Redefining the essential trafficking pathway for outer membrane lipoproteins

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The outer membrane (OM) of Gram-negative bacteria is a permeability barrier and an intrinsic antibiotic resistance factor. Lipoproteins are OM components that function in cell wall synthesis, diverse secretion systems, and antibiotic efflux pumps. Moreover, each of the essential OM machines that assemble the barrier requires one or more lipoproteins. This dependence is thought to explain the essentiality of the periplasmic chaperone LolA and its OM receptor LolB that traffic lipoproteins to the OM. However, we show that in strains lacking substrates that are toxic when mislocalized, both LolA and LolB can be completely bypassed by activating an envelope stress response without compromising trafficking of essential lipoproteins. We identify the Cpx stress response as a monitor of lipoprotein trafficking tasked with protecting the cell from mislocalized lipoproteins. Moreover, our findings reveal that an alternate trafficking pathway exists that can, under certain conditions, bypass the functions of LolA and LolB, implying that these proteins do not perform any truly essential mechanistic steps in lipoprotein trafficking. Instead, these proteins' key function is to prevent lethal accumulation of mislocalized lipoproteins.

outer membrane | lipoprotein trafficking | Lol pathway | Cpx response | NlpE

The outer membrane (OM) blocks entry of many currently available antibiotics, preventing their clinical use in treating Gram-negative infections amid rising rates of resistance to effective drugs (1, 2). Understanding the pathways that assemble the OM permeability barrier continues to be a key goal toward uncovering novel therapeutics (3). The OM is an essential organelle that consists of a phospholipid and lipopolysaccharide (LPS) asymmetric bilayer with resident transmembrane β-barrel proteins (termed OMPs) and lipid-anchored lipoproteins (4). Recent progress has identified the essential multiprotein machines responsible for transporting and assembling most of these components into a contiguous barrier: the Bam complex folds OMPs (5); the Lpt system delivers LPS to the cells surface (6); and the Lol pathway traffics lipoproteins to the OM (7). Notably, each of the assembly machines requires at least one OM lipoprotein component for function. For example, BamD and LptE are each essential for cell viability in *Escherichia coli*. In addition, OM lipoproteins are essential cofactors for peptidoglycan cell wall synthesis in many Gram-negative bacteria (8, 9), and are important virulence factors in pathogens by serving as components in protein and polysaccharide secretion systems, motility structures, and antibiotic efflux pumps (10–13). Consequently, the trafficking pathway that delivers these lipoproteins to the OM is both the linchpin of all essential OM assembly processes and a fundamental contributor to pathogenicity.

Nascent lipoproteins are directed for secretion through the Sec translocase via an N-terminal signal peptide that includes a characteristic lipobox motif (7). Following translocation, an invariant cysteine in the lipobox is diacylated and this modification permits type II signal peptidase to subsequently cleave the signal peptide (Fig. 1*A*) (14, 15). The modified cysteine becomes the first amino acid (+1) and is additionally acylated at its amino terminus (Fig. 1*A*) (16). The mature triacylated lipoprotein is now anchored into the periplasmic leaflet of the inner membrane (IM) bilayer. Most *E. coli* lipoproteins are targeted to the OM. Whether a lipoprotein is retained in the IM or trafficked to the OM is determined by the identity of the +2 amino acid: an aspartate causes retention, whereas most other residues allow OM targeting (17, 18). The extreme hydrophobicity of lipoprotein acyl chains poses a challenge for crossing the aqueous periplasm en route to the OM. A five-protein LolABCDE pathway traffics lipoproteins to the OM in *E. coli* and other Gram-negative bacteria (Fig. 1*A*) (7). An IM ATP-binding cassette (ABC) transporter formed by a LolCDE complex first extracts OM-targeted lipoproteins from the IM bilayer (19). LolA receives lipoproteins from LolCDE and shuttles them across the periplasm while shielding their lipophilic moieties (20). LolA delivers cargo lipoproteins to LolB, itself an OM lipoprotein, which catalyzes their insertion into the OM bilayer (21).

Each of the Lol proteins is considered essential because each is required to deliver essential lipoproteins, such as BamD and LptE, to the OM. Both BamD and LptE are highly conserved throughout all Gram-negative bacteria, as are the IM Lol proteins and LolA (22, 23). However, no LolB homolog is apparent among α- and ε-proteobacterial classes of the major Gram-negative phylum (24). Hence, organisms such as *Caulobacter crescentus* and *Helicobacter pylori* produce lipoproteins with essential functions in the OM, but appear to lack a complete Lol pathway to deliver them to the OM. This apparent paradox prompted us to scrutinize the essentiality of LolB for lipoprotein trafficking in *E. coli*. Here, we show that LolB—and, surprisingly, LolA—are not essential for lipoprotein trafficking. Rather, we demonstrate that the function of both proteins is to prevent toxic accumulation of OM-targeted lipoproteins in the IM. Removing two lipoprotein substrates and manipulating an envelope stress response entirely bypasses the need for LolA and LolB, but not LolCDE. Our findings reveal the existence of an alternate trafficking pathway that receives substrates from the IM LolCDE complex and delivers them to the OM.

Significance

In Gram-negative bacteria, most lipoproteins synthesized in the inner membrane (IM) are trafficked to the outer membrane (OM). The Lol pathway is the trafficking paradigm: LolCDE releases lipoproteins from the IM; LolA shuttles them between membranes to LolB in the OM. Several OM lipoproteins are essential for viability. In apparent concordance, the Lol proteins are each essential in wild-type cells. However, we show that *Escherichia coli* grows well without LolA and LolB in the absence of one nonessential substrate and appropriately engineered stress responses, revealing that LolAB do not perform truly essential mechanistic roles in trafficking. Rather, LolAB are needed to prevent toxic lipoprotein mislocalization. Our findings change the prevailing paradigm and reveal the existence of an alternate trafficking route.

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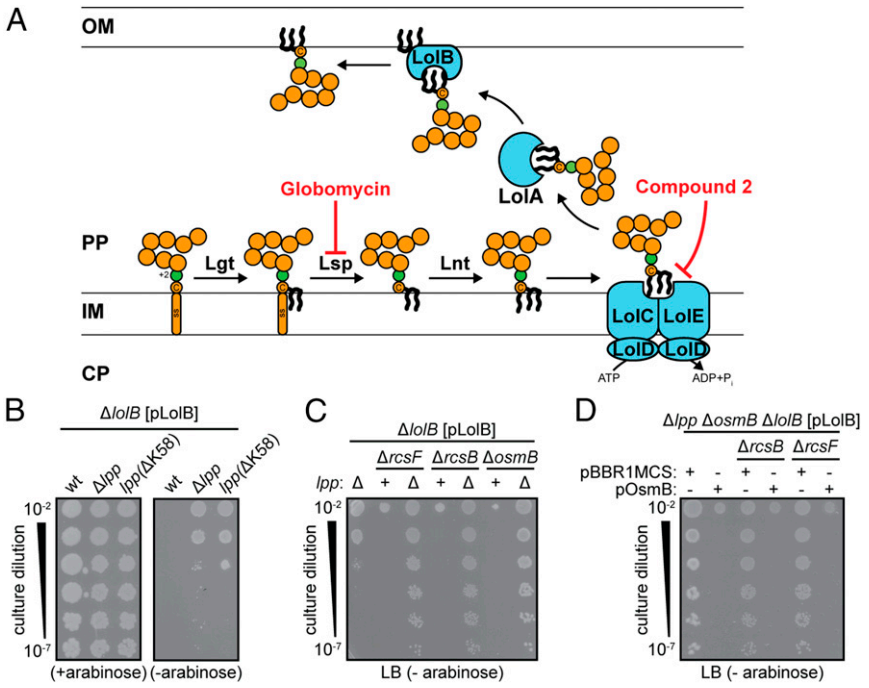

Fig. 1. Depleting LolB causes toxicity because of mislocalized Lpp and OsmB. (*A*) The OM lipoprotein biogenesis pathway. Secreted lipoproteins are acylated by the diacylglyceryltransferase Lgt at an invariant cysteine. This modification allows type II signal peptidase Lsp to release the prolipoprotein form it signal sequence (SS). The newly formed NH_2 group of the +1 cysteine is then acylated by Lnt and the mature lipoprotein enters the Lol pathway. Globomycin specifically inhibits Lsp activity (45). Compound 2 inhibits LolC and LolE function (44). (*B*) Loss of either Lpp or its cell wall-binding K58 residue increases cell viability when LolB is depleted. (*C*) Inactivation of both Lpp and the Rcs-regulated OM lipoprotein OsmB significantly improves viability during LolB depletion. (*D*) OsmB is responsible for the lethal toxicity during LolB depletion. pBBR1MCS is the vector control for pOsmB. *B*–*D* show growth of serially diluted cultures on indicated agar media.

Results

Abundant LolB Is Required to Prevent Mislocalization of an Abundant Substrate. To probe the essentiality of LolB in *E. coli*, we constructed a plasmid-based LolB depletion system where the native *lolB* locus was deleted and ectopic LolB expression was controlled from an arabinose-inducible promoter on a multicopy plasmid (pLolB). Cellular levels of LolB are depleted when cells are grown without the arabinose inducer and growth of otherwise wild-type cells is strictly inducer-dependent (Fig. 1*B*). We investigated whether these cells died directly because of a lack of LolB or because of some indirect resultant toxicity. Depleting LolB levels would compromise the Lol pathway and cause the accumulation of mislocalized lipoproteins in the IM. The most abundant protein in *E. coli* (~10^6 molecules) is Lpp (25), an OM lipoprotein that forms a covalent linkage to the peptidoglycan cell wall via its C-terminal K58 residue (26). Intentionally mislocalizing Lpp to the IM by mutating its +2 residue is lethal, but this lethality can be suppressed by deleting K58 to prevent cell wall attachment (27). We found that either Δ*lpp* or *lpp*(ΔK58) mutations modestly increased viability during LolB-depletion, confirming that a mislocalized lipoprotein contributes to the lethal toxicity caused by depleting LolB (Fig. 1*B* and Fig. S1). It seemed possible that mislocalization of other lipoproteins may similarly account for poor viability in LolB-depleted conditions.

LolB-Depletion Causes a Toxic Activation of the Rcs Stress Response. Several stress responsive signal transduction systems use OM lipoproteins as stress sensors. The Rcs two-component phosphorelay uses the OM lipoprotein RcsF to detect defects throughout the envelope (28–30). Stress conditions are thought to enable RcsF to reach across the periplasm to promote activation of the RcsC IM histidine kinase, which then phosphorylates the RcsB response regulator, allowing it to control expression of Rcs regulon genes (28, 30, 31). Mislocalizing RcsF to the IM by mutating its +2 residue causes strong activation of the Rcs response even in the absence of stress (32). We hypothesized that RcsF may mislocalize during LolB-depleted growth and inappropriately activate the Rcs response. Indeed, Δ*lpp* Δ*rcsF* cells exhibited markedly increased viability during LolB-depletion compared with Δ*lpp* cells (Fig. 1*C*). Moreover, we observed similarly robust viability in Δ*lpp* Δ*rcsB* cells that still produce the RcsF lipoprotein but cannot induce the regulon (Fig. 1*C*). Clearly, mislocalized RcsF is not itself toxic during LolB-depletion; rather, the resultant activation of the Rcs regulon is toxic.

We sought to identify the regulon members responsible for toxicity by screening knockout alleles of candidate RcsB-regulated genes (29). We found that toxicity was completely dependent upon induction of the *osmB* gene, encoding a small OM-targeted lipoprotein whose cellular function remains unclear. We observed the same robust viability in Δ*lpp* Δ*osmB* cells during LolB-depletion as in Δ*lpp* Δ*rcsB* or Δ*lpp* Δ*rcsF* cells (Fig. 1*C*). Moreover, expressing OsmB *in trans* restored the toxicity of LolB depletion to Δ*lpp* Δ*osmB* cells (Fig. 1*D*). The basis for OsmB toxicity remains to be fully elucidated.

Our results identified two sources of lethal toxicities that are caused by lowered LolB levels. Mitigating these toxicities by deleting *lpp* and *rcsF*, *rcsB*, or *osmB* significantly improved viability during LolB-depleted conditions. However, we were unable to delete *lolB* outright in these strains; they require leaky expression from pLolB. To more tightly regulate LolB production, we constructed single-copy LolB-depletion strain by placing an arabinose-inducible *lolB* at an ectopic chromosomal locus (λ*lolB*) and then deleting the native *lolB* gene. Using this tightly regulated, single-copy LolB-depletion system, cells grown without inducer exhibited poor viability even when both *lpp* and *rcsF* were deleted, confirming that these cells continue to require LolB function (Fig. S2, lane 1).

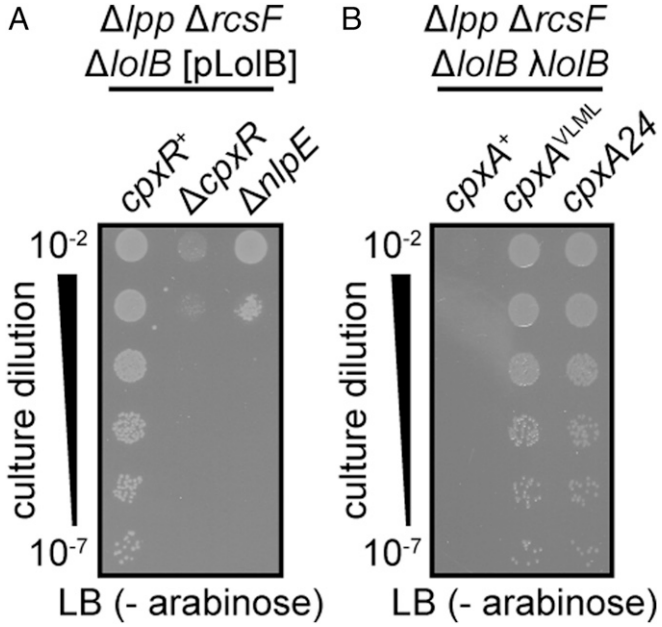

Fig. 2. The Cpx response protects the cell from impaired lipoprotein trafficking. (*A*) The Cpx response monitors trafficking efficiency via the sensor NlpE and is required for survival during LolB-depletion. (*B*) Activating mutations in CpxA allow cells to grow without LolB. Images show growth of serially diluted cultures on indicated agar media.

The Cpx Response Monitors Lipoprotein Trafficking and Protects Against Defects. A second stress response that employs an OM lipoprotein sensor is the Cpx two-component system. Adhesion of *E. coli* to abiotic surfaces is thought to allow the OM lipoprotein NlpE to reach across the periplasm to activate the CpxA IM histidine kinase, thereby increasing phosphorylation of the response regulator CpxR and enabling it to induce the Cpx regulon (33, 34). We examined the contribution of the Cpx response to growth during LolB-depleted conditions. The absence of CpxR severely reduced viability of Δ*lpp* Δ*rcsB* cells during LolB-depletion (Fig. 2*A*). Hence, rather than being harmful, the Cpx response actually protects cells against the consequences of reduced LolB levels. Mislocalizing NlpE to the IM by mutating its +2 residue induces the Cpx response, likely by facilitating CpxA interaction (35). We hypothesized that NlpE may mislocalize to IM when LolB is depleted and thus activate the Cpx response. Indeed, we found that Δ*lpp* Δ*rcsB* Δ*nlpE* cells exhibited the same reduced viability during LolB-depletion as Δ*lpp* Δ*rcsB* Δ*cpxR* cells (Fig. 2*A*). Hence, activation of the Cpx response relies entirely on signaling that originates from NlpE. Strikingly, these data identify the Cpx system as a watchdog of lipoprotein trafficking: by monitoring the biogenesis of NlpE, the Cpx system can mount a protective response when the trafficking pathway is impaired.

Activating the Cpx Response Bypasses Essentiality of LolB. We took advantage of the λ*lolB* system to more rigorously test the essentiality of LolB by searching for spontaneous mutants that could suppress the inducer-dependent growth phenotype. Two such mutant strains were isolated from media lacking inducer (Fig. 2*B*) and both carry suppressor mutations in *cpxA*. The first mutation was a 12-bp duplication that caused an insertion of VLML after amino acid 27, near the periplasmic sensor domain of the protein. CpxA sensor domain mutations commonly induce the Cpx response (36). Indeed, the second suppressor mutation was a 32-amino acid sensor domain deletion corresponding to the well-characterized activating allele *cpxA24* (36, 37). We tested whether activating *cpxA* mutations were sufficient for suppression by introducing *cpxA24* and a previously characterized activating mutation, *cpxA17* (A188E) (36, 37), into the Δ*lpp* Δ*rcsF* Δ*lolB* λ*lolB* strain. Both *cpxA* mutations enabled growth in the absence of inducer, confirming that activating CpxA permits growth despite extremely low levels of LolB (Fig. S2).

We hypothesized that the *cpxA* suppressor mutations might allow inducer-independent growth because they bypass the essentiality of *lolB* outright. We introduced *cpxA24* into Δ*lpp* Δ*rcsB* cells that expressed *lolB* only from its native locus. We then used genetic linkage analysis to quantify how readily we could introduce a Δ*lolB*::*cam* deletion by cotransduction while selecting for a nearby Δ*ychN*::*kan* allele. Astonishingly, *lolB* could be deleted from *cpxA24* Δ*lpp* Δ*rcsB* cells as readily as from cells complemented with *lolB in trans* (carrying pLolB) (Table 1). Notably, this finding indicates that there is no selective pressure to maintain *lolB* in *cpxA24* Δ*lpp* Δ*rcsB* cells. Clearly, *lolB* is no longer essential in *cpxA24* Δ*lpp* Δ*rcsB* cells. Moreover, we could also readily delete *lolB* in Δ*lpp* Δ*rcsB* cells carrying plasmid pLD404 that overexpresses NlpE (33), underscoring that activation of the Cpx response allows for complete bypass of LolB. To date we have been unable to identify any single Cpx regulon member that is required for total LolB independence.

Essential Lipoproteins Are Efficiently Trafficked in the Absence of LolB. Despite an incomplete Lol pathway, essential lipoproteins must be reaching the OM to support viability of *cpxA24* Δ*lpp* Δ*rcsB* cells that lack *lolB*. The Bam machine, responsible for the essential process of folding OMPs into the OM, is a five-member complex that relies on four OM lipoproteins (5, 38) (Fig. 3*A*): BamD is an essential lipoprotein component (39); the BamBCE lipoproteins are not individually essential, but do play important roles for efficient OMP folding (5). For example, loss of BamB results in lowered OMP levels, whereas loss of both BamB and BamE is lethal (40). The final component, BamA, is itself a β-barrel OMP.

We exploited the Bam machine's extensive reliance on OM lipoproteins to gauge the efficiency of lipoprotein trafficking in cells lacking LolB by assessing OMP levels. We observed that two nonessential OMP substrates, LamB and OmpA, were completely unaffected by loss of *lolB* (Fig. 3*B*). The Bam machine has two essential OMP substrates: the LPS insertase LptD and BamA. LptD is the cell's most complicated OMP substrate: its folding requires both assistance from an additional essential OM lipoprotein (LptE) and disulfide bond rearrangement to achieve the oxidized, functional form ($LptD_{OX}$) (41, 42). We found that *cpxA24* Δ*lpp* Δ*rcsB* cells lacking *lolB* produced the same levels of both BamA and $LptD_{OX}$ as *lolB*$^+$ controls (Fig. 3*B*). These data suggested OMP folding was not compromised in cells lacking LolB.

We also investigated Bam complex composition to assess the efficiency of Bam lipoprotein trafficking to the OM when LolB is absent. We constructed Δ*bamA* strains that were complemented with plasmid-expressed His_6-tagged BamA. The tagged BamA was affinity purified from whole cells and we then measured copurification of the BamCDE lipoproteins. Remarkably, we observed no difference in Bam machine composition between *lolB*$^+$ and

Table 1. LolB is dispensable in Δ*lpp* Δ*rcsB cpxA24* cells

Recipient strain	Δ*lolB*::*cam* cotransduction frequency (%)*
[pLolB]†	49
Δ*lpp*	0
Δ*rcsB*	0
Δ*lpp* Δ*rcsB*	0
Δ*lpp* Δ*rcsB cpxA24*	46

*P1*vir* lysates of a Δ*ychN*::*kan* Δ*lolB*::*cam* strain were used to transduce the indicated recipient strains. To determine cotransduction frequency, Kan^r transductants were first selected, and then 300 of these transductants were replica plated to determine the number of Kan^r Cam^r transductants.
†Linkage analysis was performed in media supplemented with arabinose.

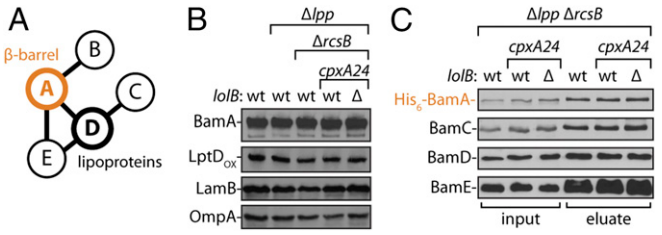

Fig. 3. The Bam machine is fully formed and fully function in cells that lack LolB. (*A*) Schematic of the five protein OM Bam machine. Solid lines indicate direct protein–protein interactions (38, 56, 57). Bam lipoproteins are in black, essential proteins are in bold. (*B*) The absence of LolB does not reduce the levels of OMPs assembled by the Bam machine. BamA and the mature, folded LptDOX are essential Bam substrates. LamB and OmpA are nonessential OMPs. OMPs and Bam lipoproteins were detected by immunoblotting of whole-cell samples from the indicated strains, probed with antisera raised against each protein. (*C*) The BamCDE lipoproteins copurify with His$_6$-BamA equally well in the presence or absence of LolB. Input samples and Ni-NTA-purified eluate samples were subjected to immunoblotting using antisera specific for each Bam protein.

Δ*lolB* cells in the *cpxA24* Δ*lpp* Δ*rcsB* background (Fig. 3*C*). This finding was particularly striking for BamC because this lipoprotein can only be copurified with BamA indirectly, through an interaction with another lipoprotein (BamD). Clearly, our data shows that BamCDE lipoproteins are efficiently delivered to the OM in *cpxA24* Δ*lpp* Δ*rcsB* cells that lack LolB. Although we do not have antisera to detect BamB, it's absence causes significant reductions in OMP levels (43). Because we did not observe any such OMP defects above, our data are consistent with BamB being present in Bam machines of *cpxA24* Δ*lpp* Δ*rcsB* Δ*lolB* cells lacking LolB. Collectively, our findings demonstrate that in cells lacking LolB, the Bam complex remains fully formed and fully functional for OMP assembly, despite the absence of a recognized pathway to traffic four of its five components to the OM.

LolA Is also Nonessential for Trafficking but LolCDE Are Required. Clearly, cells that lack LolB can deliver essential lipoproteins to the OM via some alternate LolB-independent pathway. We wondered if other Lol proteins were similarly nonessential, or if they participated in the alternate pathway and remained essential. We again used genetic linkage analysis to test *lolA* essentiality in *cpxA24* Δ*lpp* Δ*rcsB* cells. Remarkably, *lolA* could be readily deleted from Δ*lpp* Δ*rcsB* cells in a *cpxA24*-dependent manner (Table 2). Moreover, we could construct a *cpxA24* Δ*lpp* Δ*rcsB* Δ*lolA* Δ*lolB* strain that lack both LolA and LolB and only produces the IM LolCDE complex. However, we are unable to delete *lolCDE* in any background. Indeed, the *cpxA24* Δ*lpp* Δ*rcsB* background is highly sensitive to depletion of LolCDE (Fig. S3). Accordingly, we also observed that *cpxA24* Δ*lpp* Δ*rcsB* cells remained sensitive to "compound 2," a pyrazole inhibitor of LolC and LolE (Table S1) (44). The minimum inhibitory concentration (MIC) of compound 2 was significantly lower in the Δ*lolA* and Δ*lolB* derivatives of *cpxA24* Δ*lpp* Δ*rcsB*. Indeed, the Δ*lolA* and Δ*lolB* derivatives are more sensitive to a range of antibiotics (Fig. S4), suggesting increased cell permeability. We do not yet understand the basis for this permeability. The *cpxA24* Δ*lpp* Δ*rcsB* strain and its Δ*lolA*/*B*/*AB* derivatives cells also remained sensitive to the Lsp signal peptidase inhibitor globomycin (45) (Table S1). Lsp activity requires that substrate prelipoproteins are first diacylated (14). The sensitivity of *cpxA24* Δ*lpp* Δ*rcsB* cells to globomycin demonstrates that Lsp activity remains essential and we hence infer that diacylation still occurs in these cells to allow for Lsp activity.

Our data support a model in which an alternate trafficking pathway receives substrate lipoproteins from LolCDE at the IM but delivers them to the OM via a LolA- and LolB-independent mechanism. In fact, the LolAB pathway appears to compete for substrates with the alternate pathway. We noted that the *cpxA24* Δ*lpp* Δ*rcsB* strain lacking *lolA* grow equally well as its *lolA*$^+$ parent (Fig. 4*A*). In contrast, the *cpxA24* Δ*lpp* Δ*rcsB* strain that lacks *lolB* exhibit a consistent growth defect compared with its *lolB*$^+$ parent (Fig. 4*A*). Part of this growth defect might be attributable to polar effects of the knockout *lolB* allele on the essential *ispE* gene that lies downstream. However, we found that additionally deleting *lolA* improved growth of Δ*lolB* cells (Fig. 4*A*), suggesting that LolA production in the absence of LolB might be detrimental. We tested this hypothesis directly by introducing a plasmid encoding an arabinose-inducible *lolA* (pLolA) into *cpxA24* Δ*lpp* Δ*rcsB* Δ*lolB* cells. Inducing LolA overproduction was extremely toxic in cells lacking *lolB* but was well tolerated in all *lolB*$^+$ control strains (Fig. 4*B*). It appears likely that overproducing LolA when LolB is absent titrates lipoproteins into dead-end LolA-bound complexes that cannot be inserted into the OM.

Discussion

The current model of lipoprotein trafficking posits that after extraction from the IM by LolCDE, all lipoproteins transit the periplasm and reach the OM via LolA and LolB, respectively. Our findings change this paradigm. We reveal that an undiscovered, LolAB-independent, route for lipoprotein trafficking to the OM exists and is capable of supporting cell viability under certain conditions. We conclude that LolA and LolB do not perform any truly essential mechanistic steps and so are not absolutely required for lipoprotein trafficking. In contrast, LolCDE play a critical role and are fundamentally essential. The as yet undiscovered system must provide a redundant means by which to bringing lipoproteins across the periplasm and anchor them into the OM. Our findings suggest that the LolAB-dependent pathway is critically important to prevent mislocalization of OM-destined lipoproteins in the IM. Lowering LolA or LolB levels is acutely toxic for the cell not because it deprives the OM of essential lipoproteins, but because it allows two lipoprotein substrates—Lpp and OsmB—to accumulate in the IM to toxic levels. Lpp toxicity is clearly caused by the covalent attachment of the protein to the cell wall from the IM. OsmB contains glycine-zipper domains that feature in several pore-forming proteins (46). We suspect that mislocalized OsmB might form ion-permeable pores through the IM; this is a lethal event in *E. coli* because IM integrity is required to maintain the membrane potential that powers cellular energy generation. LolA and LolB are critically important in wild-type cells to protect the IM from damage that can be inflicted by lipoproteins that are en route to the OM.

Extensive biochemical evidence has established that LolA cannot insert lipoproteins into membranes but that it can receive lipoprotein substrates from LolCDE (19, 20, 47–49). On the other hand, analogous studies of LolB show that it can insert lipoproteins into membranes but it cannot receive them from LolCDE; its

Table 2. LolA and LolB are dispensable in Δ*lpp* Δ*rcsB* *cpxA24* cells

Recipient strain	Δ*lolA*::*kan* cotransduction frequency (%)*
[pLolA]†	89
Δ*lpp*	0
Δ*rcsB*	0
Δ*lpp* Δ*rcsB*	0
Δ*lpp* Δ*rcsB cpxA24*	86
Δ*lpp* Δ*rcsB cpxA24* Δ*lolB*::*cam*	91

*P1*vir* lysates of a *zca-1230*::Tn*10* Δ*lolA*::*kan* strain were used to transduce the indicated recipient strains. To determine cotransduction frequency, Tet^r transductants were first selected, and then 300 of these transductants were replica plated to determine the number of Tet^r Kan^r transductants.
†Linkage analysis was performed in media supplemented with the arabinose inducer.

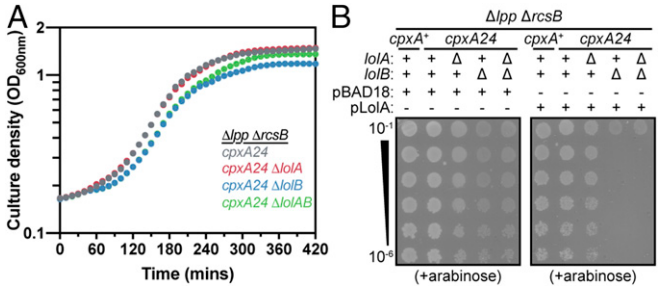

Fig. 4. LolA is toxic in cells lacking LolB. (*A*) Δ*lpp* Δ*rcsB cpxA24* cells lacking *lolB* grow to a lower final culture density than isogenic *lolB*$^{+}$ cells; the additional loss of *lolA* increases the final culture density of Δ*lpp* Δ*rcsB cpxA24* Δ*lolB* cells. (*B*) Overproduction of LolA causes lethal toxicity in Δ*lpp* Δ*rcsB cpxA24* cells that lack *lolB*. pBAD18 is the vector control for pLolA. Serial dilutions of saturated cultures were plated onto indicated solid medium.

source for lipoprotein substrates is LolA (21, 48, 49). The fact that the established trafficking pathway relies on both LolA and LolB, together, is consistent with our essentiality study. Under conditions where LolB is no longer essential, we observe that LolA is likewise nonessential. Indeed, when LolB is missing, overproducing LolA is acutely toxic, likely because of titration of lipoproteins into dead-end LolA-bound products that cannot be inserted into the OM. Such titration would deprive an alternate trafficking pathway of essential substrates to support viability. Moreover, this finding suggests that the alternate pathway is not only independent of LolA, but that it cannot use LolA.

It has long been noted that conservation of LolB is poor in α- and ε-proteobacteria (22, 24). It is possible that organisms of these classes produce a structural or functional homolog of LolB that cannot be readily identified. Alternatively, LolA might be sufficient in such cells. Curiously, genome-scale transposon mutagenesis studies of the α-proteobacterium *C. crescentus* and the ε-proteobacteria *H. pylori* hint that their *lolA* homologs might not be essential (50, 51). It is tempting to speculate that the LolAB-independent trafficking pathway in *E. coli* may be conserved in these species.

As much as our findings disprove the essentiality of LolA and LolB for lipoprotein trafficking, they underscore the fundamental requirement for the LolCDE proteins. This fact is perhaps not surprising: lipoprotein acylation remains essential in our LolAB-bypass system and the cell requires a mechanism to extract OM-destined lipoproteins from the IM as a prerequisite for their subsequent trafficking. It is remarkable that a second, LolAB-independent, trafficking pathway appears to be compatible with LolCDE so that it can receive lipoprotein substrates. Moreover, this alternate pathway has sufficient capacity to not affect the efficiency with which the four lipoproteins of the Bam machine are brought to the OM.

The σ^{E} stress response monitors—among other cell envelope processes—biogenesis of one class of OM proteins, the β-barrel OMPs, and can protect the cell against defects in the OMP assembly pathway (52, 53). A comparable protective response for the lipoprotein class of OM proteins has not previously been identified. Our findings reveal that the Cpx system fulfills this role. When lipoprotein trafficking defects occur during LolB depletion, both the Rcs and the Cpx stress-signaling pathways are induced by mislocalization of their respective lipoprotein sensors RcsF and NlpE, but the consequences of the resultant signaling through the two pathways are very different. Whereas Rcs signaling is acutely toxic and lethal to the cell, Cpx signaling significantly improves cell viability. This fact leads us to conclude that Cpx (and not Rcs) is dedicated to alleviating stress caused by lipoprotein trafficking defects. CpxA monitors the efficiency of lipoprotein trafficking through its interaction with NlpE, revealing a new physiological role for this lipoprotein. When trafficking is impaired, mislocalized NlpE accumulates in the IM and is sensed by CpxA, activating a protective response that significantly improves cell viability. Activating the Cpx system is required to bypass LolAB completely. This requirement seems consistent with our demonstration that LolAB act to protect the IM. Indeed, one of the long-established roles of the Cpx system is to maintain IM homeostasis (54). We do not yet know if Cpx activation is required to combat the toxicity caused by other mislocalized lipoproteins or if it is required to increase production of components of the LolAB-independent pathway. It may be that Cpx activation contributes in both a positive and a negative fashion. Indeed, attempts to identify a single, responsible Cpx regulon member have so far been unsuccessful. Additional studies are needed to understand the functional roles of this envelope stress response.

Materials and Methods

Strains and Growth Conditions. Strains and plasmids used in this study are provided in Tables S2 and S3, respectively. See Table S4 for oligonucleotides used. Detailed descriptions of strain and plasmid constructions are provided in *SI Materials and Methods*.

Description of the Suppressor Selection. A detailed description of suppressor selection is provided in *SI Materials and Methods*. Briefly, cultures of a Δ*lpp* Δ*rcsF* Δ*lolB*::*kan* λ*lolB* (MG2246) strain were plated onto media lacking arabinose to select for mutants capable of inducer-independent growth. Suppressor mutant strains were transduced with *nadA*::Tn*10* to test whether the nearby *bla*-marked (Amp^{R}) λ*lolB* construct could be removed. The absence of *lolB* in the resultant Amp^{S} strain was confirmed by Illumina whole-genome sequencing and with intra-*lolB* diagnostic PCR. Whole-genome sequencing identified suppressor mutations in *cpxA*.

Affinity Copurification of Bam Lipoprotein with His_6-BamA. Composition of the Bam complex assessed as previously described (55) and is detailed in *SI Materials and Methods*. Briefly, equivalent numbers of cells were taken from cultures, lysed in BugBuster (EMD Millipore) supplemented with 5 μg/mL lysozyme, 250 U of benzonase, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Debris was removed following 20-min centrifugation at 20,000 × *g* and lysates were incubated with Ni-NTA resin for 16 h at 4 °C. Resins were washed and proteins eluted from resin by washing with 200 mM imidazole.

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