The Lipid-Modifying Multiple Peptide Resistance Factor Is an Oligomer Consisting of Distinct Interacting Synthase and Flippase Subunits

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ABSTRACT **Phospholipids are synthesized at the inner leaflet of the bacterial cytoplasmic membrane but have to be translocated to the outer leaflet to maintain membrane lipid bilayer composition and structure. Even though phospholipid flippases have been proposed to exist in bacteria, only one such protein, MprF, has been described. MprF is a large integral membrane protein found in several prokaryotic phyla, whose C terminus modifies phosphatidylglycerol (PG), the most common bacterial phospholipid, with lysine or alanine to modulate the membrane surface charge and, as a consequence, confer resistance to cationic antimicrobial agents such as daptomycin. In addition, MprF is a flippase for the resulting lipids, Lys-PG or Ala-PG. Here we demonstrate that the flippase activity resides in the N-terminal 6 to 8 transmembrane segments of the *Staphylococcus aureus* MprF and that several conserved, charged amino acids and a proline residue are crucial for flippase function. MprF protects *S. aureus* against the membrane-active antibiotic daptomycin only when both domains are present, but the two parts do not need to be covalently linked and can function in *trans*. The Lys-PG synthase and flippase domains were each found to homo-oligomerize and also to interact with each other, which illustrates how the two functional domains may act together. Moreover, full-length MprF proteins formed oligomers, indicating that MprF functions as a dimer or larger oligomer. Together our data reveal how bacterial phospholipid flippases may function in the context of lipid biosynthetic processes.**

IMPORTANCE **Bacterial cytoplasmic membranes are crucial for maintaining and protecting cellular integrity. For instance, they have to cope with membrane-damaging agents such as cationic antimicrobial peptides (CAMPs) produced by competing bacteria (bacteriocins), secreted by eukaryotic host cells (defensins), or used as antimicrobial therapy (daptomycin). The MprF protein is found in many Gram-positive, Gram-negative, and even archaeal commensals or pathogens and confers resistance to CAMPs by modifying anionic phospholipids with amino acids, thereby compromising the membrane interaction of CAMPs. Here we describe how MprF does not only modify phospholipids but uses an additional, distinct domain for translocating the resulting lysinylated phospholipids to the outer leaflet of the membrane. We reveal critical details for the structure and function of MprF, the first dedicated prokaryotic phospholipid flippase, which may pave the way for targeting MprF with new antimicrobials that would not kill bacteria but sensitize them to antibiotics and innate host defense molecules.**

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Bacteria face frequently changing environmental conditions, which may threaten membrane integrity and thereby numerous essential processes involved in cell division and cell maintenance. While biosynthesis of bacterial phospholipids has been well studied, the process of phospholipid translocation to the outer leaflet of the membrane remains enigmatic, as proteins implicated in phospholipid translocation have rarely been described, and potential candidates are problematic to study or to identify because of technical difficulties in monitoring phospholipid translocation activity (1).

A major threat to bacterial membrane integrity is the class of membrane-active cationic antimicrobial peptides (CAMPs) produced by competing microorganisms or eukaryotic hosts (2). Cationic antimicrobial peptides are amphipathic molecules, which are electrostatically attracted to negatively charged phospholipids due to their positive net charge, enabling them to bind to a specific membrane-bound target, which is often the cell wall precursor lipid II (3). Subsequent insertion into the membrane can lead to perturbation of essential membrane-associated processes (4) or complete disruption of the membrane at higher concentrations

(5). Importantly, antibiotics of last resort against highly antibiotic-resistant bacteria, such as the cationic antibiotic vancomycin and the CAMP-like antibiotic daptomycin, also bind in a charge-dependent manner to the membrane of clinically relevant *Staphylococcus aureus* strains (6–9). A major strategy of bacteria to protect the membrane from the ubiquitous presence of antimicrobial peptides and antibiotics is to reduce the overall net charge of the membrane by, e.g., the modification of negatively charged phosphatidylglycerol (PG) (and in some cases also cardiolipin) with lysine or alanine residues (10, 11) or the modification of lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria with aminoarabinose (12). The modification of PG with amino acids is mediated by the bacterial MprF protein, which transfers lysine or alanine residues from aminoacyl-tRNA to PG, thereby reducing the negative net charge of the membrane and leading to resistance to CAMPs and antibiotics in various opportunistic pathogens (10). MprF is a large integral membrane protein with a hydrophilic C-terminal part. This cytosolic part plus 6 of 14 predicted transmembrane segments (TMSs) of MprF have recently been shown to be required for transferring lysine to phosphatidylglycerol in *S. aureus* (6). The core region of aminoacyl-PG synthase activity resides in the cytosolic part as demonstrated by *in vitro* activity of purified cytosolic domains from various prokaryotes (13–15), while the hydrophobic part is thought to be required for anchoring the enzyme to the membrane (14). We have recently discovered that the hydrophobic part of MprF harbors a Lys-PG flippase, which translocates Lys-PG into the outer leaflet of the membrane, thereby leading to repulsion of CAMPs and daptomycin (6). Moreover, we have shown that Lys-PG-producing MprF proteins from *S. aureus* and *Clostridium perfringens* are capable of also flipping zwitterionic Ala-PG and that Ala-PG has a similar capacity to protect the membrane of *S. aureus* against daptomycin as Lys-PG (16). The correlation of Lys-PG and Ala-PG flipping with daptomycin susceptibility in *S. aureus* substantially facilitates the ongoing elucidation of fundamental characteristics of MprF as a model lipid flippase

In this study, we characterize the MprF flippase in detail and show that it is a distinct functional subunit located at the N terminus of MprF. Together with membrane protein topology elucidation, reexamination of the extension of the synthase domain, and identification of conserved amino acid positions with crucial roles in flippase activity, our study reveals a new model of the domain architecture and function of MprF proteins. Moreover, we report multiple domain interactions that not only lead to binding of the flippase to the synthase domain but also to oligomerization of MprF, thereby providing a new view on the complex structure and mode of action of MprF proteins.

RESULTS

Topology of the transmembrane domain of MprF. The hydrophobic part of MprF is involved in both Lys-PG synthesis and Lys-PG flipping, but ca. two-thirds of it could be deleted from the N terminus without compromising synthase activity (6). So far, the exact extensions of flippase and synthase and possible regions with overlapping functions for both activities have remained unknown. We recently employed the SOSUI membrane protein topology prediction algorithm to assess the topology of MprF (6). However, the transmembrane topology prediction of MprF by SOSUI differs substantially from the prediction by the TOPCONS algorithm (17) (Fig. 1A), which combines and evaluates the results of five different algorithms, including OCTOPUS, which is based on a set of sequences of experimentally validated topologies (18).

In order to elucidate the MprF topology, we fused truncated MprF variants at 19 predicted loop regions to the marker enzymes β-galactosidase (LacZ) or alkaline phosphatase (PhoA) (Fig. 1B). PhoA is active only when itis translocated across the cytoplasmic membrane into the periplasm of *Escherichia coli* where the required intrachain disulfide bond can be formed (19). In contrast, LacZ is active only when the LacZ moiety of the hybrid protein remains in the cytoplasm (19). Profound LacZ activity but PhoA inactivity of fusions at positions 90, 125, 196, 247, 269, 273, 276, 317, 393, and 445 suggested that the loops between ca. positions 73 to 129, 182 to 201, 243 to 335, 391 to 394, and 439 to 451 are located intracellularly. In contrast, PhoA activity of fusions at positions 48, 52, 158, 161, 225, 363, 418, and 488 indicated that loops between ca. amino acids 32 to 73, 149 to 162, 221 to 223, 414 to 439, and 471 to 510 are located extracellularly (Fig. 1A and B; see Table S4 in the supplemental material). As observed in many other membrane protein topology studies using LacZ/PhoA fusions, PhoA activity was often accompanied with LacZ activity, which may be due to proteolytic release of LacZ before the protein is correctly inserted into the cytoplasmic membrane (20, 21). Many of our findings were in stark contrast to the SOSUI prediction while corresponding largely to the TOPCONS prediction, indicating that TOPCONS is much more appropriate for predicting MprF structure than SOSUI. In fact, PhoA activity was found at every second predicted loop over the entire MprF length, which is expected for an integral membrane protein and provides a solid basis for the new topology model. The only major deviation from the TOPCONS prediction was that the presence of an extracellular loop between predicted TMSs at positions 245 to 265 and 278 to 298 could not be confirmed. Instead, we observed pronounced LacZ activity but hardly any PhoA activity with fusions at three positions within the predicted loop. Therefore, we propose that a large intracellular loop encompassing ca. positions 243 to 335 is formed. The two putative hydrophobic α-helices may be associated with the membrane but appear not to fully integrate into the membrane in our experiment (Fig. 1). Moreover, our topology analysis revealed major differences at the junction between the N-terminal domain and the hydrophobic part required for synthase activity compared with the old SOSUI prediction. According to our new data, the smallest MprF variant recently found to have Lys-PG activity (6) does not consist of only six TMSs as previously thought but also includes a major part of the large central intracellular loop (Fig. 1A).

The Lys-PG synthase is ca. 50 amino acids shorter than previously thought. According to our topology analysis of MprF, the six C-terminal TMSs of MprF are in fact ca. 50 amino acids shorter than in the previous prediction by SOSUI (Fig. 1A). Since the extension of the synthase domain was recently investigated by shortening the N terminus of MprF two TMSs at a time, a shorter N-terminally truncated MprF variant, which lacks major parts of the large intracellular loop (corresponding to five SOSUI-predicted TMSs and the cyotosolic part) was not tested for Lys-PG synthase activity. However, other shorter constructs consisting of TMSs 8 to 12 (amino acids 363 to 840), TMSs 11 and 12 and the cytosolic part, or the cytosolic part alone were tested and found to be unable to synthesize Lys-PG. This prompted us to investigate whether the Lys-PG synthase domain is in fact shorter than previously determined. We expressed a truncated synthase variant

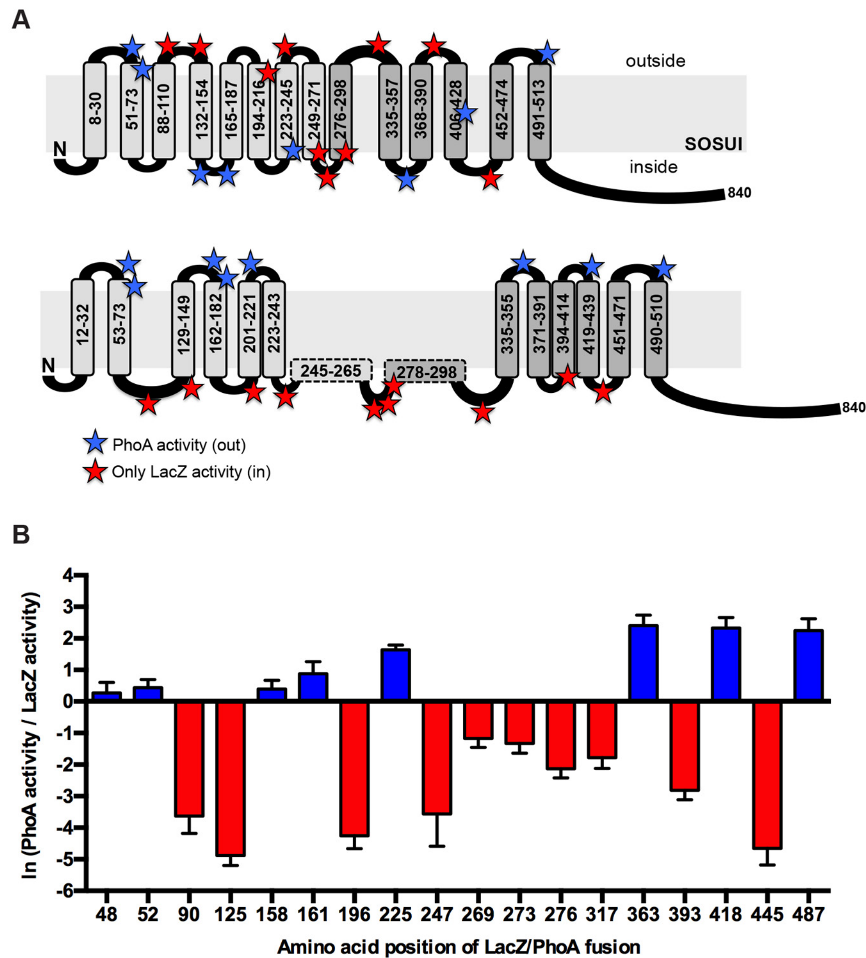

FIG 1 MprF topology analysis. (A) Predicted MprF topology by SOSUI (top panel) and model for the actual topology according to results from this study, which largely follow the TOPCONS prediction (bottom panel). LacZ/PhoA activity determined with fusion constructs (panel B) is indicated by red (LacZ) and blue (PhoA) asterisks. TMSs associated with the recently determined synthase domain are shown in dark grey. Two putative α-helices predicted by TOPCONS that were not confirmed in the topology analysis are shown with dashed lines. (B) Relative activities of LacZ and PhoA in cells with the various MprF fusion constructs. Alkaline phosphatase activity was divided by β-galactosidase activity and expressed as the natural logarithm as described recently (35). Positive values represent cells with higher PhoA activity than LacZ activity (blue bars), and negative values represent cells with higher LacZ activity than PhoA activity (red bars). The means plus standard errors of the means (SEM) (error bars) of three independent experiments are shown.

lacking the large intracellular loop completely, encompassing TMSs 7 to 12 and the C-terminal cytosolic part (amino acids 328 to 840) (Fig. 2) in the *S. aureus* mprF deletion mutant and found that it led to Lys-PG production levels similar to those of full-length MprF (Fig. 3A). Thus, the Lys-PG synthase is ca. 50 amino acids shorter than previously thought. Therefore, it is tempting to speculate that the adjacent N-terminal domain encompassing TMSs 1 to 6 and the large intracellular loop is specifically involved in Lys-PG flippase activity.

The N-terminal domain encompassing TMSs 1 to 6 and the large intracellular loop can be separated from the synthase domain and still promote flippase activity in *trans*. The N-terminal domain encompassing TMSs 1 to 6 and the large intracellular loop may be specifically involved in flippase activity in MprF, but it may require the hydrophobic part of the synthase domain to function effectively. Previous studies have demonstrated that the entire hydrophobic part can translocate Lys-PG when expressed in *trans* but have not attempted to define whether the 6 TMSs of the minimal synthase are required for flippase activity. In these previous studies, flippase activity of MprF has been found to correlate with reduced daptomycin susceptibility (6, 16). In order to determine whether the integrity of the entire hydrophobic part of MprF is required for flippase activity, we analyzed daptomycin susceptibility of *S. aureus* mprF deletion mutant strains expressing N-terminally or C-terminally truncated MprF variants coexpressed with the minimal synthase on two separate plasmids (Fig. 2). *S. aureus* mprF mutants expressing N-terminally truncated variants of MprF were hardly impaired in Lys-PG production (Fig. 3A) but were hypersusceptible to daptomycin (Fig. 3B), confirming the crucial role of the N terminus for the flippase reaction. Separate expression of the N-terminal domain (amino acids 1 to 320; TMSs 1 to 6 plus large intracellular loop) with the synthase domain (amino acids 328 to 840) led to 2-fold-higher MIC of daptomycin, while extension of the N-terminal domain with two adjacent TMSs from the synthase domain (amino acids 1 to 393) led to 5-fold-higher, wild-type level MIC of daptomycin (Fig. 4). Further C-terminal truncation of the N-terminal domain (amino acids 1 to 274; TMSs 1 to 6 plus truncated large intracel-

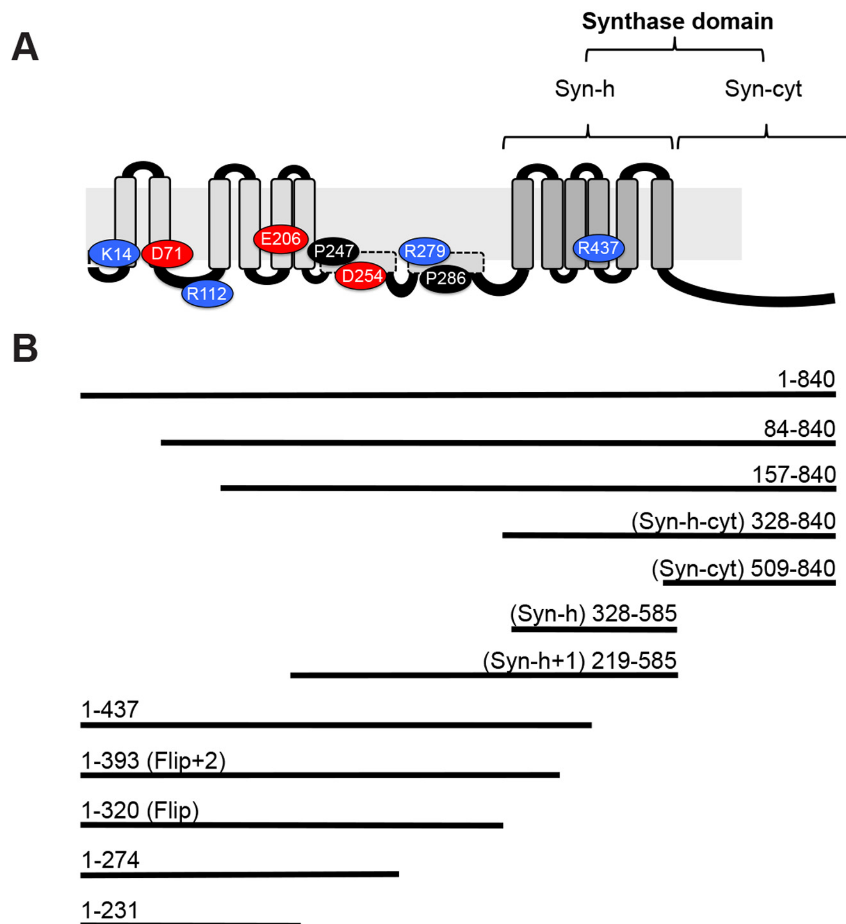

FIG 2 Conserved charged amino acid and proline residues in the transmembrane region of MprF and truncated MprF variants employed in this study. (A) Topology of MprF with conserved cationic amino acids (blue), anionic amino acids (red), and proline residues (black). (B) Extension of truncated MprF variants employed in this study. Amino acid positions are indicated.

lular loop) abolished its capacity to increase the MIC of daptomycin. Thus, the integrity of the entire hydrophobic part of MprF is not required for Lys-PG flippase activity. For full flippase activity, the N-terminal domain, together with two adjacent TMSs of the hydrophobic part of the synthase domain, are required (TMSs 1 to 8). However, robust flippase activity can be achieved by separate expression of the N-terminal domain encompassing TMSs 1 to 6 and the large intracellular loop alone, suggesting that this part represents a functional domain in MprF proteins and the core region of Lys-PG flippase activity. The N-terminal domain (TMSs 1 to 6 plus large intracellular loop) is designated the flippase (Flip) domain and the two parts of the synthase domain are designated as Syn-h (hydrophobic part) and Syn-cyt (cytosolic, hydrophilic part) from here on (Fig. 2).

Conserved charged amino acids and a conserved proline residue are crucial for flippase activity. Conserved charged amino acids in the hydrophobic part of MprF might be involved in the flippase reaction and/or could be important structural determinants. Sequence alignment of the hydrophobic parts of 15 characterized MprF proteins, which are known to synthesize Lys-PG, Ala-PG, or both, led to the identification of seven conserved charged amino acids (K14, D71, R112, E206, D254, R279, and R437) and two conserved proline residues (P247 and P286), which except for R437 are located in the flippase domain (Fig. 2; see Fig. S1 in the supplemental material). No characteristic differences could be found between sequences of Ala-PG- and Lys-PG-producing MprF proteins. P247 and D254 are part of a conserved G-P-G-F-D(E) motif, and R279 and P286, located in the large intracellular loop, belong to an R-Y-P motif. R112 is part of an R(K)-Y motif in the intracellular loop between TMSs 2 and 3. Interestingly, E206 is predicted to be located in the midst of a TMS (TMS 5), suggesting that it might accommodate the bulky, positively charged Lys-PG head group during translocation across the membrane. D254 is located in the large intracellular loop. K14 and D71 are predicted to be located at the cytoplasmic side of the respective TMS and could be important topological determinants. We exchanged all conserved charged and proline residues found in the hydrophobic part of MprF with alanine residues, expressed the constructs in the *S. aureus mprF* deletion mutant, and determined daptomycin susceptibility as a surrogate readout for flippase activity, as well as Lys-PG synthase activity as an indicator of effective membrane insertion. Strains with all tested constructs produced amounts of Lys-PG similar to that of the strain complemented with wild-type *mprF* (Fig. 5A), suggesting that Lys-PG synthase activity and membrane insertion were not impaired by the point mutations, but strains expressing MprF with exchanged

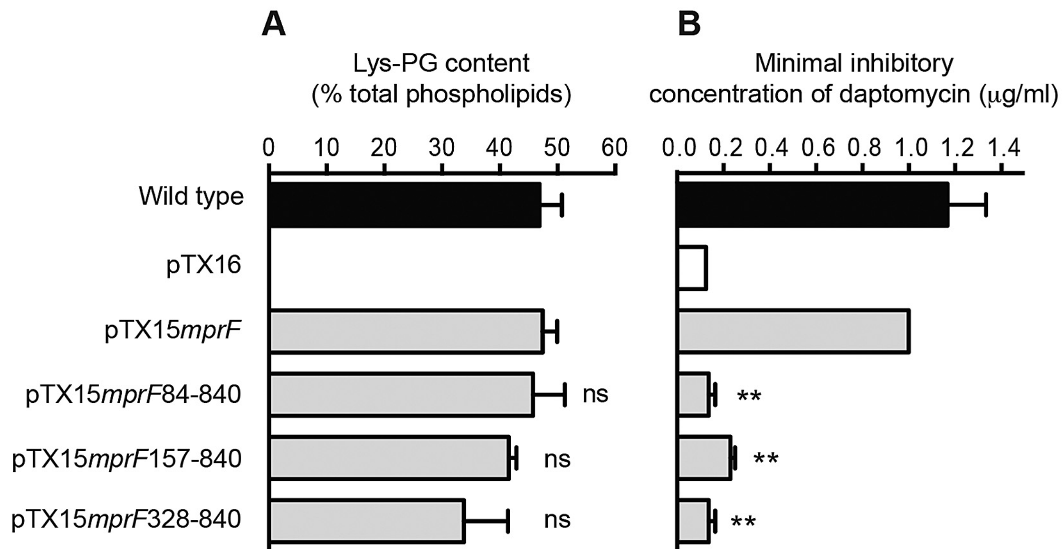

FIG 3 Impact of N-terminal truncations of MprF expressed in *S. aureus* Δ*mprF* mutant on Lys-PG production and flippase activity. (A) Percentage of Lys-PG production in relation to total phospholipid content. (B) Impact of MprF variants on the MIC of daptomycin as an indicator of flippase activity. Values that are significantly different ($P < 0.01$) from the value for the *S. aureus* Δ*mprF* mutant expressing wild-type MprF (pTX*mprF*) are indicated (**), and values that are not significantly different (ns) from the value for the *S. aureus* Δ*mprF* mutant expressing wild-type MprF (pTX*mprF*) are shown. The means plus SEM of three independent experiments are shown.

charged amino acids or P247 displayed pronounced increased susceptibility to daptomycin (Fig. 5B). Exchange of P286 located in one of the hydrophobic segments in the large intracellular amphipathic loop led to only moderately, nonsignificantly altered daptomycin susceptibility (Fig. 5A and B), indicating that this residue is dispensable for Lys-PG flippase activity. The facts that conserved charged amino acids in the hydrophobic part of MprF are predominantly located in the flippase domain and are crucial for flippase activity further support the notion that the flippase domain is indeed a distinct functional domain in MprF.

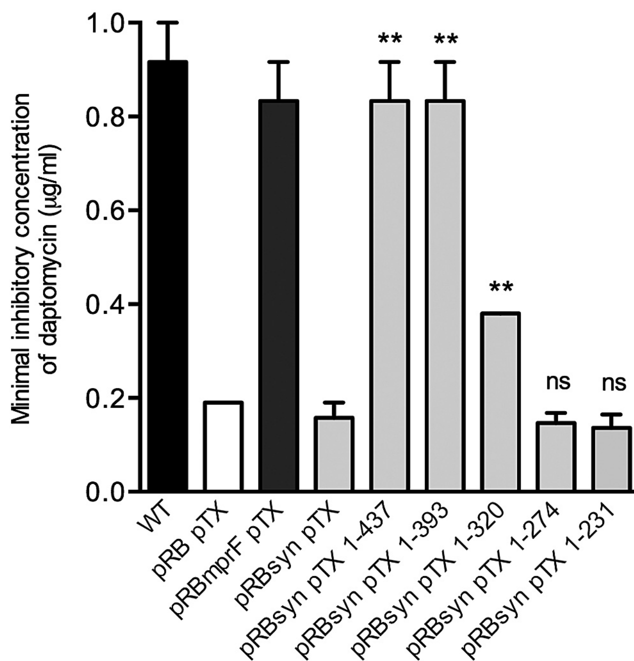

FIG 4 Impact of C-terminal MprF truncations coexpressed in *trans* with the synthase domain (pRB*syn*) in the wild-type (WT) *S. aureus* and *S. aureus* ΔmprF mutant on daptomycin susceptibility as an indicator of flippase activity. Values that are significantly different ($P < 0.01$) (**) and values that are not significantly different (ns) from the value for the *S. aureus* ΔmprF mutant expressing wild-type MprF (pRB*mprF* pTX) are shown. The means plus SEM of three independent experiments are shown.

The MprF flippase and synthase domains expressed in *trans* bind to each other. The two MprF functional domains for PG lysinylation and Lys-PG translocation might interact physically with each other, even when not covalently linked, e.g., in order to effectively transfer Lys-PG from the synthase to the flippase. In order to investigate potential interactions between the synthase and flippase domains, we cloned truncated and extended domain variants (Fig. 2) in expression plasmids from an *E. coli* two-hybrid system resulting in C- and N-terminal fusions of the domain variants with the complementary adenylate cyclase fragment T18 or T25 (see Fig. S2 in the supplemental material). Interaction of T18 with T25 results in the production of cAMP, leading to expression of β-galactosidase, which can be detected by plating bacteria on selective media, such as MacConkey agar, and its activity can be quantified by incubating permeabilized cells with *o*-nitrophenol-galactoside (ONPG) as the substrate for β-galactosidase (22, 23). Multiple combinations of domain variants and plasmid combinations were tested for interaction in the bacterial two-hybrid assay (Fig. S2) by quantifying β-galactosidase activity (22). We found that the flippase domain (Flip) interacted with the cytosolic and hydrophobic parts of the synthase domain (Syn-cyt and Syn-h), as well as with itself. The cytosolic part of the synthase domain (Syn-cyt) also interacted with itself. In contrast, the hydrophobic part of the synthase domain (Syn-h) did not interact with itself and therefore appears to be only involved in interdomain interaction (Fig. 6A and B). Of note, an extended hydrophobic part of the synthase domain (Syn-h+1) and the extended flippase domain (Flip+2) seemed to be impaired in domain interaction, which could be due

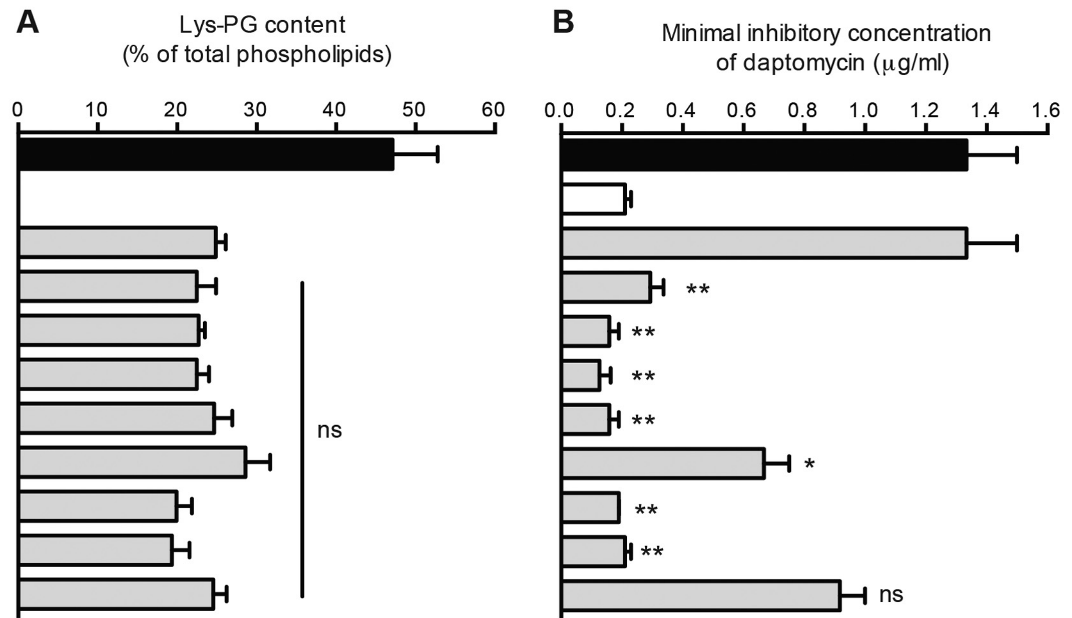

FIG 5 Impact of conserved charged amino acid and proline residues on Lys-PG production and daptomycin susceptibility. (A) Percentage of Lys-PG production in relation to total phospholipid content. (B) Impact of daptomycin on MIC as an indication of flippase activity. Values that are significantly different from the value for the *S. aureus* Δ*mprF* mutant expressing wild-type MprF (pRB*mprF*) are indicated as follows: *, $P < 0.05$; **, $P < 0.01$. Values that are not significantly different (ns) from the value for *S. aureus* Δ*mprF* mutant expressing wild-type MprF (pRB*mprF*) are also shown. The means plus SEM of three independent experiments are shown.

to steric hindrance. Thus, the flippase can interact with the synthase even when the two domains are not covalently linked.

MprF forms homo-oligomers. The multiple domain interactions within MprF observed in the bacterial two-hybrid interaction assay raised the possibility that MprF may oligomerize. In fact, we observed interaction of full-length MprF with itself in the *E. coli* two-hybrid assay (Fig. 6A), indicating that MprF forms dimers or larger oligomers. Oligomerization and complex formation of membrane proteins can be analyzed in Western blots of sodium dodecyl sulfate (SDS)-free blue native polyacrylamide gel electrophoresis (BN PAGE) (24, 25). In order to detect MprF oligomerization with anti-GFP antibodies in *S. aureus* membrane extracts, we transformed a plasmid encoding superfolder green fluorescent protein (sfGFP) fused C terminally to MprF (26) into an *S. aureus* mprF mutant bearing a second deletion in protein A (*spa*) to avoid unspecific antibody binding. After separating membrane proteins isolated from the membrane fraction of MprF-GFP-expressing *S. aureus* in a BN PAGE, we were able to detect three distinct bands at ca. 140, 300, and 600 kDa, respectively (Fig. 7). For a negative control, we analyzed the membrane fraction from *S. aureus* mprF deletion mutants harboring the empty vector and found that the band at 140 kDa was not related to GFP-MprF, while the bands at 300 and 600 kDa were specific for the GFP-MprF fusion protein (Fig. 7). The first band at 300 kDa migrated substantially higher than expected from the theoretical molecular mass of 125 kDa for monomeric MprF-GFP, however, due to bound lipids, detergent, and Coomassie blue, it is not unusual for membrane proteins to run at higher apparent molecular masses in BN PAGE (25). Importantly, the additional band exhibiting 2-fold-higher molecular mass (600 kDa) suggests that MprF proteins could oligomerize, forming homodimers or homotetramers. Moreover, this finding confirms the observed interaction

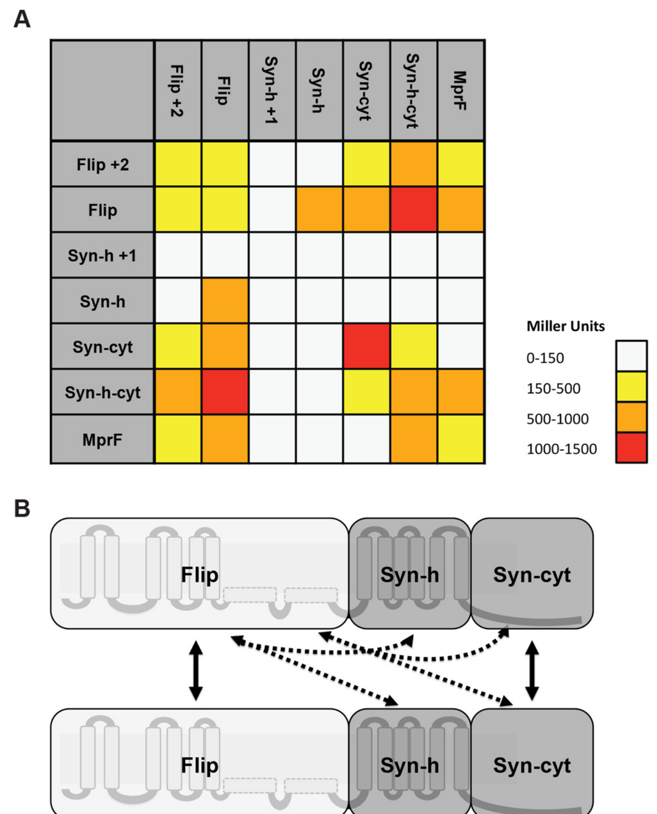

FIG 6 MprF domain interactions. (A) Maximally observed β-galactosidase activity in *E. coli* cells expressing different combinations of N- and C-terminal adenylate cyclase fragment fusions and expression plasmids. β-Galactosidase activity (in Miller units) is indicated as a heat map. (B) Overview of observed MprF domain interactions. Dashed arrows indicate multiple potential interactions of the flippase with the synthase domain in *trans* or in *cis*.

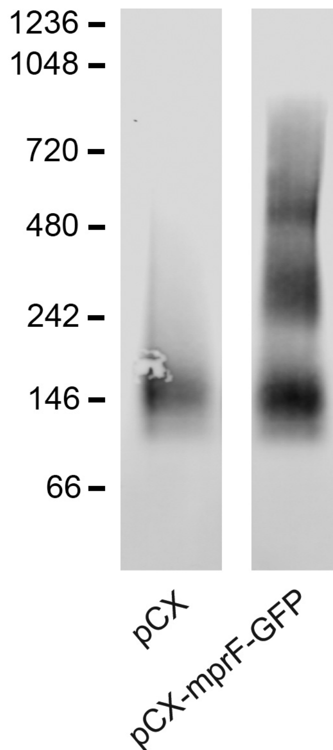

FIG 7 Detection of MprF-GFP in the membrane fraction of *S. aureus* Δ*mprF* Δ*spa* double mutant by BN PAGE. The membrane fraction from *S. aureus* ΔmprF mutant expressing the empty vector (pCX) was employed as a negative control. The molecular masses (in kilodaltons) of marker proteins are given to the right of the gel.

of MprF with itself in the *E. coli* two-hybrid assay, where MprF homodi- or -oligomerization appears to be mediated between Flip and Syn-cyt domains (Fig. 6B).

DISCUSSION

The Lys-PG flippase and synthase in the MprF of *S. aureus* are distinct, functional subunits, implying that MprF may have evolved by fusion of two different proteins. The flippase domains of MprF proteins have limited sequence identity, but certain amino acids are conserved and critical for Lys-PG translocation. One conserved negatively charged amino acid, E206, is particularly interesting because it is located in the middle of a TMS, which may facilitate the passage of charged aminoacyl-phospholipid head groups across the hydrophobic membrane. It is tempting to speculate that the two charged conserved residues D254 and R279, located in the hydrophobic region of the large intracellular loop could be part of a binding site for Lys-PG translocation. The fact that conserved charged amino acids are found in flippase domains of MprF proteins, which synthesize Lys-PG, Ala-PG, or both may indicate a general role of these residues in aminoacyl-phospholipid translocation. A recent study, which identified MprF proteins based on the highly conserved cytosolic part of the synthase domain found that most MprF proteins encompass either 12 to 14 TMSs or 4 to 8 TMSs plus the Syn-cyt domain (14), indicating that shorter MprF proteins (4 to 8 TMSs) lack the flippase domain.

The observed multiple domain interactions between the synthase and flippase indicate that both subunits are operating in a cooperative fashion, which is further supported by our observation that two TMSs from the synthase domain are required for optimal Lys-PG flippase activity. Interestingly, the observed complex intramolecular interactions in MprF appear to also result in oligomerization of MprF which may be a requirement for phospholipid translocation. Of note, homo-oligomerization has also been observed in the well-characterized ATP-dependent bacterial lipid A flippase MsbA (27). However, MprF and MsbA do not share any similarity, and it remains to be determined whether MprF is acting as a facilitator or is energy dependent. Amazingly, even though bacteria harbor many other major phospholipids aside from aminoacyl-phospholipids in their membranes, MprF is so far the only dedicated phospholipid flippase identified in bacteria. As MprF proteins are nonessential, it remains to be investigated whether bacteria translocate other major phospholipid classes unspecifically, e.g., by spontaneous passage with low efficiency, or if other dedicated phospholipid flippases exist in prokaryotes. Finally, since the MprF flippase is accessible from the outside and protects the *S. aureus* membrane against antimicrobial peptides and membrane-active antibiotics such as daptomycin and vancomycin, it represents an excellent target candidate for the development of anti-infective compounds, which may not only act synergistically with host-derived antimicrobial peptides but also with antibiotics of last resort.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5α, *E. coli* XL1-Blue, and *S. aureus* SA113 (ATCC 35556) are frequently used laboratory strains. The Lys-PG-deficient *S. aureus* SA113 *mprF* mutant has been described recently (11). *E. coli* or *S. aureus* strains were grown in LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or Mueller-Hinton broth (MHB) (0.2% beef extract, 1.75% acid hydrolysate of casein, 0.15% starch) supplemented with appropriate antibiotics unless otherwise noted. Susceptibility to daptomycin was determined by MIC test strips according to the manufacturer's advice (Liofilchem).

MprF topology. Two series of fusions were constructed in which the *phoA or lacZ* gene was fused to truncated *mprF* variants and expressed in plasmid pRB474 in *E. coli* DH5α. *phoA* and *lacZ* were amplified from *E. coli* SURE (Stratagene) chromosomal DNA, digested with XhoI and MfeI, and ligated together with *mprF* variants amplified from pRB474mprF (6) and digested with SphI and XhoI (see Table S1 and Table S3 in the supplemental material). The resulting *phoA* and *lacZ* constructs were subsequently cloned in pRB474 using the restriction sites SphI and EcoRI (Table S1), enabling constitutive expression, and transformed into *E. coli* DH5α which does not harbor functional *phoA* and *lacZ* genes. Activities of PhoA and LacZ were assayed in permeabilized cells by the methods of Manoil (28) and Miller (29) as employed by others (19, 30), with *p*-nitrophenyl phosphate (pNPP) and *o*-nitrophenyl galactoside (ONPG), respectively. Strains were grown to exponential phase (optical density at 578 nm [OD_{578}] of 0.5), harvested (2 ml), and resuspended in 1.5 ml of buffer A (1 M Tris-HCl [pH 8]) for the PhoA assay or in buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ [pH 7]) for the LacZ assay. Seventy-five microliters of sodium dodecyl sulfate (SDS) (0.1%) and 75 μl of chloroform were added, followed by 10 s of vortexing and 10-min incubation at 28°C. Two hundred microliters of the suspension was added to two sets of triplicate wells in a 96-well plate, and 40-μl quencher reagent (1 M K_2HPO_4 for PhoA; 2 M Na_2CO_3 for LacZ) was added to one set of triplicate wells as a negative control. Forty microliters of substrate (15 mM pNPP in buffer A for PhoA; 13.3 mM ONPG for LacZ) was added to each well. The reaction mixture was incubated at room temperature and quantified by measuring the absorption increment at 405 nm. Enzyme units were calculated as the turnover of micromole of substrate per minute in the linear range of the reaction.

Activities were normalized to the highest measured value of LacZ or PhoA (see Table S4 in the supplemental material) to account for different specific activities of the two enzymes.

Prediction of MprF structure and similarity. The transmembrane topology of MprF was predicted with the SOSUI program (engine version 1.11; http://bp.nuap.nagoya-u.ac.jp/sosui/) and TOPCONS program (http://topcons.cbr.su.se/). MprF was compared with homologous protein sequences using the DNASIS MAX 2.05 software (Hitachi Software Engineering) to define conserved amino acid residues in the hydrophobic part of MprF.

Expression and mutagenesis of *mprF* in *E. coli*. Selected amino acids of MprF were replaced with alanine by site-directed mutagenesis using the QuikChange kit (Stratagene, La Jolla, CA). Primers used for mutagenesis are listed in Table S2 in the supplemental material. Plasmid pRB474*mprF* was used as the template for the mutagenesis and amplification procedure. The resulting plasmids and proteins are given in Table S1.

Cloning of *mprF* in the *Staphylococcus*-specific plasmid pRB474 or pTX15. *mprF* and truncated genes were cloned in the *E. coli*/*Staphylococcus*-specific shuttle plasmid pRB474 (31) in such a way that the 5′ end was downstream of the plasmid-encoded constitutive *veg* promoter as described in Table S1 in the supplemental material. In parallel, truncated *mprF* genes were cloned in the *Staphylococcus*-specific expression vector pTX15 (32) in the right orientation to permit expression from the xylose-inducible plasmid-encoded *xyl* promoter (strains were induced with 0.5% xylose). Empty plasmids pTX16 (32) and pRB474 were used in certain control strains.

Isolation and quantification of polar lipids. Polar lipids were extracted from exponential-phase bacteria with chloroform-methanol (2:1 [by volume]) by the Bligh-Dyer procedure (33), vacuum dried, and dissolved in chloroform-methanol (2:1 [by volume]). For detection of Lys-PG, appropriate amounts of polar lipid extracts were spotted onto silica gel 60 F254 high-performance thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, Germany) using a Linomat 5 sample application unit (Camag, Berlin, Germany) and developed with chloroform-methanol-water (65:25:4 [by volume]) in an automatic developing chamber ADC 2 (Camag, Berlin, Germany). Lys-PG content was quantified as described recently (6). Phospholipids of exponentially growing strains were selectively stained with molybdenum blue spray reagent (1.3% molybdenum oxide dissolved in 4.2 M sulfuric acid [Sigma]). Integrated lipid spot intensities of molybdenum blue-stained phospholipids were determined with ImageJ (http://rsbweb.nih.gov/ij/)

Bacterial two-hybrid assay. To investigate domain interactions in MprF, the commercially available bacterial two-hybrid kit from Euromedex was employed (BACTH system kit). *mprF* variants were fused C terminally (pKT25C and pUT18C) and N terminally (pUT18N) to the adenylate cyclase fragments T25 and T18 of *Bordetella pertussis*, respectively. pKT25 is a low-copy-number vector, while pUT18C and pUT18 are high-copy-number vectors. Interaction of T25 with T18 leads to cyclic AMP (cAMP) production and expression of the *lac* and *mal* operons in *E. coli*. The resulting constructs (see Table S2 in the supplemental material) were used to cotransform chemically competent *E. coli* BTH101, and resulting transformants were tested in triplicate for β-galactosidase activity to quantify protein-protein interactions (as described in reference 22). Briefly, cells were grown overnight in LB broth containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and appropriate antibiotics at 37°C. One hundred microliters of each overnight culture was transferred into a microtiter plate (BD Biosciences, Heidelberg, Germany), and optical densities were determined at 600 nm with an μQuant microplate spectrophotometer (BioTek, Bad Friedrichshall, Germany). Another 100 μl of each culture was transferred into a 96-deep-well plate (Ritter GmbH, Kaufbeuren, Germany) containing 1 ml buffer Z (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot 1H_2O$, 10 mM KCl, 1 mM $MgSO_4 \cdot 7H_2O$, 50 mM β-mercaptoethanol) per well. Twenty microliters of 0.1% SDS and 40 μl of chloroform were added to each well, and the cells were lysed by vigorously pipetting up and down. The plates were then incubated at room temperature for 40 min to allow for phase separation. One hundred microliters of the upper phase was carefully transferred to a new microtiter plate, and 20 μl of ONPG was added to each well to start the enzymatic reaction. As soon as the samples turned yellow, reactions were stopped by adding 50 μl of 1 M Na_2CO_3, and the reaction time was noted. Optical densities at 550 and 420 nm were determined, and β-galactosidase activity was calculated according to the formula: $1{,}000 \times \{[OD_{420} - (1.75 \times OD_{550})]/(t \times v \times OD_{600})\}$ where t is the reaction time (in minutes) and v is the reaction volume (in milliliters).

Detection of MprF by blue native Western blotting. BN PAGE was carried out as described previously (34), using a Novex NativePAGE Bis-Tris 4 to 16% gradient gel (Invitrogen, Carlsbad, CA). For BN PAGE of membrane fractions, 20 ml of cells with an OD_{600} of 1 were incubated with 750 μl of lysis buffer (100 mM EDTA [pH 8.0], 1 mM $MgCl_2$, 5 μg/ml lysostaphin, 10 μg/ml DNase, proteinase inhibitor [cocktail set III from Calbiochem] in phosphate-buffered saline [PBS]) for 30 min at 37°C and lysed three times with 500 μl of zirconia beads in a high-speed homogenizer (Savant Instruments) for 30 s each time at a speed of 6,500 rpm. The beads were removed, and the cell lysate was cleared from debris by centrifugation for 20 min at 14,000 rpm and 4°C. The supernatant was transferred to microcentrifuge polypropylene tubes (catalog no. 357448; Beckman Coulter), and cytoplasmic membranes were precipitated by ultracentrifugation for 45 min at 55,000 rpm and 4°C (Beckman Coulter rotor TLA 55). The pellets were dissolved in 27 μl resuspension buffer (750 mM aminocaproic acid, 50 mM Bis-Tris [pH 7.0]), and dodecyl maltoside was added to a final concentration of 1% to solubilize. After 1-h incubation at 4°C, unsolubilized material was removed by ultracentrifugation for 30 min at 40,000 rpm and at 4°C. Twenty microliters of supernatant was transferred to fresh tubes containing 2.2 μl of 10× BN loading dye (5% [wt/vol] Serva Blue G, 250 mM aminocaproic acid, 50% glycerol) and mixed. Subsequently, 20 μl per well was run on a Novex NativePAGE 4 to 16 % Bis-Tris gel. Separated proteins were transferred to a polyvinyl difluoride membrane and detected via GFP-specific rabbit IgG antibody (Invitrogen) and goat anti-rabbit IgG Dylight 800 (Pierce) as the secondary antibody in an Odyssey imaging system of LI-COR.

Statistical methods. Statistical analyses were performed with the Prism 4.0 package (GraphPad Software) and the between-group differences were analyzed for significance with the two-tailed Student's t test. A P value of ≤ 0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02340-14/-/DCSupplemental.

Figure S1, PDF file, 2.1 MB.
Figure S2, PDF file, 0.5 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.02 MB.
Table S4, DOCX file, 0.1 MB.

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