Uncovering lipopolysaccharide regulation in bacteria via the critical lipid binding tunnel of YciS/YciM

Highlights
- Identifying a critical lipid binding tunnel of YciS/YciM
- The lipid binding tunnel could bind lipid molecules
- Mutants of lipid binding tunnel inhibit cell growth severely
Uncovering lipopolysaccharide regulation in bacteria via the critical lipid binding tunnel of YciS/YciM

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SUMMARY
Gram-negative bacteria contain an asymmetric outer membrane, in which the outer leaflet is composed of lipopolysaccharide (LPS). LPS, a drug target of polymyxin, plays an essential role in drug resistance, biofilm formation, and pathogenesis. An important inner membrane protein, YciM, may be responsible for the regulation of LPS biosynthesis and transport. Here, we report the crystal structure of YciM from Salmonella typhimurium in a complex with a non-specifically bound molecule, an ethylene glycol, which identified a tunnel that could bind lipids. Our in vitro assays showed that YciM could bind lipid molecules with affinity in the micromolar range, while mutagenic and functional studies confirmed that lipid-binding residues are critical for the function of YciM. Additionally, our data also showed that YciM accurately regulates LPS biosynthesis and transport with YciS, which could help to better understand the regulation mechanism of LPS.

INTRODUCTION
The cell envelope of Gram-negative bacteria contains three distinct structures, the inner membrane (IM), the periplasm, and the outer membrane (OM). The OM is an asymmetric lipid bilayer, of which the outer leaflet consists of LPS. The deficiency or impairment of LPS would lead to loss of membrane integrity and cell death for most Gram-negative bacteria (Wang and Quinn, 2010). Lipid A, a prominent component of LPS, has high heterogeneity that is controlled by diverse regulatory factors and growth conditions (Klein et al., 2011). Bacteria maintain OM lipid asymmetry and stability to protect themselves from the intrusion of toxic substances, such as antibiotics, detergents, and dyes (Konovalova et al., 2017; Moffatt et al., 2019). The function of OM was mainly maintained by its lipid asymmetry; the perturbation of lipid asymmetry would cause elevated cell membrane permeability and toxic substances easily invading (Henderson et al., 2016). The broken balance between LPS and phospholipids is the major factor that caused perturbation of lipid asymmetry, and the biosynthetic pathway of LPS and phospholipids has been well characterized in E. coli (Escherichia coli) (Wang et al., 2015). The Kdo2-lipid A biosynthetic pathway begins with the building block, N-acetyl glucosamine linked to the nucleotide carrier. LpxA catalyzes the first reaction; it transfers an R-3-hydroxyacyl chain to the N-acetyl glucosamine (Williams and Raetz, 2007). R-3-hydroxyacyl-ACP is also the substrate of FabZ in the phospholipid biosynthetic pathway (Zhang et al., 2016). It has been suggested to be a key factor in maintaining the dynamic equilibrium between LPS and phospholipids (Emiola et al., 2016; Heath and Rock, 1996; Williams and Raetz, 2007). As the equilibrium constant for the first reaction catalyzed by LpxA is unfavorable, LpxC performs the first committed step of lipid A biosynthesis, which strictly regulates the level of LPS, and is imperative for cell growth (Barb and Zhou, 2008). Some studies have shown that LpxC is a validated target for the development of effective inhibitors and the design of novel antibiotics (Barb et al., 2007; Jackman et al., 2000; Pizzi et al., 2017; Tomaras et al., 2014).

It has been reported that the level of LpxC is affected by the AAA + protease protein FtsH (Fivenson and Bernhardt, 2020). The unstructured C-terminal of LpxC containing the motif LAXXXXXAVLA could be recognized and degraded by FtsH. This special structure is indispensable in helping LpxC through the pore of FtsH but is not sufficient for LpxC to be degraded by FtsH (Fuhrer et al., 2007). The absence of FtsH leads to a rise in LpxC expression and an accumulation of LPS that causes cell death. Moreover, according to the studies in 2014, YciM is necessary for FtsH to regulate LPS biogenesis in E. coli (Klein et al., 2014).
YciM is an inner membrane protein with one N-terminal transmembrane domain. It contains 389 amino acid residues and the crystal structure of YciM from *E. coli* comprises nine tetratricopeptide repeat (TPR) motifs and a C-terminal domain containing two Cys-x-x-Cys motifs (Prince and Jia, 2015). Interestingly, these Cys-x-x-Cys motifs are proposed to constitute a metal-binding site (Prince and Jia, 2015). However, the mechanism of YciM regulation of LPS biosynthesis and transport remains unknown.

In this study, we have determined the structure of the YciM cytoplasmic domain of *S. typhimurium* at 2.7 Å by X-ray crystallography. Our in vitro binding assays by isothermal titration calorimetry (ITC) showed that YciM could bind LPS and 3-hydroxymyristic acid with affinity in the micromolar range, and it has a higher binding affinity to LPS than to 3-hydroxymyristic acid. Our in vitro functional assays confirmed the importance of the lipid-binding residues. Furthermore, we demonstrated that YciM and YciS form a complex to regulate LPS biosynthesis and transport. Our study not only contributes to understanding the regulative mechanism of LPS biogenesis but also might help future anti-gram-negative bacteria drug discovery.

RESULTS

The overall structure of YciM from *S. typhimurium*

YciM of *S. typhimurium* was overexpressed and crystallized using an in situ limited proteolysis method with thermolysin at 1:1000 mass ratio (proteinase: protein). The resulting crystals belonged to space group *P*2₁2₁2₁ with cell dimensions *a* = 72.08 Å, *b* = 78.96 Å, *c* = 124.03 Å, and *α* = 90°, *β* = 90°, *γ* = 90° (Table S1). The structure was determined to a resolution of 2.7 Å by molecular replacement with one protomer of 4ZLH as a searching model. Crystal data collection and refinement statistics are listed in Table S1. There are two copies of YciM per asymmetric unit with a dimension of about 81.6 Å in length and 79.7 Å in height (Figure 1A). This structure contains 254 residues, from residue Glu136 to Gln389 with the final *R* factor of 0.2480 and final *R* free of 0.2489. The YciM soluble protein can be divided into two domains; the N-terminal domain and the C-terminal domain which form a bird wing shape with dimensions of approximately 36.4 Å in length and 79.7 Å in height. The N-terminal domain contains six TPR motifs while the C-terminal domain is the rubredoxin-like domain. The structures of the two protomers are almost identical with a root-mean-square deviation (RMSD) of 1.56 Å over 254 aligned residues, with some conformational changes at the TPR3, TPR4, and TPR8 motifs.

Lipid binding site

Dimeric YciM is formed through interactions of TPR7 and TPR8 of the protomers with an interface area of 1002.9 Å². Following refinement, extra electron density was observed at the interface between the protomers of the dimer to which we successfully docked an ethylene glycol. The *F*₀-*F* and 2*F*₀-*F* map, which are respectively generated from refinement results without and with the ethylene glycol, are displayed in Figure S1. Hydrophobic residues L309, P313, M315, F318, M344, and V345 of both protomers create a hydrophobic tunnel that is responsible for the lipid binding, while residues T306, Q310, and Q348 may also interact with the lipid on the surface of the tunnel (Figures 1B and 1C). Conservation analysis showed that residues L309, P313, F318, L321, M322, and V345 are highly conserved, indicating these residues may play important roles in YciM functionality (Figure S2).
YciM prefers to bind LPS

According to the structural findings, we proposed that the hydrophobic tunnel of YciM might bind lipid molecules, like LPS intermediates. To test this hypothesis, YciM soluble domain’s binding affinities to 3-hydroxymyristic acid and LPS were determined by ITC, which revealed that 3-hydroxymyristic acid’s $K_d$ is $1.25 \pm 0.88 \text{ mM}$ and LPS’s $K_d$ is $0.65 \pm 0.26 \text{ mM}$, suggesting that YciM binds lipids selectively and prefers to LPS. (Figures 1D and 1E) We also tested the calorimetric reaction between ethylene glycol and YciM (Figure S3). The calorimetric results indicate that the thermal effect of titration is mainly derived from the heat of dilution rather than the reaction, which also confirms that the conformational changes of lipid binding tunnel are caused by the entry of lipid molecules rather than crystallization solution.

Different conformations of YciM from E. coli and S. typhimurium

We compared our structure with the E. coli YciM (PDB code: 4ZLH) to figure out if there are different conformations of YciM. Our structure contains 254 residues, from residue Glu136 to Gln389 (Figure 2A), and the previously determined structure of the soluble domain of E. coli YciM consists of residues from G67 to L389 (Figure 2B) (Prince and Jia, 2015). The structure of a single copy of YciM from S. typhimurium reported here is very similar to that from E. coli, with an RMSD of 1.12 Å over 249 aligned residues (Figures 2E and 2F). However, superimposition of one protomer of the dimeric YciM from E. coli with that of dimeric YciM from S. typhimurium showed that the second protomer of the E. coli protein is rotationally shifted by approximately 40° and 31.9 Å at the widest point compared to that from S. typhimurium (Figures 2I and 2J).

Importantly, the lipid binding tunnel present in S. typhimurium YciM is closed by the TPR8 motif in E. coli YciM, consistent with the disappearance of the tunnel in the E. coli/YciM structure (Figures 2C and 2D). The tunnel of S. typhimurium YciM was analyzed by HOLE (Smart et al., 1993), and we displayed the cavity...
running through the lipid binding tunnel of *S. typhimurium* YciM by VMD (Humphrey et al., 1996). The dots show the locus of the outer surface of a flexible sphere squeezing through the pore, and the lipid binding tunnel exists between two protomers of *S. typhimurium* YciM (Figure 2K). The radius is around 2 Å which appears in the middle of the tunnel (depths of 10 Å) (Figure 2L). The largest binding of lipid at the interface of the *S. typhimurium* YciM dimer may contribute to the observed rotational twist of the protomers compared to the *E. coli* YciM protein.

**Lipid-binding residues are critical for bacterial growth**

To study whether the tunnel residues involved in lipid binding are important for the function of YciM, a series of site-direct mutants (M315A/F318A/F318D/V345A/V345D/Q348A/L386A) were generated by the site-direct-mutagenesis and *yciM* deletion strain was generated using the pEcCas/pEcgRNA system. The functional assays were performed in the ΔyciM strain (Figures 3A and 3B). Notably, we found that
Figure 3. YciM is important for the growth and viability of *E. coli* and affects LPS biogenesis directly

(A and B) Amino acid residues in the tunnel that could interact with lipid molecules, including M315, F318, V345, Q348, and L386. The surface map of these mutated amino acids was shown in (B). The structure was displayed in PyMOL.

(C) Surface map of mutated amino acids conservation in *S. typhimurium* YciM. The deep red of M315, F318, and V345 suggests they are highly conservative. The light pink of Q348 and L386 indicates their relatively high conservation.

(D) A series of site-directed mutants (M315A/F318A/F318D/V345A/V345D/Q348A/L386A) in knockout strain ΔyciM were generated to explore the function of the lipid binding tunnel of YciM. All strains were grown overnight in LB liquid medium supplemented with chloromycetin and 0.2% L-arabinose. Cells were harvested at 3,000 × g for 5 min and washed by sterilized double distilled H2O twice. The re-suspended culture was diluted to OD600 1.0 and serially diluted at various dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}). 2 µL from each dilution were spotted on LB or M9 plates. Growth was scored after 48 h at 28°C. The functional assay was repeated in triplicate at least. Using PMB-103 (ΔyciM carrying an empty vector pBAD33) as the negative control, PMB-104 (ΔyciM carrying a plasmid pBAD33-yciM) as the positive control, the single mutant V345D and L386A caused grown defect in both LB and M9 plates, indicating the importance of lipid binding tunnel of YciM for bacteria survive.

(E) The expression level of mutants. The Western blot showed that the mutant protein expression levels are similar to that of the wild type.

(F) Growth curve of WT (MG1655), strain ΔyciS, ΔyciM, and ΔyciSM. All strains were cultured overnight in LB liquid medium at 37°C. Cells were harvested at 3,000 × g for 5 min and washed by sterilized double distilled H2O twice. The re-suspended culture was diluted to OD600 1.0 and grown at 37°C. Growth was monitored by measuring OD600 every 1 h for 9 h. The assay was repeated in triplicate at least. The growth speed of strain ΔyciS was slightly lower than that of WT, but the growth speed of strain ΔyciM was far lower than that of both WT and strain ΔyciS, indicating that deleting yciM harmed the growth of *E. coli*. Notably, strain ΔyciSM grew worst among them, which suggests yciS cooperated with yciM in bacterial survival.

(G and H) (G) and (H) displayed the growth condition of strain ΔyciM and ΔyciSM, respectively. The culture condition and experimental procedure were as same as that of the site-directed mutant and used LB plate as the indicated plate. The functional assay was repeated in triplicate at least. PMB-101 (MG1655 carrying an empty vector pBAD33) is the control in this functional assay. PMB-103 and PMB-107 (ΔyciSM carrying an empty vector pBAD33) showed severe growth defects after cultivating at 28°C for about 48 h. But those defects were made up of yciM and yciSM complemental strains (PMB-104 and PMB-108). The growth condition of PMB-103 and PMB-107 is similar, meaning yciM is more crucial than yciS in the regulation process.
Figure 3. Continued
(I) Silver-stained tricine-SDS-PAGE gel shows LPS level in MG1655 or strain ΔyciM carrying pBAD33 or pBAD33-yciM. Strains were grown in presence of 0.2% L-arabinose to induce the expression of YciM. 16.5% SDS-Tricine gel was applied in this experiment, followed by silver staining to reveal LPS. The LPS extraction and measurement were repeated in triplicate at least.

(J) The relative level of LPS was measured by Image J. Using PMB-101 as control and defining its level of LPS as 100%. The relative LPS level of PMB-102 is 91.8 ± 3.3% of that of the control group. The LPS level of strain deleting yciM (PMB-103) increased 10.7 ± 0.06 folds based on PMB-101, suggesting that yciM deletion caused an urgent LPS accumulation. The LPS level of complemental strain is 1.54 ± 0.03 folds of that of PMB-101.

mutant V345D grew very poor in both LB and M9 plates. Mutant L386A also has grown defect in M9 plates, but it is not significant in LB plates (Figure 3D). We also checked the expression level of mutant V345D and L386A. The Western blot showed that the mutant protein expression levels are similar to that of the wild type (Figure 3E). To find out whether residue V345 is critical for the interaction between YciM and lipid molecules, we used ITC to test mutant V345D’s binding affinities to 3-hydroxymyristic acid and LPS. Results reveal that mutant V345D could barely bind 3-hydroxymyristic acid or LPS (Figure S4). The negatively charged side chain of aspartic acid might disrupt the interaction between YciM and lipid molecules. Besides, L386 may play an important role through the hydrophobic interactions with lipids and the alanine mutation could abolish the interaction. It is worth noting that the conserved residue analysis using Consurf (Glaser et al., 2003) revealed that M315, F318, V345, Q348, and L386 are highly conservative (Figure 3C), indicating that our findings might be applicable to YciM homologs in other strains.

YciS can be copurified with YciM
To validate the interaction between YciS and YciM in S. typhimurium, we generated constructs to overexpress YciM and YciS. When 8 × His tag was attached to the N-terminal of YciS, we found that it can be copurified with YciM (Figure S5A). Besides, YciS and YciM were also found together in fractions, even the 8 × His tag was changed to N-terminal of YciM (Figure S6). Meanwhile, we noticed that the band of YciS on SDS-PAGE was mainly smear-like, which was different from the result Klein G et al. found in E. coli (Klein et al., 2014). It spanned from 17 kDa to approximately 27 kDa (Figure S5B). To figure out whether the smear was caused by the non-specific binding of Ni column, we changed the N-terminal 8 × His tag of YciS to the Strep tag (tag sequence WSHPQFEK). Interestingly, we also found the smear band of YciS on SDS-PAGE, indicating that smear is the characteristic of YciS (Figure S7).

YciM and YciS are critical for growth and viability of E. coli K12
From the growth curve, ΔyciM strain grew very poor on M9 liquid medium at 37°C (Figure 3F). However, the ΔyciS strain only grew slightly slower than that of the wild type. The strain ΔyciSM, containing the deletion of both yciS and yciM, grew worse than strain ΔyciM, suggesting that yciS may cooperate with yciM in the survival of E. coli. All strains grew very well on LB medium means yciM is critical for E. coli when they are living in restrictive conditions, such as M9 medium. To further examine these observations, we constructed strain PMB-104 (ΔyciM carrying a plasmid pBAD33-yciM) and PMB-108 (ΔyciSM carrying a plasmid pBAD33-yciSM). The plasmid pBAD33 is unique in that the replication of the plasmid itself is dependent on the presence of L-arabinose. Deleting yciM or deleting both yciS and yciM caused E. coli to grow extremely sick on LB plates. However, overexpressing yciM or both yciS and yciM has no effect on the growth of E. coli (Figures 4G and 4H) Together, these results showed that the yciM gene is important for E. coli to survive.

yciM negatively regulates LPS level
To figure out why the deletion of yciM caused cell death, we measured the amount of LPS using Tricine-SDS-PAGE and silver staining (Figure 3I). Our result showed that the level of LPS is much higher in yciM deletion strain (at least ten times) compared with that of the parental strain (Figures 3I and 3J), which is consistent with the previous report (Mahalakshmi et al., 2014). Besides, the amount of LPS of stain PMB-102 (MG1655 carrying a plasmid pBAD33-yciM and in the presence of 0.2% L-arabinose) was slightly lower than that of wild type, indicating that overexpressed YciM leads to a decrease in LPS level. Meanwhile, the complemental strain PMB-104 with additional copies of yciM produced a higher amount of LPS than the wild type, but still far lower than that produced by strain PMB-103 (ΔyciM carrying an empty vector pBAD33).

DISCUSSION
In this study, we sought to better understand the role of the membrane proteins YciS and YciM in LPS biogenesis. Structurally, YciM contains a TPR domain mediating protein-protein interactions and a
zinc-binding domain with four cysteine residues locking a zinc ion in site. Based on the two YciM structures, we anticipate that YciM serves as a “switcher”, which could bind and release a lipid substrate by changing conformation when anchored in the IM inner leaflet. Residues V345 and L386 in the lipid binding tunnel are highly conserved and play important roles in the functionality of YciM. Their interactions with potential substrates are essential for cell survival. Our ITC results showed that LPS and 3-hydroxytyrismyric acid appear to bind YciM’s soluble domain compared to the control molecule ethylene glycol which is accidentally observed in the crystal structure. Due to the micromolar affinity of the YciM soluble portion to LPS and LPS precursors, the threshold of LPS fully occupying the potential binding site could be quite high, and other lipid molecules, such as phospholipids, also have a great chance to bind to the site. Furthermore, the cellular environment is much more complicated, and the transmembrane domain of YciM could anchor YciM into the membrane and all the lipids inside the membrane could compete this binding site. Considering the competition of lipid from the inner membrane, the Kd of YciM to other lipid molecules should be measured by several advanced techniques in the future to further investigate this. After mutating residue V345 in the lipid binding tunnel, YciM lost the ability of binding lipid molecules, like 3-hydroxytyrismyric acid or even LPS. Those results enhance our proposed “switcher” functionality of YciM. During the pull-down experiment, YciS could be co-purified with YciM which provided important clues concerning their function in mediating LPS regulation. Mutants absenting yciS and yciM have poorer growth than that absenting yciM suggested YciS could coordinate with YciM in regulating LPS biogenesis. An extremely high level of LPS in yciM deletion mutants leads to cell death, which was matched with results observed by Klein G et al. (Klein et al., 2014). According to previous studies by Fuhrer F et al. (Fuhrer et al., 2007), FtsH cooperates with YciM in the degradation of LpxC. Therefore, in the absence of YciM, LpxC would not be degraded and caused an urgent accumulation of LPS. Besides, overexpressing YciM would lead to over degradation of LpxC and the level of LPS decreasing. Our data suggest that YciM is a finely controlled negative regulator of LPS.

Although the structure of YciM in E. coli has been identified by Prince et al., in 2015, the function of YciM has not been clarified, leaving the roles YciM played in LPS regulation unclear (Prince and Jia, 2015). FtsH and YciM have been proved to affect lipid A biogenesis directly (Fuhrer et al., 2007). IM protein YejM could interact with LPS regulation proteins (like YciM or FtsH) by its periplasmic domain (Cian et al., 2019; Clairfeuille et al., 2020). Based on the obtained results, we supposed an LPS regulation mechanism of YciS and YciM: when the level of LPS decrease, YejM would combine YciM to prevent the interaction between FtsH and LpxC, which allows LpxC to produce LPS; when the LPS level is too high in bacteria, the lipid binding tunnel could interact with lipid substrates and activate YciM, then LpxC would be delivered by YciM and degraded by protease FtsH to curb the lipid A synthesis. If the lipid binding tunnel of YciM loses its functionality, it could not combine lipid substrates and could not deliver LpxC to FtsH, which caused the LPS level to elevate. The open conformation allows YciM to bind lipid molecules and inhibit LpxC’s activity, thus reducing the LPS level. After lipid molecules detach from the lipid binding tunnel, YciM would return to closed conformation and active LpxC, which induces the production of LPS (Figure 4). YciS may play a role in stabilizing YciM in the regulation mechanism. When the balance between LPS and phospholipid of the membrane was broken, cell permeability would increase that making cells become more fragile and are hard to resist the intrusion of toxin substance.

Figure 4. Two different conformations of YciM affect LPS level
The open conformation of YciM allows lipid molecules to bind the lipid binding tunnel. When YciM was activated, it would deliver LpxC to FtsH and LpxC’s activity was inhibited, thus causing the LPS level to decrease. After lipid molecules leaves, YciM returns to its closed conformation and combines with YejM, followed by LpxC was activated to produce LPS.
In summary, our results suggest that YciM is important for cell growth and could act as a "switcher" to regulate the LPS level. It would be worthwhile to test this model and also examine the mechanistic aspects of LPS regulation by YciS/YciM.

Limitations of the study
This study promotes additional information for our understanding of the LPS regulation mechanism via YciS/YciM. But there are still some limitations. For instance, the lipid binding tunnel in YciM has affinity to several lipid molecules. There could be a possibility that YciM might even play multi-roles in other bacterial life events, but what kind of lipid molecules still needs to be explored. Furthermore, when YciM is anchored in the membrane, the binding competition from different kinds of lipid from the membrane compositions would occur; the affinity of YciM to other lipid molecules should be measured by several advanced techniques to further investigate this.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
  - Lead contact
  - Materials availability
  - Data and code availability
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
  - Strains and culture condition
- **METHOD DETAILS**
  - Plasmid construction
  - Expression and purification of membrane protein complex YciS and YciM
  - Expression and purification of cytoplasmic domain of YciM
  - Crystallization of cytoplasmic domain of YciM
  - X-ray data collection and structure determination
  - Isothermal titration calorimetry (ITC)
  - Null mutant generation
  - Growth curve
  - Growth and viability assays
  - Quantification of LPS
  - Site-directed mutagenesis
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104988.

ACKNOWLEDGMENTS
This work was supported by the National Key R&D Program of China (2021YFC2100600) and the National Natural Science Foundation of China (31800052). We thank Prof. Yuhui Sun at Wuhan University to provide us the tool plasmids used for genome editing. We thank the staffs from BL17U, BL18U, and BL19U1 at Shanghai Synchrotron Radiation Facility (SSRF, China) and staffs from I03 at Diamond Light Source (UK) for assistance during X-ray data collection.

AUTHOR CONTRIBUTIONS
L.Y. generated the yciSM deletion strains and performed mutagenesis assays; H.H., H.L., and Z.Z. generated constructs and determined *S. typhimurium* YciM crystal structure; X.L. performed protein and ligand binding affinity assays. L.Y. wrote the paper; Z.D. discussed and revised the paper; C.D. and Z.Z. conceived the study, analyzed the data, and wrote the paper.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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Received: March 31, 2022
Revised: June 4, 2022
Accepted: August 17, 2022
Published: September 16, 2022
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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse Anti-Streptavidin epitope tag (Strep-Tag) Monoclonal Antibody, Unconjugated, Clone Strep-tag II | Sigma-Aldrich | RRID:AB_1007528 |
| Goat Anti-Mouse Goat anti-mouse IgG-HRP Polyclonal, Hrp Conjugated | Sigma-Aldrich | RRID:AB_631736 |
| Bacterial and virus strains |        |            |
| *Escherichia coli* MG1655 | ATCC | ATCC 700926 |
| *Salmonella enterica* subsp. *enterica* serovar Typhimurium LT2 | ATCC | ATCC 700720 |
| Δycs | This study | N/A |
| ΔycM | This study | N/A |
| ΔycSM | This study | N/A |
| PMB-101 (MG1655::Chl/pBAD33) | This study | N/A |
| PMB-102 (MG1655::Chl/pBAD33-yciM) | This study | N/A |
| PMB-103 (ΔycSM::Chl/pBAD33) | This study | N/A |
| PMB-104 (ΔycSM::Chl/pBAD33-yciM) | This study | N/A |
| PMB-105 (ΔycSM::Chl/P_{wild}-yciM) | This study | N/A |
| PMB-106 (MG1655::Chl/pBAD33-yciSM) | This study | N/A |
| PMB-107 (ΔycSM::Chl/pBAD33) | This study | N/A |
| PMB-108 (ΔycSM::Chl/pBAD33-yciSM) | This study | N/A |
| Chemicals, peptides, and recombinant proteins |        |            |
| SUMO-YciM | This study | N/A |
| YciS-YciM | This study | N/A |
| Experimental models: Organisms/strains |        |            |
| *Escherichia coli* MG1655 | ATCC | ATCC 700926 |
| Recombinant DNA |        |            |
| Plasmid: pET-28a(+)::YciS | This study | N/A |
| Plasmid: pET-28a(+)::YciM | This study | N/A |
| Plasmid: pET-28a(+)::YciSM | This study | N/A |
| Plasmid: pBAD33-yciM | This study | N/A |
| Plasmid: pBAD33-yciSM | This study | N/A |
| Software and algorithms |        |            |
| XDSGUI | Kabsch (2010) | XDS Package (mpg.de) |
| Diffraction Anisotropy Server | Strong et al. (2006) | Services – UCLA-DOE Institute | Diffraction Anisotropy Server |
| Phaser-MR | McCoy et al. (2007) | https://phenix-online.org/ |
| CCP4i | Liebschner et al. (2019) | CCP4i: The CCP4 Graphical User Interface |
| Refmac5 | Murshudov et al. (1997) | CCP4i: The CCP4 Graphical User Interface |
| PyMol | De-Lano Scientific | PyMOL | pymol.org |
| Deposited data |        |            |
| The structure of *S. typhimurium* YciM | This study | PDB code: 7W2B |
| The structure of *E. coli* YciM | Prince and Jia (2015) | PDB code: 4ZLH |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Zhenyu Zhang (zhengyu.zhang@whu.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability

• Data
The X-ray structures of S. typhimurium YciM was deposited in the Protein Data Bank under accession codes 7WZB. All other data generated and analyzed in this study are available within the article and the Supplementary Information.

• Code
This study did not generate any code.

• Additional information
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon reasonable request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Strains and culture condition
E. coli MG 1655 was used as the experimental model for null mutant generation
Strains were cultured in Luria–Bertani (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) or M9 Minimal medium (85.52 g/L Na2HPO4·12H2O, 15 g/L KH2PO4, 2.5 g/L NaCl, 5 g/L NH4Cl) supplemented with appropriate antibiotics. The following antibiotics were used at the indicated concentrations: kanamycin (Kan), 50 μg/mL; spectinomycin (Spc), 50 μg/mL, chloromycetin (Chl), 25 μg/mL.

METHOD DETAILS

Plasmid construction
Two DNA fragments containing yciS and yciM were amplified from the genome DNA of wild-type strain S. typhimurium separately. The DNA fragments were purified with DNA gel extraction kit (Magen, China) and then cloned into pET-28a(+) vector to generate pET-28a(+)-yciS and pET-28a(+)-yciM respectively. yciS and yciM are consecutive genes on the genome of wild-type strain S. typhimurium. So DNA fragments containing yciS and yciM were amplified from S. typhimurium together to construct pET-28a(+)-yciSM, and the 8×His tag was attached to the N-terminal of yciS.

Expression and purification of membrane protein complex YciS and YciM
The plasmid pET-28a(+)-yciSM was transformed into E. coli C43(DE3) cells to express proteins. A single colony was inoculated into 10 mL LB liquid medium supplemented with kanamycin and cultured at 37°C overnight. The overnight seed culture was inoculated (1:100) into LB liquid medium supplemented with kanamycin and grown at 37°C until the OD600 reached 0.8–1.0, then the proteins were induced by adding 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) for 14 h at 20°C with shaking at 220 rpm. Cells were harvested by centrifugation at 5,000 × g and re-suspended in TBS buffer (20 mM Tris-HCl, 300 mM NaCl, pH 8.0). The cell pellets were lysed by passing through a cell disruptor (ATS, China) at 30,000 psi. The unbroken cells and cell debris were removed by centrifugation at 10,000 × g for 1 h at 4°C. The supernatant was ultracentrifuged at 140,000 × g for 1 h at 4°C, and the membrane pellet was solubilized with an extraction buffer (20 mM Tris-Cl, 300 mM NaCl, 20 mM imidazole, 1% (w/v) n-Dodecyl-β-D-Maltopyranoside (DDM), pH 8.0) at 4°C for 1 h. The supernatant was then centrifuged at 10,000 × g for 30 min before being loaded onto a 5 mL of HisTrap-HP column (GE Healthcare). The column was pre-equilibrated with a balance buffer (20 mM Tris-Cl, 300 mM NaCl, 20 mM imidazole, 0.05% DDM, pH 8.0) and washed by a wash buffer (20 mM Tris-Cl, 300 mM NaCl, 40 mM imidazole, 0.05% DDM, pH 8.0). The proteins were eluted by an elution buffer (20 mM Tris-Cl, 300 mM NaCl, 300 mM imidazole, 0.05% DDM, pH 8.0), and further purified
by the size exclusion chromatography with a Superdex 200 size-exclusion column (GE Healthcare). The purity of protein was checked using SDS-PAGE. All the purification processes were performed at 4 °C.

Expression and purification of cytoplasmic domain of YciM

The plasmid was transformed into E. coli BL21(DE3) cells to express proteins. Culture condition was as same as described previously. Cells were harvested by centrifugation at 5,000 × g and re-suspended in the lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10% glycerol, pH 8.0) added with complete EDTA-free Protease Inhibitor Cocktail Tablet, 50 µg/mL DNase I and 100 µg/mL lysozyme. The cell pellets were lysed by passing them through a cell disruptor (ATS, China) at 30,000 psi. The unbroken cells and cell debris were removed by centrifugation at 16,000 × g for 30 min at 4°C. The supernatant was loaded onto a 5 mL of HisTrap-HP column (GE Healthcare) pre-equilibrated with the balance buffer (20 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 8.0). The protein was washed by the wash buffer (20 mM Tris-HCl, 300 mM NaCl, 40 mM imidazole, 10% glycerol, pH 8.0) and eluted by the elution buffer (20 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole, 10% glycerol, pH 8.0). The protein sample was desalted using a pre-equilibrated desalting column (GE Healthcare) with the TBS buffer to remove the high concentration of imidazole. TEV protease was added to the desalted protein at a TEV to protein mass ratio 1:100 and incubated at 4°C overnight. The post-cleavage mixture was loaded onto the pre-equilibrated 5 mL HP HisTrap column (GE Healthcare) again and collected. Collected proteins were further purified by the size exclusion chromatography with a Superdex 200 size-exclusion column (GE Healthcare). The purity of protein was checked using SDS-PAGE. All the purification processes were performed at 4°C.

Crystallization of cytoplasmic domain of YciM

For crystallization, protein in the TBS buffer (20 mM Tris, 300 mM NaCl, pH 8.0) was concentrated to 10 mg/mL after being digested by thermolysin at 1:500 mass ratio at room temperature for 4 h. Crystallization conditions were primarily screened by the mosquito robot (TTP Lab Tech) with 96-well plates using commercial solution kits from Hampton Research at protein to buffer 2:1, 1:1, and 1:2 ratios. Best diffraction crystals were grown from 0.5 L of protein YciM with 0.5 µL of the reservoir solution, using the sitting drop crystallization protocol (Adachi et al., 2003). The native YciM crystal was grown at 20 °C in 21.25% Ethylene glycol and 15% glycerol over 2 weeks. Crystals were flash frozen in a stream of liquid nitrogen before data collection.

X-ray data collection and structure determination

The X-ray diffraction data were collected at Diamond Light Source beamline I02 (UK) and Shanghai Synchrotron Radiation Facility beamline BL19U1 (China). The data were subsequently indexed, integrated, and scaled by XDSGUI program suite (Kabsch, 2010). The data appeared to be slightly anisotropic and was then corrected using UCLA-DOE LAB — Diffraction Anisotropy Server XDS processed data (Strong et al., 2006). The anisotropic truncated resolutions were 2.4 Å 2.4 Å 2.9 Å along a*, b*, c* directions respectively. The initial phases were determined by molecular replacement using CCP4i suite PhaserMR program (Liebschner et al., 2019; McCoy et al., 2007) with one protomer of 4ZLH as the searching model.

The crystal structure of S. typhimurium YciM cytoplasmic domain was then built using Buccaneer CCP4i (Collaborative Computational Project, 1994) and refined using Refmac5 (Murshudov et al., 1997). The crystal structure in this study greatly differs from that of 4ZLH. Our dimeric structures shifted at a 40 degrees angle against each other compared to 4ZLH, thus opening a tunnel of potential lipid binding ability. The Crystal structure of S. typhimurium YcIM cytoplasmic domain was determined at a resolution of 2.7 Å, which belonged to P212121 space group with the cell dimensions of a = 72.08 Å, b =78.96 Å, c = 124.03 Å, and α = 90°, β = 90°, γ = 90°, and two molecules in one asymmetric unit. Structure representations were prepared with PyMOL (DeLano Scientific). The geometries of the final structure were evaluated by PHENIX (Collaborative Computational Project, 1994) and the resulting coordinates and structure factors have been deposited in the Protein Data Bank (PDB code: 7WZB).

Isothermal titration calorimetry (ITC)

Experiments were performed using the Nano-ITC (TA, USA) in the standard buffer conditions given above. In each titration 60–70 µM protein, in the cell, was titrated with 25 injections of 300 µM ligand. The volume of each injection was 10 µL. Injections were continued beyond saturation level to allow for the determination of heats of ligand dilution. The resulting data were fit to a single-site binding isotherm
With an additional term for the heat of dilution, using the NanoAnalyze software supplied with the calorimeter. Estimated errors in thermodynamic quantities are generated automatically by the software. The variation between repeated experiments at 25°C is consistent with these error estimates.

**Null mutant generation**

Complete deletion of yciS, yciM and both of yciS and yciM were constructed by pEcCas/pEcgRNA system provided by Li Q lab (Li et al., 2021). Special N20 sequence of each gene was designed using Benchling and fused with Bsu1-linearized pTargetF using Golden Gate Assembly to yield target pEcgRNA for yciS and yciM. The electroporation of competent cells of E. coli MG1655 carrying pEcCas was performed using protocol provided by Cha et al. (Cha et al., 1997; Pujol and Kado, 2000; Sharan et al., 2009). The culture medium was supplemented with a final concentration of 10 mM L-arabinose to induce the expression of λ-Red system. For genome editing, 100 µL of competent cells were mixed with 100 ng of pEcgRNA series plasmids and 400 ng of donor DNA, and the mixture was electroporated in a precooled 1 mm Gene Pulser cuvette. For genome editing, 100 µL of competent cells were mixed with 100 ng of pEcgRNA series plasmids and 400 ng of donor DNA, and the mixture was electroporated in a precooled 1 mm Gene Pulser cuvette. The electroporation mixture was immediately suspended in 1 mL of fresh LB medium. Cells were recovered by incubating at 37°C for 1 h before spreading on LB plates containing kanamycin and spectinomycin, and the plates were then incubated overnight at 37°C. Individual colonies were randomly picked and verified by colony-PCR and DNA sequencing. The pEcgRNA series plasmids of verified strains were eliminated by methods provided by Li Q lab (Li et al., 2021).

**Growth curve**

Cells of overnight cultures of bacteria grown in 3 mL LB liquid medium at 37°C were harvested at 3,000 × g for 5 min and washed by sterilized double distilled H2O twice. The re-suspended culture was diluted at 1:100 ratio into M9 liquid medium (supplemented with 0.2% D-glucose as the carbon source) and grown at 37°C with shaking at 220 rpm. Growth was monitored by measuring OD600 every 1 h for 9 h.

**Growth and viability assays**

A single colony of each strain was inoculated into 3 mL LB liquid medium supplemented with 0.2% L-arabinose and grown at 37°C overnight with shaking at 220 rpm. Cells were harvested at 3,000 × g for 5 min and washed by sterilized double distilled H2O twice. The re-suspended culture was diluted to OD600 1.0, and then serially diluted at various dilutions (10⁻¹, 10⁻², 10⁻³, 10⁻⁴, 10⁻⁵). 2 µL aliquots of each dilution were spotted on indicated plates and incubated at 28°C for 48 h.

**Quantification of LPS**

The extraction of LPS according to an improved hot aqueous-phenol method from Davis M.R lab (Davis and Goldberg, 2012). A single colony of each strain was inoculated into 10 mL M9 medium supplemented with 0.2% L-arabinose and 0.2% D-glucose and grown at 37°C with shaking at 220 rpm for 24 h. The culture was diluted to OD600 0.5 with M9 medium followed by centrifugation at 10, 600 × g for 10 min. Diluting 2 × stock (100 mM Tris-HCl, pH 6.8, 24% w/v glycerol, 8% w/v SDS, 5% v/v 2-β-mercaptoethanol, 0.02% w/v bromophenol blue) 1:1 in sterile distilled H2O to prepare the 1 × tricine sample stock. Pelleted bacteria were re-suspended with 200 µL 1 × tricine sample buffer and boiled for 10 min. Adding 50 µg of both DNase I and RNase to samples and incubating at 37°C for 30 min. Then adding 100 µg of the Proteinase K to samples and incubating at 59°C for 3 h. To each sample, adding 200 µL of ice-cold Tris-saturated phenol and vortexing each sample for approximately 5 to 10 s. Samples was incubated at 65°C for 15 min and vortexed occasionally. After incubating cool to room temperature, and then adding 1 mL of room-temperature diethyl ether to each sample and vortexing for 5 to 10 s. Centrifuging the samples at 13,300 × g for 30 min. Carefully remove the samples from the centrifuge and extract the bottom blue layer. LPS were revealed by 16.5% Tricine-SDS-PAGE and silver staining according to the manufacturer’s instructions.

**Site-directed mutagenesis**

The full length of yciM was amplified from MG1655 and cloned into vector pBAD33 to generate pBAD33-yciM. Plasmids of mutagenesis were constructed using pBAD33-yciM as the template with different primers listed in Table S1. All site-directed mutants were generated following Liu, H. and J.H. Naismith’s protocol (Liu and Naismith, 2008). Plasmids were sequenced to verify the desired mutants. The resultant plasmids were transformed into the competent cells ΔyciM using electroporation for characterization observation.
QUANTIFICATION AND STATISTICAL ANALYSIS

For LPS extraction and growth curve assays, data were plotted using Origin 2021. Pooled data from $N = 3$ separate experiments and Standard Deviation (SD) are shown.

For ITC experiments, data were processed by NanoAnalyze software supplied with the calorimeter to fit to a single-site binding isotherm. Estimated errors in thermodynamic quantities were generated automatically by the software.