Lipopeptide antibiotics disrupt interactions of undecaprenyl phosphate with UptA

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Abstract: The peptidoglycan pathway represents one of the most successful antibacterial targets with the last but critical step being the flipping of carrier lipid, undecaprenyl phosphate (C_{55}-P), across the membrane to renter the pathway. This translocation of C_{55}-P is facilitated by DedA and DUF386 domain-containing family membrane proteins via unknown mechanisms. Here we employ native mass spectrometry to investigate the interactions of UptA, a putative flippase from *Bacillus subtilis*, with C_{55}-P, membrane phospholipids and cell wall targeting antibiotics. Our results show that UptA forms monomer-dimer equilibria and binds to ligands in a pH-dependent fashion. Specifically, we show that UptA interacts more favourably with C_{55}-P over shorter-chain analogues and membrane phospholipids. Moreover, we demonstrate that lipopeptide antibiotics, amphomycin and aspartocin D, can directly intercept UptA function by outcompeting the substrate for the protein binding site. Overall, this study shows that UptA-mediated translocation of C_{55}-P is potentially regulated by anionic phospholipids and provides insights for future development of antibiotics targeting carrier lipid recycling.

Introduction

The global threat of drug-resistant bacterial pathogens calls for renewed efforts in the search for new antibiotics, along with the identification of new biochemical pathways and interactions that can be effectively intercepted. A key antibiotic target is peptidoglycan, the cell wall polymer that provides bacteria with resistance to osmotic stress and environmental assaults (1, 2). Accordingly, topline antibiotics such as amoxicillin and vancomycin (3, 4) intercept the transpeptidation step of the peptidoglycan synthesis (5-7). Moreover, promising antibiotic candidates, such as ramoplanin and moenomycin, target the transglycosylation step (8, 9). Peptidoglycan biosynthesis begins with the formation of uridine diphosphate-*N*-acetylmuramyl-pentapeptide (UM5) in the cytosol (10), followed by its coupling to the lipid carrier undecaprenyl monophosphate (C_{55}-P) on the cytosolic side of the inner membrane (11, 12). The resulting lipid I is further decorated with a GlcNAc residue to form lipid II (13) before being flipped across the inner membrane (14-16). In the periplasm, the glycopeptide moiety of lipid II is cross-linked into a nascent peptidoglycan polymer and the lipid carrier is released as undecaprenyl diphosphate (C_{55}-PP) (17-19). To participate in the next round of precursor transfer, C_{55}-PP must be dephosphorylated to form C_{55}-P (20-22) and then flipped across the inner membrane such that its phosphate headgroup returns to the cytoplasmic side. The mechanism by which C_{55}-P is translocated across the cytoplasmic membrane is the least understood of the membrane-associated steps of the peptidoglycan pathway (Fig. 1A).

Recently, proteins belonging to the DedA- and the DUF368 domain-containing families were shown to facilitate trans-bilayer transport of C_{55}-P in the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (23), and in the Gram-negative bacteria *Vibrio cholerae* (24). DedA proteins are highly conserved, having 8 homologues in the model bacteria *E. coli* and 6 homologues in *B. subtilis* (25-27). Members of the DedA transporters in *B. subtilis* include UptA and PetA, which are proposed to facilitate trans-bilayer flipping of C_{55}-P and anionic phospholipids, respectively (23, 28). Compared to the DedA family, the DUF368-domain containing proteins, exemplified by PopT, are less conserved, being found in *S. aureus* and *V. cholerae,* but are absent in *B. subtilis* and *E. coli* (29). *V. cholerae* PopT becomes important for cell survival only under alkaline conditions (24). Several Gram-positive and Gram-negative bacteria encode one or multiple UptA-type transporters suggesting that the C_{55}-P recycling process, mediated by these flippases, is broadly conserved across different bacterial species. For example, the single DedA homologue in *Borrelia burgdorferi* is an essential protein required for proper cell division in this organism (30). Therefore, the recycling step of peptidoglycan biosynthesis mediated by UptA-type flippases can be targeted by antibiotics. This necessitates a molecular-level understanding of how UptA interacts with its cognate substrate, C_{55}-P, and potentially with membrane phospholipids, to elucidate the mechanism of C_{55}-P translocation.

Herein we employ native mass spectrometry (native MS) to elucidate how *B. subtilis* UptA interacts with multiple lipidic substrates and antibiotics. We show that purified UptA exists as an equilibrium of monomers and dimers. We further show that UptA interacts more favorably with C_{55}-P than C_{55}-PP and membrane phospholipids, and then provide molecular-level evidence on the amino acid residues involved in C_{55}-P binding. We find that UptA binds to its cognate ligand C_{55}-P in a pH-dependent manner and show that lipopeptide antibiotics, such as amphomycin and aspartocin D, bind to UptA with higher affinity than other cell-wall targeting antimicrobial peptides such as bacitracin and vancomycin. Interestingly, amphomycin and its analogues outcompete the flippase UptA for C_{55}-P binding, highlighting a new mode of action and avenue for the development of novel antibiotics to intercept peptidoglycan biosynthesis.

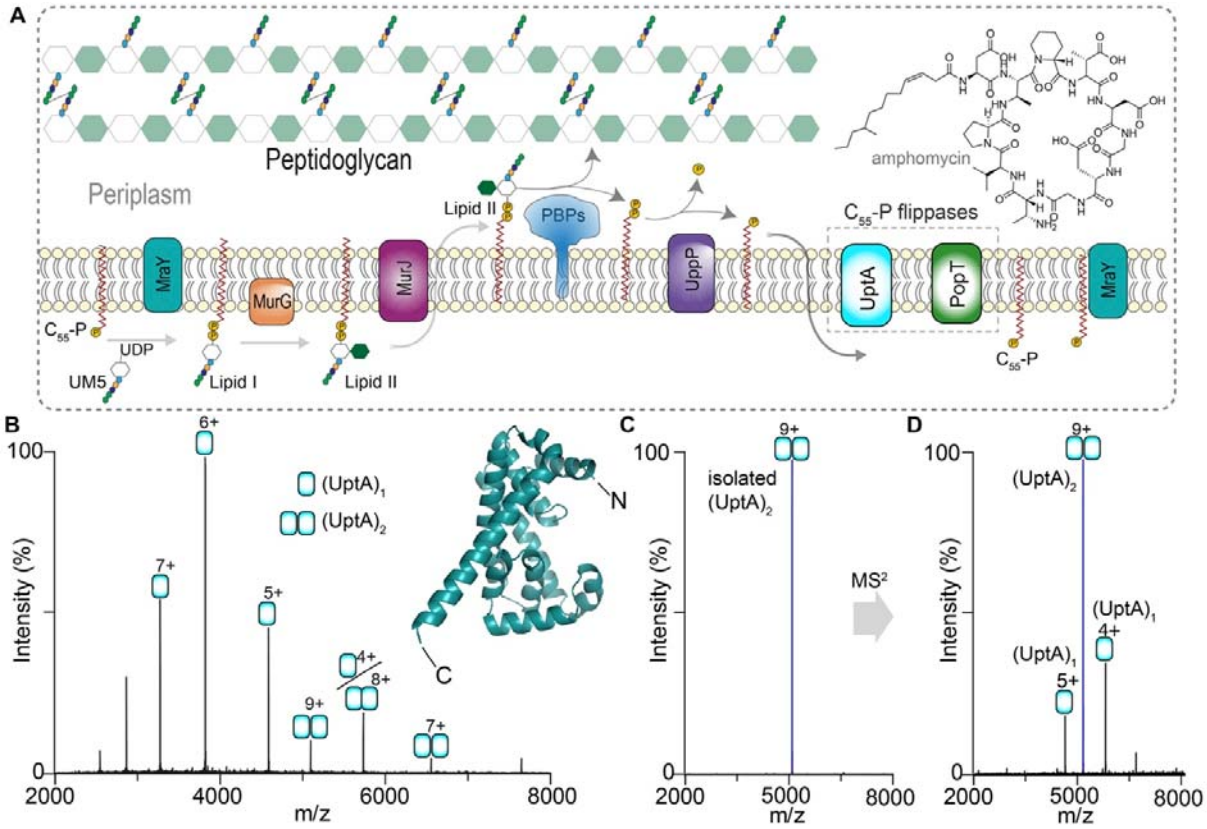


Fig. 1: UptA is primarily monomeric with a low population of dimer. A) Schematic depiction of the lipid II cycle of peptidoglycan biosynthesis. C_{55}-P, undecaprenyl monophosphate; C_{55}-PP, undecaprenyl diphosphate; UM5, UDP-*N*-acetylmuramyl-pentapeptide; PBPs, penicillin-binding proteins. **B**) Native mass spectrum of UptA (2.2 µM) liberated from a buffer containing 200 mM ammonium acetate (pH 8.0), 0.05% LDAO. Peaks are assigned to UptA in monomeric and dimeric forms. Insert, the model structure of UptA (AFO31823-F1) predicted by AlphaFold (31). Predicted N- and C- termini are indicated. **C**) Quadrupole isolation of the UptA dimer (9+ charge state). **D**)

MS/MS of the isolated UptA dimer upon collisional activation (190 V). The dimer dissociates into individual UptA protomers confirming their non-covalent association.

Results

Monomer-dimer equilibrium of UptA. We expressed *B. subtilis* UptA in *E. coli* and introduced the purified protein into the mass spectrometer from a buffer containing 0.05% LDAO and 200 mM ammonium acetate (pH 8.0). The mass spectrum of UptA displays two charge state distributions assigned to monomers (22963.49±0.70 Da) and dimers (45925.21±0.65 Da) (Fig. 1B). To confirm the non-covalent association of UptA monomers, we isolated the 9+ charge state assigned to the dimer and subjected these ions to high-energy collisional dissociation (HCD, Fig. 1C). The resulting MS^2 spectrum displays new peaks that correspond by mass to UptA protomers, and bear charges complementary to the isolated parent ions (+5 and +4) (Fig. 1D). Of note, the experimental mass for the UptA monomer is higher than the theoretical sequence mass (22936.36 Da) by 28 Da, suggesting an endogenous modification. To identify this unknown modification, we activated the monomer (6+ charge state) using HCD, yielding a plethora of *b*- and *y*-type fragment ions (Fig. S1). The observed fragments are consistent with the formylation of the N-terminal methionine of UptA (Fig. S1). Bacterial inner membrane proteins are normally co-translationally formylated on the N-terminus, however, this modification is rarely observed *in vitro* due to subsequent removal by the cytosolic deformylase (32). Therefore, retainment of N-terminal formylation on UptA suggests periplasmic localisation of the N-terminus of UptA, thus supporting the predicted N-out topology (23). Additionally, we observe UptA in monomeric and dimeric forms in other detergents (Fig. S2), confirming the oligomeric composition of UptA, which might apply to other bacterial DedA homologues.

UptA binds C_{55}-P with high affinity. We explored the binding affinity of UptA towards C_{55}-P by recording mass spectra for solutions containing 2.5 µM UptA and 0 to 40 µM C_{55}-P in 200 mM ammonium acetate, 0.05% LDAO at pH 8.0. The mass spectrum for the equimolar mixture of UptA and C_{55}-P yielded peaks corresponding to UptA in its apo form and in complex with C_{55}-P (Fig. 2A), an indication of high-affinity binding interactions. Further increase in the concentrations of C_{55}-P enhanced the intensity of the UptA:C_{55}-P complex, together with the emergence of additional binding events (Fig. 2A). We deconvoluted the spectra (33) to extract mean relative intensities of bound and unbound forms of UptA in the spectral series. The data reflected an increase in the relative abundance of UptA:$(C_{55}\text{-P})_n$ complexes as a function of the concentration of C_{55}-P (Fig. 2B). For the first binding event, the relative intensity plateaued at a protein/ligand molar ratio of ~4 (Fig. 2B). By fitting the experimentally observed intensity ratios to the Hill equation, we obtained an apparent K_d = 5.7 µM (95% CI: 5.0 to 6.4 µM) for the binding of one C_{55}-P molecule to UptA. This K_d

value for UptA/C_{55}-P binding is of the same order of magnitude as the binding interactions for MurG/lipid I (K_d = 1.89±0.6 μM) (34) and MurJ/lipid II (K_d = 2.9±0.6 μM) (14), indicating that UptA exhibits a similarly high affinity binding with respect to C_{55}-P.

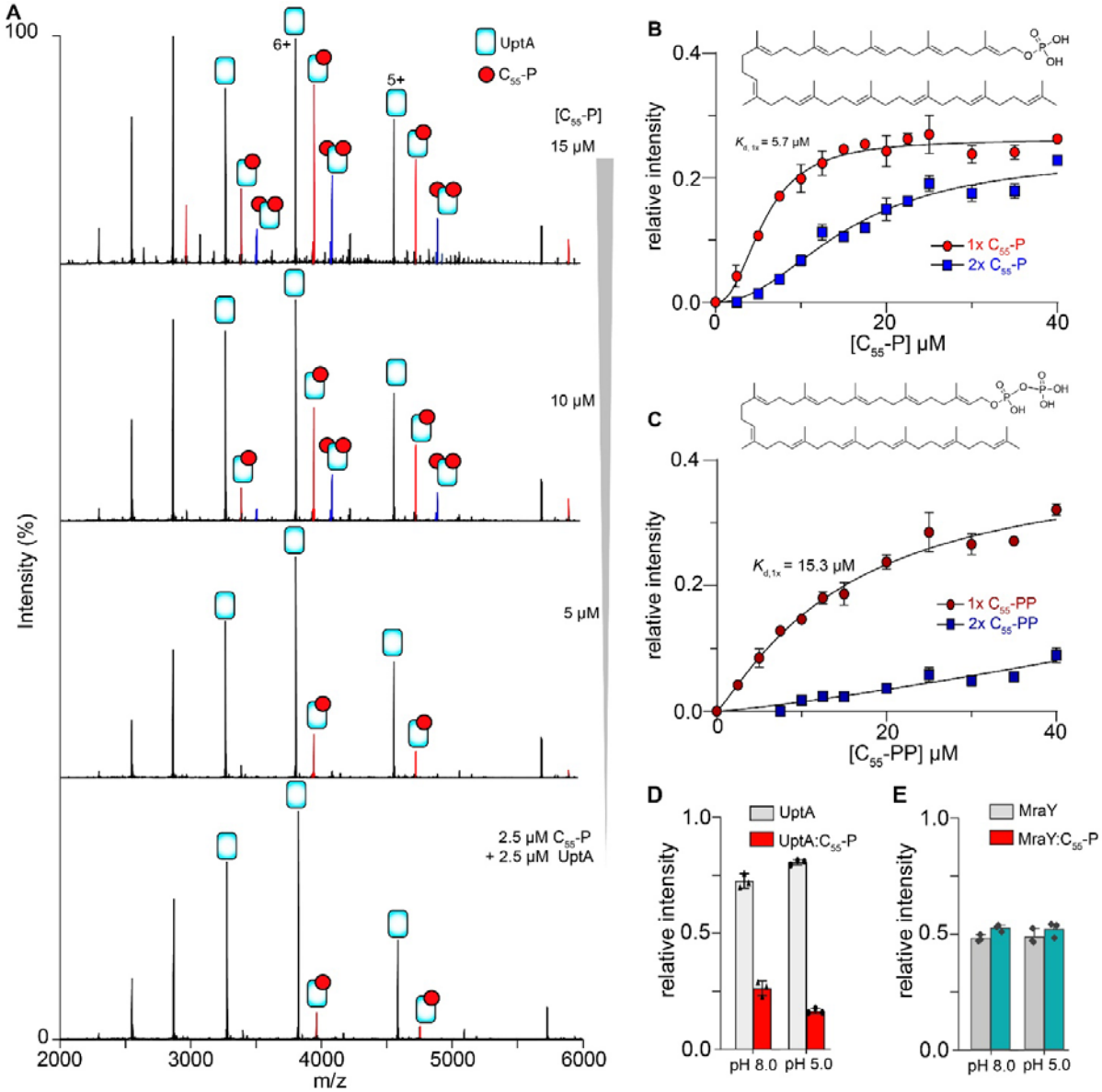


Fig 2. UptA binds C_{55}-P with a high affinity. A) Spectra for UptA incubated with different concentrations of C_{55}-P in a buffer containing 200 mM ammonium acetate (pH 8), 0.05% LDAO. **B-C**) The relative abundance of UptA bound to one (1x) or two (2x) molecules of C_{55}-P (panel B) and C_{55}-PP (panel C). Inserts, chemical structures of C_{55}-P and C_{55}-PP. Each data point represents an average of 3 replicate measurements, and the curves represent best fits of the data to Hill's equation (see methods), error bars are standard deviations. **D,E)** Relative abundance of ligand-free and C_{55}-P-bound to UptA (panel D) and MraY (panel E) at different pH. Bar represents the mean of 3 replicate measurements shown as data points, and the error bars are standard deviations.

To investigate the binding selectivity of UptA for monophosphate (C_{55}-P) versus diphosphate (C_{55}-PP) forms of the lipid carrier, we performed comparative binding assays. First, we recorded mass spectra for a mixture of UptA with increasing concentrations of C_{55}-PP (Fig. S3). We observed peaks assigned to UptA in ligand-free and C_{55}-PP-bound forms, and the relative intensity of the UptA:C_{55}-PP complex increased with increasing concentration of C_{55}-PP (Fig. 2C). We then fit the data to Hill equation to obtain an apparent K_d for this system. The relative intensities of UptA:C_{55}-PP yielded a significantly higher apparent K_d = 15.3 µM (95% CI: 9.5 to 32.8 µM) than the K_d obtained for UptA:C_{55}-P (K_d = 5.7±0.7 µM). Being within an order of magnitude, these dissociation constants suggest that UptA forms extensive contact with both forms of the lipid carriers, but that the protein interacts more favourably with C_{55}-P than C_{55}-PP. We therefore conclude that UptA is more selective towards C_{55}-P than C_{55}-PP, thus confirming its preferred endogenous substrate.

C_{55}-P binding to UptA is sensitive to pH. Membrane flippases such as MurJ, which is driven by proton-motive force (35), exhibit pH-dependency in ligand binding (14). We therefore considered the possibility that substrate interactions of UptA might exhibit pH dependency. To this end, we equilibrated C_{55}-P with UptA at a protein/ligand molar ratio of 1:4 in a buffer of the same composition but at pH 8.0 and pH 5.0. The resulting spectrum at pH 8.0 exhibits peaks consistent with monomeric UptA in an apo form, and in complex with 1 and 2 molecules of C_{55}-P (Fig. S4). We also observed UptA dimer in complex with up to two C_{55}-P molecules (Fig. S4). However, the intensity of peaks assigned to C_{55}-P-bound UptA is more intense at pH 8.0 compared to pH 5.0 (Fig. 2D). These results imply that more favourable protein-ligand interactions occur under physiologically relevant pH conditions. As a negative control, we tested for pH sensitivity of C_{55}-P binding to MraY, the latter being the downstream enzyme in the peptidoglycan pathway that couples UM5 to C_{55}-P. To this end, we equilibrated MraY and C_{55}-P at a molar ratio of 1:4 in a buffer of pH 8.0 and pH 5.0. In this case, the spectra show no significant difference in the intensity of C_{55}-P bound to MraY at pH 8.0 and at pH 5.0 (Fig. 2E, Fig. S4). We therefore conclude that environmental pH modulates the interaction of C_{55}-P with UptA, and potentially its transport mechanism.

Amino acid residues mediating C_{55}-P interactions with UptA.

Two conserved arginine residues (R112 and R118) in the membrane re-entrant loops of UptA have been shown to impair the cellular function of UptA by making the cells more susceptible to inhibition by amphomycin (23). Based on the model structure of UptA predicted by AlphaFold, the R112 residue should engage Q64 while R118 is able to form hydrogen bonds with E32, H119 and W146 to potentially stabilise the membrane re-entrant loops (Fig. 3A). To test for the impact of these residues on C_{55}-P binding, we generated the E32A, Q64A, R112A, R118A, H119A and W146A UptA point mutants. After purification of the proteins, using the same protocol as the wild type (WT), we

equilibrated aliquots of each protein variant (5 µM) with C_{55}-P (10 µM) and recorded spectra under the same instrument settings. The resulting spectra exhibited peaks consistent with C_{55}-P binding in all cases; only a modest reduction in C_{55}-P binding intensity was observed in the case of the R112A and R118A mutant compared to the wild type (Fig. S5, Fig. 3B). We surmised that single point mutation was insufficient to attenuate binding and therefore generated double mutants R112A/R118A and R118A/R119A. Comparing the C_{55}-P binding affinity of the double mutants with the WT, we found a more significant reduction in C_{55}-P binding for the R112A/R118A, suggesting that these residues are important for C_{55}-P binding. We additionally generated a double mutant R112E/R118E in which the polarity of both native arginine residues in the WT are reversed. This mutant exhibited the weakest C_{55}-P binding of all variants tested (Fig. 3B). This observation suggests that impaired network of hydrogen bonding between R118 and Q64 on the one hand, and R118 and E32 could have also disrupted the C_{55}-P binding sites, highlighting the critical roles of R112 and R118 in the interaction of UptA with C_{55}-P.

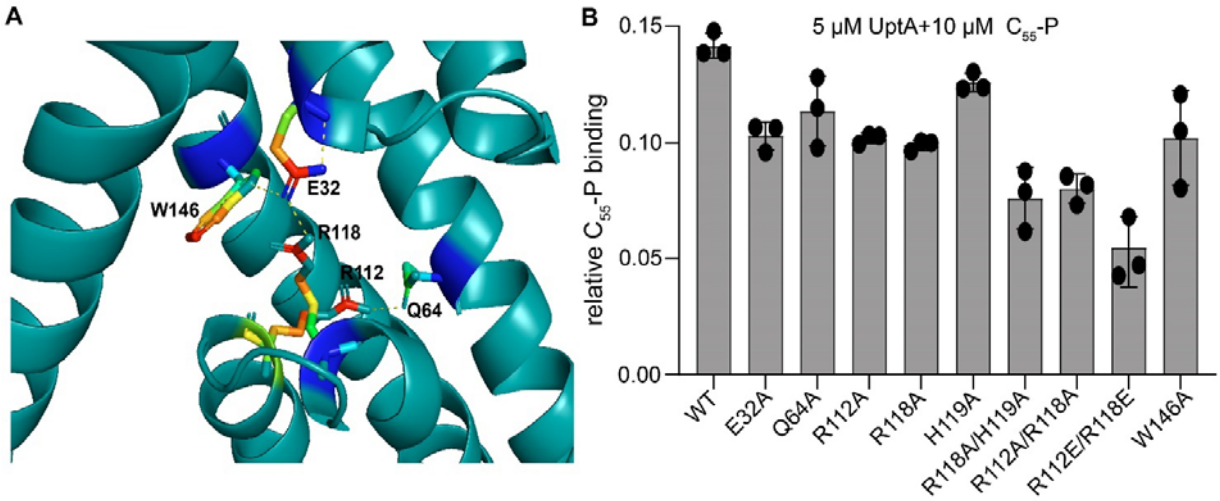


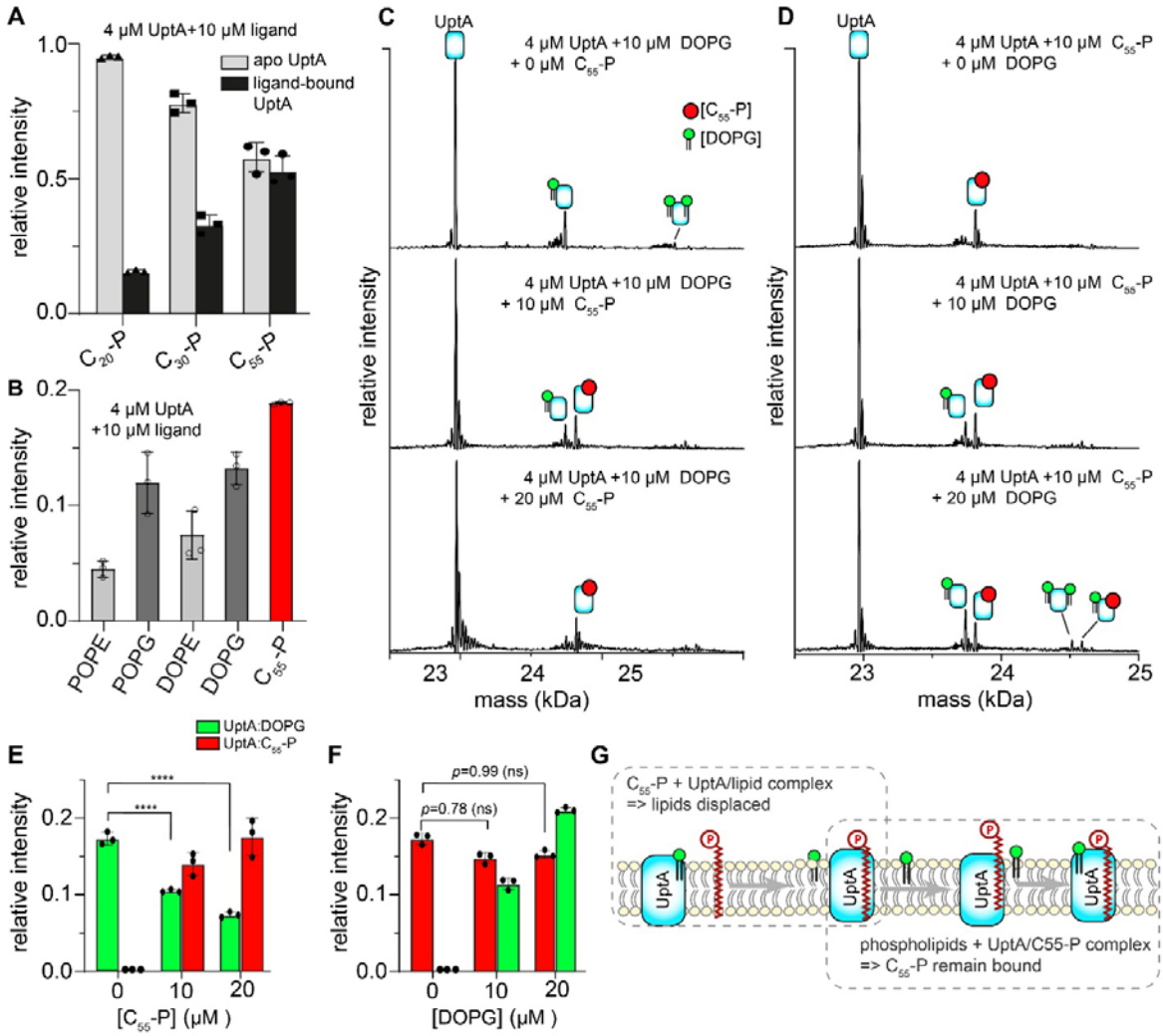
Fig. 3. Probing the amino acid residues involved in UptA-C_{55}-P binding. A. Zoomed views of UptA model structure showing hydrogen bonding networks surrounding R118 and R112. R112--Q64: 3.1 Å; R118--E32: 2.7 Å; and E32--W146: 3.5 Å. Hydrogen bonding distances were computed using ProteinTools(36) and the image was processed in PyMOL. **B.** Relative intensity of C_{55}-P bound to UptA wild type and mutants. Bars represent the mean of three biological replicates with each data point shown in black, error bars are standard deviations. The double mutant at residues R112 and R118 caused the most significant reduction in C_{55}-P binding.

C_{55}-P binds preferentially to dimeric forms of UptA. Since purified UptA displayed monomer-dimer equilibria, we considered the possibility that C_{55}-P might preferentially interact with one oligomeric species over the other. To probe this hypothesis, we used the UptA point mutant E32A

since the latter consistently retained a higher dimer population than the wild type (Fig. S6). We prepared and analysed solutions containing 2.5 µM E32A UptA and 0-20 µM C_{55}-P at pH 8.0. The resulting spectra exhibited peaks assigned to UptA monomers and dimers in complex with C_{55}-P (Fig. S6). We find that the relative intensity of ligand-bound dimers is higher than for the ligand-bound monomers, but the ligand binding does not significantly affect monomer-dimer ratios. This result indicates that the ligand C_{55}-P binds to dimeric UptA with a higher affinity than to the monomeric form.

UptA interacts more favourably with C_{55}-P than its shorter-chain analogues. Next, we investigated the chain length selectivity of UptA by testing its interaction with geranylgeranyl phosphate (C_{20}-P), hexaprenyl phosphate (C_{30}-P), and C_{55}-P. For this experiment, we incubated 4 µM UptA with a 10-fold molar excess of C_{20}-P, C_{30}-P and C_{55}-P at pH 8.0 and recorded spectra under the same conditions. For the UptA/C_{20}-P sample, the spectrum exhibits low-intensity peaks corresponding by mass to UptA:C_{20}-P complexes, leaving most of the protein in the apo form (Fig. S7). In the case of UptA/C_{30}-P, we observed more intense protein/ligand complexes, indicating a higher binding affinity of UptA to C_{30}-P compared to C_{20}-P (Fig. S7). The spectra recorded for the UptA/C_{55}-P mixture present peaks with the highest intensity of protein/ligand complexes (Fig. S7), indicating a more favourable interaction for C_{55}-P compared to the lipidic substrate with shorter aliphatic chains. Accordingly, the relative abundance of UptA in complex with C_{55}-P is higher than in the case of UptA-bound C_{30}-P and C_{20}-P (Fig. 4B). Thus, the interaction of UptA with the lipid carrier is enhanced by the hydrophobic tail of C_{55}-P, supporting our hypothesis that the long polyprenyl tail of the lipid carrier forms extensive contact with UptA. Together, these data are consistent with C_{55}-P being the preferred endogenous substrate for UptA.

UptA bind more favourable to anionic phospholipids than cationic lipids. DedA family proteins are proposed to either translocate carrier lipids or membrane phospholipids. We therefore probed the interaction of UptA with membrane phospholipids to understand if the latter could modulate its function. For this purpose, we selected the anionic lipids phosphatidylglycerols (PG) and phosphatidylethanolamines (PE), being the major classes of phospholipid headgroups found in *B. subtilis* (37). We then recorded mass spectra of solutions containing 4 µM UptA incubated with 10 µM POPE (16:0/18:1 PE), and 10 µM DOPE (18:1/18:1 PE). The spectra display peaks corresponding to UptA in apo and lipid-bound forms in both cases (Fig. S8). However, the mean relative intensity of UptA:POPG complex is higher than for UptA:POPE complexes (Fig. 4B), indicating that UptA interacts more favourably with POPG than with POPE. Similarly, DOPG (18:1/18:1 PG), binds more intensely to UptA than DOPE (18:1/18:1 PG) (Fig. 4B). These observations suggest that UptA interacts more favourably with anionic than cationic lipids.



Importantly, C_{55}-P binds to UptA with higher relative affinity than any of these diacylglycerol phospholipids (Fig. 4B), in line with C_{55}-P being its native substrate.

Fig 4. Undecaprenyl phosphate outcompetes phospholipids for UptA binding. A) Relative intensity of ligand-free and ligand-bound UptA incubated with C_{20}-P, C_{30}-P and C_{55}-P. UptA binds to C_{55}-P more intensely than the shorter chain analogues. **B)** Relative intensity of UptA bound to phospholipids and C_{55}-P. UptA interacts more favourably with C_{55}-P than with the phospholipids. Bar represents the mean of three independent replicates shown as data points, and error bars are standard deviations. **C**) Deconvoluted native mass spectra for 4 μM UptA and10 μM DOPG in the presence of increasing concentrations of C_{55}-P. DOPG-bound to UptA was significantly displaced by C_{55}-P. **D**) Spectra for 4 μM UptA and 10 μM C_{55}-P, then incubated with increasing concentrations of DOPG. C_{55}-P were not significantly displaced from UptA by DOPG. **E,F)** Relative intensities of UptA:DOPG (green) and UptA:C_{55}-P (red) from the spectra shown in panel C and D, respectively. Bar represents the mean of three independent replicates shown as data points, error bars are

standard deviations. P-values from two-tailed t-tests with n=3; ****p<0.0001; ns, not significant. **G)** Schematic illustrations of phospholipids and C_{55}-P competing for UptA binding.

C_{55}-P outcompetes phospholipids for UptA binding. To investigate whether or not C_{55}-P can outcompete phospholipids in binding to UptA, we incubated solutions containing UptA-DOPG with increasing concentrations of C_{55}-P. Spectra were recorded after aliquots of the solution were equilibrated on ice for 15 mins. The spectrum of UptA/DOPG mixture in the absence of C_{55}-P reflected the binding of 1-2 molecules of DOPG per UptA (Fig. 4C). The presence of C_{55}-P in the sample yielded new peak corresponding to UptA:C_{55}-P complex, and simultaneously caused attenuation of the intensity DOPG-bound UptA in a concentration-dependent manner. At the highest C_{55}-P concentration tested (20 µM), C_{55}-P has displaced most of DOPG from UptA (Fig. 4C,E). We therefore hypothesised that phospholipids should be unable to outcompete C_{55}-P bound to UptA. To test this hypothesis, we incubated UptA with C_{55}-P and then equilibrated aliquots with an increasing concentration of DOPG. Indeed, C_{55}-P remains bound to UptA even in the presence of excess DOPG (20 µM) (Fig. 4D,F), indicating that phospholipids could not significantly displace C_{55}-P from UptA. Analogous experiments with DOPE show that C_{55}-P effectively outcompete DOPE for UptA binding but the latter could not efficiently displace C_{55}-P from UptA (Fig. S9). Together this study shows that UptA interacts more favourably with C_{55}-P than with membrane phospholipids, thereby establishing C_{55}-P as the preferred endogenous substrate.

Lipopeptide antibiotics disrupt UptA:C_{55}-P complex. We next sought to identify an antibiotic that can intercept flippase function by disrupting its interaction with the carrier lipid substrate. We tested a range of cell-wall targeting antibiotics before selecting daptomycin, amphomycin, and aspartocin, as these lipopeptides bind to UptA with a high affinity (see below). Daptomycin is known to kill bacteria by depolarising the membrane (38) and by complexing with lipid II (39). We investigated if daptomycin affects C_{55}-P binding to UptA by recording spectra for solutions containing 2.5 µM UptA, 10 µM C_{55}-P and different concentrations of daptomycin. In the absence of antibiotics, the spectrum exhibited peaks assigned to UptA in apo form, and in complex with 1 and 2 C_{55}-P molecules (Fig. S10). In the presence of daptomycin, we observed additional peaks corresponding to the binary complex UptA:daptomycin and the ternary complex UptA:C_{55}-P:daptomycin (Fig. S10). Further increases in daptomycin concentration resulted in higher intensity of these complexes, indicating that daptomycin does not directly compete against C_{55}-P binding to UptA.

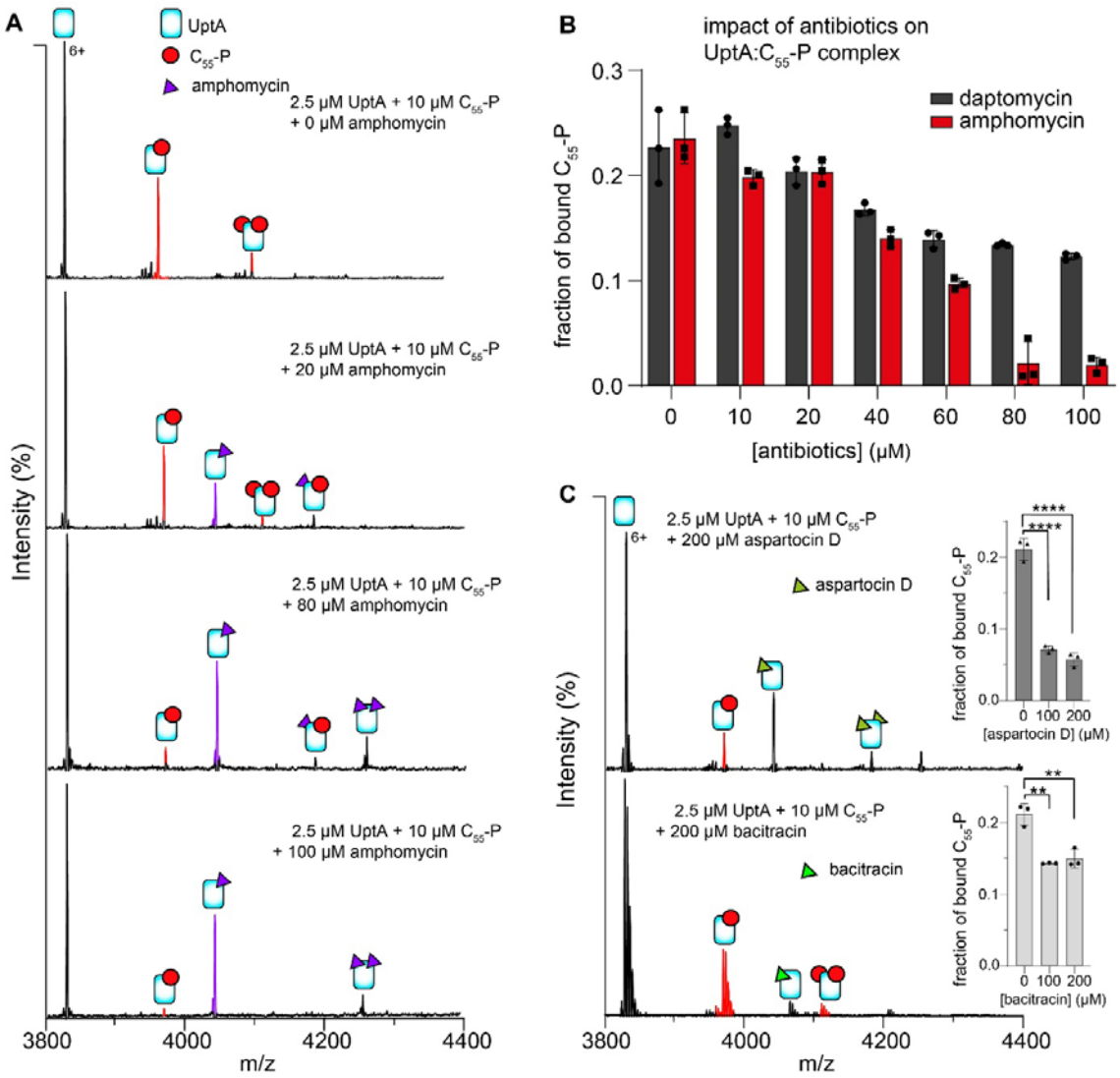


Fig. 5 UptA function can be intercepted by lipopeptide antibiotics. A) Mass spectra (6+ charge state) for solutions containing 2.5 μM UptA and 10 μM C_{55}-P incubated with 0-100 μM amphomycin. **B**) Relative intensity of C_{55}-P bound to UptA in the presence of amphomycin and daptomycin. Bar represents the mean of three independent replicates shown as data points and error bars are standard deviations. Amphomycin destabilises the UptA:C_{55}-P complex whereas daptomycin has a modest impact. **C)** Representative spectra for UptA/C_{55}-P mixtures in the presence of aspartocin D and bacitracin. Inserts, the mean fraction of C_{55}-P bound to UptA in the presence of excess aspartocin and bacitracin. Bar represents the mean of three independent replicates shown as data points, and error bar represents standard deviations. P-values from two-tailed t-tests with n=3; ****$p<0.0001$; **$p<0.006$.

Amphomycin and derivatives are known to kill bacteria by forming complexes with free C_{55}-P (40), however, whether or not this involves a direct interaction with a protein cellular target is not known. We therefore investigated how amphomycin affects the UptA:C_{55}-P complex. We recorded spectra for solutions containing 2.5 µM UptA, 10 µM C_{55}-P and then equilibrated aliquots with increasing concentrations of amphomycin. The spectra exhibited peaks consistent with amphomycin binding to UptA, in addition to C_{55}-P binding (Fig. 5A). We find that the peak intensities of the UptA:C_{55}-P complex reduce with respect to an increase in the concentration of amphomycin (Fig. 5B). Compared to the case of daptomycin, little-to-no UptA:C_{55}-P complexes remained in the presence of 10-fold molar excess of amphomycin (Fig. 5B), suggesting that this effect is unique to amphomycin. We then performed similar experiments with aspartocin D, an analogue of amphomycin. The resulting spectrum shows that aspartocin D similarly binds to UptA and also induces dissociation of UptA:C_{55}-P complex (Fig. 5C). To verify these results, we studied the impact of bacitracin and vancomycin on UptA:C_{55}-P interactions. Bacitracin and vancomycin are peptide antibiotics whose mode of action involves sequestration of C_{55}-PP and lipid II, respectively (41, 42). To this end, we prepared UptA/C_{55}-P mixtures and then equilibrated aliquots with bacitracin and vancomycin. In line with the specificity of bacitracin for C_{55}-PP rather than C_{55}-P, bacitracin binds to UptA only with low intensity and modestly competes against C_{55}-P binding to UptA, even at elevated concentrations of 200 µM (Fig. 5C). By contrast, vancomycin has no observable impact on the binding intensity of C_{55}-P with UptA. Taken together, these data indicate that amphomycin and aspartocin D induce dissociation of UptA:C_{55}-P complexes, and can also interfere with peptidoglycan pathway by binding directly to UptA in addition to their known propensity to form complexes with free C_{55}-P molecules (40).

Discussions

In this study, using native MS, we investigated the interplay of multiple lipidic substrates and antibiotics with UptA (summarised in Fig. 6). Specifically, we report that purified UptA exists as an equilibrium of monomers and dimers, suggesting that DedA proteins might form dimers in the native membrane when lateral pressures in the lipid bilayers (43) might prevent their dissociation. In line with the dependency of DedA family proteins on proton motive force (44), we observed less C_{55}-P binding to UptA in the presence of excess H^+ and that the monomer-dimer ratio of UptA is sensitive to pH rather than to C_{55}-P binding. This feature contrasts with those of the upstream peptidoglycan enzymes MraY (34, 45) and UppP (46, 47) whose dimerization are promoted by lipid binding.

Our studies also show that UptA selectively binds to C_{55}-P over C_{55}-PP and that the protein interacts more favourably with C_{55}-P than the shorter chain analogues C_{30}-P and C_{20}-P. This implies that the hydrophobic tail of C_{55}-P makes extensive contact with UptA, promoting the binding interactions and

potentially facilitating the transport of carrier lipids. The higher affinity of UptA towards the monophosphate form of the carrier lipid, rather than to the diphosphate form, favours a mechanism in which UptA mediates unidirectional flipping of C_{55}-P rather than acting as a scramblase for both C_{55}-P and C_{55}-PP. We further show that UptA interacts more favourably with the anionic lipids DOPG and POPG than the cationic lipids DOPE and POPE and that these lipids are readily displaced by C_{55}-P. This binding preference agrees with UptA being responsible for C_{55}-P rather than the phospholipid translocation (23). Competition between anionic lipids, which are more abundant in the native lipid bilayer than C_{55}-P, suggests that both lipids bind near the same sites on UptA and that the local lipid environment of UptA can potentially modulate its cellular function.

We provided molecular-level evidence that the conserved arginine (R112 and R118) residues in the putative membrane re-entrant helices of UptA are important for its substrate binding interactions, confirming earlier reports that *B. subtilis* strains bearing these mutations on UptA are more susceptible to inhibition by MX2401, a derivative of amphomycin (23). Despite the combined cellular pool of C_{55}-PP and C_{55}-P being limited to ~1.5×10^5 molecules per cell (48), they mediate the transport of glycan-bearing components of the cell wall peptidoglycan, teichoic acid and capsular polysaccharide (49). Therefore, antibiotics that intercept C_{55}-P and its flippases are potential drug targets that can potentially intercept multiple pathways and thus mitigate drug resistance. Accordingly, bacitracin and teixobactin form complexes with C_{55}-PP (50, 51), while amphomycin analogues form complexes with C_{55}-P to intercept bacterial cell wall formation (23, 40, 52, 53). Herein we show that amphomycin and aspartocin D can additionally bind directly to UptA and induce dissociation of the UptA: C_{55}-P complex, a finding that can therefore guide the development of new antibiotic analogues to intercept the flippase function of UptA and its homologues.

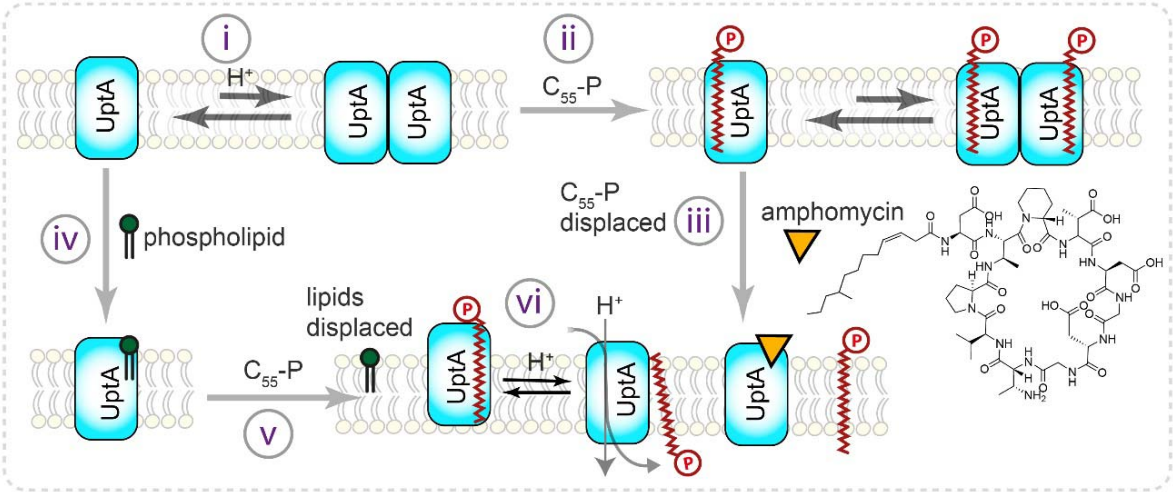


Fig. 6 Insights from native MS into the interaction of UptA with lipidic substrates and antibiotics. (i) UptA exhibits monomer-dimer equilibria, with more dimer at pH 8.0 than pH 5.0. (ii) UptA interacts with C_{55}-P more favourably than C_{55}-PP, C_{30}-P, and C_{20}-P. (iii) Amphomycin destabilises the UptA:C_{55}-P complex, effectively out-competing the flippase for its carrier lipid substrate. (iv) UptA binds phospholipids, with a higher preference for the anionic than cationic lipids. (v) C_{55}-P is able to displace UptA-bound phospholipids, suggesting that both ligands interact with UptA near the same sites. (iv) Binding of H^+ to UptA enables translocation of C_{55}-P and its subsequent release for the next round of PG precursor synthesis.

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Supporting information

Lipopeptide antibiotics disrupt interactions of undecaprenyl phosphate with UptA

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Methods

Chemicals

C_{55}-PP, C_{55}-P, C_{30}-P and C_{20}-P were purchased from Larodan. Phospholipids – POPE, DOPE, POPG, DOPG – were purchased from Avanti Polar Lipids. Amphomycin and aspartocin D were purchased from Cayman; while Daptomycin and bacitracin were purchased from Merck. *n*-dodecyl-*β*-D-maltoside (DDM), *n*-decyl-*β*-D-maltoside (DDM), n-octylglucoside (OG) and *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (LDAO) were from Anatrace.

Plasmids

A codon-optimised version of *Bacillus subtilis* UptA (yngC) (Integrated DNA Technologies) was inserted into a modified pET15b vector between NdeI and NheI endonuclease restriction sites by In-Fusion HD cloning kit (Takara Bio). The resulting plasmid encodes UptA fused to GFP-His_6 on the C-terminus via a TEV-protease cleavable site. Plasmids for UptA mutants were generated by site directed mutagenesis with CloneAmp HiFi PCR Premix (Takara Bio) using pairs of oligonucleotides listed in Table S1. The plasmid pET26-MBP-MraYaa bearing HRV3C protease cleavage site between the MBP and the wild-type MraY was a kind gift from S-Y Lee (Addgene plasmid #100166) (1).

Table S1: Oligonucleotides using to generate plasmids for the expression of UptA mutants.

Primers	Sequence
E32AF	TCCAAGCGCAATTGTATTGGCCTACGGTGG
E32AR	ACAATTGCGCTTGGAATAATTTCAATCATCAGT
Q64AF	GATCGCCGCAATTTTCATTTACTGGATTGGCCGT
Q64AR	AAAATTGCGGCGATCGTTCCCCCGATA
R112AF	TTCTGCTGCCTTCATCCCTGTAGTCCGTCATGC
R112AR	ATGAAGGCAGCAGAAAACACAACCCCCG
R118AF	TGTAGTCGCACATGCTATTTCGATCCCAGCCG
W146AF	CATTCCTGCCTCCATCCTGTTCGTTTACCTGG
W146AR	ATGGAGGCAGGAATGATGGCAAGTACTGTC
R112A/R118A_Fwd	TTCTGCTGCCTTCATCCCTGTAGTCGCACATGCTATTTCGATCCCAGCCG
R112A/R118A_Rev	GCATGTGCGACTACAGGGATGAAGGCAGCAGAAAACACAACCCCCG
R112E/R118E_Fwd	ATTCATCCCTGTAGTCGAACATGCTATTTCGATCCCAGCCG
R112E/R118E_Rev	ACTACAGGGATGAATTCAGCAGAAAACACAACCCCCG

Expression and purification of UptA

Plasmids were used to transform chemically competent *E. coli* C43(DE3) cells (Lucigen) and selected on LB/Agar containing 100 µg/ml ampicillin at 37 °C. Single colonies were used to inoculate 60 mL LB containing 100 µg/ml ampicillin and were grown at 37 °C overnight for 16 h. For large-scale protein expression, 10 mL of the overnight culture was aseptically transferred into 1 L of LB/ampicillin and cells were allowed to grow at 37°C until the $OD_{600\ nm}$ reached 0.6-0.8 after which the temperature was lowered to 18 ^{o}C. Expression of UptA-GFP was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM UptA-GFP and shaking for 20 h at 18 °C. Cells were harvested by centrifugation at 5000 × *g* and lysed immediately or stored at -80 ° C until required.

Cells were thawed on ice and resuspended in a buffer containing 50 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 µg/mL deoxyribonuclease I (DNAse I), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and EDTA-free protease inhibitor cocktail tablets. The cells were then disrupted with a microfluidiser (Microfluidics) operated at 20,000 psi. Non-lysed cells and debris were pelleted by centrifugation at 20,000 × *g* for 20 min and the clarified lysate was retained. The membranes fraction was then pelleted by ultracentrifugation at 100,000 x g for 1 h. They were resuspended in a buffer containing 20 mM Tris-HCl (pH 8.0), 300 mM NaCl, 20% glycerol. Membrane aliquots were either solubilised immediately or flash-frozen in liquid nitrogen and stored at -80°C. The membrane was solubilised by incubation with DDM at a final concentration of 1% (w/v) for 60 min. Non-solubilised aggregates were removed by centrifugation at 20,000×g for 20 min and the supernatant was loaded onto a 5-ml HisTrap HP column preequilibrated in buffer A (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, 0.03% DDM and 20 mM imidazole). After binding of the His-tagged protein, the column was washed with 100 mL of buffer A, and then with 50 mL of buffer B (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, 0.03% DDM and 100 mM imidazole). UptA-GFP was eluted from the column with buffer C (20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 10% glycerol, 0.02% DDM and 300 mM imidazole). To remove imidazole and to cleave the GFP fusion, eluted protein was mixed with TEV protease and dialysed against buffer D (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 0.02% DDM) at 4°C for 16 h.

The digestion mixture was subsequently incubated with 2-mL bed volume of nickel nitriloacetic acid (NTA) agarose resin to remove His-tagged GFP and TEV protease. Tag-free UptA was collected as the flowthrough, concentrated using an Amicon centrifugal concentrator with nominal molecular weight cut-off 50 kDa. The protein was finally purified by size-exclusion chromatography (SEC) using a 24-mL Superdex 200 Increase column. The SEC buffer was 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.02% DDM. UptA was concentrated to ~200 uM, aliquoted, flash-frozen flash in liquid nitrogen, and stored at -80 °C until use. Concentration was measured using a Biomate UV spectrophotometer at 280 nm using extinction coefficients calculated from their predicted amino acid

sequences. UptA mutants were purified in 3 biological replicates, using the same procedures as for the wild type.

Expression and purification of MraY.

MraY from *Aquifex aeolicus* was purified as previously described (3). Briefly, the plasmid pET26-HisMBP-MraYaa was used to transform chemically competent *E. coli* C41(DE3) cells (Lucigen) and selected on LB/Agar containing 50 µg/ml kanamycin at 37 °C. Cells were grown in Terrific Broth media supplemented with 50 µg/ml kanamycin at 37°C until the $OD_{600\,nm}$ reached 0.8. Protein expression was induced by adding IPTG at a final concentration of 1 mM for 4 h at 37 °C. Cells were harvested by centrifugation at 5000 × *g* and lysed and solubilized by incubation with 1% DDM for 1 h. His-tagged MBP-MraY was purified by affinity chromatography and digested by HRV3C protease. MraY was isolated by SEC into a buffer containing 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.02% DDM.

Sample preparation for native MS

Before measurements, the protein was buffer-exchanged into 0.05% LDAO, 200 mM ammonium acetate ("MS buffer") at the desired pH using a centrifugal buffer exchange device (Micro Bio-Spin 6, Bio-Rad). Stock solutions of lipids were prepared from chloroform/methanol solution by evaporating aliquots of known volume in a SpeedVac. After drying, the lipid films were weighed and resuspended to a final concentration of 0.5 mM in 200 mM ammonium acetate (pH8) and 0.05% LDAO by vertexing. Amphomycin and aspartocin D were purchased from Cayman while Daptomycin and bacitracin were purchased from Merck. Stock solutions of 0.5 mM amphomycin, aspartocin D, daptomycin and bacitracin, were made in the same buffer. All experiments were repeated three times from newly prepared stock solutions.

Native mass spectrometry

About 3 µL protein aliquot was transferred into a gold-coated borosilicate capillary (Harvard Apparatus) and was mounted on the nano ESI source of a Q-Exactive hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The instrument settings were 1.2 kV capillary voltage, S-lens RF 200%, argon UHV pressure 3.3×10^{-10} mbar, the capillary temperature was set to 200 °C, and resolution of the instrument was set to 17,500 at a transient time of 64 ms. Voltages of the ion transfer optics –injection flatapole, inter-flatapole lens, bent flatapole, and transfer multipole were set to 5, 3, 2, and 30 V respectively. The noise level was set at 3. Unless otherwise stated, proteins were activated by applying 75 V in the high-energy collisional dissociation cell without in-source trapping. For the MS^2 spectra, parent ions were isolated using an in-source trapping voltage of -200 V, and then fragmented by applying 150-200 V in the high-energy collision-induced dissociation cell. To identify endogenous modifications using native top-down mass spectrometry, the 6+ charge state of monomeric UptA was isolated (3828 ± 5 Th) and activated using HCD (200 V) with reduced trapping pressures. The resulting fragments were detected in the

Orbitrap (R = 280,000 @ *m*/*z* 200), averaging ~500 transients to maximise the signal-to-noise ratio. Theoretical fragments from N-terminally formylated UptA were matched to the MS^2 spectrum (3 ppm) using TDValidator (Proteinaceous) and manual validation. Data were visualised and exported for processing using the Qual browser of Xcalibur 4.1.31.9 (Thermo Scientific). Spectral deconvolution was performed using UniDec 6.0.2 (2). Relative binding affinities were obtained from deconvoluted spectra by dividing the intensity of ligand-bound protein peaks by the sum of the intensities of ligand-bound and ligand-free protein peaks. The mean and standard deviation of these fractional binding intensities from three independent experiments were plotted against the concentrations of the ligand C_{55}-P or C_{55}-PP. To obtain the apparent dissociation constant K_d, the mean relative intensities at different concentrations were fitted globally using GraphPad Prism 10.2.0 to Eq. (1):

$$y = \frac{B_{max} x^h}{x^h + K_d{}^h} \qquad (1)$$

Where y is the mean relative intensity and x is the corresponding ligand concentration. B_{max} is the maximum specific binding, K_d is the apparent dissociation constant, and h is the Hill coefficient.

Supplementary Figures

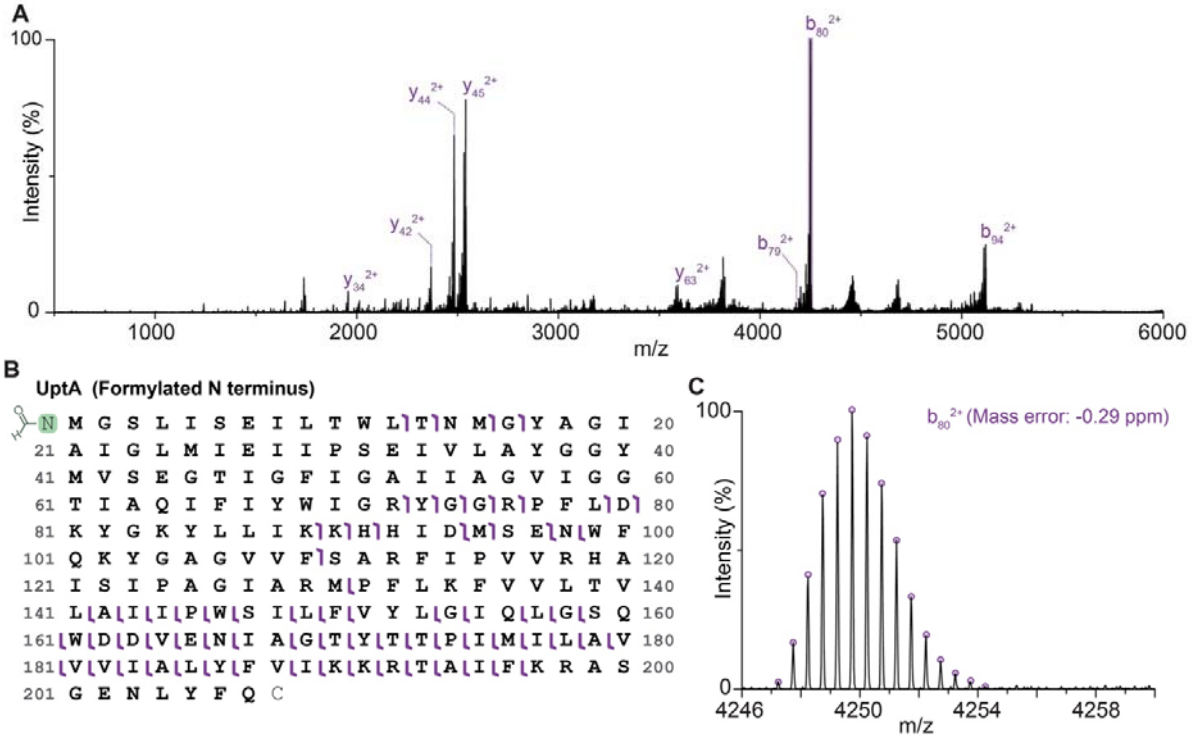

Fig. S1. UptA is formylated at the N-terminus. A. HCD MS/MS spectrum of UptA (6+). The eight most abundant assigned ions are labelled. **B**. Sequence map of the UptA construct used in this study. Terminal *b*- and *y*-type fragment ions observed in the MS/MS spectrum are indicated. **C.** Expanded view of the peak corresponding to b_{80}^{2+} ion**.** The theoretical isotopic envelope is overlaid.

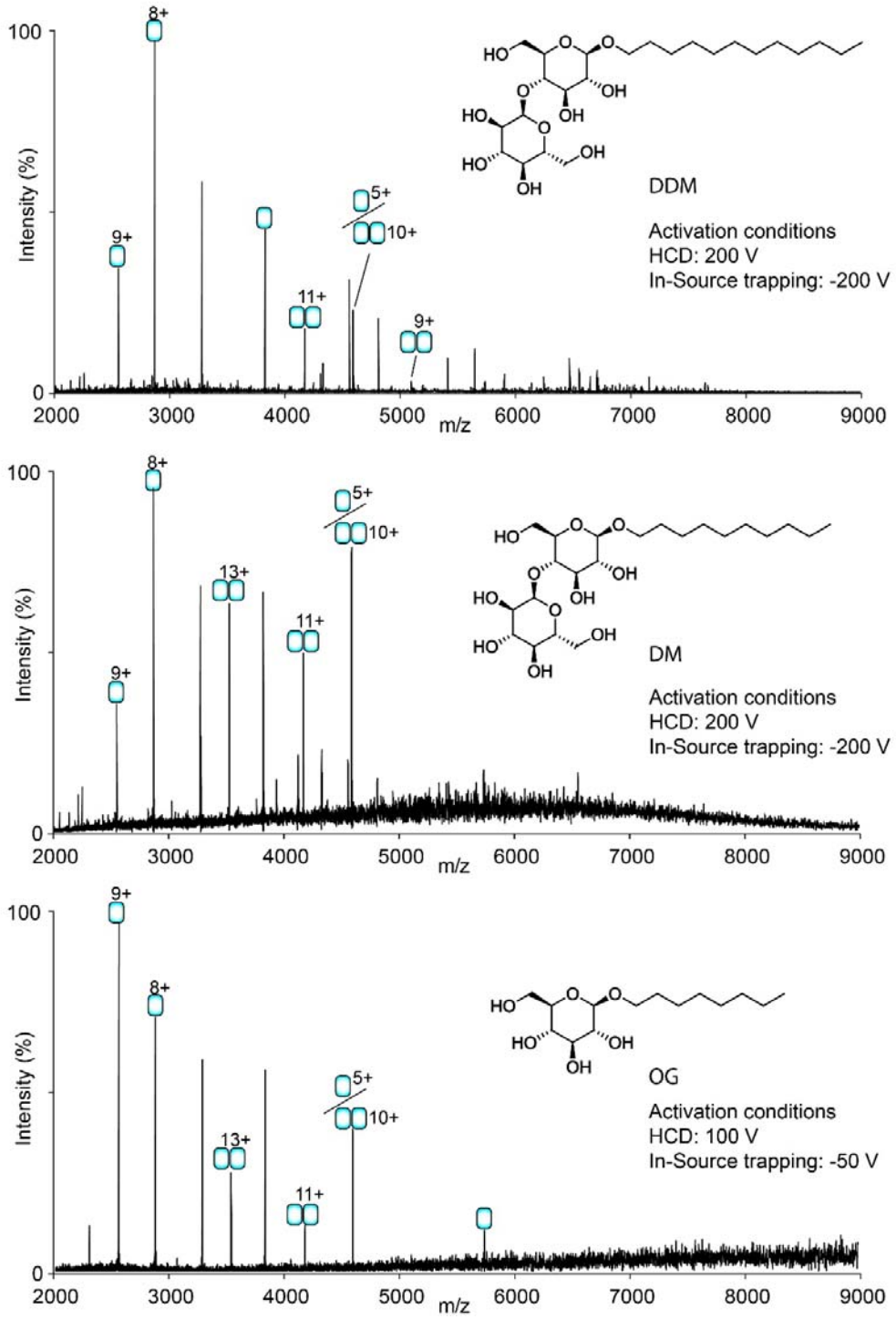

Fig. S2. Oligomeric state of UptA in different detergents. Shown are the spectra recorded for UptA in buffer containing 200 mM Ammonium acetate (pH 8.0) supplemented with indicated detergents. The instrument was tuned differently in each case to maximise the transmission of dimeric species.

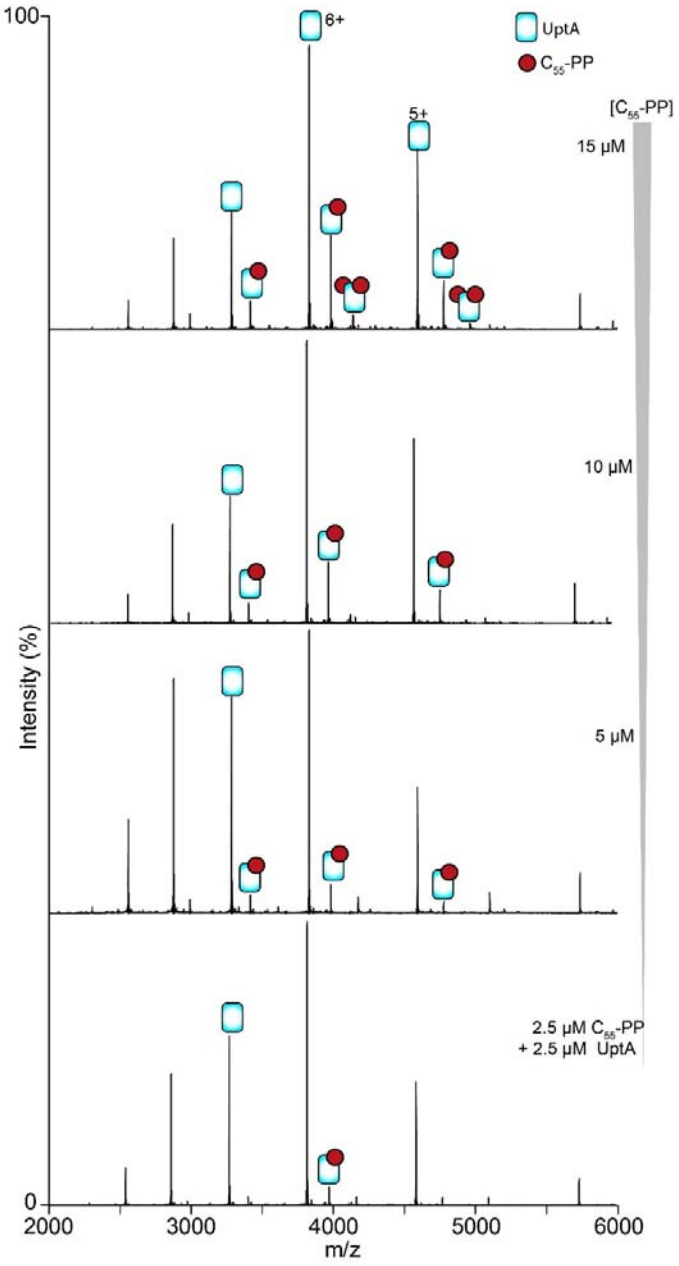

Fig. S3. UptA:C_{55}-PP binding interactions. Spectra for UptA incubated with different concentrations of C_{55}-PP in a buffer containing 200 mM ammonium acetate (pH 8), 0.05% LDAO. UptA bind C_{55}-PP but with lesser intensities compared to C_{55}-P (cf. Fig. 2A).

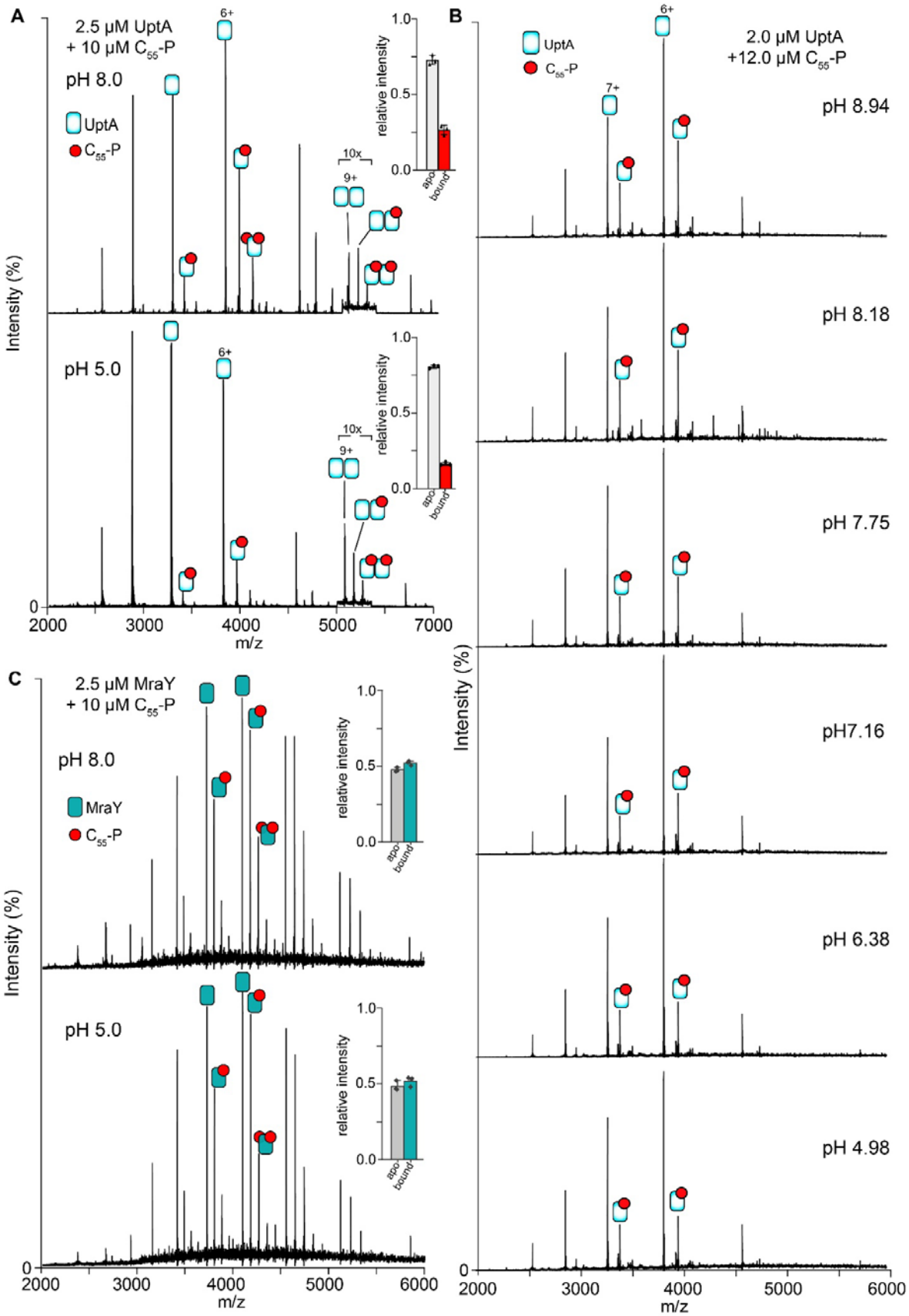

Fig. S4. pH dependency of C_{55}-P binding interactions with UptA and MraY. A. Spectra for

UptA/C_{55}-P mixtures at indicated pH. Inserts, the relative abundance of apo UptA and C_{55}-P bound forms. **B.** Spectra for UptA:C55-P mixtures as a function of pH. UptA (20 µM) and C_{55}-P (120 µM) were prepared in 200 mM ammonium acetate (pH 8.9) and 0.5% LDAO to allow formation of UptA: C_{55}-P complex at pH 8.9. Sample aliquots were then prepared from this stock solution by 10-fold dilutions with a buffer of indicated pH. Decrease in pH caused a release of C_{55}-P bound to UptA**.** Samples were incubated for 30 min at 20°C. **C.** Spectrum for MraY/C_{55}-P mixture at pH 8.0 and pH 5.0. There is no pH-dependency in C_{55}-P binding by MraY.

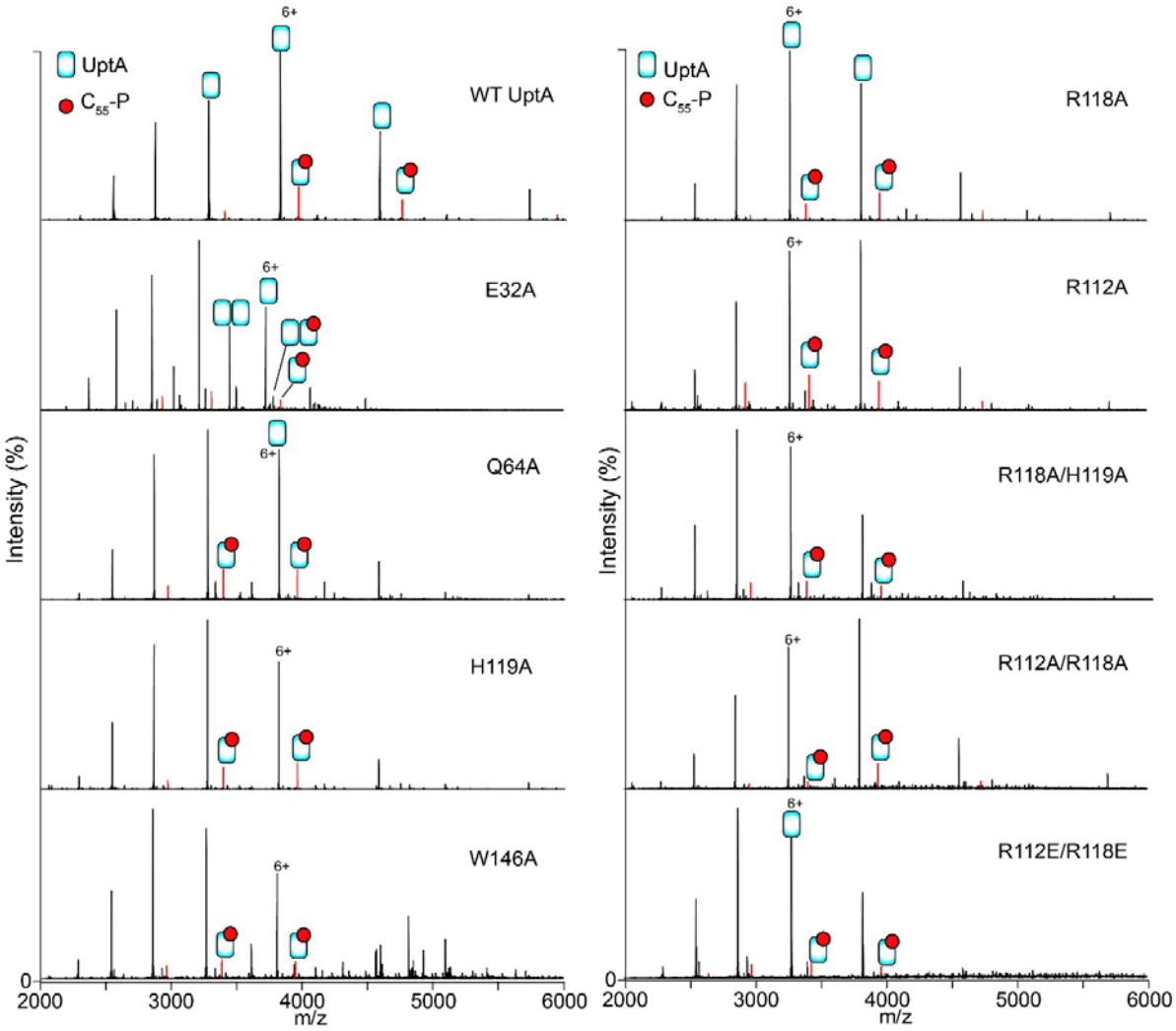

Fig. S5. Native MS analysis of C_{55}-P binding to UptA mutants. Shown are the representative spectra for UptA wild type and mutants (5 uM) incubated with C_{55}-P (10 uM). Proteins were liberated from a buffer containing 0.05% LDAO and 200 mM ammonium acetate (pH8. 02) using a collisional activation of 100 V.

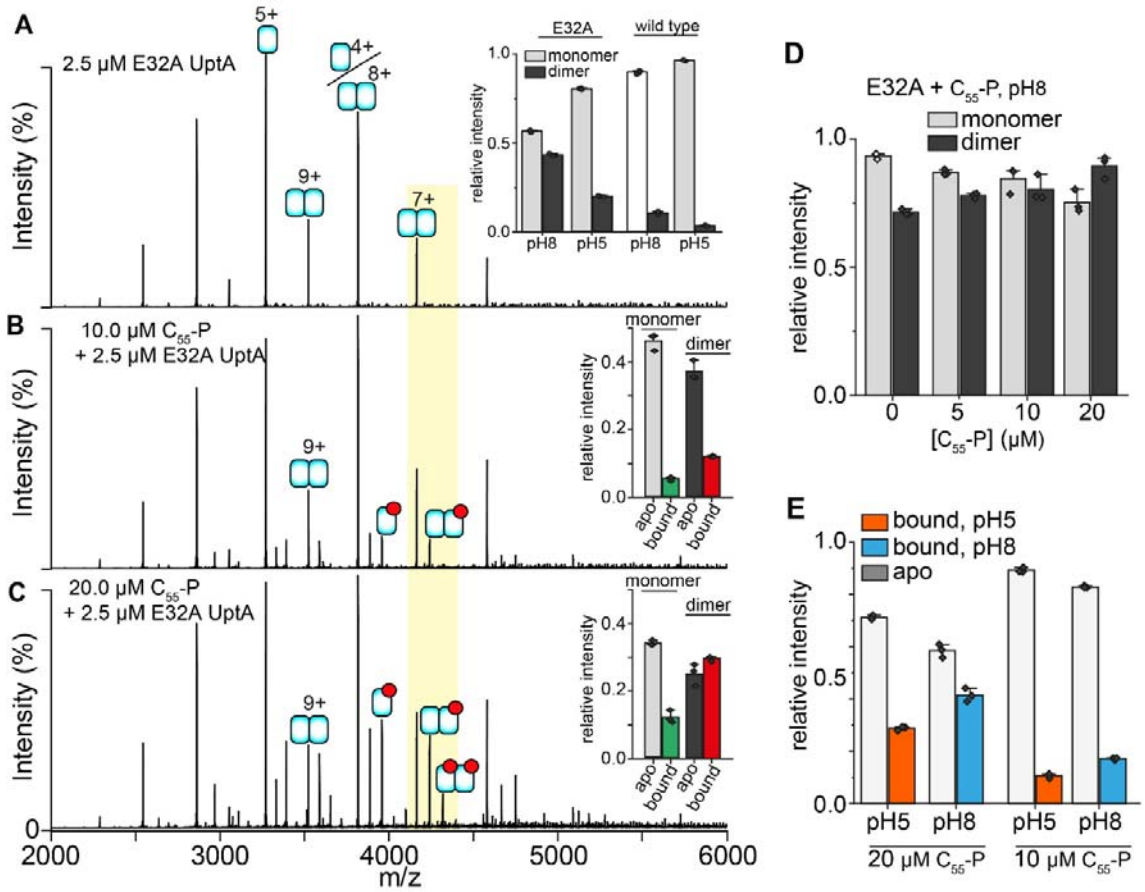

Fig. S6. Dimer fraction of UptA binds C_{55}-P more than the monomeric form. **A.** Spectra from UptA E32A mutant display peaks corresponding to monomers and dimers. Insert, the relative intensity of monomer-dimer populations in the E32A mutant and the wild-type UptA**.** More dimers are observed at pH 8.0 than at pH 5.0. **B-C.** Spectrum of UptA E32A mutant equilibrated with 10 μM and 20 μM C_{55}-P. Insert, the relative intensity of apo and C_{55}-P bound proteins in each spectrum. Dimer fractions bind the ligand more than the monomers. **D.** The total proportion of monomers and dimers in a mixture of E32A UptA and 0-20 μM C_{55}-P. The ligand C_{55}-P does not cause a significant difference in the monomer-dimer ratios. **E.** Mean relative intensity of apo and C_{55}-P bound E32A UptA at pH5 and pH8. C_{55}-P bind to UptA more intensely at pH 8.0 than at pH 5.0.

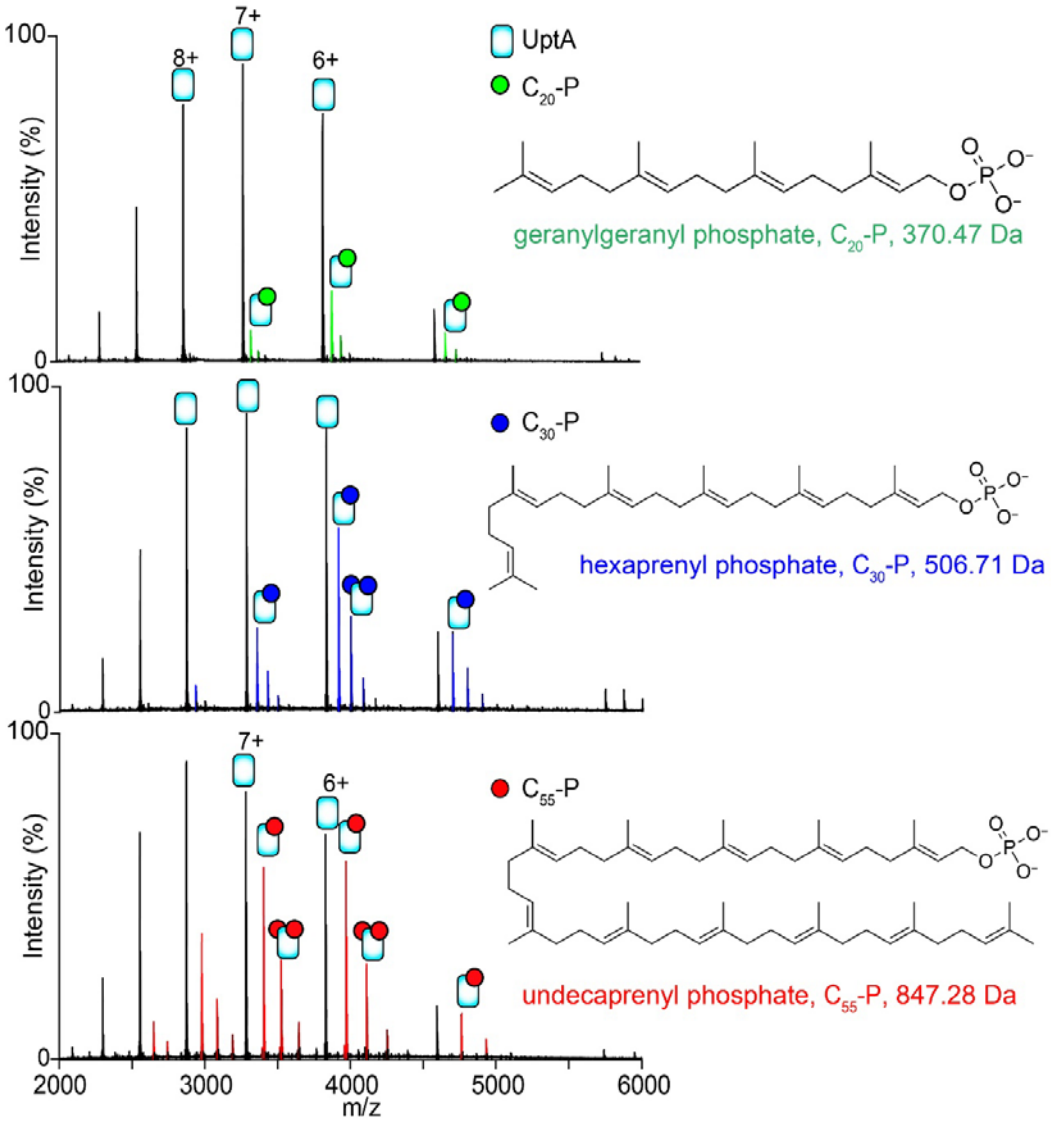

Fig. S7. UptA binds carrier lipids of longer chain lengths more intensely than those of shorter chain, and phospholipids. Shown are the representative spectra for 4 µM UptA equilibrated with 40 µM undecaprenyl phosphate, C_{55}-P hexaprenyl phosphate, C_{30}-P and geranylgeranyl phosphate, C_{20}-P. Samples were prepared in a buffer containing 200 MM ammonium acetate (pH8.0) and 0.05% LDAO.

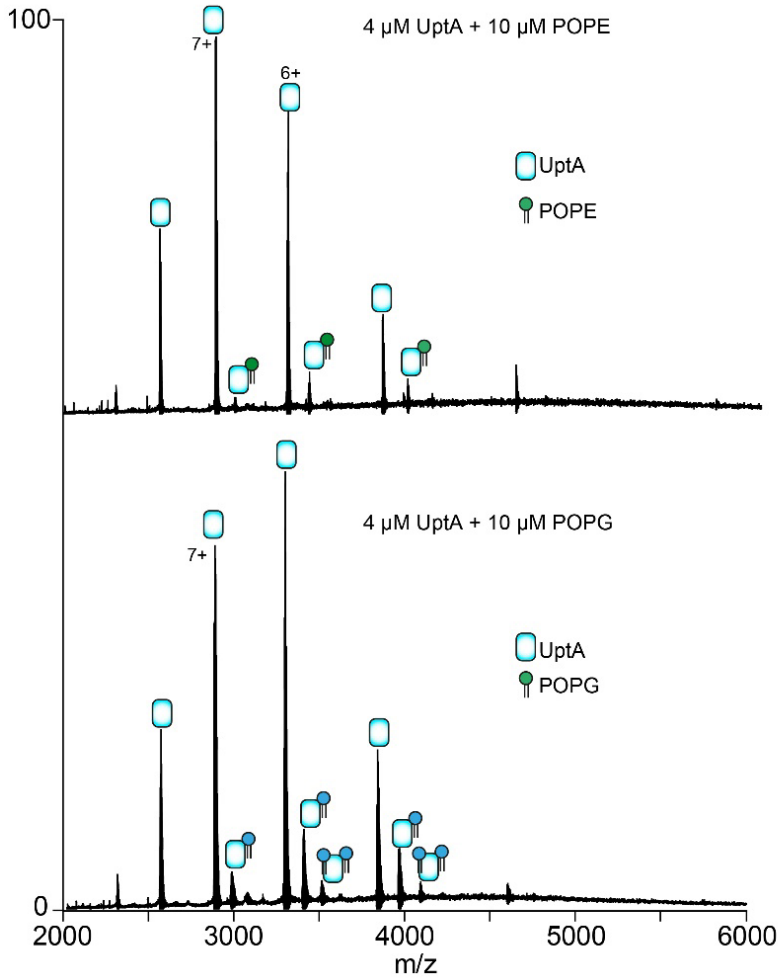

Fig. S8. UptA interacts more favourably with anionic lipid POPG than the cationic lipid POPE. Spectra for UptA incubated with POPE and POPG and measur'ed using the collisional activation voltage 75 V.

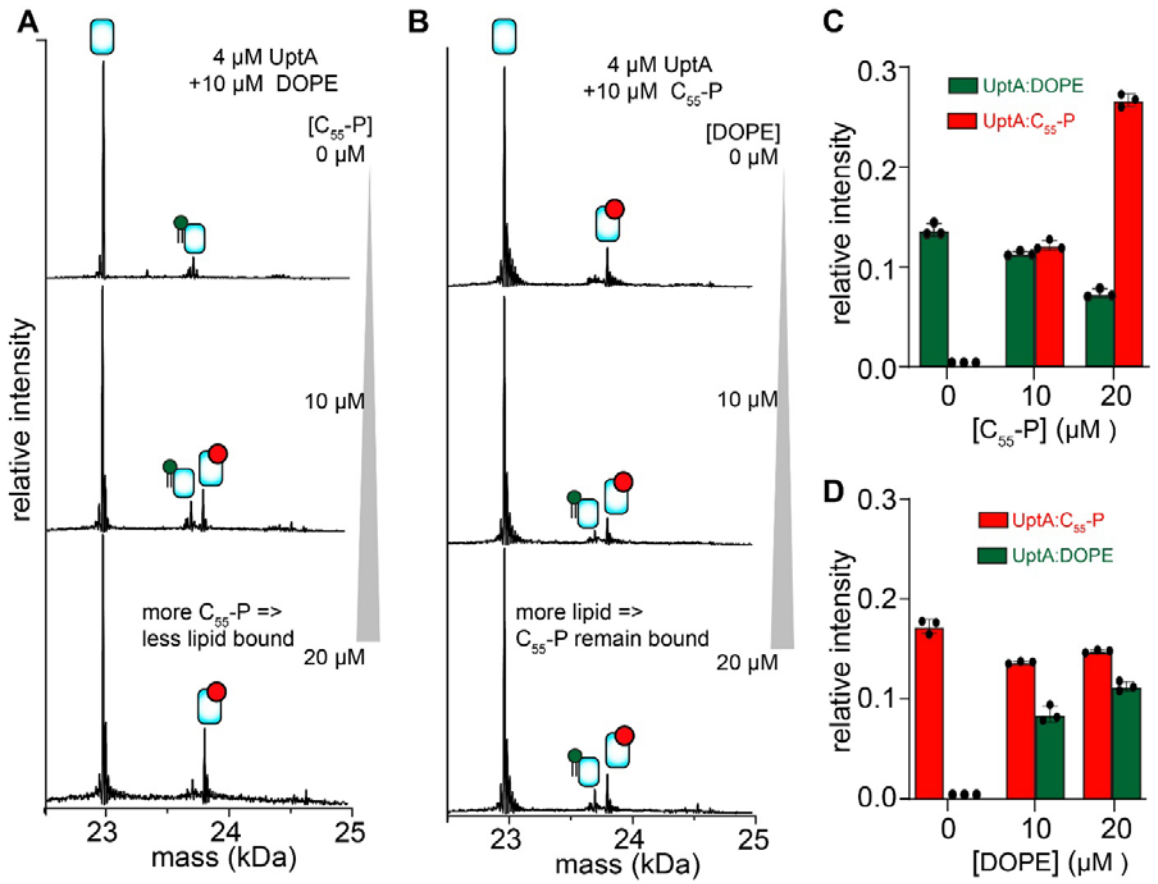

Fig. S9. Competition for UptA between DOPE and C_{55}-P. A. Deconvoluted mass spectra for solutions containing 4 μM UptA and 10 μM DOPE but different concentrations of C_{55}-P. The DOPE bound to UptA was displaced by the added C_{55}-P. **B.** Spectra for solutions containing 4 μM UptA and 10 μM C_{55}-P but different concentrations of DOPE. C_{55}-P remains bound to UptA in the presence of added phospholipids. C,D) Relative intensities of UptA:DOPE and UptA:C_{55}-P in the data shown in panels A and B, respectively.

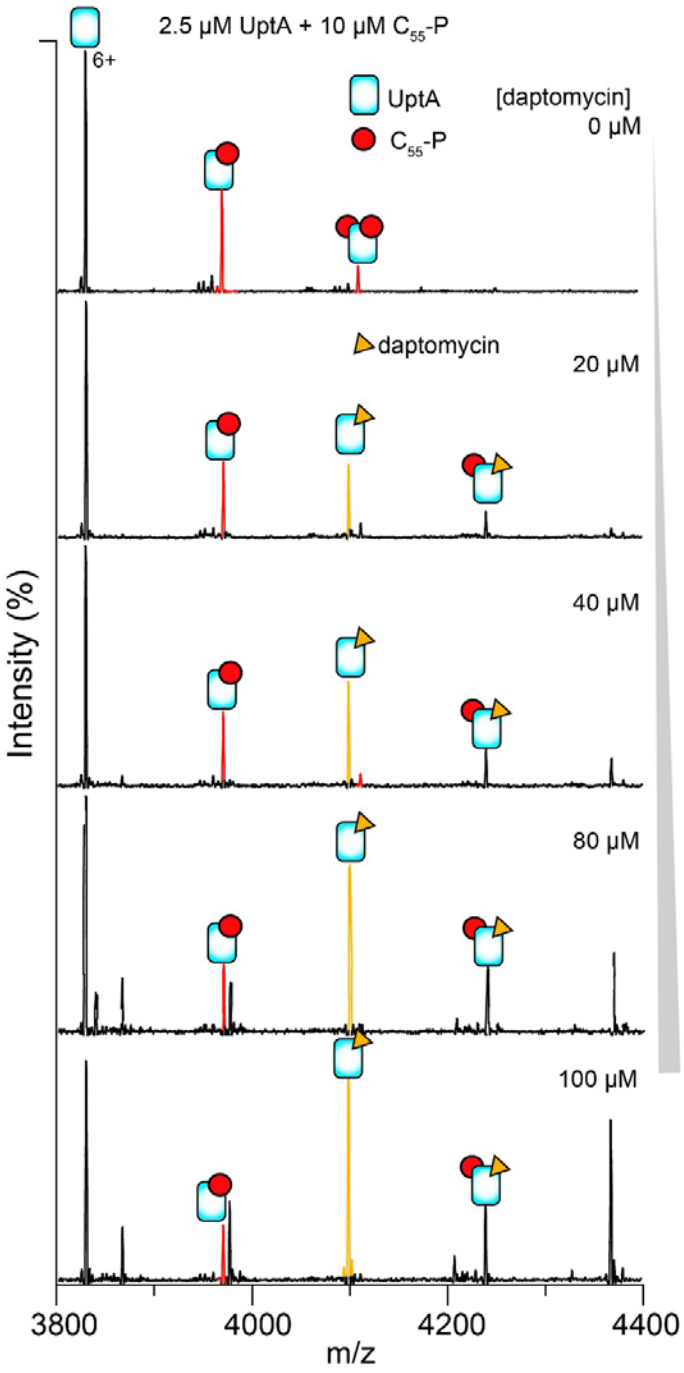

Fig. S10. Daptomycin binds to UptA and partially disrupts UptA:C_{55}-P interactions. Shown are the spectra (6+ charge state) of 2.5 μM UptA and 10 μM C_{55}-P in the presence of different daptomycin concentrations.

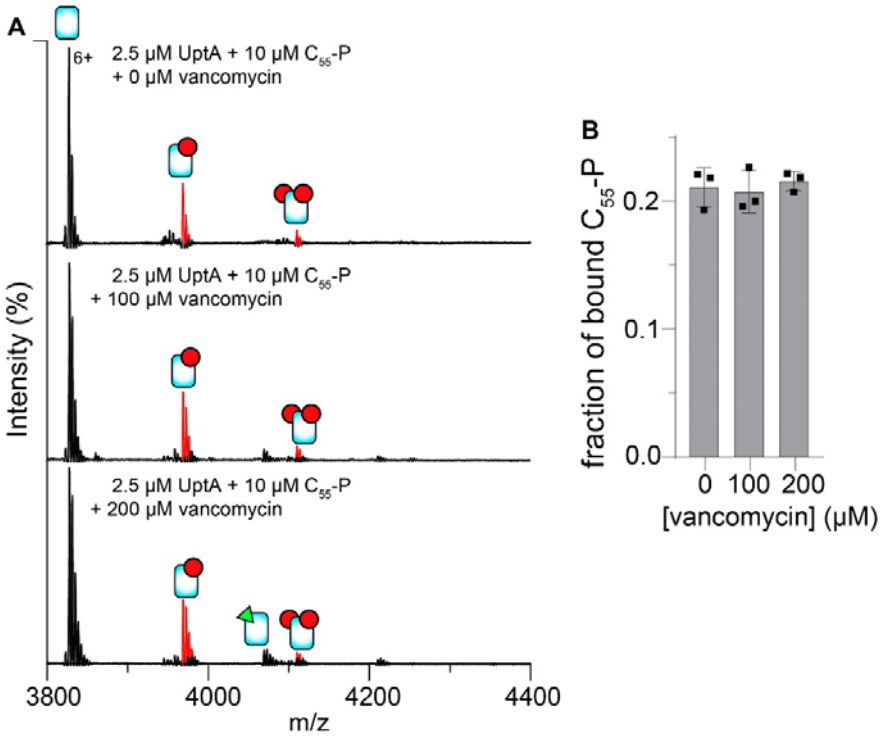

Fig. S11. Vancomycin binds to UptA only weakly and does not significantly affect C_{55}-P binding. A. Spectra recorded for 2.5 μM UptA and 10 μM C_{55}-P but in the presence of 0, 100 and 200 μM vancomycin. **B.** Relative intensity of UptA-bound C_{55}-P in the spectra shown in panel A.

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