Toxin Mediates Sepsis Caused by Methicillin-Resistant Staphylococcus epidermidis

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Abstract

Bacterial sepsis is a major killer in hospitalized patients. Coagulase-negative staphylococci (CNS) with the leading species Staphylococcus epidermidis are the most frequent causes of nosocomial sepsis, with most infectious isolates being methicillin-resistant. However, which bacterial factors underlie the pathogenesis of CNS sepsis is unknown. While it has been commonly believed that invariant structures on the surface of CNS trigger sepsis by causing an over-reaction of the immune system, we show here that sepsis caused by methicillin-resistant S. epidermidis is to a large extent mediated by the methicillin resistance island-encoded peptide toxin, PSM-mec. PSM-mec contributed to bacterial survival in whole human blood and resistance to neutrophil-mediated killing, and caused significantly increased mortality and cytokine expression in a mouse sepsis model. Furthermore, we show that the PSM-mec peptide itself, rather than the regulatory RNA in which its gene is embedded, is responsible for the observed virulence phenotype. This finding is of particular importance given the contrasting roles of the psm-mec locus that have been reported in S. aureus strains, inasmuch as our findings suggest that the psm-mec locus may exert effects in the background of S. aureus strains that differ from its original role in the CNS environment due to originally "unintended" interferences. Notably, while toxins have never been clearly implied in CNS infections, our tissue culture and mouse infection model data indicate that an important type of infection caused by the predominant CNS species is mediated to a large extent by a toxin. These findings suggest that CNS infections may be amenable to virulence-targeted drug development approaches.
Author Summary

Coagulase-negative staphylococci (CNS) are the leading cause of sepsis in hospitalized patients, causing a significant number of deaths. This situation is further worsened by a limitation of therapeutic options due to the fact that most CNS infectious isolates are resistant to methicillin. CNS sepsis has been assumed to be due to an over-reacting immune response triggered by invariant bacterial surface structures. By using tissue culture and animal infection model-based evidence, we here show that in contrast to that notion, the PSM-mec toxin produced by methicillin-resistant strains of the leading CNS species Staphylococcus epidermidis has a strong impact on the severity of sepsis and its outcome. This is the first report to link a toxin to the pathogenesis of the most frequent bacterial cause of sepsis. Notably, these findings pave the way for anti-virulence strategies against this widespread and deadly type of infection.

Introduction

Bacterial sepsis is a frequent cause of death in hospitalized patients. Coagulase-negative staphylococci (CNS) are the leading cause of nosocomial sepsis, especially in neonates [1–3]. CNS sepsis most often originates from the infection of indwelling medical devices, such as catheter-related bloodstream infections (CRBSIs) or central line-associated blood stream infections (CLABSIs) [4]. Most prominent among CNS infections are those due to the skin commensal Staphylococcus epidermidis [5]. However, the bacterial factors contributing to the development of sepsis, in particular in CNS, are poorly understood.

Given that toxins have long been assumed to be widely absent from CNS [6], sepsis caused by S. epidermidis and other CNS, similar to other Gram-positive bacteria, has so far been believed to be due predominantly to an overwhelming immune reaction directed against invariable, pro-inflammatory cell surface molecules, such as teichoic acids and lipopeptides [7]. Recently, the notion that CNS do not commonly produce toxins had to be revised with the discovery of the pro-inflammatory and cytolytic phenol-soluble modulin (PSM) staphylococcal toxin family [8]. However, due to the difficulties associated with genetic manipulation of S. epidermidis and other CNS, the roles of PSMs in CNS infections, including most notably sepsis, have hitherto remained unexplored.

Most S. epidermidis blood infections are caused by methicillin-resistant strains (MRSE), with methicillin resistance rates even exceeding those found among S. aureus [9]. Methicillin resistance is encoded on so-called staphylococcal chromosome cassette (SCC) mec mobile genetic elements, which are believed to have originated from CNS, from where they were transferred to S. aureus [10]. While other PSMs are core-genome encoded [8], one PSM toxin, called PSM-mec, is encoded within SCCmec elements of subtypes II, III, and VIII [11, 12]. The psm-mec gene is embedded in a short regulatory (sr) RNA, which in S. aureus has been reported to down-regulate the production of other PSMs and thereby decrease virulence [13, 14]. While this effect has been claimed to generally explain lower virulence of hospital-associated MRSA strains [13], it is quite moderate and extensively strain-dependent [11, 13]. Recently, the psm-mec locus has been introduced on a plasmid into some CNS that naturally lack psm-mec, and was reported to trigger gene regulatory changes [15]; but the roles that the PSM-mec peptide or the psm-mec srRNA naturally play in CNS including S. epidermidis are unknown.

Here we analyzed the role of the psm-mec locus in S. epidermidis sepsis by using tissue culture and animal infection models. Our findings show for the first time that a toxin can have a
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strong impact on CNS sepsis, setting the stage for anti-virulence strategies directed against this frequent and deadly infection.

### Results and Discussion

To analyze the impact of the *psm-mec* locus on *S. epidermidis* sepsis, we produced isogenic *psm-mec* deletion mutants (Δ*psm-mec*) in two MRSE strains, a clinical isolate (SE620) and the genome-sequenced strain RP62A. PSM-mec production in these strains is representative of clinical PSM-mec-positive MRSE (S1 Fig), which we determined in a clinical *S. epidermidis* strain collection from Norway to occur in ~ 2/3 (59/91) of the ~ 50% (91/180) methicillin-resistant *S. epidermidis*. We also introduced a point mutation in the start codon of the *psm-mec* gene in the genome of strain SE620 to differentiate between effects mediated by the PSM-mec peptide versus those due to the *psm-mec* srRNA (*psm-mec*). Notably, the stability of the *psm-mec* RNA was not significantly altered by introduction of the 1-basepair start codon mutation (S2 Fig).

We first analyzed those mutants in a murine sepsis model. Mortality was significantly reduced in the Δ*psm-mec* mutants of both strains (Fig 1A and 1B). There was no significant difference between the Δ*psm-mec* mutant and the *psm-mec* start codon mutant (Fig 1A). Furthermore, CFU in the blood and the kidneys were strongly reduced in the Δ*psm-mec* mutants of both strains and the *psm-mec* start codon mutant (Fig 1C–1F). These results demonstrate a strong contribution of the PSM-mec toxin to bacteremia and mortality due to *S. epidermidis* sepsis, while the *psm-mec* srRNA did not show any impact.

We showed previously that synthetic PSM-mec peptide is strongly pro-inflammatory and has moderate to strong cytolytic capacity [12]. To analyze the contribution that the *psm-mec* locus has to pro-inflammatory and cytolytic capacity in the *S. epidermidis* background, we measured cytokine concentrations during experimental murine sepsis and determined cytolytic capacity of the bacterial strains toward human neutrophils in vitro. Cytokine concentrations during sepsis are the result of a systemic reaction due to several immune cell types, and are thus best determined in vivo, while cytolytic capacity can be most accurately measured in vitro.

The PSM-mec peptide, but not the *psm-mec* srRNA, had a strong and significant impact on the production of cytokines during murine sepsis (Fig 2). At 12 h after infection, the mouse IL-8 homologue CXCL1 was significantly reduced when mice were infected with the Δ*psm-mec* or *psm-mec* start codon mutant of strain SE620, to about half the concentration measured in mice infected with the wild-type strain (Fig 2A). Concentrations of IL-1β and TNF-α were even more strongly reduced to levels not significantly different from those measured in mock (PBS) infected animals (Fig 2B and 2C). In the RP62A background, the phenotypes were similar, with differences being more pronounced during earlier stages of the infection (measured at 2 versus 12 h) (Fig 3). These results showed that the cytokine storm that commonly accompanies bacterial sepsis is strongly dependent on the PSM-mec toxin in *S. epidermidis*.

In addition to being pro-inflammatory, the PSM-mec toxin has pronounced cytolytic capacity [12]. Cytolysis by PSMs is believed to be most important for infection when bacteria are engulfed in the phagosome of neutrophils and other phagocytes [16, 17]. Survival of bacteria when incubated with human neutrophils and survival in whole human blood was significantly higher with the *S. epidermidis* wild-type strain than with Δ*psm-mec* or *psm-mec* start codon mutants, as was killing of neutrophils when incubated with whole bacteria (Fig 4), emphasizing the role of the PSM-mec toxin in evasion of neutrophil killing and resistance to the strong bactericidal capacities of immune defense mechanisms in human blood. Together, these results indicate that the both the pro-inflammatory and cytolytic capacities of the PSM-mec peptide contribute to the development of *S. epidermidis* sepsis.
In *S. aureus*, the *psm-mec* locus has also been implicated in biofilm-forming capacity, although effects were generally minor and highly strain-dependent [12]. Similar to *S. aureus*, biofilm formation in *S. epidermidis* was affected only slightly by the *psm-mec* locus, and as this was seen only in one strain, similarly strain-dependent (Fig 5). In that strain, SE620, the effect was due to the PSM-mec peptide, not the *psm-mec* srRNA. These findings indicate that during indwelling medical device-associated bloodstream infections by *S. epidermidis*, the impact of PSM-mec generally is by contributing to the development of sepsis, as we have shown here, rather than by promoting biofilm formation on the device itself.

Our results showed that the *psm-mec* srRNA is not involved with sepsis or other relevant virulence phenotypes in *S. epidermidis*. As a previous study suggested that the *psm-mec* srRNA...
leads to gene regulatory changes in \textit{S. epidermidis} \cite{15}, based on the introduction of a \textit{psm-mec} expressing plasmid into \textit{S. epidermidis}, we also directly investigated whether the \textit{psm-mec} locus has a gene regulatory impact in \textit{S. epidermidis}. The most important regulatory effect of the \textit{psm-mec} locus in \textit{S. aureus}, by which the sometimes negative impact of the \textit{psm-mec} locus on virulence in \textit{S. aureus} was explained, has been reported to consist in the alteration of the expression of other, core genome-encoded PSMs \cite{18}. PSM expression was altered only to a very low extent in the \textit{psm-mec}-negative as compared to the wild-type \textit{S. epidermidis} strains, with changes only significant for some PSMs and never exceeding a factor of \textasciitilde 1.5 (Fig 6).

This demonstrates that there is only a very minor effect of the \textit{psm-mec} srRNA on PSM
expression when analyzed directly in the *S. epidermidis* background. Furthermore, we analyzed genome-wide gene expression in the *psm-mec* mutants of both strains by microarray analysis (Tables 1 and 2). For microarray analysis, strains were grown to the maximum of PSM-mec expression as determined by qRT-PCR (10 h) (Fig 7). While we observed gene regulatory changes that were due to the *psm-mec* srRNA, they mostly comprised metabolic (e.g., riboflavin and purin/pyrimidine synthesis) rather than virulence genes, and were inconsistent between the two strains. Notably, the results of the previously claimed impact of the *psm-mec* locus on virulence would be negative [13, 15, 18], contrasting the positive effect we observed in

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**Fig 3. Mouse sepsis model, cytokine concentrations, strain RP62A.** Cytokine concentrations in the blood of mice infected with RP62A or its *psm-mec* deletion mutant at 2 and 12 h after infection. Statistical analysis is by 1-way ANOVA; multiple comparisons using Bonferroni post-tests. Error bars show ±SEM. *, P<0.05; **, P<0.01; ***, P<0.001. Statistical results are only shown for the RP62A versus *psm-mec* mutant comparison. Note TNF-α concentrations were below the detection limit at 12 h. Δ*psm-mec*, isogenic *psm-mec* deletion mutant; *psm-mec**, *psm-mec* gene start codon mutant.

doi:10.1371/journal.ppat.1006153.g003
the mouse sepsis model. Such a gene regulatory mechanism can thus be ruled out as underlying psm-mec-mediated development of *S. epidermidis* sepsis.

Our results may explain the highly inconsistent phenotypes that have been attributed to psm-mec in *S. aureus* [11–13], inasmuch as the psm-mec locus may exert effects in the background of *S. aureus* strains that differ from its original role in the CNS environment. One such possibility that remains to be investigated is that the highly expressed psm-mec mRNA interferes with other DNA or RNA sequences in *S. aureus*. Furthermore, the psm-mec srRNA barely
exceeds the limits of the psm-mec gene [14], which contrasts the only other case of an srRNA with an embedded peptide toxin in staphylococci, namely the well-described regulatory RNAIII of the staphylococcal accessory gene regulator (Agr) system. RNAIII significantly exceeds the boundaries of the embedded PSM peptide gene, hld [19]. Together, these observations suggest that the psm-mec srRNA does not serve a well-defined general purpose in virulence gene regulation.

Fig 5. Impact of psm-mec on biofilm formation in S. epidermidis. Biofilm formation by S. epidermidis strains and isogenic psm-mec deletion and psm-mec start codon mutants. Biofilm formation was measured using a semi-quantitative microtiter plate assay. 24 wells per group were measured. ****, P<0.0001 (1-way ANOVA, Bonferroni post tests vs. wild-type strain); N.S., not significant. Error bars show ±SEM. Δpsm-mec, isogenic psm-mec deletion mutant; psm-mec*, psm-mec gene start codon mutant.

doi:10.1371/journal.ppat.1006153.g005
In conclusion, our study reveals that sepsis due to MRSE is mediated to a large extent by the PSM-mec peptide toxin, representing the first example of a toxin being made responsible for the development of CNS sepsis. Our study was largely based on the investigation of isogenic \( \Delta \)psm-mec mutants in clinical strains of \( S. epidermidis \), using tissue culture and animal infection models. Future clinical work is needed to assess whether PSM-mec and/or other toxins contribute to sepsis in humans. Importantly, our results suggest that CNS sepsis may be amenable to virulence-targeted therapeutic approaches, such as those targeting the quorum-sensing system Agr [20], which strictly regulates PSM expression [21], or monoclonal antibody-based therapy directed against the toxin.

**Methods**

**Bacterial strains and growth conditions**

Strain RP62A is a genome-sequenced clinical MRSE isolate [22]. Strain SE620 is an MRSE clinical isolate from Norway [23]. Isogenic \( \Delta \)psm-mec deletion mutants and the psm-mec* start codon mutant were produced with the constructs previously used for \( S. aureus \) [12, 14], using
Table 1. Microarray results, RP62A.

| Gene    | Number   | Function                                                                 | RP62AΔpsm-mec vs. RP62A |
|---------|----------|--------------------------------------------------------------------------|-------------------------|
| pyrB    | SERP0766 | aspartate carbamoyltransferase                                           | 18.14                   |
| ribE    | SERP1327 | riboflavin synthase subunit alpha                                        | 16.04                   |
| ribBA   | SERP1326 | 3,4-dihydroxy-2-butaneone-4-phosphate synthase                          | 15.16                   |
| purM    | SERP0656 | phosphoribosylaminomimidazole synthetase                                | 14.94                   |
| purF    | SERP0655 | amidophosphoribosyltransferase                                           | 14.77                   |
| purN    | SERP0657 | phosphoribosylglicinamidase formyltransferase                           | 14.72                   |
| serS    | SERP2545 | seryl-tRNA synthetase                                                    | 13.55                   |
| purH    | SERP0658 | bifunctional phosphoribosylaminomimidazolecarboxamide formyltransferase/IMP cyclohydrolase | 13.45                   |
| purD    | SERP0659 | phosphoribosylamine—glycine ligase                                      | 11.92                   |
| arcB-2  | SERP2351 | ornithine carbamoyltransferase                                           | 11.33                   |
| ribD    | SERP1328 | riboflavin biosynthesis protein RibD                                    | 10.99                   |
|         | SERP2546 | hypothetical protein                                                     | 9.88                    |
| ribH    | SERP1325 | 6,7-dimethyl-8-ribityllumazine synthase                                  | 9.50                    |
| arcC    | SERP2352 | carbamata kinase                                                        | 9.07                    |
| purL    | SERP0654 | phosphoribosylformylglicinamidine synthase II                            | 7.33                    |
|         | SERP2381 | NADH:flavin oxidoreductase/fumarate reductase, flavoprotein subunit       | 7.27                    |
| #N/A    | SE2245   |                                                                           | 7.00                    |
|         | SERP2279 | hypothetical protein                                                     | 6.65                    |
|         | SERP2278 | hypothetical protein                                                     | 6.50                    |
|         | SERP2380 | drug transporter                                                         | 6.26                    |
| cysH    | SERP2192 | phosphoadenyl-sulfate reductase                                          | -95.16                  |
| sat     | SERP2186 | sulfate adenylyltransferase                                              | -18.63                  |
|         | SERP0094 | cysteine synthase/cystathionine beta-synthase family protein             | -13.93                  |
| cysK    | SERP0152 | cysteine synthase                                                        | -9.84                   |
|         | SERP0095 | trans-sulfuration enzyme family protein                                  | -8.77                   |
|         | SERP2196 | MarR family transcriptional regulator                                   | -8.64                   |
|         | SERP1478 | GntR family transcriptional regulator                                   | -8.26                   |
|         | SERP2003 | amino acid ABC transporter ATP-binding protein                           | -7.99                   |
|         | SERP2187 | hypothetical protein                                                     | -7.65                   |
|         | SERP2195 | alpha keto acid dehydrogenase complex, E3 component, lipoamide dehydrogenase | -7.46                   |
| cysC    | SERP2185 | adenylylsulfate kinase                                                   | -7.41                   |
|         | SERP2004 | amino acid ABC transporter permease                                      | -6.80                   |
| lacE    | SERP1790 | PTS system, lactose-specific IIIBC components                            | -6.80                   |
|         | SERP1980 | nitrite extrusion protein                                                | -5.78                   |
| cysI    | SERP2190 | sulfite reductase subunit beta                                           | -5.74                   |
| lacG    | SERP1789 | 6-phospho-beta-galactosidase                                             | -5.70                   |
| lacF    | SERP1791 | PTS system, lactose-specific IIA component                              | -5.07                   |
|         | SERP0056 | hypothetical protein                                                     | -4.91                   |
|         | SERP2005 | amino acid ABC transporter amino acid-binding protein                     | -4.86                   |
| lacA    | SERP1795 | galactose-6-phosphate isomerase subunit LacA                             | -4.81                   |

1 The top 20 down- and up-regulated genes are shown.
2 Gene numbers are for strain RP62A, unless a specific gene is not annotated or exists in that strain, in which case the number for strain ATCC12228 is shown.
3 Up-regulation is shown by positive numbers, down-regulation by negative numbers.

doi:10.1371/journal.ppat.1006153.t001
Table 2. Microarray results, SE620.1

| Gene    | Number | Function                                                                 | SE620Δpsm-mec vs. SE620 | SE620psm-mec* vs. SE620 | SE620Δpsm-mec vs. SE620psm-mec* |
|---------|--------|--------------------------------------------------------------------------|--------------------------|--------------------------|---------------------------------|
| -       | SERP2245 | tributyrin esterase EstA                                                  | 4.25                     | 1.24                     | 3.42                            |
|ribE     | SERP1327 | riboflavin synthase subunit alpha                                         | 4.18                     | 16.69                    | -3.99                           |
|ribBA    | SERP1326 | 3,4-dihydroxy-2-butanone-4-phosphate synthase                           | 3.62                     | 12.67                    | -3.50                           |
| -       | SERP2546 | hypothetical protein                                                      | 3.41                     | 1.41                     | 2.42                            |
|ribD     | SERP1328 | riboflavin biosynthesis protein RibD                                      | 3.06                     | 10.33                    | -3.38                           |
| -       | SERP0664 | hypothetical protein                                                      | 3.00                     | 1.89                     | 1.59                            |
| -       | SERP2321 | hypothetical protein                                                      | 2.92                     | 5.19                     | -1.77                           |
|rbsK     | SERP2100 | Ribokinase                                                               | 2.82                     | 2.29                     | 1.23                            |
|ribH     | SERP1325 | 6,7-dimethyl-8-ribityllumazine synthase                                   | 2.79                     | 8.78                     | -3.15                           |
|purS     | SERP0652 | phosphoribosylformylglycinamidine synthase, PurS protein                  | 2.76                     | 2.95                     | -1.07                           |
| -       | SERP1933 | hypothetical protein                                                      | 2.71                     | 1.65                     | 1.64                            |
| -       | SERP2364 | succinyl-diaminopimelate desuccinylase                                   | 2.66                     | 3.38                     | -1.27                           |
| -       | SERP2354 | tributyrin esterase EstA                                                  | 2.64                     | 1.88                     | 1.40                            |
| -       | SERP1498 | ammonium transporter                                                     | 2.58                     | 4.47                     | -1.74                           |
| -       | SERP1933 | hypothetical protein                                                      | 2.53                     | 1.63                     | 1.55                            |
|mraY     | SERP0747 | phospho-N-acetylmuramoyl-pentapeptide-transferase                         | 2.49                     | 1.27                     | 1.96                            |
|purQ     | SERP0653 | phosphoribosylformylglycinamidine synthase I                             | 2.40                     | 2.48                     | -1.03                           |
| -       | SERP2357 | amino acid ABC transporter permease                                       | 2.39                     | 2.17                     | 1.10                            |
| -       | SERP1529 | hypothetical protein                                                      | 2.34                     | -1.90                    | 4.46                            |
|purF     | SERP0655 | amidophosphoribosyltransferase                                            | 2.32                     | 2.85                     | -1.23                           |
| -       | SERP0473 | hypothetical protein                                                      | -6.11                    | -1.43                    | -4.28                           |
| -       | SERP0473 | hypothetical protein                                                      | -5.46                    | -1.31                    | -4.17                           |
| -       | SERP1474 | hypothetical protein                                                      | -5.27                    | -11.93                   | 2.26                            |
| -       | SERP1478 | GntR family transcriptional regulator                                     | -4.32                    | -11.37                   | 2.63                            |
| -       | SERP2158 | amino acid permease                                                       | -3.75                    | -3.27                    | -1.14                           |
| -       | SERP0494 | ThiJ/Ppl family protein                                                   | -3.71                    | -1.71                    | -2.16                           |
|trpG     | SERP0938 | anthranilate synthase component II                                       | -3.65                    | -1.87                    | -1.96                           |
| -       | SERP1475 | ABC transporter ATP-binding protein                                       | -3.46                    | -8.38                    | 2.42                            |
|czzA     | SERP1755 | CzzA family transcriptional regulator                                     | -3.30                    | -2.32                    | -1.42                           |
| -       | SERP0273 | alpha/beta hydrolase                                                     | -3.08                    | -1.73                    | -1.78                           |
| -       | SERP0507 | CBS domain-containing protein                                             | -3.06                    | -1.48                    | -2.07                           |
| -       | SERP2091 | hypothetical protein                                                      | -2.94                    | -1.32                    | -2.22                           |
| -       | SE0735   | hypothetical protein                                                      | -2.91                    | -1.12                    | -2.60                           |
| -       | SERP1476 | hypothetical protein                                                      | -2.91                    | -9.68                    | 3.33                            |
| -       | SERP1477 | ABC transporter ATP-binding protein                                       | -2.89                    | -9.52                    | 3.30                            |
| -       | SERP0620 | hypothetical protein                                                      | -2.80                    | -1.04                    | -2.69                           |
| -       | SERP2129 | short chain dehydrogenase/reductase family oxidoreductase                | -2.78                    | -1.28                    | -2.17                           |
| -       | SE0082   | ABC transporter ATP-binding protein                                       | -2.71                    | -1.12                    | -2.42                           |
| -       | SERP0385 | ABC transporter ATP-binding protein                                       | -2.69                    | -1.56                    | -1.73                           |

(Continued)
a strategy with the allelic exchange vector pKOR1 [24]. The *psm-mec* locus and adjacent DNA do not differ between *S. aureus* and *S. epidermidis* [25]. For construction of the *psm-mec* mutant, the start codon mutation was created by introducing a ClaI restriction site (introducing ATC instead of the ATG start codon) using primer PSMEClarev GAGGGTATGCATATCGATTTCACTGGTGTTATTA CAAGC and primer PSMECladir (reverse complement of PSMEClarev). Two PCR fragments were amplified using those primers and primers psmEatt1 and psmEatt2, respectively [12], cut with ClaI, ligated, and cloned into pKOR1. The resulting plasmid was used for allelic replacement as described [24]. Growth patterns of the mutants were indistinguishable from those of the wild-type (S3 Fig). Strains were grown in tryptic soy broth (TSB), unless otherwise noted.

**Mouse sepsis model**

Female, 6–10 weeks old, C57BL/6NCRI (Charles River) mice were used. The mice were injected via the tail vein with 5 x 10^8 CFU in 100 μl phosphate-buffered saline (PBS) of the indicated bacterial strains grown to mid-exponential growth phase and monitored for disease development every 8 h for up to 120 h. This dosis was determined to be minimally necessary to achieve mortality and production of inflammatory cytokines (S4 Fig). Animals were

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**Table 2. (Continued)**

| Gene Number | Function | SE620Δpsm-mec vs. SE620 | SE620psm-mec vs. SE620 | SE620Δpsm-mec vs. SE620psm-mec* |
|-------------|----------|--------------------------|-------------------------|---------------------------------|
| SERP1479    | hypothetical protein | -2.66                    | -2.94                   | 1.11                            |

1 The top 20 down- and up-regulated genes are shown. Sorting was by the SE620Δpsm-mec vs. SE620 comparison and the gene expression changes for the same genes for the other comparisons are shown.
2 Gene numbers are for strain RP62A, unless a specific gene is not annotated or exists in that strain, in which case the number for strain ATCC12228 is shown.
3 Up-regulation is shown by positive numbers, down-regulation by negative numbers.
euthanized immediately if showing signs of respiratory distress, mobility loss, or inability to eat and drink. Cytokine concentrations were measured at 2 and/or 12 h, as indicated, using commercially available ELISA kits (IL-1β, TNF-α, BD BioSciences; CXCL1, R&D Systems).

Interaction of bacteria with human neutrophils and bacterial survival in human blood

For survival in whole blood experiments, about 10⁸ bacteria in 100 μl Dulbecco’s PBS from mid-exponential growth phase were added to 500 μl heparinized human blood and mixtures were incubated for 6 h. Aliquots were taken at 2-h intervals, and CFU were determined by plating and incubating plates overnight at 37°C.

For neutrophil interaction experiments, neutrophils were isolated from the venous blood of human volunteers as described [26]. Bacteria from mid-exponential growth phase were mixed with neutrophils at an MOI (bacteria/neutrophils) of 10:1. Bacteria/neutrophil mixtures were incubated at 37°C, 5% CO₂, 90% humidity for 6 h. At 2-h intervals, 50 μl of Triton X-100 was added to the 200-μl bacteria/neutrophil suspensions, aliquots were plated, and plates incubated at 37°C overnight for CFU counting. Alternatively, the rate of neutrophil lysis promoted by the bacteria was determined after 4–h incubation using a lactate dehydrogenase (LDH) assay at an MOI of 100:1.

Biofilm formation

Biofilm formation was assessed in a semi-quantitative 96-well microtiter plate assay as previously described [27], using TSB + 0.5% glucose.

PSM measurement

Relative PSM concentrations in culture filtrates were determined as described using reversed-phase high-pressure liquid chromatography/electrospray mass spectrometry (RP-HPLC/ESI-MS) [28].

Quantitative real-time (RT)-PCR and microarray analysis

Quantitative RT-PCR was performed as previously described [29] with the following oligonucleotides: psm-mecF, TGCATATGATTTCACTGGTGTTA, psm-mecR, CGTTGAATATTTCCTCTGTTTTTATGGTG, psm-mec probe, ATTTAATCAAGACTTGACTTCTCAG. Expression was measured relative to that of 16S RNA. Cultures were grown to the maximum of psm-mec expression as determined by qRT-PCR (10 h). Total RNA and cDNA were prepared as described [30]. Biotinylated S. aureus cDNA was hybridized to custom Affymetrix GeneChips (RMLChip 3) with 100% coverage of chromosomal genes from strains S. epidermidis RP62A and scanned according to standard GeneChip protocols (Affymetrix). Each experiment was replicated 3 times. Affymetrix GeneChip Operating Software was used to perform the preliminary analysis of the custom GeneChips at the probe-set level. Subsequent data analysis was performed as described [30]. The complete set of microarray data was deposited in NCBI’s Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and is accessible through GEO Series accession number GSE85265.

Analysis of mRNA stability

To determine psm-mec mRNA stability in strains S. epidermidis SE620 and the psm-mec start codon mutant, bacteria were cultured at 37°C for 10 hours. At time t = 0 min, rifampicin (50 mg/ml stock in DMSO) was added to the cultures to a final concentration of 100 μg/ml. One-
ml aliquots were taken and immediately centrifuged at 4°C to pellet cells, which were then frozen at -70°C. Remaining cultures were further incubated at 37°C with shaking; one-ml aliquots were taken at the indicated times and RNA was subsequently isolated from all cell pellets as described [29]. Samples were analyzed by qRT-PCR using primers psm-mecR and psm-mecF with a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) according to the manufacturer’s instructions. Expression was measured relative to that of 16S RNA.

Statistics
Statistical analysis was performed using GraphPad Prism Version 6.0. Comparisons were by 1-way or 2-way ANOVA for comparisons of three and more, and by unpaired t-tests for comparisons of 2 groups. Error bars show ±SEM.

Ethics statement
The animal protocol (LB1E) was reviewed and approved by the Animal Care and Use Committee at the NIAID, NIH, according to the animal welfare act of the United States (7 U.S.C. 2131 et. seq.). All mouse experiments were performed at the animal care facility of the NIAID, Building 50, in accordance with approved guidelines. All animals were euthanized by CO2 at the end of the studies. Human neutrophils were isolated from blood obtained under approved protocols at the NIH Blood Bank or with a protocol (633/2012BO2) approved by the Institutional Review Board for Human Subjects, NIAID, NIH. All subjects were adult and gave informed written consent.

Supporting Information
S1 Fig. PSM-mec production in clinical MRSE strains. A clinical strain collection was analyzed for PSM-mec production by RP-HPLC/MS of stationary-phase culture filtrates. The horizontal line shows the mean. Colored dots show the production in the strains used in this study.

S2 Fig. Stability of psm-mec and psm-mec’ RNA. Data are not significantly different between the two groups at any time point.

S3 Fig. Growth curves in TSB of S. epidermidis strains and isogenic psm-mec deletion and psm-mec start codon mutants.

S4 Fig. Determination of the minimal dose in the mouse infection model. (A,B) Mortality in the mouse bacteremia model at different doses of strains SE620 and RP62A, respectively. (C) Concentration of the inflammatory cytokine CXCL-1 (TNF-α) in mouse blood at 12 h. Note only one mouse could be used for the group infected with strain SE620, as the others died very early. (A-C) n = 5 in every group.

Author Contributions
Conceptualization: MO.
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Formal analysis: LQ JWM MO.
Funding acquisition: MO.
Investigation: LQ FD DCST THN CLF VYT JWM DES HSJ SYQ GYCC ELF.
Methodology: LQ GYCC THN SYQ MO.
Project administration: MO.
Supervision: MO.
Validation: LQ THN GYCC MO.
Visualization: MO.
Writing – original draft: MO.
Writing – review & editing: MO.

References
1. Becker K, Heilmann C, Peters G. Coagulase-negative staphylococci. Clin Microbiol Rev. 2014; 27 (4):870–926. PubMed Central PMCID: PMCPMC4187637. doi: 10.1128/CMR.00109-13 PMID: 25278577
2. Cheung GY, Otto M. Understanding the significance of Staphylococcus epidermidis bacteremia in babies and children. Curr Opin Infect Dis. 2010; 23(3):208–16. PubMed Central PMCID: PMCPMC2874874. doi: 10.1097/QCO.0b013e328337fecd PMID: 20175994
3. Marchant EA, Boyce GK, Sadarangani M, Lavoe PM. Neonatal sepsis due to coagulase-negative staphylococci. Clin Dev Immunol. 2013; 2013:586076. PubMed Central PMCID: PMCPMC3674645. doi: 10.1155/2013/586076 PMID: 23762094
4. Vassallo M, Dunais B, Roger PM. Antimicrobial lock therapy in central-line associated bloodstream infections: a systematic review. Infection. 2015; 43(4):389–98. doi: 10.1007/s15010-015-0738-1 PMID: 25657033
5. Otto M. Staphylococcus epidermidis—the 'accidental' pathogen. Nat Rev Microbiol. 2009; 7(8):555–67. PubMed Central PMCID: PMCPMC2807625. doi: 10.1038/nremicro2182 PMID: 19609257
6. Otto M. Virulence factors of the coagulase-negative staphylococci. Front Biosci. 2004; 9:4–11. PMID: 14766414
7. Bochud PY, Calandra T. Pathogenesis of sepsis: new concepts and implications for future treatment. BMJ. 2003; 326(7383):262–6. PubMed Central PMCID: PMCPMC1125122. PMID: 12560281
8. Cheung GY, Joo HS, Chatterjee SS, Otto M. Phenol-soluble modulins—critical determinants of staphylococcal virulence. FEMS Microbiol Rev. 2014; 38(4):698–719. PubMed Central PMCID: PMCPMC4072763. doi: 10.1111/1574-6976.12057 PMID: 24372362
9. Raad I, Alrahwan A, Rolston K. Staphylococcus epidermidis: emerging resistance and need for alternative agents. Clin Infect Dis. 1998; 26(5):1182–7. PMID: 9597250
10. Otto M. Coagulase-negative staphylococci as reservoirs of genes facilitating MRSA infection: Staphylococcal commensal species such as Staphylococcus epidermidis are being recognized as important sources of genes promoting MRSA colonization and virulence. Bioessays. 2013; 35(1):4–11. PubMed Central PMCID: PMCPMC3755491. doi: 10.1002/bies.201200112 PMID: 23165978
11. Chatterjee SS, Chen L, Joo HS, Cheung GY, Kreiswirth BN, Otto M. Distribution and regulation of the mobile genetic element-encoded phenol-soluble modulin PSM-mec in methicillin-resistant Staphylococcus aureus. PLoS One. 2011; 6(12):e28781. PubMed Central PMCID: PMCPMC3236207. doi: 10.1371/journal.pone.0028781 PMID: 22174895
12. Queck SY, Khan BA, Wang R, Bach TH, Kretschmer D, Chen L, et al. Mobile genetic element-encoded cytolyisin connects virulence to methicillin resistance in MRSA. PLoS Pathog. 2009; 5(7):e1000533. PubMed Central PMCID: PMCPMC2712073. doi: 10.1371/journal.ppat.1000533 PMID: 19649313
13. Kaito C, Saito Y, Ikou M, Omae Y, Mao H, Nagano G, et al. Mobile genetic element SCC/mec-encoded psm-mec RNA suppresses translation of agrA and attenuates MRSA virulence. PLoS Pathog. 2013; 9(4):e1003269. PubMed Central PMCID: PMCPMC3617227. doi: 10.1371/journal.ppat.1003269 PMID: 23592990
14. Cheung GY, Villaruz AE, Joo HS, Duong AC, Yeh AJ, Nguyen TH, et al. Genome-wide analysis of the regulatory function mediated by the small regulatory psm-mec RNA of methicillin-resistant *Staphylococcus aureus*. Int J Med Microbiol. 2014; 304(5–6):637–44. PubMed Central PMCID: PMCPMC4087065. doi: 10.1016/j.ijmm.2014.04.008 PMID: 24877726

15. Ikuo M, Nagano G, Saito Y, Mao H, Sekimizu K, Kaito C. Inhibition of exotoxin production by mobile genetic element SCCmec-encoded psm-mec RNA is conserved in staphylococcal species. PLoS One. 2014; 9(6):e100260. PubMed Central PMCID: PMCPMC4057442. doi: 10.1371/journal.pone.0100260 PMID: 24926994

16. Surewaard BG, de Haas CJ, Vervoort F, Rigby KM, DeLeo FR, Otto M, et al. Staphylococcal alpha-phe-nol soluble modulins contribute to neutrophil lysis after phagocytosis. Cell Microbiol. 2013; 15(8):1427–37. PubMed Central PMCID: PMCPMC4784422. doi: 10.1111/cmi.12130 PMID: 23470014

17. Grosz M, Kolter J, Papatkia K, Winkler AC, Schafer D, Chatterjee SS, et al. Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulin alpha. Cell Microbiol. 2014; 16(4):451–65. PubMed Central PMCID: PMCPMC3969633. doi: 10.1111/cmi.12233 PMID: 2416701

18. Kaito C, Saito Y, Nagano G, Ikuo M, Omae Y, Hanada Y, et al. Transcription and translation products of the cytolysin gene psm-mec on the mobile genetic element SCCmec regulate *Staphylococcus aureus* virulence. PloS Pathog. 2011; 7(2):e1001267. PubMed Central PMCID: PMCPMC3033363. doi: 10.1371/journal.ppat.1001267 PMID: 21304931

19. Novick RP, Ross HF, Projan SJ, Kornblum J, Moghazeh S. Synthesis of staphylococcal RNAIII upon phagosomal escape. Mol Cell. 2008; 32(1):150–8. PubMed Central PMCID: PMCPMC2575650. doi: 10.1116/j.molcel.2008.08.005 PMID: 18851841

20. Khan BA, Yeh AJ, Cheung GY, Otto M. Investigational therapies targeting quorum-sensing for the treatment of *Staphylococcus aureus* infections. Expert Opin Investig Drugs. 2015; 24(5):689–704. doi: 10.1517/13543784.2015.1019062 PMID: 25704585

21. Queck SY, Jameson-Lehee M, Villaruz AE, Bach TH, Khan BA, Sturdevant DE, et al. RNAIII-independent target gene control by the agr quorum-sensing system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. Mol Cell. 2008; 32(1):150–8. PubMed Central PMCID: PMCPMC2575650. doi: 10.1116/j.molcel.2008.08.005 PMID: 18851841

22. Gill SR, Fouts DE, Archer GL, Mongodin EF, Deboy RT, Ravel J, et al. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus aureus* strain. J Bacteriol. 2005; 187(7):2426–38. PubMed Central PMCID: PMCPMC1065214. doi: 10.1128/JB.187.7.2426-2438.2005 PMID: 15774886

23. Klingenberg C, Ronnestad A, Anderson AS, Abrahamsen TG, Zorman J, Villaruz A, et al. Persistent strains of coagulase-negative staphylococci in a neonatal intensive care unit: virulence factors and inva-siveness. Clin Microbiol Infect. 2007; 13(11):1100–11. doi: 10.1111/j.1469-0691.2007.01818.x PMID: 17850346

24. Bae T, Schneewind O. Allelic replacement in *Staphylococcus aureus* with inducible counter-selection. Plasmid. 2006; 55(1):58–63. doi: 10.1016/j.plasmid.2005.05.005 PMID: 16051359

25. Qin L, Mccausland JW, Cheung GY, Otto M. PSM-Mec-A Virulence Determinant that Connects Transcriptional Regulation, Virulence, and Antibiotic Resistance in Staphylococci. Front Microbiol. 2016; 7:1293. PubMed Central PMCID: PMCPMC4992726. doi: 10.3389/fmicb.2016.01293 PMID: 27597849

26. Voyich JM, Otto M, Mathema B, Braughton KR, Whitney AR, Welty D, et al. Is Panton-Valentine leukocidin the major virulence determinant in community-associated methicillin-resistant *Staphylococcus aureus* disease? J Infect Dis. 2006; 194(12):1761–70. doi: 10.1086/505906 PMID: 17109350

27. Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M. Quorum-sensing control of biofilm factors in *Staphylococcus epidermidis*. J Infect Dis. 2003; 188(5):706–18. doi: 10.1086/377239 PMID: 12934187

28. Joo HS, Otto M. The isolation and analysis of phenol-soluble modulins of *Staphylococcus epidermidis*. Methods Mol Biol. 2014; 1106:93–100. doi: 10.1007/978-1-62703-736-5_7 PMID: 24222457

29. Yao Y, Vuong C, Kocianova S, Villaruz AE, Lai Y, Sturdevant DE, et al. Characterization of the *Staphy-lococcus epidermidis* accessory-gene regulator response: quorum-sensing regulation of resistance to human innate host defense. J Infect Dis. 2006; 193(6):841–8. doi: 10.1086/500246 PMID: 16479519

30. Li M, Lai Y, Villaruz AE, Cha DJ, Sturdevant DE, Otto M. Gram-positive three-component antimicrobial peptide-sensing system. Proc Natl Acad Sci U S A. 2007; 104(22):9469–74. PubMed Central PMCID: PMCPMC1890518. doi: 10.1073/pnas.0702159104 PMID: 17517597