The cell envelope of the Gram-negative bacterium, *Escherichia coli* consists of two membranes, the outer and the inner membrane, separated by a rigid peptidoglycan layer. The peptidoglycan containing space between the two membranes is the periplasm. Before reaching their final destination, outer membrane (OM) proteins must first cross the inner membrane (IM) and periplasmic space. Much is known concerning the translocation of proteins across the inner membrane of *E. coli* from their site of synthesis in the cell cytoplasm to the periplasm, at equilibrium with the external pH.

The protein sequence responsible for lipoprotein membrane localization in *Escherichia coli* exhibits remarkable specificity.†

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Structural information defining an N-terminal sequence required for the membrane sorting of bacterial lipoproteins has been previously garnered through the study of a hybrid outer membrane (OM) lipo-β-lactamase (LL) (Ghayeb and Inouye (1984) J. Biol. Chem. 259, 463–467). Introduction of an aspartate as the second residue of mature LL (D2 mutant) causes an inner membrane (IM) localization of this protein (Yamaguchi, K., Yu, F., and Inouye, M. (1988) Cell 53, 423–432). Introduction of asparagine at the third residue of mature LL (D3 mutant) causes a weaker IM sorting signal and when present as the fourth residue (D4), normal OM sorting occurs. A positively charged residue at the second position (K2) has no effect on OM localization. Remarkably, glutamate substitution at either the second (E2) or third (E3) position does not interfere with OM sorting. Sorting of the mutant D2 LL can be partially suppressed by introduction of a positively charged histidine (D2H3) or lysine (D2K3) at residue 3 of the mature protein. These results indicate that both the negative charge of the aspartate residue and some structural feature not present in a glutamate residue are required for sorting to the IM. The suppression of IM localization of the D2H3 LL double mutant can be eliminated by growing *Escherichia coli* at pH 8.4 to reduce the histidine partial positive charge. This result supports the essentiality of a negative charge in IM localization and indicates that a committed step in lipoprotein sorting is made in a cellular compartment, the periplasm, at equilibrium with the external pH.

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The abbreviations used are: OM, outer membrane; LL, lipo-β-lactamase; IM, inner membrane; TRICINE, N-tris(hydroxy-methyl)methylglycine; MES, 2-(N-morpholino)ethanesulfonic acid; IPTG, isopropyl-β-D-thiogalactopyranoside.

The protein sequence responsible for lipoprotein membrane localization in *Escherichia coli* exhibits remarkable specificity. In general, secreted proteins must be synthesized with an N-terminal extension, known as the signal sequence, which is proteolytically removed during the translocation process (see Ref. 1 for a review). Many of the proteins required for secretion have been identified, and their role in protein translocation is being characterized (see Ref. 2 for a review). In contrast, very little is known about the process by which newly synthesized proteins are sorted to the outer membrane. A region of the outer membrane protein OmpA between residues 154–180 has been proposed to act as a sorting sequence (3). This region contains a predicted β-strand which is a putative membrane spanning sequence (see Ref. 4 for a review). A double mutant OmpA protein which was expected to alter the predicted structure of this β-strand was indeed incapable of OM insertion (5). Experiments utilizing lacZ fusions have lead to the suggestion that a region within the first 49 mature residues of the LamB protein is required for OM localization (6). Alternatively, the C-terminal region may be important for LamB trimerization which has been proposed to be a prerequisite for LamB assembly in the OM (7). In contrast, no specific region responsible for OM localization of the OmpC (8), or PhoE (9) proteins has been found.

Lipoprotein sorting is probably the best characterized example of protein localization to the OM. Lipoprotein is a generic term referring to a large number of structurally and functionally distinct proteins which share in common a particular lipid modification at the N-terminal cysteine of the mature protein (see Ref. 10 for a review). Two fatty acids and one fatty acid are linked by ester and amide bonds, respectively, to the N-terminal glycerylcysteine residue. This lipid modification is believed to functionally tether the lipoprotein to the membrane. Consequently, a fundamental physical difference exists between lipoproteins and non-lipid modified OM proteins. It is therefore possible that the OM sorting of lipoproteins proceeds via a mechanism which is essentially distinct from that in operation for localization of non-lipid-modified OM proteins. Indeed, structural features common to many non-lipid-modified OM proteins which may be important in protein localization are absent in lipoproteins (11).

A short N-terminal sequence of the major OM lipoprotein of *E. coli* is the only region required for membrane interaction (12). A 9-residue N-terminal portion of this lipoprotein, when fused to the otherwise soluble β-lactamase, is capable of directing the resultant lipid-modified fusion protein, lipo-β-lactamase (LL) to the OM in *vivo* (13). The residue adjacent to the N-terminal lipid-modified cysteine of the LL fusion is a serine derived from the major OM lipoprotein. When this serine residue is replaced with aspartate, a residue found adjacent to the N-terminal cysteine of OM-localized lipoproteins (14, 15), the resulting LL mutant is localized to the IM in *vivo* (16). Alteration of this single residue completely blocks the function of the OM sorting sequence derived from the N
terminus of the major lipoprotein. In the present study the role of charged residues in the function of this sorting sequence is characterized.

MATERIALS AND METHODS

Bacterial Strain, Plasmids, and Growth Media—The E. coli strain SB211 (16) was utilized in all experiments. Both pJG311 (13) and pKY702 (16) have been previously described. Unless otherwise indicated, bacteria were grown at 37 °C in M9 minimal medium (17) supplemented with 0.4% glucose, 0.2% casamino acids, 0.81 mM MgSO₄, 20 µg/ml thiamine, 50 µg/ml tryptophan, and 34 µg/ml chloramphenicol. MT medium consists of 25 mM TRICINE, 25 mM MES, 62 mM potassium phosphate, 15 mM NH₄SO₄, and 5 µg/ml FeSO₄, plus the supplements used in M9 and adjusted to pH 6.25 with NaOH. T medium is equivalent to MT lacking MES with the pH adjusted to 8.4. At the time of culture harvest, the pH of growth media was determined to be 7 for M9, 6 for MT, and 8.2 for T.

Mutagenesis—Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 380B DNA synthesizer. The lipolactamase gene from either pJG311 or pKY702 was subcloned into M13mp18 and employed as template for oligonucleotide-directed site-specific mutagenesis using the in vitro mutagenesis system provided by Amersham Corp. following the manufacturer's protocol. All mutations were verified by sequencing with Sequenase (Amersham Corp.) following the manufacturer's protocol. The mutant proteins generated are shown in Fig. 1.

Membrane Fractionation and Electrophoresis— Cultures (500 ml) were grown to a Klett reading of 60 (blue filter) (this is equivalent to an OD₅₀₀ of 0.2) at which point IPTG was added to a final concentration of 2 mM. Following 5 min of induction at 37 °C, cultures were harvested at 7,000 × g for 15 min and resuspended in 5 ml of 0.75 M sucrose, 10 mM Tris-HCl, pH 7.8. Spheroplasts were prepared and sonicated and the cell envelope isolated according to the method described by Osborn et al. (18). Inner and outer membrane fractions were isolated by overnight centrifugation at 35,000 rpm (150,000 × g) in an SW41Ti rotor (Beckman) at 6 °C through a discontinuous sucrose gradient as described by Schnaitman (19). The IM fraction bands at the interface between the 24 and 42% sucrose steps, while the OM fraction was collected as the 55% sucrose cushion. A membrane fraction of intermediate density (designated M) observed just above the 42–55% sucrose interface was collected separately. Proteins of the various membrane fractions were resolved on 17.5% Laemmli polyacrylamide gels (20) and stained with Coomassie Brilliant Blue. The quantity of lipo-β-lactamase and OmpA present in each fraction was determined by densitometry.

RESULTS

Membrane Localization of Lipo-β-lactamase Mutants— Sorting of LL to the OM of E. coli can be blocked by the replacement of the second mature residue, serine (S2) by aspartate (D2) (16). To characterize more thoroughly the region of LL responsible for sorting to the OM, the mutants shown in Fig. 1 were constructed by oligonucleotide-directed site-specific mutagenesis. A 5-min incubation with IPTG was used to induce mutant LL production. No toxic effects due to protein overproduction were apparent at this early time period (see discussion below). E. coli expressing each mutant LL protein was fractionated into IM and OM using a discontinuous sucrose density gradient. The quantity of LL and the OM protein OmpA in each fraction was determined by densitometry of Coomassie Brilliant Blue-stained proteins resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Under the induction conditions employed, the 31-kDa LL is a major envelope protein, being present at approximately the same level as OmpA. The amount of OmpA in the IM fraction is an indication of cross-contamination between fractions.

The results presented in Fig. 2 and quantified in Table I illustrate the remarkable specificity of the sorting process. Whereas the presence of aspartate at position 2 of the mature protein (D2) caused almost complete localization of LL to the IM, movement of this residue to position 3 (D3) caused this

FIG. 1. Lipo-β-lactamase sorting mutations. The signal peptide and N-terminal mature sequence derived from the major outer membrane lipoprotein is indicated by a closed box. The open box designates mature β-lactamase sequences. Changes to the wild-type protein sequence are indicated, with base alterations underlined. The signal peptide cleavage site is indicated by an arrow. The S2 sequence is wild-type.

FIG. 2. Membrane localization of mutant lipo-β-lactamase proteins. E. coli cell envelopes (E) from cultures expressing wild-type (S2) and mutant lipo-β-lactamase were fractionated into outer membrane (O), inner membrane (I), and a fraction of intermediate density (M) as described under "Materials and Methods." Control cultures harbored a plasmid carrying an insertion inactivated lipo-β-lactamase gene. One equivalent of envelope was loaded in lanes O, I, and M as compared to 0.9 equivalents in lane E. OmpA and lipo-β-lactamase (LL) are indicated by arrows at the left. The position of molecular weight markers is shown at the right.
mutant protein to be distributed in both membranes. The quantity of D2 LL found in OM fractions (10-20%) could be due to contamination with IM. When located further from the N terminus at position 4 (D4), the aspartate residue had no effect on sorting. As with the wild-type S2 protein, virtually all of the mutant D4 LL was found in the OM. The presence of the mature LL. This should neutralize at least partially the negative charge of the aspartate. Indeed, the presence of either a histidine (D2H3) or lysine (D2K3) residue at position 3 had no suppressive effect (Fig. 3 and Table I). These results clearly indicate that the negative charge of the aspartate residue is essential for IM lipoprotein localization.

**Effect of Lipo-β-lactamase Overproduction on Cell Growth**—In the course of these experiments we observed that, in all cases, the overexpression of LL induced by IPTG was toxic (Fig. 4). However, the nature of the IPTG-induced cell death was dependent upon the membrane localization of the overproduced LL. Cultures expressing protein which localized like wild-type LL to the OM (S2, D4, E2, E3, and K2) ceased growth approximately 1 h after IPTG addition (Fig. 4). Microscopic examination indicated that cells from these cultures were intact but could not be rescued from IPTG-induced death by growth on minimal plates lacking IPTG (not shown). In contrast, cultures expressing an IM-localized LL (D2, D2Q3, and D2Y3) exhibited a rapid decline in culture turbidity following IPTG addition (Fig. 4). Cells from such cultures had visibly lysed with consequent release of protein into the growth media (data not shown). Although not evident in the first two panels of Fig. 4, cultures expressing the D2 mutant protein continue to grow normally immediately after IPTG addition and only lyse between 30 min to 1 h later. Variations between experiments are probably due to slight differences in growth rate and cell density at the time of induction. Cultures expressing mutant LL which distributed between inner and outer membrane (D3, D2H3, and D2K3) show an intermediate phenotype. Following IPTG addition, the culture turbidity declined more slowly and not to as great an extent as cultures producing the IM-localized LL (Fig. 4). Apparently, when the protein is distributed between both membranes, the level required to cause lysis is attained relatively late. In addition, fewer cells in the culture eventually lyse. The lysis observed with IM-localized LL mutants suggests some perturbation in peptidoglycan structure. It is conceivable that accumulation of LL in the IM, but not the OM, inhibits the synthesis or assembly of some essential peptidoglycan component. It should be noted that these differences are not due to altered levels of LL production in the various mutants (Figs. 2 and 3). It is important to note that membrane localization determinations were carried out with cultures which were induced for only 5 min at an OD<sub>600</sub> of 0.2. Under these conditions, cultures continue to grow normally for 1 h before exhibiting the toxic effects characteristic of membrane localization illustrated in Fig. 4 (data not shown). Therefore, the membrane localizations were made with normally growing cultures. In addition, the mutations described here had no effect upon the normal lipid modification of LL (see Ref. 16 for D2; the data for other mutants are not shown). This observed difference in growth phenotype reflects the altered localization of mutant LL proteins in living cells, and as such is an in vivo corroboration of the in vitro membrane localization as determined by gradient fractionation.

**Effect of pH on the Localization of the Lipo-β-lactamase D2H3 Suppressor Mutant**—Although it is clear that the mutant D2 LL is sorted to the IM, the stage in the sorting process at which the localization decision is made remains unknown. Final LL membrane localization may be predetermined while the precursor protein remains on the cytoplasmic side of the IM. Alternatively, the potential for routing to either the inner or outer membrane may remain viable until the LL protein is...
translocated to the periplasm and processed. The observation that an adjacent histidine residue is able to suppress the IM localization caused by the aspartate D2 mutation offered a method to differentiate between these two possibilities.

The cytoplasm of growing *E. coli* is buffered against changes in the pH of the external growth medium (21, 22), whereas the periplasm is not. If the D2 mutation predisposes LL to IM localization while the precursor is still on the cytoplasmic side of the membrane, the suppressive effect of an adjacent positively charged histidine residue (D2H3 mutant) should not be subject to titration by external pH. Only if the sorting committed step occurs in the periplasm should the membrane localization of the D2H3 mutant LL be titrated by alteration of the growth medium pH.

At pH 6.25 the wild-type S2 protein was localized normally to the OM (Fig. 5 and Table II). At this pH most of the D2 mutant LL was localized in the IM. However, a small but significant amount of this protein was found in the OM (Fig. 5 and Table II). Low pH was the only condition found to cause any significant OM sorting of the D2 mutant. The reason for this remains obscure. In agreement with experiments conducted at pH 7 (Fig. 3), the D2H3 mutant protein was primarily localized in the OM (Fig. 5 and Table II). Consistent with the importance of charge for suppression, there was a marginal increase in the quantity of D2H3 LL found in the OM at pH 6.25 (Table II) when compared with pH 7 (Table I).

Membrane localization of the wild-type S2 and D2 mutant LL to outer and inner membrane, respectively, was unaltered at pH 8.4 (Fig. 5 and Table II). However, the D2H3 mutant protein was found entirely in the IM (Fig. 5 and Table II). At a pH where the histidine residue was expected to be uncharged, no suppressive effect on IM localization of the D2H3 mutant protein was observed. It should be noted that at pH 8.4 a significant proportion of each LL protein (S2, D2, and D2H3), but not OmpA, was found associated with a membrane fraction of density intermediate to that of inner or outer membrane (M in Table II). The significance of this observation remains unknown.

The culture growth phenotype associated with LL overpro-

**Fig. 4.** Growth of *E. coli* cultures following induction of lipo-β-lactamase overproduction. The control shown is an uninduced culture harboring the plasmid encoding wild-type lipo-β-lactamase (S2). Growth of uninduced cultures harboring plasmids encoding mutant lipo-β-lactamase proteins was not different from this control and is not shown. The point of IPTG addition to induce lipo-β-lactamase production (approximately 0.1 OD unit) is indicated by an arrow. In the second panel, IPTG was added at an early (S2, D2, E2) and a later (E2, D2K3, D2H3) time point due to slow growth of the latter cultures. The mutant lipo-β-lactamase produced by a given culture following induction is indicated at the right of the relevant growth curve (S2, D2, D3, D4, E2, E3, K2, D2H3, D2K3, D2Q3, D2Y3).

![Fig. 5. Membrane localization of the D2H3 lipo-β-lactamase double mutant at high and low pH. Cultures were grown in MT medium, pH 6.25, or T medium, pH 8.4. Induction with IPTG was for 15 min. Cell envelopes were fractionated as described under “Materials and Methods.” Only 0.45 equivalents of envelope from T medium grown cells were loaded in lane E. Otherwise all sample loadings and abbreviations are as described in the legend to Fig. 2.](image-url)
TABLE II

Effect of pH on the membrane localization of the D2H3 lipo-β-lactamase suppressor mutant

<table>
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<tr>
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*a* The amount of lipo-β-lactamase and OmpA in gradient fractions was quantified by densitometry of Coomassie blue stained electrophoretic gels and is reported as a percentage of the total LL and OmpA recovered in these fractions.

*b* The percentage of envelope LL and OmpA recovered in gradient fractions.

Abbreviations used are: LL, lipo-β-lactamase; OM, outer membrane; IM, inner membrane; M, gradient fraction of intermediate density.

The amount of lipo-β-lactamase and OmpA in gradient fractions was quantified by densitometry of Coomassie blue stained electrophoretic gels and is reported as a percentage of the total LL and OmpA recovered in these fractions. The percentage of envelope LL and OmpA recovered in gradient fractions. Abbreviations used are: LL, lipo-β-lactamase; OM, outer membrane; IM, inner membrane; M, gradient fraction of intermediate density.

![Graph](image)

**Time (hr)**

![Graph](image)

**DISCUSSION**

The data presented here indicate that a negatively charged aspartate residue at the second position of LL causes sorting to the IM. An adjacent positively charged residue can partially suppress IM localization and do so in a pH-dependent manner when histidine provides the neutralizing charged residue. The aspartate residue must be positioned close to the protein N terminus and cannot be replaced by glutamate. These findings point to a remarkable positional and structural specificity in the process of LL sorting. This specificity clearly points to the involvement in lipoprotein membrane localization of other presently unidentified cellular proteins.

The membrane localization of the overexpressed prototypic OM (S2) and IM (D2) LL proteins has been corroborated by Sarkosyl detergent fractionation (16). We demonstrate here a strong correlation between membrane localization and the lethal outcome of LL overexpression (Fig. 4). As the mutational variations studied are subtle (S2 to D2 to E2) and no change in β-lactamase activity is caused by these alterations, a major change in LL conformation is unlikely to be respon-
sible for differences in toxicity. Instead, such a dramatic change in growth can more readily be explained as altered membrane localization, and as such, the data in Fig. 4 provides an in vivo corroboration of the in vitro localization determinations. At the time of membrane isolation, no toxic effects due to LL overexpression are evident. Furthermore, the membrane localization of S2, D2, and D2H3 LL mutants is unaltered when the level of protein production is reduced by 50% (data not shown). All available evidence suggests that the membrane localization determinations presented here accurately reflect the situation in vivo.

It is possible that the N terminus of the major OM lipoprotein contains an OM sorting signal which is disrupted by the introduction of an aspartate residue. Consistent with this hypothesis is the observation that only a limited variety of amino acids are found as the second residue of OM-localized bacterial lipoproteins (Table III). The second residue is serine, glycine, or alanine in nine of the 13 recognized OM lipoproteins. A glutamine or asparagine is the second residue in three of these proteins. Of the 12 lipoproteins for which membrane localization remains unknown, this second residue is occupied by serine, glycine, or alanine in 11 cases, with the remaining protein containing an asparagine residue (10). Most lipoproteins in E. coli are localized to the OM (24 and Table III). Should these proteins be found to occupy sites in the OM, fully 20 of 25 OM-localized lipoproteins would contain a structurally similar serine, glycine, or alanine residue adjacent to the N-terminal cysteine. This may suggest a specific conformation is required for OM localization.

Alternatively, an aspartate residue may actively function as IM sorting signal. In this scenario, lipoproteins lacking an appropriately positioned aspartate residue will by default be localized to the OM. Our findings emphasize the important role of aspartate charge as opposed to major conformation changes and would therefore tend to support the idea of active sorting to the IM. However, the data presented here do not permit a definitive distinction between these two possibilities. It is, however, noteworthy that at pH 6.25 some D2 LL does localize to the OM (Fig. 5, Table II). This could indicate that there is a pH-sensitive component of the cellular sorting machinery. At low pH the decreased activity of this putative protein may not be able to maintain the high rate of D2 LL sorting to the IM required under conditions of lipoprotein overproduction. Consequently, some D2 LL enters the OM sorting pathway. This interpretation would support the latter hypothesis.

It should be emphasized that we are studying the localization of an artificial hybrid protein. In the case of LL the sole region of the mature protein responsible for membrane localization is the lipid-modified nine residue N-terminal sequence derived from the major OM lipoprotein. The remainder of this hybrid, β-lactamase, has no affinity for membranes. In contrast, naturally occurring lipoproteins may contain other regions capable of membrane interaction. The use of this hybrid protein therefore permits a clear examination of the structural requirements for N-terminal sorting sequence function without the complications inherent in proteins containing other regions of membrane affinity. Obviously, however, one should not ignore other sequences of potential sorting activity which may be present in naturally occurring lipoproteins.

In spite of this limitation, the results obtained here with LL appear quite relevant for the sorting of natural lipoproteins. E. coli lipoprotein-28 (14) and Klebsiella pneumoniae pullulanase (15, 36) which contain aspartate residues adjacent to the N-terminal cysteine are localized to the IM when expressed in E. coli. However, pullulanase is found in the OM of K. pneumoniae (37), but additional proteins which are absent in E. coli are required for pullulanase exposure on the K. pneumoniae cell surface (15). For pullulanase, replacement of the aspartate residue with serine or asparagine causes pullulanase to become localized to the OM of E. coli.2 In remarkable agreement with our results is the observation that a lipoprotein endogluconase from Pseudomonas solanacearum is localized to the OM when expressed in E. coli (29). This lipoprotein contains an aspartate as the fifth mature residue, a position which our data argue is outside the IM sorting sequence. In addition, no naturally occurring OM lipoprotein has been found to contain an aspartate residue at position 2.

The situation is not as clear with the other two lipoproteins which have been identified in the IM of Gram-negative bacteria. The cytochrome subunit of the photosynthetic reaction center of Rhodopseudomonas viridis IM has been shown to be a lipoprotein (37). This lipoprotein contains as third residue of the mature protein a glutamate and, unique among bacterial lipoproteins, a phenylalanine at position 2. The cytochrome lacks the N-terminal amide-linked fatty acyl group present in other lipoproteins. In addition, this protein interacts strongly with the other three IM subunit proteins of the photosynthetic reaction center. Based upon results derived from LL, one would wrongly predict that the cytochrome, lacking an aspartate residue at position 2, should be localized to the OM. However, it is possible that the incomplete lipid modification or interaction with other IM proteins is responsible for the localization of this lipoprotein. Although our results clearly point to the inability of the glutamate to replace aspartate, it is interesting to note that a small quantity of the E3 mutant LL did localize to the IM (Fig. 2). This IM-localized E3 LL cannot be accounted for by cross-contamination of gradient fractions since the amount of E3 LL in the IM appears to be significantly greater than the amount of OmpA in this same fraction (Table I). This indicates that the E3 residue may exert a minimal IM sorting effect, which in the case of the cytochrome, may be strengthened by association with IM proteins.

2 A. Pugsley, personal communication.

### Table III

<table>
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<tr>
<th>Lipoprotein</th>
<th>Source</th>
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* The N-terminal 5 residues are shown (see Ref. 10 for references).
* Unless otherwise indicated by an asterisk, membrane localization was determined in E. coli.
Identified as a protein which can be labeled by radioactive fatty acid, new lipoprotein-3 (Nlp-3) has been shown to be present in the IM (24). The N-terminal sequence of a mature lipoprotein designated NlpB which is thought to be identical to Nlp-3 has been determined to contain an aspartate residue at position 2 (10). If NlpB is indeed located in the IM, this observation will be in sharp contrast to our finding that the D4 mutant LL is entirely in the OM. It could indicate that other regions of the mature lipoprotein may affect the N-terminal sorting sequence or play an entirely independent role in the localization process. Indeed, we have recently found that an aspartate replacement of the glutamine residue at position 2 of the pCloDF13-encoded lipoprotein bacteriocin release protein (lysine protein H), which is localized primarily to the E. coli OM (32, 33, 39), does not cause sorting of this lipoprotein to the IM. The bacteriocin release protein is embedded in the OM in a protease-resistant manner and may therefore be an integral protein exhibiting strong affinity for OM components which cannot be overcome by a single amino acid replacement.

The observation that the membrane localization of the mutant D2H3 LL can be titrated by altering the growth medium pH clearly indicates that membrane sorting must occur in a compartment accessible to the growth medium. This compartment is most likely the periplasm. The sorting decision would then probably be made as the mature lipoprotein resides on the periplasmic face of the IM following translocation and processing of the precursor protein. To support this contention we have observed that uninduced cells expressing either the OM localized S2 LL or IM retained D2 mutant protein exhibit the identical minimal inhibitory concentration for ampicillin (6 mg/ml) and produce equivalent amounts of LL protein (data not shown). This indicates that the β-lactamase portion of the hybrid assumes a native, biologically active conformation in the periplasm. It follows that the S2 and D2 LL proteins must reside on the periplasmic face of the outer and inner membrane, respectively.

The mechanism by which this lipoprotein would then move to the OM remains essentially a black box. However, several mechanisms are possible. Since the lipoprotein is tethered to the membrane via a lipid moiety, two models proposed for the membrane transfer of phospholipids (40) may be applicable for lipoprotein. Soluble periplasmic proteins analogous to the well-characterized class of eukaryotic phospholipid exchange proteins (41) may be employed. Recent findings have shown such a protein to be essential in yeast (42) for normal protein secretion. Alternatively, lipoproteins may be able to diffuse through the lipid bilayer to the OM across sites of inner-outter membrane adherence (43). A third model envisions vesicle-mediated lipoprotein transfer analogous to the pathway in operation for protein transport through the eukaryotic golgi (44). Finally, the spontaneous association of individual lipoproteins with specific IM or OM localized receptor sites is possible.

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