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Dominant Negative *lptE* Mutation That Supports a Role for LptE as a Plug in the LptD Barrel

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Lipopolysaccharide (LPS) is the major outer leaflet constituent of the Gram-negative outer membrane (OM) bilayer. A bipartite protein complex of LptD and LptE assembles LPS into the OM. It has been established that LptE assists folding and assembly of its β-barrel partner LptD, yet reported biochemical evidence suggested additional LptE functions. Here, we isolated dominant negative *lptE* mutations, seeking to inform these functions. The *lptE14* mutation increased OM permeability to erythromycin, even when the wild-type *lptE* gene was present. We show that the *lptE14* mutation does not cause a defect in either LptD assembly or LPS export. A spontaneous IS*1* insertion in *secA* suppressed *lptE14* erythromycin sensitivity by removing the C-terminal SecB-binding domain of SecA. While this suppressor mutation broadly impeded SecB-dependent secretion of preproteins, we show that suppression was a direct and specific consequence of reduced LptD levels in the OM. We suggest that *lptE14* causes poor plugging of the LptD β barrel and that a reduction of ineffectively plugged LptD-LptE14 complexes in the OM decreases permeability to erythromycin. Hence, *lptE14* supports a proposed plug-and-barrel LptE-LptD arrangement.

The cell envelope of Gram-negative bacteria consists of an inner membrane (IM) and an outer membrane (OM) that are separated by an aqueous periplasmic space. The OM is an essential organelle, serving as a selective permeability barrier that permits influx of nutrients while excluding toxins and hydrophobic antibiotics (1). The glycolipid lipopolysaccharide (LPS) is central to OM barrier function, forming the outer leaflet of this lipid bilayer (2, 3). LPS is synthesized at the IM and must traverse the aqueous periplasmic environment to reach the OM, where it is assembled at the cell surface (4). Seven essential LPS transport (Lpt) proteins involved in this pathway have now been identified in *Escherichia coli* (5). The Lpt proteins collectively form a transenvelope complex that delivers LPS from the IM to the OM (6). The current model of LPS transport posits that LptBFG, which constitute an ABC transporter, extract LPS from the IM (5, 7–11), and then the molecule is passed to the periplasmic domain of LptC (8, 10–12). Next, additional ATP hydrolysis by LptBFG facilitates passage of LPS from LptC to LptA in the periplasm (6, 11–13). Multiple LptA proteins polymerize in end-on-end fashion to span the periplasm, from the IM LptBCFG components to the OM LptDE complex (14–16). The soluble N-terminal domain of LptD is homologous to LptA and likely serves as the final subunit in the LptA multimer (6, 13). As a consequence of this docking, LPS is thought to flow from LptA to LptD (11). The final steps in the Lpt pathway insert LPS into the OM outer leaflet and are accomplished by the LptDE complex but remain mechanistically poorly understood.

LptD is a large β-barrel outer membrane protein (OMP), and the biogenesis of this protein is an especially complex process. Once secreted into the periplasm, LptD is largely reliant on the chaperone function of SurA, which maintains the molecule in an assembly-competent state and delivers it to the Bam complex, which assembles β-barrel proteins into the OM (17–19). Before LptD reaches Bam, a pair of disulfide bonds forms in a process that requires DsbA (20–22). Late in assembly, once the protein is inserted in the OM, these disulfide bonds are rearranged (22). The properly oxidized LptD protein ($LptD_{OX}$) comprises the mature form that is functional in LPS export (20, 22).

Genetic analysis of the *lptE6* mutation demonstrated that LptE is required for the biogenesis of mature LptD (23). This mutation caused a severe reduction in LptD levels and a concomitant increase in OM permeability to antibiotics. OM permeability could be suppressed by mutations in *bamA* and *lptD* that restored levels of mature LptD. More recently, it has emerged that LptE is required to guide the proper oxidation of LptD, once assembly of the β-barrel domain into the OM is complete (22). At the OM, LptE forms a high-affinity stable 1:1 complex with LptD (24). Indeed, cross-linking experiments demonstrated extensive interaction sites across the surface of LptE and prompted a model of the LptE-LptD plug-and-barrel arrangement where LptE resides in the lumen of the LptD β barrel (25). In addition to its role in LptD assembly, purified LptE has been shown to specifically bind LPS, hinting that the protein may also function directly in LPS assembly into the OM (24).

While the isolation of *lptE6* was valuable in understanding LptD assembly, no mutations isolated to date have further informed the other functions of LptE. In particular, the function of LptE in the LPS assembly process *in vivo* is wholly unknown. This study set out to identify and characterize novel mutations that affect the LptD assembly-independent functions of LptE.

The LptDE complex is extremely stable *in vivo*, so we reasoned that a properly assembled LptDE complex that contained a nonfunctional LptE protein could probably not be rescued by functional LptE provided in *trans*. If so, then an excess of functionally compromised mutant LptE could then decrease the levels of functional LptDE complex even in the presence of $lptE^+$, thereby dis-

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Reference or source
Escherichia coli strains		
DH5α	F$^-$ *endA1 thi-1 recA1 relA1 gyrA96 deoR nupG* ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 hsdR17*(r_K^- m_K^+) λ$^-$	
MC4100	F$^-$ *araD139* Δ(*arg-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 thi*	26
NR754	MC4100 Ara$^+$	27
JW3584	BW25113 Δ*secB*::*kan*	28
JY17	NR754/p*lptE6*	23
JY18	NR754 Δ*lptE2*::*kan*/p*lptE6*	23
JY32	NR754/p*lptE*$^+$	This study
JY33	NR754 Δ*lptE2*::*kan*/p*lptE*$^+$	This study
JY34	NR754/p*lptE14*	This study
JY35	NR754 Δ*lptE2*::*kan*/p*lptE14*	This study
JY105	JY33 *secA*$^+$ *leuA*::Tn*10*	This study
JY106	JY33 *secA827*::IS*1 leuA*::Tn*10*	This study
JY107	JY35 *secA*$^+$ *leuA*::Tn*10*	This study
JY108	JY35 *secA827*::IS*1 leuA*::Tn*10*	This study
MG934	NR754 Δ*lptE secB*$^+$/p*lptE*$^+$	This study
MG935	NR754 Δ*lptE secB*$^+$/p*lptE14*	This study
MG949	NR754 Δ*lptE* Δ*secB*::*kan*/p*lptE*$^+$	This study
MG950	NR754 Δ*lptE* Δ*secB*::*kan*/p*lptE14*	This study
MG1053	NR754 Δ*lptE* Δ*lptD*::*kan2 secA*$^+$ *leuA*::Tn*10*/pBAD33::*lptE*$^+$/p*lptD*	This study
MG1054	NR754 Δ*lptE* Δ*lptD*::*kan2 secA827*::IS*1 leuA*::Tn*10*/pBAD33::*lptE*$^+$/p*lptD*	This study
MG1055	NR754 Δ*lptE* Δ*lptD*::*kan2 secA*$^+$ *leuA*::Tn*10*/pBAD33::*lptE14*/p*lptD*	This study
MG1056	NR754 Δ*lptE* Δ*lptD*::*kan2 secA827*::IS*1 leuA*::Tn*10*/pBAD33::*lptE14*/p*lptD*	This study
MG1057	NR754 Δ*lptE* Δ*lptD*::*kan2 secA*$^+$ *leuA*::Tn*10*/pBAD33::*lptE*$^+$/p*sfmC-lptD*	This study
MG1058	NR754 Δ*lptE* Δ*lptD*::*kan2 secA827*::IS*1 leuA*::Tn*10*/pBAD33::*lptE*$^+$/p*sfmC-lptD*	This study
MG1059	NR754 Δ*lptE* Δ*lptD*::*kan2 secA*$^+$ *leuA*::Tn*10*/pBAD33::*lptE14*/p*sfmC-lptD*	This study
MG1060	NR754 Δ*lptE* Δ*lptD*::*kan2 secA827*::IS*1 leuA*::Tn*10*/pBAD33::*lptE14*/p*sfmC-lptD*	This study
JCM537	MC4100 Ara$^{r/-}$ Δ*pldA* Δ*mlaC*	29
Plasmids		
p*lptE*$^+$	*lptE*$^+$ cloned into pBAD18	30
pBAD33::*lptE*$^+$	*lptE*$^+$ cloned into pBAD33	This study
pBAD33::*lptE14*	*lptE14* cloned into pBAD33	This study
p*lptD*	*lptD* cloned into pET23/42	30
p*sfmC-lptD*	SfmC signal sequence fused to LptD mature sequence, cloned into pET23/42	N. Ruiz

rupting OM integrity. Such mutations would therefore be dominant negative. On the other hand, *lptE* mutations impairing early steps in LptD biogenesis—before the LptDE complex is formed—such as *lptE6*, would be recessive. Hence, we sought to isolate dominant negative *lptE* mutations as a means of enriching for genetic alterations that affect the LptD assembly-independent functions of LptE.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. All strains and plasmids used in this study are listed in Table 1. Cultures were routinely grown at 37°C in Luria-Bertani (LB) medium. Where appropriate, growth media were supplemented with the following: 50 μg/ml ampicillin (Amp), 25 μg/ml kanamycin, 25 μg/ml tetracycline, 50 μg/ml erythromycin (Ery), 10 μg/ml rifampin, 625 μg/ml bacitracin, 0.2% (wt/vol) glucose, and 0.2% (wt/vol) L-arabinose (Ara).

Random PCR mutagenesis and antibiotic sensitivity screening. The oligonucleotide primers used in this study are provided in Table 2. Random PCR mutagenesis was performed using a GeneMorphII random mutagenesis kit (Agilent) and 125 ng each of primers 5lptE670828 and 3lptE671409 to amplify *lptE* from 0.1 ng of the p*lptE*$^+$ template in 40 cycles. A heavy mutagenesis was achieved, yielding an average of 2 to 5 coding changes per *lptE* amplicon. The MEGAWHOP cloning method was used to generate mutagenized plasmid pools (31). Briefly, the mutagenized amplicons were purified and 250 ng was used to prime PCR with about 50 ng of p*lptE*$^+$, generating a pool of nicked plasmids that contained mutagenized *lptE* which was subsequently treated with DpnI to digest the p*lptE*$^+$ template. Plasmid pools were passaged through *E. coli* DH5α with transformants selected on LB medium supplemented with ampicillin and 0.2% glucose. Plasmid pools were then isolated from DH5α and electroporated into competent strain NR754, and transformants were selected on LB medium supplemented with ampicillin and 0.2% glucose.

TABLE 2 Oligonucleotides used in this study

Name	Sequence (5′–3′)
5lptE670828	TTAATCACCGCCGGGTGT
3lptE671409	CGGGTACAACCGAATCATCA
lptE_g71a_F	GCTGGCATCTGCATGATACCACGCAG
lptE_g71a_R	CTGCGTGGTATCATGCAGATGCCAGC
lptE_c159g_F	GTGCGGTGCGTAAGCAGTTACGTCTG
lptE_c159g_R	CAGACGTAACTGCTTACGCACCGCAC
lptE_t192a_F	GGTGTCGAGTTGCTTGAAAAAGAAACCACGCGTAAG
lptE_t192a_R	CTTACGCGTGGTTTCTTTTTCAAGCAACTCGACACC
lptE_t302c_F	CAGAGTATCAGATGACCATGACGGTTAATGC
lptE_t302c_R	GCATTAACCGTCATGGTCATCTGATACTCTG
lptE_t408c_F	CAAATGGCGTTAGCGAACGATAACGAACAAGACATG
lptE_t408c_R	CATGTCTTGTTCGTTATCGTTCGCTAACGCCATTTG
lptE_c556t_F	AACGCCTGCATGCGTCTCCACCACGCTGGGTAACT
lptE_c556t_R	AGTTACCCAGCGTGGTGGAGACGCATGCAGGCGTT
JY10	ATGCTGATGCACCACGTAACG
JY18	TTGCAGCTTTTTCATTGTCTTAAACC
SacI_lptE	AAAGAGCTCGCGCGGGAGGAAGC
lptE_R_XbaI	GATGCCTCTAGAACCGAATCATCAGTTACCCA

Individual NR754 transformant colonies were then identified, picked with a Genetix Qpix2 XT colony picker, and transferred to 96-well plates filled with 200 μl LB broth supplemented with ampicillin and 0.2% glucose. In total, 3,700 transformants were picked. Plates were incubated at 37°C for 3 h. Plate cultures were replica plated onto LB medium-ampicillin plates additionally supplemented with either 0.2% glucose, 0.2% arabinose, 0.2% arabinose with erythromycin, 0.2% arabinose with rifampin, or 0.2% arabinose with bacitracin. Plates were incubated overnight at 37°C. Plates were examined to identify antibiotic sensitivity, and mutant strains were isolated.

Haploid *lptE* strains were generated following P1*vir* transduction of Δ*lptE2*::*kan*. The kanamycin resistance cassette was cured from this disruption with plasmid pCP20, as previously described (32).

Plasmid construction. Site-directed mutagenesis of p*lptE*$^+$ was used to construct plasmids carrying individual mutations of *lptE14*. The primers used are listed in Table 2. To generate pBAD33::*lptE*$^+$ plasmids, the *lptE* allele was PCR amplified from p*lptE*$^+$ plasmids using primers SacI_lptE and lptE_R_XbaI, digested with SacI and XbaI, and cloned into correspondingly digested pBAD33.

Immunoblot analysis. For logarithmic-phase protein samples, culture density was determined by measurement of the A_{600}, and 1 ml of the culture was pelleted and resuspended in a volume of SDS-PAGE sample buffer corresponding to the A_{600} divided by 7. For stationary-phase protein samples, 0.25 ml of the culture was pelleted and resuspended in SDS-PAGE sample buffer corresponding to the A_{600} divided by 40. When appropriate, samples were prepared under nonreducing conditions where SDS-PAGE sample buffer lacked β-mercaptoethanol. Samples were boiled for 5 min and then subjected to SDS-PAGE. To detect LptD, 20 μl of protein sample was loaded; for detection of all other proteins, samples were first diluted 1:25 in SDS-PAGE sample buffer and then 20 μl was loaded. The oxidation state of LptD was assessed by comparing the migration of the protein when subjected to SDS-PAGE under nonreducing conditions. Reduced LptD species migrate further than the oxidized protein under these conditions, and incorrectly oxidized LptD also displays altered migration, as described elsewhere (20).

Resolved proteins were transferred to nitrocellulose membranes, which were then blocked in milk and incubated overnight with polyclonal rabbit antisera: anti-LptD (1:5,000), anti-LptE (1:30,000), anti-DegP (1:30,000), anti-OmpA (1:20,000), and anti-LamB (1:20,000). Membranes were subsequently incubated with donkey anti-rabbit secondary antibody conjugated to horseradish peroxidase (used at 1:10,000) for 2 h. Blots were developed with enhanced chemiluminescence (Amersham) and visualized by exposure of X-ray film (Denville).

Linkage mapping. Linkage mapping using pools of random Tn*10* insertions was used to isolate transposon insertions linked to suppressing loci, as previously described (29). The *secA* IS*1* insertions were found to be approximately 40% linked to *leuA*::Tn*10*. The mutation was confirmed by PCR amplification of the *secA* locus with primers JY10 and JY18 and sequencing of the resultant amplicon. The IS*1* insertion increased the amplicon size by 777 bp compared to that for the *secA*$^+$ strain.

Lipid A palmitoylation assay. Overnight cultures were subcultured 1:100 into 5 ml of fresh LB broth supplemented with 0.2% arabinose to which 5 μCi/ml of $^{32}PO_4$ was added. Cultures were grown at 37°C for 2.5 h. LPS was purified from radiolabeled bacteria, and lipid A was released by mild acid hydrolysis, as previously described (33). Equal quantities of radiolabeled lipid A were spotted onto a silica thin-layer chromatography (TLC) plate (Macherey-Nagel) and developed with chloroform, pyridine, 88% formic acid, and water (50:50:16:5, vol/vol). Plates were then dried and exposed to a phosphor screen overnight. Samples were visualized using a Typhoon scanner (GE).

RESULTS

Screen for isolating dominant negative *lptE* alleles identifies *lptE14*. To generate mutant *lptE* alleles, random error-prone PCR mutagenesis was used to mutagenize p*lptE*$^+$. Pools of mutagenized plasmids were transformed into strain NR754 with wild-type *lptE*. Increased OM permeability to hydrophobic antibiotics is a hallmark of OM biogenesis defects (34). To identify dominant negative alleles, *lptE* diploid transformants were screened for sensitivity to hydrophobic antibiotics erythromycin, rifampin, and bacitracin in media supplemented with arabinose (to fully induce expression of the plasmid-encoded mutagenized *lptE* allele).

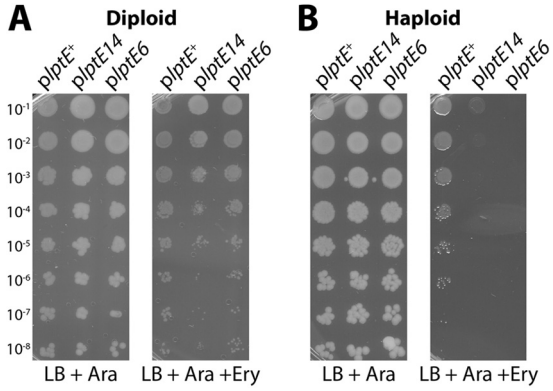

FIG 1 LptE14 expression induces OM permeability to erythromycin antibiotics. Stationary-phase cultures of *lptE* diploid strains expressing chromosomally encoded *lptE*$^+$ and either p*lptE*$^+$ (JY32), p*lptE14* (JY34), or p*lptE6* (JY17) (A) or cultures of Δ*lptE2*::*kan* strains carrying either p*lptE*$^+$ (JY33), p*lptE14* (JY35), or p*lptE6* (JY18) (B) were serially diluted and replica plated onto LB plates supplemented with ampicillin and Ara or plates additionally supplemented with 50 μg/ml Ery. Plates were incubated overnight at 37°C.

One isolated, haploid-viable allele, *lptE14*, showed significant sensitivity to erythromycin when expressed in diploid with chromosomally encoded *lptE*$^+$ (Fig. 1A). Hence, this mutant was dominant negative, compromising the OM barrier, despite the presence of a functional LptE protein. Notably, the LptD assembly-defective mutation *lptE6* was recessive in diploid analysis, despite its reported acute antibiotic sensitivity in haploid (23). In haploid strains which contain the chromosomal Δ*lptE2*::*kan* null allele, the p*lptE14*-carrying mutant was viable and actually showed no growth defect compared to the p*lptE*$^+$-carrying control (see Fig. S1 in the supplemental material). Clearly, the LptE14 protein was proficient in the essential functions required of LptE. However, when OM permeability was assessed in the *lptE14* haploid strain, sensitivity to erythromycin was more pronounced (Fig. 1B). Additionally, the *lptE14* mutation caused increased sensitivity to rifampin (not shown).

LptE14 does not impair LptD assembly. We sought to isolate mutations that affect LptE function in OM biogenesis, but not because they caused defects in LptD assembly. Mature, OM-assembled LptD forms two intramolecular disulfide bonds, and LptE is required for this oxidation (20, 23). Therefore, the oxidized form of the protein ($LptD_{OX}$) can be used to assess the efficiency of the LptD assembly process. We examined the levels of $LptD_{OX}$ from stationary-phase *lptE* haploid culture samples processed under nonreducing conditions. The levels of $LptD_{OX}$ in the p*lptE14* mutant were the same as those in the p*lptE*$^+$ control strain (Fig. 2). Additionally, we did not detect activation of DegP proteolytic activity, which has been described as a hallmark of defective LptD assembly in the p*lptE6* mutant (23) (Fig. 2). These results demonstrated that the *lptE14* mutation did not impair LptD assembly. Rather, the increased OM permeability phenotype of

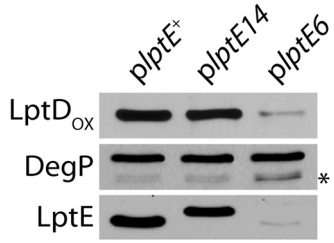

FIG 2 LptE14 is stable and supports LptD assembly. Protein samples from stationary-phase cultures of Δ*lptE2*::*kan* strains expressing p*lptE*$^+$ (JY33), p*ltE14* (JY35), or p*lptE6* (JY18) were subjected to SDS-PAGE and immunoblotting with anti-LptD, anti-DegP, and anti-LptE antibodies. Samples in $LptD_{OX}$ were prepared under nonreducing conditions. LptE14 displays altered migration in SDS-PAGE. *, a DegP degradation product that appears upon protease activation.

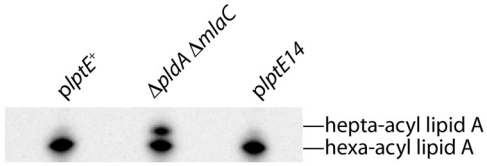

FIG 3 LptE14 does not induce palmitoylation of lipid A by PagP. LPS was extracted from stationary-phase cultures of Δ*lptE2*::*kan* strains carrying p*lptE*$^+$ (JY33) and p*lptE14* (JY35), grown in LB medium supplemented with 0.2% arabinose, and radiolabeled with ^{32}P. Samples were developed by TLC and then visualized by phosphorimaging. The Δ*pldA* Δ*mlaC* mutant (JCM537) was included as a positive control for hepta-acyl lipid A.

the p*lptE14* mutant must have been due to a deficiency in some other LptE function. Hence, the *lptE14* allele had satisfied the criteria of our screen.

The mutations in *lptE14* introduced six amino acid substitutions throughout the protein: R24H, N53K, D64E, I101T, K136N, and R186C. These mutations did not affect the stability of LptE14, with the protein expressed at levels comparable to those of wild-type LptE (Fig. 2). We wondered if any individual substitution was directly responsible for the OM permeability caused by *lptE14*. Using site-directed mutagenesis, we generated single substitution mutations in plasmid p*lptE*$^+$, and the resultant strains were assessed in both diploid and haploid for sensitivity to erythromycin. No single mutation was sufficient to confer the p*lptE14* mutant phenotype (see Fig. S2 in the supplemental material). Owing to the many possible combinations of substitutions, we did not further pursue identification of a minimal subset of changes sufficient to induce OM permeability.

LptE14 does not affect LPS export. Biochemical evidence suggests that the wild-type *E. coli* LptE protein directly and specifically binds LPS (24). Since the increased OM permeability associated with *lptE14* could not be attributed to faulty LptD assembly, another possible cause for permeability might be that the LptE14 protein has been functionally compromised in the process of LPS export. Under normal conditions, LPS is the predominant lipid species of the OM outer leaflet (3); phospholipids are actively excluded from the outer leaflet but can become mislocalized when Lpt components are depleted and LPS export is impaired (7, 30, 35). Mislocalization of phospholipids in the outer leaflet of the OM activates the OM enzyme PagP, which then uses these phospholipids as the substrate for palmitoylation of the hexa-acyl form of lipid A to produce a hepta-acyl lipid A form (33). This modification has previously been used to detect LPS export defects (30, 35). To determine if PagP activity was stimulated by the *lptE14* mutation, we extracted lipid A from the haploid p*lptE14* mutant and the corresponding control p*lptE*$^+$ strain. Lipid A was also extracted from a Δ*pldA* Δ*mlaC* mutant strain defective in removing mislocalized phospholipids as a positive control for detection of hepta-acyl lipid A species (29). Cultures were grown to logarithmic phase in medium radiolabeled with ^{32}P, and lipid A was extracted, developed by thin-layer chromatography, and finally detected by autoradiography (33). Hexa-acyl lipid A was detected in all strains examined (Fig. 3). While hepta-acyl lipid A was readily apparent in the control Δ*pldA* Δ*mlaC* strain, we were unable to detect this PagP-modified species in lipid A prepared from either the mutant p*lptE14* strain or the p*lptE*$^+$ control (Fig. 3). The absence of lipid A palmitoylation suggested that LPS export was not significantly impaired in the p*lptE14* mutant, at least not to a level at which PagP becomes activated.

Inhibition of SecB-dependent secretion suppresses p*lptE14* OM permeability. Since the p*lptE14* mutant appeared to be neither defective in LptD assembly nor impaired in LPS export, it must have been deficient in a distinct function. To determine this function, we sought to isolate and identify second-site mutations that restore the integrity of the OM permeability barrier and suppress the sensitivity of the haploid p*lptE14* strain to erythromycin. Spontaneous suppressors were selected from medium supplemented with 50 μg/ml erythromycin. Mutations suppressing the erythromycin sensitivity of p*lptE14* arose at frequencies of approximately 10^{-6}. Linkage mapping was used to determine the chromosomal loci of suppressors.

One class of erythromycin-resistant suppressors mapped to the *secA* locus and was found to have an IS*1* insertion in the 3′ region of *secA*; such IS*1* insertion mutations were isolated independently three times. These suppressors fully restored the erythromycin resistance of the p*lptE14* mutant to the levels of the p*lptE*$^+$ strain (Fig. 4A). Additionally, these suppressors also restored resistance to rifampin (not shown). The effect of the IS*1* insertions was to truncate the SecA protein at either amino acid 827 (*secA827*::IS*1*) or amino acid 830 (*secA830*::IS*1*), thereby removing the final 95 or 92 amino acids, respectively. The region removed included the entire SecB-binding domain (36). SecB is a secretion-specific cytoplasmic chaperone that maintains preproteins in a secretion-competent form (37). SecB binds with high affinity to SecA at the SecYEG translocon (38); in this way, SecB delivers preproteins to the secretion machinery.

It seemed likely that the *secA827*::IS*1* mutation would inhibit the SecB-dependent secretion pathway by removing the SecB-binding site in SecA. Indeed, a deletion-insertion Δ*secB*::*kan* allele was also able to fully suppress p*lptE14* sensitivity to erythromycin (Fig. 4B). Thus, inhibition of SecB-dependent secretion seemed central to restoring OM barrier integrity in p*lptE14*. To directly confirm that the *secA827*::IS*1* mutation impeded secretion, protein samples from logarithmic-phase cultures of *secA*$^+$ and *secA827*::IS*1* strains were immunoblotted with antibodies to detect OMPs LamB and OmpA and the periplasmic protein maltose-binding protein (MBP). The secretion of these proteins has been directly shown to proceed via the SecB pathway (39–41). Upon export from the cytoplasm, the signal sequence is cleaved from the N terminus of these proteins. We observed clear accumulation of the signal sequence-carrying preprotein forms of these proteins in the *secA827*::IS*1* strains, confirming that secretion of these pro-

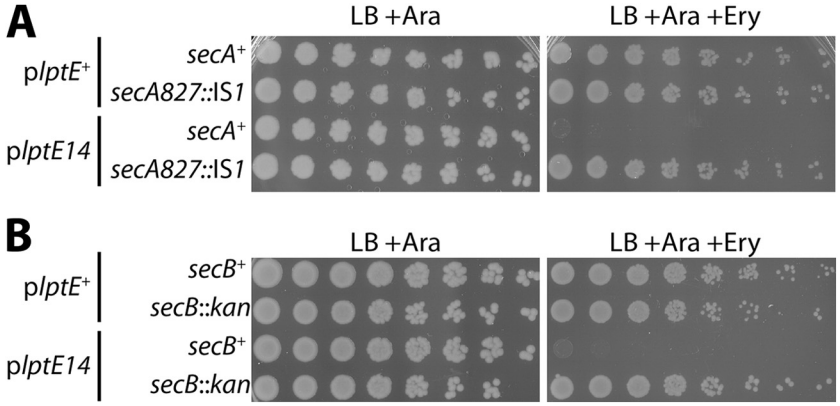

FIG 4 Mutations in *secA* or *secB* suppress p*lptE14* OM permeability. (A) Stationary-phase cultures of Δ*lptE2*::*kan* strains carrying p*lptE*$^+$ with *secA*$^+$ (JY105) or with *secA827*::IS*1* (JY106) and strains carrying p*lptE14* with *secA*$^+$ (JY107) or with *secA827*::IS*1* (JY108) were serially diluted and replica plated onto LB medium with 0.2% arabinose plates or plates additionally supplemented with 50 μg/ml erythromycin. (B) Likewise, cultures of Δ*lptE* strains carrying p*lptE*$^+$ with *secB*$^+$ (MG934) or with Δ*secB*::*kan* (MG949) and strains carrying p*lptE14* with *secB*$^+$ (MG935) or Δ*secB*::*kan* (MG950) were serially diluted and replica plated. Plates were incubated at 37°C.

teins was being impaired in this background (see Fig. S3 in the supplemental material). As expected in the *secA827*::IS*1* background, preproteins were detected in strains carrying either p*lptE14* or functional p*lptE*$^+$. Collectively, the data confirmed that reducing the secretion of SecB-dependent substrates was an effective strategy to restore the barrier function of the OM of the p*lptE14* mutant.

***secA827*::IS*1* does not affect LptE14 levels but lowers LptD$_{OX}$.** Clearly, the *secA827*::IS*1* suppressor was affecting a broad range of secreted proteins destined for the periplasm or OM, possibly including LptE and/or LptD. One possibility was that *secA827*::IS*1* restored OM barrier function by lowering the amount of LptE14 protein that reached the OM. However, immunoblotting indicated that *secA827*::IS*1* did not reduce the levels of LptE or LptE14 compared to those for the *secA*$^+$ controls, and no preprotein forms were detected (Fig. 5). Likewise, Δ*secB*::*kan* did not lower the levels of LptE compared to those for the *secB*$^+$ strains (Fig. 5). Simply, restoration of OM integrity could not be attributed to lowered levels of the mutant protein. More broadly, the data demonstrated that LptE secretion is SecB independent.

The SecB-dependent secretory pathway is favored by OMPs; since LptD is an OMP, it seemed likely that the *secA827*::IS*1* and Δ*secB*::*kan* suppressor mutations would affect LptD biogenesis. We examined the levels of LptD$_{OX}$ by immunoblotting stationary-phase protein samples. Both suppressor mutations, *secA827*::IS*1* and Δ*secB*::*kan*, drastically lowered the levels of LptD$_{OX}$ in comparison to those for the *secA*$^+$ *secB*$^+$ strains (Fig. 5). Although we did not detect the precursor form of the LptD protein, we think it likely that these suppressors were reducing the amount of LptD that was secreted from the cytoplasm.

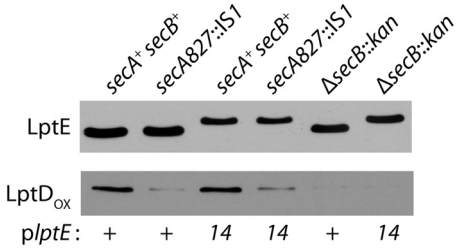

FIG 5 LptD$_{OX}$ levels are decreased by *secA* and *secB* suppressor mutations. Δ*lptE* strains carrying plasmid p*lptE*$^+$ with *secA*$^+$ *secB*$^+$ (JY105), with *secA827*::IS*1 secB*$^+$ (JY106), or with *secA*$^+$ Δ*secB*::*kan* (MG949) and Δ*lptE* strains carrying p*lptE14* with *secA*$^+$ *secB*$^+$ (JY107), with *secA827*::IS*1 secB*$^+$ (JY108), or with *secA*$^+$ Δ*secB*::*kan* (MG950) were grown to stationary phase, and protein samples were prepared under reducing (LptE) or nonreducing (LptD$_{OX}$) conditions and immunoblotted with anti-LptE or anti-LptD antisera.

Restoring LptD$_{OX}$ levels abrogates *secA827*::IS*1* suppression. The *secA827*::IS*1* suppressor must certainly cause broad alterations to the composition of both the OM and the periplasm, and suppression might require such nonspecific reductions of envelope proteins. However, the striking reduction in LptD$_{OX}$ caused by *secA827*::IS*1* and Δ*secB*::*kan* raised the more intriguing possibility that these suppressors might remedy the OM permeability defect caused by LptE14 by specifically reducing the levels of LptD$_{OX}$ and correspondingly lowering the number of LptD-LptE14 complexes in the OM. To test this hypothesis, we sought to increase LptD$_{OX}$ levels in the *secA827*::IS*1* background and determine whether this restored the erythromycin sensitivity conferred by *lptE14*.

Preproteins are directed to the Sec machinery for translocation from the cytoplasm by either the posttranslational, SecB pathway or the cotranslational, signal recognition particle (SRP) pathway (42). The secretory route taken by nascent preproteins is determined by the composition of their signal sequence; a heterologous signal sequence can redirect the secretory route of a protein (43, 44). The signal sequence of the periplasmic pilin chaperone SfmC has been shown to direct secretion via the SRP pathway (45). An SfmC-LptD fusion where the native LptD signal sequence has been replaced by the SfmC signal sequence was expressed from plasmid p*sfmC-lptD*. The SfmC-LptD fusion was fully able to complement a Δ*lptD*::*kan2* null mutation. This was unsurprising, since the protein sequence of the mature, secreted SfmC-LptD fusion was identical to that of the wild-type LptD protein.

We then constructed haploid p*lptE*$^+$ and p*lptE14* strains carrying either *secA827*::IS*1* or *secA*$^+$ and expressing either the plasmid-encoded SfmC-LptD fusion or wild-type LptD. We then deleted the native *lptD*$^+$ gene in these strains by introducing Δ*lptD*::*kan2* so that only plasmid-encoded LptD was expressed. To maintain plasmid compatibility, *lptE* alleles were expressed from pBAD33.

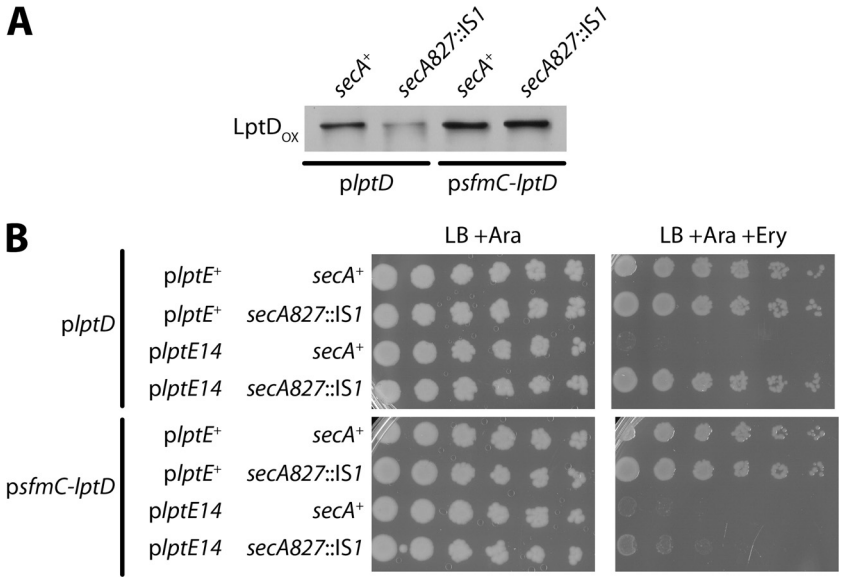

FIG 6 Routing of LptD secretion via the SRP pathway restores levels of $LptD_{OX}$ and abolishes *secA827*::IS*1* suppression. (A) Protein samples were obtained from stationary-phase cultures of Δ*lptE* Δ*lptD*::*kan2* strains carrying pBAD33::*lptE14* with p*lptD* and either *secA*$^+$ (MG1055) or *secA827*::IS*1* (MG1056) or carrying pBAD33::*lptE14* with p*sfmC-lptD* and either *secA*$^+$ (MG1059) or *secA827*::IS*1* (MG1060). Samples were prepared under nonreducing conditions and immunoblotted with anti-LptD. (B) Stationary-phase cultures were serially diluted and plated onto LB plates with 0.2% arabinose or plates additionally supplemented with 50 μg/ml erythromycin. Plates were incubated overnight at 37°C. Cultures were of Δ*lptE* Δ*lptD*::*kan2* strains carrying pBAD33::*lptE*$^+$ and either p*lptD* with *secA*$^+$ (MG1053), p*lptD* with *secA827*::IS*1* (MG1054), p*sfmC-lptD* with *secA*$^+$ (MG1057), or p*sfmC-lptD* (MG1058); alternatively, strains carried pBAD33::*lptE14* and either p*lptD* with *secA*$^+$ (MG1055), p*lptD* with *secA827*::IS*1* (MG1056), p*sfmC-lptD* with *secA*$^+$ (MG1059), or p*sfmC-lptD* (MG1060).

Immunoblots were performed to determine whether rerouting LptD to the SRP-dependent pathway would increase $LptD_{OX}$ levels in the *secA827*::IS*1* background. In the pBAD33::*lptE14* mutant, we observed that $LptD_{OX}$ levels from plasmid p*lptD*$^+$ were significantly reduced by *secA827*::IS*1* (Fig. 6A). On the other hand, the levels of the $LptD_{OX}$ from p*sfmC-lptD* were unaffected by *secA827*::IS*1* and were equivalent to the levels in *secA*$^+$ (Fig. 6A). Clearly, routing LptD secretion via SRP was able to circumvent the effect of the *secA* suppressor mutation and maintain wild-type levels of functional LptD at the OM.

We next examined if the increased $LptD_{OX}$ derived from p*sfmC-lptD* altered the *secA827*::IS*1* suppression of pBAD33::*lptE14* mutant OM permeability. We found that *secA827*::IS*1* was still able to suppress erythromycin sensitivity when wild-type LptD was expressed from p*lptD*$^+$ (Fig. 6B). However, when the SfmC-LptD fusion was expressed, suppression was abolished and erythromycin sensitivity was restored (Fig. 6B). The SfmC-LptD fusion itself did not seem to cause any increase in OM permeability, since the pBAD33::*lptE*$^+$ strain remained fully resistant to erythromycin. We conclude that the *secA827*::IS*1* and Δ*secB*::*kan* suppressors act by specifically reducing the amount of LptD-LptE14 complexes in the OM.

DISCUSSION

While LptE is central to the correct assembly and oxidation of LptD, biochemical evidence suggested that it has additional roles, including active involvement in the LPS insertion process carried out by the OM LptDE complex (24). To gain insight into these additional roles, we screened for dominant negative *lptE* mutations. The *lptE14* mutation satisfied the criteria of our screen: it exhibited compromised OM integrity, despite the presence of functional *lptE*$^+$ in diploid analysis, yet it was fully competent in assisting LptD assembly and oxidation in haploid strains. In these regards, *lptE14* was quite different from previously characterized mutations such as *lptE6*, which is recessive in diploid analysis and where impaired LptD assembly is the underlying cause of increased OM permeability (23). For these reasons, we believe that *lptE14* defines a new class of mutations that affects a distinct function of LptE.

Purified LptE binds specifically to LPS molecules (24). Thus, it is possible that *lptE14* impaired this binding or otherwise interfered with LPS transport. To compensate for such LPS assembly defects, phospholipids flip from the inner leaflet of the OM to the outer leaflet, and this triggers the enzymatic activity of PagP (30, 35). However, we could not detect PagP activation in the *lptE14* mutant strain. If LptE14 was impairing LPS export, it was doing so at levels where PagP activation could not be detected. Such a mild defect does not seem to account for the significant sensitivity to erythromycin that we observe in the mutant strain. Hence, it is unlikely that LptE14 is defective in LPS transport.

We suspected that the nature of the *lptE14* defect might be informed by examining suppressor mutations. The erythromycin sensitivity of *lptE14* could be suppressed by IS*1* insertions in *secA*. Specifically, *secA827*::IS*1* restored OM barrier integrity by inhibiting the posttranslational SecB-dependent secretion pathway that is utilized preferentially by OMPs. Indeed, a *secB* null allele was also a suppressor of *lptE14*. These suppressors answered our selection because they specifically reduced the levels of mature LptD. We demonstrated that rerouting the secretion of LptD via the cotranslational SRP pathway, circumventing the effect of *secA827*::IS*1*, abrogated suppression and restored the OM permeability to erythromycin caused by *lptE14*.

The very existence of the *secA827*::IS*1* and Δ*secB*::*kan* suppressors provides additional support to the argument that the mutant

LptE14 protein is functional for LPS transport. If LptE14 was compromised in an LPS transport function, reducing the number of LptE14-LptD complexes would exacerbate the defect and not suppress it. We conclude that the LptE14 mutant protein is competent to assemble LptD and that—in the properly assembled LptD-LptE14 complex—LptE14 functions properly in LPS transport. What, then, is the defect cause by *lptE14*?

We propose a model whereby LptE14 is defective in plugging the LptD β-barrel lumen. The inability of LptE14 to properly plug LptD would provide a direct avenue for antibiotic entry into the cell. This model is consistent with the dominance of the *lptE14* mutation: complexes of LptE14-LptD would permit antibiotic entry into the cell, despite the presence of wild-type, effectively plugged LptE-LptD complexes. The suppressors of *lptE14* antibiotic sensitivity further support the model. These mutations reduce the levels of LptE14-LptD complexes within the OM. Decreasing the number of improperly plugged LptE14-LptD complexes would reduce the avenues for entry of antibiotics and restore higher levels of resistance. The *lptE14* mutation provides genetic evidence for the plug-barrel architecture and supports biochemical studies that identified extensive LptE-LptD interactions and first proposed this LptE-LptD arrangement (25).

The *lptE14* model poses important questions for the biogenesis and function of OM Lpt components. It has been suggested that the folded LptE plug is a template for LptD β-barrel assembly, hence the requirement of LptE for LptD assembly (25). LptE14 remains fully functional in its role during LptD biogenesis, yet our data suggest that LptE14 is a poor plug of the resulting β barrel. We think that this suggests that LptE assists LptD folding in a manner that is distinct from the plugging function. Indeed, the emerging landscape suggests that LptE has three distinct functions—LPS export, LptD assembly, and LptD plugging—each of which might be genetically separable.

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