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The free and bound forms of Lpp occupy distinct subcellular locations in *Escherichia coli*

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Summary

The lipoprotein Lpp is the most numerically abundant protein in *Escherichia coli*, has been investigated for over 40 years, and has served as the paradigmatic bacterial lipoprotein since its initial discovery. It exists in two distinct forms: a “bound-form”, which is covalently bound to the cell’s peptidoglycan layer, and a “free-form”, which is not. Although it is known that the carboxyl-terminus of bound-form Lpp is located in the periplasm, the precise location of free-form Lpp has never been determined. For decades, it has been widely assumed that free-form Lpp is associated with bound-form. In this work, we show that the free and bound forms of Lpp are not largely associated with each other, but are found in distinct subcellular locations. Our results indicate that free-form Lpp spans the outer membrane and is surface-exposed, whereas bound-form Lpp resides in the periplasm. Thus, Lpp represents a novel example of a single lipoprotein that is able to occupy distinct subcellular locations, and challenges models in which the free and bound forms of Lpp are assumed to be associated with each other.

Keywords

Braun’s lipoprotein; peptidoglycan; murein lipoprotein; surface-exposed lipoprotein

Introduction

Surface-exposed lipoproteins play important biological roles in many Gram-negative pathogens (Cornelissen *et al.*, 1998, Dashper *et al.*, 2000, Jin *et al.*, 2001, Leuzzi *et al.*, 2005). Even though three lipoproteins have been demonstrated to be surface-exposed in *Escherichia coli* (Manning *et al.*, 1980, Drummelsmith & Whitfield, 2000, Robinson *et al.*, 2006), it is commonly assumed in many descriptions of this model organism’s cell envelope that the vast majority of lipoproteins reside in the periplasm (Ruiz *et al.*, 2006, Tokuda, 2009). Surface proteolysis of intact cells has long been the favored method to demonstrate a protein’s surface-exposure, but *E. coli* has evolved to thrive in the protease-rich environment of the mammalian intestinal tract. Indeed, many surface-exposed proteins in *E. coli* are protease-resistant in intact cells (Beher *et al.*, 1980, Bolla *et al.*, 1990, Cole *et al.*, 1983, Manning et al., 1980, Ronco *et al.*, 1988). Moreover, none of the surface-exposed lipoproteins in *E. coli* were discovered using protease-based methods (Manning et al., 1980, Drummelsmith & Whitfield, 2000, Robinson et al., 2006). Thus, novel, protease-

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independent methods must be developed in order to adequately test the prevalence of surface-exposed lipoproteins in *E. coli*.

Lpp (a.k.a. lipoprotein, Braun's lipoprotein, murein lipoprotein, and MlpA) is the best-characterized bacterial lipoprotein. It is a small, outer membrane (OM) lipoprotein in *E. coli* that physically tethers the OM to the peptidoglycan layer (Braun & Rehn, 1969, Braun & Bosch, 1972, Braun & Wolff, 1970, Braun & Sieglin, 1970). Lpp is estimated to be present at as many as 750,000 copies per cell, making it the most numerically abundant protein in *E. coli* (Nikaido, 1996, Neidhardt, 1996). Cells lacking Lpp exhibit numerous OM defects, including increased OM permeability to antibiotics and other toxic small molecules, leakage of periplasmic contents into the extracellular environment, and heightened production of outer membrane vesicles (Yem & Wu, 1977, Hirota *et al.*, 1977, Suzuki *et al.*, 1978).

In vivo, Lpp exists in two separate forms: a "bound-form", which is covalently attached by its carboxyl-terminal lysine to the peptidoglycan layer, and a "free-form", which is not attached (Inouye *et al.*, 1972, Braun & Bosch, 1972, Braun & Rehn, 1969). In the cell, the free and bound forms of Lpp exist in an approximate 2:1 ratio, respectively (Inouye et al., 1972). Although it has long been known that the carboxyl-terminus of bound-form Lpp is located in the periplasm, published models omit description of the location of free-form Lpp (Nikaido, 1996, Nikaido & Nakae, 1979), or predict it to be physically associated with the bound form of the protein (Shu *et al.*, 2000, Inouye, 1974). However, a physical association *in vivo* between free-form and bound-form Lpp subunits has never been demonstrated, and the location of free-form Lpp remains unclear.

Here, we describe a widely applicable method to label surface-exposed proteins in *E. coli*. Surprisingly, our results show that free-form Lpp is surface-exposed. We provide evidence that bound- and free-form Lpp occupy two separate and distinct subcellular locations: bound-form Lpp is located entirely within the periplasm, whereas free-form Lpp represents a transmembrane topology that spans the OM and is exposed at the cell surface. As such, Lpp provides a unique example of a lipoprotein that stably occupies two distinct subcellular locations: one, which spans the OM lipid bilayer, and another, which exists in the periplasm.

Results

A method for labeling surface-exposed proteins in E. coli

Numerous surface-exposed *E. coli* OM proteins are highly resistant to proteolysis from the extracellular surface (Bolla et al., 1990, Manning et al., 1980, Ronco et al., 1988, Beher et al., 1980, Cole et al., 1983). An alternative method to identify surface-exposed proteins lies in labeling cells with OM-impermeable reagents, however this strategy requires careful consideration of the unique barrier properties of the OM. In general, the Gram-negative OM functions as a barrier to prevent the entry of hydrophobic compounds of any molecular weight, as well as high-molecular-weight, hydrophilic compounds, while also allowing the non-specific entry of low-molecular-weight, hydrophilic compounds (Nikaido & Rosenberg, 1981, Nikaido, 2003).

We sought to find a protein-labeling reagent that would not cross the OM and enter the periplasm. NHS-LC-LC-biotin is a hydrophobic, primary amine-reactive biotinylation reagent (Fig. 1A). Although the manufacturer describes this reagent as being generically membrane-permeable, we reasoned that the *E. coli* OM might exclude it, owing to its hydrophobicity and relatively high molecular weight. If this reagent does enter the periplasm, then it should not be able to label OM proteins with a periplasmic orientation, without also labeling some of the more abundant soluble periplasmic proteins.

To examine the OM permeability of NHS-LC-LC-biotin, we labeled whole cells and examined the distribution of the biotin label in samples that were subsequently fractionated into soluble (containing mostly cytoplasmic and periplasmic proteins) and insoluble (containing mostly membrane proteins) fractions. Because the label was almost exclusively detected in insoluble-fraction proteins (Fig. 1B), we concluded that the labeling reagent may not have entered the periplasm to a significant degree.

We were concerned that this reagent might selectively partition into membrane lipid bilayers or selectively label membrane proteins for unforeseen reasons, two situations that could also yield the observed result (Fig. 1B). To test these possibilities, we labeled a cell-free lysate and subsequently separated the lysate into the soluble and insoluble fractions, as above. Upon doing so, we observed numerous, novel labeled proteins in the soluble fraction, thereby eliminating the possibilities that NHS-LC-LC-biotin selectively partitions into membranes or labels only insoluble proteins (Fig. 1C).

Even though our controls seemed to indicate that NHS-LC-LC-biotin labels only surface-exposed proteins, we were deeply concerned that our labeling method could damage or disrupt the OM permeability barrier. To examine the effects of our labeling method on OM permeability, we compared labeled and unlabeled cells in several ways. However, labeled cells showed no detectable decrease in viability (as measured by determining the efficiency of plating on solid SDS/EDTA medium) or growth rate, as compared to unlabeled controls (data not shown). In addition, labeled and unlabeled cells had indistinguishable zones of clearing around EDTA-treated filter discs placed in the center of solid media containing SDS (data not shown). Finally, cells labeled by our labeling method were no more permeable to Sytox Green than their unlabeled counterparts (77/2289 labeled cells were permeable to Sytox Green, as compared 101/2048 unlabeled cells). These results demonstrate that our labeling method does not damage or disrupt the *E. coli* OM permeability barrier in a detectable way.

To further investigate the surface-specificity of NHS-LC-LC-biotin, we sought to examine known surface-exposed proteins using null mutants lacking these proteins. One such protein is OmpA, an abundant transmembrane β-barrel protein that serves as a surface receptor for TuII* bacteriophage (Datta *et al.*, 1977). By comparing the biotin label profiles of wild-type and *ompA*-null strains, we observed the loss of a major surface-exposed protein in the *ompA*-null strain that migrates with the same electrophoretic mobility as OmpA (Fig. 1D). We were also interested in the identity of the low molecular weight protein that is intensely labeled by this reagent. Lpp is an extremely abundant, low molecular weight OM protein with this same electrophoretic mobility. Indeed, an *lpp*-null strain showed no labeled proteins with this electrophoretic mobility (Fig. 1D). These results suggest that at least a portion of the Lpp molecules in *E. coli* are surface-exposed.

A soluble, high-molecular-weight labeling reagent also labels Lpp

Lpp was not previously thought to be surface-exposed in *E. coli*. Therefore, although our control experiments indicate that NHS-LC-LC-biotin labels only surface-exposed molecules, and that Lpp is labeled by this reagent, we were hesitant to conclude that Lpp is truly surface-exposed. To more rigorously test whether Lpp is surface-exposed, we synthesized a novel, primary-amine-reactive, hydrophilic, high-molecular-weight labeling reagent, PEG-NHS-biotin. The polyethyleneglycol backbone of this molecule makes it hydrophilic, yet of sufficiently high molecular weight so as to preclude its entry into the periplasm (Decad & Nikaido, 1976, Nakae & Nikaido, 1975) (Fig. 2A). After labeling intact cells with PEG-NHS-biotin and fractionating the sample into soluble and insoluble fractions, we again observed the vast majority of the label in insoluble fraction proteins with a pattern similar to

the pattern of proteins labeled by NHS-LC-LC-biotin (Fig. 2B). Moreover, Lpp was still intensely labeled by this reagent.

Because PEG-NHS-biotin labels proteins less intensely than NHS-LC-LC-biotin, we used longer exposure times in developing our blots. In doing so, we observed a single biotinylated protein in the soluble fraction. However, this protein was found to share a similar electrophoretic mobility to that of BCCP, a cytoplasmic biotin carrier protein with a covalently attached biotinyl prosthetic group (Li & Cronan, 1992). Furthermore, this protein was observed to be uniformly biotinylated in unlabeled cells (data not shown). Thus, we can conclude that PEG-NHS-biotin cannot enter the *E. coli* periplasm. Because two different primary amine-reactive labeling reagents that we predicted to be OM-impermeable for different reasons both labeled Lpp, we more seriously considered the possibility that some of the Lpp molecules in *E. coli* may, in fact, be surface-exposed.

The LC-LC-biotin label can be removed by surface proteolysis

The LC-LC-biotin moiety has two internal isopeptide bonds and creates a third when it attaches to a primary amine. Although *E. coli* surface-exposed proteins are highly resistant to proteolysis by extracellular proteases, we postulated that the surface-exposed LC-LC-biotinyl group might be protease-sensitive. Indeed, when we treated intact LC-LC-biotinylated cells with either proteinase K or trypsin, significant amounts of the biotin label were removed from both OmpA and Lpp (Fig. 2C) with no concomitant proteolytic degradation of either of the proteins to which it was attached. As a control, OmpA is especially informative in that it is known to be susceptible to proteolysis from the periplasmic face of the OM but not from the extracellular surface (Chen *et al.*, 1980). Since OmpA levels were not affected by the protease treatment, we can eliminate the possibilities that the proteases entered the periplasmic space or that the LC-LC-biotin modification renders proteins susceptible to surface proteolysis. Therefore, we conclude that NHS-LC-LC-biotin primarily labels surface-exposed molecules, and, additionally, that a portion of the Lpp molecules in *E. coli* are surface-exposed.

A labeling control using lipoproteins with known topologies

To determine if NHS-LC-LC-biotin can penetrate the OM and label periplasmic lipoproteins from this location, we examined whether we could label a known periplasmic lipoprotein, NlpB (Wu *et al.*, 2005), or a known surface-exposed lipoprotein, TraT (Manning et al., 1980). Although TraT was clearly labeled, we were unable to detect labeled NlpB, even when this protein was produced at over 6-fold higher levels (Fig. 2D). These results establish that NHS-LC-LC-biotin specifically labels surface-exposed lipoproteins.

A label-independent confirmation of Lpp surface-exposure

In other organisms, surface-exposed lipoproteins can deliver fluorescent proteins, to the cell surface (Schulze & Zuckert, 2006). If Lpp can function similarly to deliver an epitope tag to the cell surface, it would provide a labeling reagent-independent means to confirm the surface-exposure of free-form Lpp. Even though the Lpp-FLAG construct has a non-native carboxyl-terminus (DYKDDDDK), the epitope-tagged protein was present in cells as both the free and bound forms (Fig. 3A). Although the amount of bound-form is quite low (bound-form Lpp migrates more slowly than free-form, usually as a ladder of bands, because a heterogeneous population of peptidoglycan fragments are attached to its carboxyl-terminus), this allele complemented an *lpp*-null strain for SDS/EDTA resistance on solid media as well as the wild-type allele when expressed from a plasmid, indicating that it is physiologically functional (data not shown).

Even though Lpp is not susceptible to proteolysis from the extracellular surface (Fig. 2C), we discovered that we could remove the FLAG epitope from Lpp-FLAG using whole cell surface proteolysis. Indeed, we were able to use trypsin to remove the epitope from approximately one third of the cells' Lpp-FLAG molecules without affecting OmpA (Fig. 3B). We conclude from these results that the carboxyl-terminus of free-form Lpp is exposed at the cell surface.

We wished to determine whether our inability to remove the FLAG epitope from all of the cells' Lpp-FLAG molecules was due to incomplete proteolysis, the FLAG epitope interfering with the protein's localization to the cell surface, or a reflection of the native distribution of the protein in the cell. To address this, we labeled intact, surface-proteolysed Lpp-FLAG-expressing cells with NHS-LC-LC-biotin to determine whether both the epitope-containing and truncated forms of the protein could be labeled. Both forms of the protein were labeled by LC-LC-biotin (Fig. 3C), thereby suggesting that our inability to remove the FLAG epitope from all of the cells' Lpp-FLAG proteins is simply due to incomplete proteolysis.

The free and bound forms of Lpp exist in different subcellular compartments

Lpp exists in two different forms in *E. coli*: a "bound-form" in which the protein's carboxyl-terminus is covalently attached to the peptidoglycan layer, and a "free-form", which is not (Inouye et al., 1972). Because bound-form Lpp is known to be located in the periplasm, surface-labeling reagents should not label it. To test this, we labeled cells with NHS-LC-LC-biotin, fractionated the two forms of Lpp, and compared the amount of biotin label attached to each of the two forms, relative to the amount of Lpp protein present in each fraction. We found that the biotin label was predominantly associated with free-form Lpp (Fig. 4A). This result provides further evidence that NHS-LC-LC-biotin cannot readily enter the *E. coli* periplasm.

We considered the possibility that bound-form Lpp might not be susceptible to labeling by NHS-LC-LC-biotin *in vivo*. To test this, we labeled cells with a high concentration of NHS-LC-LC-biotin and over-exposed blots to determine whether we could detect any biotin-labeled bound-form Lpp, whatsoever. Indeed, under these conditions we observed biotin-labeled bound-form Lpp, thereby indicating that bound-form Lpp can be modified by NHS-LC-LC-biotin *in vivo* (Fig. 4B). Furthermore, bound-form Lpp prepared from a similarly-labeled *imp4213* strain (a strain with severe OM permeability defects) showed noticeably more biotin label than did bound-form from the wild-type strain (Fig. 4B) (Braun & Silhavy, 2002). These results provide additional evidence that NHS-LC-LC-biotin is excluded by the OM in wild-type strains. More importantly, these results suggest that the label's bias towards the free-form is due to a topological difference between the two forms.

Our results indicate that at least some of the cell's free-form Lpp molecules are labeled by OM-impermeable labeling compounds (Fig. 4A), yet we also observed that free-form Lpp is insensitive to proteolysis from the extracellular surface (Fig. 2C). In contrast, bound-form Lpp is not labeled by OM-impermeable labeling compounds to a significant degree. Therefore, bound-form Lpp, which is periplasmic, as well as any periplasmic free-form Lpp molecules, should be sensitive to proteases introduced into the periplasm. To test this possibility, we disrupted cells' OM permeability barrier with EDTA and passed proteinase K into the periplasm. Upon doing so, we observed degradation of only bound-form Lpp, whereas free-form Lpp was not noticeably affected (Fig. 4C). Thus, both label-dependent and label-independent methods indicate that the free and bound forms of Lpp occupy distinct subcellular compartments.

The carboxyl-terminus of Lpp is surface-exposed

In order to better understand the orientation and position of the free-form Lpp molecules that are labeled by our method, we employed mass spectrometry (MS) to identify where LC-LC-biotin attaches to the protein. Three LC-LC-biotin-modified peptides, LDNMATK*, K*, and LDNMATK*YRK* (where '*' denotes modification by LC-LC-biotin), were detected by MALDI LTQ Orbitrap MS analysis with accuracies of 0.8 ppm, 0 ppm, and 1.3 ppm, respectively. Thus, the two carboxyl-terminal lysines of Lpp (Lys_{55} and Lys_{58}) seem to be modified with LC-LC-biotin (Fig. 5A). To verify that Lpp is modified at these positions, we subjected the dually LC-LC-biotin-modified tryptic peptide corresponding to the 10, carboxyl-terminal amino acid residues (LDNMATK*YRK*, Fig. 5A) to MS^2 and MS^3 analyses. The resulting fragment ions confirmed the presence of LC-LC-biotin modifications attached to the two carboxyl-terminal lysines (Fig. S1).

Our MS analyses indicate that the carboxyl-terminus of free-form Lpp is exposed at the cell surface, and suggest that most of the remainder of the protein is not. However, we were unable to detect all of the tryptic peptides by this method, including the acylated, amino-terminal tryptic lipopeptide. Because the amino-terminal tryptic lipopeptide ends at a lysine residue, it is a potentially modifiable position of the protein. Thus, our MALDI MS analysis results alone cannot exclude the possibility that Lpp also exists in an orientation with its amino-terminus exposed at the cell surface. In addition, it is possible that other positions of the protein are labeled, but not observed in our MALDI MS experiment due to low levels or other unforeseen reasons.

To further investigate whether Lys_{55} and Lys_{58} are the only labeled positions of the protein, we cloned and expressed wild-type *lpp*, an *lpp* allele lacking the carboxyl-terminal lysine ($lpp_{\Delta K58}$), and an *lpp* allele lacking the four carboxyl-terminal-most amino acids, KYRK ($lpp_{\Delta 55–58}$). When expressed at equal levels in an *lpp*-null strain (Fig. 5B), wild-type Lpp showed the highest level of biotin labeling, whereas the variant lacking the carboxyl-terminal-most lysine showed reduced levels of labeling (Fig. 5B). In agreement with our MALDI MS/MS results, the Lpp variant that lacks the four carboxyl-terminal-most amino acids showed no detectable biotin labeling (Fig. 5B). From these results, we conclude that surface-exposed free-form Lpp molecules adopt a topology with their carboxyl-termini, but not their amino-termini, exposed at the cell surface.

Mutant lpp alleles alter the levels of surface-exposed Lpp

Two different mutant alleles of *lpp* that produce either entirely free-form or mostly bound-form Lpp have been described (Zhang *et al.*, 1992, Zhang & Wu, 1992). By reconstructing these alleles in an isogenic strain background at the native chromosomal locus, free- and bound-form Lpp levels can be altered to examine the effects on its surface-exposure.

Bound-form Lpp is covalently bound to the cell's peptidoglycan layer via its carboxyl-terminal lysine residue by a linkage between its e-amino group and the carboxyl group at the L-center of meso-diaminopimelate (Braun & Wolff, 1970). Thus, the mutant allele lacking this lysine codon produces a protein that exists entirely as the free form (Zhang & Wu, 1992). As expected, a strain carrying this allele ($lpp_{\Delta K58}$) expressed only free-form Lpp (Fig 6A).

The *lpp* allele that expresses mostly bound-form Lpp has an internal deletion of codons 17–37 of the mature, wild-type protein (Fig. 5A). In agreement with a previous study (Zhang et al., 1992), a strain carrying this allele ($lpp_{\Delta 17–37}$) produced almost entirely bound-form Lpp with very little free-form (Fig. 6A). To determine whether the levels of free-form Lpp in these strains were reflective of the amount of surface-exposed Lpp, we surface-labeled cells with NHS-LC-LC-biotin and examined the amount of biotinylated Lpp. As expected if only

free-form Lpp is surface-exposed, the extent of the biotin labeling directly paralleled the levels of free-form Lpp in each of these strains (Fig. 6B).

Lpp levels in the $lpp_{\Delta 17–37}$ strain appear lower than in wild-type when examined by immunoblot (Fig. 6A). However, we know from metabolic labeling experiments that the mutant and wild-type proteins are produced at similar levels (See Supplementary Information). The fact that the mutant protein is missing 1/3 of the amino acids that are present in the wild-type protein likely explains the observed difference in antibody binding.

The oligomeric state of free-form Lpp

A physical association *in vivo* between free- and bound-form Lpp subunits has not been reported, although a trimer comprised of only free-form Lpp subunits has been observed (Choi *et al.*, 1986). If the free and bound forms of Lpp are physically associated with each other *in vivo*, and if a substantial number of trimers have one or more bound-form Lpp monomers present, then the number of crosslinked trimers will increase substantially if covalent binding of Lpp to the peptidoglycan is prevented. However, if the two forms are not physically associated, then preventing Lpp from being covalently bound to the peptidoglycan should have little effect on the levels of crosslinked free-form Lpp trimer. It is important to note that in the absence of lysozyme treatment to digest the cell's peptidoglycan layer, bound-form Lpp does not enter an acrylamide gel. Therefore, by not treating samples with lysozyme, one can be certain that only free-form Lpp will be observed. To investigate the potential physical association between free- and bound-form Lpp *in vivo*, we exposed wild-type and $lpp_{\Delta K58}$ cells to increasing concentrations of disuccinimidyl suberate (DSS) and examined Lpp trimer levels by immunoblot analysis. Both strains revealed similar amounts of the free-form trimer that increased as the level of crosslinker was increased, confirming that the free and bound forms of Lpp are not largely associated *in vivo* (Fig. 6C). Slightly more Lpp trimer was observed in the $lpp_{\Delta K58}$ strain at all crosslinker concentrations, likely reflecting the fact that this strain produces approximately 50% more Lpp than the wild-type.

Discussion

It has long been known that there are two forms of Lpp: a "bound-form," which is covalently bound to the peptidoglycan, and a "free-form," which is not (Inouye et al., 1972, Braun & Bosch, 1972, Braun & Rehn, 1969). Some studies have assumed that the two forms are associated with each other (Inouye, 1974, Shu et al., 2000), however such an association has never been experimentally demonstrated. Instead, we have discovered that most or all of the free-form molecules are surface exposed. Free-form Lpp can be labeled by an NHS-activated biotinylation reagent, which is excluded by the OM, whereas bound-form labels poorly. Conversely, bound-form Lpp can be degraded by proteases introduced into the periplasm, whereas free-form cannot. If a FLAG tag is added at the carboxyl-terminus of Lpp, the mutant protein still retains its biological function. Most strikingly, the tagged protein is covalently attached to the peptidoglycan, and a substantial fraction of the FLAG-tagged molecules are surface-exposed. The simplest explanation for all of these results is that the free and bound forms of Lpp are not largely associated with each other, but, instead, occupy distinct subcellular locations.

When biosynthesis of Lpp is complete, the molecule is tethered to the periplasmic face of the inner membrane by its amino-terminal lipid moieties. The Lol system then transports individual Lpp monomers across the periplasm to the OM, leaving them tethered to the inner surface of the OM (Yokota *et al.*, 1999). Our data indicate that the carboxyl-terminus, but not the amino-terminus of free-form Lpp, is exposed at the cell surface. Accordingly, we suspect that the lipid moieties remain embedded in the inner leaflet of the OM and that the

molecule assumes a transmembrane conformation. Such a topology would explain why free-form Lpp is resistant to proteases present on either side of the OM. Because Lpp is present in two distinct subcellular locations, we suggest that it may be more accurate to refer to the 'periplasmic' and 'transmembrane' forms of Lpp in certain contexts.

Although it may be very transient, all bound-form molecules must initially exist as periplasmic free-form molecules, prior to being covalently bound to the peptidoglycan layer. In addition, it is possible that a small fraction of the cell's free-form Lpp molecules remain in the periplasm, perhaps associated with bound-form molecules. In either case, it is possible that a small subpopulation of the cell's free-from molecules resides in the periplasm. It is important to note that we do not suspect that we would be able to detect this putative subpopulation with the experiments that we have performed. Nevertheless, our results suggest that the vast majority of the free-form molecules in *E. coli* are exposed at the cell surface. On average, *E. coli* cells express as many as 500,000 free-form Lpp monomers. Considered from the perspective that there are approximately 1.4–3.5 million copies of LPS per cell in the OM (Neidhardt, 1996, Nikaido, 1996), our results imply that free-form Lpp molecules are a significant feature of the *E. coli* OM bilayer.

We do not yet understand how free-form Lpp is inserted into the OM. It is possible that the Bam complex, which assembles β-barrel proteins in the OM, is involved (Ruiz et al., 2006). Alternatively, there may be a dedicated translocase or a folding catalyst that can interconvert the periplasmic and transmembrane forms before the former is covalently attached to the cell wall. Given the small size of Lpp it is also possible that membrane insertion happens spontaneously. Regardless of the mechanism, it is clear that it can translocate peptide tags, such as the FLAG epitope (DYKDDDDK), that are attached to the carboxyl-terminus of the molecule. This is particularly striking because the FLAG epitope is highly charged and, thus, would likely present a challenging translocation substrate.

Previous reports indicated that free-form Lpp is trimeric (Choi et al., 1986, Shu et al., 2000). We have confirmed this result and have extended it to show that the transmembrane form is also likely to be trimeric. We do not know the structure of the transmembrane form. However, the amino acid sequence of Lpp is not incompatible with a transmembrane form. Indeed, models depicting oligomers of the protein in transmembrane form have been proposed (Inouye, 1974). The crystal structure of an unlipidated Lpp peptide, corresponding to residues 2–57 of the mature protein, has been determined and reveals a trimeric, coiled-coil structure (Shu et al., 2000). This trimer's surface is studded with numerous polar and charged residues and it is unlikely that such a structure could stably span the OM. However, it is possible that this structure represents the periplasmic topology of Lpp. Regardless of the structure, our results suggest that most of the periplasmic monomers are crosslinked to the cell wall because we see little evidence indicating substantial levels of periplasmic free-form Lpp molecules. Unfortunately, since the oligomeric state of the periplasmic form of Lpp is not known the biological significance of the published structure is not clear.

Bound-form Lpp tethers the OM to the peptidoglycan cell wall, and pronounced OM defects are apparent in strains carrying the $lpp_{\Delta K58}$ mutation, which breaks this tether, but leaves free-form undisturbed (Zhang & Wu, 1992). One way to address the function of free-form Lpp would be to phenotypically characterize a strain that produces only bound-form Lpp. As noted above, the $lpp_{\Delta 17–37}$ mutation virtually eliminates free-form Lpp. However, this mutation confers a mucoid phenotype on solid media, and is dominant in diploid analysis, demonstrating that the truncated mutant protein is toxic. Thus, the physiological function of free-form Lpp remains unclear. It may be that cells produce an excess of free-form Lpp simply to ensure sufficient levels of the protein are available to be covalently bound to the peptidoglycan. It is also possible that the transmembrane form of the protein can be

converted into the periplasmic form. Therefore, the transmembrane topology may represent a neutral location where reserves of free-form Lpp can be stored for immediate use at a later time.

We do not understand what determines the ratio of the periplasmic and transmembrane forms. There may be a regulatory mechanism to evaluate and adjust this ratio or it may be an intrinsic property of the protein, itself. Our results with the $lpp_{\Delta K58}$ mutation suggest that the transmembrane form is the default state. This may indicate that the enzyme(s) that covalently attach Lpp to the cell wall may be limiting (Magnet *et al.*, 2007). Does the ratio of the two topological forms vary under any growth conditions? Can the transmembrane topological form be converted into the periplasmic form? The answers to these questions may provide insights into the physiological role of the transmembrane form of Lpp.

Certain other bacterial membrane proteins have dual properties. For example, secreted bacterial protein toxins, such as colicins and hemolysins, can transition between soluble and membrane-inserted topologies (Parker & Feil, 2005). Proteins with dual membrane orientations, such as EmrE, also exist (Rapp *et al.*, 2007). Finally, integral OM proteins, such as OmpA and OprF can exist in either “open” or “closed” barrel conformations (Sugawara *et al.*, 2006, Sugawara & Nikaido, 1994). By existing in two distinct subcellular locations, our work adds yet another example of a bacterial membrane protein that has dual properties. Lpp has been a useful model lipoprotein for decades and there is clearly more to be learned from this simple, 58 amino acid lipoprotein.

Experimental procedures

Bacterial strains and cell culture conditions

Bacterial cells were grown in Luria-Bertani (LB) broth or on LB agar at 37°C unless specified otherwise. Bacterial strains, most of which are derivatives of *E. coli* MC4100 (Silhavy *et al.*, 1984), are listed in Table S1.

Whole cell surface labeling

Cells were subcultured 1:1000 from overnight-grown cultures into 5 mL cultures with 0.02% (w/v) arabinose, when necessary to induce expression from plasmid constructs. Cultures were grown with aeration for 3 hours, after which time the cells were pelleted, washed 3 times in PBS, and normalized to $O.D._{600}$ = 10.0 in PBS. NHS-LC-LC-biotin (Pierce, cat. # 21343) was added to a final concentration of 2% (v/v) from a 25 mg/mL stock in DMSO and the cells were held for 20 minutes at room temperature. PEG-NHS-biotin was added to a final approximate concentration of 10% (v/v) from a 5% (w/v) stock in 50% DMSO and reacted for 20 minutes at room temperature. The reactions were quenched by the addition of Tris, pH=7.5 to a final concentration of 250 mM. Finally, the labeled cells were washed in PBS and resuspended directly in the original reaction volume of SDS/PAGE loading buffer.

Subcellular fractionation

To separate the soluble and insoluble subcellular fractions, a 100 mL mid-exponential culture was washed, normalized to $O.D._{600}$ = 10.0 in PBS, and labeled as above. The cells were then lysed by passage through a French pressure cell at 20,000 PSI. The lysates were spun in a microcentrifuge for 1 minute to remove intact cells and 1 mL of the supernatant was spun for 30 minutes at 100,000 rpm in a Beckman TLA 100.2 ultracentrifuge rotor. The supernatant was saved as the soluble fraction and the pellet was washed with water and saved as the insoluble fraction by solubilizing the pellet in 1 mL of 1% SDS. In order to examine whether NHS-LC-LC-biotin partitions into membranes, the same process was

followed, except that cells were first washed, normalized, and mechanically lysed, and subsequently labeled and fractionated by ultracentrifugation.

Tests of the effects of NHS-LC-LC-biotin labeling on OM integrity

In the following experiments, a single mid-exponential culture of cells was washed in PBS, normalized to $O.D._{600}$ = 10.0, and split into two identical aliquots. One aliquot was labeled with NHS-LC-LC-biotin as described above, whereas the other aliquot was left untreated.

To examine the effects on viability, cells were diluted through 6, 10-fold serial dilutions in LB and approximately 1 μL of each dilution was spotted onto triplicate LB agar plates or LB agar plates containing 0.5% SDS. The highest dilution at which single colonies appeared was recorded and used to calculate the efficiency of plating. No difference was observed between the two treatments.

To examine the effects of NHS-LC-LC-biotin labeling on OM permeability, cells were plated on triplicate LB agar plates containing 0.5% SDS, and a filter disc containing 2, 5, or 10 μL of 0.5M EDTA was placed in the center of the plate. The plates were incubated overnight at 37°C and the zones of clearing were recorded. No difference in the size of the zone of clearing was observed between the labeled and unlabeled trials at any EDTA concentration.

To examine the effects of NHS-LC-LC-biotin labeling on OM permeability, the proportion of cells that were permeable was determined using Sytox Green at a final concentration of 5 mM according to the manufacturer's instructions (Invitrogen). Over two thousand cells for each condition were examined using light and fluorescence microscopy, and the proportion of fluorescent, permeable cells was calculated.

To examine the effects of NHS-LC-LC-biotin labeling on growth rate, aliquots of labeled and unlabeled cells were diluted 100-fold into triplicate broth cultures and the optical density was monitored over several hours. The averaged treated and untreated cells grew with indistinguishable kinetics.

Fractionation of the free and bound forms of Lpp

To separate the free- and bound-form Lpp fractions, 1mL of cells were labeled, washed, resuspended in PBS + 1% SDS, and boiled for 20 minutes. The intact peptidoglycan sacculi were then pelleted by spinning for 30 minutes at 100,000 rpm in a Beckman TLA 100.2 ultracentrifuge rotor. Following this, the supernatant fraction was saved as the free-form fraction. The peptidoglycan pellets were gently washed 4 times with 10 mM Tris, pH=7.5. Next, the pellets were resuspended in 1mL of 10 mM Tris, pH=7.5 and dispersed using a brief pulse from a probe tip sonicator. Following this, the sacculi were incubated overnight at 37°C in the absence or presence of 0.2 mg/mL lysozyme to yield bound-form fraction samples. No proteins were ever observed in bound-form samples that were not treated with lysozyme, indicating that the peptidoglycan sacculi were intact and were not contaminated with free-form fraction proteins.

To examine the whether bound-form Lpp could be labeled by NHS-LC-LC-biotin *in vivo*, mid-exponential cultures of CC01 and CC14 were washed and normalized as above and NHS-LC-LC-biotin was added to a final concentration of 5% (v/v). Cells were reacted, quenched, and fractionated into the free- and bound-form fractions, as above. Following fractionation, blots were detected with streptavidin-HRP at 1:10,000 and exposed to film for an extended length of time.

Immunoblot analyses

α-Lpp and α-biotin immunoblot analyses were carried out with standard protocols with nitrocellulose membranes in TBS-T. Prior to detection, blots were blocked for 1 hour with 5% milk in TBS-T. Lpp was detected with polyclonal rabbit primary α-Lpp antiserum (kindly provided by M. Inouye) at 1:400,000 and a donkey, α-rabbit, HRP-conjugated secondary antibody at 1:8,000 (GE Healthcare, cat. # NA934V). Biotin and LC-LC-biotin were detected with a streptavidin-HRP conjugate (Rockland Immunochemicals, cat. # S000-03) in TBS-T + 1% milk used at 1:20,000 from a 1 mg/mL stock. OmpA was detected with an α-LamB polyclonal rabbit primary antiserum that cross-reacts with OmpA at 1:20,000 in TBS-T. NlpB was detected with α-NlpB polyclonal rabbit primary antiserum at 1:20,000 in TBS-T (Wu et al., 2005). TraT was detected with α-TraT polyclonal rabbit primary antiserum at 1:20,000 in TBS-T (see below).

Synthesis of PEG-NHS-biotin

PEG 3500-NHS-biotin was synthesized using tricarballylic acid, *O*-benzylhydroxylamine hydrochloride, PEG 3500, and biotin (Acros) using both previously published (Schlemminger *et al.*, 2003) and standard chemical procedures. Briefly, tricarballylic acid was condensed onto *O*-benzylhydroxylamine to yield a benzyl-protected NHS ester derivative. The free carboxylic acid of this product was esterified with PEG 3500. The product was then deprotected, activated, and biotin was linked to the activated NHS ester. A more detailed description of this synthesis is shown in Figure S2.

Proteolysis methods

The susceptibility of the LC-LC-biotin label attached to Lpp and OmpA was examined by treating labeled and washed whole cells with 0.5 mg/mL trypsin or proteinase K at 37°C for 1 hour in 100 mM NaCl, 20 mM Tris, pH=7.5. The proteases were inactivated by adding PMSF to 5 mM and washing the cells 4 times in 100 mM NaCl, 20 mM Tris, pH=7.5 + 1 mM PMSF. The cells were then normalized to an equivalent $O.D._{600}$ = 5.0 by resuspending the cell pellet in SDS/PAGE loading buffer + 1 mM PMSF.

To examine the protease susceptibility of Lpp-FLAG, *lpp* null cells (strain CC03) harboring pBAD/lpp or pBAD/lpp-FLAG plasmids were grown to mid-log phase in LB broth supplemented with 0.02% arabinose to induce expression of the Lpp constructs. Cells were washed in 100mM NaCl, 20mM Tris, pH=7.5, normalized to $O.D._{600}$ = 10.0, and incubated in the presence of 0.5 mg/mL trypsin + 2% glycerol at 37°C for 1 hour. The cells were then washed three times in PBS, normalized to $O.D._{600}$ = 10.0 in PBS, and labeled with NHS-LC-LC-biotin, as described above.

To examine the susceptibility of free- and bound-form Lpp to periplasmic proteolysis, cells were washed in 100mM NaCl, 20mM Tris, pH=7.5 and normalized to $O.D._{600}$ = 10.0. EDTA was added to a final concentration of 5 mM and the cells were split into two identical aliquots. Proteinase K was added to one aliquot to a final concentration of 0.5 mg/mL and the cells were incubated at 37°C for 1 hour. Following this, PMSF was added to a final concentration of 5 mM and the cells were separated into free- and bound-form fractions, as above, except that 1mM PMSF was added to the overnight lysozyme digestion reactions.

Purification of TraT and production of α-TraT antibodies

To purify TraT, *traT* was amplified using oligonucleotides TraTNsolFor and TraTSalSTOPRev, digested with NdeI and SalI, and cloned into similarly-digested pET28a. The resulting plasmid, pET/TraTNsol, was inserted into BL21(λDE3) cells. An overnight culture of the expression strain was subcultured 1:150 into 6L of LB + 20 μM IPTG and the cells were grown for 3 hours with shaking. Cells were pelleted, washed, and mechanically

lysed using a pressure lysis device (Microfluidics). His-TraT was batch-purified from the cells using 12 mL Ni-NTA resin, according to the manufacturer (Qiagen). The 100 and 250 mM imidzaole eluate fractions were pooled and dialyzed against 2 large volumes of 100mM NaCl, 50mM Tris, 0.5mM EDTA, 5% glycerol, pH=7.75. Finally, the dialyzed protein was concentrated by passage through a 50 kDa MWCO Centricon filter (Millipore) and retention on a 30 kDa MWCO Centricon filter.

1 mg purified His-TraT was mixed with Freund's complete adjuvant and injected into a New Zealand white rabbit (Covance). Serum was collected at 28 days and reacted with a 25 kDa protein in F+ cells, but showed no cross-reactivity with other *E. coli* proteins in F- cells.

Molecular biological methods

The oligonucleotides used in this study are listed in Table S1. Chromosomal mutant *lpp* alleles were constructed in CC05 using single oligo-mediated mutagenesis (Costatino & Court, 2003) with oligos "delta17–37ultramer" and "lppdeltaK58." CC06 (an $lpp_{\Delta 17–37}$ strain) was identified by screening candidate colonies with PCR reactions using "lppF2" and "lppseqrev" primers to identify strains with PCR amplicons that were shorter than the wild-type *lpp* product. CC07 (an $lpp_{\Delta K58}$ strain) was identified by screening candidate colonies for sensitivity to 0.5% SDS + 1 mM EDTA. The sequence of both alleles was determined by PCR amplifying the *lpp* gene using primers "lppF2" and "lppseqrev" and then sequencing the product with primer lpp "lppF3" at GENEWIZ, Inc. (South Plainfield, NJ) to verify the expected sequence from the gene's promoter to the stop codon. Following this, the alleles were mobilized from CC06 and CC07 into CC01 by P1 transduction to create strains CC08 and CC09 ($lpp_{\Delta 17–37}$ and $lpp_{\Delta K58}$ strains, respectively).

Standard molecular biological methods were used for plasmid construction. Plasmids pBAD/lpp, pBAD/$lpp_{\Delta K58}$, and pBAD/$lpp_{\Delta 55–58}$ were constructed by amplifying the *lpp* open reading frame and ribosome binding site using primers pBADlppFor and either pBADlppRev, pBADlpp-CKRev, or pBAD/lpp-C2K rev, respectively. The correct sequence of all plasmids was determined by directly sequencing with both pBAD-Forward and pBAD-Reverse primers at GENEWIZ, Inc.

Examination of NHS-LC-LC-biotin surface-specificity using NlpB and TraT control lipoproteins

To examine the surface-specificity of NHS-LC-LC-biotin using lipoproteins with known orientations, CC11 (*ompA*-null, *nlpB*-null) cells were transformed with pBAD/nlpB or pBAD/traT. An *ompA*-null strain was used, as NlpB co-migrates with OmpA. NlpB expression was induced with 0.02% arabinose and TraT expression was induced with 0.001% or 0.002% arabinose, and the cells were surface-labeled with NHS-LC-LC-biotin, as described above. Known quantities of purified NlpB-His (kindly provided by J. Malinverni) or His-TraT were used as references for immunoblot quantification of each protein's expression level in the labeled samples. Reference protein standards were varied through several, twofold dilutions, and were used in ranges that flanked the concentration of the experimental proteins. Duplicate quantifications were performed for each sample.

Mass spectrometric analyses

For mass spectrometric analyses, whole cells were surface-labeled with NHS-LC-LC-biotin and insoluble-fraction SDS/PAGE samples were prepared as described above. The samples were separated using 10–20% SDS/PAGE gels and the 10–12 kDa region of the gel was excised and diced into 1-mm^3 pieces. The sample was digested with 12.5 ng/mL sequencing grade modified trypsin (Promega), and the resulting peptides were extracted on reverse-phase resin (Poros 20 R2, PerSeptive Biosystems), as previously described (Cristea *et al.*,

2005). The peptide mixtures were eluted directly onto a MALDI target in 50% methanol, 20% acetonitrile, 0.1% trifluoroacetic acid + 2 mg/mL α-Cyano-4-hydroxycinnamic acid matrix. MALDI MS and MS/MS analyses were performed using a MALDI LTQ Orbitrap XL, as previously described (Luo *et al.*, 2010). Lists of the expected *m*/*z* for tryptic peptides and ion fragments were generated using Prowl (http://prowl.rockefeller.edu/) with K +452.24571 for modification by LC-LC-biotin.

For MALDI LTQ MS^2 analysis, the ion of interest (LDNMATK*YRK*, *m*/*z* 2144.143) was isolated in the ion trap with a 2 Da mass range window, and subjected to collision induced dissociation (CID), as described (Luo et al., 2010), using a normalized collision energy of 35%. As expected from the primary sequence of this ion, the predominant ion was the y_8 ion, resulting from the preferential cleavage at the carboxyl-terminus of the aspartic acid (Qin & Chait, 1995). Thus, this ion (NMATK*YRK*, *m*/*z* 1916.11) was further subjected to MALDI LTQ CID MS^3 analysis with the same settings as above (Fig. S1).

in vivo crosslinking

To crosslink Lpp *in vivo*, cells were subcultured 1:1000 and grown for 3 hours, washed three times in PBS, and normalized to $O.D._{600}$ = 10.0. Whole cells were crosslinked with a range of concentrations of disuccinimidyl suberate (DSS) concentrations for 20 minutes at room temperature. Following this, the reactions were quenched by the addition of Tris, pH=7.5 to a final concentration of 250 mM. Finally, the crosslinked cells were washed in PBS and resuspended in SDS/PAGE loading buffer at their original concentration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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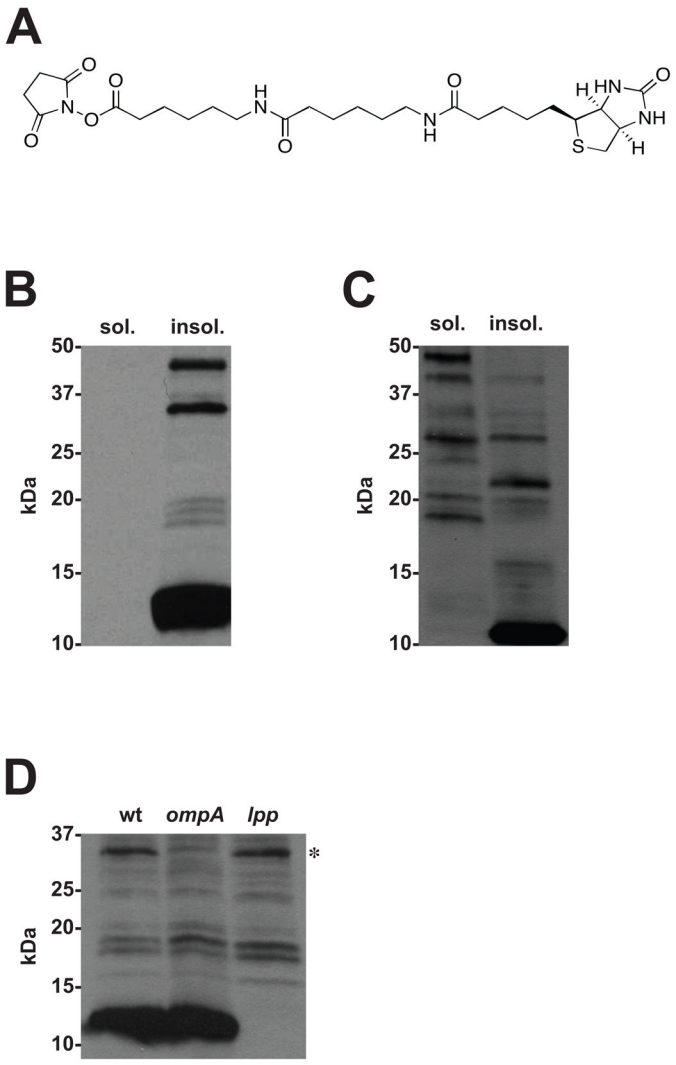

Figure 1. A method for labeling surface-exposed proteins in *E. coli*

(A) Structure of NHS-LC-LC-biotin.

(B) Western blot probed for biotin of soluble (sol.) and insoluble (insol.) fraction proteins from whole cells labeled with NHS-LC-LC-biotin.

(C) Western blot probed for biotin of soluble (sol.) and insoluble (insol.) fraction proteins from a lysate labeled with NHS-LC-LC-biotin.

(D) Western blot probed for biotin of cellular proteins from wild-type, "wt"; *ompA*-null, "*ompA*"; and *lpp*-null, "*lpp*" strains. The protein band that is suspected to be biotin-labeled OmpA is indicated with an asterisk.

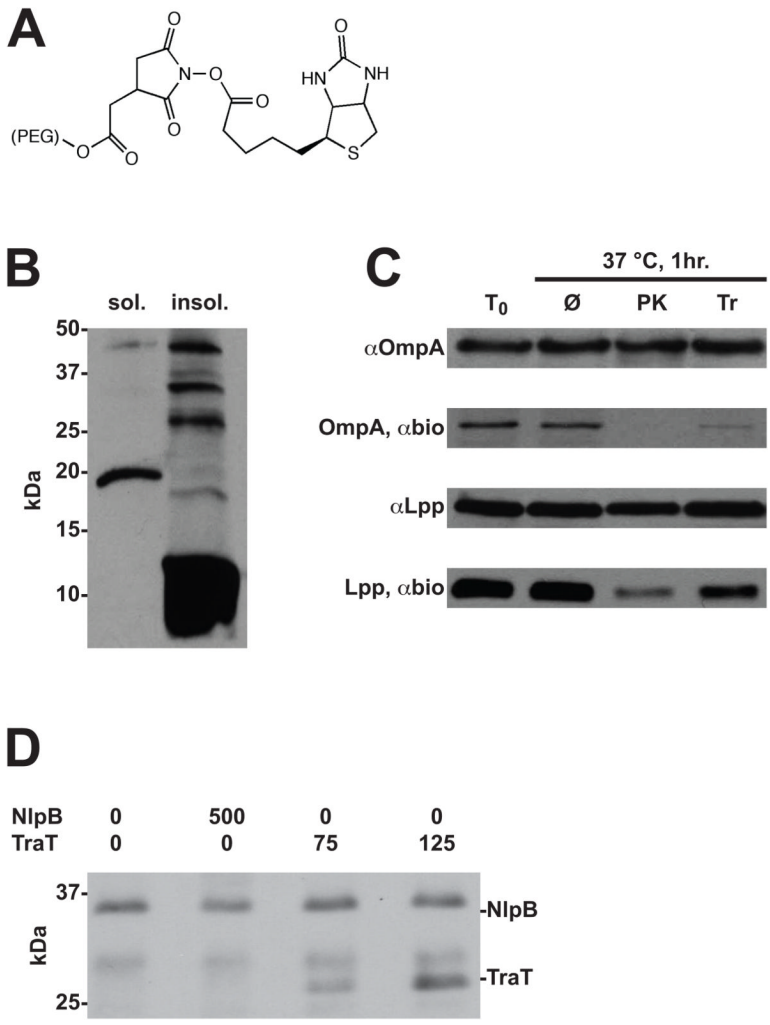

Figure 2. Lpp is surface-exposed in *E. coli*

(A) Structure of PEG-NHS-biotin. An identical N-hydroxysuccinimidyl-biotin moiety is found at either end of the PEG 3500 backbone.

(B) Western blot probed for biotin of soluble and insoluble fraction proteins from whole cells labeled with PEG-NHS-biotin.

(C) Western blots, cropped to show the OmpA and Lpp regions, probed for specific proteins or biotin, as indicated. Samples were frozen immediately after labeling (T_0), or treated with no protease (Ø), proteinase K (PK), or trypsin (Tr), as indicated.

(D) Western blot probed for biotin of cells expressing the indicated quantities (in ng) of NlpB or TraT, labeled with NHS-LC-LC-biotin. The known positions of NlpB and TraT are indicated to the right.

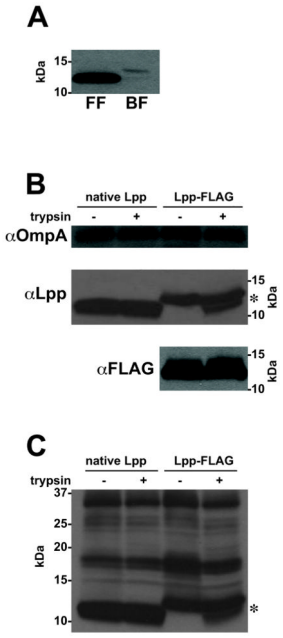

Figure 3. Label-independent confirmation of Lpp surface-exposure

(A) Western blot of Lpp from cells expressing Lpp-FLAG, separated into the free-form (FF) and bound-form (BF) subcellular fractions. The FF sample was diluted ten-fold prior to loading.

(B) Western blots of whole cells expressing native Lpp or Lpp-FLAG untreated (−) or treated (+) with trypsin, as indicated. Blots were probed for OmpA, Lpp, or the FLAG epitope, as indicated. The novel Lpp species that results from trypsin treatment of the Lpp-FLAG protein is indicated with an asterisk.

(C) Western blot probed for biotin of whole cells expressing native Lpp or Lpp-FLAG untreated (−) or treated (+) with trypsin, as indicated, then labeled with biotin. The novel Lpp species that results from trypsin treatment of the Lpp-FLAG protein is indicated with an asterisk.

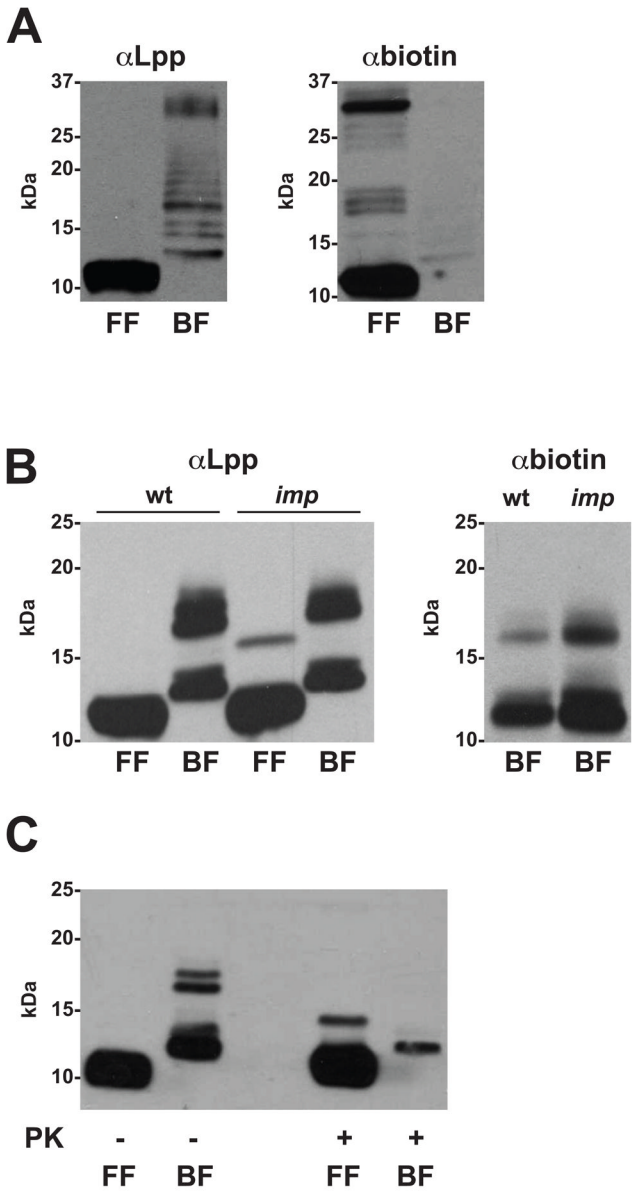

Figure 4. The free and bound forms of Lpp occupy distinct subcellular locations

(A) Western blots of samples from cells labeled with NHS-LC-LC-biotin and separated into free-form (FF) and bound-form (BF) Lpp fractions. Blots were probed for Lpp or biotin, as indicated.

(B) Western blots of samples from wild-type (wt) or *imp4213* (*imp*) cells labeled with a high concentration of NHS-LC-LC-biotin and separated into free-form (FF) and bound-form (BF) Lpp fractions. Blots were probed for Lpp or biotin, as indicated.

(C) Western blots of Lpp from EDTA-treated cells left untreated (−) or treated (+) with proteinase K (PK) and separated into free- and bound-form Lpp fractions.

Figure 5. The carboxy-terminus of Lpp is surface exposed

(A) Schematic of Lpp depicting the acylated amino-terminus, internal deletion from the $Lpp_{\Delta 17–37}$ mutant (central gray box), deleted carboxy-terminal lysine from the $Lpp_{\Delta K58}$ mutant (boxed in gray at right), and peptide that was isolated and used in MS^2 and MS^3 analyses (underlined). Asterisks indicate the lysine residues modified by LC-LC-biotin. (B) Western blots of LC-LC-biotin-labeled *lpp*-null cells expressing different forms of Lpp. Blots were probed for Lpp or biotin, as indicated. Cells harbored pBAD18 plasmid vector (v), pBAD/lpp (wt), pBAD/$lpp_{\Delta K58}$ (ΔK58), or pBAD/$lpp_{\Delta 55–58}$ (Δ55–58).

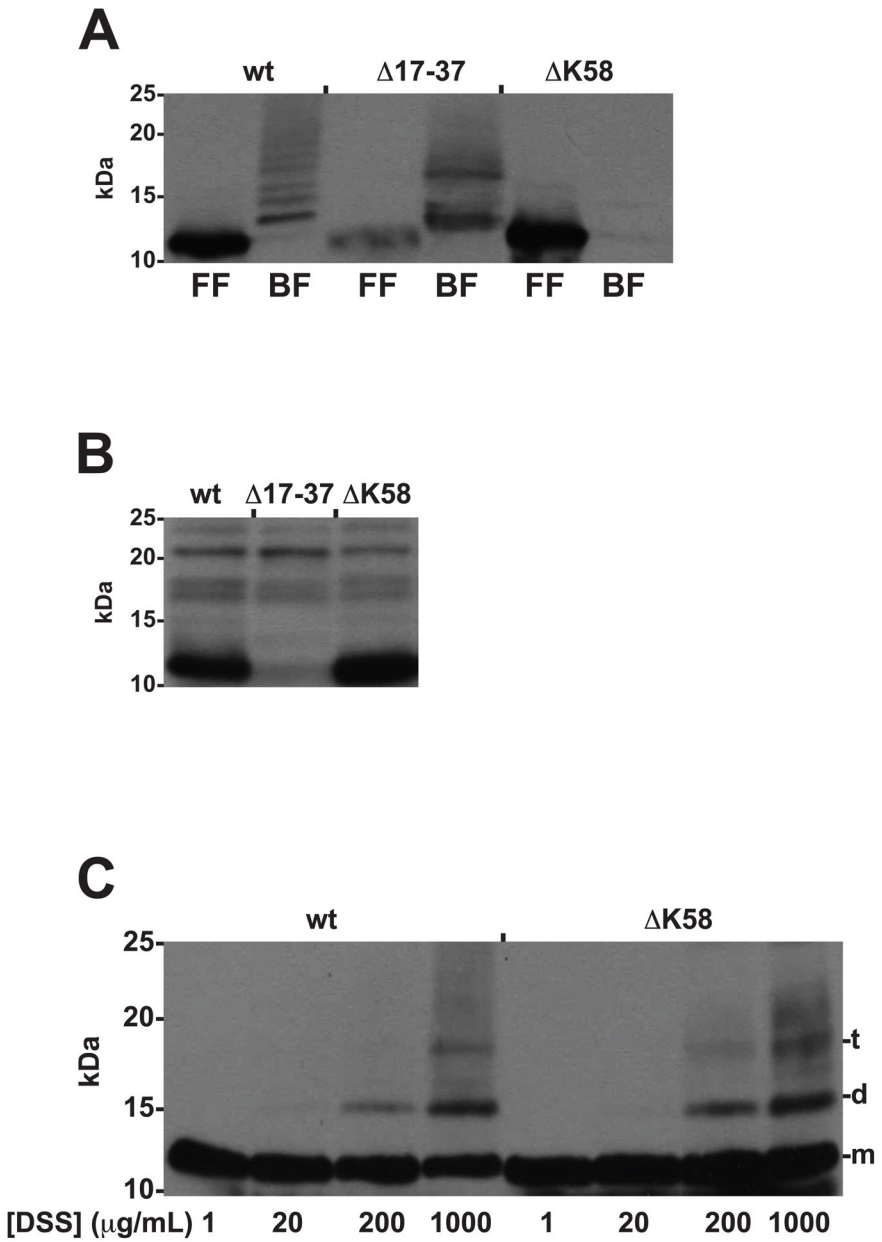

Figure 6. Mutant alleles alter levels of surface-exposed Lpp

(A) Western blot probed for Lpp of samples from cells expressing the indicated *lpp* alleles, separated into the free-form (FF) and bound-form (BF) subcellular fractions. Because the $Lpp_{\Delta 17–37}$ mutant protein is not detected well by the antibody used in these studies, twice the sample volume was loaded for this strain, relative to the amounts loaded from the other 2 strains.

(B) Western blot probed for biotin of samples from cells expressing the indicated forms of Lpp, labeled with LC-LC-biotin. Equal volumes of cell density-normalized samples were loaded in each lane.

(C) Western blot of Lpp from cells expressing the indicated forms of Lpp, crosslinked with the indicated concentrations of disuccinimidyl suberate (DSS). The apparent monomeric (m), dimeric (d), and trimeric (t) complexes are indicated.