Phospholipid translocation captured in a bifunctional membrane protein MprF

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As a large family of membrane proteins crucial for bacterial physiology and virulence, the Multiple Peptide Resistance Factors (MprFs) utilize two separate domains to synthesize and translocate aminoacyl phospholipids to the outer leaflets of bacterial membranes. The function of MprFs enables Staphylococcus aureus and other pathogenic bacteria to acquire resistance to daptomycin and cationic antimicrobial peptides. Here we present cryo-electron microscopy structures of MprF homodimer from Rhizobium tropici (RtMprF) at two different states in complex with lysyl-phosphatidylglycerol (LysPG). RtMprF contains a membrane-embedded lipid-flippase domain with two deep cavities opening toward the inner and outer leaflets of the membrane respectively. Intriguingly, a hook-shaped LysPG molecule is trapped inside the inner cavity with its head group bent toward the outer cavity which hosts a second phospholipid-binding site. Moreover, RtMprF exhibits multiple conformational states with the synthase domain adopting distinct positions relative to the flippase domain. Our results provide a detailed framework for understanding the mechanisms of MprF-mediated modification and translocation of phospholipids.
Amoacyl phospholipids, such as lysylphosphatidylglycerol (LysPG), are an important class of lipids with crucial biological functions in antibiotic resistance, pathogenicity, stress response, and motility of bacterial cells. They are widely distributed in microbes such as Staphylococcus aureus, Mycobacterium tuberculosis, and many other pathogenic bacteria. An integral membrane protein known as Multiple Peptide Resistance Factor (MprF) catalyzes biosynthesis of amoacyl phosphatidylglycerol (aaPG) by using phosphatidylglycerol and amoacyl-tRNA as substrates. MprF orthologs from different species can modify phosphatidylglycerol (PG) or cardiolipin with distinct amoacyl groups including lysyl, alanyl, arginyl, and ornithyl groups. When mprf was knocked out in S. aureus, the bacteria became highly susceptible to daptomycin and cationic antimicrobial peptides (CAMPs) including defensins from human neutrophil and bacteriocins. Daptomycin and cationic antimicrobial peptides (CAMPs) including defensins from human neutrophil and bacteriocins.

Overall structure and oligomeric state of RmpF. Recombinant RmpF protein with a hexahistidine tag fused to its carboxyterminal region was expressed in E. coli cells, and the protein was purified through immobilized metal affinity chromatography in solutions with either dodecyl-β-D-maltoside (β-DDM) or glycodeoxigenin (GDN) (see “Methods” for more details). For single-particle cryo-EM analysis, the purified RmpF protein was further reconstituted with PG into lipid nanodiscs, a nanoscale complex consisting of a small patch of lipid bilayer and the target protein surrounded by engineered membrane-scaffold protein (Supplementary Figs. 1a and 2a). Two-dimensional (2D) and three-dimensional (3D) classes of the single-particle images indicate that RmpF exists mainly as homodimers in nanodiscs (Supplementary Figs. 1b, c and 2b-d). The cryo-EM maps for RmpF(DDM)-nanodiscs and RmpF(GDN)-nanodiscs (full-length RmpF protein purified in β-DDM/GDN and reconstituted in nanodiscs) were refined to 3.7 and 2.96 Å resolution, respectively (Supplementary Figs. 1d–f and 2e–g). The cryo-EM map of RmpF(GDN)-nanodiscs exhibits well-defined features allowing construction of a structural model with ~94.4% amino acid residues of the full-length RmpF protein and identification of four lipid molecules per monomer (Supplementary Fig. 3 and Table 1). The structure of RmpF(DDM)-nanodiscs contains three lipid molecules and represents a state different from that of RmpF(GDN)-nanodiscs as discussed below. The crystal structure of the catalytic domain of RmpF in the C-terminal region has been solved at 2.0 Å resolution (Supplementary Fig. 4), and serves as the initial model for building the corresponding region in the cryo-EM structures of the full-length RmpF.

While the MprF protein from S. aureus (SaMprF) may oligomerize into homodimers or homotetramers, RmpF in nanodiscs mainly exists as an arch-shaped homodimer with the C2 symmetry axis running through the dimerization interface (Fig. 1a–d). The function of RmpF is related to polymyxin B (a lipopeptide antibiotic) resistance, acid tolerance, nodulation phenotype of E. coli, and development of anti-infective strategies against drug-resistant S. aureus infections caused by methicillin-resistant and vancomycin-resistant S. aureus (MRSA and VRSA) as well as other multidrug-resistant gram-positive bacteria. Numerous mutations of mprf have been identified in the daptomycin-resistant (DAP-R) S. aureus and among them, several gain-of-function mutations were verified as the causes of DAP-R phenotype.

MprF is a bifunctional protein with two separate domains and the antimicrobial peptide resistance of S. aureus requires the presence of both domains. While the cytoplasmic domain of MprF functions as an aaPG synthase, the membrane-spanning domain serves as a phospholipid flipase mediating translocation of aaPG from the inner leaflet to the outer leaflet of the membrane. Occurrence of LysPG in the outer leaflet of bacterial membrane might help to repel CAMPs from reaching the membrane surface through electrostatic repulsion, modulate the membrane with recombinant RmpF protein was prepared and incubated with a bifunctional amine-reactive crosslinking reagent (disuccinimidyl suberate, DSS). After crosslinking, the products were solubilized in β-DDM solution, separated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and detected through western blot by using the anti-His-Tag antibody. The crosslinking result demonstrates that the dimeric form of RmpF protein is present in the membrane (Fig. 1e). Dimerization of RmpF is mainly mediated by the transmembrane domain and a total surface area of 12080.9 Å² is buried in the RmpF dimer with 4263.3-Å² interface area between two adjacent monomers, indicating the dimeric state of RmpF is stable in lipid nanodiscs, according to the result of quantitative analysis through PISA (proteins, interfaces, structures, and assemblies) program. Curiously, four lipid molecules, namely PG1, PG2, and their symmetry-related molecules PG1′ and PG2′, are located at the monomer–monomer interface (Fig. 1f). They collectively contribute to dimerization of RmpF by forming polar and hydrophobic interactions with two adjacent monomers simultaneously. While the protein–protein contact within the dimer contributes merely 629.5-Å² interface area, the four PG molecules form 1646.3 and 1641.1-Å² interface area with the two adjacent monomers, respectively, suggesting that these interfacial lipid molecules have crucial roles in stabilizing the dimeric state of RmpF. In RmpF(GDN)-nanodisc, the hydrophobic group of a GN D molecule occupies the binding site of the 2-acyl chain of PG2 molecule observed in RmpF(DDM)-nanodisc, while the other PG molecule (PG3) at the peripheral region of the dimerization interface is located nearby GDN (Supplementary Fig. 3c).
Subdomain 2 (Ser334-Arg530) includes TM9–14. Subdomain 1 harbors a core unit composed of two similar motifs with inverse membrane topology, namely the TM2–TM3a–TM3b–TM4 motif and the TM6–TM7a–TM7b–TM8 motif (Fig. 2c). The two motifs are related to each other through a pseudo-C2 axis running approximately parallel to membrane plane (Supplementary Fig. 5b–d). The overall structure of the RtMprF flippase domain represents a distinctive membrane protein fold and does not resemble any other lipid transporters with known structures, such as TMEM16F (a lipid scramblase)\textsuperscript{30}, Lipid II flippase MurF\textsuperscript{31}, the ATP-binding cassette transporter MsbA\textsuperscript{32}, and P4-ATPase lipid-flippase Drsp-Cdc50p\textsuperscript{33} (Supplementary Fig. 6). The carboxyl-proximal region of RtMprF forms a water-soluble synthase domain active in synthesizing aaPG on the cytoplasmic
side. It contains the binding sites for aminoacyl-tRNA and PG according to the previous work on the synthase domain structures of MprF homologs from *Pseudomonas aeruginosa* and *Bacillus licheniformis* (*Pa*MprF and *Bl*MprF)\(^1\). The synthase domain of *Rt*MprF (Pro539-Gly860) superposes well with those of *Pa*MprF and *Bl*MprF (root-mean-square deviation of α-carbons at 0.977 and 1.297 Å, respectively). They share similar tandem repeats of the General Control Nonderepressible 5 (a histone acetyltransferase of a transcriptional regulatory complex) related N-acetyltransferase (GNAT) folds (GNAT folds 1 and 2) (Supplementary Fig. 4). The GNAT fold 1 of the synthase domain is covalently linked to TM14 in the flippase domain through a flexible loop (residues 531–539, invisible in the map). Meanwhile, it associates closely with the flippase domain through...
non-covalent interactions (Fig. 2d). First, amino acid residues from α3 (Asp604 and Glu611), α4 (Asp635), and β5 (Arg639) regions of the GNAT fold 1 in RTMprF form close interactions (salt bridges and hydrogen bond) with Arg456 from the TM10–TM11 loop, Arg136 from TM3b, and Thr145 from TM4 (Fig. 2e). Their interactions contribute to the formation of Interface 1 between the synthase domain and the flipase domain. Second, the loop region before α10 of GNAT fold 2 contacts with the β-hairpin loop between TM5 and TM6 from the cytoplasmic surface of Subdomain 1 of the flipase domain. This interface (Interface 2) is mainly stabilized by three pairs of hydrogen bonds (Fig. 2f). Among the amino acid residues involved in the inter-domain interactions, Arg456 of RTMprF is highly conserved in other MprF homologs (Supplementary Fig. 7).

The flipase domain of RTMprF harbors two internal lipid-binding sites within membrane-embedded cavities. Remarkably, the flipase domain of RTMprF contains two deep cavities located on the cytoplasmic and periplasmic sides of the membrane, respectively (Cavities C and P, Fig. 3a). Cavity C opens to the inner leaflet of the lipid bilayer through a lateral portal measuring 6–8 Å wide (Fig. 3b). Meanwhile, the cavity penetrates deep into a central region of Subdomain 1 near the estimated middle plane of lipid bilayer. The wall of Cavity C is mainly shaped by the transmembrane helices in Subdomain 1, namely TM2, TM3a–3b, TM4, TM5, TM6, TM7a–7b, and TM8. On the other side, Cavity P is surrounded by TM1, H1, TM7b, and TM8 from Subdomain 1 as well as TM9, H3, TM10, TM11, TM12, and TM13 from Subdomain 2. While the internal pocket of Cavity P also extends deep into the central region close to the tip of Cavity C, it has a lateral portal on the other side opening 6–8 Å wide toward the outer leaflet of the lipid bilayer (Fig. 3a). In the central region, the two cavities are separated from each other by a barrier around Arg304 on TM8 (Fig. 3c). Arg304 forms a salt bridge with Glu280 and is hydrogen-bonded to Ala274 and Gly275 from TM7a–7b loop region. The hydrogen bonds between Arg304 and the carbonyl groups of Gly275 and Ala274 might serve to stabilize the side chain of Arg304 in a favorable orientation for establishing ionic interaction with the side chain of Glu280.

Strikingly, one LysPG molecule each (LysPG1 and LysPG2) is trapped in Cavity C and P of the RTMprF(GDN)-nanodisc structure (Fig. 3a). The cryo-EM density of LysPG1 is well-defined and matches well with the model, while the putative LysPG2 shows well-resolved density for the fatty-acyl chains and relatively weak density for the head group. The density of LysPG1 exhibits three arms with similar shape and length (Supplementary Fig. 3b). The first arm is buried inside Cavity C and surrounded by polar amino acid residues, such as Asn117, Asp234, Ser238, and Arg304. The second arm is sandwiched between TM7a and TM4, and surrounded by hydrophobic residues. The density of the third arm is the weakest among the three and it is located at the outmost region exposed to the hydrophobic area of lipid bilayer. Although the local resolution of the three individual arms may appear insufficient for distinguishing the phospho-[3-lysyl (1-glycerol)] head group and two fatty-acyl groups, the interpretation of the lipid molecule is assisted by considering the compatibility of the individual groups with their local environments. As a result, the first arm is assigned as the phospho-[3-lysyl(1-glycerol)] head group and the other two arms most likely belong to the fatty-acyl chains of LysPG molecule. The model is further verified through mutagenesis and biochemical analysis (described below). As shown in Fig. 3b, the LysPG1 molecule in Cavity C has the characteristic hook-shaped polar head group inserted deeply toward the center of Subdomain 1, while the hydrophobic fatty-acyl chains of LysPG1 extend outwardly to the inner leaflet of lipid bilayer. The fatty 2-acyl chains of LysPG1 crawl upward along the external surface of RTMprF and form hydrophobic interactions with non-polar amino acid residues from TM7a and TM4. The head group of LysPG1 bends upward to a position near the middle plane of lipid bilayer, instead of pointing downward to the cytoplasmic surface. In comparison, PG molecules at the dimerization interface adopt the head-group-down inward-facing configuration common to bulk phospholipids in the inner leaflet (Fig. 1f).

The lysyl group of LysPG1 molecule binds to Asp234 and Tyr307 through its side-chain ε-amino group (Fig. 3d). The ε-amino group of LysPG is sandwiched between Ala274 and Tyr303, and located merely ~4.4 Å from the barrier site (Arg304) between Cavities C and P. Between the ε-amino group of LysPG and Ala274, there is a water molecule serving as a bridge connecting them through hydrogen bonds (Supplementary Fig. 3d). Asp234 of RTMprF is conserved in the homolog from Pseudomonas aeruginosa and is replaced by a similar residue (Glu, also an acidic residue favorable for lysyl group binding) in some other species. Besides, Tyr303, Arg304, and Tyr307 in RTMprF are part of the XRXXY motif highly conserved among various MprF homologs (Supplementary Fig. 7). When they are individually mutated to alanine in RTMprF, the amount of LysPG co-purified with the D234A and R304A mutant protein samples is reduced significantly compared to the wild type (Fig. 3e, f), indicating that these two charged residues are crucial for LysPG binding. The results are consistent with the current model of LysPG1 molecule with its lysyl head group buried inside Cavity C. Besides, the head-group glycerol of LysPG1 is hydrogen-bonded to Asn117 and forms van der Waals contact with Phe155. Thereby, the lipid-binding site in Cavity C functions to stabilize the head group of LysPG1 in the upward position through specific interactions. The characteristic hook-like shape of LysPG1 in Cavity C indicates that flipping of LysPG may begin at the initial stage of translocation process on the inner leaflet side instead of the outer leaflet side.

Unlike LysPG1, LysPG2 located in Cavity P has an extended conformation (Supplementary Fig. 3b). Its head group is positioned on the periplasmic surface and both fatty-acyl chains extend deep into the cavity toward the center of Subdomain 1 (Fig. 3a). There are actually three well-resolved fatty-acyl chain densities inside Cavity P of the RTMprF(GDN)-nanodisc structure (Supplementary Fig. 8a). Among the three acyl chains, the two long ones join each other at the head-group region near
periplasmic surface and belong to a phospholipid molecule tentatively assigned as LysPG2 in the model. The third acyl chain is much shorter than the other two, likely belonging to a detergent molecule. While the density for the two fatty-acyl chains of the phospholipid molecule are fairly strong and clear, the head-group density is relatively weak. When the map contour level is lowered, the density corresponding to the lysyl group becomes visible and appears to be connected to the glycerol group (Supplementary Fig. 8b). Therefore, the lipid density feature in Cavity P is interpreted as a LysPG molecule with highly flexible lysyl group. Alternatively, a PG molecule may also occupy the site. The backbone glycerol-3-phosphate group of LysPG2 may bind to adjacent residues through hydrogen bonds, whereas the fatty-acyl chains form van der Waals and hydrophobic interactions with
Fig. 3 Cavities and internal lipid-binding sites in the flippease domain of RTMprF. a Two intrinsic cavities (Cavities C and P) found in the flippease domain. A cross-sectional view of the electrostatic potential surface model is shown; Blue, positive potential; white, neutral; red, negative potential. The LysPG1 molecule (shown as stick model in yellow) has its head group buried in deep Cavity C. In contrast, the LysPG2 molecule has its head group positioned outside, while two long fatty-acyl chains are buried deep in Cavity P. The cryo-EM densities of the LysPG molecules in Cavities C and P are shown as green meshes (contoured at 3.0 σ level). b A zoom-in-view of LysPG1 molecule in Cavity C showing its fatty-acyl chains extending into membrane region through the lateral portal. c The contribution of Arg304–Glu280 ionic pair as the barrier between Cavity C and Cavity P. Arg304, Glu280, Ala274, and Gly275 are shown as stick models. d Interactions between the head group of LysPG1 and adjacent amino acid residues. e Analysis of LysPG co-purified with wild-type RTMprF and mutants through the TLC experiment. The same amount of WT or mutant protein was used for extraction of lipid samples for TLC. The plate was stained by ninhydrin (an amino group-specific dye). PE phosphatidylethanolamine. The lipid spots are identified according to the standard samples of LysPG, PE, and other phospholipids shown in Supplementary Fig. 9d. f Quantification of the relative amount of LysPG co-purified with four RTMprF mutants in comparison with the wild type (WT). The error bars indicate the standard errors of the mean values (n = 3). p = 0.000388 between WT and D234A, ***, p = 0.2153 between WT and Y303A, ns not significant; p = 0.000105 between WT and R304A, ***, p = 0.1283 between WT and Y307A, ns (two-sided unpaired t-test between WT and various mutants). The experiment was repeated independently twice with similar results. g Interactions of LysPG2 with nearby amino acid residues. The residues in van der Waals contacts or hydrophobic interactions with LysPG2 are shown as silver stick models. The images of a–d and g represent the structure of RTMprF(GDN)-nanodiscs at 2.96 Å with two internal LysPG molecules. h Superposition of the structures of RTMprF at two different states. Color codes: blue, RTMprF(DDM)-nanodiscs; red, RTMprF(GDN)-nanodiscs. The arrows indicate the putative motion of TM12 and TM14 when the protein switches from one state to the other. In c, d, and g, the numbers labeled nearby the green dashes indicate the distances (Å) between two adjacent groups.

nearby residues including Arg304, Phe276, and several other hydrophobic residues (Fig. 3g). Such a well-resolved lipid feature found in Cavity P is only present in RTMprF(GDN)-nanodisc but not in RTMprF(DDM)-nanodisc.

In the RTMprF(DDM)-nanodisc structure, Cavity C is also occupied by a LysPG molecule similar to the one observed in RTMprF(GDN)-nanodiscs (Supplementary Fig. 3e, b). In contrast, Cavity P only contains some detergent-like density features much weaker than that of LysPG2 in RTMprF(DDM)-nanodiscs. Thin-layer chromatography (TLC) and mass spectrometry analysis results indicate that the RTMprF protein sample does contain LysPG in the lipids co-purified along with the protein (Supplementary Fig. 9a, b). As the E. coli cell does not produce endogenous LysPG by itself4,19, the LysPG molecules bound to RTMprF should be its own product. Through the TLC experiments, the stoichiometry of LysPG co-purified with RTMprF (DDM) protein sample is estimated to be ∼1.2 LysPG molecules per RTMprF monomer (Supplementary Fig. 9c). The strong lipid density in Cavity C indicates that it may take up one LysPG molecule, whereas Cavity P in RTMprF(DDM)-nanodiscs is likely occupied by detergent or lipid molecule at very low occupancy. In comparison, the LysPG:protein stoichiometry of the RTMprF (GDN) sample is ∼2.6 (Supplementary Fig. 9f, g), much higher than the LysPG:protein stoichiometry of the RTMprF(DDM) sample. Such difference may account for the strong phospholipid density in Cavity P of RTMprF(GDN)-nanodisc due to higher occupancy of LysPG, consistent with the interpretation of the lipid as LysPG2.

Evident conformational differences exist between RTMprF (DDM)-nanodisc and RTMprF(GDN)-nanodisc structures in the regions around Cavity P, despite that their overall structures are similar (Fig. 3h). By superposing them, it is apparent that TM12 moves 2.2 Å closer to TM11 and TM14 moves 4.2 Å closer to TM9 upon binding of LysPG2 in Cavity P. Besides, the amino acid residues involved in binding the fatty-acyl chains of LysPG2 are also adjusted slightly. Previously, it was found that a truncation mutant of SaMprF lacking the bulk region of Subdomain 1 is inefficient in translocating LysPG to the outer leaflet and failed to confer CAMP resistance, whereas the production of LysPG was unaffected18. As Subdomain 1 is involved in the formation of lipid-binding sites in both Cavities C and P, the absence of Subdomain 1 will abolish the lipid-translocating function of MprF mainly because the mutant protein cannot either bind LysPG from the inner leaflet or host it on the outer leaflet side.

The lipid-binding site in Cavity C of RTMprF can accept different aAPGs. In the flippease domain of RTMprF, the amino acid residues (D234, Y303, R304, Y307) involved in binding the head group of LysPG directly or indirectly are identical or highly similar among various homologs (Supplementary Fig. 7). While MprF homologs from R. tropici and S. aureus (and many others) catalyze biosynthesis of LysPG, the one from P. aeruginosa and one of the two homologs from C. perfringens (CpMprF2) produce alanyl-phosphatidylglycerol (AlaPG) instead of LysPG,34. To find out whether the flippease domain of RTMprF can accept AlaPG or not, we have constructed an MprF chimera (RTPaMprF) by fusing the flippease domain of RTMprF with the synthase domain of PaMprF (Fig. 4a). The chimeric RTPaMprF protein was expressed in E. coli and can be purified in sufficient amount for lipid extraction and analysis. The lipid analysis result indicates that the RTPaMprF is active in synthesizing AlaPG but not LysPG, and AlaPG could be co-purified along with the RTPaMprF protein (Fig. 4b). Mutation of the four key residues involved in binding LysPG results in significant decrease of the amount of AlaPG co-purified with the protein (Fig. 4c). Therefore, it is apparent that the preference for different aAPG is mainly conferred by the synthase domain of RTMprF, presumably by the selective binding of different aminocarboxy-TRNA in its active site.

For the flippease domain of RTMprF, the substrate specificity appears to be broad, allowing it to accept either LysPG or AlaPG. Similarly, the flippease domains of MprF homologs from S. aureus and C. perfringens also exhibit relaxed substrate specificities35. The head groups of LysPG and AlaPG most likely share the same binding pocket in Cavity C of the flippease domain. Binding of AlaPG in the pocket is more sensitive (than LysPG) to the mutation of the four key residues involved in aAPG binding, as the alanyl group contains a small side chain and may require the presence of the bulky side chains of Tyr303 and Tyr307 in the flippease domain of RTMprF for its binding through van der Waals interactions.

The LysPG-binding site in Cavity C is involved in regulation of LysPG synthesis and translocation. What is the functional role of the LysPG-binding site in Cavity C of RTMprF protein? Do the mutations of LysPG-binding sites affect the overall level of LysPG production and the flippease function under in vivo condition? To answer the questions, we have analyzed the overall LysPG levels of E. coli cells expressing D234A, Y303A, R304A, and Y307A mutants of RTMprF and compared them to the cells expressing wild-type protein. Remarkably, the cells expressing the mutant
proteins have higher level of LysPG (per unit protein) than those with the wild-type protein (Fig. 4d, e), while the protein expression levels are slightly variable among the wild type and mutants (Fig. 4e). Among the four mutants, the cells expressing R304A mutant exhibit the highest level of total LysPG, while those with Y307A mutant have lower level of total LysPG than the other three mutants but are still 1.5 times of those with wild-type protein. Similarly, the levels of LysPG on the outer leaflet of membrane (accessible by the fluorescamine dye) appear to be also higher for the mutants than for the wild type (Fig. 4f, g). Fluorescamine is a membrane-impermeable dye mainly reacting with the LysPG exposed on cell surface (on the outer leaflet) and was previously used for characterizing the in vivo flippase function of SaMprF in S. aureus9.
To further analyze the functional role of Glu280 in RtMprF, three point mutants, namely E280A, E280K, and E280Q, were generated. While the protein expression level of E280A mutant is about 1.5 times that of the wild type (Fig. 4i). Nevertheless, the E280K and E280Q mutants exhibit more than 30 times increase in the total LysPG level per unit protein relative to the wild type, despite that the two mutant proteins express at much lower levels than the wild type (Fig. 4h, i). Similarly, the amounts of fluorescamine-labeled LysPG per unit protein also increase significantly in the two mutants (Fig. 4j, k).

Moreover, the proportion of LysPG in total phospholipids extracted from cells expressing the six mutants mentioned above are about 1.5–5 times of the LysPG/total phospholipids ratio in cells expressing wild-type RtMprF (Supplementary Fig. 9d, e). Therefore, the mutations on the LysPG-binding sites in Cavities C or the Glu280–Arg304 ionic pair of RtMprF have dramatic stimulating effect on its synthase function, presumably by removing the potential inhibitory effect imposed by LysPG bound to Cavity C. The mutations may also enhance its synthase function leading to increased level of LysPG on the outer leaflet of the membrane.

RtMprF exhibits variable conformations with the synthase domain rearranged relative to the flipase domain. MprFs utilize aminoacyl-tRNA from cytosol and PG from membrane as the substrates for aminoacyl phospholipid synthesis7. The potential binding site of lysyl-tRNA in the synthase domain of RtMprF is located at the cleft between GNAT folds 1 and 2 (Supplementary Fig. 10a, b), according to the previous studies on an alanyl transferase FemX involved in pep-tidyoglycan biosynthesis (in complex with an aminoacyl-tRNA analog) and BmMprF in complex with L-lysine amide (LYN)19,36. Apparently, the active site of the synthase domain is separated from the LysPG-binding sites in the flipase domain by a large distance at 47.0 (within the same subunit) or 71.4 Å (between adjacent subunits). Such a large gap is apparently unfavorable for direct translocation of lipid molecules (PG or LysPG) between the two domains. To overcome the problem, RtMprF may go through large conformational changes and rearrange the synthase domain to a position close to membrane surface in order to acquire PG from the membrane and release LysPG back to the membrane.

In addition to the major class (class A) of symmetrical RtMprF dimers, there are minor classes (classes B–D) exhibiting asymmetric conformations (Supplementary Fig. 10c). Within the asymmetric RtMprF dimers, one monomer rearranges the synthase domain more dramatically than the adjacent one. When the flipase domains of classes B–D are superposed with that of class-A symmetrical dimer, it becomes apparent that the synthase domains adopt distinct positions in the minor classes (Supplementary Fig. 10d–f). For class A, the long axis of the synthase domain forms 115.2° angle with the membrane plane. In comparison, the corresponding ones of classes B–D form much smaller angles (12.8–32.4°) with the membrane plane (Supplementary Fig. 10c). In these cases, the synthase domains may rotate from the upright position to nearly horizontal positions and detach from the cytoplasmic surfaces of the flipase domains. Consequently, the restraints restricting their movement are greatly reduced so that the synthase domain can readily move across long distance through Brownian motion. The inter-domain flexibility of RtMprF, as reflected by the variable positions of the synthase domain, may allow it to change conformation so as to acquire hydrophobic substrate (PG) from the membrane and to release LysPG back to the membrane for translocation by the flipase domain.

Discussion
Single-nucleotide polymorphisms of SaMprF are frequently found to be associated with daptomycin resistance of S. aureus,
whereas the action mechanism of SaMprF in causing daptomycin resistance remains largely unclear \(^1\). A recent work reported that T345A single-nucleotide polymorphism of SaMprF can cause daptomycin resistance in \(S. aureus\) reproducibly, while the mutation did not affect LysPG synthesis or translocation\(^4\). To analyze the locations of DAP-R-related mutations on SaMprF\(^14,37\), a structural model of SaMprF is generated through the comparative protein structure modeling method\(^38\). The mutation sites are mainly located in the flipase domain, while only three of them are in the synthase domain (Supplementary Fig. 11a, b). Among the 24 sites in the flipase domain of SaMprF, eighteen are located in Subdomain 2 and the remaining six are in Subdomain 1. Interestingly, TM9 in Subdomain 2 contains a region of high-frequency DAP-R mutations including two gain-of-function mutations (T345A and V351E) responsible for significantly enhanced DAP-R phenotype\(^14\) (Supplementary Fig. 11c). It was proposed that the mutations may modulate specific interactions of SaMprF with the antibiotic molecule instead of affecting LysPG synthesis or translocation\(^4\).

Computational docking analysis suggests that daptomycin molecule can fit well in Cavity P of SaMprF (Supplementary Fig. 11d). The daptomycin molecule inserts its N-terminal decanoyl fatty-acyl group and tryptophan side chain in two deep pockets of Cavity P (Supplementary Fig. 11e). Several DAP-R mutations are located near the entrance of Cavity P (Supplementary Fig. 11f) or on the wall of its internal pockets (Supplementary Fig. 11g). The mutations may influence the interactions between daptomycin and SaMprF by altering the overall shape and surface property of Cavity P. While the earlier work\(^4\) and our hypothetical model both suggest that daptomycin may interact with SaMprF, more biochemical and other evidences are needed to verify their interaction and to address the question about whether SaMprF can directly translocate daptomycin or not. The structural model serves as a preliminary framework to guide further experiments in order to reveal the mechanism of daptomycin resistance caused by SaMprF variants. Moreover, the model might also be useful for discovery and development of SaMprF inhibitors as anti-infective agents. In practice, the SaMprF model could potentially be used in the structure-based virtual screening of antibiotic drugs for treating MRSA or VRSA infections.

As shown in the structure and crosslinking experiment (Fig. 1), RtMprF protein forms homodimer and larger oligomers, consistent with previous biochemical study on SaMprF\(^2\). There might be potential functional advantages for the RtMprF proteins to form homo-oligomers (dimer or tetramer). First, the dimerization interface of RtMprF homodimer contains four PG molecules, which may also serve as substrate for the synthase domain. Upon dimerization, the lipid substrate (PG) of RtMprF may be enriched in the milieu to promote synthesis of aaPG. Second, the two monomers may help each other in translocating aaPG if it is released to the membrane after being produced by the synthase domain. Dimerization or tetramerization of MprF may help to concentrate multiple flipase domains in a local region enriched with LysPG so that the translocation of LysPG can occur more efficiently as free diffusion of LysPG in the membrane is a relatively slow process and translocation of lipid molecule across the membrane is also a rate-limiting process\(^19\).

In bacterial cells, MprFs carry out dual functions by synthesizing aaPG and translocating it to the outer leaflet of bacterial membrane\(^9\). Although the synthase domain of MprF alone can catalyze synthesis of LysPG by itself under in vitro conditions, efficient production of LysPG in vivo requires the presence of both domains, and it was suggested that the transmembrane domain of MprF may help to position the catalytic domain in the cytosol during aaPG synthesis\(^8\). The cryo-EM structure of RtMprF in complex with LysPG reveals the close relationship between the two domains and apparently, the membrane-embedded flipase domain does serve to position the catalytic domain in the cytosol by forming specific interactions with it (Fig. 2d). As shown in Fig. 2e, f, the catalytic domain interacts with amino acid residues from the cytoplasmic surfaces of Subdomains 2 and 1 mainly through salt bridges and hydrogen bonds. Such specific interactions enable the catalytic domain to approach membrane surface and acquire the lipid substrate (PG) from the membrane more efficiently than the catalytic domain expressed alone (not attached to the Subdomain 2). Although the in vitro activity assay reveals that the purified catalytic domain of MprF is active in the absence of transmembrane domain\(^15\), the function of isolated catalytic domain of MprF relies on delivering of lipid substrate through detergent (Triton X-100) micelles in the solution, a condition which does not exist in the in vivo environments. Therefore, the catalytic domain expressed alone in \(E. coli\) is not functional in producing of aaPG\(^8\), mainly because it is inefficient in acquiring the lipid substrate from the membrane through random diffusion process.

The characteristic hook-shaped LysPG1 and its binding site in Cavity C observed in the structures shed light on the mechanism of lipid selectivity in the flipase domain of MprF. The aminoacyl head groups of LysPG or AlaPG are specifically recognized by amino acid residues on the wall of Cavity C as observed in the RtMprF structure (Fig. 3d). The electronegative surface within Cavity C (Fig. 3b) is favorable for the binding of positively charged aminoacyl group of LysPG or AlaPG, but unfavorable for the anionic phospholipids (such as PG and cardiolipin (CL)) to bind. Moreover, the head groups of phosphatidylethanolamine (PE), phosphatidylserine (PS), and PG are much smaller in size and lacks the lysyl or alanyl group when compared with LysPG and AlaPG. Even if PE, PS, or PG from the membrane can enter Cavity C in MprF, they cannot form stable interactions with nearby residues. As for CL, it contains four fatty-acyl chains and is too big to fit in Cavity C of MprF. Therefore, the unique phospholipid-binding site in Cavity C of MprF likely selects LysPG or AlaPG against the other phospholipids through compatible shape, size, and surface charge property.

Near the interface between the two domains of RtMprF, LysPG is found within the inner cavity (Cavity C) of the flipase domain instead of binding to the active site of the synthase domain. Therefore, the RtMprF(DDM)-nanodisc structure may represent an intermediate state of the RtMprF protein after LysPG is synthesized and before it is translocated to the outer leaflet. How does MprF coordinate the two domains to facilitate synthesis of LysPG at the interface between cytosol and membrane surface (on the intracellular side) under physiological conditions? After LysPG is synthesized, how is it translocated by MprF from the inner leaflet to the outer leaflet of the membrane? To address the questions, here we propose a mechanistic model to account for the LysPG synthesis and translocation process mediated by MprF based on the structural and biochemical analysis results of RtMprF (Fig. 5). For MprF to acquire the substrate PG molecules from membrane, the synthase domain may need to approach the cytoplasmic surface of the membrane in a nearly horizontal position (State 1). After LysPG is synthesized and tRNA is released, the synthase domain returns to the upright position (State 2) and LysPG in the inner leaflet of membrane diffuses into the binding site in Cavity C (State 3, as observed in RtMprF (DDM)-nanodisc structure with one LysPG bound). Diffusion of LysPG into Cavity C and its translocation is likely driven by the electrostatic repulsive force from the positively charged cytoplasmic surface near the entrance of Cavity C. Subsequently, the gate around the Arg304–Glu280 ionic pair might open transiently so that LysPG can migrate from Cavity C to Cavity P through a...
Fig. 5 A mechanistic model of LysPG synthesis and translocation mediated by MprF. While each monomer in the homodimer may mediate the entire cycle of LysPG synthesis, translocation and release independently, the other monomer may also serve to stabilize the adjacent one in the optimal position (relative to lipid bilayer) for synthesis and translocation of LysPG. The cavities and Arg304-Glu280 pair are only shown in the active monomer and the other monomer is left out for clarity. State 0 represents a resting state of MprF. At State 1, the curved solid arrow indicates the nucleophilic attack of the PG head-group hydroxyl on the activated α-carbon of lysyl-tRNA and the dash arrow denotes the break of covalent bond between lysyl group and tRNA. At States 2 and 4, the dash arrows indicate the lateral diffusion of LysPG into and out of the cavities in RtMprF. Loading of LysPG into Cavity C leads to State 3 as observed in the RtMprF(DDM)-nanodisc structure. During the transition from State 3 to State 4, a channel connecting Cavity C and Cavity P may emerge transiently to facilitate translocation of LysPG. When the Cavity C in State 4 is refilled with a second LysPG molecule, both cavities are occupied by LysPG as observed in the RtMprF(GDN)-nanodisc structure (State 5). Further releasing of LysPG from Cavity P to the membrane leads to switching of State 5 to State 3.
putative channel connecting the two cavities (State 4). A second molecule of LysPG may further enter Cavity C, so that Cavities C and P are both loaded with LysPG as observed in the RtMprF (GDN)-nanodisc structure (State 5). On the outer leafllet side, the LysPG molecule in Cavity P might be attracted by the negatively charged periplasmic surface around the lateral portal so that it can be released to the outer leafllet of the membrane. The LysPG translocation process may cycle among States 3–5 till the LysPG pool in the inner leafllet side is depleted. Afterwards, MprF returns to the initial apo-state (State 0) for the next round of LysPG synthesis-translocation process.

In the RtMprF(GDN)-nanodisc structure, there is an additional lipid-like density nearby LysPG1 (Supplementary Fig. 12). The density is sandwiched by LysPG1 and the β-hairpin loop between TM5 and TM6. As the molecule is located at the entrance of Cavity C, it may serve as a potential site for LysPG loading after it is synthesized and released from the synthase domain. Alternatively, in case that a PG molecule is captured at this site, it may serve as the substrate for the synthase domain if it could diffuse laterally to the active site of the synthase domain in a horizontal position close to the membrane surface. The third possibility is that it may belong to the bulk lipid (such as PE or elses) from the membrane, serving to stabilize LysPG1 in Cavity C and prevent unloading of LysPG1 on the cytoplasmic side by blocking the cytoplasmic portal of Cavity C.

While the head group of LysPG1 is blocked from entering Cavity P by the putative gate around the Arg304–Glu280 ionic pair in RtMprF (Figs. 3c and 5, State 3), the remaining parts of LysPG1 (including the glycerol-3-phosphate backbone, two fatty-acyl chains and the head-group glycerol group) are prevented from entering Cavity P by the steric hindrance effects of amino acid residues from TM7a, TM7b, TM3a, TM3b, as well as the TM7a–TM7b and TM3a–TM3b loops. Among them, His271 on TM7a–7b loop region serves to block the 1-acyl chain of LysPG from entering the cleft between the TM7a–TM7b and TM3a–TM3b loops. Such a narrow cleft is closed at a contact point where Pro273 and Ala274 (in the TM7a–TM7b loop region) form van der Waals interaction and hydrogen bond with the membrane, as well as the R279A mutant exhibited significantly increased daptomycin susceptibility (suggesting a reduced flipase activity of SaMprF) compared to the wild type. Moreover, the R279A mutation (corresponding to Arg304 in RtMprF) also leads to similar loss-of-function effect. For RtMprF, while the E280A mutant does not express in E. coli (presumably due to high toxicity of the target protein), the E280K and E280Q mutants (Fig. 4h–k) as well as the R304A mutant (Fig. 4d–g) can be expressed in E. coli and exhibit enhanced level of both synthase and flipase activities relative to the wild type. The distinct functional phenotypes between R304A of RtMprF and R279A of SaMprF may be due to the differences in protein expression level and host species.

As the Glu280–Arg304 pair is located at the gate between the two cavities, protonation of Glu280 may lead to weakening of Glu280–Arg304 interaction and trigger further conformational changes nearby to open the gate and allow the substrate (LysPG) to go through. In this case, the proton gradient across the membrane might be exploited by MprF to stimulate or drive the translocation of LysPG molecule.

As LysPG is initially synthesized on the cytoplasmic side and inserted to the inner leafllet of the membrane, the concentration of LysPG should be higher in the inner leafllet side than [LysPG] in the outer leafllet. Moreover, there is a LysPG hydrolase found in some bacterial periplasm serving to hydrolyze LysPG and lower its concentration in the outer leafllet constantly. Therefore, there should be a concentration gradient of LysPG between the inner leafllet and the outer leafllet of the membrane at the initial stage. In this case, MprF likely serves as a uniporter to facilitate diffusion of LysPG down its concentration gradient and the process might not need to consume energy, but it could probably be stimulated by protonation of Glu280. Alternatively, it may function like a lipid scramblase, such as TMEM16, which harvest the energy of the phospholipid gradient and may utilize a hydrophilic cavity on the surface of membrane-embedded domain for transport of lipids. For future works, analyzing the flipase activity of MprF through the liposome-based transport assay under asymmetric (and symmetric) pH conditions across the membrane will be helpful in addressing the question about whether the flipase activity of MprF is regulated by proton/ion gradient or not.
Methods

Protein expression and purification. The mpfR gene from R. tropici (RMpFR) was cloned into the pET21b vector and the recombinant plasmid was used to transform E. coli C41(DE3) cells. The cells were cultured in Terrific Broth media containing 50-μg mL⁻¹ ampicillin, and protein expression was induced with 0.5-mM isopropyl β-D-thiogalactopyranoside (IPTG) overnight at 16 °C after OD₆₀₀ reached 1.0. For purification of RMpFR, cells were harvested through centrifugation, resuspended in a buffer containing 25-mM Tris-HCl (pH 8.0), 300-mM NaCl, and 1.5% β-DMM (Anatrace) and Incubated at 4 °C for 30 min. The suspension was sonicated and centrifuged at 11,000 g (IL-25.50 rot, Beckman) for 30 min. The supernatant was ultracentrifuged at 158,000 g. After centrifugation at 40,000 g, for 30 min, the supernatant was loaded onto a Ni-NTA column and the protein was purified at 4 °C by a step-wise elution method with three different buffers containing 25-mM Tris-HCl (pH 7.5), 300-mM NaCl, 20, 50, or 300-mM imidazole, and 0.05% β-DMM for MpfR/DDM sample or 0.02% GDN for MpfR/GDN (GDN) sample. The fraction eluted in the buffer containing 300-mM imidazole was pooled and concentrated to 10 mg·mL⁻¹ in 100-kDa molecular weight cutoff (MWCO) concentrator (Millipore) for further experiments.

To construct the RfPaMpfR chimera, the cDNA sequence of RMpFR synthase domain (residues 542–869) on the pET21b-RmmpR vector was replaced by the coding sequence of PuMP synthase domain (residues 554–881, Alcaligenes) through two PCR reactions. In detail, the region encoding PuMpfR synthase domain was amplified through the first PCR reaction by using a pair of primers with sequences of 5′-CCGCAAGACGGGCGGCAAATGCTAGCAGGCAAGCCGCCGCAAGCCTTCTACCAAA-3′. The DNA product contains 30-bp nucleotides in the 5′- and 3′-terminal regions matching with the regions upstream and downstream of the coding sequence of RMpFR synthase domain on the vector pET21b, respectively. The replacement was further accomplished through a second PCR reaction by using the first PCR product as template and a pair of primers (5′-TCCGGCAGAACGGGCGGCAAATGCTAGCAGGCAAGCCGCCGCAAGCCTTCTACCAAA-3′) as primers and the pET21b-RmmpR vector as template. The second PCR reaction adopted the protocol of the Quick Change method. After digestion of the template with DpnI, the product of the second round of PCR was used for transformation of DH5α E. coli competent cells for plasmid amplification. After transformation and antibiotics resistance screening, the clone with target plasmid was selected and verified through DNA sequencing. The protocols for RfPaMpfR protein expression and purification were the same as the one used for wild-type RMpFR.

The DNA encoding the domain of RmmpR (RmmpR-SD, residues 542–869) was cloned into the pET21b vector and transformed into the E. coli BL21 (DE3) cells for protein expression. For purification of the recombinant RmmpR-SD protein expressed in BL21(DE3) cells, the cell pellets harvested through centrifugation were resuspended in a buffer with 25-mM Tris-HCl (pH 8.0), 300-mM NaCl, and 1.5% β-DMM (Anatrace) and incubated at 4 °C for 30 min. After centrifugation at 40,000 g, for 30 min, the supernatant was loaded onto a Ni-NTA column and the protein was purified at 4 °C by a step-wise elution method with three different buffers containing 25-mM Tris-HCl (pH 7.5), 300-mM NaCl, and 0.05% β-DMM for MpfR/DDM sample or for MpfR/GDN (GDN) sample. The fraction eluted in the buffer containing 300-mM imidazole was pooled and concentrated to 10 mg·mL⁻¹ in 100-kDa molecular weight cutoff (MWCO) concentrator (Millipore) for further experiments.

Cryo-EM sample preparation and data acquisition. The purified RMpFR protein in nanodiscs was concentrated to 6 mg·mL⁻¹ for RMpFR/DDM-nanodiscs or 13 mg·mL⁻¹ for RMpFR/GDN-nanodiscs using a 100-kDa MWCO Amicon concentrator (Millipore). The Quantifoil 1.2/1.3-mm holey carbon grids (300 mesh, copper) were glow discharged for 60 s at 30 Pa and then placed into a 200-kV cryo-electron microscope with dose-weighting using MotionCor2 program with 5 by 5 patches and a B-factor of 250 resulting in a super-resolution pixel size of 0.5 Å (physical pixel size of 1.0 Å). Movies (32 frames per movie file) were captured with a defocus value at a range of −1.5 to −2.0 μm in the super-resolution mode using a dose rate of −9.6 e⁻⁻⁻¹ μm⁻² s⁻¹ over 52 s yielding a cumulative dose of −50 e⁻⁻⁻² Å⁻¹.

Image processing. For RMpFR/DDM-nanodiscs, a total of 2291 cryo-EM movies were aligned with dose-weighting using MotionCor2 program with 3 by 3 patches and a B-factor of 250. Micrograph contrast transfer function (CTF) estimations was performed using CTFFIND 4.1.15 program. Particle picking, 2D classification, and ab initio 3D reference generation were performed in cryoSPARC v2.9 program. After manual inspection of the micrographs, 2549 were selected and −100 particles were picked manually from the micrograph and sorted into 2D classes. The best classes were selected and used as references for sub-classification during data collection at a nominal magnification of x20,000, resulting in a super-resolution pixel size of 0.5 Å (physical pixel size of 1.0 Å). Micrographs (32 frames per movie file) were captured with a defocus value at a range of −1 to −2 μm in the super-resolution mode using a dose rate of −9.6 e⁻⁻⁻¹ μm⁻² s⁻¹ over 52 s yielding a cumulative dose of −50 e⁻⁻⁻² Å⁻¹.

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Model building and refinement. The structural model of the flippase domain of RmMprF was built manually in Coot program38, guided mainly by the cryo-EM map. The initial 3D model prediction from PyMOL and the transmembrane helix prediction from THMMH Server54 (ver. 2.0) were used as references during model building. While most of the transmembrane helices are identified in the map and the models are registered with amino acid sequences, the density for TM14 is too weak for MprF(DDM)-nanodiscs and it is tentatively interpreted with a poly-Alanine α-helix model. For the remaining cryo-EM density, the crystal structure was docked manually into the corresponding region of the cryo-EM map of the full-length RmMprF, subject to rigid body refinement and local adjustment in Coot, and then merged with the flippase domain. The structural model of RmMprF was refined using Coot and phenix.refine55,56 on HPTLC maps for F254 plates (Merck) and 17.8-nmol protein was used and extracted in 40-μl chloroform. The standard samples of 1.0, 2.0, 4.0, and 8.0-nmol LysPG were loaded on the plate as references. Lipid spots were visualized through the iodine staining procedure. It is noteworthy that the data obtained through iodine staining generates a linear standard curve better than those stained with ninhydrin. For quantifying the relative amount of LysPG/AlaPG co-purified with RmMprF/RmPurMprF mutants, lipids extracted from same amount of purified RmMprF/RmPurMprF mutant protein were subjected to TLC analysis. The protein concentration was measured through the bicinchoninic acid method (TransGen Biotech, Beijing). The phase plate chloroform:methanol:acetic acid solvent (80:12:15) were utilized to separate lipids extracted from RmMprF and RmPurMprF mutant protein, respectively. The image of the iodine- or ninhydrin-stained TLC plate was processed by Image J and analyzed by the GraphPad program. For the data presented in Figs. 3e, f, 4c and Supplementary Fig. 9b, f, three aliquots of the sample of the same type are loaded on the TLC plates and the measurements of the three parallel spots are used for statistical analysis.

Crosslinking of RmMprF. The oligomeric state of the RmMprF protein was analyzed through chemical crosslinking experiment by using the membrane preparation from three independent repeats of the full-length RmMprF. The cells were disrupted in a buffer consisting of 20-mM HEPES (pH 7.5) and 300-mM NaCl (buffer A). After the cells were lysed by passing through a high-pressure homogenizer (ATS Engineering), the cell debris was removed through low-speed centrifugation at 10,000 ×g for 30 min. The membrane fraction was resuspended in buffer A and sonicated with 1 s on, 5 s off for 2 min to homogenize the sample. The membrane suspension was aliquoted and then treated with di-3-cyanoimidyl suberate (DSS) at 0.5–5.0 mM final concentration for 1 h at 30 °C with constant mixing on a shaker. The reactions were quenched by adding 100-mM Tris-HCl (pH 7.5). The crosslinked samples were solubilized by adding 1% FDDM for 1 h in the shaker. Subsequently, the samples were centrifuged at 18,000 ×g for 10 min and the supernatant was mixed with 5× SDS-PAGE loading buffer, and then loaded on the SDS-PAGE gel for electrophoresis. The bands on the gel were transferrred to polyvinylidene difluoride membrane and then detected with primary antibodies to RmMprF (infusion antibodies on 11H1 and 11H2) and Goat anti-Mouse IgG (H+L)-HRP (1:2000 dilution). After being developed with the western blotting Ultra ECL horseradish peroxidase substrate (Perkin–Elmer), the blots were imaged on a chemiluminescence CCD system (ChemiScope 3500 mini imager, Clinx Science Instruments).

TLC and mass spectrometry. The lipids from E. coli membrane expressing recombinant RmMprF/RmPurMprF or from the purified RmMprF protein samples were extracted according to Bligh and Dyer procedures28,29. In detail, 12-mg chloroform:methanol (1:2, v/v) mixture was added to 3.2-ml sample and mixed well through vortex. Subsequently, 4-ml chloroform was added to the sample and vortexed again to mix. Finally, 4-ml water was added to the sample and vortexed well. The mixture was centrifuged at 1000 ×g for 5 min to get a two-phase system with aqueous phase at the bottom and organic phase at the top. The bottom phase was washed twice with an aqueous upper phase solution (freshly made by mixing chloroform, methanol, and water at 2:2:1.8 (v/v/v) ratio and centrifuging the mixture). Finally, the bottom phase was recovered, dried under vacuum, and dissolved in 100-μl chloroform. The lipid samples were separated on the HPTLC silica gel 60 F254 plates (Merck) in a mobile phase of chloroform:methanol:water mixture (65:25:4). Lipid spots were visualized through staining with iodine or ninhydrin. For separation of AlaphG and PE, a mobile phase of chloroform:acetic acid:water (80:12:15) mixture was used. The liquid chromatography-mass spectrometry (LC-MS)/MS analysis was performed on a Thermo Scientific Dionex UHPLC system coupled with a Thermo Scientific LTQ–Orbitrap Discovery mass spectrometer. Data on the identification of the lipid extracts were obtained using the cryo-EM structure of MprF as the template and the amino acid sequence alignment data of the two homologs as the other input. Virtual docking of dipotymin molecule on SaMprF was carried out through the Autodock Vina program (v1.1.2) by providing the homologous model of SaMprF and the structure of dipotymin downloaded from the Protein Data Bank [PDB code: 1T5M (https://www.rcsb.org/structure/1T5M)]. A cubic box with 60 × 60 × 60 grid points (in the x, y, z dimensions) and 0.375-Å spacing was applied to define the search region on the SaMprF model during the Autodock analysis.

Computational modeling analysis. The model of SaMprF is constructed through the Modeller 9.23 program38 by using the cryo-EM structure of RmMprF as the template and the amino acid sequence alignment data of the two homologs. The other input. Virtual docking of dipotymin molecule on SaMprF was carried out through the Autodock Vina program (v1.1.2) by providing the homologous model of SaMprF and the structure of dipotymin downloaded from the Protein Data Bank [PDB code: 1T5M (https://www.rcsb.org/structure/1T5M)]. A cubic box with 60 × 60 × 60 grid points (in the x, y, z dimensions) and 0.375-Å spacing was applied to define the search region on the SaMprF model during the Autodock analysis.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary Information file. The coordinates and the cryo-EM maps for RmMprF(DDM)-nanodiscs and RmMprF(GDN)-nanodiscs have been deposited in the PDB [6LVF and 7DUW] and EMDB [EMD-0992 and EMD-30869]. The crystal structure and the structure factors for RmMprF-SD have been deposited in the PDB [6LV0]. The other PDB files used for analysis and comparison in this work were downloaded from the RCSB PDB website (http://www.rcsb.org/), including the structures of dipotymin [accession code: 1T5M], FemX-aminoacyl-tRNA complex [accession code: 4HP], BLP-M1NY complex [accession code: 4VM6], TMSA16F [accession code: 609P6], Mtr...
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Acknowledgements

We thank J. J. Wu for his initial efforts in cloning and purifying various MprF homologs. The cryo-EM data were collected at the Center for Biological Imaging (CBI), Core Facilities for Protein Science at the Institute of Biophysics, Chinese Academy of Sciences, and the beamtime on Talos Arctica was sponsored by the National Laboratory of Biomacromolecules and CBI. We also thank X. J. Huang, B. L. Zhu, L. H. Chen, and F. Sun at CBI for their assistance in cryo-EM data collection; Z. S. Xie for her technical assistance on the mass spectrometry analysis of lipid samples; the staffs at the Photon Factory (BL1A and NW12A), Shanghai Synchrotron Radiation Facility (BL17U and BL18U1), and the Protein Science Research Facility of IBP (Y. Han) for their assistance on crystal screening and X-ray data collection; X. B. Liang for her support in sample preparation, data collection, and storage. The project is funded by the National Natural Science Foundation of China (31670749 and 31925024), the Basic Frontier Science Research Program of CAS (ZDBS-LY-SM003-02), and the Strategic Priority Research Program of CAS (XDB37020101 and XDB08020302).

Author contributions

D.S. purified the RtMprF protein, reconstituted the nanodisc sample, prepared cryo-EM grids, collected cryo-EM data, processed the data, built the model and refined the structure, and performed quantitative analysis on the proportion of LysPG in total phospholipids extracted from E. coli cells through the TLC method. H.J. optimized the protocol for RtMprF expression and purification, solved the crystal structure of RtMprF synthase domain, performed site-directed mutagenesis, and carried out lipid extraction and TLC experiments. D.S., H.J., and Z.L. analyzed the structure and wrote the manuscript. Z.L. conceived and supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-23248-z.

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Peer review information Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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