Lipoprotein LptE is required for the assembly of LptD by the β -barrel assembly machine in the outer membrane of *Escherichia coli*

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Most Gram-negative bacteria contain lipopolysaccharide (LPS), a glucosamine-based phospholipid, in the outer leaflet of the outer membrane (OM). LPS is unique to the bacterial OM and, in most cases, essential for cell viability. Transport of LPS from its site of synthesis to the cell surface requires eight essential proteins, MsbA and LptABCDEFG. Although the key players have been identified, the mechanism of LPS transport and assembly is not clear. The stable LptD/E complex is present at the OM and functions in the final stages of LPS assembly. Here, we have identified the mutant allele *lptE6*, which causes a two-amino-acid deletion in the lipoprotein LptE that affects its interaction with LptD. Highly specific suppressor mutations were isolated not only in *lptD* but also in *bamA*, which encodes the central component of the β -barrel assembly machine. We show that *lptE6* and both suppressor mutations affect the assembly of the LptD/E complex and suggest that the lipoprotein LptE interacts with LptD while this protein is being assembled by the β -barrel assembly machine.

β -barrel protein | protein folding | protein targeting | allele-specific suppressor

The cell envelope of Gram-negative bacteria such as *Escherichia coli* consists of an inner membrane (IM), an aqueous peptidoglycan-containing periplasm, and an outer membrane (OM) (1). Unlike the IM, which is composed entirely of phospholipids, the OM is an asymmetric lipid bilayer with an inner leaflet composed of phospholipids and an outer leaflet composed of lipopolysaccharide (LPS) (2). Strong lateral interactions between neighboring LPS molecules make the OM more impermeable than a phospholipid bilayer to a wide range of hydrophobic dyes, hydrophobic antibiotics, and detergents (including bile salts), allowing Gram-negative bacteria to survive in environments such as the human intestine (1, 3).

The LPS biosynthetic pathway is well understood (4), but the process of LPS transport to the OM and assembly at the cell surface is just beginning to be characterized (5). LPS is synthesized at the inner leaflet of the IM (6). In the first step of LPS transport, the essential ABC transporter MsbA flips LPS from the cytoplasmic to the periplasmic face of the IM (7–9). Seven essential lipopolysaccharide transport (Lpt) proteins transport LPS from the IM to the cell surface (10–14). When any one of the Lpt proteins is depleted, de novo synthesized LPS can no longer reach the cell surface and abnormal membrane structures accumulate in the periplasm (10–13). There is at least one transport pathway component in each cellular compartment and their locations suggest order of function in LPS transport. LptB/C/F/G (formerly YhbG/YrbK/YjgP/YjgQ) form an ABC transporter at the IM that is believed to extract LPS from the periplasmic leaflet of the IM (11, 13, 15). The periplasmic protein LptA (formerly YhbN) binds LPS (16) and is thought to aid in the transport of the amphipathic LPS across the aqueous periplasm (10). LPS is transported to the OM in spheroplasts even though soluble periplasmic factors have been lost (17). In addition, it has recently been reported that all seven

Lpt proteins form a transenvelope complex and are found in a light OM fraction containing both the IM and the OM (18). Therefore, LPS transport across the periplasm most likely takes place at membrane contact sites through a protein bridge. The LptD/E complex (formerly Imp/RlpB) at the OM presumably functions in the final stages of assembling LPS into the outer leaflet of the OM (12). LptE binds LPS in a specific manner and could be playing a role in receiving LPS from the periplasm (19). Although the individual roles of these OM proteins are yet to be established, some insights have begun to emerge through recent developments in the biochemical characterization of this two-protein complex.

LptD is an outer membrane protein (OMP) with a soluble N-terminal domain (residues 25–202) and an integral C-terminal β -barrel domain (residues 203–784) (19, 20). Two nonconsecutive intramolecular disulfide bonds covalently join the two domains (21, 22). LptE is a lipoprotein anchored to the OM via N-terminal lipid moieties (23). Both OMPs and lipoproteins pass through the Sec translocon after synthesis in the cytoplasm but their biogenesis pathways diverge at the periplasmic face of the IM. After signal sequence cleavage, OMPs are thought to be protected in their unfolded state by periplasmic chaperones until they are delivered to the BamABCDE complex for assembly into the OM (24). Indeed, biogenesis of LptD is dependent on both the periplasmic chaperone SurA (25) and the Bam complex (12). In the case of lipoproteins, after lipid modifications to the N-terminal cysteine residue and signal sequence cleavage, those destined for the OM, like LptE, are released from the IM by LolCDE and handed off to the periplasmic chaperone LolA, which delivers them to LolB for assembly into the OM (26). At the OM, LptD and LptE form an extremely stable 1:1 complex and the β -barrel domain of LptD is sufficient for this interaction with LptE (19). Not only does LptE stabilize LptD but also it is required for the proper oxidation of LptD, suggesting a crucial role for LptE in the folding of its complex partner (19, 22).

Here we describe the isolation of highly specific suppressor mutations of a partial loss-of-function, 6-bp deletion mutation in *lptE*. Characterization of these suppressors provides insights into the structure and biogenesis of the LptD/E complex. These suppressors also provide a rational explanation for chemical conditionality, the genetic approach that was used to identify mutations in genes that specify components of the complex that assembles β -barrel proteins in the OM (27, 28).

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(Fig. 2). This result demonstrates that the *lptE6* mutation directly affects the interaction between LptD and LptE.

***lptE6* Impairs LptD Biogenesis.** LptD has two intramolecular disulfide bonds and mature, oxidized LptD (LptD_{OX}) migrates slower than reduced LptD (LptD_{RED}) during SDS/PAGE (21, 22). Unfolded LptD_{RED} runs at ~83 kDa and LptD_{OX} runs at ~120 kDa. When LptE is depleted, LptD_{OX} can no longer be detected even though LptD_{RED} is present at wild-type levels. Therefore, the interaction with LptE is required for the proper oxidation of LptD (22). Because LptE6 has lowered affinity for LptD, we wanted to determine if LptD biogenesis is impaired in the *plptE6* mutant, using the oxidation state of LptD as a diagnostic tool.

It was previously observed that levels of LptD4213, which carries a 23-amino-acid deletion and is severely destabilized, are higher in logarithmic-phase cultures compared with stationary-phase cultures (27). Therefore, to evaluate LptD assembly in the *plptE6* mutant, we examined the levels of LptD_{RED} and LptD_{OX} in late logarithmic-phase cells. LptD_{RED} levels in the *plptE6* mutant, like those of LptD4213, are higher during the logarithmic phase of growth (Fig. 3, *Upper*, lanes 1 and 2) but strikingly, most of the LptD present is not properly oxidized (Fig. 3, *Lower*, lanes 1 and 2). The misfolded protein must be present in some disulfide-bonded aggregate that either does not enter the gel or runs as a diffuse smear. This result demonstrates an LptE6-dependent LptD biogenesis defect.

To determine whether the biogenesis defect is at the level of complex stability, complex formation, or both, we compared the stability of the LptD/E6 complex with that of the wild-type complex using our previously described methods (19). The wild-type LptD/E complex is very protease resistant and it does not dissociate upon SDS/PAGE provided the samples are not heated. In both types of experiments, the LptD/LptE6 complex behaves similarly (Fig. S1). These results suggest that the primary defect caused by LptE6 is in complex formation.

Mutations in *lptD* and *bamA* Suppress *lptE6*. We hoped to genetically identify the regions of LptD that interact with LptE by finding conformational suppressor mutations of *lptE6* in *lptD* that restore the barrier function of the OM. To select for suppressors, we used bacitracin instead of rifampicin to avoid the high number of antibiotic-resistant mutations we would isolate in *rpoB* (29). Specifi-

Fig. 2. The *lptE6* mutation directly affects the interaction between LptE and LptD. Ni-NTA affinity purification is shown, using AM604 containing pET23/42 (lane 1), pET23/42scT-*lptE-his* (lane 2), or pET23/42*lptE6-his* (lane 3). Samples before (whole-cell lysates) and after (Ni-NTA eluates) purification were subjected to immunoblotting using anti-LptD and anti-His antibodies. Positions of relevant molecular markers are indicated in kilodaltons.

Fig. 3. The suppressors of *lptE6* improve LptD assembly. Whole-cell samples from AM604 Δ *lptE* (*plptE*), AM604 Δ *lptE* (*plptE6*), AM604 Δ *lptE* *lptD7* (*plptE6*), and AM604 Δ *lptE* *bamA8* (*plptE6*) grown to an OD₆₀₀ of ~1.0 were boiled in the presence (*Upper*) or the absence of β -ME (*Lower*) and Western blot analysis of LptD was carried out to detect reduced LptD (LptD_{RED}) or oxidized LptD (LptD_{OX}), respectively.

cally, we used bacitracin at a concentration normally lethal to the *plptE6* mutant strain, but not to the *plptE*⁺ strain. Suppressors arose spontaneously at a frequency of 8×10^{-8} .

A total of 11 independent suppressors were characterized. On the basis of colony morphology, these suppressors could be divided into two classes. The 3 suppressor strains in class I form small colonies, whereas the 8 suppressor strains in class II form normal-sized colonies. Genetic mapping showed that the class I suppressor mutations were linked to *lptD*, whereas the class II mutations were linked to *bamA*. *bamA* encodes a conserved integral membrane protein that is an essential component of the machinery involved in assembling β -barrel OMPs into the OM (30).

Allele Specificity of the *lptE6* Suppressors. DNA sequence analysis of the chromosomal *lptD* gene in the class I suppressors revealed that all three independent members of this class carry the same AT:GC transition mutation at codon 633, which results in a D633G substitution. This allele is called *lptD7*. Asp633 lies in the predicted β -barrel domain of LptD (19).

DNA sequence analysis showed that all eight independent suppressor strains in class II harbor mutations in *bamA*. Strikingly, all of them alter codon 159. Five suppressors have a P159L change and there is one suppressor each with a P159S, P159A, or P159Q change. Because all of the suppressors behave similarly, we went forward with the analysis of just the P159L mutant in this work and the allele is called *bamA8*. Pro159 is in the second polypeptide transport-associated (POTRA) domain of BamA. BamA has five periplasmic POTRA domains and they are thought to template β -strand formation during the folding of substrates (31, 32).

Both *lptD7* and *bamA8* improve the barrier function of the *plptE6* mutant to similar extents, although not to wild-type levels, determined by a two- to fourfold increase in resistance to rifampicin and bacitracin (Table 1, rows 4–6). The fact that we repeatedly found suppression by mutating the same two codons suggests that the suppression is allele specific. Although we do not have a large collection of *lptE* mutants to combine with *lptD7* and *bamA8* and exhaustively test for allele specificity, we did check

whether the suppressors of *lptE6* could suppress *lptD4213* and vice versa. *bamA8* does not suppress the MacConkey sensitivity defect of *lptD4213*. Similarly, *bamA6* (formerly *yaeT6*), which carries a two-amino-acid insertion in POTRA3 of BamA and suppresses *lptD4213* (28), does not suppress the bacitracin sensitivity of *lptE6*. Null mutations in *bamB* can suppress *lptD4213* under conditions of slow growth (27). However, a *bamB* null allele does not suppress permeability defects in the *plptE6* mutant. In fact, both *bamB*[−] and *bamA6* aggravate the defects conferred by *lptE6* (Table 1, rows 7–10). Therefore, the suppressors of *lptE6* work by correcting a specific problem and not by ameliorating generalized OM defects.

***lptE6* Suppressors Improve LptD Assembly.** The isolation of a suppressor mutation that affects a POTRA domain of BamA strongly suggested that the LptD assembly defect conferred by *lptE6* might be corrected by the suppressor mutation. Examination of LptD_{OX} levels in the suppressor strains during the late-logarithmic growth phase showed that there is more correctly oxidized LptD in the presence of either *bamA8* or *lptD7* (Fig. 3, Lower, lanes 2–4). Levels of LptD_{RED} are comparable in the *plptE6* mutant and the suppressor-containing strains under these experimental conditions (Fig. 3, Upper, lanes 2–4). This result confirms that the difference in the levels of LptD_{OX} is a result of improved LptD assembly and not just a reflection of total protein levels.

When we examined the levels of LptD_{RED} in stationary phase, we found that LptD levels are higher in the suppressor strains (Fig. 1, Middle, lanes 4–6). *lptD* is regulated by σ^E , a transcription factor that responds to envelope stress (33). Because defects in OMP biogenesis and LPS biosynthesis can induce σ^E (34, 35), there is a possibility that the suppressor mutations in *bamA* and *lptD* result in increased LptD synthesis due to σ^E induction. However, the levels of a member of the σ^E regulon, the periplasmic protease DegP (36), are not increased in the suppressor-containing strains relative to the *plptE6* mutant (Fig. 1, Bottom, lanes 4–6), ruling out further σ^E induction by the suppressor mutations. In fact, on the basis of the amounts of proteolyzed DegP (37), the suppressor mutations lower the σ^E stress response that is induced in the *plptE6* mutant. The increased levels of LptD in the presence of the suppressor mutations in stationary phase, therefore, reflect an increase in LptD stability due to improved LptD/E biogenesis.

Phenotypic Characterization of *lptE6* Suppressors. *lptD7* and *bamA8* do not cause any permeability defects in an otherwise wild-type background (Table 1, rows 11 and 12). The *bamA8* strain has wild-type levels of LptD and LptE, whereas the *lptD7* strain appears to have slightly lowered levels of both LptD and LptE (Fig. 4). The reduced presence of the LptD/E complex at the OM in the *lptD7* strain probably leads to induction of the σ^E stress response as seen by the up-regulation of DegP (Fig. 4). The D633G substitution in LptD, therefore, confers LptD/E complex instability in combination with LptE⁺ but has the opposite effect in combination with LptE6.

Discussion

The selection used in this study to isolate suppressors of *lptE6* was quite demanding. The *lptE6* mutation is a deletion; thus, reversion is not possible. In addition, there are no chromosomal mutations that would confer resistance to the antibiotic used, bacitracin, under the conditions used. Consequently, mutants that survive selection are rare and all of them contain bona fide suppressors. All of these suppressors fall into one of only two phenotypic classes, and each class carries alterations in one particular codon in one of two genes. Because these suppressor mutations have been isolated multiple times, we believe that the selection is saturated. Because of this striking specificity, we suggest that an

Fig. 4. Effects of *lptD7* and *bamA8* on LptD and LptE levels. Western blot analysis is shown of LptD and LptE and DegP on whole-cell samples prepared from AM604, AM604 *lptD7*, and AM604 *bamA8* after overnight growth. The asterisk indicates degradation products of DegP.

understanding of these suppressors will provide clear insights into the nature of the defect caused by *lptE6*.

LptE is known to form a stable complex with the β -barrel protein LptD with a stoichiometry of 1:1 (19). As shown by Freinkman et al., LptE has an extensive interaction surface with LptD, suggesting that LptE is deeply embedded in the barrel (38). Although the residues altered in LptE6 do not directly contact LptD (38), LptE6 destabilizes LptD, suggesting that this mutation weakens an interaction between the two proteins. The suppressor allele *lptD7*, isolated three independent times, results in a single-amino-acid substitution in the β -barrel portion of LptD. The specificity of this suppressor mutation suggests that we have indeed found a classic interactive suppressor. The D633G mutation in *lptD7* is either restoring a lost contact between LptE6 and LptD or relieving a constraint imposed by the YPISA to YRA change caused by *lptE6*. Because the *lptE6* mutation does not destabilize the mature LptD/E complex, we suggest that these two mutations alter an interaction that is important for complex assembly. This hypothesis could also explain the phenotypes of *lptD7* in an otherwise wild-type background. This mutation likely alters the assembly process with wild-type LptE.

LptE and LptD are targeted to the OM by different pathways (Fig. 5B) and they must interact to form a stable, properly oxidized complex at the OM. LptD must be at least partially folded to bring the Cys residues in the N- and C-terminal domains into close proximity for disulfide bond formation and interaction with LptE is required for this process (22). Suppressor mutations of *lptE6* that improve LptD biogenesis were also found in *bamA*, which encodes the essential β -barrel component of the machinery involved in assembling β -barrel OMPs such as LptD into the OM. Eight independent selections yielded Ser, Leu, Ala, or Gln substitutions at the same residue, Pro159. The varied nature of the substitutions suggests that the loss of the Pro residue is vital for the suppression.

The astonishingly specific genetic interaction identified here between *lptE* and *bamA* suggests that LptE interacts with the rudimentary barrel domain of LptD while it is being held in the Bam complex. The suppressor mutation in *bamA8* is perhaps able to bias the folding of LptD such that the interaction between LptE6 and LptD is strengthened. LptE could participate in LptD folding by either acting as a scaffold or stabilizing the newly folded protein. In either case, completion of disulfide bond formation in LptD must take place at the Bam machine or after the LptD/E complex is released from the machine.

The *lptD* gene was first identified in a genetic selection for mutations that disrupt the OM permeability barrier. One mu-

Fig. 5. Genetic interactions between *lpt* and *bam*. (A) Genetic interactions (dashed lines) between genes encoding members of the Lpt and Bam complexes exist. *lptD4213* is suppressed by mutations in *bamA* and *bamB* (27, 28). *lptE6* is suppressed by mutations in *lptD* and *bamA*. Physical interactions in the LptDE (12) and BamABCDE (30, 40) complexes are shown with solid lines. (B) LptE and LptD are targeted to the OM by different pathways. Lipoprotein LptE is targeted to LolB by the Lol pathway (26). OMP LptD is targeted to the BamABCDE complex (24). Defects in LptD assembly caused by the presence of LptE6 are suppressed by mutations in the gene encoding BamA, suggesting that LptD and LptE interact at the Bam complex during biogenesis of the LptDE complex.

tation that answered this selection, *lptD4213* (formerly *imp4213*), renders *E. coli* sensitive to a wide variety of antibiotics and detergents (39). As summarized in Fig. 5A, components of the Bam complex, which assembles OMPs (30, 40), were identified by mutations that suppress the permeability defects caused by *lptD4213*, using a genetic strategy termed chemical conditionality (27, 28, 41). Basically, it was shown that different toxic small molecules could be used to identify suppressors that correct the *lptD4213* defects to varying degrees. Suppressor phenotypes correlated with the physical properties of the small molecules and the suppressor mutations identified the structural genes for BamA and BamB. Why mutations that alter the complex that assembles LPS at the cell surface could be suppressed by mutations that alter the complex that assembles OMPs was not clear. One possible explanation suggests that the activity of both machines must be balanced. Another possibility suggests that the activity of one machine may be regulated by directed interaction with the other machine.

Results presented here provide a more attractive explanation for chemical conditionality (Fig. 5B). We started out with a defect in LptE, the lipoprotein component of the LPS assembly machinery, and found a suppressor mutation that alters BamA, a part of the OMP assembly machinery. The permeability defects caused by the mutation in LptE are due to defects in the biogenesis of the β -barrel component of the LPS assembly machinery, LptD, and suppressor mutations in *lptD* and *bamA* both fix this assembly defect. In analogous fashion, we suggest that the *lptD4213* mutation also causes a defect in the assembly of LptD and suppressors that alter components of the Bam complex address this problem. Different suppressors that correct the OM permeability defect to varying degrees do so by fixing the LptD/E assembly defect to varying degrees.

Our results argue that *lptD4213*, *lptE6*, and *lptD7* all affect assembly of the LptD/E complex. Using a completely different, but complementary approach, Freinkman et al. (38) have identified another deletion in the β -barrel domain of LptD that affects assembly as well. We suggest that suppressors will be useful not only

for identifying important genes but also for probing the mechanism of β -barrel assembly.

Materials and Methods

Bacterial Strains and Growth Conditions. All strains (Table S1), plasmids, growth conditions, and antibiotic sensitivity assays are described in SI Materials and Methods.

Construction of the $\Delta lptE2::kan$ Allele. Primers ACM143 and GC28 (Table S2), with homologies to regions upstream and directly downstream of *lptE*, were used to amplify the kanamycin cassette from pKD4 (42) by PCR. The resulting PCR product was used to replace the entire *lptE* gene with the kanamycin cassette in a recombining reaction (43) to obtain the $\Delta lptE2::kan$ allele. Insertion of the kanamycin cassette was verified by PCR.

PCR Mutagenesis of *lptE*. Random mutations were generated by error-prone PCR using the GeneMorph kit (Stratagene). Primers ACM146 and ACM147 (Table S2) were used at 125 ng each with 77 ng pBAD18/*lptE* (12) as the template in a 50- μ L reaction. The PCR conditions were 95 °C for 4 min followed by 30 cycles of 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min followed by a final extension for 10 min at 72 °C. The product was digested with EcoRI and XbaI (New England Biolabs), ligated into similarly cut pBAD18 (44), and transformed into AM604. Transformants were selected on LB agar with ampicillin.

Identification of Mutant *lptE* Plasmid Constructs. To replace the endogenous *lptE* in the transformants with $\Delta lptE2::kan$, the transformants carrying the mutagenized *lptE* on pBAD18 (~100 per ligation reaction) were pooled and treated as the recipient culture in a P1 transduction reaction in the presence of arabinose. Transductants (~100 per reaction) were selected on LB agar with kanamycin and patched onto both LB agar with kanamycin and LB agar with rifampicin. Patches that grew in the presence of kanamycin but not rifampicin were purified further on kanamycin and their sensitivities to rifampicin, bacitracin, erythromycin, and novobiocin were measured using the BBL Sensi-Disk Antimicrobial Susceptibility Test Discs (BD) as previously described (27). Candidates that showed significant zones of inhibition around the 6-mm discs were selected for further analysis and the plasmids were sent to Genewiz for DNA sequencing.

Affinity Purification. Affinity purification experiments were performed as previously described (19).

Suppressor Selection and Mapping. Overnight cultures of GC190 were grown from single colonies. One hundred microliters (~ 10^8 cells) of each culture were spread on LB agar with bacitracin. Suppressors were allowed to develop spontaneously at 37 °C overnight. Suppressors were purified in the presence of bacitracin and grouped into two classes on the basis of colony size. To find out if the suppressor mutations were in *lptD*, linkage to *carB::Tn10*, a marker that is 40% linked to *lptD*, was calculated. To map the mutation in a representative class II suppressor strain, the strategy outlined in Ruiz et al. (28) was used, using a pool of MC4100 mutants containing random *mini-Tn10* insertions to donate wild-type alleles (45). We found that a *yafD::miniTn10* allele was 27% linked to the suppressor mutation. Linkage analysis with *yafD::miniTn10* showed that all members of class II were most likely carrying a suppressor mutation in *bamA*. The *lptD* and *bamA* genes were sequenced to identify the suppressor mutations in the respective mutants. The suppressor strains were rebuilt to ensure that the mutations in *lptD* and *bamA* were sufficient for suppression.

Immunoblot Analysis. To analyze the levels of proteins in stationary phase, 250 μ L of overnight cultures was pelleted at 13,200 rpm for 1.5 min and resuspended in a volume of SDS sample buffer equal to OD₆₀₀/24. To analyze the oxidation state of LptD, 1:200 dilutions of overnight cultures were grown to an OD₆₀₀ of 1. Either 1 mL or 500 μ L of culture was pelleted and resuspended in a volume of SDS sample buffer equal to OD₆₀₀/12 in the presence or absence of 5% (vol/vol) β -ME, respectively. Samples were boiled for 10 min before being subjected to SDS/PAGE. Rabbit polyclonal antisera against LptD (1:7,000 dilution) (21), LptE (1:40,000 dilution) (19), and DegP (1:50,000 dilution) (46) and donkey ECL horseradish peroxidase conjugate anti-rabbit IgG (GE Life Sciences) (1:10,000 dilution) were used for immunoblotting. To visualize the bands, the ECL antibody detection kit (Amersham Pharmacia Biotech) and HyBlot CL film (Denville Scientific) were used. Suitable controls are shown in Fig. S2.

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