The major outer membrane lipoprotein (Lpp) of *Escherichia coli* possesses serine at position 2, which is thought to function as the outer membrane sorting signal, and lysine at the C terminus, through which Lpp covalently associates with peptidoglycan. Arginine (R) is present before the C-terminal lysine in the wild-type Lpp (LppSK). By replacing serine (S) at position 2 with aspartate (D), the putative inner membrane sorting signal, and by deleting lysine (K) at the C terminus, Lpp mutants with a different residue at either position 2 (LppDK) or the C terminus (LppSR) or both (LppDR) were constructed. Expression of LppSR and LppDR little affected the growth of *E. coli*. In contrast, the number of viable cells immediately decreased when LppDK was expressed. Prolonged expression of LppDK inhibited separation of the inner and outer membranes by sucrose density gradient centrifugation, whereas short-term expression did not. Pulse-labeled LppDK and LppDR were localized in the inner membrane, indicating that the amino acid residue at position 2 functions as a sorting signal for the membrane localization of Lpp. LppDK accumulated in the inner membrane covalently associated with the peptidoglycan and thus prevented the separation of the two membranes. Globomycin, an inhibitor of lipoprotein-specific signal peptidase II, was lethal for *E. coli* only when Lpp possessed the C-terminal lysine. Taken together, these results indicate that the inner membrane accumulation of Lpp per se is not lethal for *E. coli*. Instead, a covalent linkage between the inner membrane Lpp having the C-terminal lysine and the peptidoglycan is lethal for *E. coli*, presumably due to the disruption of the cell surface integrity.

So far, more than 10 species of lipoproteins have been reported to be present in either the outer or inner (cytoplasmic) membrane of *Escherichia coli* (1, 2, 7, 9, 13, 14, 20, 24, 34). Irrespective of their locations, all lipoproteins are synthesized as precursors with signal peptides at their N termini and then translocated across the inner membrane in a Sec machinery-dependent manner (8, 35, 39). Mature lipoproteins possess a fatty acid-modified cysteine at the N terminus (4, 9). Both the modification and the signal peptide cleavage take place in the inner membrane, leading to the generation of mature lipoproteins (11, 12, 15, 33, 38, 42). The sorting of lipoproteins for final localization then follows (32). A single amino acid present at the N-terminal second position has been proposed to be a determinant of the membrane localization of lipoproteins (41). The major outer membrane lipoprotein (Lpp) and the inner membrane lipoprotein NlpA possess serine and aspartate, respectively, at this position (9). The replacement of serine at this position by aspartate causes the localization of an Lpp-Bla fusion protein in the inner membrane (41). Furthermore, upon the substitution of aspartate for serine at the same position, an NlpA-Bla fusion protein is localized in the outer membrane. A periplasmic protein, LplA (p20), can recognize the lipoprotein sorting signal and specifically releases the outer membrane-directed lipoprotein from the inner membrane by forming an LplA-lipoprotein complex (26). When the complex interacts with a receptor on the outer membrane, the incorporation of a lipoprotein into the outer membrane takes place (27).

Lpp localized in the outer membrane exists as a trimer and interacts with the peptidoglycan both covalently and noncovalently (5, 16). The interaction with the peptidoglycan contributes to the maintenance of the integrity of the cell surface structure (10, 36). Covalent bonding occurs between the C-terminal lysine of Lpp and diaminopimelic acid of the peptidoglycan (3, 6, 43, 44). Since the lipoprotein sorting signal was determined with the Lpp-Bla fusion protein possessing only the N-terminal region of Lpp, it is not clear whether the interaction between Lpp and the peptidoglycan plays any role in the Lpp localization.

We report here that the amino acid residue at the N-terminal second position indeed functions as the sorting signal for intact Lpp and that the C-terminal lysine plays little role in Lpp localization but is lethal for *E. coli* if Lpp is mislocalized in the inner membrane.

**MATERIALS AND METHODS**

**Materials.** Globomycin (17), a gift from Sankyo Co., Ltd., was dissolved in methanol (50 mg/ml) prior to use. Tran 35S-label (a mixture of 70% [35S]methionine and 20% [35S]cysteine [total, 1,000 Ci/mmol]) was obtained from ICN. Antiserum against SecG, Lpp (11), and OmpA (37) were raised in rabbits against the respective purified proteins.

**Bacterial strains and media.** *E. coli* JM38 (F− ara D[(lac-proAB) rpsL Δ80 lacZΔM15]) was used for plasmid construction. The cells were grown on L broth or M63 (NaCl)-maltose, which contained 0.4% (wt/vol) maltose, as a sole source of carbon, 0.5% (wt/vol) NaCl, thiamine (20 μg/ml), thymine (20 μg/ml), and 40-μg/ml concentrations of each amino acid except methionine and cysteine. Lpp derivatives were expressed in JESS505 (F′ lpp pps pps proA argE thi gal lac xyl mtl r) (10) obtained from the National Institute of Genetics collection. Where specified, chloramphenicol (25 μg/ml) and ampicillin (50 μg/ml) were added to the medium.
Construction of plasmids. The construction of pMAN885 was carried out as follows. pSTV29 (Takara Shuzo Co., Ltd.) was digested with Tth111, blunt ended, and further digested with Sall to generate a 2.3-kb Tth111 (blunt-ended)-Sall fragment carrying the chloramphenicol resistance gene and its replication origin. pQNS05 (22) was digested with SacI, blunt ended, and further digested with Sall to generate a 1.7-kb Sall-SacI (blunt-ended) fragment carrying the PBAD promoter and the araC gene. These two fragments were then ligated together to construct pMAN885. To construct pJY111 carrying the wild-type lpp gene (lppX) under the control of P araBAD (28) was digested with MluI, blunt ended, and further digested with XbaI to generate a 0.33-kb XbaI-MluI (blunt-ended) fragment. This fragment carrying lppSK was then ligated with a large XbaI-Smal fragment of pMAN885. To construct pJY116 carrying lppSK under the control of P araBAD, a 0.23-kb SacI-MluI (blunt-ended) fragment of pKEN125 encoding the C-terminal region of Lpp and a 0.1-kb XbaI-Smal fragment of pKY702 (41) encoding the signal peptide and N-terminal region of an Lpp derivative, which possesses aspartate instead of serine at the N-terminal second position, were ligated with a large XbaI-Smal fragment of pMAN885.

To construct pJY111 carrying lppSK under the control of tacPO, a 0.33-kb XbaI-MluI (blunt-ended) fragment of pKEN125 was ligated with a large XbaI-Smal (blunt-ended) fragment of pTTQ18 (Amersham Life Science) carrying tacPO, lacI, and the ampicillin resistance gene. Construction of pJY116 carrying lppSK under the control of tacPO was performed by ligating a 0.23-kb SacI-MluI (blunt-ended) fragment of pKEN125 and a 0.1-kb XbaI-Smal fragment of pKY702 together with a large XbaI-Smal (blunt-ended) fragment of pTTQ18. To construct pJY151 carrying lppSR or pJY156 carrying lppSK under the control of tacPO, a 0.1-kb XbaI-Smal fragment of pKEN125 (for pJY151) or pKY702 (for pJY156) was ligated with a 0.23-kb SacI-MluI fragment (blunt-ended) fragment of pDOC006 (6), which encodes an Lpp derivative lacking the C-terminal lysine, and a large XbaI-Smal (blunt-ended) fragment of pTTQ18. Plasmids carrying lppSR or lppSK under the control of P araBAD were constructed as follows. The multiple-cloning site of pUC18 was introduced into the EcoRI and SalI sites of pMAN885 to construct pMAN885EH by using a pair of oligonucleotides synthesized with a Beckman DNA synthesizer: 5'-TCG(A)ATT(CGA)GCT(T)GGG(ATT)CTC(TAG)AGT(CGA)GCT(TGC)AA(GCT)TAA-3' and 5'-ATT(AA)GAT(TAC)GCT(TGC)ATG(CCT)GC(A)GCT(T)GGG(ATT)CTC(TAG)AGT(CGA)GCT(TGC)AA(GCT)TAA-3'. The 3.4-kb KpnI-PstI fragment of pJY111 or pJY156 was ligated with a large KpnI-PstI fragment of pMAN885EH to construct pJY851 (lppSR under the control of P araBAD), pJY856 (lppSK under the control of P araBAD), respectively. The deletion of the C-terminal lysine (i.e., mutation from 5'-CGG ATG AA-3' to 5'-CGG ATG TA-3') and the substitution of serine by aspartate at position 2 (i.e., mutation of 5'-TG GCC ACA GCC-3' to 5'-TG GCC AGC GCC-3') were confirmed by sequencing of the corresponding region.

Membrane localization of Lpp derivatives. JE5505 harboring pJY111, pJY151, or pJY156 was grown on L broth at 37°C to the mid-exponential phase of growth, and then the lpp gene was expressed for 1 h after the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were harvested from 30 ml of culture by centrifugation (10,000 × g, 10 min), washed once with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, and then resuspended in the same buffer. Conversion of the cells into spheroplasts was performed as described previously (38). The spheroplast suspension (3 ml) was sonicated, and the total membrane fraction was obtained by centrifugation (100,000 × g, 30 min) after the removal of unbroken cells. The total membranes were resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and then applied to a 30 to 55% (wt/wt) linear sucrose gradient containing 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. After centrifugation at 80,000 × g (wt/wt) linear sucrose gradient containing 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA and then applied to a 30 to 55% sucrose density gradient, the bands on the immunoblot were quantitated with an Atto Densitometer 7500 (Atto Co., Ltd.). Labeling with Tran 35S-label was carried out for 1 min and then the membranes were fixed in 2.5% (wt/vol) glutaraldehyde for 5 min. Membrane localization of pulse-labeled Lpp derivatives. JE5505 harboring pJY111, pJY116, pJY151, or pJY156 was grown on L broth at 37°C to the mid-exponential phase of growth, and then the lpp gene was expressed for 1 h after the addition of IPTG (isopropyl-β-D-thiogalactopyranoside). The cells were harvested from 30 ml of culture by centrifugation (10,000 × g, 10 min), washed once with 50 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, and then resuspended in the same buffer. Conversion of the cells into spheroplasts was performed as described previously (38). The spheroplast suspension (3 ml) was sonicated, and the total membrane fraction was obtained by centrifugation (100,000 × g, 30 min) after the removal of unbroken cells. The total membranes were resuspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA and then applied to a 30 to 55% (wt/vol) linear sucrose gradient containing 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA, respectively. The deletion of the C-terminal lysine (i.e., mutation from 5'-CGG ATG AA-3' to 5'-CGG ATG TA-3') and the substitution of serine by aspartate at position 2 (i.e., mutation of 5'-TG GCC ACA GCC-3' to 5'-TG GCC AGC GCC-3') were confirmed by sequencing of the corresponding region.

Membrane localization of pulse-labeled Lpp derivatives. JE5505 harboring pJY111, pJY116, pJY151, or pJY156 was grown on M63 (NaCl)-maltose medium at 37°C to the mid-exponential phase of growth. After the temperature was shifted to 30°C, the lpp gene was expressed after the addition of 0.2% (wt/vol) arabinose for 5 min. Labeling with Tran 35S-label was carried out for 1 h and then terminated by the addition of nonradioactive methionine and cysteine. The cells harvested from 1 ml of culture fluid were converted into spheroplasts and then disrupted by sonication. The total membrane fraction was then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The Lpp derivatives in each fraction were immunoprecipitated (25) and analyzed by SDS-PAGE and fluorography.

Covalent linkage between Lpp derivatives and peptidoglycan. The method reported by Zhang and coworkers (43, 44) was slightly modified. Cells containing Lpp derivatives were disrupted with a French pressure cell at 15,000 lbf/in². The total membranes were resuspended in 1 ml of 10 mM Tris-HCl (pH 7.5), solubilized with 1% (wt/vol) SDS at a protein concentration of 1 mg/ml, and then boiled for 5 min. The supernatant and pellet were obtained by centrifugation at 100,000 × g for 1 h. The pellet was resuspended in 0.9 ml of 50 mM Tris-HCl (pH 7.5). Aliquots (80 µl) of this suspension were mixed with 90 µl of lysosyme (2 mg/ml) and then incubated at 37°C overnight. The SDS supernatant and SDS insoluble pellet with or without lysozyme treatment were analyzed by SDS-PAGE and immunoblotting with an anti-Lpp antibody.

RESULTS AND DISCUSSION

Construction of Lpp derivatives and the effect of their expression on the growth of E. coli. Lpp derivatives having the putative inner membrane sorting signal with either lysine (LppDK) or arginine (LppDR) at the C terminus and the putative outer membrane sorting signal with either lysine or arginine (LppSR) or arginine (LppDR) at the C terminus and the outer membrane sorting signal with the C-terminal arginine (LppSR) were constructed. The genes encoding these three derivatives and the wild-type Lpp (LppSK) were placed under the control of either tacPO on a multicopy plasmid or P araBAD, the promoter for the araBAD operon, on a low-copy-number plasmid. Lpp-deficient E. coli JE5505 harboring one of these plasmids was grown on L broth or M63 (NaCl)-maltose medium with or without an inducer. JE5505 exhibited poor growth on M63-maltose medium when 0.5% NaCl was not added as a supplement (data not shown). Whether the medium was L broth (Fig. 1A) or M63 (NaCl)-maltose medium (B) containing chloramphenicol (25 µg/ml) with (●) or without (○) 0.2% arabinose. Growth was monitored as OD 660.

![FIG. 1. Effects of Lpp derivatives on the growth of E. coli.](http://jb.asm.org/) (Figure 1 of the original article)
maltose, expression of the tacPO-controlled Lpp derivatives could not be examined with this medium.

By means of quantitative immunoblotting with an anti-Lpp antibody (data not shown), the level of Lpp materials expressed from tacPO on a multicopy plasmid was found to be about 50% of that of chromosomally encoded wild-type Lpp, irrespective of the Lpp derivative species. The level of Lpp material expressed from PBAD on a low-copy-number plasmid was only about 20% of that of Lpp expressed from the chromosome. The reason why the level of the plasmid-encoded Lpp material was lower than that of the chromosomally encoded Lpp is not known at present. In any event, the results in Fig. 1 indicate that the expression of an Lpp derivative possessing the putative inner membrane sorting signal is toxic for E. coli and that this toxic effect is abolished upon deletion of the C-terminal lysine, through which the wild-type Lpp covalently associates with peptidoglycan.

Expression of LppDK immediately decreases the number of viable cells. The effect of the expression of LppDK on growth was examined in more detail by determining the number of viable cells. JE5505 harboring a plasmid (PBAD-controlled lp- pDK) was grown on L broth. When the optical density at 660 nm (OD_{660}) reached 0.5, LppDK was expressed by the addition of arabinose. The OD_{660} ceased to increase at 1.5 h after arabinose addition, whereas it continued to increase in the absence of arabinose (Fig. 2A). On the other hand, the number of viable cells immediately decreased after the addition of arabinose (Fig. 2B). When M63 (NaCl)-maltose medium was used instead of L broth, the number of viable cells also decreased. In contrast, the expression of other Lpp derivatives had no effect on the number of viable cells with either medium. Since the agar plates used for determination of the numbers of viable cells did not contain arabinose, these results indicate that the lethality is irreversible and specific to LppDK.

Membrane localization of the Lpp derivatives. The Lpp derivatives were induced for 4 h in JE5505 growing on L broth. Membranes were isolated from the cells and fractionated by means of sucrose density gradient centrifugation (Fig. 3). The amounts of the inner membrane protein, SecG (29), the outer membrane protein, OmpA (21), and Lpp derivatives were determined in each fraction by means of quantitative immunoblotting. The derivatives having the putative outer membrane sorting signal (LppSK and LppSR) were recovered in the outer membrane fraction, in which OmpA was also recovered (Fig. 3A and B). The distribution profiles of these two derivatives were essentially the same but distinct from that of SecG, indicating that the C-terminal lysine plays little role in the outer membrane localization of Lpp. On the other hand, OmpA, SecG, and LppDK were recovered in an intermediate fraction with essentially the same distribution profile when the membr-
Each fraction were immunoprecipitated and then detected by SDS-PAGE and fraction were analyzed by immunoblotting after SDS-PAGE. Lpp derivatives in which was followed by fractionation into 11 fractions. OmpA and SecG in each branes were prepared and analyzed by sucrose density gradient centrifugation, PBAD was grown at 37°C on M63 (NaCl)-maltose medium. After the temperature boring a plasmid carrying the gene for an Lpp derivative under the control of arabinose (0.2%), followed by labeling for 1 min with Tran35S-label. Membrane-containing LppDK were fractionated (Fig. 3C), indicating that the replacement of the outer membrane sorting signal with the inner membrane sorting signal somehow causes tight association of the two membranes. Deletion of the C-terminal lysine from LppDK significantly improved the membrane separation (Fig. 3D). The distribution profile of LppDR was similar to that of SecG, although a considerable portion of LppDR was still recovered in the intermediate fraction, in which about 50% of SecG and most of OmpA were recovered. Taken together, these results suggest that most likely an aspartate residue at the N-terminal second position causes the localization of LppDK and LppDR to the inner membrane, where the Lpp derivatives associate with the peptidoglycan either covalently (LppDK) or noncovalently (LppDR), thereby preventing separation of the outer and inner membranes. Interaction between the peptidoglycan and outer membrane proteins (31) such as OmpA, OmpC, and OmpF may also contribute to this outer membrane-peptidoglycan-inner membrane association.

Lysozyme treatment seems to be insufficient for breaking this association.

To confirm the inner membrane localization of LppDR and LppDK, the Lpp derivatives were expressed for a short time and pulse-labeled to minimize the association between the derivatives and the peptidoglycan. The distribution profiles of OmpA and SecG in fractions obtained by sucrose density gradient centrifugation did not differ with the Lpp derivative species (Fig. 4), indicating that short-term expression of Lpp derivatives has little effect on the separation of the two membranes. Both LppDR and LppDK were found to be localized in the inner membrane fraction (Fig. 4C and D), in which SecG was recovered. On the other hand, LppSK was found in higher-density fractions, which contained OmpA. LppSR expressed for 1 h was localized in the outer membrane (Fig. 3), whereas the distribution profile of the pulse-labeled LppSR coincided with that of neither OmpA nor SecG (Fig. 4B). This may suggest that the outer membrane localization of Lpp slows down upon deletion of the C-terminal lysine, although LppSR is eventually localized in the outer membrane. In any event, the results in Fig. 3 and 4 indicate that the membrane sorting signal indeed exists at the N-terminal second position of Lpp.

**Covalent linkage between inner membrane Lpp and peptidoglycan.** The tacPO-controlled Lpp derivatives were expressed for 1 h in JE5505 growing on L broth. After disruption of the cells with a French pressure cell, membranes were prepared and boiled in SDS, followed by centrifugation. The pellet was further treated with lysozyme or not treated. The Lpp derivatives in the SDS supernatant and pellet were then analyzed by SDS-PAGE and immunoblotting with the anti-Lpp antibody (Fig. 5). The Lpp derivatives recovered in the SDS supernatant represent the free form of Lpp, whereas the distribution profile of the pulse-labeled LppSK and LppDK in the lysosome-treated pellet fraction were heterogeneous and migrated slower than the free forms of the respective derivatives, indicating that not only LppSK but also LppDK in the inner membrane contains covalently associated peptidoglycan fragments of different sizes. When the pellet fraction was not treated with lysozyme, neither LppSK nor LppDK was detected since the derivatives are too large to enter the gel (data not shown). On the other hand, neither LppSR nor LppDR covalently associated with peptidoglycan. These derivatives were only recovered in the SDS supernatant since they lack the C-terminal lysine which is essential for the covalent linkage (6, 43, 44). These results, taken together, indicate that LppDK mislocalized in the inner membrane co-
Globomycin requires the C-terminal lysine of Lpp for its lethality. Globomycin, an inhibitor of signal peptidase II, causes the accumulation of the precursor form of Lpp in the inner membrane and is lethal for E. coli (18). It has been shown that the Lpp precursor in the inner membrane covalently binds to peptidoglycan (19). As described above, the mature Lpp mislocalized in the inner membrane was not lethal unless Lpp was covalently bound to the peptidoglycan. To determine whether or not the C-terminal lysine of Lpp is also required for the lethality of globomycin, JE5505 was grown on M63 (NaCl)-maltose medium containing arabinose for the ex- 

VOL. 179, 1997 LETHALITY OF MISLOCALIZED Lpp 2861 

From the results presented here, we conclude that E. coli cells cannot survive, presumably due to disruption of the integrity of the cell surface structure, when a covalent linkage is formed between the inner membrane Lpp and the peptidogly- 

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