Depletion of Apolipoprotein N-Acyltransferase Causes Mislocalization of Outer Membrane Lipoproteins in Escherichia coli*

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Lipoproteins in Gram-negative Enterobacteriaceae carry three fatty acids on the N-terminal cysteine residue, two as a diacylglyceride and one through an N-linkage following signal peptide cleavage. Most lipoproteins are anchored in the outer membrane, facing the periplasm, but some lipoproteins remain in the plasma membrane, depending on the amino acid at position +2, immediately after the fatty-acylated cysteine. In vitro, the last step in lipoprotein maturation, N-acylation of apolipoproteins by the plasma membrane apolipoprotein N-acyltransferase (Lnt), is necessary for efficient recognition of outer membrane lipoproteins by the Lol system, which transports them from the plasma to the outer membrane (Fukuda, A., Matsuyama, S.-I., Harai, T., Nakayama, J., Nagasawa, H., and Tokuda, H. (2002) J. Biol. Chem. 277, 43512–43518). To study the role of Lnt in vivo, we constructed a conditional int mutant of Escherichia coli. The apo-form of peptidoglycan-anchored major lipoprotein (Lpp) and two other outer membrane lipoproteins accumulated in the plasma membrane when int expression was reduced. We also found that Lnt is an essential protein in E. coli and that the lethality is partially because of the retention of apoLpp in the plasma membrane. Topology mapping of Lnt with β-galactosidase and alkaline phosphatase fusions indicated the presence of six membrane-spanning segments. The lnt gene in a mutant of Salmonella enterica displaying thermosensitive Lnt activity (Gupta, S. D., Gan, K., Schmid, M. B., and Wu, H. C. (1993) J. Biol. Chem. 268, 16551–16556) was found to carry a mutation causing a single glutamate to lysine substitution at a highly conserved position in the last predicted periplasmic loop of the protein.

The Escherichia coli K12 genome encodes almost a hundred putative lipoproteins (1), a unique class of exported proteins, most of which are anchored in the inner leaflet of the outer membrane by their N-terminal fatty acids (2). The best characterized lipoprotein, Lpp, is present in two forms in the outer membrane. Approximately one-third of the Lpp molecules in the cell are cross-linked to the peptidoglycan via the C-terminal lysine residue (3), thereby contributing to outer membrane integrity (4). The remainder of the Lpp exists as a free (unbound) form. Lpp has been extensively used to study bacterial lipoprotein biogenesis and sorting to the outer membrane. Three fatty acids are bound to its N-terminal cysteine residue, two in a diacylglyceride that is linked via a thioether bond to the sulfhydryl group and one to the amine group that is liberated upon signal peptide cleavage (5, 6). The fully matured lipoprotein is then transferred from the plasma membrane to the periplasmic lipoprotein chaperone LolA (7) by the ABC transporter LolCDE (8), and then delivered to the outer membrane docking protein LolB (9), whereupon it inserts into the inner lipid leaflet of the outer membrane. It seems reasonable to assume that most if not all E. coli outer membrane lipoproteins follow exactly the same route.

A relatively small number of lipoproteins remain in the E. coli plasma membrane. In the two characterized plasma membrane lipoproteins, the endogenous protein NlpA (10) and the Klebsiella oxytoca amylolytic enzyme pullulanase (11), retention in the plasma membrane requires the aspartate residue at position +2, immediately after the fatty acylated cysteine. Furthermore, the introduction of an Asp residue into outer membrane lipoproteins or its presence in artificial lipoproteins (formed by fusing the signal peptide and first few amino acids of a lipoprotein to a reporter protein) causes their retention in the plasma membrane (12, 13). Other residues except proline and aromatic amino acids cause lipoproteins to be routed to the outer membrane (12, 13). The ability of Asp to cause efficient plasma membrane retention may be influenced both by amino acids in the adjacent sequence (14, 15) and by the structure of the polypeptide of which it is part (16).

Asp lipoproteins differ from outer membrane lipoproteins in being unable to activate the LolCDE ATPase in proteoliposomes, suggesting that Asp functions as an Lol avoidance signal (8, 17, 18). Other details of the mechanism by which lipoproteins are retained in the plasma membrane are unclear. The unique physicochemical properties of aspartate seem to be important for this function (19), which makes it all the more surprising that the structurally unrelated aromatic amino acids and proline at position +2 in an artificial lipoprotein can also function as efficient plasma membrane retention signals (13). Furthermore, it has not been established whether Asp functions as an Lol avoidance signal in other species of bacteria.

The plasma membrane enzyme that carries out the third and final step in lipoprotein processing and acylation (apolipoprotein N-acyltransferase or Lnt) was first identified in E. coli (20). A gene (lnt) encoding this enzyme was subsequently identified by the same group in Salmonella enterica sv. Typhimurium through studies of a temperature-sensitive mutant in which apoLpp (lacking the N-acyl group) accumulated at the...
nonpermissive temperature (21). The lnt gene E. coli (initially called cutE) to reflect its known function and to distinguish it from the S. enterica gene, IntEC (22) was independently identified in studies of a copper-sensitive mutant (22).

Sequence alignments reveal that homologues of Lnt are present in Gram-negative bacteria but apparently absent from most Gram-positive bacteria, including Bacillus subtilis and Staphylococcus aureus (23, 24). This observation led to the proposal that lipoproteins produced by Gram-positive bacteria might be incompletely fatty-acylated and, since all of them are proposal that lipoproteins produced by Gram-positive bacteria Staphylococcus aureus (23, 24). This observation led to the

E. coli LntEc araB

E. coli LntEc araB

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For radiolabeling, cells were grown exponentially in LB medium as above, washed, and resuspended in minimal medium supplemented with 0.4% glucose or 0.5% glycerol (when cells were grown in medium containing arabinose) for 15 min at 30 °C. Proteins from 1 ml of each culture were labeled with [35S]methionine for 5 min at 30 °C and then precipitated with 10% trichloroacetic acid. Precipitated proteins were dissolved and immunoprecipitated with anti-Lpp (provided by H. Tokuda), according to Ref. 36, resuspended in 50 μl of SDS sample buffer, and analyzed by urea SDS-PAGE.

**Immunoprecipitation of apoLpp**—For radiolabeling, cells were grown exponentially in LB medium as above, washed, and resuspended in minimal medium supplemented with 0.4% glucose or 0.5% glycerol (when cells were grown in medium containing arabinose) for 15 min at 30 °C. Proteins from 1 ml of each culture were labeled with 50 μCi of [35S]methionine for 5 min at 30 °C and then precipitated with 10% trichloroacetic acid. Precipitated proteins were dissolved and immunoprecipitated with anti-Lpp (provided by H. Tokuda), according to Ref. 36, resuspended in 50 μl of SDS sample buffer, and analyzed by urea SDS-PAGE.

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**Table II**

| Plasmid no. | Characteristics | Resistance | Source/Ref. |
|-------------|----------------|------------|-------------|
| pKD46       | araC repA101ts p_marB γβ-exo | Ap* | 28 |
| pJY111      | p_mar-lpp | Ap | 27 |
| pJY151      | p_mar-lppSR | Ap | 27 |
| pCHAP1447   | pSU18 p_mar-pulA-CAmalE | Cm | This study (13) |
| pCHAP1447   | pBS18 p_mar-pulA-CAmalE | Km | This study (13) |
| pCHAP1447   | pBS18 p_mar-pulA-CAmalE | Km | This study (13) |
| pCHAP1444   | pBS18 p_mar-pulA-CFmalE | Km | This study (13) |
| pCHAP1444   | pBS18 p_mar-pulA-CFmalE | Km | This study (13) |
| pCHAP1445   | pBS18 p_mar-pulA-CmalE | Km | This study (13) |
| pCHAP1446   | pBS18 p_mar-pulA-CmalE | Km | This study (13) |
| pCHAP6560   | pGPT04-potClE | Ap | This study |
| pCHAP6561   | pCHAP6560-kan | Ap | This study |
| pCHAP6563   | pCHAP6561-paraB | Ap | This study |
| pCHAP6571   | pUC18 p_mar-lnt | Ap | This study |
| pCHAP6573   | pUC18 p_mar-lnt(3'E435K) | Ap | This study |
| pCHAP6574   | pUC18 p_mar-lnt(3'E435K) | Ap | This study |
| pCHAP6576   | pUC18 p_mar-lnt(3'E435K) | Ap | This study |
| pCHAP6577   | pBAD33 p_mar-lacZ | Cm | This study |
| pCHAP6578   | pBAD33 p_mar-phaA | Cm | This study |
| pCHAP6580   | pBAD33 p_mar-lnt(3'30-phaA) | Cm | This study |
| pCHAP6581   | pBAD33 p_mar-lnt(3'53-phaA) | Cm | This study |
| pCHAP6582   | pBAD33 p_mar-lnt(3'80-phaA) | Cm | This study |
| pCHAP6583   | pBAD33 p_mar-lnt(3'117-phaA) | Cm | This study |
| pCHAP6584   | pBAD33 p_mar-lnt(3'154-phaA) | Cm | This study |
| pCHAP6585   | pBAD33 p_mar-lnt(3'190-phaA) | Cm | This study |
| pCHAP6586   | pBAD33 p_mar-lnt(3'218-phaA) | Cm | This study |
| pCHAP6587   | pBAD33 p_mar-lnt(3'476-phaA) | Cm | This study |
| pCHAP6588   | pBAD33 p_mar-lnt(3'512-phaA) | Cm | This study |
| pCHAP6589   | pBAD33 p_mar-lnt(3'30-lacZ) | Cm | This study |
| pCHAP6590   | pBAD33 p_mar-lnt(3'53-lacZ) | Cm | This study |
| pCHAP6591   | pBAD33 p_mar-lnt(3'80-lacZ) | Cm | This study |
| pCHAP6592   | pBAD33 p_mar-lnt(3'117-lacZ) | Cm | This study |
| pCHAP6593   | pBAD33 p_mar-lnt(3'154-lacZ) | Cm | This study |
| pCHAP6594   | pBAD33 p_mar-lnt(3'190-lacZ) | Cm | This study |
| pCHAP6595   | pBAD33 p_mar-lnt(3'218-lacZ) | Cm | This study |
| pCHAP6596   | pBAD33 p_mar-lnt(3'476-lacZ) | Cm | This study |
| pCHAP6597   | pBAD33 p_mar-lnt(3'512-lacZ) | Cm | This study |

* Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.

**Table III**

| Name | Sequence (5' to 3') |
|------|-------------------|
| 5'-kan | GAGTTCAGGAAAATCTGATGATAAAC |
| kan-3' | TAATGACCGGGCGTGTACGGCGGC |
| 5'-para | TCTAGACGTCATACAAAGGGGACC |
| para-3' | CCCTGTTTTTTGGGTGATTGCAG |
| 5'-ybe-k | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| p-lntE3'-3' | CCCTGTTTTTTGGGTGACTGCAACCCCGAACACTTGATGATAAATACGCTG |
| 5'-cutE | GAATTCCGAAAATCTGATGATAAAC |
| cutE3'-3' | CTCCGTCAGGCGACTAAACATCACCATACCATACCATATAAAGGATCC |
| 5'-int | GAATTCCGAAAATCTGATGATAAAC |
| int-3' | CTCCGTCAGGCGACTAAACATCACCATACCATATAAAGGATCC |
| 5'-cutE435 | GAATTCCGAAAATCTGATGATAAAC |
| cutE3'-3' | CTCCGTCAGGCGACTAAACATCACCATACCATATAAAGGATCC |
| 5'-phaA | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| phaA-3' | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| 5'-lacZ | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| lacZ-3' | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| 5'-intE-3' | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-30 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-53 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-80 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-117 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-154 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-190 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-218 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-476 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |
| int-3'-512 | GTCTAAATCCCCGATGATCCCAGCCGAACTGATGATAAATACGCTG |

The primers included in this work are listed in Table III.
Construction and Analysis of Int^{lac}-lacZ and Int^{phoA} Gene Fusions—The *phoA* gene encoding alkaline phosphatase, *PhoA*, lacking the region coding for the signal peptide (from amino acid +19) was PCR-amplified from plasmid pCHAP6092, using primers 5′-phoA and phoA-3′ (Table III), and cloned into *PstI* and HindIII sites in pBAD33 to obtain pCHAP6578. This plasmid was used to construct 3 plasmids, pCHAP6580 to pCHAP6588 (Table II), encoding *IntEc*({eq}50\)–*PhoA* to *IntEc*({eq}512\)–*PhoA* (numbers in parentheses correspond to the amino acid positions—below the genes indicate the orientation with primers 5′-IntEc3–5′ and 3′-IntEc117–3′, IntEc190–3′, IntEc218–3′, IntEc476–3′, and IntEc513–3′ (Table III), was inserted into the XbaI and PstI sites in pCHAP6578. The same procedure was employed to construct the 9 plasmids, pCHAP6589 to pCHAP6597, encoding *IntEc*({eq}50\)–*LacZ* to *IntEc*({eq}512\)–*LacZ*. First, the *lacZ* gene encoding β-galactosidase, *LacZ* (from amino acid +9), was PCR-amplified from plasmid pH552 (37) using primers 5′-lacZ and lacZ-3′ (Table III) and cloned into the PstI and HindIII sites in pBAD33 to obtain pCHAP6577. An *IntEc* DNA fragment obtained by PCR amplification with primers 5′-IntEc1′ and one of the nine 3′ primers IntEc30–3′ to IntEc512–3′ (Table III) was inserted into the XbaI and PstI sites in pCHAP6578.

Derivatives of strain RS272 producing the *IntEc*-*PhoA* and *IntEc*-*LacZ* chimeras or carrying pCHAP6577 or pCHAP6578 as controls were grown in LB medium at 30 °C supplemented with chloramphenicol and arabinose. To measure alkaline phosphatase activity, cells were diluted in Z-buffer (33) and permeabilized with octylpolyoxyethylene as above. The reaction was started by adding 200 μl of orthonitrophenyl β-D-galactoside (ONPG) (Fluka) in 50 mM HEPES (pH 7.4), and then placed at the bottom of a centrifuge tube. Steps (600 μl) were created using 56.2, 53.2, 50.2, 47.1, 44.2, 41.2, 38.1, and 35.9% sucrose solutions, and the tubes were centrifuged in a swing-out rotor for 36 h at 230,000 × g at 10 °C. Twenty fractions (250 μl) were collected from the top of the tubes and analyzed by SDS-PAGE and immunoblotting with appropriate antibodies. The concentration of sucrose in each fraction was determined from the refractive index.

Edman Degradation of Lipoproteins—To purify the histidine-tagged lipolipAe proteins for sequencing, bacteria from 100 ml of saturated LB broth culture containing 1 mM IPTG (to induce the expression of the *malE* gene, which is under *p_{lacZ}* control) were disrupted in a French pressure cell, and the cell envelope was collected by centrifugation at 160,000 × g for 60 min. Membrane proteins were dissolved in 2% SDS (N,N-dimethylyristeaminommonium/propane sulfonate (Fluka) in 50 mM Tris-HCl (pH 8.0) and then adsorbed onto Talon cobalt affinity resin (Clontech) from which the lipolipAe proteins were eluted with 200 mM imidazole (note that MalE cannot be purified on amylase resin in the presence of detergents). Purified proteins were precipitated with 10% trichloroacetic acid and then separated by SDS-PAGE on 10% acrylamide gels and electrophoratted onto Immobilon-P™ polyvinylidene difluoride membranes (Millipore) for automated Edman sequencing on an Applied Biosystems 477A gas phase Sequenator.

RESULTS

*IntEc* Is Essential in *E. coli*—To study the consequences of *IntEc* depletion in *E. coli*, a conditional mutant was constructed in which the chromosomal *IntEc* gene (originally called *cutE* (22), see Introduction) is expressed only from the arabinose-inducible AraC-dependent promoter *p_{arab}*. The *IntEc* gene is located in an operon downstream from the *ybeX* gene, whose function is unknown. The 25 nucleotides between *ybeX* and *IntEc* were replaced by a cassette containing a selectable kanamycin resistance gene (*kan*), the *rpoC* transcription terminator (*rpoCter*; to prevent transcription read-through from *p_{ybeX}*), and *paraB* (Fig. 1; see “Materials and Methods”) by homologous recombination. The resulting *p_{arab}-IntEc* strain (PAP8504) consistently ceased growth and began to lyse after 8 generations of growth in LB medium without arabinose and with glucose (to repress *p_{arab}*-expression), and did not form colonies on minimal glucose or LB agar (data not shown). The integration of the cassette between *ybeX* and *IntEc* was first verified by PCR amplification using primers flanking the entire cassette and then by transduction into the *E. coli* strain PAP105 using phage P1 with selection for the kanamycin resistance cassette (see “Materials and Methods”). Donor and transductants exhibited the same arabinose dependence and were complemented by a cloned copy of *IntEc* expressed from *p_{lacZ}* in a pUC18 derivative (pCHAP6571; see below), demonstrating that *IntEc*
Bacterial Lipoprotein Acylation and Localization

![Diagram](image)

**Fig. 2. Synthesis and accumulation of apoLpp upon LntEc depletion in E. coli.**

A, steady-state levels of apoLpp and Lpp. Crude extracts from PAP8504 (paraB-lntEc) grown in LB broth containing arabinose or glucose for 8 generations at 37 °C were analyzed by urea SDS-PAGE and by immunoblotting with anti-Lpp antibodies. B, synthesis of apoLpp. PAP8504 was grown in LB broth containing arabinose or glucose for the indicated number of generations. The cells were then washed and resuspended in minimal medium and labeled for 15 min with [35S]methionine. Lpp was immunoprecipitated and analyzed by urea SDS-PAGE and autoradiography.

is essential for the viability of E. coli, as shown previously (21) in S. enterica. Although the paraB-lntEc was integrated into the chromosome, the amount of LntEc produced is probably higher than the levels achieved with the wild-type strain, but this does not affect its use in the experiments described below.

Immunoblotting with antibodies against the major outer membrane lipoprotein Lpp revealed the gradual appearance of the apolipoprotein form (apoLpp) when paraB-lntEc was repressed by glucose in LB cultures (Fig. 2A), confirming that the LntEc activity declines under these conditions. ApoPal could not be detected by immunoblotting (data not shown; see “Discussion”). Immunoprecipitation of [35S]methionine-labeled proteins with anti-Lpp revealed almost complete absence of N-acetylation as the arabinose-deprived cultures approached the time when they lyse (Fig. 2B).

LntEc Depletion Induces Juxtaposition of Plasma and Outer Membranes—To analyze the localization of apolipoproteins, membranes from cells of strain PAP8504 grown in LB containing arabinose or glucose were separated by sucrose gradient centrifugation. Lpp and Pal in membranes from arabinose-grown cells were detected almost exclusively in dense fractions containing the outer membrane porins, well separated from less dense fractions containing SecG, an integral plasma membrane protein. In contrast, distinct outer and plasma membrane peaks were not detected when membranes from LntEc-depleted cells (6.5 generations after repression of paraB-lntEc) were examined (Fig. 3). In these gradients, Lpp and apoLpp were both detected exclusively in the middle of the gradient, as were SecG, Pal, the porins, and all other membrane proteins detected by Coomassie Blue staining (Fig. 3). These data indicate that the architecture of the cell envelope is drastically altered by LntEc depletion, probably because the outer and plasma membranes become juxtaposed (see below) shortly before the onset of lysis.

Cell envelope instability was also observed by phase contrast microscopy of LntEc-depleted E. coli, which were oval and swollen (data not shown). A similar phenotype was reported for the S. enterica lntEc mutant SE5312 at 42 °C (21). Furthermore, our sucrose flotation gradient analysis of membranes from this mutant grown at 42 °C revealed that plasma and outer membrane proteins were in the same fractions in the center of the gradient, whereas these two classes of proteins were clearly separated in membranes from the wild-type strain (LT2) or from the mutant grown at 30 °C (data not shown). Thus, LntEc depletion in E. coli has the same effects on envelope architecture as LntEc inactivation in S. enterica.

A similar association of plasma and outer membranes to that caused by LntEc depletion or inactivation was shown previously (27) to occur in E. coli after production of Lpp carrying the Asp^2 signal. It was proposed that this protein (LppDK) prevents the separation of the two membranes according to their density and causes lysis because it is both anchored in the plasma membrane and covalently linked to the peptidoglycan by its C-terminal lysine residue (27). Most interestingly, introduction of an lpp::Tn10 mutation into S. enterica strain SE5312 abolished temperature sensitivity caused by the lntEc mutation (21). Therefore, a major factor contributing to the lethality caused by LntEc deletion in the strain carrying the paraB-lntEc allele could be the proposed retention of peptidoglycan cross-linked apoLpp in the plasma membrane (25).

To test this idea, we analyzed 12 independent revertants of strain PAP8504 (paraB-lntEc) selected on LB agar without arabinose in a search for extragenic suppressor mutations in lpp. The presence of the kan-rpoCter-paraB cassette in the revertants was verified by PCR using primers flanking the cassette, which was then transduced into strain BW25113 by P1 phage. Three of the mutants were resistant to P1 phage, probably due to changes in surface lipopolysaccharide composition, whereas a further six had acquired a mutation in paraB that rendered them AraC-independent. Two of the remaining three mutants were found to be devoid of Lpp when examined by SDS-PAGE and immunoblotting, whereas the third produced a form of Lpp that migrated aberrantly upon SDS-PAGE (slower migrating monomeric form and abundant dimeric form; not shown). Sequence analysis revealed that the lpp gene in this mutant encodes a protein with glycine to aspartate substitution at position 14 in the signal peptide. Most interestingly, the same mutation was previously identified in an E. coli K12 mutant producing unmodified and unprocessed Lpp that was not cross-linked to the peptidoglycan and, therefore, was phenotypically Lpp^- (38). These results corroborate the idea that cross-linking of plasma membrane apoLpp to the peptidoglycan is partially responsible for the lysis resulting from LntEc depletion.

Finally, an lpp::Tn10 derivative of the paraB-lntEc strain PAP8504 was constructed by P1 transduction (PAP8505). This strain, like the revertants (see above) grew on LB agar but did not produce colonies upon drastic repression by glucose. In LB liquid cultures, PAP8505, like the Lpp^- parent strain, lysed 8 generations after replacement of arabinose by glucose. When pJY111 carrying the wild-type allele of lpp (27) was introduced into strain PAP8505, the transformants lost their ability to grow on agar in the absence of arabinose. In contrast, introduction of pJY151 (27), which carries an lpp allele encoding a variant of Lpp (LppSR) in which the C-terminal lysine that is normally cross-linked to the peptidoglycan is replaced by arginine, still allowed growth on agar in the absence of arabinose (data not shown). These data confirm that the toxicity caused by failure to express paraB-lntEc can be partially relieved by preventing Lpp synthesis, export, or cross-linking to the peptidoglycan.

**Apollp Is Localized in the Plasma and Outer Membranes—** Although PAP8505 cells lacking Lpp are fragile and leak periplasmic proteins (4), their membranes can be separated by sucrose gradient centrifugation (not shown). Synthesis of LppSR did not affect this separation (Fig. 4A), allowing it to be used as a marker for localizing apolipoproteins after LntEc depletion. LppSR was detected in the outer membrane, whereas the apoLppSR was detected mainly in the plasma membrane fractions (Fig. 4A), although some apoLppSR also appeared in fractions enriched in outer membrane proteins (Fig. 4B). As already noted, apoPal could not be detected under these conditions, and Pal was exclusively present in the outer membrane fraction of LntEc-depleted cells (Fig. 4A).

**Other Outer Membrane Lipoproteins Also Accumulate in the Plasma Membrane When LntEc Levels Are Decreased—** To de-
termine whether the localization of other outer membrane lipoproteins was affected by LntEc depletion, strain PAP8505 was transformed with pCHAP1447, encoding a fatty acylated variant of the E. coli periplasmic maltose-binding protein MalE (13) with C-terminal hexahistidine extension. This protein (lipoCA-MalE) has an alanine residue at position 11001 and is normally localized to the outer membrane (13). However, when strain PAP8505(pCHAP1447) was grown in glucose (Lnt-), a substantial amount of lipoCA-MalE accumulated in the plasma membrane. Sequence analysis of lipoCA-MalE purified by cobalt affinity chromatography from the plasma and outer membrane peaks of the glucose-grown cells revealed the presence of protein with a free N terminus, indicating the presence of apoLipoCA-MalE in both peaks. The same protein purified from cells with Lnt did not have a free N terminus. These data are consistent with the expected consequences of Lnt depletion and indicate that failure to N-acylate the N-terminal cysteine residue of a lipoprotein can be detected by N-terminal sequencing of the purified protein.

Substantial amounts of the endogenous outer membrane lipoprotein NlpD (39) also accumulated in the plasma membrane fraction of the glucose-grown cells, although Pal was again located exclusively in the outer membrane in both glucose and arabinose-grown cells (Fig. 5). Although it seems probable that the plasma membrane-associated NlpD was in the apo-form, it could not be separated from the mature (outer membrane-associated) form by SDS-PAGE.

Alternative Plasma Membrane Retention Signals Do Not Operate by Impeding LntEc—Aromatic amino acids and proline at position +2 in the artificial lipoprotein, lipoMalE, cause its retention in the plasma membrane (13). To test whether these amino acids, unlike Asp² (25), function by impeding LntEc, we determined whether the N terminus of five lipoMalE derivatives that accumulate in the plasma membrane (Asp², Phe², Pro², Trp², or Tyr²) (13) was blocked to Edman degradation. Like the lipoCA-MalE described above, the proteins were tagged with a C-terminal hexahistidine that allowed them to be affinity-purified from detergent-solubilized envelope preparations. All five proteins were found to have a blocked N terminus, indicating the presence of an N-acetyl group (see above) or, less likely, some other N-terminal modification. Thus, none of the known plasma membrane retention signals appears to operate by preventing N-acylation of the cysteine residue by LntEc, as already demonstrated for Asp² (25).

Characterization of the Mutation in Int⁵ Gene of the S. enterica ts Mutant—Because the mutation in S. enterica strain

![Fig. 3. Localization of membrane proteins in E. coli PAP8504.](image)
E435K substitution in LntSe. Most interestingly, both firming that the temperature sensitivity is caused by the growth of LntSe(E435K) and LntEc(E435K) (pCHAP6576) prevented strain LT2. Most interestingly, Glu435 is located in a highly conserved region of LntSe. (21) conservative substitutions and 28 nonconservative substitutions out of 512 residues) to be functionally interchangeable. (26)

S. enterica gene amplified from E. coli K12 and the activity of the reporter proteins was determined in permeabilized cells. Immunoblotting using anti-PhoA (alkaline phosphatase) and anti-LacZ (β-galactosidase) antibodies revealed that the chimeras were stable and that their estimated sizes were as predicted from knowledge of the fusion site (Fig. 6). Hybrid proteins with junction sites after Pro53, Pro117, Pro154, 190, or 512 (Fig. 6), indicating that these sites are exposed to the cytoplasm (PhoA is only active when exported to the periplasm, whereas LacZ is active only when retained in the cytoplasm (40)). When the reporters were placed after Ala80, Pro218, and Pro476, LacZ activities were at least 10 times lower, and PhoA activities at least 22 times higher than when the junction sites were at positions 53, 117, 154, 190, or 512 (Fig. 6), indicating that these sites are exposed on the periplasmic side of the plasma membrane. We note, however, that the specific β-galactosidase activity of LacZ fusions at positions 53, 117, 154, and 190 was considerably lower than at position 512, although it was reproducibly higher at positions 80, 219, and 476 (Fig. 6). Most surprisingly, fusions constructed after Pro30 of LntEc gave relatively high PhoA and LacZ activities. The N-terminal hydrophobic segment of LntEc presumably acts as a signal sequence to drive PhoA export, consistent with the presence of three arginine residues on its N-terminal (cytoplasmic) side (41), but cannot promote LacZ export, as reported previously for similar signal peptide-LacZ constructs (42). Similarly high enzyme activities were reported for other LacZ chimeras in which the fusion site was located after the first transmembrane segment of different LntEc polypeptide membrane proteins (43–45).

We conclude that LntEc probably possesses six rather than the predicted eight transmembrane segments interconnected by hydrophilic loops, with the N and C termini located in the cytoplasm (Fig. 7) and the predicted transmembrane segments between positions 112 and 195 being in the cytoplasm. This predicted topology is entirely consistent with von Heijne’s pos-

FIG. 4. ApoLpp is localized in both membranes in LntEc-depleted cells. Membranes from strain PAP8505 (paraB-lntEc, lpp::Tn10) grown in LB medium containing arabinose (Lnt +) or glucose (Lnt −) for 6.5 generations were separated by flotation sucrose density gradient centrifugation. A, 20 fractions from each gradient were analyzed by urea SDS-PAGE and immunoblotting with anti-FhuA, anti-SecG, anti-Pal, and anti-Lpp. B, fractions 6 and 7, and 15 and 16 corresponding to the plasma and outer membrane fractions from the two gradients were analyzed by urea SDS-PAGE and immunoblotting with anti-Lpp. PM, plasma membrane; OM, outer membrane.

SE5312 that causes the temperature-sensitive phenotype (21) was not characterized, the lntEc gene from this mutant was PCR-amplified and sequenced. A single mutation causing a glutamate to lysine substitution at position 435 was found in comparison with the lntSe gene amplified from S. enterica strain LT2. Most interestingly, Glu435 is located in a highly conserved region of LntSe.

The same mutation was introduced in the E. coli K12 lntEc gene by directed mutagenesis, thereby producing the LntEc(E435K) allele. Trans-complementation tests were then performed in S. enterica strain SE5312 and in E. coli PAP8504 with lnt or lnt(E435K) from E. coli or S. enterica cloned in high copy plasmids. Wild-type alleles of lntEc (pCHAP6571) and lntSe (pCHAP6573) complemented both the lntEc mutation in strain SE5312 at 37 and 42 °C and the paraB-lntEc mutation in strain PAP8504 grown in glucose (Table IV), indicating that the Lnt enzymes from these two species are sufficiently similar (26 conservative substitutions and 28 nonconservative substitutions out of 512 residues) to be functionally interchangeable. The paraB-lntEc mutant PAP8504 failed to grow at either 37 or 42 °C when LntSe(E435K) (pCHAP6574) was produced, confirming that the temperature sensitivity is caused by the E435K substitution in LntSe. Most interestingly, both LntSe(E435K) and LntEc(E435K) (pCHAP6576) prevented growth of S. enterica wild-type strain LT2 at 42 °C, suggesting that overproduction of either protein in LT2 is either toxic or is dominant negative over the activity of chromosome-encoded functional LntSe at this temperature (Table IV). However, LntEc(E435K) and LntSe(E435K) were not toxic at 42 °C in E. coli strain PAP8504 on LB agar containing arabinose, possibly because more LntEc is produced when lntEc is expressed from paraB in E. coli than when the lntSe is expressed from its own promoter in S. enterica. Moreover, in contrast to LntSe(E435K), LntEc(E435K) restored arabinose-independent growth of E. coli PAP8504 even at 42 °C. These data show that, even though LntEc and LntSe proteins are functionally interchangeable, the E435K substitution resulted in a temperature-sensitive enzyme only in LntSe, implying that other amino acid differences between these two proteins determine whether or not this substitution has an effect on enzyme activity or stability.

Plasma Membrane Topology of LntEc—Bioinformatic analyses of lnt genes from Gram-negative bacteria predicted the presence of 8 segments of sufficient hydrophobicity to adopt a transmembrane topology ((Gln10–Ala27)I, (Trp34–Asn50)II, (Ala57–Val70)III, (Pro88–Leu112)IV, (Trp127–Leu138)V, (Leu163–Leu187)VI, (Leu195–Ile211)VII, and (Trp488–Leu507)VIII; amino acid positions according to LntEc). This predicted topology of LntEc was tested by constructing a series of lntEc−lacZ gene fusions. The lacZ and phoA genes (encoding β-galactosidase LacZ and alkaline phosphatase PhoA, respectively) lacking their 5′-translation start signals (and, in the case of phoA, lacking a part of the region coding for the signal peptide) were PCR-amplified and fused to nine selected sites in regions of lntEc encoding loops between the predicted transmembrane segments, in plasmids under the paraB promoter. Each fusion was expressed in E. coli under arabinose induction, and the activity of the reporter proteins was determined in permeabilized cells. Immunoblotting using anti-PhoA (alkaline phosphatase) and anti-LacZ (β-galactosidase) antibodies revealed that the chimeras were stable and that their estimated sizes were as predicted from knowledge of the fusion site (Fig. 6). Hybrid proteins with junction sites after Pro53, Pro117, Pro154, Arg190, and Lys512 of LntEc showed relatively high or moderate LacZ and low PhoA activities, indicating that these sites are exposed to the cytoplasm (PhoA is only active when exported to the periplasm, whereas LacZ is active only when retained in the cytoplasm (40)). When the reporters were placed after Ala80, Pro218, and Pro476, LacZ activities were at least 10 times lower, and PhoA activities at least 22 times higher than when the junction sites were at positions 53, 117, 154, 190, or 512 (Fig. 6), indicating that these sites are exposed on the periplasmic side of the plasma membrane. We note, however, that the specific β-galactosidase activity of LacZ fusions at positions 53, 117, 154, and 190 was considerably lower than at position 512, although it was reproducibly higher than at positions 80, 219, and 476 (Fig. 6). Most surprisingly, fusions constructed after Pro30 of LntEc gave relatively high PhoA and LacZ activities. The N-terminal hydrophobic segment of LntEc presumably acts as a signal sequence to drive PhoA export, consistent with the presence of three arginine residues on its N-terminal (cytoplasmic) side (41), but cannot promote LacZ export, as reported previously for similar signal peptide-LacZ constructs (42). Similarly high enzyme activities were reported for other LacZ chimeras in which the fusion site was located after the first transmembrane segment of different E. coli polypeptide membrane proteins (43–45).
itive-inside rule (41). According to this prediction, residue Glu435 is located in the large periplasmic loop between transmembrane segments 5 and 6 (Fig. 7).

**DISCUSSION**

The data reported here provide in vivo evidence that LntEc-mediated N-acylation of major outer membrane lipoprotein Lpp and probably also of outer membrane lipoprotein NlpD and the artificial lipoprotein lipoCA-MalE is required for their efficient release from the plasma membrane. These data corroborate previous in vitro studies showing that LolA cannot promote apoPal release from proteoliposomes by LolCDE (25). In view of the substantial structural differences between alternative lipoprotein plasma membrane retention signals (Phe^H110012, Pro^H110012, Trp^H110012, and Tyr^H110012) (13) and the canonical plasma membrane retention signal or Lol avoidance signal (Asp^H110012), it was speculated that one or all of them might operate by preventing N-acylation of normally outer membrane lipoproteins. We demonstrated that this is not the case by showing that lipoMalE variants possessing these signals have blocked N termini by Edman degradation, suggesting the presence of an N-acyl group. The way these amino acids prevent Lol-mediated lipoprotein transport to the outer membrane remains to be clarified.

Even though apoLppSR was clearly visualized after LntEc depletion, the apo-form of another abundant outer membrane lipoprotein, Pal, could not be detected (data not shown), possibly because, like the apo-forms of lipoCA-MalE and NlpD, it cannot be separated from the mature form by SDS-PAGE. Furthermore, Pal was reproducibly found exclusively in the outer membrane in LntEc-depleted cells, whereas apoLpp and two other outer membrane lipoproteins, NlpD and lipoCA-MalE accumulated in the plasma membrane (Figs. 4 and 5).

There are four possible explanations for these observations.

(i) ApoPal has a higher affinity than apoLpp for LntEc. Therefore, trace amounts of LntEc remaining after long periods of p_nuc(IntEc) repression are sufficient for N-acylation of apoPal.
(ii) ApoPal is transported to the outer membrane by the Lol system, although this would be in contradiction to the observation that LolCDE cannot release apoPal from proteoliposomes in the presence of LolA (25).

(iii) ApoPal is retained in the plasma membrane but is degraded.

(iv) ApoPal associates with the peptidoglycan or with outer membrane components such as OmpA (46), and its association with the plasma membrane is consequently disrupted when the cells are broken in the French press.

Cross-linking of the plasma membrane apo-form of Lpp to the peptidoglycan could explain the cofractionation of the plasma and outer membrane pools from envelopes of LntEc-depleted cells in sucrose gradients, because the peptidoglycan would be linked to both the outer membrane (via mature Lpp) and to the plasma membrane (via apoLpp). An identical phenomenon occurs when proLpp (uncleaved and unacylated) accumulates in the plasma membrane because of the absence of phosphatidylglycerol caused by a mutation in the pgdA gene (47) or when Lpp (LppDK) is directed to the plasma membrane by a Asp12 (27). In all cases, rupture of the bacteria in a French press would result in the formation of outer and plasma membrane vesicles that are both linked to the peptidoglycan, causing them to float to an intermediate density in the sucrose gradients.

The disorganization of the cell envelope that results from the mislocalization of peptidoglycan-bound apoLpp (Fig. 3) is presumably a major cause but not the only cause of lysis that ensues LntEc depletion. Membrane disorganization might also explain why some apoLppSR, which is not linked to the peptidoglycan, was observed in fractions containing outer membrane proteins when LntEc was depleted (Fig. 4B). Bulk mixing of plasma and outer membrane can be ruled out as a possible explanation because the integral plasma membrane protein SecG did not appear in increased amounts in the dense (outer) membrane fractions under these conditions (Fig. 4). One possible explanation for this observation could be the formation of heterotrimers of Lpp containing a mixture of mature and apo-forms (48), although we cannot rule out that apoLpp is transported, albeit inefficiently, to the outer membrane by the Lol system. ApoLipoCA-MalE was also found in outer membrane fractions when Lnt was depleted, but this observation is more difficult to interpret because the assay used (detection of a free N terminus that allows Edman degradation) is not quantitative. Therefore, only trace amounts of apoLipoCA-MalE, comparable with those of SecG, might be present on the outer membrane fraction because of mixing of membrane components.

The inhibition of growth caused by severe glucose repression of pavoR-lntEc in E. coli was not overcome by elimination of Lpp, whereas lpp::Tn10 restored growth of the S. enterica lnt Ec mutant at 42 °C (21), probably because the lnt Ec mutation does not completely inactivate Lnt Ec at 42 °C. Furthermore, the fact that glucose repression of pavoR-lntEc in E. coli is lethal even in the absence of Lpp suggests that lnt expression is completely blocked under these conditions, leading to the inactivation of essential outer membrane lipoproteins (LolB, for example (49)) by delocalization (in the apo-form) to the plasma membrane (49, 50).

Mutations in the lnt (cutE/actA) genes have been reported to confer copper sensitivity (22, 51). This is an interesting observation, especially in view of the presence of a putative copper-binding site, HXXMXXM (amino acids 425–431; HFQARM in E. coli Lnt (22)), in the large, well conserved periplasmic loop of LntEc between transmembrane segments 5 and 6. Copper binding by Lnt has not been investigated, however, and only the first amino acid of this putative motif (His) is highly conserved in Lnt from different bacterial species (as are the glutamine and arginine that precede and follow it, respectively), whereas the two methionines are often replaced by nonconservative amino acids in predicted Lnt homologues from bacteria distantly related to E. coli (data not shown). Thus, the effects of lnt mutations on copper sensitivity are probably due to incorrect localization or inactivation of one or more outer membrane lipoproteins (as

![Fig. 6](image-url)  
**Fig. 6. Detection by immunoblotting and enzymatic activities of LntEc-PhoA and LntEc-LacZ hybrid proteins in E. coli.** Crude extracts from KS272 producing LntEc-PhoA and LntEc-LacZ hybrid proteins or carrying plasmids without lnt Ec-derived inserts (control) were analyzed by SDS-PAGE and by immunoblotting with anti-alkaline phosphatase antibodies. Cells were grown in LB medium containing arabinose to induce expression of the gene fusions. Alkaline phosphatase and anti-alkaline phosphatase antibodies. Cells were grown in LB medium containing arabinose to induce expression of the gene fusions. Alkaline phosphatase and anti-alkaline phosphatase antibodies.

![Fig. 7](image-url)  
**Fig. 7. Proposed topology of Lnt Ec in the plasma membrane.** Arrows and numbers indicate the amino acid after which the LacZ and PhoA reporter proteins are fused to Lnt Ec. The position of the E435K substitution in the lnt Ec allele of S. enterica is also indicated (diamond).
apolipoproteins) involved in copper homeostasis (e.g., NlpE (52)), rather than to direct copper-Lnt interactions.

The coding sequence of the mutant int (cutE) gene amplified from the copper-sensitive E. coli mutant reported by Rogers et al. (22) was found to be devoid of mutations (data not shown). This suggests that the mutation in this strain is not in int itself but might be in the upstream ybeX promoter and, consequently, might diminish int expression. On the other hand, the transposon insertion in the R. meliloti int (actA) gene that causes copper and acid sensitivity would be expected to inactivate the gene, implying that Lnt function is not essential in Rhizobium (51). Most interestingly, the amino acid substitution that inactivates the S. enterica Lnt at 42 °C (E435K) (52) affects one of several totally conserved amino acids in Lnt homologues and might define part of the catalytic site of the enzyme. The mechanisms by which Lnt hydrolyzes phospholipids and transfers the resulting fatty acids onto the N-terminal cysteine residue of lipoproteins should be investigated.

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