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Alanyl-Phosphatidylglycerol and Lysyl-Phosphatidylglycerol Are Translocated by the Same MprF Flippases and Have Similar Capacities To Protect against the Antibiotic Daptomycin in *Staphylococcus aureus*

Christoph J. Slavetinsky, Andreas Peschel, and Christoph M. Ernst

Interfaculty Institute of Microbiology and Infection Medicine, Cellular and Molecular Microbiology Division, University of Tübingen, Tübingen, Germany

The lysinylation of negatively charged phosphatidylglycerol by MprF proteins reduces the affinity of cationic antimicrobial peptides (CAMPs) for bacterial cytoplasmic membranes and reduces the susceptibility of several Gram-positive bacterial pathogens to CAMPs. MprF of *Staphylococcus aureus* encompasses a lysyl-phosphatidylglycerol (Lys-PG) synthase and a Lys-PG flippase domain. In contrast, *Clostridium perfringens* encodes two MprF homologs which specifically synthesize alanyl-phosphatidylglycerol (Ala-PG) or Lys-PG, while only the Lys-PG synthase is fused to a putative flippase domain. It remains unknown whether cationic Lys-PG and zwitterionic Ala-PG differ in their capacities to be translocated by MprF flippases and if both can reduce CAMP susceptibility in Gram-positive bacteria. By expressing the MprF proteins of *C. perfringens* in an *S. aureus mprF* deletion mutant, we found that both lipids can be efficiently produced in *S. aureus*. Simultaneous expression of the Lys-PG and Ala-PG synthases led to the production of both lipids and slightly increased the overall amounts of aminoacyl phospholipids. Ala-PG production by the corresponding *C. perfringens* enzyme did not affect susceptibility to CAMPs such as nisin and gallidermin or to the CAMP-like antibiotic daptomycin. However, coexpression of the Ala-PG synthase with flippase domains of Lys-PG synthesizing MprF proteins led to a wild-type level of daptomycin susceptibility, indicating that Ala-PG can also protect bacterial membranes against daptomycin and suggesting that Lys-PG flippases can also translocate the related lipid Ala-PG. Thus, bacterial aminoacyl phospholipid flippases exhibit more relaxed substrate specificity and Ala-PG and Lys-PG are more similar in their capacities to modulate membrane functions than anticipated.

The lysinylation of negatively charged phosphatidylglycerol (PG) by the multiple peptide resistance factor (MprF) is a major strategy of bacterial pathogens, including *Staphylococcus aureus* (22), *Listeria monocytogenes* (32), *Mycobacterium tuberculosis* (18), *Bacillus anthracis* (28), and *Rhizobium tropici* (30), to resist cationic antimicrobial peptides (CAMPs) encountered during colonization and infection of human or other eukaryotic hosts (17, 21). Inactivation of MprF leads to hypersusceptibility not only to CAMPs but also to the membrane-targeting antibiotic daptomycin (10, 12), which has many features in common with host CAMPs, as well as to increased susceptibility to cationic antibiotics such as vancomycin (27) and gentamicin (20). The minor impact of phospholipid lysinylation on the membrane proteome of *S. aureus* (29) and the direct impact of lysinylated phospholipids on antimicrobial peptide interaction with model membranes (1) underline the specific role of MprF in reducing susceptibility to antimicrobial peptides. Since bacterial phospholipids are crucial interaction partners in the complex processes leading to bacterial damage by CAMPs (5, 36) or membrane-active antibiotics, MprF represents a major line of bacterial defense against membrane-targeting antimicrobials. Because of its crucial role in bacterial infections and its widespread occurrence in many pathogens, MprF has been proposed as a promising target for the development of novel drugs that would render bacterial pathogens highly susceptible to host defenses and antibiotics (11, 34).

MprF lysinylates PG by transferring lysine residues from lysyl-tRNA to PG (26, 31). Every MprF protein identified to date consists of a hydrophobic N terminus of variable length and a highly conserved cytosolic C terminus (9, 25). Recently, we have shown that a large portion of the N terminus is dispensable for lysyl-PG (Lys-PG) biosynthesis in *S. aureus* but is required for Lys-PG-mediated reduced CAMP and daptomycin susceptibility because of its ability to facilitate the translocation of Lys-PG from the inner to the outer leaflet of the cytoplasmic membrane (10). Without the flippase mechanism of MprF, *S. aureus* is highly susceptible to CAMPs and daptomycin (10). *In vitro* experiments with crude cell lysates have revealed that the catalytic part of the aminoacyl phospholipid synthase is located in the C-terminal cytosolic part of MprF (14, 25, 26) while *in vivo* Lys-PG synthesis in *Escherichia coli* required some of the adjacent transmembrane segments of MprF of *S. aureus* and *B. subtilis* (10, 28).

Interestingly, point mutations leading to amino acid exchanges in the MprF proteins of *S. aureus* isolates with increased daptomycin resistance have been frequently reported (3, 7), suggesting that MprF can alter its function to confer daptomycin resistance. The impact of the mutations on MprF activity remains unclear but might involve increased Lys-PG synthesis (35), increased Lys-PG flipping (15), or both (19).

Aside from the production of Lys-PG, some MprF proteins synthesize alanyl-PG (Ala-PG), such as MprF homologs from *Enterococcus faecium* (25), *Clostridium perfringens* (26), *Bacillus sub-*

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Address correspondence to Andreas Peschel, andreas.peschel@uni-tuebingen.de.
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tilis (25), or *Pseudomonas aeruginosa* (16). While the MprF homologs of *P. aeruginosa* and one of the two homologs of *C. perfringens* (MprF1) strictly synthesize Ala-PG, the MprF of *E. faecium* is capable of synthesizing Lys-PG, Ala-PG, and Arg-PG (25). The impact of the production of zwitterionic Ala-PG on susceptibility to antimicrobial peptides has so far been studied only in *P. aeruginosa*, which alanylates a maximum of 6% of the phospholipids (16). Ala-PG deficiency in the Gram-negative bacterium *P. aeruginosa* has led to increased susceptibility to certain CAMPs (protamine sulfate, poly-L-lysine, and polymyxin E), β-lactam antibiotics (ampicillin, oxacillin, and cefsulodin), the lipopeptide antibiotic daptomycin, and chromium ions (2, 16), indicating that MprF is of relevance for reducing CAMP susceptibility in both Gram-positive and Gram-negative organisms. The impact of the production of zwitterionic Ala-PG on antimicrobial peptide susceptibility has so far not been investigated in Gram-positive bacteria.

The functional expression of the Ala-PG synthase of *C. perfringens* in *S. aureus* presented in this study enabled us to investigate the impact of Ala-PG on CAMP susceptibility in a Gram-positive pathogen and led to the unexpected observation that zwitterionic Ala-PG is as effective as cationic Lys-PG in protecting bacterial membranes against daptomycin. However, the Lys-PG flippase domains of MprF turned out to be required for Ala-PG to confer a wild-type level of daptomycin susceptibility, indicating that Lys-PG flippases can translocate both types of aminoacyl phospholipids.

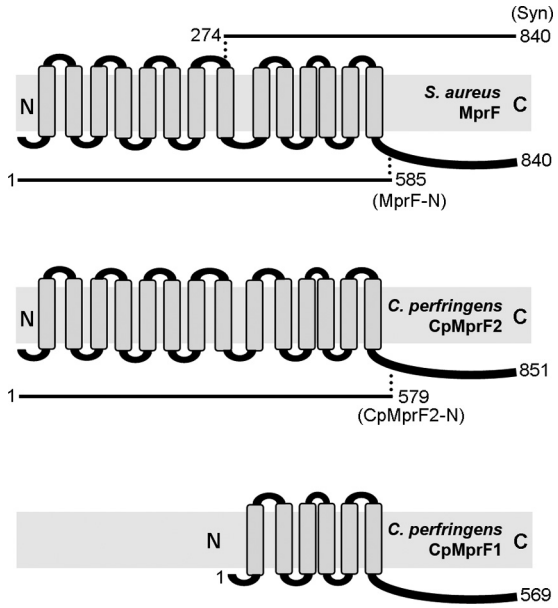

FIG 1 Structures of *S. aureus* and *C. perfringens* MprF proteins. The lengths and predicted transmembrane segments of MprF (*S. aureus*), CpMprF1, and CpMprF2 (*C. perfringens*) are shown. Extensions of truncated MprF variants MprF-N and CpMprF2-N and of the previously characterized *S. aureus* synthase domain (Syn) are indicated.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. aureus* SA113 (ATCC 35556) is a frequently used laboratory strain. Lys-PG-deficient strain SA113 Δ*mprF* has been described recently (22). Strains were grown in Mueller-Hinton broth (MHB; 0.2% beef infusion solids, 1.75% casein hydrolysate, 0.15% starch); overnight cultures were supplemented with appropriate antibiotics. For coexpression experiments, the medium was supplemented with 0.5% xylose to allow the expression of genes under the control of the pTX15 plasmid.

Prediction of MprF structure. The transmembrane topology of MprF proteins from *S. aureus* and *C. perfringens* was analyzed with the topology prediction algorithm PRO-TMHMM (http://topcons.cbr.su.se/) (4, 33).

Cloning of *cpmprF1* and *cpmprF2* into *Staphylococcus*-specific plasmid pRB474 or pTX15. The *C. perfringens* MprF genes *cpmprF1* and *cpmprF2* were amplified from *C. perfringens* ATCC 13124 as described below and cloned into *S. aureus* Δ*mprF*. *cpmprF1* and *cpmprF2* correspond to recently described *mprF1* and *mprF2* of *C. perfringens* SM101 (26). CpMprF2 is annotated as CPF_1456 and is 100% identical to MprF2 of *C. perfringens* SM101 (CPR_1258). CpMprF1 has been annotated as two proteins, CPF_1845 and upstream CPF_1846, in *C. perfringens* ATCC 13124. However, genomic sequencing of the upstream region of CPF_1845 revealed that CPF_1846 forms a single protein together with CPF_1845 (data not shown). The protein shares 98.8% identity and 100% similarity with the recently described MprF1 protein (CPR_1564) from strain SM101 (26) and was named CpMprF1 in this study. DNA was isolated from *C. perfringens* ATCC 13124 and amplified with primers 5′-ATTGGATCCAGGAGGTAGATATTATGTGGGATTCACTAAAAAAA AGTTATAGAC-3′ and 5′-GCTACTAAGAAAACCTATAAAGTTGTAT AGAATTCTT-3′ and primers 5′-TTGGATCCAGGAGGTACAAAAGG TGAAGTTAAATATAAAGATAAGTG-3′ and 5′-GTATAGTTGTAA GAAACTTAAAAAGTTTAATTAAATAAGAATTCTA-3′, respectively. The truncated variant *cpmprF2-N* was amplified with the additional primer 5′-GGCAATAATGAAAATCTATTTAGGGAAATATAAGAATT CTG-3′. *cpmprF1* was cloned into *E. coli-Staphylococcus*-specific shuttle plasmid pRB474 (8) in such a way that the 5′ end was downstream of the plasmid-encoded constitutive *veg* promoter. In parallel, *cpmprF1*, *cpmprF2*, and truncated variants were cloned into *Staphylococcus*-specific expression vector pTX15 (23) in the correct orientation to permit expression from the xylose-inducible plasmid-encoded *xyl* promoter. The plasmid constructs expressing *S. aureus mprF* and truncated variants have been described recently (10). Empty plasmid pTX16 (23) or pRB474 was used in certain control strains.

Isolation and quantification of polar lipids. Polar lipids were isolated from *S. aureus* cultures grown to the logarithmic phase (3 h) and extracted with chloroform-methanol (2:1, vol/vol) by the Bligh-Dyer procedure (6), vacuum dried, and dissolved in chloroform-methanol (2:1, vol/vol). Amino groups or phosphate groups containing lipids were identified by ninhydrin or molybdenum blue staining, respectively. Aminoacyl phospholipids were quantified in relation to total phospholipid content by determining lipid spot intensities of molybdenum blue-stained lipids as described recently (10).

Determination of susceptibility to antimicrobial peptides. MICs of gallidermin and nisin were determined by diluting overnight cultures to an optical density at 600 nm (OD_{600}) of 0.1 in fresh MHB medium containing serial dilutions of antimicrobial peptides without further antibiotics as described recently (10, 24). Briefly, serial 2-fold dilutions of gallidermin (3.2, 1.6, 0.8, 0.4, and 0.2 μg/ml) and nisin (32, 16, 8, 4, and 2 μg/ml) in MHB were inoculated with precultures adjusted to an OD_{600} of 0.1. Twenty-four-well plates with 1-ml aliquots were shaken at 37°C, and the MIC of gallidermin was determined after 24 h while the MIC of nisin was determined after 48 h. The MIC was defined as the antibiotic concentration causing a 90% reduction of growth as calculated by interpolation. The assay was repeated at least three times. Susceptibility to daptomycin was determined by epsilometer test (Etest) according to the manufacturer's advice (Alere).

RESULTS

Lysyl- and alanyl-PG synthases of *C. perfringens* are functional in *S. aureus*. The MprF protein of *S. aureus* synthesizes Lys-PG (22), while *C. perfringens* has two MprF proteins, one synthesizing Ala-PG and a second synthesizing Lys-PG (26). The Lys-PG synthase CpMprF2 of *C. perfringens* is very (54%) similar in sequence

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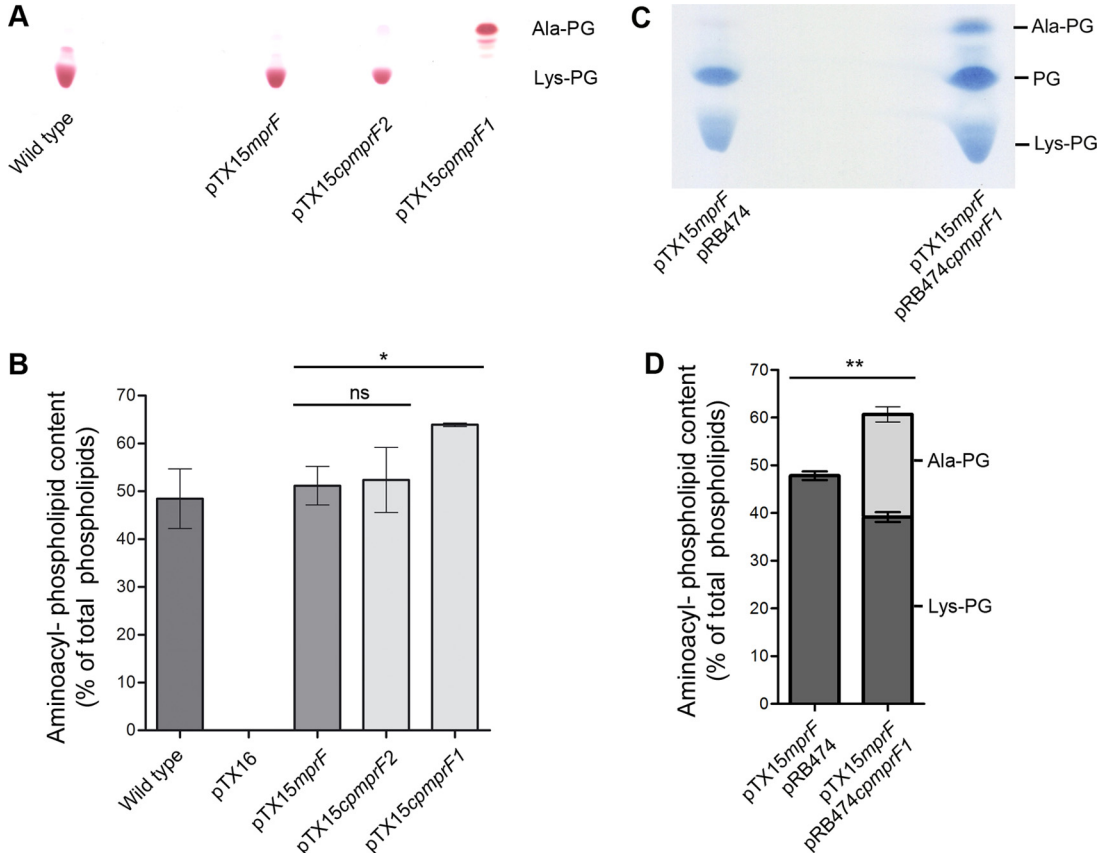

FIG 2 Aminoacyl phospholipid production by *C. perfringens* MprF proteins in *S. aureus*. (A) Detection of aminoacyl phospholipids from wild-type *S. aureus* and an *S. aureus mprF* deletion mutant strain expressing empty plasmid pTX16, *S. aureus* MprF (pTX15*mprF*), or *C. perfringens* MprF protein (pTX15*cpmprF2* or pTX15*cpmprF1*). Polar lipids were separated by TLC and stained with the amino group-specific dye ninhydrin. (B) Quantification of aminoacyl phospholipid content. Polar lipids were separated by TLC, stained with the phosphate group-specific dye molybdenum blue, and quantified densitometrically. (C) Detection of phospholipids from *S. aureus mprF* deletion mutants expressing *S. aureus* MprF together with empty plasmid pRB474 or *S. aureus* MprF together with *C. perfringens* CpMprF1 on separate plasmids. Polar lipids were separated and detected as described for panel B. (D) Quantification of aminoacyl phospholipids as described for panel B. The Ala-PG and Lys-PG contents of an *S. aureus mprF* deletion mutant expressing *mprF* and *cpmprF1* or *mprF* alone are indicated. Means and standard errors of the means of at least three independent experiments are shown. *, $P < 0.05$; **, $P < 0.005$; ns, not significantly different from *S. aureus* Δ*mprF* containing plasmid pTX15*mprF* (B) or plasmids pTX15*mprF* and pRB474 (D).

and predicted topology to *S. aureus* MprF, while the Ala-PG synthase CpMprF1 lacks the first 8 of 14 predicted transmembrane segments implicated in the flippase activity of *S. aureus* MprF (10) (Fig. 1).

To investigate whether the MprF proteins of *C. perfringens* can be functionally expressed in *S. aureus*, we cloned *cpmprF1* and *cpmprF2* into plasmids, expressed them in an *S. aureus mprF* deletion mutant, and analyzed the aminoacyl phospholipid contents of the resulting strains. Expression of *cpmprF2* led to Lys-PG biosynthesis, while the *cpmprF1*-containing strain produced an aminoacyl phospholipid that migrated much faster on thin-layer chromatography (TLC) plates than Lys-PG at a position described recently for Ala-PG (26) (Fig. 2A). The CpMprF2- or CpMprF1-expressing *S. aureus* strain produced aminoacyl phospholipids at a level similar to or 15% higher than that of the *S. aureus* wild type, respectively (Fig. 2B), thereby demonstrating that Ala-PG and Lys-PG synthases of *C. perfringens* are fully functional and are integrated effectively into the phospholipid-biosynthetic pathways of *S. aureus*. In line with this notion, the CpMprF1- or CpMprF2-expressing strain exhibited growth behavior and a microscopic appearance similar to those of the *S. aureus* wild type (data not shown).

CpMprF1-mediated Ala-PG production in *S. aureus* does not lead to reduced CAMP and daptomycin susceptibility. The absence of Lys-PG in *S. aureus* leads to hypersusceptibility toward CAMPs (22), including the CAMP-like antibiotic daptomycin (10). To investigate whether Ala-PG affects CAMP susceptibility, we tested the *cpmprF1*-expressing *S. aureus* strain for susceptibility to the CAMPs gallidermin and nisin, as well as daptomycin, and found that CpMprF1 did not affect susceptibility to gallidermin and nisin and led to only a minor decrease in daptomycin susceptibility (Fig. 3 A to C). In contrast, CpMprF2-mediated Lys-PG production conferred a wild-type level of CAMP susceptibility with 6-fold decreased daptomycin susceptibility or 4-fold decreased gallidermin and nisin susceptibility compared to the *S. aureus mprF* deletion mutant (Fig. 3A to C). Thus, the inability of CpMprF1 to reduce CAMP susceptibility may be due either to differences in the capacities of Ala-PG and Lys-PG to prevent the interaction of CAMPs with the cytoplasmic membrane or to the absence of the aminoacyl phospholipid flippase domain at the CpMprF1 N terminus because of which Ala-PG might not be able to reach the outer leaflet of the cytoplasmic membrane (Fig. 1).

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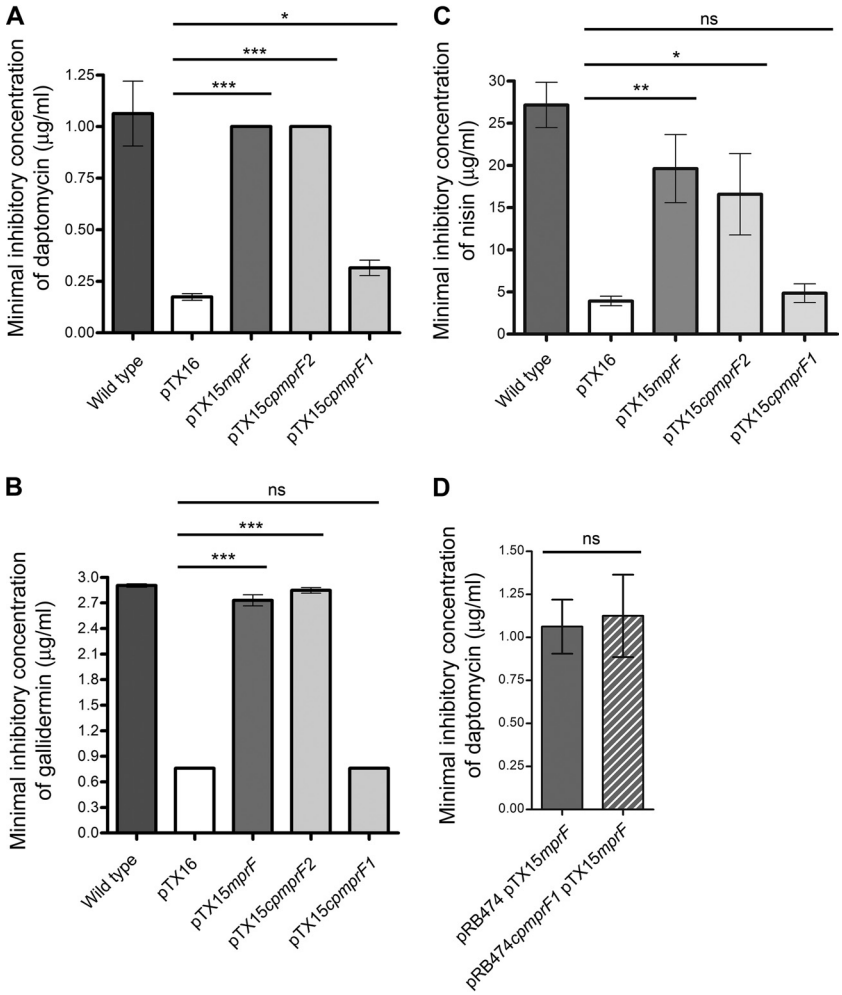

FIG 3 Impact of *C. perfringens* MprF proteins on antimicrobial peptide resistance. (A to C) MICs of daptomycin (A), gallidermin (B), and nisin (C) were determined with the *S. aureus* wild-type strain and *mprF* deletion mutants expressing empty plasmid (pTX16) and *S. aureus* MprF (pTX15*mprF*) and *C. perfringens* MprF proteins (pTX15*cpmprF2* and pTX15*cpmprF1*). (D) MIC of daptomycin determined with *mprF* deletion mutants expressing *S. aureus* MprF together with empty plasmid pRB474, as well as *S. aureus* MprF together with *C. perfringens* CpMprF1 on separate plasmids. Means and standard errors of the means of at least three independent experiments are shown. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$; ns, not significantly different from *S. aureus* Δ*mprF* containing plasmid pTX16 (A to C) or plasmids pTX15*mprF* and pRB474 (D).

Simultaneous expression of MprF and CpMprF1 leads to production of both Lys-PG and Ala-PG but does not lead to further reduced daptomycin susceptibility. The finding that *S. aureus* can produce Lys-PG or Ala-PG raised the questions of whether both lipids can be produced simultaneously in similar amounts and whether the presence of two aminoacyl phospholipids affects membrane properties. Therefore, we coexpressed *cpmprF1* of *C. perfringens* together with *mprF* of *S. aureus* on separate plasmids and analyzed the aminoacyl phospholipid content of the resulting strain. Coexpression of *cpmprF1* with *mprF* led to significant Ala-PG levels, accounting for one-third of the overall aminoacyl phospholipid content (Fig. 2C and D), but it had no additional impact on daptomycin susceptibility (Fig. 3D). However, coproduction of Ala-PG with Lys-PG led to 13% increased overall aminoacyl phospholipid content compared to that of a strain with *mprF* alone (Fig. 2D), in agreement with our above-mentioned findings concerning the strain expressing the Ala-PG synthase alone (Fig. 2B). Thus, even though CpMprF1 is derived from a bacterial species that is only distantly related to *S. aureus*, it is able to compete effectively with MprF in aminoacyl phospholipid biosynthesis. The achievable level of Lys-PG appears to be limited to ca. 50% of the total phospholipids, while Ala-PG can be produced in slightly larger amounts.

Evidence that Lys-PG flippases can translocate Ala-PG, thereby leading to levels of reduced daptomycin susceptibility similar to those obtained with Lys-PG. The similarity of MprF to CpMprF2 (Fig. 1) and the ability of CpMprF2 to confer a wild-type level of CAMP susceptibility (Fig. 3) suggested that CpMprF2 may be able to flip Lys-PG too. Recently, the N-terminal hydrophobic part of MprF without the cytosolic part has been shown to lack the capacity to synthesize Lys-PG but to be able to flip Lys-PG and reduce susceptibility to CAMPs (10). To verify that the corresponding domain of CpMprF2 has a similar capacity, we constructed a truncated variant of CpMprF2 lacking the cytosolic part (Fig. 1) and expressed it in *trans* with the Lys-PG synthase domain of MprF (10). In accord with our previous findings, the Lys-PG synthase domain of MprF alone

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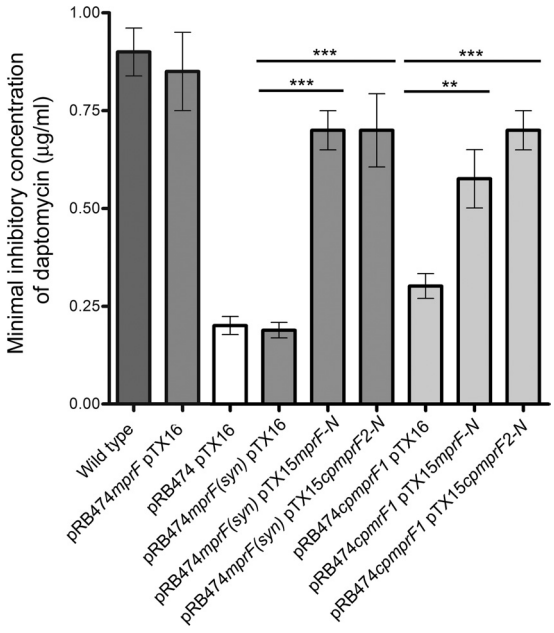

FIG 4 Impact on daptomycin resistance of Lys-PG flippases expressed in *trans* with Ala-PG or Lys-PG synthase in *S. aureus*. MICs of daptomycin were determined for a set of *mprF* deletion mutants expressing the Lys-PG synthase domain of *S. aureus* MprF [pRB474*mprF*(*syn*)] together with the empty plasmid (pTX16), the Lys-PG flippase domain of *S. aureus* (pTX15*mprF-N*), and the Lys-PG flippase domain of *C. perfringens* (pTX15*cpmprF2-N*). Another strain set consisted of the *mprF* deletion mutant expressing the Ala-PG synthase of *C. perfringens* (CpMprF1) together with the empty plasmid (pTX16) and the Lys-PG flippase domain of *S. aureus* (pTX15*mprF-N*) or *C. perfringens* (pTX15*cpmprF2-N*). Wild-type *S. aureus* and the complemented *mprF* deletion mutant (pRB474*mprF*/pTX16) served as positive controls, while the *mprF* deletion mutant expressing empty plasmids pTX16 and pRB474 served as a negative control. Means and standard errors of the means of at least three independent experiments are shown. Statistically significant differences from *S. aureus* Δ*mprF* expressing respective aminoacyl phospholipid synthases lacking Lys-PG flippase domains [pRB474*mprF*(*syn*)/pTX16; pRB474*cpmprF1*/pTX16] are indicated as follows: ***, $P < 0.0001$; **, $P < 0.01$.

did not affect daptomycin susceptibility. However, coexpression with the putative flippase domain of CpMprF2 led to significantly reduced daptomycin susceptibility comparable to coexpression with the flippase domain of MprF from *S. aureus*, which confirms that CpMprF2 bears a Lys-PG flippase domain that is functional in *S. aureus* (Fig. 4).

We hypothesized that the Lys-PG flippase domain of CpMprF2 might also be responsible for flipping Ala-PG in *C. perfringens* and tested this idea by coexpressing CpMprF1 with the flippase domain of CpMprF2 and monitoring the daptomycin susceptibility of the resulting strains. The sole expression of CpMprF1 affected daptomycin susceptibility only slightly, as before. However, coexpression of CpMprF1 with the flippases of the *C. perfringens* and *S. aureus* MprF proteins on separate plasmids led to the same level of reduced daptomycin susceptibility as strains coexpressing the Lys-PG synthase domain from *S. aureus* with the flippase domain from *S. aureus* or *C. perfringens*, attaining MIC levels similar to those for the *S. aureus* wild type (Fig. 4). Thus, the Lys-PG flippases appear to have relaxed substrate specificities and to be also able to flip Ala-PG in addition to Lys-PG.

DISCUSSION

The ability of MprF homologs to synthesize different types of aminoacyl phospholipids has raised the question of how specific the aminoacyl phospholipid synthases and flippase domains may be and which structural features may govern specificity. Broad-range substrate specificity of aminoacyl phospholipid synthase domains of MprF has previously been described for *E. faecium* MprF, which accepts various aminoacyl-tRNA species such as lysyl-tRNA, alanyl-tRNA, and arginyl-tRNA. Moreover, the recognition of lysyl- and alanyl-tRNA by the MprF from *B. subtilis* has been reported (25). Importantly, a recent study has come to the conclusion that the preference of MprF proteins for either Ala-tRNA or Lys-tRNA cannot be deduced from the protein sequence (2). The observation that only Lys-PG synthases but not Ala-PG synthases appear to exhibit relaxed substrate specificity has led to the hypothesis that the affinity of the enzymes for larger substrate residues such as lysine groups is a prerequisite for the recognition of smaller substrates such as alanine groups (25). In contrast to *B. subtilis* and *E. faecium*, *C. perfringens* produces Ala-PG and Lys-PG with two separate MprF proteins, which might be advantageous for the differential regulation of membrane properties under changing environmental conditions (26). We found that both *C. perfringens* MprF homologs are functional in *S. aureus*, as they led to the production of both lipids. The reason for the observed higher levels of production of Ala-PG than of Lys-PG remains unclear. They might be due to different allosteric regulation of the respective synthases. Why some Gram-positive bacteria produce both Ala-PG and Lys-PG remains unclear. It may be a strategy to increase the overall aminoacylation levels of negatively charged phospholipids or to fine-tune membrane fluidity and permeability. Of note, the production of more than one aminoacyl-lipid species in one strain has so far been reported only in Gram-positive bacteria (*E. faecium*, *B. subtilis*, and *C. perfringens*) (25, 26).

While the specificities of aminoacyl synthase domains have been elucidated to some extent, those of the flippase domains in MprF proteins have remained unknown. Our study provides evidence that the flippase domains of Lys-PG synthesizing *S. aureus* and *C. perfringens* MprF homologs do not discriminate between Lys-PG and Ala-PG and translocate both lipids. We also report that the Ala-PG synthase of *C. perfringens* lacks a flippase domain, which appears not to be required because of the presence of a Lys-PG flippase with relaxed aminoacyl-PG specificity in CpMprF2. It is tempting to speculate that the affinity of Lys-PG flippases for larger substrates such as Lys-PG is a prerequisite for the affinity of smaller substrates such as Ala-PG, as observed for the recognition of various aminoacyl-tRNA species by Lys-PG synthases. However, studies with a dedicated Ala-PG producing and translocating MprF protein in a Gram-positive background are required to verify this hypothesis. Of note, expression of the dedicated Ala-PG producing MprF of *P. aeruginosa* in *S. aureus* did not appear to lead to a functional protein, as no aminoacyl phospholipid production could be observed and no impact of coexpression with Lys-PG or Ala-PG synthase on daptomycin susceptibility could be observed (data not shown).

The finding that Lys-PG and Ala-PG are equally effective in reducing daptomycin susceptibility in *S. aureus* was unexpected. Zwitterionically charged Ala-PG should be less effective in electrostatic repulsion of CAMP-like compounds such as daptomycin than cationic Lys-PG. Nevertheless, both types of lipids should lead to a reduced affinity for CAMPs, which may be sufficient to prevent CAMP-mediated membrane damage. Since both types of lipid aminoacylation are capable of reducing daptomycin susceptibility in *S. aureus*, one might speculate that the reduction of PG

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content *per se* leads to reduced daptomycin susceptibility, which is in line with the previous observation that reduced PG synthesis results in reduced daptomycin susceptibility in *B. subtilis* (12, 13), indicating that increased PG aminoacylation resulting in decreased PG content might be contributing to reduced daptomycin susceptibility in a similar manner in *S. aureus*. The finding that a cationic net charge is not strictly required for aminoacyl phospholipids to protect *S. aureus* against daptomycin may not only be important for understanding the molecular basis of MprF-mediated elevated daptomycin resistance in clinical *S. aureus* isolates with point mutations in *mprF* but may also be applicable to Ala-PG producing vancomycin-resistant enterococci, which are increasingly treated with daptomycin.

In conclusion, our study indicates that Ala-PG and Lys-PG have overlapping functions and can be translocated by the same MprF flippase domains, thereby presenting a basis for more detailed studies on the structure and function of MprF proteins.

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