AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

YejM Controls LpxC Levels by Regulating Protease Activity of the FtsH/YciM Complex of *Escherichia coli*

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ABSTRACT LpxC is a deacetylase that catalyzes the first committed step of lipid A biosynthesis in *Escherichia coli*. LpxC competes for a common precursor, *R*-3-hydroxymyristoyl-UDP-GlcNAc, with FabZ, whose dehydratase activity catalyzes the first committed step of phospholipid biosynthesis. To maintain the optimum flow of the common precursor to these two competing pathways, the LpxC level is controlled by FtsH/YciM-mediated proteolysis. It is not known whether this complex or another protein senses the status of lipid A synthesis to control LpxC proteolysis. The work carried out in this study began with a novel mutation, *yejM1163*, which causes hypersensitivity to large antibiotics such as vancomycin and erythromycin. Isolates resistant to these antibiotics carried suppressor mutations in the *ftsH* and *yciM* genes. Western blot analysis showed a dramatically reduced LpxC level in the *yejM1163* background, while the presence of *ftsH* or *yciM* suppressor mutations restored LpxC levels to different degrees. Based on these observations, it is proposed that YejM is a sensor of lipid A synthesis and controls LpxC levels by modulating the activity of the FtsH/YciM complex. The truncation of the periplasmic domain in the YejM1163 protein causes unregulated proteolysis of LpxC, thus diverting a greater pool of *R*-3-hydroxymyristoyl-UDP-GlcNAc toward phospholipid synthesis. This imbalance in lipid synthesis perturbs the outer membrane permeability barrier, causing hypersensitivity toward vancomycin and erythromycin. *yejM1163* suppressor mutations in *ftsH* and *yciM* lower the proteolytic activity toward LpxC, thus restoring lipid homeostasis and the outer membrane permeability barrier.

IMPORTANCE Lipid homeostasis is critical for proper envelope functions. The level of LpxC, which catalyzes the first committed step of lipopolysaccharide (LPS) synthesis, is controlled by an essential protease complex comprised of FtsH and YciM. Work carried out here suggests YejM, an essential envelope protein, plays a central role in sensing the state of LPS synthesis and controls LpxC levels by regulating the activity of FtsH/YciM. All four essential proteins are attractive targets of therapeutic development.

KEYWORDS *Escherichia coli*, FtsH protease, lipid homeostasis, LpxC, YciM, YejM, lipopolysaccharide

Integrity of the outer membrane (OM) is vital for Gram-negative enteric bacteria, since this structure is directly exposed to hostile environments—such as in the mammalian gut—where enteric bacteria such as *Escherichia coli* live. The bacterial OM is an asymmetric lipid bilayer, because its two major lipid constituents, lipopolysaccharide (LPS) and phospholipids (PLs), are distributed asymmetrically, with LPS and PLs occupying the outer and inner leaflets, respectively (1, 2). β-Barrels and lipoproteins are the two major proteinaceous constituents of the OM, with the former class frequently making channels to allow diffusion of nutrients from the external milieu or transport of proteins/LPS into or across the OM (1). Unlike β-barrels, lipoproteins are involved in

Citation Nguyen D, Kelly K, Qiu N, Misra R. 2020. YejM controls LpxC levels by regulating protease activity of the FtsH/YciM complex of *Escherichia coli*. J Bacteriol 202:e00303-20. https://doi.org/10.1128/JB.00303-20.

Editor Conrad W. Mullineaux, Queen Mary University of London

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For a commentary on this article, see https://doi.org/10.1128/JB.00370-20.

Received 19 May 2020
Accepted 9 June 2020
Accepted manuscript posted online 15 June 2020
Published 25 August 2020

providing mechanical strength to the envelope structure (3) or in the transport of OM constituents (4, 5). A thin peptidoglycan layer beneath the OM is connected to it through a specific lipoprotein (Lpp) (6) and a β-barrel (OmpA) (7).

The OM function of maintaining the selective permeability barrier is compromised when its lipid asymmetry is perturbed (1, 2). Many factors contribute to this perturbation, including altered LPS packing due to interrupted bridging between LPS's phosphate groups and cations (8) and processes that are defective in removing PL from the outer leaflet of the OM (9). LPS and PL synthesis is intimately tied to a common acyl-donating precursor, *R*-3-hydroxymyristoyl-acyl carrier protein (ACP), whose distribution into these two competing pathways is critically controlled (10, 11). LpxA catalyzes the first reversible step of UDP-GlcNAc acylation by *R*-3-hydroxymyristoyl-ACP (12). The second nonreversible step, involving LpxC-mediated deacetylation of acylated UDP-GlcNAc, commits the pathway to subsequent additions of acyl chains and the completion of the lipid A molecule (13). FabZ is a dehydratase that competes for *R*-3-hydroxymyristoyl-ACP for PL synthesis (10).

Mutations in *lpxC*, called *envA*, were first isolated among smooth ampicillin-sensitive derivatives of a rough ampicillin-resistant parent strain (14). The original *envA* mutation conferred sensitivity to several antibiotics (15, 16) and carried a missense mutation, resulting in an H19 to Y substitution in the 305-residue LpxC (EnvA) protein (17). Additional missense mutations in *lpxC*, called *asmB*, were later obtained among isolates that reversed the assembly defect of a mutant OmpF protein (18). To reveal the genetic interactive network of *lpxC*, revertants overcoming sensitivity to antibiotics or bile salts were obtained, and the extragenic suppressor alleles called *sefA1* (15, 19) and *sabA1* (20) were subsequently mapped to *fabZ*. Independently, mutations in *fabZ* were also identified as suppressors of a temperature sensitive allele of *lpxA* (10). These studies provided the genetic evidence of interactions between the two competing pathways involving LPS and PL biosynthesis. Indeed, when mutations in *lpxC* lowered LPS synthesis, a greater pool of acyl-ACP was driven toward PL synthesis (20). Inhibition of lipid A synthesis either by an *lpxA* mutation or an inhibitor of LpxC's deacetylase activity elevated LpxC levels (21). This led to the hypothesis of the existence of a novel feedback loop that senses lipid A precursors to regulate the rate of lipid A biosynthesis (21). To coordinate this regulation, the authors also postulated the existence of a lipid A sensor in the OM or the periplasmic side of the inner membrane (IM). Consistent with the prediction of posttranscription regulation of LpxC, Ogura et al. (11) showed that an IM-bound essential protease, FtsH, is responsible for degrading LpxC and that FtsH's essentiality may stem from elevated LPS synthesis.

A mutation in the putative lipid A sensor, *yejM*, was first obtained among antibiotic-sensitive isolates with reduced lipid A levels (22). However, the identity of the affected gene in the original isolate LH530 remained unknown until a decade later when De Lay and Cronan (23) identified it to be *yejM*. The original *yejM* mutation caused a truncation of the protein segment thought to reside in the periplasm (23). Moreover, it was determined that the IM-anchored region of YejM, predicted to fold into five transmembrane helices, was essential for growth (23). Recently, additional mutations in *yejM* were isolated through a genetic strategy that sought bypass suppressors to allow iron-chelating enterobactin to enter cells defective in its transport across the OM (24). These mutations also caused truncations of the putative periplasmic domain and conferred hypersensitivity to antibiotics, such as vancomycin, that are normally precluded from crossing the intact OM barrier. Based on the observations of increased OM permeability, elevated levels of lyso-PLs, and a synthetic growth phenotype without PldA, which is responsible for degrading PL from the outer leaflet of the OM, it was concluded that the essentiality of YejM stems from its role in lipid homeostasis (24). YejM is also implicated in cardiolipin transport in *Salmonella* (25, 26) and *Shigella* (27); however, YejM's essentiality is unlikely to be due to this proposed function given that cardiolipin is not essential in these enteric bacteria.

In 2014, an IM-bound modulator of FtsH, YciM, was identified (28–30). *yciM* is essential, but the gene deletion is possible when cells are grown on a minimal medium

TABLE 1 MICs of vancomycin and erythromycin in various genetic backgrounds

Strain name	Relevant genotype	Codon (no.) change	MIC (μg/ml) of:	
			Erythromycin	Vancomycin
RAM3199	Δ*tonB*	NA[a]	96	128
RAM3200	Δ*tonB yejM1163*	NA	3	4
RAM3201	Δ*tonB yejM1163 ftsH*-E367D	GAA (367)→GAC	48	64
RAM3202	Δ*tonB yejM1163 yciM*-W377G	UGG (377)→GGG	48	12
RAM3203	Δ*tonB yejM1163 yciM*-A126V	GCG (126)→GUG	96–128	≥128
RAM3204	Δ*tonB yejM1163 yciM*-L95R	CUA (95)→CGA	96–128	≥128
RAM3205	Δ*tonB yejM1163 ftsH*-L78R	CUG (78)→CGG	64–96	96-128
RAM3206	Δ*tonB yejM1163 ftsH*-D74A	GAU (74)→GCU	32–48	64-96

[a]NA, not applicable.

at 30°C (29) or on a rich medium in the presence of a suppressor mutation mapping in *lpxC* (29, 30). These studies implicated that YciM regulates LPS synthesis by modulating FtsH-mediated degradation of LpxC and concluded that Δ*yciM*'s lethality, like that of Δ*ftsH*, may be linked to LPS overproduction due to unregulated LpxC levels. However, it remained unclear as to how cells sensed excess LPS to activate FtsH/YciM-mediated degradation of LpxC levels and reestablish LPS/PL homeostasis. As concluded in this and two recent studies (31, 32), YejM likely plays a sensory role for LPS synthesis by monitoring the accumulation of LPS intermediates in the IM and coordinates with the FtsH/YciM complex to regulate LpxC levels.

The study presented here took advantage of the antibiotic hypersensitivity phenotype of the mutant *yejM1163* allele (24) and sought antibiotic-resistant revertants. Suppressor mutations in these revertants were found in the *ftsH* and *yciM* genes. It was shown that *yejM1163* caused a dramatic reduction in the LpxC level, while suppressor mutations reelevated LpxC levels to various degrees to normalize lipid homeostasis and repair a breach in the OM permeability barrier.

RESULTS AND DISCUSSION

Isolation of antibiotic-resistant revertants of the Δ*tonB yejM1163* strain. The antibiotic hypersensitivity phenotype of the Δ*tonB yejM1163* strain allowed the opportunity to isolate and characterize suppressor mutations that confer antibiotic resistance. Vancomycin and erythromycin were chosen as selective agents, since both are large antibiotics (>700 Da) and thus are useful in probing outer membrane permeability defects. Erythromycin- and vancomycin-resistant mutants arose at a frequency of 10^{-7} to 10^{-8}, indicating the prevalence of possible missense mutations in genes indispensable under the applied selective condition. Six independently obtained resistant isolates were characterized in some detail for this study.

Whole-genome sequence (WGS) analysis was carried out to locate the mutation responsible for the antibiotic-resistant phenotype. The initial WGS analysis involved four revertants with distinct growth phenotypes on selective and nonselective media. Once mutations were located, they were confirmed by PCR amplification and Sanger sequencing of the affected gene. Genetic location of suppressor mutations in the remaining two revertants were first determined by P1 transductional analysis using markers linked to the targeted genes, followed by direct Sanger sequencing of the amplified DNA fragment. All six suppression mutations were unique and mapped within the two essential genes, *ftsH* and *yciM* (*lapB*) (Table 1). The location of all six suppressors in two essential genes and their missense nature were consistent with the low frequency of occurrence of the antibiotic-resistant revertants.

Phenotypic characterization of revertants. We first determined MICs against the two antibiotics used for selection, erythromycin and vancomycin, using Etest presoaked antibiotic strips. As expected, all six revertants had significantly higher MICs against erythromycin and vancomycin than the parental strain carrying the *yejM1163* allele (Table 1). In two revertants, carrying YciM-L95R and YciM-A126V substitutions, the resistance against these two OM permeability-probing antibiotics reached similar levels as the strain carrying the wild-type *yejM* allele.

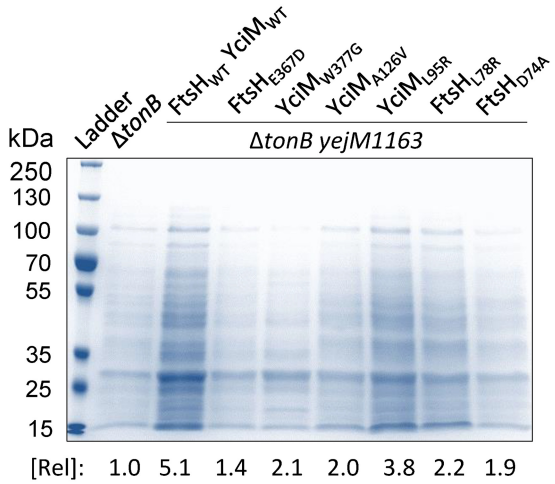

FIG 1 Examination of cell-free culture supernatants by SDS-PAGE. Cells from overnight-grown cultures were spun down, and the supernatants, after filtration and concentration, were analyzed by SDS-PAGE. Supernatant proteins were visualized after staining with Coomassie brilliant blue. Protein bands were quantified using Bio-Rad Quantity One software (amounts relative to that in the Δ*tonB* strain are shown). The key genetic characteristics of strains used are shown at the top.

Strains used in this study contained a Δ*tonB* mutation, which significantly impaired cell growth on a medium not supplemented with 40 μM ferric chloride (24). *yejM1163* was isolated among revertants that grew more rapidly than the parent Δ*tonB* strain on a medium not supplemented with ferric chloride (24). *yejM1163* was subsequently shown to increase OM permeability, allowing iron to enter the cell and relieving growth defects on an unsupplemented medium (24). Based on these observations, it stands to reason that suppressor mutations that overcome the antibiotic sensitivity phenotype of the Δ*tonB yejM1163* strain would simultaneously reduce the OM permeability defect of the strain. If so, we expect to see a reduced leaky phenotype and somewhat impaired growth on a medium not supplemented with ferric chloride.

To assess the leaky phenotype, cell-free supernatants were isolated from overnight cultures grown with ferric chloride. Iron was added to ensure normal growth of the Δ*tonB* control strain and revertants that increase MICs against erythromycin and vancomycin. After separating cells by centrifugation, any remaining cells were removed by filtering supernatants through 0.2-μm-pore size syringe filters. Supernatants were concentrated by centrifugation under vacuum and reconstituted in roughly one-tenth the original volume. Samples of the supernatants, normalized to the culture optical density at 600 nm (OD_{600}), were analyzed by SDS-PAGE, and protein bands were visualized and quantified after Coomassie brilliant blue staining (Fig. 1). As expected, the presence of the *yejM1163* mutation in the Δ*tonB* background caused a dramatic increase the amount of proteins leaked through the outer membrane. It should be noted that we previously showed that leaked proteins in the *yejM1163* background are not the result of cell lysis, since, unlike the highly leaky Δ*tolA* strain (33), there was no trace of the cytoplasmic enzyme in the culture supernatant (24). All six revertants derived from the Δ*tonB yejM1163* strain had reduced levels of leaked proteins (Fig. 1), thus supporting the hypothesis that the suppressor mutations mapping in *ftsH* or *yciM* lower the OM permeability of the *yejM1163* mutant. Based on antibiotic resistance data (Table 1), we were expecting YciM-L95R and YciM-A126V mutants to display the lowest leaky phenotype, but this was not the case (Fig. 1). One possibility for this discrepancy may be that higher protein contents in the culture supernatants of the two YciM mutants in Fig. 1 reflect a combined result of perturbed OM integrity and increased OM vesiculation. In contrast, the antibiotic resistance phenotype in Table 1 mainly reflects restored OM permeability barrier.

Next, we tested the growth of strains on a medium unsupplemented with ferric chloride (Fig. 2). The Δ*tonB* strain grew poorly, and faster-growing revertants were

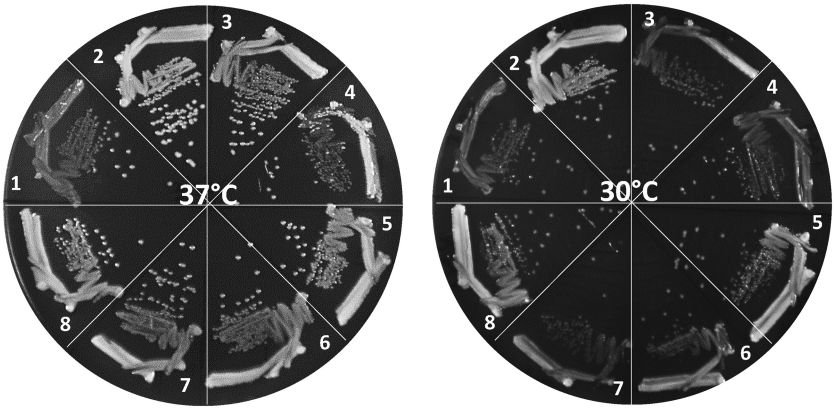

FIG 2 Bacterial growth on LBA was recorded after incubating petri plates at 37°C and 30°C for 24 h. Bacterial strains used are as follows: 1, RAM3199 (Δ*tonB*); 2, RAM3200 (Δ*tonB yejM1163*); 3, RAM3201 (RAM3200 *ftsH*-E367D); 4, RAM3202 (RAM3200 *yciM*-W377G); 5, RAM3203 (RAM3200 *yciM*-A129V); 6, RAM3204 (RAM3200 *yciM*-L95R); 7, RAM3205 (RAM3200 *ftsH*-L78R); 8, RAM3206 (RAM3200 *ftsH*-D74A).

occasionally visible after 24 h growth at both 30°C and 37°C. In contrast, the Δ*tonB yejM1163* strain grew much faster than the Δ*tonB* strain, forming larger colonies, and produced no visible revertants. All six revertants had growth patterns in between those of the Δ*tonB* and Δ*tonB yejM1163* strains, with more pronounced growth differences at 30°C than at 37°C (Fig. 2). It is worth noting that we did not determine bacterial growth rates by monitoring growth of liquid cultures due to the concern that faster growing revertants, as seen in the Δ*tonB* strain and one of the revertants carrying YciM-W377G, may take over the population and thus artificially display better-than-expected growth. Nevertheless, combined with higher MIC (Table 1) and reduced protein leakage data (Fig. 1), the qualitative growth data (Fig. 2) are consistent with the notion that all six suppressor mutations reduce the OM permeability defect of the *yejM1163* mutant.

Effect of suppressor mutations on LpxC levels. We previously concluded that YejM plays an essential role in lipid homeostasis independent of its putative nonessential role in transporting cardiolipin from the inner to the outer membrane (24). This conclusion was further corroborated by the identification of *yejM1163* suppressor mutations in two genes—*ftsH* and *yciM*—whose products coordinate proteolysis of LpxC (11, 29, 30, 34), an essential enzyme that operates at a critical juncture in the biogenesis of LPS and PL (10). Based on the central role LpxC plays in lipid biogenesis and the fact that *yejM1163* suppressors map to genes whose products control LpxC levels, we hypothesized that YejM positively regulates LpxC levels. Accordingly, LpxC would be destabilized in the *yejM1163* background but restabilized by suppressor mutations in the *ftsH* and *yciM* genes. This hypothesis is also consistent with the membrane permeability data, since destabilization in LpxC is expected to drive the common pool of lipid intermediate toward PL synthesis and interfere with the OM asymmetry and permeability (Table 1 and Fig. 1).

To test our hypothesis, we examined LpxC levels by Western blot analysis using the commercially available LpxC polyclonal antibodies (Fig. 3A). In the wild-type *yejM* background, the LpxC band, migrating at the 34-kDa position, was present just below a cross-reacting band running above it. Remarkably, no LpxC was detected from cell extracts of the *yejM1163* strain. Different levels of LpxC were detected in strains carrying suppressor mutations in the *ftsH* or *yciM* gene. Although LpxC was barely visible in four of the suppressor-carrying strains, its levels rose dramatically in two strains carrying the A126V and L95R alterations in the YciM protein (Fig. 3A). The absence of LpxC in the *yejM1163* strain supported the hypothesis that wild-type YejM is required for LpxC's stability. On the other hand, suppressor mutations appear to stabilize LpxC and reestablish lipid homeostasis by downregulating the activities of two negative modulators of LpxC, FtsH and YciM.

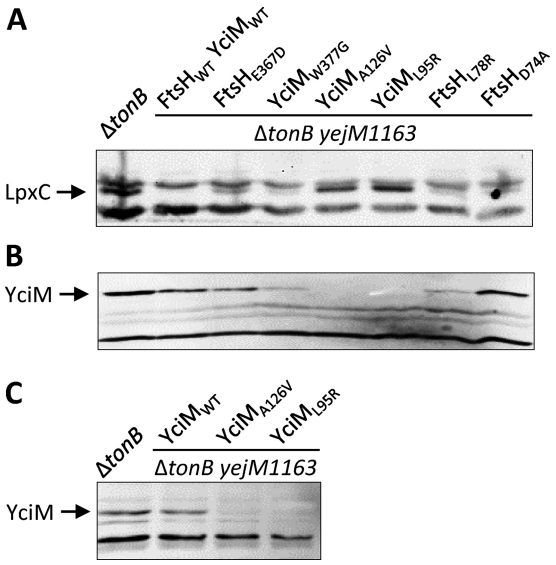

FIG 3 Effects of *ftsH* and *yciM* suppressor mutations on LpxC and YciM levels. Western blot analyses were carried out to detect LpxC (A) and YciM (B and C). LpxC was examined from whole-cell extracts, while YciM was examined from either purified envelopes (B) or whole-cell extracts (C).

Effect of suppressor mutations on YciM levels. The strong suppressive effects of YciM-A126V and YciM-L95R prompted the examination of YciM levels to determine whether suppressors broadly affect YciM's folding and stability or its specific function. YciM levels were examined from Western blots of envelopes purified from overnight cultures (Fig. 3B). Remarkably, all three *yciM* suppressor mutants showed significantly reduced levels of YciM, and no YciM was detected in strains carrying the YciM-A126V or YciM-L95R substitution. It is important to note that LpxC levels were the highest in the same two suppressor strains with no detectable YciM (Fig. 3A), thus showing a strong negative correlation between the levels of the two proteins. Curiously, the level of YciM was also significantly reduced in one of the suppressors carrying the FtsH-L78R substitution. Although the reason for this is not clear, it may reflect altered substrate specificity of FtsH (see below).

Because YciM levels in strains carrying the A126V and L95R suppressor alterations were dramatically low, we considered the possibility that mutant YciM was lost during the lengthy envelope isolation procedure. Therefore, YciM levels in these two strains were reexamined from cell extracts obtained by directly boiling pelleted cells in an SDS-based sample buffer (Fig. 3C). Again, unlike the control strains, no YciM was detected in the two suppressor carrying strains, thus confirming the notion that *in vivo* misfolding/destabilization of the mutant YciM proteins likely reduces FtsH-mediated degradation of LpxC.

Phenotypic verification of the mutant YciM data. The data presented above suggested that reduced levels of the mutant YciM proteins led to LpxC stabilization and restoration of lipid homeostasis in the *yejM1163* background. If so, expression of wild-type YciM in the mutant *yciM* background should once again stimulate LpxC degradation and destabilize lipid homeostasis. We can phenotypically test this balance/imbalance in lipid biogenesis by determining MICs against erythromycin and vancomycin. The wild-type gene and a mutant *yciM* allele, *yciM*-L95R, were cloned into pBAD24 in which an arabinose-inducible promoter controls the expression of the cloned gene (35). Plasmids with cloned *yciM* genes and an empty vector were transformed into the *yejM1163* strain carrying the *yciM*-L95R mutation, and MICs against erythromycin and vancomycin were measured. The presence of a plasmid carrying the wild-type *yciM* gene restored the drug sensitivity phenotype in the *yejM1163 yciM*-L95R strain (Table 2), indicating that (i) *yciM*-L95R is recessive to the wild-type allele and (ii)

TABLE 2 MICs of vancomycin and erythromycin in various genetic backgrounds containing an empty pBAD24 vector or the cloned wild-type or mutant *yciM* gene

Strain name	Relevant genotype	MIC (μg/ml) of:	
		Erythromycin	Vancomycin
RAM3212	Δ*tonB* (pBAD24)	64–96	128
RAM3213	Δ*tonB* (pBAD-*yciM*)	64–96	128
RAM3214	Δ*tonB yejM1163* (pBAD24)	6	4
RAM3215	Δ*tonB yejM1163* (pBAD-*yciM*)	3	1.5
RAM3207	Δ*tonB yejM1163 yciM*-L95R (pBAD24)	64–96	>128
RAM3208	Δ*tonB yejM1163 yciM*-L95R (pBAD-*yciM*)	4	2
RAM3209	Δ*tonB yejM1163 yciM*-L95R (pBAD-*yciM*-L95R)	96	>128

the presence of wild-type YciM triggers an imbalance in lipid homeostasis, presumably by inducing FtsH-mediated LpxC proteolysis. As expected, a plasmid carrying the mutant *yciM*-L95R allele or the empty vector failed to reverse the drug-resistant phenotype of the *yejM1163 yciM*-L95R strain (Table 2), reflecting the preservation of balanced lipid homeostasis. These data further support the notion that the unstable YciM-L95R and presumably YciM-A126V proteins fail to stimulate FtsH-mediated LpxC proteolysis, thus suppressing the *yejM1163* defect. The presence of the pBAD24-*yciM* plasmid in the Δ*tonB* background did not significantly change MICs against erythromycin or vancomycin, while the *yejM1163* strain carrying this plasmid grew sickly and displayed elevated MICs against the two antibiotics, presumably due to overexpression of YciM (Table 2). It is worth mentioning that during MIC tests, leaky *yciM* expression from the plasmid was sufficient for complementation. The presence of 0.02% arabinose caused a severe growth defect in the strain expressing wild-type *yciM* but not the mutant *yciM* gene (data not shown). We assume that this growth defect likely stems from enhanced proteolysis of LpxC, whose levels are already extremely low due to *yejM1163*, thus further curtailing the flow of the common lipid precursor pool toward LPS synthesis.

YciM structure and the possible mechanism of suppression. Modeling (28, 29) and structural (36) analyses revealed that YciM has two distinct domains, with its N terminus anchored to the inner membrane, while the soluble cytoplasmic domain is folded into 9 tetratricopeptide repeat (TPR) motifs, known for their ability to mediate protein-protein interactions (37), and a rubredoxin-like metal-binding motif (38) at its C terminus. The three novel suppressor mutations isolated in this work affect highly conserved residues in TPR-2 (L95R), TPR-3 (A126V), or the metal-binding motif (W377G) (Fig. 4 and 5). The L95R and A126V substitutions drastically lower YciM levels, while the effect of W377G on YciM levels is less severe (Fig. 3). Although the three substitutions could affect specific activities of YciM, e.g., its interaction with FtsH or metal-binding capacity, lower YciM levels in all three cases preclude us from suggesting a specific functional defect. Instead, it is possible that the suppressive effect of these substitutions stems from their lower levels. Since YciM interacts with FtsH, a broad-specificity envelope protease, it is conceivable that structural changes brought upon by the three substitutions make the mutant YciM proteins targets of FtsH. The net result of lower YciM levels would be reduced FtsH-mediated proteolysis of LpxC and, consequently, its stabilization in the *yejM1163* background.

It is important to note that YciM is an essential protein, yet at least in two mutants, YciM-L95R and YciM-A126V, YciM levels were undetectable by Western blotting. Previous work has shown that a deletion of *yciM* is tolerated in a background containing a missense mutation in *lpxC* (29, 30). Essentiality of YciM in the wild-type background is thought to stem from LPS overproduction due to LpxC stabilization without activated FtsH (29, 30). Consequently, mutations such as those mapping to *lpxC*, which decrease the flow of the common lipid precursor pool to LPS, are expected to be tolerated in a Δ*yciM* background. Due to extremely low protein levels, *yciM*-L95R and *yciM*-A126V mimic a *yciM*-null allele. *yejM1163* dramatically reduces LpxC levels/stability, thus creating an environment where *yciM*-L95R and *yciM*-A126V alleles will be tolerated.

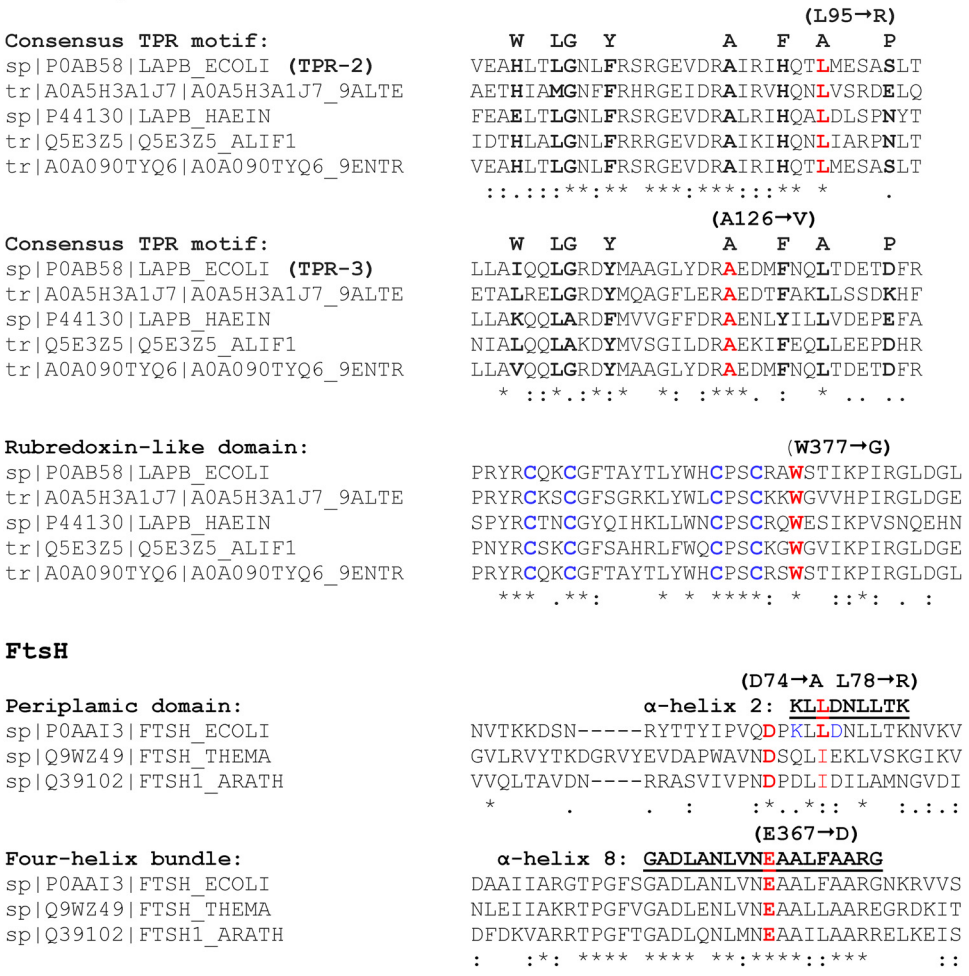

FIG 4 Alignment of selected regions of YciM/LapB and FtsH proteins from different species by ClustalW2 analysis. The YciM sequence alignment included the TPR2, TPR3, and rubredoxin-like domains, while the FtsH sequence alignment included the periplasmic and four-helix bindle domains. YciM/LapB protein sequences used were from *E. coli* (P0AB58), *Alteromonas* sp. (A0A5H3A1J7), *Haemophilus influenzae* (P44130), *Aliivibrio fischeri* (Q5E3Z5), and *Citrobacter werkmanii* (A0A090TYQ6). FtsH protein sequences used were from *E. coli* (P0AAI3), *Thermotoga maritima* (Q9WZ49), and *Arabidopsis thaliana* (Q39102).

Indeed, *yciM*-L95R and *yciM*-A126V elevate LpxC levels in the *yejM1163* background, but this increase simply normalizes the flow of the common lipid precursor pool between LPS and PL pathways and repairs the OM permeability defect caused by *yejM1163*.

FtsH structure and the mechanism of suppression. FtsH is an assemblage of several topologically, structurally, and functionally distinct domains (39–41). The three novel suppressor mutations isolated in this study affect relatively conserved residues (Fig. 5) and map in two distinct domains: D74A and L78R are present in the periplasmic domain (PD), while E367D affects the cytoplasmic 4-helix-bundle domain, which is sandwiched between the nucleotide-binding AAA domain and the protease domain (Fig. 5). Although the primary sequence of the periplasmic domain shows little conservation, the two affected residues, D74 and L78, are relatively conserved within α-helix 2 (Fig. 4). The periplasmic domain of FtsH is shown to be essential neither for oligomerization nor for proteolytic activity; instead, it is proposed to play a role in the recognition of membrane-bound substrates (42). This domain of FtsH interacts with the negative modulators HflKC that are thought to influence proteolysis of membrane-bound substrates from the periplasmic side (42). LpxC is a cytoplasmic protein, and so it is unlikely that the two suppressor alterations in the periplasmic domain of FtsH

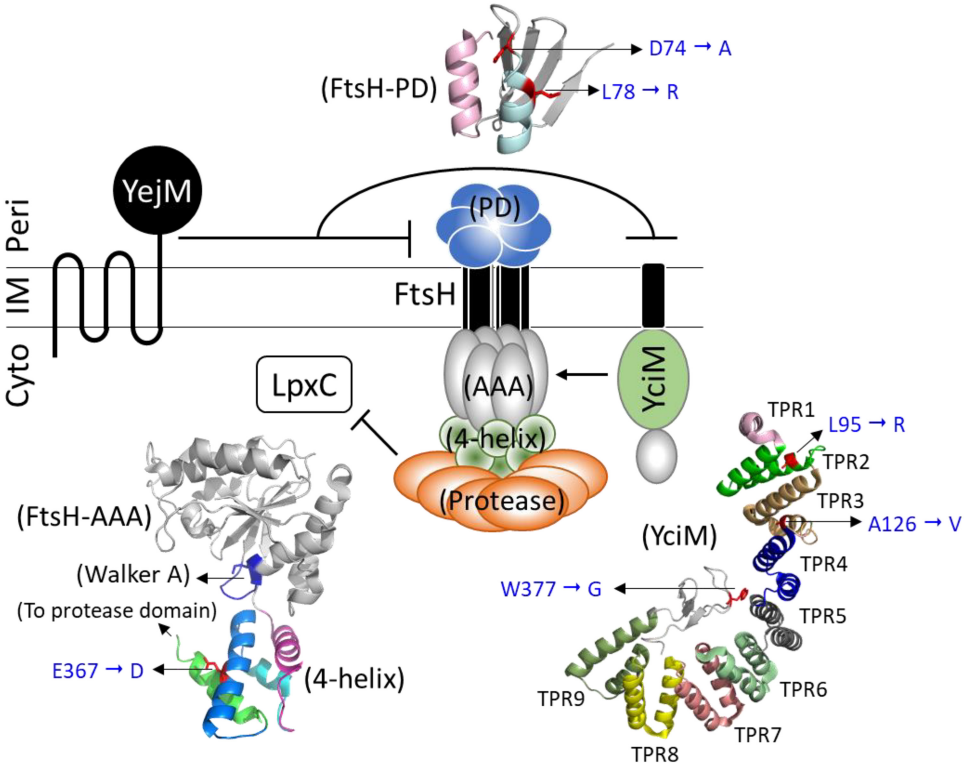

FIG 5 A cartoon showing the working model of the involvement of YejM in regulating LpxC levels. It is hypothesized that the periplasmic domain of YejM interacts with the FtsH/YciM complex to lower its proteolytic activity against LpxC. Also shown are the various domains of YciM and FtsH, where six suppressor alterations isolated in this study are located. FtsH-PD, periplasmic domain; FtsH-AAA, ATPase associated with diverse cellular activities; 4-helix, four-helix bundle; TPR, tetratricopeptide repeat; Cyto, cytoplasm; IM, inner membrane; Peri, periplasmic.

directly affect LpxC recognition. We consider an alternative that the periplasmic domain of YejM negatively modulates FtsH through interactions with FtsH's periplasmic domain. If so, a truncation of the periplasmic domain in YejM1163 may activate FtsH and enhance LpxC proteolysis. D74A and L78R may indirectly lower FtsH's protease activity or LpxC recognition so that stabilized LpxC can direct the common lipid precursor pool toward LPS synthesis.

Function of the 4-helix-bundle domain, where the E367D alteration lies, remains poorly understood. Residues of the E367-containing α-helix 8 are highly conserved (Fig. 5), indicating structural/functional importance. Due to its positioning between the nucleotide-binding and protease domains (Fig. 5), the 4-helix-bundle domain may be crucial for allosteric communications between the two domains during ATP hydrolysis and substrate binding/release. The E367D change may interfere with this communication and thus the proteolytic activity of FtsH. It is also worth considering that E367D may affect FtsH's interaction with YciM, lessening FtsH activation. Regardless of the precise mechanism, E367D appears to lower FtsH's protease activity to partially stabilize LpxC (Fig. 3) and mend the OM permeability defect (Table 1).

Comparison of our findings with those from other laboratories. After our initial conclusion of the essential role of YejM in lipid homeostasis (24), two subsequent publications (31, 43) and one recently submitted manuscript (32) provided independent verifications of this thesis. The work carried out here and that in three other laboratories began with either mutant *yejM* alleles producing C-terminal truncated proteins (24, 31, 43) or with a strain in which *yejM* expression could be depleted (32). All four studies sought revertants with improved growth phenotypes, and in all cases, *yciM* turned out to be a common site of suppressor mutations. Since YciM is a positive modulator of FtsH that degrades LpxC (Fig. 5), it is not surprising that work conducted

here (Table 1) and by Cian et al. (43) also found suppressor mutations mapping to *ftsH*. Moreover, as LpxC is the target of the YciM-FtsH complex, overexpression of LpxC (32) or missense mutations in *lpxC* (43) also overcame the growth defect caused by YejM truncation or depletion. These genetic findings revealed a crucial role of YejM in stabilizing LpxC, which sits in a critical juncture of LPS and PL synthesis (10, 13, 20, 21).

The question remains as to exactly how YejM stabilizes LpxC. One hypothesis posits direct binding of YejM to the FtsH/YciM complex to lower its activity toward LpxC (Fig. 5). Indeed, the work carried out here (Fig. 3) and that by Fivenson and Bernhardt (32) showed LpxC destabilization in the YejM mutant or YejM-depleted background. Colocalization studies have indicated that YejM interacts with YciM (32). Curiously, however, no studies have suggested any significant part of YciM being exposed to the periplasm. Instead, the available data suggest that YciM is anchored to the inner membrane through its first 20 amino acids, while the rest of the protein resides in the cytoplasm (28). Given this topology, it is difficult to see how YejM's periplasmic domain, which is partially deleted in YejM1163, would directly interact with YciM to interfere with its function. On the other hand, the periplasmic domain of FtsH, located between its two transmembrane (TM) helices, has been shown to be important for its function and interaction with its negative modulators, HflKC (42). Moreover, two of the three novel *ftsH* suppressor mutations, which appear to lower FtsH's activity against LpxC, map to the periplasmic domain of FtsH (Fig. 5). Based on the observations that YciM apparently lacks any significant periplasmically exposed region and that FtsH's periplasmic domain influences its function, it is worth considering that YejM may interact with FtsH's periplasmic domain to modulate its activity. While experimental verification for this awaits, a common theme has emerged from the recent studies that YejM plays a crucial role in regulating lipid homeostasis by modulating FtsH/YciM-mediated proteolysis of LpxC. Future work on how an imbalance in lipid homeostasis affects YejM activity will provide a clearer picture on its role as an LpxC regulator.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and antibiotic sensitivity assays. All *Escherichia coli* K-12 strains used in this study are derived from RAM1292 (MC4100 Δ*ara714*) (44). Lysogenic broth (LB) was prepared from Difco LB EZMix powder. LB agar (LBA) was prepared using LB plus 1.5% agar (Becton, Dickinson and Company). When required, kanamycin (25 μg/ml), ampicillin (50 μg/ml), and $FeCl_3$ (40 μM) were added to LB or LBA. Unless specified, all cultures were incubated at 37°C for 18 h. All chemicals were of analytical grade. Antibiotic sensitivity was assessed by determining MICs of vancomycin and erythromycin. Etest strips (bioMérieux) soaked with a concentration gradient of an antibiotic were placed on LBA plates overlaid with 4 ml of soft agar containing 100 μl of overnight-grown bacterial cultures and ferric chloride. When strains containing a plasmid were used, soft agar was also supplemented with ampicillin. Plates were incubated for 24 h at 37°C, after which the inhibition zones were measured. Two independent cultures were used, and each culture was tested with two duplicates of antibiotics strips.

Genetic and DNA methods. Bacteriophage P1-mediated transductions, using antibiotic resistance markers, were carried out as described previously (45). The promoterless *yciM* gene was cloned into pBAD24 (35) by ligating a PCR-amplified *yciM* open reading frame (ORF). Cloning was enabled by the introduction of NcoI and HindIII restriction sites during PCR amplification of the wild-type or mutant *yciM* ORF and ligating into pBAD24 that was also restriction digested with NcoI and HindIII. The ligated mixture was transformed into transformation-competent *E. coli* JM109 cells, and transformed colonies were selected for on LBA containing ampicillin. Plasmids containing the correct-size *yciM* gene clone were extracted using a QiaPrep Spin MiniPrep kit (Qiagen) and sequenced. pBAD24-*yciM* was then transformed into appropriate strains for complementation analysis. Sanger sequencing and whole-genome sequencing, as described previously (33), were carried out at the Arizona State University DNA Core Facility. The *yciM* NcoI forward primer sequence was 5′-ATTCCATGGTGGAGTTGTTGTTTCTGCTTTTG CC-3′; the *yciM* HindIII rev primer sequence was 5′-ATAAGCTTAGTGGTGGTGGTGGTGGTGCAGGCCATCA AGACCGCG-3′.

Protein methods. To isolate whole-cell envelopes, cells were lysed by the French press method (46). Envelopes were pelleted by centrifuging cell-free lysates for 1 h at 105,000 × *g* and resuspended in an SDS-based sample buffer. For detection of specific proteins, Western blot analysis was carried out as described previously (46). LpxC and YciM primary rabbit antibodies (purchased from LifeSpan Biosciences, Inc.) were diluted 1:5,000 prior to use. To isolate cell-free supernatant, cells from 1-ml overnight cultures were pelleted by centrifugation at 16,000 × *g* for 5 min. Eight hundred microliters of the supernatant was collected and passed through a 0.22-μm filter. Filtrate, roughly 600 μl, was dried in a SpeedVac and reconstituted in 50 μl of sterile water. Concentrated supernatants (9 μl) were analyzed by SDS-polyacrylamide (11%) gels, and proteins were visualized by staining the gel with Coomassie brilliant blue.

ACKNOWLEDGMENTS

We thank the Research and Training Initiatives office of the School of Life Sciences and NIH grant R21 AI117150 for partly funding this work.

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