Identification of an amphipathic peptide sensor of the Bacillus subtilis fluid membrane microdomains

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Regions of increased fluidity are newly found bacterial membrane microdomains that are composed of short, unsaturated and branched fatty acyl chains in a fluid and disordered state. Currently, little is known about how proteins are recruited and localized to these membrane domains. Here, we identify a short amphipathic α-peptide in a previously unreported crystal structure and show that it is responsible for peripheral localization of the phosphate acyl-transferase PIsX to the fluid microdomains in Bacillus subtilis. Mutations disrupting the amphipathic interaction or increasing the nonpolar interaction are found to redistribute the protein to the cytosol or other part of the plasma membrane, causing growth defects. These results reveal a mechanism of peripheral membrane sensing through optimizing nonpolar interaction with the special lipids in the microdomains. This finding shows that the fluid membrane microdomains may take advantage of their unique lipid environment as a means of recruiting and organizing proteins.
Results

Crystal structure. *B. subtilis* PlsX was crystallized in the presence of the product analog palmitoyl phosphoramide and the crystal structure was determined at 2.30 Å resolution. However, the product analog was not found in the solved structure, in which the asymmetric unit contains two largely symmetric subunits in a mushroom architecture with a four-helix bundle stem formed at the dimeric interface (Fig. 1a). The structure is closely similar to the previously solved structure of the same protein with a root-mean-squared deviation of 0.56 Å over all comparable Cα carbon atoms, forming two symmetric large cavities suspected to be the active sites at the dimeric interface (circled in Fig. 1a). The only obvious difference with the previous structure lies in a 13-residue fragment (residues 250–262) at the exposed end of the helix bundle stem (Fig. 1b). This fragment is fully ordered with good electron density in the structure, while it is largely disordered in the previously determined structure of the same protein14,15. In the crystal structure of *E. faecalis* PlsX (1U7N15), most residues of this fragment form part of the α-helices in the helix bundle stem with four disordered residues in the middle, which correspond to residues 253–256 in *B. subtilis* PlsX.

The 13-residue fragment is structurally different in the two subunits of the dimeric structure. It is comprised of a 9-residue (residues 253–261) α-helix and a 2-residue loop in chain B and a largely random coil loop in chain A (Fig. 1c). Interestingly, the 9-residue α-helical peptide in chain B is amphipathic with Thr253, Thr255, and Lys257 at the interface and Leu254, Leu258, and Ala261 forming the nonpolar surface together with the hydrophobic part of the interfacial residues (Fig. 1d). Ala259 and Ala260 are also close to the polar–nonpolar interface without a polar functional group like other interfacial residues. The N-terminus of the α-peptide is connected to the end of one vertical helix through the 2-residue loop (Thr251 and Ser252) and its C-terminus is connected to another vertical helix of the four-helix bundle stem via a one-residue sharp turn (Val262). This amphipathic α-peptide was formed in the crystal likely due to the presence of the product analog, palmitoyl phosphoramidate, which is an anionic detergent by nature. Its absence in previously determined crystal structures might be a result of the lack of a similar detergent in the crystallization buffer14,15.

Since amphipathic α-peptides are well known to recognize various membrane features for peripheral localization to biological membranes16-24, the identified 9-residue amphipathic α-peptide is expected to be involved in the subcellular localization of PlsX to RIFs. Nonetheless, a monomeric green fluorescent protein (GFPm) with the peptide at its N-terminus failed to attach to the RIFs, suggesting that a single peptide is unable to localize to the fluid membrane microdomains (Supplementary Fig. 1). In consideration of the overall symmetry of the dimeric structure, this short amphipathic α-peptide is also expected to be formed in the other protomer under physiological conditions. However, repetitive crystallization attempts failed to observe the two expected amphipathic α-peptides in an alternate structure in the presence of other detergents or palmitoyl phosphoramidate at an increased concentration.

**Amphipathicity required for the RIFs localization.** To investigate the role of the identified amphipathic α-peptide in its RIFs localization, PlsXm was fused to GFPm at the N-terminus and induced for expression by xylose from the amyE locus in *B. subtilis* in the presence of the untagged PlsX expressed from its native locus exactly as previously reported3. RIFs puncta were readily detected on bacterial cells at the early log phase by staining with the DiIC12 reporter dye and optimally co-localized with the expressed GFPm::PlsX fusion protein in widefield fluorescent microscopy (Fig. 2a). From the confocal microscope image (Fig. 3), it is obvious that a majority of the fusion protein is associated with the DiIC12-stained RIFs with negligible amount in the cytosol. To disrupt the amphipathicity of the identified α-peptide, the polar interfacial residues Thr253, Thr255, and Lys257 were mutated individually to alanine and the resulting
mutants were not localized to RIFs (Fig. 2a) but were mostly found in cytosol (Fig. 3). Noticeably, no free GFP<sub>m</sub> was detected in the cells (Supplementary Fig. 2) and thus the observed delocalization was not due to degradation of the mutant proteins. In comparison, similar alanine mutation of the non-interfacial Ser256 or Thr251 in the 2-residue connecting loop (Fig. 1c) had no effect on the subcellular localization (Fig. 2a). In addition, delocalization of the fusion protein to cytosol was also observed when a serine was introduced to the position of Leu254 on the nonpolar face of the amphipathic α-peptide (Figs. 2a and 3). However, when L254E, L258E, or A261D was introduced to the nonpolar face, the RIFs localization was not changed (Fig. 2a). This unexpected result is likely due to snorkeling of the introduced side-chain carboxylate to the polar–nonpolar interface, which is allowed by the close proximity of the mutated residues to the interface. To test this, L254D mutant was made with a shorter side chain compared to L254E and was indeed found to delocalize to cytosol like other amphipathicity-disrupted mutants (Fig. 2a). Moreover, recombinant PlsX was found to directly bind to protein-free total lipids from <i>B. subtilis</i> and the K257A mutation was found to weaken this interaction (Supplementary Fig. 4). Taken together, these results provide strong evidence that PlsX interacts directly with the interface. To test this, L254D mutant was made with a shorter side chain compared to L254E and was indeed found to delocalize to cytosol like other amphipathicity-disrupted mutants (Fig. 2a).

### Hydrophobic interaction in the RIFs localization

To understand how the RIFs localization is affected by the hydrophobic interaction of the amphipathic α-peptide with the membrane, Leu254 and Leu258 on the nonpolar face were mutated separately to alanine. The resulting mutant fusion proteins were both delocalized into the cytosol (Figs. 2c and 3). For comparison, the subcellular localization of the fusion protein showed no change for similar alanine mutation of Met250 and Val262 at the two ends of the 13-residue fragment (Fig. 2c). The unchanged RIFs localization of the V262A-containing mutant strongly suggests that the Val262 side chain makes no contact with the membrane although this residue is spatially close to Ala261 and makes the sharp transition from the amphipathic α-peptide to the vertical helix (Fig. 1c, d).

To explore the effect of increased hydrophobic interaction, the interfacial residue Ala259 was mutated to threonine while Thr253 and Thr255 were replaced with tryptophan, a snorkeling residue with the largest hydrophobic moiety for interaction with acyl groups of the membrane lipid. Under widefield fluorescence microscopy, the A259T-containing mutant was not visible due to poor expression (Supplementary Fig. 2), whereas both T253W and T255W-containing mutants are mostly distributed throughout the whole cell but remain partially enriched on the DiIC12-stained RIFs (Fig. 2b). Under confocal fluorescence microscopy, a majority of the T253W-containing mutant was delocalized to the cytosol but the T255W-containing mutant remains associated with the lateral membrane and the midcell region (Fig. 3). In corroboration of this result, the GFP<sub>m</sub>-T255W protein was found to be associated with the membrane at a level similar to the wild-type GFP<sub>m</sub>-PlsX but higher than GFP<sub>m</sub>-T257A (Supplementary Fig. 5), which was mainly delocalized to cytosol. On the other
hand, several point mutations were introduced to the nonpolar surface of the amphipathic α-peptide to increase the hydrophobic interaction at the polar-nonpolar interface. Effect of mutations disrupting amphipathicity of the α-peptide. b Effect of mutations increasing the hydrophobic interaction at the polar-nonpolar interface. c Effect of mutations varying the hydrophilicity of the residues on the non-polar side of the amphipathic α-peptide. Yellow color in the merged image indicates co-localization of the fluorescent labels DiIC12 and GFPm, which emit red and green lights, respectively. The scale bar is 2 μm and the same for all images, which are cut from a larger area that include at least 10 cells as shown in Supplementary Fig. 3. The larger images are used to calculate the Pearson’s correlation coefficient (R) that is an indicator of the extent of co-localization and is provided in the merged images.

was also found to be partly associated with the RIFs foci with the highest proportion for the L254W-containing mutant, the second for the A261L-containing mutant, and a negligible amount for the A261W-containing mutant (Fig. 2c). Noticeably, all the mutant proteins were expressed as a full-length fusion with GFPm (Supplementary Fig. 2) and thus their delocalization to the cytosol was not due to free green fluorescent protein released from their degradation.

Effect of the RIFs localization on cell growth. Detachment of PlsX and other peripheral proteins from the RIFs has been suggested to be a major contributor to the daptomycin antibacterial effect of inhibiting synthesis of phospholipids and the cell wall³. However, this proposed role of the subcellular localization is complicated by the multiple membrane-disrupting effects of daptomycin²⁷,²⁸. To determine the putative functional effect of the RIFs localization, we used a mutant plsX gene containing the S256A, K257A, or T255W point mutation to replace the wild-type gene and expressed the mutant proteins under the native promoter. In the meantime, the wild-type plsX gene was used as a control in the gene replacement to avoid polar effect. The resulting B. subtilis strains were readily obtained. As shown in Fig. 4, the strains with the gene replacement with the wild-type plsX gene or its S256A mutant with unchanged RIFs localization are almost indistinguishable from the wild-type B. subtilis strain 168 in their vegetative growth curves except a higher plateau for the latter, suggesting a mild polar effect for the gene insertion. In contrast, the strain expressing either the K257A or T255W mutant shows a similar two-hour delay in transition to the exponential phase and a plateau lower than the S256A mutant.
This growth impairment is greater for the K257A mutant, which is mostly delocalized to the cytosol, than the T255W mutant, which is mostly redistributed to the non-RIFs membrane, due to the apparent different plateau level in their growth curves. Since the point mutations are very far from the suspected active site (Fig. 1a) and should not affect the catalytic activity, this growth impairment provides unambiguous evidence for a crucial role for the subcellular localization in the physiological function of PlsX. This is further supported by the unaffected catalytic activity of the pure recombinant K257A protein in comparison to the non-mutated PlsX (Supplementary Fig. 6).

Discussion

Amphipathic α-peptides are structural motifs commonly found in many proteins for specific recognition of and binding to biological membranes according to their structural features, such as curvature, lipid composition, and charges. We have identified a amphipathic peptide in the phosphate acyltransferase PlsX which specifically recognizes and binds membrane regions according to their fluidity, a previously unreported membrane feature. It locates in the middle of the protein and works as a dimer that caps the exposed end of the four-helix bundle stem in the enzyme’s mushroom-like structure. This peptide contains nine residues in two and a half α-helical turns and is too short to be predicted from its amino acid sequence by HeliQuest. The small size is comparable to the membrane targeting peptide of MinD, which also works as a dimer to involve in placement of the bacterial cell division site. Importantly, the peripheral localization mediated by this short amphipathic peptide is shown to be important for the physiological function of PlsX by the growth impairment caused by its putative disruption, which also shows the importance of RIFs in phospholipid biosynthesis.

The two-peptide working model of the identified amphipathic α-peptide is consistent with several lines of experimental observation. Besides being in line with the overall symmetry of the PlsX crystal structure (Fig. 1a), this model is supported by the failure of localization of the monomeric amphipathic α-peptide in fusion with GFPm to RIFs (Supplementary Fig. 1). In addition, this model is able to explain the subcellular localization of many GFPm PlsX mutants, such as K257A, T253A, and T255A, which are mostly delocalized to the cytosol or other parts of cell membrane but yet retain residual co-localization with RIFs (Figs. 2 and 3). Although this partial delocalization from RIFs can be explained by reduced strength in the interaction of the mutated peptide with membrane lipids, it is better understood on the basis of the model that allows formation of a hybrid dimer composed of a wild type PlsX monomer without fusion and a PlsX mutant in fusion with GFPm. Such hybrid dimers could be formed from subunit swapping between a wild-type PlsX dimer and a GFPm-PlsX mutant dimer that are both produced with the former expressed at a higher amount according to a previous estimation in a closely similar expression system. These hybrid dimers are less affected by the point mutations in their interaction with membrane lipids in comparison to the fluorescent homodimers and may still be able to localize to RIFs. Therefore, the residual RIFs localization of the mutants is likely an experimental indicator of the two-peptide working model for the peripheral recognition and specific localization to the fluid membrane microdomains.

Specific localization of RIFs mediated by the short symmetric amphipathic α-peptides is a result of their balanced interaction with the polar head groups and the nonpolar acyl chains of the lipids in the fluid microdomains. The polar interaction is apparently kept to a minimal strength barely enough for adhesion of the short α-peptides to RIFs, because their membrane association is lost when a pair of hydrogen bonds are removed by mutating Thr253 or Thr255 into alanine. However, strengthening this interfacial polar interaction appears to be tolerated as seen in the unchanged RIFs localization for proteins containing the A261D, L254E, and L258D mutations (Fig. 2a), in which the sidechain carboxylate snorkels to the polar–nonpolar interface to strengthen the polar interaction with the head groups of membrane lipids. This tolerance may greatly increase the structural diversity for the RIFs-recognizing amphipathic α-peptides, allowing them to have a polar residue on its hydrophobic surface.

In contrast, the nonpolar interaction is more tightly controlled in the RIFs localization of the symmetric amphipathic α-peptides. A decrease in this interaction is not tolerated as seen from delocalization of the fusion protein to cytosol when a leucine residue (Leu254 or Leu258) on the nonpolar face is mutated to alanine (Figs. 2 and 3). An increase in this nonpolar interaction is also not tolerated since the protein is at least partially delocalized from the RIFs foci, no matter whether the increase happens at the interface (for T253W and T255W) or the nonpolar surface of the amphipathic peptide (for A261L, A261W, and L254W). Interestingly, the extent of delocalization from the RIFs appears to correlate with the magnitude of hydrophobicity increase on the nonpolar surface. The fusion protein is totally retained on the RIFs when the hydrophobicity increase is moderate as seen for the A261L and L254W mutants, whilst the delocalization is complete when the hydrophobicity increase is bigger in the A261W mutant (Figs. 2 and 3). In comparison, the interfacial mutations T253W and T255W should cause a similar hydrophobicity increase as the A261W mutation, but these mutants exhibit a different subcellular distribution with partial RIFs co-localization, which is likely due to the difference in the site of the hydrophobicity increase. More interestingly, the T255W mutant is mostly re-distributed from the RIFs to other parts of the membrane because it is still associated with the membrane in confocal fluorescence microscopy (Fig. 3 and Supplementary Fig. 5), while the T253W mutant is mainly delocalized to the cytosol (Fig. 3). One possible explanation for this difference is that Trp255 (in the T255W mutant) is located on the side facing the amphipathic α-peptide of the other subunit in the functional dimer (Fig. 1b, c) to allow the two symmetric tryptophan residues to interact with each other while Trp253 (in the T253W mutant) is located on the opposite side of the peptide.
with similar hydrophobicity in most sequences. Interestingly, is replaced by a serine and Leu254 is replaced by a phenylalanine sequences, the interfacial lysine is strictly conserved while Thr253 consistent with its high level of conservation in the sequence alignment (Fig. 5a). Among the 12 non-redundant sequences, the revealed limitations on the amphipathic α-peptide keep its hydrophobicity in a narrow range to achieve specific localization to the fluid membrane microdomains.

The limitations on the nonpolar face of the amphipathic α-peptide are apparently imposed by the RIFs lipids to maximize the preferential interaction to achieve the subcellular localization. Although RIFs are suggested to comprise of lipids with short, branched and unsaturated fatty acyl chains1,3, their exact lipid composition is not known. This unknown lipid composition is further complicated by the fact that phospholipids are formed at an optimal level. As a result, it is possible that RIFs recognize a specific lipid composition to modulate specific localization effects. However, how this reveals the limitations on the nonpolar face of the amphipathic α-peptide is not well conserved despite the high sequence conservation for the RIFs-recognizing motif outside the α-peptide.

α-peptide and unable to interact similarly. However, how this probable difference in the side chain interaction leads to different subcellular distribution is not clear. Nevertheless, the RIFs localization effect of the hydrophobicity-increasing mutations strongly support that the amphipathic α-peptide keeps its hydrophobicity in a narrow range to achieve specific localization to the fluid membrane microdomains.

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When compared with the PlsX orthologues from other bacterial genera, the amino acid sequence of the identified amphipathic α-peptide is not well conserved despite the high sequence conservation in other structural motifs (Fig. 5b). This lack of conservation may be due to the absence of RIFs in other bacteria, which is however unlikely because of the finding of these membrane microdomains in Escherichia coli2. Alternatively, it can also be explained if PlsX is not specifically localized to RIFs in most other bacterial genera. More likely, it may be due to the difference in the lipid composition of RIFs in different bacterial genera. As mentioned earlier, the fatty acyl composition is distinct in the Bacillus genus and very different from that of all other bacterial genera1,3, raising the possibility that the amphipathic α-peptide has to change in order to adapt to the altered lipid composition of RIFs in other bacteria. Further investigation is needed to fully understand the low sequence conservation for the RIFs-recognizing motif outside the Bacillus genus.

**Fig. 5** Sequence conservation of the PlsX amphipathic α-peptide. a Alignment of the orthologues from the *Bacillus* genus. PlsX sequences were reviewed, non-redundant sequences deposited in the UniProt database. b Alignment of the orthologues from different bacterial genera. PlsX sequences were chosen from 95 reviewed PlsX sequences filtered at 50% sequence identity from the UniProt database, according to the number of PlsX orthologues they represent. The sequences are named directly by their UniProtKB mnemonic identifiers and aligned with the *Bacillus subtilis* PlsX structure determined in the current study (PDB ID: 6A1K). α9 is the RIFs-recognizing amphipathic α-peptide.
In summary, we have found a short amphipathic α-peptide in the phosphate acyltransferase PlsX, which is responsible for sensing RIFs for specific localization in B. subtilis. Through mutational analysis, the amphipathic peptide is found to recognize the fluid membrane microdomains through limiting its hydrophobic interaction with the membrane in a narrow range and close to the minimum level. This requirement of strictly restricted hydrophobic interaction may underlie the small size of the peptide and the minimum level of interfacial polar interaction also found through the mutational analysis. This stringent restriction is apparently required for optimal interaction of the amphipathic α-peptide with the special lipids in RIFs, suggesting that the fluid membrane microdomains may take advantage of their unique lipid environment as a means of recruiting and organizing proteins. It would be interesting to see whether similar restriction of hydrophobic interaction plays a role in the RIFs localization of transmembrane proteins or other monotopic proteins.

Methods

Synthesis of 1-oxohexadecyl phosphoramidic acid. The product analog was synthesized using a reported method. Briefly, 3 mmol palmitoyl chloride and 12 mmol of 1-oxohexadecyl phosphoramidate were dissolved in 12 ml dry tetrahydrofuran and cooled down to −78 °C. 12 mmol n-BuLi was added dropwise and the reaction was stirred overnight before being quenched by 1 ml acetic acid. The mixture was washed three times with brine and dried over anhydrous Na2SO4. Then the intermediate product was further purified by silica gel column chromatography using ethyl acetate:n-hexane (1:3) as eluent and the residue was removed to yield 1-mono 1-oxohexadecyl phosphoramidate as a white powder. In the second step, 50 mg 1-oxohexadecyl phosphoramidate was dissolved in 2 ml CH2Cl2, to which 200 μl of trimethylsilyl bromide was then added dropwise. Subsequently the reaction mixture was stirred at room temperature overnight. After trimethylsilyl bromide and CH2Cl2 were removed in vacuo, 1 ml 95% ethanol was added to the residue and the mixture was stirred for 1.5 h at room temperature. Then ethanol was removed and 8 ml ether was added to wash the white solid product C16NP, which was dried in vacuo.

PlsX expression and purification. The plxX gene was amplified from the B. subtilis genomic DNA using the primers PlsX-for and PlsX-rev listed in Supplementary Table S1 and integrated into the pET28a (Novagen) between NcoI and Xhol. The inserted gene was sequenced in full-length by Beijing Genomics Institute (BGI, Shenzhen, China) to ensure that no mutation was introduced. The recombinant plasmid was transformed into E. coli C43 (DE3) (Lucigen) and the resulting cells were incubated overnight at 37 °C on Luria-Bertani broth (LB) agar plates supplemented with 50 μg/ml kanamycin. For expression of the protein with a C-terminal hexahistidine tag, a single colony of the recombinant cells was inoculated to 4 L LB medium supplemented with 50 μg/ml kanamycin and the cells were grown at 37 °C with shaking at 250 rpm until OD600 reached 0.4. The harvested cells were then re-suspended in the start buffer A (25 mM Tris–HCl, pH 8.0, 500 mM NaCl, 20 mM imidazole, 1% glycerol) for 15 min to dissolve the cells. The cell debris and the supernatant was loaded onto a 5 ml HisTrap HP column (GE Healthcare) that was pre-equilibrated with the storage buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol). The eluted protein solution was concentrated with Millipore YM-30 and further purified by cation exchange using a HiPrep 16/60 Sephacryl S-200 column (GE Healthcare) that was pre-equilibrated with the storage buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol). The eluted protein was collected and concentrated to 15 mg/ml before crystallization and stored at −20 °C. All steps in this purification were carried out at 4 °C.

PlsX structure determination and analysis. Crystallization was carried out using the hanging drop method by mixing a protein solution and reservoir solution at 1:1 ratio under room temperature (22 °C). The protein solution was prepared by dissolving the product analog 1-oxohexadecyl phosphoramidic acid in the PlsX solution with a final protein concentration of 2.5–7.5 mg/ml. Rod-like crystals appeared over a week and grew to the full size within 3 weeks in a solution containing 5 mg/ml PlsX, 1 mM 1-oxohexadecylphosphoramidic acid, 0.08 M Tris–HCl (pH 8.0), 0.16 M LiSO4, 21% PEG 4000, 0.002 mM CymAL 7 and 5% β-galactosidase. Crystals were cryoprotected with the mother liquor supplemented with 15% PEG 400 and flash-cooled and stored in liquid nitrogen and diffraction data were collected up to 2.30 Å at 100 K with an ADSC Quantum 310R charge-coupled device detector at beamline BL17U1 and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF) and processed with iMosflm and Aimless in the CCP4 suite. The structure was solved by molecular replacement by Phaser-MR in PHENIX, using the previously solved structure of B. subtilis PAX (PDB ID: 1IV1) as the model. The structure model was further built using COOT and refined by PHENIX-Refine. MolProbity was used to assess the overall quality of the structural model. Data collection and refinement statistics are shown in Table 1 and the structure has been deposited in the Protein Data Bank (PDB ID: 6A1K). In structural analysis, all graphics were generated by PyMOL Version 1.3. Multiple sequence alignment was performed with Clustal Omega for structure-based presentation using ESPript 3.0.

Activity assay and direct lipid binding of PlsX and its K257A mutant. To introduce the K257A mutation, the plasmid in pET28 used in PlsX expression was used as a template for site-directed mutagenesis using the primers in Supplementary Table 1. The K257A mutant was expressed and purified exactly like the wild-type PlsX as described above. The palmitoyl-CoA substrate was prepared enzymatically from palmitoyl-CoA and apo-ACP according to a reported method. The enzymatic activity was determined based on coupling with 5, 5-dithio-bis-(2-nitrobenzoic acid), which reacted with the holo-ACP product to yield 2-nitro-5-thiobenzoic acid for UV–Vis measurement at 412 nm with an extinction coefficient of 14,130 M−1 cm−1. In our activity assay, a 200 μl reaction mixture contained 1 mM MgCl2, 500 μM 5, 5-dithio-bis-(2-nitrobenzoic acid), 500 μM phosphate, and 10 μM palmitoylACP in 50 mM Tris buffer (pH 7.5). The enzyme was then added to a final concentration of 1 μM and the reaction was monitored for absorbance change at 412 nm a UV–VIS spectrometer (Shimadzu). Assays were performed in triplicate at 25 °C.

The protein-free total lipids were prepared from B. subtilis strain grown in LB medium under aerobic condition at 37 °C and harvested when OD600 reached 0.5. Cells from 2 L liquid culture were washed by 50 ml start buffer and weakly bound impurities were removed by washing with 50 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 500 mM NaCl, 100 mM imidazole, and 1% glycerol. The eluted protein solution was concentrated with Millipore YM-30 and further purified by gel filtration using a HiPrep 16/60 Sephacryl S-200 column (GE Healthcare) that was pre-equilibrated with the storage buffer (10 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol). The eluted protein was collected and concentrated to 15 mg/ml before crystallization and stored at −20 °C. All steps in this purification were carried out at 4 °C.

Table S1 Data collection and refinement statistics

| Data collection | Space group | Cell dimensions (Å) | Resolution (Å) | Rmerge (% of | Completeness (%) | Redundancy |
|----------------|-------------|--------------------|----------------|---------------|-----------------|------------|
| PlsX (PDB code: 6A1K) | C222 | a, b, c (Å) | α, β, γ (°) | | | |
| | | 108.52, 144.76, 84.84 | 90, 90, 90 | 33.29–2.30 (2.382–2.30) | 0.100 (0.428) | 96.2 (90.9) | 6.4 (5.6) |

Refinement

| Resolution (Å) | No. of reflections | Rwork/Rfree | No. of atoms | Protein | Ligand/ion | Water | R.m.s. deviations |
|----------------|-------------------|-------------|--------------|--------|------------|-------|------------------|
| PlsX (PDB code: 6A1K) | 33.29–2.30 | 29,907 | 0.2025/0.2379 | 4889 | 4620 | 72.95 | 36.86 |

| Bond lengths (Å) | Bond angles (°) |
|------------------|-----------------|
| 0.007 | 0.96 |

*Values in parentheses are for the highest-resolution shell.
Expression of GFPm-PlsX and its mutants in *B. subtilis*. *B. subtilis* 168 strain and the vector pSG1729 for N-terminal fusion of green fluorescent protein (GFP) were acquired from Bacillus Genetic Stock Center (BGSC). The GFPm gene in the vector was introduced the A260K mutation to avoid dimerization 49, using the QuickChange II XL site-directed mutagenesis kit (Agilent) and the primers GFP-for and GFP-rev (Supplementary Table 1). The encoded protein, called monomeric fluorescent protein (GFPm) was then fused to the N-terminus of PlsX by amplifying the plx gene from the genomic DNA using the primers WT-for and WT-rev (Supplementary Table 1) and inserting it into the modified pSG1729 vector between BamHI and XhoI. The GFPm-PlsX fusion protein expressed from the resulting plasmid is the same as the GFP-PlsX protein (labeled as PlsX-GFP) in a previous study 3. To introduce point mutations into the amphipathic α-peptide of the fusion protein, its gene was mutated using the QuickChange II XL site-directed mutagenesis kit (Agilent) and the primers listed in Supplementary Table 1. For T253W, L254W, A261L, and A261W, a different protocol as reported earlier was used to introduce the mutations 60. For fusion with the amphipathic α-peptide, the coding sequence of the peptide with the residues 250-262 was inserted to the 3'-end of the green fluorescent protein gene in the vector pSG1729 with or without the A260K mutation between BamHI and XhoI using the primers HX1 and HX2. The inserted genes of the fusion proteins were sequenced in full-length by Beijing Genomics Institute (BGI, Shenzhen, China) to ensure that no unwanted mutation was introduced. For analysis of the expression level of the fusion protein by Western-blotting, the plasmid carrying its gene was transformed into *B. subtilis* 168 according to a reported method 51. The transformants were selected overnight on LB agar plates supplemented with 50 μg/ml tetracyclin and 100 μg/ml spectinomycin at 37 °C to allow genomic integration of the fusion gene and the spectinomycin resistance. The transformants were then cultured to a reported method 7. Saha, S., Anilkumar, A. A. & Mayor, S. GPI-anchored protein organization, Pearson’s correlation coefficient was calculated by the Intensity Correlation Analysis plugin of ImageJ for an area containing at least 10 cells as shown in Supplementary Fig. 3 after background subtraction 54. Statistics and reproducibility. All the imaging experiments were repeated at least once at a different time or by a different researcher. All the reported images and data were successfully reproduced. The error bars were derived from at least three independent experiments for the growth curves (Fig. 4), the membrane association data were successfully reproduced. The error bars were derived from at least three independent experiments for the growth curves (Fig. 4), the membrane association

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Author contributions
Z.G. and Y.J. conceived and designed the experiments; Y.J. solved the crystal structure; Y.J., X.D., and M.Q. mutated and imaged the fusion protein; Z.G. and Y.J. analyzed and interpreted the data; F.D. contributed to the discussion; and all authors were involved in revising the manuscript.

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