Multidrug-Resistance Transporter AbcA Secretes Staphylococcus aureus Cytolytic Toxins

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Phenol-soluble modulins (PSMs) are Staphylococcus aureus cytolytic toxins that lyse erythrocytes and neutrophils and have important functions in the S. aureus infectious process. The molecular mechanisms of PSM secretion, however, are not well understood. Here we report that knockout of the multidrug-resistance ABC transporter AbcA, which contributes to S. aureus resistance against antibiotics and chemicals, diminished the secreted amount of PSM, leading to the accumulation of PSM in the intracellular fraction. The amount of PSM in the culture supernatants of the abcA knockout mutants was restored by introduction of the wild-type abcA gene, whereas it was not completely restored by introduction of mutant abcA genes encoding AbcA mutant proteins carrying amino acid substitutions in the adenosine triphosphate binding motifs. The abcA knockout mutant exhibited attenuated virulence in a mouse systemic infection model. These findings suggest that the multidrug resistance transporter AbcA secretes PSMs and contributes to S. aureus virulence.

Keywords. multidrug resistance transporter; Staphylococcus aureus; phenol-soluble modulins; cytolytic toxins

Staphylococcus aureus is a pathogenic bacterium that causes various human diseases. Methicillin-resistant S. aureus (MRSA), in particular, which has acquired resistance against a broad range of antibiotics, causes serious clinical problems. In the United States, MRSA causes 18 000 deaths per year [1]. MRSA carries the Staphylococcus chromosome cassette SCCmec containing the mecA gene, which confers resistance against β-lactams. Furthermore, MRSA carries >30 drug pumps, and their increased expression may contribute to its resistance to multiple drugs [2, 3]. Expression of drug pumps is also increased in multidrug-resistant Pseudomonas aeruginosa and Acinetobacter baumannii [4–6]. Understanding the functions of drug pumps is important toward establishing clinical strategies against MRSA and other antibiotic-resistant pathogenic bacteria.

S. aureus AbcA comprises 6 transmembrane domains and an adenosine triphosphate (ATP) hydrolyzing domain in a single polypeptide (575 aa), which is a type III ABC transporter found in the export system of many bacteria [7, 8]. AbcA has a high similarity to multidrug-resistant ABC transporters, such as Lactococcus lactis LmrA [9], Escherichia coli MsbA [10], and S. aureus Sav1866 [11]. Overexpression of abcA leads to resistance against β-lactams, such as methicillin and cefotaxime; the phosphoglycolipid moenomycin; the lipopeptide antibiotic daptomycin; and dyes, such as rhodamine and ethidium bromide [12, 13]. Deletion of abcA leads to susceptibility to moenomycin [13]. These findings suggest that AbcA is a multidrug efflux system against various antibiotics and chemicals in S. aureus. AbcA expression is regulated by NorG, a transcription factor involved in antibiotic resistance, and by other virulence regulators, including agr, Rot, SarA, SarZ, and MgrA [13]. AbcA expression is also induced by antibiotic treatment, as well as during starvation or the stationary phase, in which many S. aureus virulence factors are produced [13]. These findings raise the possibility that AbcA has roles in S. aureus virulence other than drug efflux. The endogenous molecule that AbcA exports and the functions of AbcA in S. aureus virulence, however, have not been elucidated.

S. aureus produces phenol-soluble modulin (PSM) subtypes Hld (α-toxin), PSmα1, PSmα2, PSmα3, PSmα4, PSmβ1, and PSMβ2 [14]. Within these PSMs, Hld, PSmα1, PSmα2, and PSMα3 have high lytic activity against neutrophils and erythrocytes [14]. Deletion of genes encoding Hld or PSmα1–4 decreases S. aureus virulence in a mouse infection model [14]. PSM is considered to be a determinant of the high virulence of community-acquired MRSA (CA-MRSA), which infects healthy persons in communities, because the expression of PSM is higher in CA-MRSA than in hospital-associated MRSA (HA-MRSA) and because CA-MRSA is more virulent than HA-MRSA [14]. Recognition of PSM by human formyl peptide receptor 2 (FPR2/ALX) is important for the progression of S. aureus infection [15]. Degranulation of mast cells by Hld is suggested to have a role in S. aureus–induced allergic skin diseases [16]. PSM also has lytic activity against bacterial cells [17, 18]. In addition, PSM has a role in S. aureus biofilm formation and colony spreading [19–21]. Therefore, elucidating the secretory mechanism of PSM is important for understanding the molecular mechanisms of the S. aureus infectious process.
Hld, PSMα1, PSMα2, PSMα3, and PSMα4 are short polypeptides (20–26 aa) with an amphipathic α-helical structure and a high isoelectric point [14, 22]. These characteristics of PSM are similar to those of antimicrobial peptides produced by various bacteria, referred to as bacteriocins [23]. To identify the PSM secretory machinery, we focused on secretory systems of bacteriocins. Some S. aureus strains produce a bacteriocin (30–31 aa) called aureocin A70, which has a high isoelectric point and is secreted by a type-III ABC transporter, AurT (571 aa) [24]. Some Staphylococcus epidermidis strains produce a bacteriocin (34 aa) called Pep5, which has an amphipathic rod-like conformation and a high isoelectric point [25, 26] and is secreted by PepT (571 aa), a type-III ABC transporter with a structure similar to that of AurT [27]. In the present study, we hypothesized that the transporter-secreting PSM has a structure similar to that of AurT or PepT. We identified a transporter, AbcA, with 58% and 56% amino acid identity with AurT and PepT, respectively, and revealed that AbcA is required for PSM secretion.

Multidrug-resistant transporters are reportedly involved in the virulence of several pathogenic bacteria, including Salmonella enterica serovar Typhimurium, Pseudomonas aeruginosa, and Vibrio cholera [28–30]. The molecular mechanisms of the drug transporters that contribute to bacterial virulence, however, are unclear. The present study revealed that a drug resistance transporter secretes S. aureus cytolytic toxins, providing the link between drug resistance and virulence expression.

MATERIALS AND METHODS

Ethics Statement

Mouse infection protocols followed the regulations for animal care and use of the University of Tokyo and were approved by the Animal Use Committee at the Graduate School of Pharmaceutical Science at the University of Tokyo (experimental approval P24–49).

Bacterial Strains and Culture Conditions

S. aureus strains were aerobically cultured in tryptic soy broth (Becton Dickinson) at 37°C, and kanamycin (50 µg/mL) or chloramphenicol (12.5 µg/mL) was added to maintain plasmids. L. lactis strains were statically cultured at 30°C in Difco M17 Broth (BD) supplemented with 0.5% glucose (GM17 broth). To maintain pRH100 in L. lactis strains, erythromycin (5 µg/mL) was added to the medium. Details of bacterial strains and plasmids are listed in Supplementary Table 1.

DNA and RNA Manipulations

DNA amplification by polymerase chain reaction (PCR), transformation of E. coli, extraction of plasmid DNA, and Southern blot analysis were performed according to previously described methods [31]. Electroporation of S. aureus or L. lactis with plasmid DNA was performed as previously described [32, 33]. To knock out S. aureus genes, S. aureus RN4220 strain or the agr-null mutant [34] was electroporated with a suicide plasmid carrying 400–600-bp DNA fragments identical to the internal DNA sequence of the target gene and spread on agar plates containing 12.5 µg/mL chloramphenicol, 50 µg/mL kanamycin, or 10 µg/mL erythromycin. The developing colony was cultured and infected with phage 80α. The phage lysate was used to transduce the disrupted gene locus to S. aureus strain Newman as previously described [35]. Genomic DNA was extracted using a QIAmp DNA Blood Kit (Qiagen) and lysostaphin (Wako), and gene knock out was confirmed by Southern blot analysis.

To express S. aureus AbcA in L. lactis, the open-reading frame of the S. aureus abcA gene and the promoter and Shine-Dalgarno sequences of the L. lactis noxE gene, which are constitutively active [36], were fused by splice overlap extension PCR. The DNA fragment was cloned into pRH100, resulting in pRH100-abcA. The L. lactis MG1363 strain [37] was transformed with pRH100 [38] or pRH100-abcA. PCR primers used in this study are listed in Supplementary Table 2.

Quantitative reverse transcription–PCR to measure the levels of abcA and pmtC messenger RNA (mRNA) was performed as previously described [39].

Measurement of PSMs

Preparation and measurement of PSM were performed according to previous methods [40] with minor modifications. S. aureus overnight culture (50 µL) was inoculated into 5 mL of tryptic soy broth and aerobically cultured at 37°C for 15 hours. S. aureus overnight cultures (2 mL) were centrifuged at 21 500 rpm for 2 minutes, and the supernatant was collected. The supernatant was evaporated and dissolved by 1 mL of 40% acetonitrile. The sample was centrifuged at 21 500 rpm for 5 minutes, and 800 µL of the supernatant was evaporated. The sample was dissolved by 1 mL of water and subjected to reverse-phase high-performance liquid chromatography (HPLC). Chromatography was performed using Source 5RPC ST 4.6/150 (GE Healthcare) and 50% acetonitrile in 0.1% trifluoroacetic acid for 3 minutes and a 50%–90% acetonitrile gradient in 0.1% trifluoroacetic acid for 20 minutes at a flow rate of 1 mL/minute (Waters, Milford, Massachusetts). Absorbance at 215 nm was detected by a 2998 Photodiode Array Detector (Waters). The molecular masses in the respective peaks of PSMs were determined using liquid chromatography–electrospray ionization mass spectrometry [40]. The amounts of respective PSMs were measured as the peak areas (calculated as µV · seconds), using Empower 2 software (Waters).

The amount of intracellular PSMs was measured according to the previously described method [41] with minor modifications. S. aureus overnight culture (50 µL) was inoculated into 5 mL of tryptic soy broth and aerobically cultured at 37°C for 15 hours. S. aureus overnight culture (5 mL) was centrifuged at 2300g for 20 minutes, and the cell pellet was collected. Cells were washed 2 times by vortexing in 6 M guanidinium chloride for 10 minutes. The cells were collected by centrifugation at 21 500g for 2 minutes and suspended in 300 µL of 6 M guanidinium chloride and...
100 µL of 0.1 mm zirconia/silica beads (ZSB-01, Tomy, Tokyo, Japan). The cells was destroyed at 2500 rounds/minute for 12 minutes, using a shaking machine (Beads Crusher μT-12, Taitec, Tokyo, Japan), and centrifuged at 21 500 g for 15 minutes. The cleared supernatants were evaporated and dissolved by 1 mL of 40% acetonitrile. The sample was centrifuged at 21 500 g for 5 minutes, and 800 µL of the supernatant was evaporated. The evaporated product was dissolved in 300 µL of water and subject-ed to reverse-phase HPLC. The chromatography conditions were the same as those described above.

Figure 1. Alignment of amino acid sequences of AbcA and AurT. A, The abcA gene and the 17 genes that show similarity with AbcA were disrupted, and the amounts of phenol-soluble modulin α3 (PSMα3) in the culture supernatants were measured. The vertical axis represents relative amount of PSMα3 to that in the Newman strain. B, Amino acid sequences of AbcA and AurT were aligned using Clustal W software. Asterisks or dots under the sequences indicate identical or similar amino acids, respectively. Six transmembrane domains (cyan), extracellular region (apricot), Walker A and Walker B motifs (yellow), signature C (purple), and switch region (moss green) are presented. Arrowheads indicate amino acids substituted in this study.
Additional Methods
Other methods for the Western blot analysis, mouse infection experiment, and PSM sensitivity assay are provided in the Supplementary Materials.

RESULTS
AbcA Is Required for PSM Secretion
We searched for a protein similar to AurT from the S. aureus N315 genome database by using protein BLAST and identified AbcA (SA0599), which had the highest homology with AurT among S. aureus gene products. In addition, we found 17 S. aureus gene products having similarity to AbcA with E values of <E-10 that contained Sav1866 (SA1683 in N315 genome), a multidrug-resistant ABC transporter [11]. S. aureus strain Newman does not carry the aurT gene and expresses comparable amounts of PSM as CA-MRSA [40]. We constructed knockout mutants of these genes in S. aureus strain Newman and measured the amount of PSMα3 in the culture supernatants. The amount of PSMα3 in the culture supernatant of the abcA knockout mutant was decreased, compared with that for the

Figure 2. The amounts of phenol-soluble modulins (PSMs) in the culture supernatant were decreased in the abcA knockout mutant. A. The parent strain transformed with an empty vector (pND50) and the abcA knockout mutants transformed with pND50 or pND50 harboring the abcA gene (pWabcA) were cultured for 15 hours. PSMs in the culture supernatants were analyzed by reverse-phase high-performance liquid chromatography. Dotted lines indicate respective PSMs. In this condition, Hld and PSMα1 were not separately eluted and are indicated as Hld+PSMα1. B and C. The amounts of PSMα3 (B) or Hld+PSMα1 (C) in the culture supernatants were measured and are presented. Data are means ± standard errors of 3 independent experiments. Asterisks indicate significant differences between parent/pND50 and ΔabcA/pND50 (P < .05, by the Student t test). D. Growth curves of the parent strain transformed with pND50 and the abcA knockout mutant transformed with pND50 or pWabcA are presented. The S. aureus strains were aerobically cultured in tryptic soy broth at 37°C, and absorbance at 600 nm was measured.
parent strain (Figure 1A). AbcA showed 58% amino acid identity with AurT and had high identities with AurT in the transmembrane domain, the Walker A and Walker B motifs that are predicted to bind ATP, the signature C motif, and the switch region, which are all conserved among bacterial ABC exporters (Figure 1B). In the culture supernatant of the abcA knockout mutant, the amounts of PSMα1+Hld, PSMα2, and PSMα4, as well as PSMα3, were decreased, compared with those in the parent strain (Figure 2A–C). The amounts of PSMs in the abcA knockout mutant were restored by introducing the intact abcA gene (Figure 2D). We hypothesized that PSMs would accumulate in the cytosolic fraction of the abcA knockout mutant if PSM
secretion was blocked. In the intracellular fraction of the \textit{abcA} knockout mutant, the amounts of PSMs were increased, compared with findings for the parent strain (Figure 3A–C). The intracellular amounts of PSMs in the \textit{abcA} knockout mutant were decreased by introduction of the intact \textit{abcA} gene (Figure 3A–C). These findings suggest that AbcA is required for PSM secretion.

The ATP-Binding Motif of AbcA Is Required for PSM Secretion

Because the ABC transporter uses energy released by ATP hydrolysis, amino acid substitutions in the Walker A or Walker B motifs, which function in ATP hydrolysis, lose the transporting activity of the ABC transporter [42–45]. We constructed mutant \textit{abcA} genes encoding mutant AbcA proteins harboring an amino acid substitution in the Walker A or Walker B motifs (Figure 1B). We added a His tag-coding sequence at the C-terminus of wild-type \textit{abcA} and mutant \textit{abcA} genes to measure the expression level. We examined their complementation activity in the \textit{abcA} knockout mutant. Introduction of the wild-type \textit{abcA} gene restored the amounts of PSM\textit{α}\textsubscript{3} and Hld+PSM\textit{α}\textsubscript{1} in the culture supernatant of the \textit{abcA} knockout mutant to the parent level (Figure 4A and 4B). In contrast, mutant \textit{abcA} genes encoding K374R or D497R mutant proteins decreased the restoration activities compared with the wild-type \textit{abcA} gene (Figure 4A and 4B). The expression levels of mutant AbcA proteins were comparable to that of wild-type AbcA protein (Figure 4C). These findings suggest that the ATP-binding motif of AbcA is required for PSM secretion.

\textbf{AbcA Is Required for \textit{S. aureus} Virulence}

Based on the requirement of \textit{abcA} for PSM secretion, we investigated whether \textit{abcA} has a critical role in \textit{S. aureus} virulence expression, using a mouse systemic infection model. Survival of mice injected with the \textit{abcA} knockout mutant was higher than that of mice injected with the parent strain (Figure 5). Introduction of the wild-type \textit{abcA} gene restored the killing activity of the \textit{abcA}-knockout mutant to the parent level (Figure 5). In contrast, the mutant \textit{abcA} gene encoding the K374R mutant protein attenuated the restoration of the killing activity of the \textit{abcA}-knockout mutant, compared with the wild-type \textit{abcA}.

Figure 4. Effect of amino acid substitutions in the Walker A or Walker B motifs of AbcA against phenol-soluble modulin (PSM) secretion. \textit{A} and \textit{B}, The parent strain transformed with pND50 and the \textit{abcA} knockout mutants transformed with pND50 or plasmids expressing His-tagged wild-type AbcA (pWabcA-His), His-tagged K374R AbcA (pK374R-His), or His-tagged D497R AbcA (pD497R-His) were cultured. The amounts of PSM\textit{α}\textsubscript{3} (\textit{A}) and Hld+PSM\textit{α}\textsubscript{1} (\textit{B}) in the culture supernatants were measured. Data are means ± standard errors from 3 independent experiments. Asterisks indicate significant differences between Δ\textit{abcA}/pWabcA-His and Δ\textit{abcA}/pK374R-His or between Δ\textit{abcA}/pWabcA-His and Δ\textit{abcA}/pD497R-His (\textit{p}< .05, by the Student \textit{t} test). \textit{C}, Expression of AbcA in the \textit{abcA} knockout mutants transformed with pND50, pWabcA-His, pK374R-His, and pD497R-His was visualized by Western blot analysis, using anti-His antibody. Each lane contains 10 µg of protein from cell extracts.
gene (Figure 5). The mutant abcA gene encoding the D497R mutant protein did not significantly decrease the restoration of the killing activity (Figure 5). These findings indicate that AbcA has a critical role in S. aureus infection in mammals via the ATP-binding Walker A motif.

**AbcA Expression Is Positively Regulated by an agr Quorum-Sensing System**

Transcriptions of the psmA operon and the hld gene are positively regulated by the accessory gene regulator agr, which controls the expression of S. aureus various virulence factors by quorum sensing [46]. The expression of abcA is under the positive control of agr in the S. aureus NCTC8325 strain [47]. Because our findings indicated that abcA is required for PSM secretion, it was assumed that expression of PSM and the PSM transporter is simultaneously regulated by agr. To confirm that the regulation exists in S. aureus strain Newman, which was used as the parent strain in this study, we measured abcA expression in the agr-deleted mutant and the parent strain. The abcA expression was decreased in the agr-deleted mutant, compared with findings for the parent strain (Figure 6A). Introduction of the agr locus restored abcA expression in the agr-deleted mutant (Figure 6A). These findings suggest that AbcA expression is under the positive control of agr in S. aureus.

**AbcA Is Required for PSM Secretion in a PmtABCD-Independent Manner**

During the course of our study, Chatterjee et al reported that PmtABCD is a transporter that secretes PSMs in CA-MRSA strains [41]. Thus, S. aureus uses 2 different transporters, AbcA and PmtABCD, to secrete PSMs. We examined the relative importance of AbcA and PmtABCD for PSM secretion. Knockout of pmTC in strain Newman decreased the amount of PSMo3 and Hld+PSMa1 in the culture supernatant (Figure 6B and 6C). Double knock out of pmTC and abcA decreased the amount of PSMo3 and Hld+PSMa1, compared with the respective single-knockout mutants (Figure 6B and 6C). These results suggest that AbcA and PmtABCD are independently required for PSM secretion.

We further examined whether AbcA and PmtABCD affect the expression of each other. In the abcA knockout mutant, the amount of pmTC mRNA did not differ significantly from that of the parent strain (Figure 6D). In the pmTC knockout mutant, the amount of abcA mRNA did not differ significantly from that of the parent strain (Figure 6D).

**Heterologous Expression of AbcA in L. lactis Increases Resistance Against PSMs**

PmtABCD, which is required for PSM secretion, also functions to protect S. aureus from PSMs which was administered in the extracellular milieu [41]. If AbcA directly secretes or extrudes PSMs, we assumed that heterologous expression of AbcA functions in protecting the bacteria from PSMs. We examined whether heterologous expression of AbcA makes L. lactis resistant to PSMs. We transformed L. lactis with a plasmid carrying the C-terminal His-tagged AbcA gene and confirmed that the strain expressed AbcA (Figure 7A). When 16 or 32 µg/mL PSMo3 was added to the culture, the L. lactis strain expressing AbcA had a higher survival rate than the L. lactis strain transformed with an empty vector (Figure 7B). These findings suggest that heterologously expressed AbcA protects L. lactis from extracellularly administered PSMs.

**DISCUSSION**

Our findings revealed that a multidrug resistance transporter, AbcA, is required for secretion of the S. aureus cytolytic toxin PSM. Heterologous expression of AbcA in L. lactis was functional to protect the cells against extracellular PSM. Therefore, AbcA secretes self-synthesized PSMs and extrudes extracellularly administered PSM. Furthermore, a mouse infection experiment demonstrated that AbcA has a role in S. aureus virulence. These findings provide the first evidence that a multidrug transporter has dual functions to secrete toxins and protect cells from foreign substances. These offensive and defensive functions, respectively, are important for the bacterial infectious process.

The findings of the present study, combined with those of a previous study of PmtABCD, suggest that S. aureus uses at least 2 transporters to secrete PSMs. We demonstrated that the abcA
knockout mutant decreased PSM secretion to almost the same level as that for the pmtC knockout mutant, suggesting that the contribution of AbcA to PSM secretion is comparable to that of PmtABCD. We further revealed that abcA/pmtC double-knockout mutant secretes less PSM than the respective single-knockout mutants. Thus, AbcA and PmtC independently function in PSM secretion. Expression of both AbcA and PmtABCD is positively regulated by the agr system, which positively regulates PSM expression. Regulation of the 2 transporters by agr is meaningful to synchronize the expression of the export system and substrate synthesis. PmtABCD is encoded by a polycistronic operon [41]; PmtB and PmtD each possess 6 transmembrane domains, and PmtA and PmtC each possess ATP-binding

Figure 6. Interrelationship between abcA, agr, and pmtABCD. A, The Staphylococcus aureus parent strain, the agr deletion mutant, and the agr deletion mutant transformed with pW carrying an intact agr region were cultured to an A600 of 1 or 2, and the cells were collected to extract total RNA. Quantitative reverse transcription–polymerase chain reaction was performed to measure the amount of the abcA messenger RNA (mRNA). The data were normalized to the amount of 16S ribosomal RNA (rRNA) and are represented as values relative to that of the parent strain at an A600 of 1. Means ± standard errors of triplicate experiments from one of 3 independent experiments are presented. Single or double asterisks indicate significant differences between parent and Δagr and between Δagr and Δagr/pW, respectively (P < .05, by the Student t test). B and C, The parent strain, the abcA knockout mutant, the pmtC knockout mutant, or the abcA/pmtC knockout mutant was cultured for 15 hours. The amounts of phenol-soluble modulin α3 (PSMα3; B) and Hld+PSMα1 (C) in the culture supernatants were measured. Data are represented as mean ± standard error from 4 independent experiments (parent or abcA) or 6 independent experiments (pmtC or abcA/pmtC). Asterisks indicate Student t test P values of < .05. D, The S. aureus parent strain, the abcA knockout mutant, and the pmtC knockout mutant were cultured to an A600 of 1, and the cells were collected to extract total RNA. The amounts of the pmtC mRNA or the abcA mRNA were normalized to the amount of 16S rRNA and are represented as values relative to that of the parent strain. Data are represented as mean ± standard error of 4 independent experiments. Student t test P values between parent and ΔabcA and between parent and ΔpmtC were each > .05.

Figure 7. Effect of heterologous expression of AbcA in Lactococcus lactis against phenol-soluble modulin (PSM) resistance. A, L. lactis MG1363 strain was transformed with an empty vector (pRH100) or a plasmid carrying the His-tagged AbcA gene (pRH100-abcA). Overnight-cultured cells were lysed and subjected to Western blot analysis using anti-His antibody. B, L. lactis strain transformed with pRH100 or pRH100-abcA was cultured for 3 hours in the presence of 16 or 32 µg/ml PSMα3. The sample was spread on GM17 agar plates, and the number of colony-forming units (CFU) was counted. Vertical axis represents the survival percentage. Means ± standard errors of triplicate experiments from one of 3 independent experiments are presented. Asterisks indicate significant differences between pRH100 and pRH100-abcA (P < .05, by the Student t test).
motifs, suggesting that a heterodimer consisting of PmtABCD forms a transporter complex to secrete PSM [41]. The amino acid sequences of PmtB-PmtA and PmtD-PmtC exhibit 14% identity with that of AbcA, which is very low compared with that between AurT and AbcA. AbcA is encoded by a monocistronic operon and the single polypeptide contains both 6 transmembrane domains and an ATP-binding motif. Because most ABC transporter complexes comprise 12 transmembrane domains that form a pore across the membrane, a homodimer of AbcA may constitute transporter machinery. Because deletion of pmtABCD was successful in the PSM knockout mutant or the agr-negative mutant of USA300 or MW2 strains but not in the wild-type strains, Chatterjee et al suggested that accumulation of PSMs in the cytosol is lethal to S. aureus [41]. Consistent with their reports, we found that knockout of pmtC severely decreased the growth of strain Newman but not that of the agr-null RN4220 strain (data not shown). In contrast, the abcA knockout mutant was viable with growth indistinguishable from that of the parent strain. The different effects of abcA and pmtABCD knockouts on S. aureus growth might be due to differences in the accumulation of PSM in the cytosol or to the requirement of PmtABCD for other essential processes of S. aureus. Further studies are needed to clarify the roles of the 2 different transporters in S. aureus.

The abcA knockout mutant has an increased frequency of autolysis [47]. In the abcA knockout mutant, the expression levels of autolytic enzymes are not different from those in the parent strain, indicating that the increased autolysis in the abcA knockout mutant is not due to altered expression of autolytic enzymes [47]. This study revealed that PSM accumulates in the intracellular fraction of the abcA knockout mutant. The accumulated intracellular PSM in the abcA knockout mutant is a possible trigger for autolysis.

In this study, we examined the effects of amino acid substitutions in Walker A and Walker B motifs of AbcA against PSM secretion and found that both K374R and D497R mutant AbcA proteins decreased the activity to restore PSM secretion in the abcA knockout mutant. Both the lysine residue in Walker A and the aspartate residue in Walker B are important for ATP hydrolysis of most ABC transporters [42–45]. Thus, AbcA secretes PSMs by using the energy released by ATP hydrolysis. We further demonstrated that the K374R mutant AbcA protein attenuated the restoration of the killing activity of the abcA knockout mutant in mice. The result suggests the requirement of the Walker A motif of AbcA in the S. aureus infectious process.

Previous studies suggested that AbcA extrudes β-lactam antibiotics with lipophilic side chains; moenomycin, a glycocephalosporin; daptomycin, a lipopeptide antibiotic; and dyes such as rhodamine, ethidium bromide, and Hoechst 33 342 [12]. Although the molecular weights of PSMs are higher than those of these molecules, both PSMs and these molecules have a hydrophobic structure. This finding is consistent with the fact that multidrug transporters extrude various molecules with different structures [48]. Comparison with AbcA and other multidrug transporters would further our understanding of the molecular mechanisms underlying substrate recognition and extrusion. Most multidrug resistance transporters are not known to secrete endogenous molecules; thus, further investigation is required to determine how AbcA has evolved to secrete endogenously synthesized molecules and extrude invading foreign chemicals and whether similar multidrug transporters secrete endogenous PSM-like molecules. Transporters locate on the cell surface, which is easily accessed by chemicals. Thus, because inhibition of S. aureus AbcA is expected to lead to sensitization to antibiotics, increased autolysis, and decreased virulence, AbcA is a promising target for novel antibiotics against S. aureus.

Supplementary Data
Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes
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