, by demonstrating that antibodies against PSMβ peptides inhibited bacterial spread from indwelling medical devices, we have provided proof of principle that interfering with biofilm detachment mechanisms may prevent dissemination of biofilm-associated infection.

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Biofilms are surface-attached agglomerations of microorganisms embedded in an extracellular matrix. Biofilm-associated infections are difficult to eradicate and represent a significant reservoir for disseminating and recurring serious infections. Infections involving biofilms frequently develop on indwelling medical devices in hospitalized patients, and Staphylococcus epidermidis is the leading cause of infection in this setting. However, the molecular determinants of biofilm dissemination are unknown. Here we have demonstrated that specific secreted, surfactant-like S. epidermidis peptides — the β subclass of phenol-soluble modulins (PSMs) — promote S. epidermidis biofilm structuring and detachment in vitro and dissemination from colonized catheters in a mouse model of device-related infection. Our study establishes in vivo significance of biofilm detachment mechanisms for the systemic spread of biofilm-associated infection and identifies the effectors of biofilm maturation and detachment in a premier biofilm-forming pathogen. Furthermore, by demonstrating that antibodies against PSMβ peptides inhibited bacterial spread from indwelling medical devices, we have provided proof of principle that interfering with biofilm detachment mechanisms may prevent dissemination of biofilm-associated infection.

Introduction
Surface-attached cellular agglomerations of microorganisms called biofilms are an important virulence determinant in bacteria (1), mainly because biofilm formation significantly increases resistance to antibiotics and host defenses (2). Many biofilm-associated infections occur in the hospital setting by contamination of indwelling medical devices from the epithelial flora of the patient or health care personnel. Staphylococcus epidermidis, a normal inhabitant of human skin, is the predominant causative agent of these infections (3). Importantly, bacterial dissemination from biofilm-infected catheters represents the most frequent source of severe S. epidermidis infections such as sepsis (4). Especially neonates often develop sepsis from S. epidermidis catheter infection, and S. epidermidis infection is a significant global source of morbidity and death, particularly in very low birth weight infants (5).

The formation of a biofilm begins with the attachment of bacteria to a surface and is followed by proliferation and maturation, which ultimately leads to the characteristic 3D biofilm structure with mushroom-shaped bacterial agglomerations surrounded by fluid-filled channels (6). Later, cells may detach from the biofilm in a process believed to be of crucial importance for the dissemination of a biofilm-associated infection. The molecular basis of biofilm maturation and detachment is poorly understood, but presumably involves mechanisms to disrupt cell-cell adhesion. In vitro evidence obtained in Pseudomonas aeruginosa and Bacillus subtilis indicates cell disruption may be accomplished by surfactants (7–10), while enzymatic digestion of biofilm matrix molecules appears to promote biofilm detachment in Actinobacillus actinomycetemcomitans (11). These mechanisms are commonly under control of cell density (“quorum sensing”) and are likely to ascertain a tightly regulated degree of biofilm expansion (12, 13). In staphylococci, the molecular effectors of cell-cell disruption during biofilm development are not known. Furthermore, whether biofilm detachment mechanisms contribute to the in vivo dissemination of biofilm-associated infection has not been investigated in any bacterium.

We and others have identified a family of short staphylococcal peptides, the phenol-soluble modulins (PSMs) (14, 15), which are strictly regulated by the quorum-sensing system agr (16–18) and whose amphiphilic α-helical structure suggests surfactant-like properties. This prompted us to analyze the role of PSMs in biofilm development. In the present study, we demonstrate that the β-type PSMs represent key effectors of S. epidermidis biofilm maturation and detachment. Furthermore, we show that these peptides facilitate the dissemination of biofilm-associated infection, providing evidence for in vivo significance of biofilm detachment.

Results
PSMβ peptides are the main PSM type produced in S. epidermidis biofilm culture. To investigate the role of PSMs in biofilms, we first determined production of PSM peptides in planktonic versus biofilm culture. We found that PSM production was overall lower in biofilm culture, while relative production of β-type PSMs was significantly increased (Figure 1). Of note, β-type PSMs were virtually the only PSM type produced in the biofilm mode of growth. We detected similar production patterns in all S. epidermidis strains in our collec-

Authorship note: Rong Wang, Burhan A. Khan, and Shu Y. Queck contributed equally to this work.

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tion, with the exception of 24 strains that completely lacked production of PSMs, including the agr-encoded δ-toxin. Thus, they are most likely naturally occurring mutants in the agr quorum-sensing system, whose deletion or lack of functionality leads to absence of PSM production (16–18). Of note, all those 24 strains were shown to contain psmβ genes by analytical PCR (data not shown).

The psmβ locus forms a transcriptional unit. δ-type PSMs are distinguished from α-type PSMs and δ-toxin (~20–25 amino acids) by their larger size (~45 amino acids) and absence of cytolytic activity at physiological concentrations (18). As PSM production patterns suggested that especially the β-type PSMs may play a role in biofilm development, we first analyzed organization and transcription of the psmβ genes (Figure 2A). The psmβ operon is composed of 4 genes in strains *S. epidermidis* ATCC12228 and RP62A (19, 20), whose genome sequences are available, while the clinical isolate 1457 (21) used in our study has only 3 psmβ genes. This is due to an exact duplication of the psmβ1 gene in strains ATCC12228 and RP62A, while we determined by DNA sequencing that this duplication is missing from strain 1457. The remaining sequence of the psmβ locus of strain 1457 was exactly the same as in ATCC12228. In the shotgun-sequenced strain M23864, the psmβ operon appears to consist of 5 genes with slightly different peptide products. In keeping with the psmβ1 gene duplication, strains RP62A and ATCC12228 secreted more PSMβ1 than PSMβ2, but combined production of the PSMβ peptides was not higher than in strain 1457 (data not shown). Furthermore, we never detected a peptide in culture filtrates of any *S. epidermidis* strain corresponding to the gene product of the psmβ3 gene, indicating that the hypothetical PSMβ3 peptide is not secreted. To analyze whether the psmβ genes form an operon, and thus are transcribed together, we performed Northern blot analysis (Figure 2B). We detected 1 predominant band, whose size corresponded exactly to the postulated size of a transcript comprising all 3 psmβ genes of strain 1457, which therefore form an operon.

**PSM peptides influence *S. epidermidis* biofilm formation in vitro.** To investigate whether PSMβ peptides impact biofilm development, we first assayed in vitro biofilm formation with synthetic PSMβ peptides. The peptides were added to growing biofilm cultures of an agr mutant of the *S. epid.* 1457 strain used in this study. Construction of the psmβ1 mutant allelic replacement strain is shown at the bottom. (B) Northern blot analysis of *S. epidermidis* 1457 RNA from different growth phases using a psmβ1 probe. The psmβ signal is marked by an arrow.
of strain 1457 (22), which owing to strict regulation of PSMs by agr (16–18), is devoid of PSM production. The PSMβ peptides promoted biofilm formation in a medium concentration range, while they inhibited biofilm formation at higher concentrations (Figure 3A). Of note, this phenotype is in accordance with a putative function of PSM peptides in cell-cell disruption on the molecular level (3, 23): at lower concentrations, this process is thought to facilitate the formation of channels, which increases biofilm formation on the macroscopic level, whereas higher concentrations promote detachment and thus a reduction in biofilm mass (Figure 3B).

Biofilm formation in staphylococci has been shown to be exopolysaccharide dependent or independent (24, 25). To analyze whether PSMβ peptides mediate biofilm detachment in both exopolysaccharide-dependent and -independent S. epidermidis biofilms, we first determined presence of the ica genes responsible for production of the main biofilm exopolysaccharide polysaccharide intercellular adhesin (PIA, or poly-N-acetylglucosamine [PNAG]) in the 24 agr-negative S. epidermidis strains. Then we determined in vitro biofilm formation by these strains. Interestingly, biofilm-forming capacity was significantly higher among the strains that contained ica genes than among the ica-negative strains (Figure 4A), confirming the importance of PIA/PNAG for efficient biofilm formation in S. epidermidis (24). We selected the 2 ica-negative strains with the highest biofilm-forming capacity and 1 ica-positive strain, in addition to strain 1457, to confirm the universal importance of PSMβ peptides for biofilm detachment in vitro. In all strains, PSMβ1 had a biofilm detachment effect very similar to that observed with strain 1457 (Figure 4D), demonstrating that the impact of PSMβ peptides on biofilm detachment in vitro is not dependent on whether biofilm formation is exopolysaccharide dependent or independent.

To confirm the specificity of PSMβ detachment activity and analyze the importance of the PSMβ amphipathic α-helix in the observed detachment process, we synthesized a PSMβ1 derivative, PSMβ1*, in which 5 amino acids were altered to prevent the formation of an amphipathic α-helix (Figure 4, B and C). As the formation of an amphipathic α-helix is the basis of surfactant properties, these experiments were also conducted to gain insight into whether the detachment mechanism is dependent on the surfactant properties of PSMβ1. According to analysis by HeliQuest (http://heliquest.ipmc.cnrs.fr), the amphipathic α-helical part of PSMβ1 extends from amino acids 17 to 44 (C terminus). This was predicted by the hydrophobic face that is characteristic of an amphipathic α-helix and was found for 18–amino acid stretches comprising those, but not amino acids N-terminal of position 17 (stretches 1–18 to 16–43). In the C-terminal part of PSMβ1, 2 lysine residues (positive charge) were changed to proline residues (K22P, K40P), and 3 uncharged amino acids were altered to negatively charged amino acids (W20E, I27E, V35E) to abolish α-helicity and amphipathy in PSMβ1* (Figure 4, B and C). With PSMβ1*, the detachment effect was absent (in strains 1457, BM26, and BM33) or strongly decreased (strain BM32) compared with PSMβ1 (Figure 4D), indicating that the impact of PSMβ1 on biofilm detachment is specific and dependent on the PSMβ amphipathic α-helix. Furthermore, these results strongly suggest that the mechanism by which PSMβ peptides contribute to biofilm maturation and detachment is based on their surfactant properties.

Biofilm structuring and detachment by PSMβ peptides is likely accomplished during biofilm development, while mature S. epidermidis biofilms that are stabilized by an exopolysaccharide network may be largely resistant to macroscopic decomposition by externally added PSMβ peptides. To test this hypothesis, we added PSMβ1 peptide at 1 mg/ml to a 24-hour biofilm of the PIA/PNAG-producing strain 1457. We observed only a very minor impact on the biofilm (data not shown). In agreement with a surfactant-based mechanism of PSMβ detachment, these results suggest that PSMβ peptides must be present during biofilm development to promote biofilm structuring and detachment, while the presence of an established matrix network prevents the large-scale detachment of biofilm clusters by PSMβ peptides.

PSMβ peptides promote biofilm structuring and detachment in vitro. To validate the hypothesis that PSMβ peptides promote biofilm detachment, we analyzed biofilm development under flow using confocal laser scanning microscopy (CLSM). To measure expression of the psmβ operon, we constructed a psmβ promoter-egfp transcriptional fusion. The egfp gene was synthetically assembled using codon usage optimized for staphylococci (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42520DS1) to increase fluorescence intensity. Expression of psmβ was limited to the outer layers of the biofilm.
(Figure 5A) in accordance with a function in detachment and control by agr, for which we have previously shown a similar spatial expression pattern in S. epidermidis biofilms (26). Importantly, we observed that strong psmβ expression was followed by the appearance of void spaces (Figure 5, B and C). Furthermore, there were significantly more psmβ-expressing cells in the effluent compared with the biofilm cells (Figure 5D). These results confirmed our hypothesis that psmβ expression leads to biofilm cluster detachment during biofilm development.

To further investigate the role of PSMβ peptides in S. epidermidis biofilm development, we constructed a deletion mutant of the entire psmβ operon in S. epidermidis 1457 (Figure 2A). CLSM images revealed that static biofilms of the psmβ operon deletion mutant in comparison with the isogenic WT strain lacked channel formation and were thicker (Figure 6, A and C). Genetic complementation restored the phenotype observed in the WT (Figure 6D). These results confirmed our hypothesis that psmβ expression leads to biofilm cluster detachment during biofilm development.

In addition, they further substantiated that PSMβ peptides facilitate detachment, as the lack of detachment leads to biofilm expansion.

Furthermore, we compared the phenotype of the psmβ deletion mutant with that of an isogenic agr mutant. The biofilm phenotypes of these strains were very similar (Figure 6C), indicating that PSMβ peptides represent the main effector molecules of quorum-sensing regulated biofilm maturation in S. epidermidis.

PSMβ peptides promote dissemination from in vivo biofilms. To analyze the role of β-type PSMs in biofilm detachment in vivo, we performed a murine model of device-related infection (Figure 7 and Table 1). The development of human sepsis is a rare event compared with the frequency of catheter infection and possibly due to yet unknown roles of host factors; it is thus difficult to mimic in an animal model. We therefore focused on analyzing bacterial spread from the catheters to the lymph and organs. Furthermore, we used immune-compromised Nu/Nu mice, as we showed previously that those mice develop more pronounced and longer lasting catheter-related infection (27).

In this model, 2 catheters, precolonized with equal numbers of either the psmβ mutant or WT strain, were inserted s.c. on the right and left dorsum, respectively. In a first experiment, we measured systemic dissemination into body fluids as assessed by taking body
fluid samples from the peritoneal cavity. Bacteria harvested from body fluids were overwhelmingly and significantly more of the WT than \( \textit{psm}^{\beta} \) mutant strain (Table 1), indicating dissemination is favored in the WT over the mutant strain. Bacteria were rarely found in organs, but those that were found were almost exclusively of the WT. Owing to low and strongly varying numbers, differences in the organs only reached statistical significance in the liver sample at day 4 (Table 1). Finally, bacteria on the WT infected catheter were only of the WT strain, while on the \( \textit{psm}^{\beta} \) mutant–infected catheter, rare infiltration of WT bacteria was detected, in accordance with dissemination occurring only for WT bacteria.

In a second experiment, we focused on determining bacterial dissemination into the lymph nodes, as those would be first encountered by bacteria after dissemination. Bacteria in all lymph nodes were almost exclusively of the WT strain (Figure 7 and Table 1). Only bacteria in the right brachial and axillary lymph nodes were mainly of the mutant strain, most likely due to involvement of adjacent tissue during surgical insertion of the catheters. In contrast, no bacteria were found in the blood. This suggests that bacteria found in the body fluids from the peritoneal cavity in the first experiment originated from the lymph, while host defenses had cleared bacteria in the blood. Together, these data demonstrated that systemic dissemination from the indwelling device was strongly and significantly favored in the WT compared with the \( \textit{psm}^{\beta} \) deletion strain and thus, PSM\( ^{\beta} \) peptides play a key role in the dissemination of biofilm-associated infection. Finally, results from a bacteremia control experiment indicated that PSM\( ^{\beta} \) peptides do not have an impact on the development of disease that is unrelated to biofilm formation, as no significant difference was detected when monitoring death of animals injected with WT or \( \textit{psm}^{\beta} \) mutant bacteria, respectively (Supplemental Figure 2).

**Antibodies to PSM\( ^{\beta} \) peptides block dissemination of biofilm-associated infection.** To further substantiate the involvement of PSM\( ^{\beta} \) peptides in the dissemination of biofilm-associated infection,
we investigated whether antibodies against PSMβ peptides prevent dissemination. To that end, we first produced antibodies to PSMβ1 and PSMβ2 in mice. PSMβ peptides proved immunogenic and elicited a strong IgG response (Supplemental Figure 3). Antisera were treated using extracts from the psmβ deletion mutant, and the resulting blocked antisera were highly specific for PSMβ1 and PSMβ2, respectively (Supplemental Figure 3). We pooled the PSMβ1 and PSMβ2 antisera and analyzed the efficacy of the obtained serum in reducing the dissemination of biofilm-associated infection using the same device-related infection model. In contrast to animals treated with PBS or control serum, no bacteria were found in the livers, spleens, or kidneys of animals treated with the anti-PSMβ antibodies, and bacterial load in the lymph nodes was significantly reduced (Figure 8). Reduced bacte-

**Figure 6**

Role of PSMβ peptides and agr in *S. epidermidis* biofilm development: channel formation and biofilm expansion. (A) CLSM pictures of *S. epidermidis* 1457 WT, isogenic psmβ mutant, and psmβ-complemented strains. The WT and psmβ mutant strains were transformed with the control plasmid pT181 mcs to ensure comparability. Static biofilms were grown for 24 hours, stained with propidium iodide, and imaged using CLSM. View is from the top. (B) Analyses of total and average biovolumes, which are measures of total biofilm and degree of channel formation, respectively, using IMARIS software of the biofilm samples shown in part A. Note that increased average biofilm volume corresponds to decreased channel formation. **P < 0.01; ***P < 0.001, 1-way ANOVA with Bonferroni’s post tests. Error bars depict mean ± SEM. (C) CLSM pictures of *S. epidermidis* 1457 WT, isogenic agr, and psmβ operon deletion mutant biofilms. Growth conditions are as in A.
biofilm development is of utmost importance for potential therapeutic use.

Detailed understanding of the molecular determinants governing biofilm development is of utmost importance for potential therapeutics interfering with staphylococcal virulence, as many toxins are under positive control of agr (32–34). On the other hand, it has been noted that Agr-dysfunctional and thus, PSM-negative strains are more frequently found among strains isolated from biofilm-associated infection (26). Our present results suggest that such strains have lost the capacity to detach and owing to the lack of PSM production may form more compact and extensive biofilms.

Blocking the agr quorum-sensing system has been frequently proposed as a potential basis for novel therapeutics interfering with staphylococcal virulence, as many toxins are under positive control of agr (32–34). On the other hand, it has been noted that this may lead to increased biofilm formation, owing to negative agr control of biofilm expansion (33), which is in accordance with results achieved in the present study. However, our results also indicate that such treatment may inhibit bacterial dissemination from biofilms, showing that interference with agr might potentially be beneficial also during biofilm-associated infection. In addition, we provide proof of principle that the specific inhibition of biofilm detachment surfactants may prevent dissemination, a strategy we believe should be further evaluated for potential therapeutic use.

Surfactants are not the only molecules that have been proposed to contribute to biofilm maturation and detachment in staphylococci. According to an alternative hypothesis, these processes may also be accomplished by enzymatic degradation of biofilm matrix molecules. In that regard, recent research indicated that staphylococcal biofilm formation is accomplished by protein and/or exopolysaccharide biofilm matrix components (24, 25). Hypothetically, enzymatic degradation of these polymers may thus contribute to biofilm maturation; and a proteolytic detachment mechanism has been proposed for protein-dependent biofilms of S. aureus (28). However, direct evidence for such a mechanism is scarce and no evidence has been produced for clinical strains or in vivo. Furthermore, it has been shown that such enzymes specifically degrade only those staphylococcal biofilms that are

Biofilm maturation and detachment are commonly under quorum-sensing regulation to ascertain a well-controlled degree of channel formation and biofilm expansion (12, 26, 28, 29). This is also the case for staphylococci (26, 28–31). Importantly, our findings indicate that the PSM peptides are the main determinants that exert the impact of quorum-sensing control on these mechanisms of biofilm development in S. epidermidis on the molecular level. This is in good agreement with our previous observation in S. aureus showing that the mechanism of psm control by agr is different from, and likely evolved before, agr control of other staphylococcal virulence determinants (16). Our finding that PSMs have a key role in biofilm development as a basic phenotype of staphylococcal physiology during both commensal life and infection provides a plausible explanation for the stringency and early evolution of that regulation. Interestingly, the relatively higher concentration of PSM peptides compared with other PSM peptides in biofilm versus planktonic culture indicates that the psm operon is regulated by a yet unknown regulator in addition to the psm master regulator agr. Furthermore, we have previously shown that Agr-dysfunctional and thus, PSM-negative strains are more frequently found among strains isolated from biofilm-associated infection (26). Our present results suggest that such strains have lost the capacity to detach and owing to the lack of PSM production may form more compact and extensive biofilms.

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1457 is a biofilm-forming clinical isolate frequently used for biofilm research (21). Further, using S. epidermidis as an example, our study provides evidence for the importance of biofilm detachment molecules in the dissemination of biofilm-associated infections, thus identifying a potential target for therapeutics aimed at preventing complication and spread of such infections.

Methods

Bacterial strains, plasmids, and growth conditions. S. epidermidis 1457 is a biofilm-forming clinical isolate frequently used for biofilm research (21). The S. epidermidis 1457 psmβ mutant was constructed by replacement with a spectinomycin resistance cassette (39, 40) (for all oligonucleotides, see Table 2). Fidelity of the deletion was determined by DNA sequencing and reversed phase HPLC/electrospray ionization mass spectrometry (RP-HPLC/ESI-MS) of culture supernatants, which demonstrated the specific absence of the PSMβ peptide. For complementation, the psmβ operon from S. epidermidis 1457 was PCR amplified and cloned in plasmid pTi181ms (41). The psmβ promoter (egfp) fusion was produced by cloning the putative psmβ promoter in front of an egfp gene that was synthesized with optimized codon usage for staphylococci (Supplemental Figure 1). Bacteria were grown in tryptic soy broth (TSB) to which 0.5% glucose was added for biofilm experiments. Antibiotics were added when appropriate (12.5 μg/ml tetracycline, 400 μg/ml spectinomycin, 10 μg/ml chloramphenicol). Spectinomycin was only used during construction of, but not in experiments using the psmβ deletion strain.

Analytical PCR to test for presence of psmβ and ica genes. Genomic DNA was isolated from all S. epidermidis strains that did not show PSM peaks in HPLC analysis. The strain collections originated from Paris and Shanghai (42, 43). Presence of psmβ genes was tested using primers designed to amplify both psmβ1 and psmβ2, and either psmβ1 or psmβ2 alone (Table 2). Strains showing at least one positive signal were regarded as positive for the psmβ locus. One strain showing no signal was determined not to be S. epidermidis by Api Staph (bioMérieux) analysis. Presence of ica genes was determined using primers icaBF and icaBR, which amplify a portion of the icaB gene (44).

s.c. device-related biofilm infection model. Segments of i.v. catheters (Abbott Ireland) 1.0 cm in length, were precolonized with overnight cultures of S. epidermidis 1457 or the isogenic psmβ deletion strain for 3 hours at 37°C. After precolonization, each catheter segment was rinsed with 2 ml sterile PBS and air dried to remove residual bacterial cultures. Bacteria on several catheter pieces that were treated equally to those inserted in the animals were counted (using the method described below), showing that equal numbers of WT and mutant bacteria adhered. Nu/Nu B/C CRL female mice (Charles River Laboratories) weighing 18–25 g were anesthetized with isoflurane, their flanks shaved, and the skin cleansed with betadine and ethanol. A 5.0-mm incision was made and dissected to create a s.c. tunnel into which a precolonized catheter segment was implanted at a distance of at least 2 cm from the incision. The incision was closed with surgical sutures and the skin disinfected. Two catheter segments were inserted into each animal, 1 on each side, 1 precolonized with S. epidermidis 1457, and the other with the isogenic psmβ deletion strain. After catheter implantation, animal general health condition and disease advancement were monitored every 24 hours for a total of 4 days. Animals were euthanized via exsanguinations under deep surgical anesthesia (isoflurane) either on day 2 or day 4 after catheter implantation. Animal organs (kidneys, livers, spleens) and implanted catheters or lymph nodes were harvested for further analysis. Animal body fluid samples were obtained from the peritoneal cavity after organ removal. Pure blood samples were obtained via heart puncture. Each harvested catheter...

Table 1

Catheter-related animal infection model

<table>
<thead>
<tr>
<th>Analyzed organ, body, fluid or tissue</th>
<th>Day 2, total numbers (n = 9, except for lymph nodesa)</th>
<th>Day 4, total numbers (n = 6)</th>
<th>Percentage WT bacteria among total, day 2b</th>
<th>Percentage WT bacteria among total, day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter, WT side (left)</td>
<td>1.29 ± 0.42 × 10^5</td>
<td>0.67 ± 0.46 × 10^5</td>
<td>99.8</td>
<td>100</td>
</tr>
<tr>
<td>Catheter, Δpsmβ side (right)</td>
<td>1.02 ± 0.42 × 10^5</td>
<td>1.09 ± 0.49 × 10^5</td>
<td>6.3</td>
<td>6.1</td>
</tr>
<tr>
<td>Blood/body fluids (i.p. cavity)</td>
<td>1.2 ± 0.43 × 10^5/ml</td>
<td>1.1 ± 0.46 × 10^5/ml</td>
<td>97.2 (P &lt; 0.0001)</td>
<td>98.1 (P &lt; 0.0001)</td>
</tr>
<tr>
<td>Liver</td>
<td>100 ± 57</td>
<td>150 ± 33</td>
<td>93.3</td>
<td>96.7 (P = 0.045)</td>
</tr>
<tr>
<td>Kidney</td>
<td>17 ± 4</td>
<td>42 ± 12</td>
<td>100</td>
<td>96.0</td>
</tr>
<tr>
<td>Spleen</td>
<td>31 ± 22</td>
<td>20 ± 4</td>
<td>96.6</td>
<td>91.9</td>
</tr>
<tr>
<td>Blooda (heart puncture)</td>
<td>0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lymph nodes (n = 7a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brachial/axillary, left (WT side)</td>
<td>0.33 ± 0.10 × 10^3</td>
<td>ND</td>
<td>92.1</td>
<td>ND</td>
</tr>
<tr>
<td>Brachial/axillary, right (Δpsmβ side)</td>
<td>1.31 ± 0.36 × 10^3</td>
<td>ND</td>
<td>28.6</td>
<td>ND</td>
</tr>
<tr>
<td>Cervical</td>
<td>2.12 ± 1.48 × 10^3</td>
<td>ND</td>
<td>70.1</td>
<td>ND</td>
</tr>
<tr>
<td>Inguinal, left (WT side)</td>
<td>0.10 ± 0.10 × 10^3</td>
<td>ND</td>
<td>95.8</td>
<td>ND</td>
</tr>
<tr>
<td>Inguinal, right (Δpsmβ side)</td>
<td>0.21 ± 0.12 × 10^3</td>
<td>ND</td>
<td>66.0</td>
<td>ND</td>
</tr>
<tr>
<td>Paravertebral</td>
<td>0.80 ± 0.74 × 10^3</td>
<td>ND</td>
<td>98.7</td>
<td>ND</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>2.18 ± 1.41 × 10^3</td>
<td>ND</td>
<td>92.8</td>
<td>ND</td>
</tr>
</tbody>
</table>

aDissemination into lymph nodes and blood was investigated in a separate experiment under the same experimental conditions. bFifty-two clones, or the total number of available clones, were assayed for spectinomycin sensitivity to calculate WT versus Δpsmβ percentages. cSEM. dStatistics: WT versus Δpsmβ bacteria, unpaired t tests.
was rinsed with 2 ml sterile saline, air dried, then sonicated in 1 ml sterile saline for 1 minute, and vigorously vortexed for 10 minutes to remove bacterial cells from the catheter. Obtained cell solutions were diluted 1:10. Each organ was ground in 3 ml saline using a conical tissue grinder (VWR). Lymph nodes (only harvested at day 2 after infection) were surgically excised and ground using disposable pellet pestles in 100 μl saline. The left and right brachial/axillary and the inguinal lymph nodes were analyzed separately, while the cervical nodes were combined for analysis. Then 100 μl of all obtained solutions, blood, or body fluid samples were plated on TSB plates and counted. Colonies were transferred to TSB plates containing 400 μg/ml spectinomycin in order to distinguish colonies of the psmβ mutant strain (spectinomycin resistant) from those of the WT strain, and percentages of each colony type were determined.

For passive immunization, each mouse received 100 μl of purified mouse anti–PSMβ-IgG via i.p. injection. Control animals received injections of sterile PBS or purified serum IgG obtained from BALB/c mice immunized with adjuvant only. Precolonized catheters were s.c. inserted into the animals 24 hours after passive immunization. The infection model was performed as above, except both catheter pieces were colonized with WT bacteria, and bacterial loads were determined in the organs and lymph nodes. All animal protocols were reviewed and approved by the Animal Use Committee at Rocky Mountain Laboratories, NIAID, NIH.

Opsonophagocytosis assay. An overnight culture of S. epidermidis 1457 was inoculated at an OD600 of 0.1 into 50 ml of TSB (in 125 ml conical glass flasks) at 37°C and grown for 3 hours. The bacteria were washed once in ice-cold PBS and then harvested by centrifugation at 4°C. Dilutions of bacteria in PBS were opsonized with purified mouse anti–bodies against PBS or PSMβ1/PSMβ2 in a 1:1 ratio for 30 minutes at 37°C. Female Nu/Nu B/C RIL mice (Charles River Laboratories) were exsanguinated. The obtained mouse blood was incubated for 60 minutes at 37°C with the opsonized bacteria at a ratio of antibody/bacteria/blood of 1:1:16 in a total volume of 100 μl. In preliminary experiments, a bacterial dose of 5 × 10⁴ was determined to be optimal, as the Figure 8 Blocking dissemination of biofilm-associated infection using anti-PSMβ serum. Specific antisera against PSMβ1 and PSMβ2 were mixed 1:1 and used to protect mice in the biofilm infection/dissemination model. *P < 0.05, versus PBS and control serum for the lymph nodes, versus PBS for the kidney results; 1-way ANOVA with Bonferroni’s post-tests. For spleen and liver, differences were not statistically significant owing to the fact that dissemination into organs only occurred in a limited number of mice and therefore SEM values were high. However, dissemination into the organs of mice that received anti-PSMβ serum was never observed (average CFU/100 μl ≤ 1). Number of mice: controls, n = 9; anti-PSMβ, n = 7. Control serum was from animals that received injections containing CFA and IFA. Error bars depict mean ± SEM.

Table 2 Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betasac</td>
<td>GAAACTTTTGTGAGCTCCTATACAACCTGAAATATACC</td>
</tr>
<tr>
<td>Betabam</td>
<td>CTCCAACACGTGACTGTCAGGATCC</td>
</tr>
<tr>
<td>Betasal</td>
<td>GTGAAAGTGGGTACCATTACATGTCAGGATCC</td>
</tr>
<tr>
<td>Betapst</td>
<td>CAAGCGTTTGCCTAACACTAAGGATCC</td>
</tr>
<tr>
<td>psmβ complementation plasmid</td>
<td></td>
</tr>
<tr>
<td>22N</td>
<td>GCTTTTTTCTCAGCATCATAAGGATCCACTAATGTATCGTC</td>
</tr>
<tr>
<td>28N</td>
<td>GCTTTTTTCTCAGCATCATAAGGATCCACTAATGTATCGTC</td>
</tr>
<tr>
<td>Cloning of psmβ promoter region in egfp fusion vector</td>
<td></td>
</tr>
<tr>
<td>22N</td>
<td>GTAAACACCTAAGAAATTTTACATTAGTAATCTTAATATTTATAG</td>
</tr>
<tr>
<td>Egfbetabam</td>
<td>CCGGTGTTTATCCTGTTGAATCTATTGTTATATTTACG</td>
</tr>
<tr>
<td>Probe for Northern blot</td>
<td></td>
</tr>
<tr>
<td>PSMβ probe</td>
<td>GCGGTGTTTATCCTGTTGAATCTATTGTTATATTTACG</td>
</tr>
<tr>
<td>Oligonucleotides to test for presence of psmβ genes</td>
<td></td>
</tr>
<tr>
<td>Amplifying parts of psmβ2 and psmβ3</td>
<td>TATTTGACGCAATTAGAAGTGTAG</td>
</tr>
<tr>
<td>PSMβtestfor</td>
<td>GCTAACCACCTTTCCTGAGTTC</td>
</tr>
<tr>
<td>Amplifying parts of psmβ2 and psmβ1</td>
<td>TTAGCAGAAGCTATTGCAAATACA</td>
</tr>
<tr>
<td>PSMβ1bfor</td>
<td>TTAGAAACCGAAGATTTTACCTAATACGCT</td>
</tr>
<tr>
<td>PSMβ1arev</td>
<td>TTAGAAACCGAAGATTTTACCTAATACGCT</td>
</tr>
<tr>
<td>Amplifying parts of psmβ3</td>
<td>TATTTGACGCAATTAGAAGTGTAG</td>
</tr>
<tr>
<td>PSMβ2testfor</td>
<td>AATAATTGAAATAATAACTAACA</td>
</tr>
</tbody>
</table>
largest difference between control (blood) and antibody-treated samples was observed at that dose. Surviving bacteria were counted by spotting 400 μl (16 × 25 μl aliquots) onto TSB plates.

CLSM. For static biofilm assays, 300 μl TSB/0.5% glucose was inoculated from precultures grown overnight at a dilution of 1:100 in 8-well polystyrene chambers (Lab-Tek II; Nunc). After 24 hours of incubation at 37°C, supernatants were gently removed, and biofilm layers were washed twice with PBS, resuspended in 400 μl PBS, and stained with propidium iodide (4 μM, 400 μl, 10 minutes). Flow cells (Stowell FLCSAS0001) were inoculated from precultures grown overnight, cells were let to settle for 1 hour, and biofilms were grown under a constant flow of TSB/0.5% glucose/4 μM propidium iodide, using an Ismatec IP Precision Pump at setting 9.0. Images were acquired on a Zeiss LSM 5 Pascal laser-scanning confocal unit equipped with an argon laser, a helium-neon laser, and an Axiovior 100 microscope (Zeiss) with a 63 × 1.4–NA oil-immersion objective. Zeiss 3D (Image VisArt) software was used for the 3D visualization of biofilm structures. Further image analysis was performed using IMARIS 7.0.0 software (Bitplane).

Synthesis and detection of PSM peptides. PSM peptides were synthesized by a commercial vendor at a purity of greater than 95% with the naturally occurring N-terminal N-formyl methionine present. RP-HPLC/ESI-MS was used to measure PSM concentrations in culture filtrates as described previously (17) with slight modifications. An Agilent Technologies Zorbax SB-C8 2.3 × 30 mm reversed-phase column was used. A gradient from 0.1% trifluoroacetic acid in water to 0.1% trifluoroacetic acid in acetonitrile was run at a flow rate of 0.5 ml/min. The LC-MS system consisted of an Agilent 1100 series HPLC system connected to an Agilent Trap VL Ion Trap–Type Mass Spectrometer. 10 or 100 μl culture filtrate was injected for each sample run. Calibration was performed using synthetic PSM peptides. The 2 major peaks of each PSM ESI mass spectrum were used to compute PSM concentrations using Agilent Quant Analysis Software.

Generation, purification, and determination of immunogenicity of PSMβ-specific mouse antisera. To generate S. epidermidis PSMβ peptide–specific antibodies, BALB/c female mice (6–8 weeks / 20–25 g; Charles River Laboratories) were s.c. injected with 50 μg of N-formylated PSMβ1 or PSMβ2 in 100 μl sterile PBS emulsified with CFA for primary immunization. For secondary immunizations, which contained incomplete Freund adjuvant (IFA) instead of CFA, were given to the mice on day 14 and day 28 after the primary immunization. The control animals received injections of sterile PBS and adjuvant only. Blood samples were collected before each immunization. All animals were sacrificed 14 days after the booster immunization for the terminal blood samples. Serum samples were collected for peripheral blood antibody evaluation. To block non-specific binding, anti-PSMβ1 and anti-PSMβ2 sera were pooled, diluted 1:50 in TBS buffer (Tris-buffered saline: 10 mM Tris-HCl, 150 mM NaCl, pH 7.4), and incubated with overnight culture supernatant of the S. epidermidis 1457 isogenic psmβ deletion strain for 16 hours at 4°C with gentle shaking. Precipitated material was sedimented by centrifugation at 28,000 g for 30 minutes at 4°C, and PSMβ-specific IgG in the supernatant was purified using a HiTrap Protein G Affinity Column (GE Healthcare). Control serum samples obtained from animals immunized with adjuvants only were purified following the same procedure. The eluted IgG fractions were concentrated using Amicon Ultra-15 centrifugal filter units (Millipore) and dialyzed against sterile PBS using Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific).

ELISA assays with synthetic S. epidermidis PSMβ peptides were used to determine whether the peptides were immunogenic. Microtiter plates (Nunc 96-well flat-bottom MaxiSorp plates) were coated with 20 μg/ml of each synthetic PSMβ peptide in PBS plus 0.05% Na3C03 and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% Tween-20 and blocked for 1 hour at room temperature with 1% BSA (Sigma-Aldrich) and 0.05% Na3C03 in PBS. Serum samples were diluted in assay diluent (Tris-buffered saline containing 0.1% BSA and 0.05% Tween-20, pH 7.2) and added to the washed wells at 100 μl/well for an incubation of 2 hours at room temperature. Plates were washed again and HRP-labeled goat anti-mouse IgG (R&D Systems) or goat anti-mouse IgM (Jackson Immunoresearch Laboratory) were diluted in assay diluent and added to the plates at 100 μl/well for an incubation of 1 hour at room temperature in order to detect PSMβ1- or PSMβ2-specific mouse IgG and IgM, respectively. Plates were washed again and a substrate solution containing equal volumes of tetramethylbenzidine and H2O2 was added to the plates at 100 μl/well for color development. The reaction was terminated by adding 50 μl of 1 M H2SO4 to each well, and OD was measured at 450 nm using an ELISA plate reader.

Northern blot analysis. Total RNA (10 μg) was prepared from mid-log, late-log, and stationary growth phase of S. epidermidis, electrophoresed in a formaldehyde agarose gel (1.2%), transferred to a nylon membrane, and cross-linked with UV radiation. The DNA probe was PCR generated using specific primers (Table 2), covering the entire psmβ operon from psmβ1 to psmβ3, and labeled with digoxigenin (DIG) using a Roche DIG labeling kit according to the manufacturer’s instructions. The hybridized probe was incubated with an alkaline-phosphatase–conjugated anti-DIG antibody and detected using NBT/BCIP.

Microtiter plate biofilm assays. In vitro biofilm assays were performed in 96-well polystyrene microtiter plates as described previously (31). Plates were incubated after inoculation at 37°C for 24 hours without shaking. Biofilm formation was made visible by staining with 0.1% safranin (Sigma-Aldrich) for 30 seconds and quantified using a Safire microtiter plate reader (Tecan) and Magellan Version 3.00 software (Tecan). The reader was set to multiple read mode (circle pattern, 6 × 6 number of reads), and absorbance was measured at 490 nm.

Statistics. 1-way ANOVA with Bonferroni’s post-tests or unpaired t tests was used to calculate 2-tailed P values using Graph Pad Prism 5 software. P < 0.05 was considered significant. Error bars depict mean ± SEM.

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