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Essential *Staphylococcus aureus* toxin export system

Som S. Chatterjee[1], **Hwang-Soo Joo**[1], **Anthony C. Duong**[1], **Thomas D. Dieringer**[1], **Vee Y. Tan**[1], **Yan Song**[2], **Elizabeth R. Fischer**[3], **Gordon Y. C. Cheung**[1], **Min Li**[2], and **Michael Otto**[1,*]

[1]Pathogen Molecular Genetics Section, Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases, US National Institutes of Health, Bethesda, Maryland 20892

[2]Department of Laboratory Medicine, Huashan Hospital, Shanghai Medical College, Fudan University, 12 Central Urumqi Road, Shanghai 200040, China

[3]Microscopy Unit, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, US National Institutes of Health, Hamilton, Montana 59840

Abstract

Widespread antibiotic resistance among important bacterial pathogens such as *Staphylococcus aureus*[1] calls for alternative routes of drug development. Interfering with critical virulence determinants is considered a promising novel approach to control bacterial infection[2]. Phenol-soluble modulins (PSMs) are peptide toxins with multiple key roles in pathogenesis[3–5] and a major impact on the ability of highly virulent *S. aureus* to cause disease[3,6]. However, targeting PSMs for therapeutic intervention is hampered by their multitude and diversity. Here, we report that an ABC transporter with previously unknown function is responsible for the export of all PSM classes, thus representing a single target to interfere simultaneously with the production of all PSMs. The transporter had a strong effect on virulence phenotypes, such as neutrophil lysis, and the development of *S. aureus* infection, similar in extent to the sum of all PSMs. Furthermore, it proved essential for bacterial growth. Moreover, it protected the producer from the antimicrobial activity of secreted PSMs and contributed to defense against PSM-mediated bacterial interference. Our study reveals a non-canonical, dedicated secretion mechanism for an important toxin class and identifies this mechanism as a comprehensive potential target for the development of drugs efficiently inhibiting growth and virulence of pathogenic staphylococci.

Several observations point to the value of targeting PSMs for drug development. First, PSMs have multiple crucial roles in pathogenesis. They efficiently lyse red and white blood cells[3,7,8], control biofilm development[4,9,10], and trigger receptor-mediated inflammatory responses[3,11]. Importantly, despite recent discoveries indicating that PSMs – like other important staphylococcal virulence molecules[12,13] – are targeted by non-specific

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*corresponding author: Michael Otto Tel.: (301) 443 5209 Fax: (301) 480 3632 motto@niaid.nih.gov.

Author contributions HJ and MO performed HPLC measurements for PSM detection. EF performed electron microscopy. SC, GC, AD, VT, and TD performed animal experiments. YS and ML designed and performed experiments with neutrophils and human blood. SC performed all other experiments. SC and MO analyzed data, designed the study and wrote the paper.

mechanisms of human innate host defense[14,15], they have a major impact on disease progression[3,6]. Second, essentially all pathogenic staphylococci secrete members of the PSM family[3,7,11,16]. Third, PSMs are encoded in the core genome and strongly expressed by most strains of a species[3,11,16]. However, staphylococci commonly secrete a series of PSMs that differ considerably in amino acid sequence[3,11,16–18]. For example, *S. aureus* produces four different PSMα peptides (encoded in the *psm*α operon), two different PSMβ peptides (encoded in the *psm*β operon), and the *hld*-encoded δ-toxin[3]. Consequently, it is extremely difficult to develop strategies to interfere broadly with PSM production or toxicity. Yet, all PSMs have structural features (for example, lack of a signal peptide) suggesting that they share a hitherto unidentified, dedicated mechanism of secretion[3,7] that would provide a way to simultaneously target production of the entire PSM family. Therefore, we here set out to identify the PSM export mechanism.

Inasmuch as the Agr virulence regulator exerts a particularly strong control over PSM production[19], we hypothesized that the PSM exporter is also regulated by the Agr system. Among Agr-controlled genes with potential transport functions[20], we found only one candidate that, like *psm* genes, was present in all staphylococcal species, but absent from other bacteria (Fig. 1a, Supplementary Fig. 1 online). Assuming that it participates in PSM secretion, we named this system Pmt for Phenol-soluble modulin transporter. It consists of four genes (*pmtA, pmtB, pmtC, pmtD*), encoding an ABC transporter with two separate membrane parts (PmtB, PmtD) and two separate ATPases (PmtA, PmtC) (Fig. 1a, Supplementary Figs. 2 and 3 online). Among the Pmt proteins, especially the PmtB and PmtD sequences are highly specific for *Staphylococcus spp*.: A BlastP search for PmtB and PmtD showed pronounced similarity among *Staphylococcus spp*. (e-value of 1e-19 or 1e-22, respectively), whereas there was no similarity with proteins from non-staphylococcal organisms (maximal e-value of 0.12 or 4.1, respectively).

To provide evidence for the involvement of *pmt* in PSM secretion, we first attempted to delete the *pmt* locus by allelic replacement in two *S. aureus* strains with high clinical significance and different genetic backgrounds (USA300 LAC, USA400 MW2)[21] (Supplementary Table 1 online). However, failure to find a *pmt* deletion clone without forcing an *agr* mutation led us to hypothesize that the Pmt transporter not only facilitates PSM export but also is also essential for bacterial survival when PSM peptides are expressed. To analyze the function of *pmt*, we therefore first removed all *psm* genes from the two *S. aureus* strains, upon which deletion of *pmt* was successful, and then introduced plasmids for the expression of *psm* genes (Supplementary Table 1 online). This system proved optimally suited to analyze the contribution of Pmt to PSM export without over-expression effects, because PSM production levels were about equal or lower compared to those in the wild-type strains (Supplementary Fig. 4 online), in which PSMs are expressed at extremely high levels (Supplementary Fig. 5a,b online).

We found that secretion of all *S. aureus* PSMs was strongly dependent on Pmt (Fig. 1b, Supplementary Fig. 6 online). Notably, analysis of secreted protein profiles indicated that PSMs are the primary substrate of the Pmt transporter (Supplementary Fig. 5 online). Furthermore, except for the barely cytolytic, low-abundance PSMβ peptides[3,7,9], expression of PSMs led to significant growth defects in the absence of *pmt* (Fig. 1c, Supplementary Fig.

7 online), confirming our hypothesis on *pmt* essentiality. Pmt function was dependent on Walker A and B boxes, which are conserved nucleotide-binding domains crucial for the catalytic function of ABC transporters[22,23], demonstrating energy dependence and direct involvement of Pmt in PSM export (Fig. 1d,e, Supplementary Fig. 8 online). Moreover, similar to the results obtained in *S. aureus*, secretion of PSMs was dependent on the presence of *pmt* in the heterologous host *Lactococcus lactis* (Fig. 1f, Supplementary Fig. 9 online), and *pmt* was necessary for growth of *L. lactis* with concomitant expression of *psm* genes (Fig. 1g, Supplementary Fig. 9 online). Altogether, our findings show that Pmt facilitates PSM export in a specific, energy-driven fashion, and is essential for growth under conditions of PSM production.

We then further investigated the underpinnings of the PSM-induced growth defects that developed in the absence of *pmt*. First, we found that under those circumstances PSMs accumulate in the cytosol (Fig. 2a, Supplementary Fig. 10 online). This was demonstrated for the most potent cytolysins among *S. aureus* PSMs, PSMα2, PSMα3, and the δ-toxin[3], while detection of other PSMs was difficult, probably owing to low production levels, degradation, or non-specific adhesion to cellular material. Second, cytosolic accumulation of PSMs was accompanied by abnormal cell division and severe damage to the cytoplasmic membrane (Fig. 2b–d). Most likely, the multitude and severity of the observed effects are due to the extraordinarily strong production and distinctive surfactant characteristics of PSMs[3,4,7], overwhelming the cytosolic protein degradation machinery and causing disruption of vital interactions between macromolecules.

Some PSMs, such as PSMδ and the δ-toxin produced by the abundant human colonizer *S. epidermidis*[24], are known to act as pore-forming antimicrobials against a series of microorganisms[25,26]. Remarkably, Pmt efficiently protected *S. aureus* from the antimicrobial activities of the PSMs it secretes and from those produced by *S. epidermidis* (Fig. 3). These findings indicate that Pmt ascertains producer immunity toward PSMs and provides resistance to PSMs of non-self that may function as weapons of bacterial interference during co-colonization of the human host[25].

Next, we investigated the role of Pmt in virulence-associated phenotypes. Using expression of PSMα1–4 peptides as the predominant cytolytic PSMs[3], we show that Pmt significantly affects lysis of human neutrophils and erythrocytes and survival in human blood (Fig. 4a–c; Supplementary Fig. 11 online). Of note, the results of the neutrophil lysis experiments, which were performed using live bacteria, indicate that PSMα peptides contribute significantly to lysis of neutrophils after phagocytosis, a phenotype that has remained poorly understood on a mechanistic level. In the light of reports indicating inhibition of PSMs and other cytolytic agents by serum[14,27], these findings underscore the significant role that PSMs and Pmt have in circumventing elimination by human neutrophils. Finally, Pmt strongly impacted biofilm development (Supplementary Fig. 12 online). Importantly, these results show that Pmt affects key virulence-associated phenotypes to a similarly strong extent as the sum of all PSMs.

As the majority of *S. aureus* infections present as infections of the skin[28], we performed a mouse skin infection model to analyze the contribution of Pmt to disease progression. To

that end, we used constitutive expression of the PSMα1–4 peptides in isogenic *pmt*-positive and -negative strains. Abscesses formed by the *pmt*-positive strain were significantly larger (during the first five days after infection) than those formed by the corresponding strain lacking the *pmt* genes (Fig. 4d). Furthermore, in accordance with the strong contribution of PSMα peptides to abscess formation[3], and the somewhat smaller PSMα production levels obtained with the used expression system compared to the USA300 wild-type strain (Supplementary Fig. 4 online), the *pmt*-positive strain formed abscesses only barely smaller than those formed by the USA300 wild-type strain. Moreover, abscesses formed by the *pmt*-negative strain were indistinguishable in size from those formed by the corresponding control strain USA300 with all *psm* genes deleted (See Supplementary Table 2 for complete 2-way ANOVA results). Bacterial numbers in the abscesses were significantly different and confirmed the differences seen with abscess sizes (Fig. 4e). These experiments demonstrated that Pmt is indispensable for the development of *S. aureus* skin infection. Of note, Pmt may have an even stronger effect on the pathogenic potential of the infecting *S. aureus* strain in a natural situation with wild-type level expression of all PSMs.

In conclusion, in this study we identified the system required for export of the PSM class of staphylococcal toxins. While our experiments were performed in *S. aureus*, high similarity of *pmt* genes among staphylococci strongly suggests that the Pmt system has the same role in other species. Given the extremely high amount of PSM peptides secreted by staphylococci, the Pmt ABC transporter represents a bacterial peptide exporter with extraordinarily high efficiency. Interestingly, our finding that PSM production in the absence of the Pmt export system leads to significant growth defects indicates that PSM evolution was only possible with the co-evolution of a dedicated transporter. This may explain why PSMs are limited to the genus *Staphylococcus*, despite their relatively simple structure and the value that they presumably would have for virulence in many other bacterial pathogens. Finally, we believe that the PSM exporter represents a drug target with exceptional value, inasmuch as it not only facilitates secretion of an entire class of widespread staphylococcal toxins with key functions in virulence, but also is essential for microbial growth and defense against bacterial interference (see model in Fig. 4f,g).

Methods

For additional procedures, see Supplementary Methods (Supplementary Methods online).

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Supplementary Table 1 online. Transformation of *S. aureus* and *L. lactis* was performed by electroporation as described[29,30]. *E. coli* and *S. aureus* strains were grown in tryptic soy broth (TSB) with appropriate antibiotic selection at 37 °C and shaking at 180 rpm. *L. lactis* strains were grown in GM17 media supplemented with 0.5% glucose with appropriate antibiotic selection at 30 °C and shaking at 180 rpm. For induction, xylose (0.5%) or nisin (10 ng ml^{-1}) was added. Bacterial culture media were inoculated with overnight pre-cultures to an optical density at 600 nm of 0.1 (for *S. aureus*) or 0.05 (for *L. lactis*).

Quantitative PSM detection

PSM amounts were measured by reversed-phase high pressure liquid chromatography/electrospray mass spectrometry (RP-HPLC/ESI-MS) using an 1100 HPLC system connected to an MSD Trap SL mass spectrometer (Agilent). Samples (100 μl) were injected onto a Zorbax SB-C8 2.3 × 30 mm column (Agilent) and a 0.1% trifluoroacetic acid (TFA) water/0.1% TFA acetonitrile gradient was applied as follows (flow rate, 0.5 ml min^{-1}); 10% acetonitrile from 0 to 2 min, 50% acetonitrile from 2 to 4 min, 50 to 100% acetonitrile linear gradient from 4 to 9 min, 100% acetonitrile from 9 to 13 min, 0% acetonitrile from 13 to 16 min. For the measurement of cytosolic PSMs, samples were dissolved in 6 M guanidinium chloride and 20 μl of sample were injected onto a μRPC C2/C18 ST 4.6/100 column (GE Healthcare) and a 0.1% TFA water/0.1% TFA linear acetonitrile gradient was run over 50 min at 0.5 ml min^{-1}. Relative PSM production was determined by measuring the peak area of the extracted ion chromatogram corresponding to multiply-charged m/z values of each PSM by using QuantAnalysis software (Agilent). All PSM measurements were performed on cultures grown for 12 h (*S. aureus*), 8 h (*L. lactis* PSMα peptides and δ-toxin), or 6 h (*L. lactis* PSMβ peptides).

Construction of *pmt* deletion mutants, *psm* complementation and expression plasmids

Deletion of the *pmt* genes (*pmtABCD*) was performed using a previously described allelic replacement procedure[31]. Briefly, ~ 1-kb up- and downstream regions of *pmtABCD* were amplified from MW2 genomic DNA as template using the PCR primer pairs pmtA-D-P1/pmtA-D-P2, and pmtA-D-P3/pmtA-D-P4, respectively (Supplementary Table 3 online), introducing *att1* and *att2* sites at the distal ends. The PCR fragments were fused together by overlap PCR and subsequently cloned in the vector pKOR1[31] using a BP clonase kit (Invitrogen). The resulting vector was transformed into the strains of interest. Then, the standard allelic replacement procedure was performed as described[31].

Complementation plasmids were constructed by amplification of the *pmtABCD* genes from MW2 genomic DNA with primers P3 and P4, digestion with Kpn1 and Hind3, and cloning into pRB473 or pMG36c. The natural promoter of *pmtABCD* was amplified with primers P5 and P6 and fused with the *pmtABCD* PCR fragment prior to cloning into pRB473. Walker A and B site mutations in pRB*pmt* plasmids were constructed using 4-fragment fusion PCR, analogous subsequent digestion, and cloning into pRB473.

The various *psm* genes were amplified using the primers indicated in Supplementary Table 3 online; PCR fragments were digested with the indicated enzymes and cloned into appropriate vectors. For generation of pTXα1–4, the *psm*α1–4 fragment was obtained from vector pTX_{Δ}α1–4 by digestion with BamH1 and Mlu1 and cloned into pTX15.

Animal model of skin infection

The mouse skin infection model was performed as described previously[3]. Briefly, female Crl:SKH1-hrBR mice were injected subcutaneously with approximately 2×10^7 bacterial cells in 50 μl of PBS in the left flank of the mouse. The length (L) and width (W) of the abscess or lesion caused by the bacterial infection was measured with an electronic caliper daily for 14 d post infection and calculated using the formula L × W. Bacterial numbers in

the abscesses were determined using isolation of genomic DNA of harvested tissue samples and subsequent quantitative real-time PCR[32] of the *gyrB* gene[33]. All mouse experiments were performed at the animal care facility of the NIAID, Building 33, in compliance with the guidelines of the NIAID/NIH Institutional Animal Care and Use Committee.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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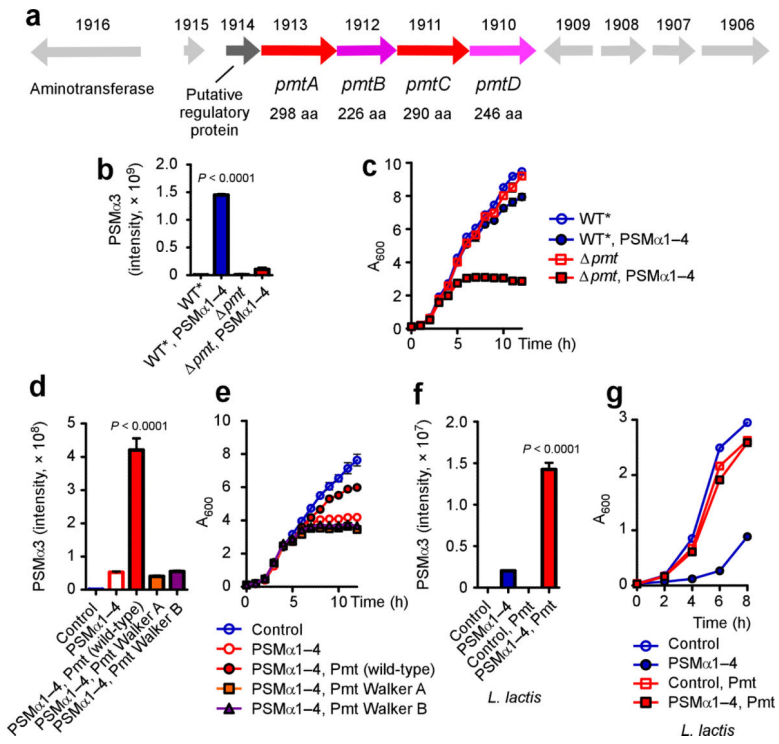

Fig. 1.

The Pmt PSM exporter. (**a**) Location and arrangement of the *pmt* operon in the *S. aureus* USA300 FPR3757 genome. aa, amino acids. (**b**) Dependence of PSM secretion on Pmt. (**c**) Dependence of growth on Pmt. (**d,e**) Energy dependence of Pmt function. PSMα3 secretion is shown in (d) and growth in (e). All tested strains are Δ*pmt* with induced expression of PSMα peptides (PSMα1–4); control, without induction. The *pmt* genes were expressed in their natural form (wild-type) or with mutated Walker A or B boxes ("Pmt Walker A", "Pmt Walker B"). (**f,g**) PSM secretion by Pmt in the heterologous host *L. lactis*. PSMα3 secretion is shown in (f) and growth in (g). Control, without induction.

In all panels: WT, USA300; WT*, USA300 with all *psm* genes deleted; Δ*pmt*, *pmt* deletion mutant, isogenic with WT*; PSMα1–4, induced expression of PSMα peptides (*S. aureus*, with xylose; *L. lactis*, with nisin). Pmt, constitutive expression of *pmt*. (**b,c,f,g**) WT*, *L. lactis*, and Δ*pmt* controls harbor the respective PSMα1–4 expression vector, but samples were not induced. (**d–g**) Control and PSMα1–4 strains also harbor the empty Pmt expression vector. See Supplementary Table 1 for complete designations of used strains and plasmids. See Supplementary Figures 6–9 online for results with other PSMs and USA400. Statistical analyses are by 1-way ANOVA and Bonferroni post-tests, in (b) and (f) shown for the PSMα1–4-expressing WT* versus all other samples.

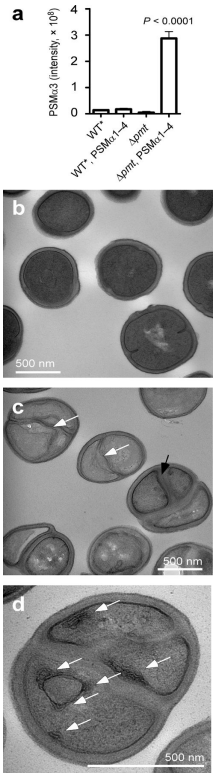

Fig. 2.

Absence of Pmt leads to intracellular accumulation of PSMs and major cellular defects. (**a**) Intracellular accumulation of PSMs. Strain designations are as in Fig. 1. PSMα3 is shown as example. Statistical analysis is by 1-way ANOVA and Bonferroni post-test shown for the PSMα1–4-expressing Δ*pmt* strain versus all other samples. See Supplementary Figure 10 online for other PSMs. (**b–d**) Effects on subcellular morphology, transmission electron microscopy (TEM) images. (**b**) control: strain WT*, PSMα1–4. (**c**) Δ*pmt*, PSMα1–4. Note disruption of membrane integrity (white arrows) and abnormal cell division, for example the additional septum marked by a black arrow. (**d**) Δ*pmt*, PSMα1–4, higher magnification. Note formation of abnormal macromolecular assemblies (white arrows) and higher electron density of the cytosolic compartment of control (b) versus Δ*pmt*, PSMα1–4 samples (c,d).

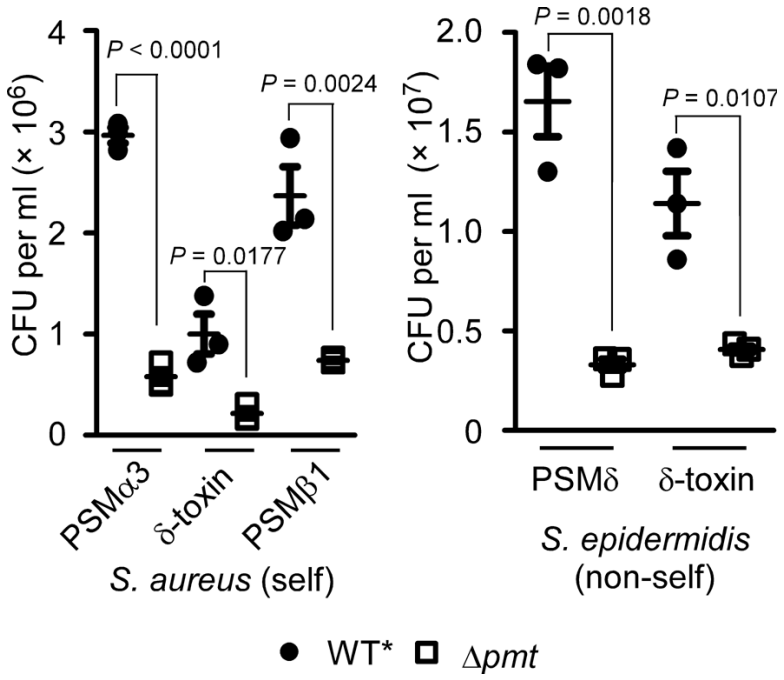

Fig. 3.

Pmt contributes to producer immunity and defense against PSM-based bacterial interference. Protective effects against *S. aureus* and *S. epidermidis* PSMs. Killing assays were performed with 3-h incubation of 10^6 CFU of bacteria and addition of different concentration of the respective peptides in carbonate-containing buffer[34], which mimics *in-vivo* conditions and was used in previous studies on PSM antimicrobial activities[25,26]. WT*, USA300 with all *psm* genes deleted; Δ*pmt*, isogenic *pmt* deletion mutant; Statistical analysis is by t-tests versus the corresponding WT* sample.

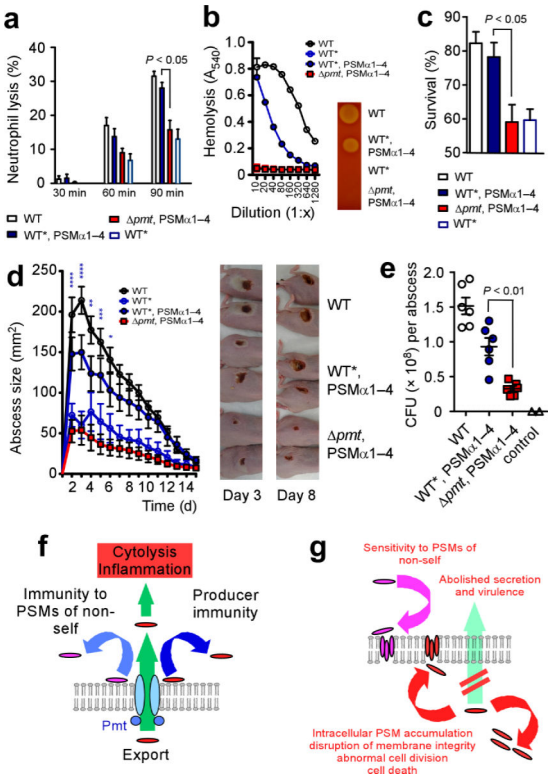

Fig. 4.

Pmt promotes virulence phenotypes and progression of *S. aureus* skin infection. (**a**) Neutrophil lysis after phagocytosis. Lysis of human neutrophils was determined microscopically after addition of equal numbers of live bacteria. Phagocytosis rates were measured under the same conditions. At > 60 min, virtually all bacteria were ingested (Supplementary Fig. 11 online). (**b**) Hemolysis. Culture filtrates were concentrated and butanol-extracted (to remove the overlaying hemolytic activity of α-toxin[8]), and hemolysis was determined using incubation with human erythrocytes (left) or human blood agar plates (right). (**c**) Survival in human blood. Bacteria were incubated with heparinized blood (10^6 CFU per ml) for 60 min. (**d**) Skin infection model. Groups of mice (n = 12) were subcutaneously injected at the dorsum with the indicated strains. Abscess sizes (lesions and surrounding inflamed areas) were measured daily. Representative images of formed abscesses are shown on the right. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; 2-way ANOVA with Bonferroni post-tests; only results comparing the two PSMα1–4-expressing strains are shown (see Supplementary Table 2 online for complete ANOVA results). (**e**) Bacterial numbers in abscesses at day 4. CFU are calculated from the performed measurement of bacterial genomic DNA by qRT-PCR of the *gyrB* gene. (**f**) Model depicting the multitude of Pmt functions. Pmt facilitates PSM export, provides producer immunity to PSMs, and protects from the antimicrobial activity of PSMs of non-self. (**g**) Model of consequences of Pmt absence. PSMs are not secreted, leading to strongly decreased virulence. In addition, PSMs accumulate in the cytosol, leading to the disruption of membrane integrity, disruption of vital macromolecular interactions, abnormal cell division

and cell death. Finally, there is sensitivity to the antimicrobial activity of PSMs of non-self. (a–e) Strain designations are as in Fig. 1.