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A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of *Escherichia coli*

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The *Escherichia coli* major outer membrane lipoprotein (Lpp) is released from the inner membrane into the periplasm as a complex with a carrier protein, LolA (p20), and is then specifically incorporated into the outer membrane. An outer membrane protein playing a critical role in Lpp incorporation was identified, and partial amino acid sequences of the protein, named LolB, were identical to those of HemM, which has been suggested to play a role in 5-aminolevulinic acid synthesis in the cytosol. In contrast to this suggested role, the deduced amino acid sequence of HemM implied that the gene encodes a novel outer membrane lipoprotein. Indeed, an antibody raised against highly purified LolB revealed its outer membrane localization, and inhibited *in vitro* Lpp incorporation into the outer membrane. Furthermore, LolB was found to be synthesized as a precursor with a signal sequence and then processed to a lipid-modified mature form. An *E.coli* strain possessing chromosomal *hemM* under the control of the *lac* promoter–operator required IPTG for growth, indicating that *hemM* (*lolB*) is an essential gene. Outer membrane prepared from LolB-depleted cells did not incorporate Lpp. When the Lpp–LolA complex was incubated with a water-soluble LolB derivative, Lpp was transferred from LolA to LolB. Based on these results, the outer membrane localization pathway for *E.coli* lipoprotein is discussed with respect to the functions of LolA and LolB.

Keywords: *Escherichia coli*/lipoprotein/LolA (p20)/LolB (HemM)/outer membrane

Introduction

Lipid-modified proteins are present in cells of organisms from bacteria to eukaryotes. Lipoproteins in bacteria (Pugsley, 1993) and small GTP-binding proteins such as Rab (Simons and Zerial, 1993) in eukaryotes have been studied extensively with respect to post-translational modification pathways, physiological importance and membrane targeting mechanisms. Cysteine residues at the N-terminus of lipoproteins and in the C-terminal region of Rab are modified with lipid, which functions as an anchor to the target membranes. The mislocalization of lipoprotein is lethal for *Escherichia coli* (Yakushi *et al.*, 1997), and correct membrane targeting of Rab is thought to be critical for vesicular transport (Simons and Zerial, 1993).

More than 10 species of lipoproteins have so far been reported in *E.coli* (Hayashi and Wu, 1990). Some of them are localized exclusively in the outer membrane whereas others are specific to the inner membrane. Irrespective of the final localization, each lipoprotein is synthesized as a precursor with a signal peptide at the N-terminus and then translocated across the cytoplasmic (inner) membrane through a protein translocation machinery (Pugsley, 1993). Lipid modification and processing to mature lipoprotein take place in the inner membrane. Lipoprotein-specific signal peptidase II, which is sensitive to globomycin, cleaves the signal peptide after the cysteine residue has been modified with diglyceride (Hussain *et al.*, 1980; Tokunaga *et al.*, 1982; Sankaran and Wu, 1994). Further fatty acylation of the cysteine residue then takes place to complete the processing.

The membrane specificity of lipoprotein localization is determined by the residue next to the modified cysteine residue (Yamaguchi *et al.*, 1988). Lipoproteins possessing aspartic acid at this position are localized in the inner membrane, whereas those possessing amino acids other than aspartic acid are destined for the outer membrane. We previously reported the discovery of a periplasmic factor which recognizes the lipoprotein sorting signal (Matsuyama *et al.*, 1995). This protein (p20) is encoded by a gene located at 20.4 min on the *E.coli* chromosome. The gene was named *lplA*, but we later found that *lplA* had been used for the lipoate–protein ligase gene, which is located at 99.6 min on the linkage map (Morris *et al.*, 1994). Since the p20 homolog of *Haemophilus influenzae* was named *lolA* (SWISS PROT; P45263), the gene encoding p20 of *E.coli* has been renamed *lolA*.

When lipoprotein possessing either the outer or inner membrane sorting signal was expressed in spheroplasts in the presence and absence of purified LolA, the outer membrane-directed lipoprotein was released into the spheroplast medium in a LolA-dependent manner (Matsuyama *et al.*, 1995). On the other hand, the inner membrane-directed lipoprotein remained in the inner membrane of spheroplasts regardless of the presence or absence of LolA. The major outer membrane lipoprotein (Lpp) released into the spheroplast medium was found to exist in a 1:1 complex with LolA. These results indicated that the LolA-dependent release of the outer membrane-directed lipoprotein is a critical step in lipoprotein sorting. Furthermore, when the Lpp–LolA complex was incubated with the inner and outer membranes, Lpp was incorporated specifically into the outer membrane. The incorporated Lpp exists as a trimer, as does *in vivo* incorporated Lpp (Choi *et al.*, 1986). Since both the inner leaflet of the outer membrane and the outer leaflet of the inner membrane consist of phospholipids, the outer membrane-specific

incorporation of Lpp strongly suggested the presence of an outer membrane factor involved in the Lpp incorporation.

We report here the discovery of a novel outer membrane lipoprotein, LolB, which is a receptor for the Lpp–LolA complex and plays an essential role in the outer membrane-specific incorporation of Lpp. Thus, two factors, LolA and LolB, functioning in the release and incorporation of lipoproteins, respectively, have now been characterized. LolB was found to be the product of *hemM*, which has been suggested to be important for 5-aminolevulinic acid synthesis (Ikemi *et al.*, 1992). However, the results shown here indicate that this is highly unlikely.

Results

Involvement of an outer membrane protein in the localization of Lpp to the outer membrane

The outer membrane, digested or not digested with trypsin, was incubated with [^{35}S]Lpp, which had been labeled in spheroplasts and released into the spheroplast medium as a complex with LolA (Matsuyama *et al.*, 1995), and then precipitated by centrifugation. Almost all Lpp molecules were recovered in the precipitate after 20 min incubation with the trypsin-undigested outer membrane (Figure 1). The trypsin digestion strongly inhibited the Lpp incorporation into the outer membrane, indicating that a proteinaceous factor in the outer membrane participates in the incorporation of Lpp.

When the [^{35}S]Lpp–LolA complex was incubated with proteoliposomes reconstituted from *E.coli* phospholipids and solubilized outer membrane proteins, Lpp was recovered with the reconstituted proteoliposomes on centrifugation (Figure 2). On the other hand, when outer membrane proteins were not reconstituted, Lpp remained in the supernatant. To purify the factor, outer membrane proteins solubilized with 2% octylglucoside were subjected to anion-exchange column chromatography. Proteoliposomes reconstituted with the flow-through fraction from this chromatography were found to contain Lpp incorporation activity (data not shown). This fraction was fractionated further by cation-exchange column chromatography (Figure 3A). The proteins in each fraction were analyzed by SDS–PAGE (Figure 3B), and then reconstituted into proteoliposomes. [^{35}S]Lpp was incorporated into proteoliposomes when the eluates after fraction 17 were used for the reconstitution (Figure 3C). The elution profile of the incorporation activity coincided with that of a 23 kDa protein band (Figure 3B). This protein, designated LolB, was characterized further as a candidate for the outer membrane factor involved in Lpp incorporation.

LolB is the product of hemM and possesses Lpp incorporation activity

Amino acid sequencing of LolB was unsuccessful, suggesting the modification of its N-terminus. We therefore digested LolB with a limited amount of V8 protease and obtained two peptide fragments. The N-terminal sequences of these fragments coincided with partial sequences of HemM (Figure 4A), which has been suggested to be involved in the synthesis of 5-aminolevulinic acid, an intermediate of porphyrin biosynthesis (Ikemi *et al.*, 1992).

To determine whether or not the *hemM* gene encodes LolB, we constructed pMAN650, which carries *hemM*

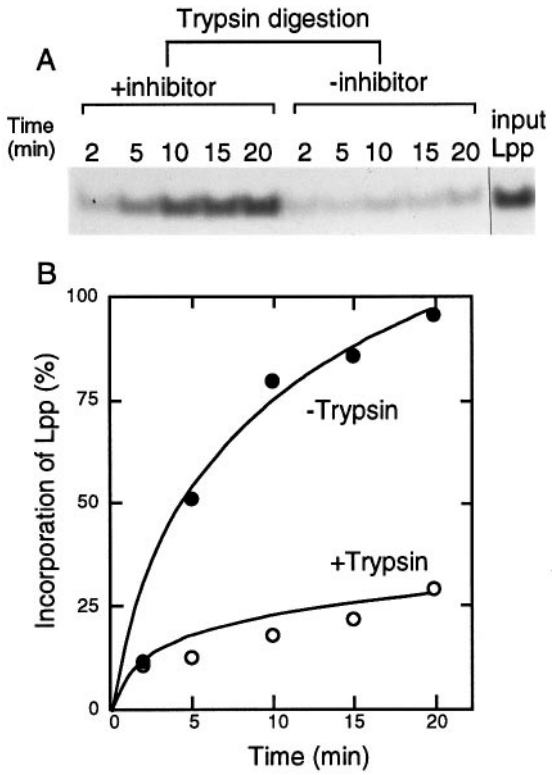

Fig. 1. Inhibition of the outer membrane incorporation of Lpp by trypsin. (**A**) The outer membrane (400 μg) prepared from Q13 was treated with trypsin (50 μg/ml) in 400 μl of 50 mM K phosphate (pH 7.5) with or without 45 μl of 1 mg/ml soybean trypsin inhibitor at 4°C for 30 min. The same amount of soybean trypsin inhibitor was added to the samples which had been trypsin treated without soybean trypsin inhibitor. Incubation was continued for a further 5 min. The outer membrane was recovered by centrifugation at 100 000 *g* for 30 min and resuspended in 40 μl of 50 mM K phosphate (pH 7.5) containing 100 μg/ml soybean trypsin inhibitor. The spheroplast medium (1.2 ml) containing the [^{35}S]Lpp–LolA complex was incubated with 24 μl of the outer membrane at 30°C. Aliquots (200 μl) were removed at the indicated times and treated with 6 M urea at 4°C to terminate the incorporation reaction. After centrifugation at 200 000 *g* for 30 min, the precipitates were analyzed by SDS–PAGE, followed by fluorography. The amount of the input Lpp is shown in the right lane. (**B**) The amount of Lpp incorporated into the outer membrane was determined by densitometrically scanning the fluorogram in (A) and plotted by taking the amount of the input Lpp as 100%. Open and closed circles represent samples treated with trypsin in the absence and presence of soybean trypsin inhibitor, respectively.

under the control of the *tac* promoter–*lac* operator, and *lacI*^{q}. When the *hemM* gene was overexpressed in *E.coli* JM83 harboring pMAN650, on addition of isopropyl-β-D-thiogalactopyranoside (IPTG) a 23 kDa protein was overproduced. This protein was purified to homogeneity (Figure 4B) and used to raise an antibody. *In vitro* Lpp incorporation into the outer membrane was then examined in the presence of this antibody or an anti-OmpA antibody (Figure 4C). The antibody raised against the *hemM* gene product, but not the anti-OmpA antibody, strongly inhibited the Lpp incorporation, indicating that LolB involved in the Lpp incorporation is a product of *hemM*. Furthermore, when proteoliposomes reconstituted from various amounts of purified LolB were used for the *in vitro* Lpp incorporation, the amount of Lpp incorporated into proteoliposomes increased with an increase in the amount of reconstituted LolB (Figure 4D). Proteoliposomes were

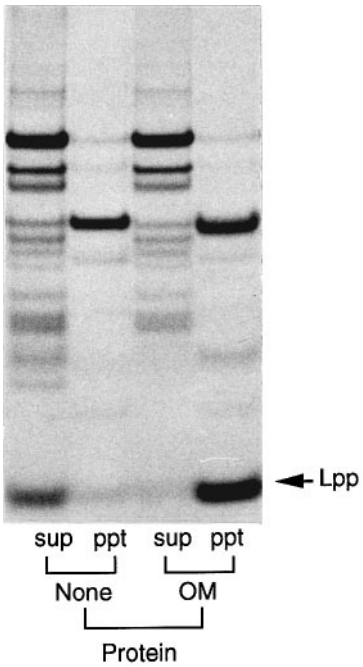

Fig. 2. Solubilization and reconstitution of the Lpp incorporation activity. Proteoliposomes were reconstituted with solubilized outer membrane proteins as described in Materials and methods. The spheroplast medium (200 µl) containing the [^{35}S]Lpp–LolA complex was incubated with 50 µl of proteoliposomes (right two lanes) or liposomes containing no protein (left two lanes) at 30°C for 30 min, and then the reaction mixture was fractionated into the supernatant (sup) and precipitate (ppt) by centrifugation at 200 000 *g* for 1 h. Samples were analyzed by SDS–PAGE and fluorography. The position of Lpp is indicated.

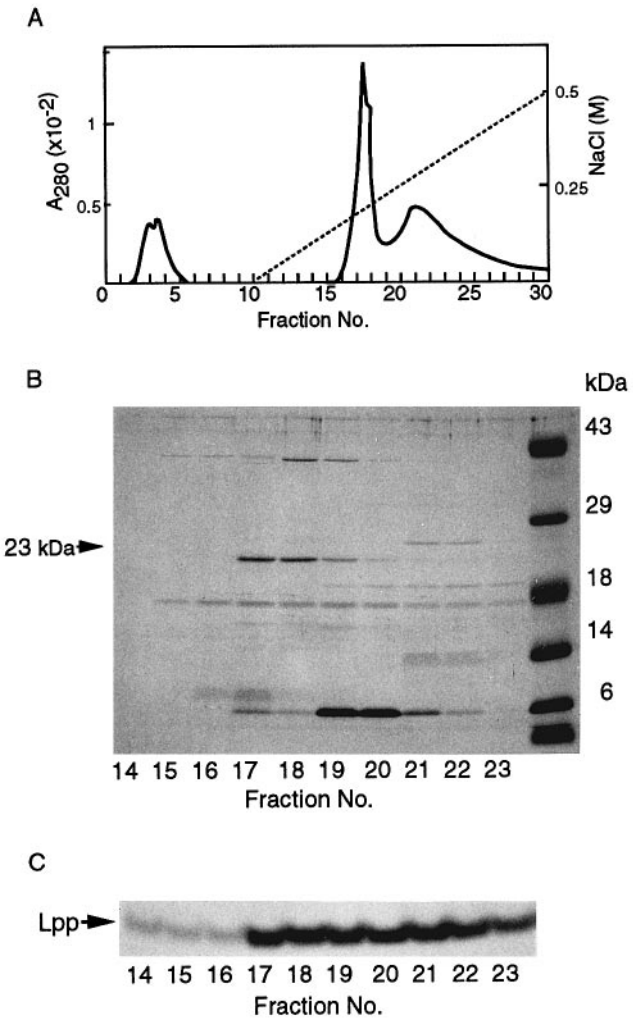

Fig. 3. Identification of an outer membrane protein involved in the incorporation of Lpp. (**A**) Solubilized outer membrane fractions, which passed through a MonoQ column and contained the Lpp incorporation activity, were fractionated further on a MonoS column as described in Materials and methods. The column was developed with a linear gradient of NaCl. Fractions of 2 ml were collected and the absorbance at 280 nm was recorded. (**B**) The indicated fractions in (A) were analyzed by SDS–PAGE followed by staining with Coomassie brilliant blue. The position of a 23 kDa protein is indicated. The positions of molecular weight marker proteins are shown on the right. (**C**) Proteoliposomes reconstituted from aliquots (80 µl) of the indicated fractions were incubated with the spheroplast medium containing the [^{35}S]Lpp–LolA complex at 30°C for 30 min. Proteoliposomes were recovered by centrifugation at 200 000 *g* for 1 h, and then analyzed by SDS–PAGE and fluorography.

also reconstituted with solubilized outer membrane proteins. The amounts of Lpp incorporated into these proteoliposomes were plotted as a function of the amount of reconstituted LolB, which was determined by quantitative immunoblotting (Figure 4D). LolB, whether it was purified or not, exhibited essentially the same incorporation activity, indicating that only LolB among the outer membrane proteins is important for the Lpp incorporation.

LolB is a novel outer membrane lipoprotein

HemM has been assumed to function in the cytosol (Ikemi *et al.*, 1992), whereas LolB was purified from the solubilized outer membrane proteins. The subcellular localization of LolB was examined by means of immunoblotting with the anti-LolB antibody (Figure 5A). LolB (HemM) was localized exclusively in the outer membrane, suggesting that LolB is a secretory protein synthesized as a precursor with a signal peptide at its N-terminus and then processed to the mature form. Indeed, we found a possible signal peptide in the deduced amino acid sequence of HemM (Figure 4A). Moreover, the lipoprotein box, a consensus sequence around the signal peptide cleavage site of lipoproteins (Hayashi and Wu, 1990), was found (Figure 4A).

To examine the processing of LolB, *E.coli* IQ85 (*secYts*) and its parental strain, IQ86, were transformed with pMAN650 carrying the *tac–lolB* gene, and then labeled with Tran^{35}S-label in the presence of IPTG at 41°C, a non-permissive temperature for IQ85. The precursor form of LolB, as well as those of OmpA and Lpp, was accumulated in IQ85 (Figure 5B, lane 1), indicating that LolB is a secretory protein possessing a signal peptide. When the labeling of IQ86 was carried out in the presence of globomycin (Hussain *et al.*, 1980), a specific inhibitor of signal peptidase II, a protein band migrating slightly more slowly than that of the LolB precursor in IQ85 was detected, as in the case of Lpp (Figure 5B, lane 3). These bands most probably represent precursor proteins modified with fatty acids (Hussain *et al.*, 1980). In contrast, the processing of proOmpA catalyzed by signal peptidase I (Date and Wickner, 1981) was insensitive to globomycin. Furthermore, when IQ86 was labeled with [^{3}H]palmitic acid, IPTG-dependent incorporation of palmitic acid into the 23 kDa band was observed (Figure 5C, lanes 1 and

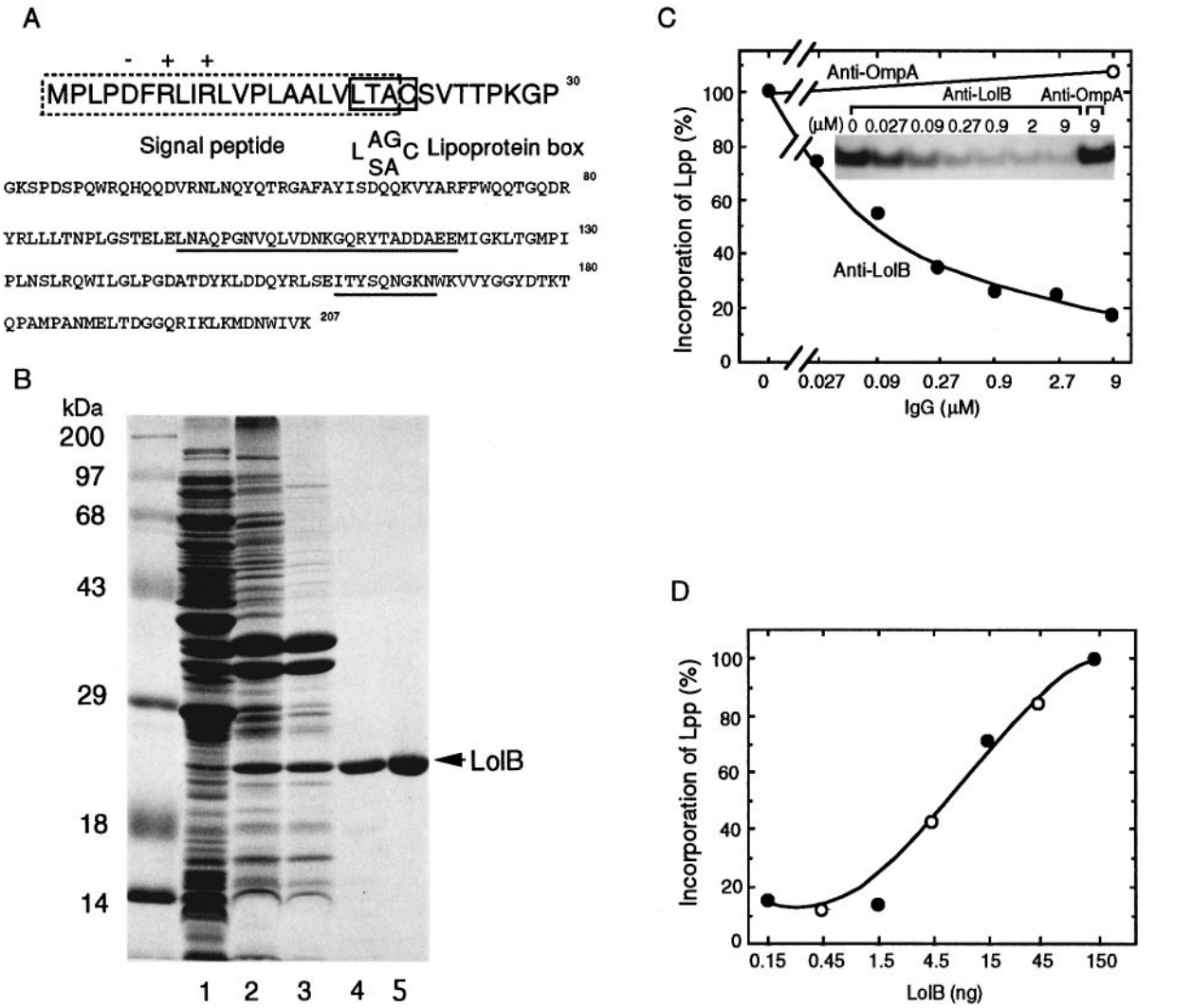

Fig. 4. Characterization of HemM (LolB) as the Lpp incorporation factor. (**A**) The amino acid sequence deduced from the nucleotide sequence of the *hemM* gene (Ikemi *et al.*, 1992) is shown. The amino acid sequences determined with proteolytic fragments of LolB are underlined. A possible signal peptide is boxed by a dotted line. A sequence similar to the lipoprotein box is indicated by a solid lined box, below which the consensus sequence is shown. Charged residues in the signal peptide are also indicated. (**B**) The *hemM* gene was overexpressed, and its product, LolB (HemM), was purified as described in Materials and methods. Fractions containing LolB at each purification step were analyzed by SDS–PAGE, followed by staining with Coomassie brilliant blue. The samples analyzed were: whole cell (lane 1), total membrane (lane 2), outer membrane (lane 3), MonoQ fraction (lane 4) and MonoS fraction (lane 5). The migration positions of the molecular weight marker proteins are indicated on the left. The position of LolB is also indicated. (**C**) An antibody was raised against highly purified LolB as described in Materials and methods. The *in vitro* incorporation of Lpp into the outer membrane prepared from MC4100 was examined with the [^{35}S]Lpp–LolA complex in the presence of various amounts of the anti-LolB (●) or anti-OmpA (○) IgGs. The Lpp incorporation was analyzed by fluorography (inset) as described in the legend to Figure 1, and the results were plotted as a function of the IgG concentration, taking the amount of Lpp incorporated in the absence of an IgG as 100%. (**D**) Proteoliposomes reconstituted with various amounts of purified LolB (●) or solubilized outer membrane proteins (○) were incubated with the [^{35}S]Lpp–LolA complex at 30°C for 30 min. The Lpp incorporation into the proteoliposomes was analyzed by fluorography as described in the legend to Figure 2. The amount of incorporated Lpp was expressed as a function of the LolB content in the reconstituted proteoliposomes, taking the maximum value as 100%.

2). This material was immunoprecipitated with the anti-LolB antibody (Figure 5C, lane 3), and migrated to the same position as ^{35}S-labeled LolB (Figure 5C, lane 4). Taking all the results together, we concluded that LolB is a novel outer membrane lipoprotein. Furthermore, these results also indicate that the involvement of HemM in 5-aminolevulinic acid synthesis is unlikely or, at most, indirect.

Depletion of LolB is lethal for E.coli

To determine the *in vivo* role of LolB, a mutant strain, SM602, in which the *lolB* gene on the chromosome is placed under the control of the *lac* promoter–operator, was constructed. The *lolB* (*hemM*) gene and the immediately downstream gene (*orf2*) constitute an operon, and the disruption of either gene was reported to be unsuccessful (Post *et al.*, 1993). To render the expression of *orf2* independent of IPTG, pMAN655 carrying the P_{BAD}-*orf2* gene was constructed and transformed into SM602. When SM602 harboring pMAN655 was grown in the presence of IPTG and then transferred to fresh medium without IPTG, growth of the cells was soon arrested (Figure 6A). Arabinose was always present for expression of *orf2*, which has been reported to be essential (Post *et al.*, 1993).

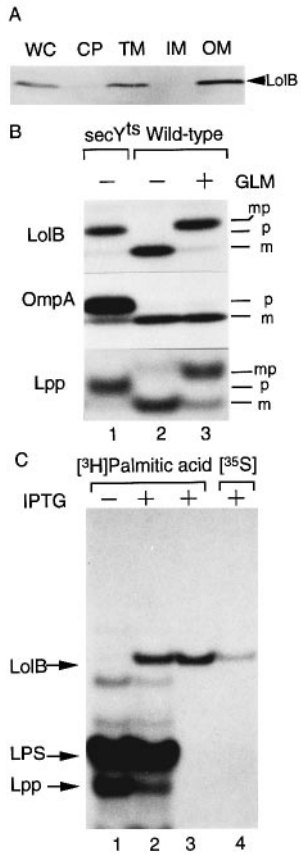

Fig. 5. LolB is a novel outer membrane lipoprotein. (**A**) MC4100 cells (WC) were disrupted by sonication, and then fractionated into soluble (CP) and total membrane (TM) fractions. The total membrane fraction was subjected to sucrose density gradient centrifugation to separate the inner (IM) and outer (OM) membranes. An equivalent amount of each fraction was analyzed by SDS–PAGE, followed by immunoblotting with the anti-LolB antibody. (**B**) IQ85 (*secYts*) harboring pMAN650 and IQ86 (wild-type) harboring pMAN650 were grown at 30°C to 2×10^8 cells/ml in M63 minimal medium supplemented with 10 μg/ml thiamine, 2% glycerol and 20 μg/ml each of 18 amino acids other than methionine and cysteine. Prior to labeling, the culture was incubated at 41°C for 2 h. After incubation with 1 mM IPTG for 5 min, 1 ml of culture was labeled with 10 μCi of Tran^{35}S-label (1000 Ci/mmol) at 41°C for 1 min in the presence (+) or absence (–) of globomycin (GLM), and then chased for 1 min. Samples were immunoprecipitated with the anti-LolB, anti-OmpA and anti-Lpp antibodies, and then analyzed by SDS–PAGE and fluorography as described previously (Matsuyama *et al.*, 1995). The mature (m), precursor (p) and modified precursor (mp) forms are indicated. (**C**) IQ86 harboring pMAN650 was grown at 37°C in M63 minimal medium in the presence (lanes 2–4) or absence (lane 1) of IPTG for 5 min. Each culture (5 ml) was labeled with 50 μCi of [9,10 (*n*)-^{3}H]palmitic acid (60 Ci/mmol, Amersham) for 30 min (lanes 1–3) or with Tran^{35}S-label at 37°C for 2 min (lane 4). The labeled cells were analyzed by SDS–PAGE and fluorography (lanes 1 and 2). The samples in lanes 3 and 4 were analyzed after immunoprecipitation of solubilized cellular proteins with the anti-LolB antibody. The positions of LolB, Lpp and lipopolysaccharide (LPS) are indicated.

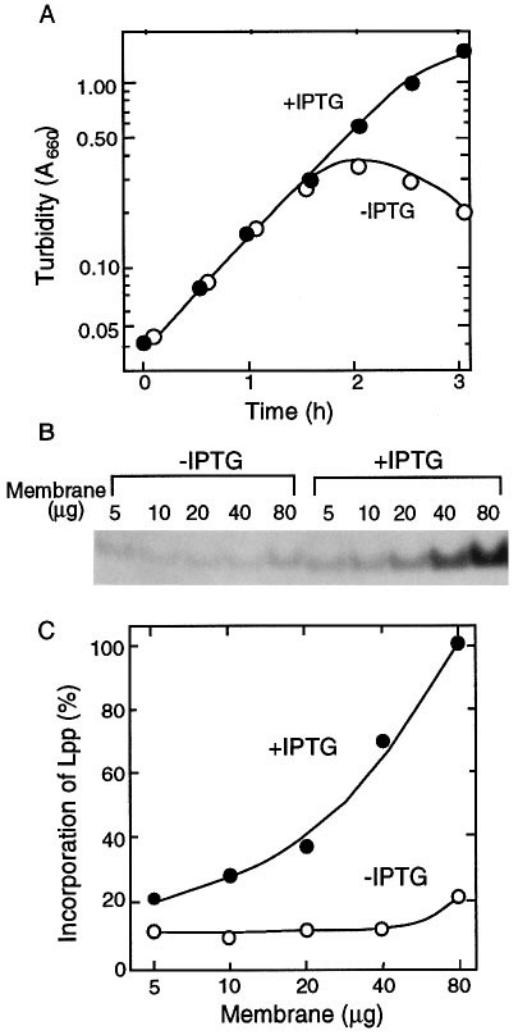

Fig. 6. Effects of LolB depletion on growth and *in vitro* membrane incorporation of Lpp. (**A**) SM602 harboring pMAN655 was grown in L-broth supplemented with 0.05% L-arabinose and 1 mM IPTG at 37°C. At time zero, the cells were washed twice with L-broth, and then transferred to fresh medium containing arabinose with or without IPTG. Growth was followed by monitoring the optical density at 660 nm. (**B**) The cells were harvested from a portion of the 2 h culture shown in (A) and used to prepare total membrane fractions containing or not containing LolB. The specified amounts of the total membrane fractions were incubated with the [^{35}S]Lpp–LolA complex at 30°C for 30 min. The Lpp incorporation was analyzed by fluorography. (**C**) The amount of Lpp incorporated was expressed as a function of the amount of membrane, taking the maximum value as 100%.

Immunoblotting with the anti-LolB antibody revealed that LolB was undetectable in the cells when growth was arrested (data not shown). These results indicate that *lolB* is an essential gene for *E.coli*.

The membrane fractions prepared from the mutant grown in the presence and absence of IPTG were used to examine the *in vitro* incorporation of Lpp. The LolB depletion significantly inhibited the incorporation of Lpp into the membrane (Figure 6B and C). The outer membrane lipoproteins Pal and NlpB also required LolB for incorporation into the membrane (data not shown), suggesting that the localization of outer membrane lipoproteins generally requires LolB.

Molecular interaction between the Lpp–LolA complex and LolB

The results mentioned above indicate that Lpp released into the periplasm on formation of the complex with LolA is incorporated into the outer membrane upon interaction with LolB, which exists in the outer membrane. Our attempt to analyze the interaction between LolB and the Lpp–LolA complex in the presence of octylglucoside, which was added to solubilize LolB, was unsuccessful

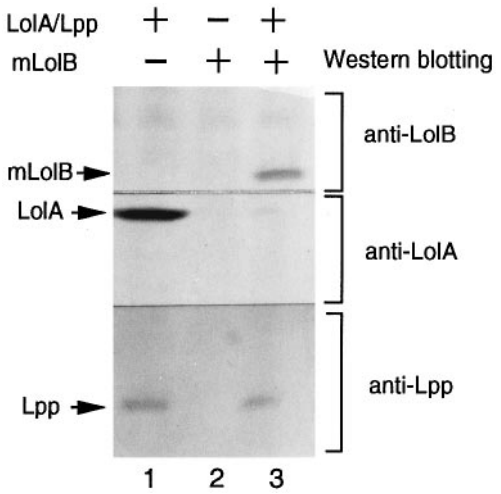

Fig. 7. Transfer of Lpp from LolA to LolB. Spheroplasts of MC4100 cells (2×10^9) were incubated in 1.8 ml of L-broth containing 180 μg of LolA and 0.25 M sucrose at 30°C for 1 h. The spheroplast medium (600 μl) containing the non-radioactive Lpp–LolA complex was obtained by centrifugation and incubated with 600 μl of 100 μg/ml of the partially purified mLolB in 25 mM Tris–HCl (pH 8.0), 0.25 M sucrose at 37°C for 45 min. After centrifugation at 16 000 *g* for 5 min, the supernatant was incubated at room temperature for 1.5 h with 100 μg of the anti-Lpp IgG, which had been purified on a protein A–Sepharose column, and then centrifuged at 100 000 *g* for 10 min. The supernatant was mixed with protein A–Sepharose resin and then stirred for 30 min. The resin was collected, washed alternately with 25 mM Tris–HCl (pH 8.0) and 25 mM Tris–HCl (pH 8.0), 0.5 M NaCl several times, and then treated with the sample buffer for SDS–PAGE at 100°C. Samples were analyzed by SDS–PAGE and immunoblotting. The blot was divided into three parts, with visualization with the anti-Lpp, anti-LolA and anti-LolB antibodies, respectively.

since the Lpp–LolA complex was unstable in octylglucoside (data not shown). We therefore engineered the *lolB* gene to obtain a water-soluble LolB derivative by replacing the N-terminal cysteine of the mature region with alanine and the signal peptide with that of OmpF. The N-terminal cysteine residue is essential for the fatty acid modification of lipoproteins (Braun, 1975). When a LolB derivative, named mLolB, was overproduced, mature mLolB was found in the periplasm in a soluble form. Furthermore, size exclusion chromatography revealed that mLolB exists as a monomer (data not shown). The overproduction only slightly inhibited growth. The mLolB protein was partially purified and used to examine the interaction with the Lpp–LolA complex.

The non-radioactive Lpp–LolA complex was formed by incubating spheroplasts with a large amount of LolA. The spheroplast medium containing the Lpp–LolA complex was then incubated with or without a near equimolar amount of mLolB at 37°C. The reaction mixture was then subjected to immunoprecipitation with the anti-Lpp antibody, and the precipitate was analyzed by SDS–PAGE, followed by immunoblotting with the anti-Lpp, anti-LolA and anti-LolB antibodies (Figure 7). When the Lpp–LolA complex incubated without mLolB was immunoprecipitated with the anti-Lpp antibody, LolA and Lpp were co-immunoprecipitated (lane 1). The mLolB protein alone was not precipitated with the anti-Lpp antibody (lane 2). In marked contrast, when the Lpp–LolA complex was incubated with mLolB, the anti-Lpp antibody co-immunoprecipitated mLolB instead of LolA (lane 3). These results indicate that Lpp is transferred from LolA to mLolB, and thus forms a Lpp–mLolB complex. Since the overproduction of mLolB only slightly inhibited growth, the affinity of mLolB for Lpp seems to be lower than that of intact LolB.

Discussion

We showed in a previous study that LolA functions as a periplasmic carrier protein for lipoproteins possessing the outer membrane sorting signal (Matsuyama *et al.*, 1995). LolA releases the outer membrane lipoproteins from the inner membrane by forming a soluble complex and then carries them to the outer membrane, where their incorporation takes place. In this study, we investigated the determinant for the outer membrane-specific incorporation of lipoproteins, and found that LolB, a new outer membrane lipoprotein, plays a critical role in the lipoprotein incorporation. The following evidence supports our conclusion: (i) the anti-LolB antibody inhibited the outer membrane incorporation of Lpp; (ii) Lpp was incorporated into proteoliposomes reconstituted from purified LolB and phospholipids; and (iii) the LolB-deficient outer membrane did not exhibit incorporation activity.

Lipopolysaccharide (LPS) is localized in the outer leaflet of the outer membrane, and plays an important role in the assembly of outer membrane proteins such as OmpF, LamB and PhoE (Mizushima, 1987). When we purified LolB from the solubilized outer membrane fraction, we found that LPS was adsorbed to a MonoQ column whereas LolB was not. Consequently, the purified LolB fraction used for the reconstitution experiment did not contain LPS. Therefore, LPS plays little part in the incorporation of outer membrane lipoproteins.

How does LolB mediate the outer membrane localization of lipoproteins? We observed Lpp–mLolB complex formation after incubation of the Lpp–LolA complex with mLolB. This indicates that Lpp is transferred from LolA to mLolB. The Lpp–mLolB complex most probably represents an intermediate of the outer membrane localization. The number of outer membrane lipoprotein molecules in one *E.coli* cell exceeds 10^5 (Braun, 1975); on the other hand, that of LolB (data not shown), as well as that of LolA (Matsuyama *et al.*, 1995), was estimated, by means of quantitative immunoblotting, to be 150–300. Therefore, to complete the outer membrane localization, Lpp must be transferred further from LolB to the outer membrane. We speculate that both LolA and LolB function catalytically to cycle the localization of the outer membrane-directed lipoproteins (Figure 8). Since mLolB was localized in the periplasm as a soluble monomer, the protein moiety of LolB seems to be exposed to the periplasm, thereby interacting with the Lpp–LolA complex in the periplasm, while the lipid moiety anchors LolB to the outer membrane. The mechanism underlying the final step of localization, which leads to the lipoprotein anchoring, remains to be clarified.

Our preliminary experiments suggested that the release of LolB from the inner membrane is also dependent on LolA (N.Yokota, S.Matsuyama and H.Tokuda, unpublished observation). LolB is therefore likely to form a soluble complex with LolA, presumably at its modified N-terminal region. Indeed, mLolB lacking the modified

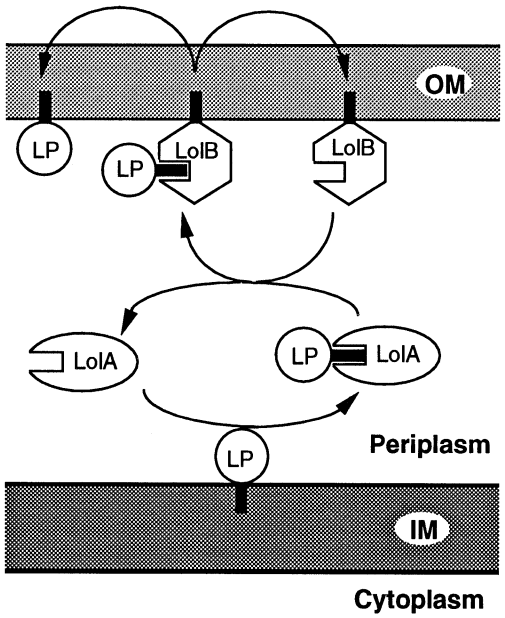

Fig. 8. Outer membrane localization of *E.coli* lipoproteins. IM, inner membrane; OM, outer membrane; LP, outer membrane-directed lipoprotein. For details, see text.

N-terminal region existed in the periplasm as a monomer. On the other hand, the N-terminal region of LolB was not required for the interaction with Lpp since mLolB formed a complex with Lpp upon interaction with the Lpp–LolA complex. The Lpp–mLolB complex was soluble, as is the Lpp–LolA complex (Matsuyama *et al.*, 1995), suggesting that the lipid-modified N-terminal region of Lpp in both complexes is shielded from the aqueous environment by LolA or LolB (Figure 8). However, no apparent homology was found between the amino acid sequences of LolA and LolB. The regions of LolA and LolB involved in the Lpp interaction therefore remain to be clarified. The details of the molecular interaction involving Lpp, LolA and LolB are currently under examination.

The phospholipid composition affects various membrane functions, for example protein translocation across the *E.coli* cytoplasmic membrane (de Kruijff, 1994; Rietveld *et al.*, 1995). Acidic phospholipids are required specifically for the translocation (Lill *et al.*, 1990; de Kruijff, 1994). LolB reconstituted into liposomes consisting of phosphatidylethanolamine and phosphatidylcholine was as active as that reconstituted into liposomes consisting of *E.coli* phospholipids (data not shown), suggesting that acidic phospholipids are not essential for the LolB function. Lpp incorporated into the outer membrane *in vitro* was resistant to 6 M urea treatment, as was the case *in vivo* (Matsuyama *et al.*, 1995). On the other hand, the urea treatment significantly reduced the amount of proteoliposomes recovered by centrifugation. Therefore, it is not completely clear whether Lpp recovered with proteoliposomes is anchored directly to the membrane or simply associated with LolB.

We found that LolA was required for the release of Lpp, Pal, NlpB and LolB. The outer membrane incorporation of these lipoproteins, other than of LolB, took place in a LolB-dependent manner. LolA and LolB therefore seem to be involved in the outer membrane localization of most lipoproteins in *E.coli*. It is not clear at present how LolB is localized to the outer membrane after the LolA-dependent release.

HemM (LolB) homologs have been found in several bacteria, although none of them has been characterized with regard to function. The identities of the HemM homologs of *Salmonella typhimurium* (Post *et al.*, 1993), *Haemophilus influenzae* (SWISS PROT; P45270) and *Pseudomonas aeruginosa* (Hungerer *et al.*, 1995) with *E.coli* LolB are 92, 25 and 24%, respectively. Furthermore, a LolA homolog was found in *H.influenzae* (SWISS PROT; P45263). These observations indicate that LolA and LolB are generally involved in the localization of outer membrane lipoproteins in Gram-negative bacteria. It is noteworthy that five tryptophan residues of *E.coli* LolB are conserved in all HemM homologs, suggesting that these tryptophan residues are important for the function of LolB.

The *hemM* gene has been suggested by genetic analysis to be involved in the synthesis of 5-aminolevulinic acid (Ikemi *et al.*, 1992). It was later found that *hemA*, but not *hemM*, is involved directly in 5-aminolevulinic acid synthesis (Chen *et al.*, 1994; Hungerer *et al.*, 1995), although co-expression of the two genes caused more efficient synthesis of 5-aminolevulinic acid (Chen *et al.*, 1994). The *hemA* and *hemM* genes are 213 bp apart from each other and are transcribed divergently from the region between the two genes (Post *et al.*, 1993). This region contains a promoter and a possible regulatory element for each gene. It is therefore likely that *hemM* expression affects the expression of *hemA*, and vice versa. Since LolB, a product of *hemM*, is an outer membrane lipoprotein, direct involvement of LolB in 5-aminolevulinic acid synthesis is highly unlikely. It was reported very recently that 5-aminolevulinic acid synthesis is increased by *hemA* overexpression whereas co-expression of *hemA* and *hemM* does not cause a further increase (Verderber *et al.*, 1997).

There seems to be a striking mechanistic resemblance between the localization of lipid-modified proteins in *E.coli* and eukaryotes. For instance, Rab proteins are transported to the target membrane as a soluble complex with a Rab GDP dissociation inhibitor, Rab GDI (Sasaki *et al.*, 1990; Ullrich *et al.*, 1994). Rab is then anchored to the target membrane in an energy-independent manner, most likely through its lipid moiety (Stenmark *et al.*, 1994). A proteinaceous factor in the target membrane was suggested recently to play roles in the recognition of the Rab–Rab GDI complex and the localization of Rab (Dirac-Sverjstrup *et al.*, 1997)

Materials and methods

Bacterial strains

The *E.coli* K-12 strains MC4100 [Δ*lacU169, araD, rpsL, relA, thiA, fibB*] (Casadaban, 1976), Q13 [Hfr, *pnp13, tyr, met,* RNaseI$^-$] (Reiner, 1969), FS1576 [C600 *thr, leu, thi, lac, thy, recD1009*] (Stahl *et al.*, 1986; Ogura *et al.*, 1989), IQ86 [MC4100 *zhd-33*::Tn*10 rpsE*] (Shiba *et al.*, 1984) and IQ85 [IQ86 *secYts24*] (Shiba *et al.*, 1984) were used in this work.

Construction of plasmids

A λ phage clone, 247 (15A7), in the Kohara library (Kohara *et al.*, 1987) was used to clone the *hemM* (*lolB*) gene as reported previously (Ikemi *et al.*, 1992). Based on the reported nucleotide sequence around the *hemM* (*lolB*) gene (Post *et al.*, 1993), a 1.7 kb *Nhe*I–*Hin*dIII DNA

fragment was isolated from the λ DNA. This fragment, which contains the *hemM* (*lolB*)-*orf2* operon but not its promoter region, was inserted into the *Xba*I–*Hin*dIII site of pTTQ18 (Amersham) carrying the *tac* promoter–*lac* operator and the *lacI*^{q} gene to construct pMAN650.

A 1.8 kb *Sal*I–*Pvu*II fragment containing the *orf2* gene was isolated from the λ DNA and then inserted into the *Sal*I–*Sma*I site of pMAN885 (Yakushi *et al.*, 1997) carrying the P_{BAD} promoter and the *araC* gene to construct pMAN655.

A 6.2 kb *Kpn*I fragment carrying the 5′- and 3′-flanking regions of *hemM* (*lolB*) was obtained from the λ DNA and then inserted into the *Kpn*I site on pUC19 (Yanisch-Perron *et al.*, 1985) to construct pMAN651. Synthetic DNA linkers d(5′CTAGGCCCCTAGGCCTCGAGCACTA-GTCCCCCC3′) and d(3′CGGGGATCCGGAGCTCGTGATCAGGGG-GGGATC5′) were inserted into the *Nhe*I site, which is located within the promoter region of the *hemM* (*lolB*)-*orf2* operon, on pMAN651 to construct pMAN652. pSY343 (Yasuda and Takagi, 1983) was digested with *Hin*dIII, treated with T4 DNA polymerase and then digested with *Xho*I to obtain a 1.5 kb DNA fragment carrying the *kan* gene. pUC19 was digested with *Pvu*II to obtain a 0.32 kb DNA fragment carrying *lacPO*. The two fragments were ligated with the large *Xho*I–*Xba*I fragment of pSP72 (Pierce) to construct pMAN653. A 1.8 kb *Xho*I–*Xba*I fragment of pMAN653 carrying the *kan* gene and *lacPO* in opposite directions was inserted into the *Xho*I–*Spe*I site on pMAN652 to construct pMAN654, which was used to construct a *lac–lolB* mutant.

To construct a water-soluble LolB (HemM) derivative, mLolB, an oligonucleotide (48mer) containing the signal cleavage region of *lolB* (*hemM*) was synthesized. The sequence at the cleavage site, 5′-CTGCCTGT-3′, was changed to 5′-CTGCAG*CT*-3′ to create a *Pst*I site (underlined), and to introduce a Cys→Ala mutation at position 1 (italicized). The synthetic oligonucleotide was incubated with 5.8 kb *Kpn*I–*Sal*I and 5.2 kb *Sca*I–*Apa*I fragments of pMAN650, followed by treatment with Klenow enzyme and T4 DNA ligase to form a hetero-duplex. After transformation into MC4100, a plasmid, pYKT101, possessing the new *Pst*I site was selected. To replace the signal peptide of mLolB with that of OmpF, a 6.1 kb *Kpn*I–*Pst*I fragment of pYKT101 was ligated with a pair of synthetic oligonucleotides (84mer), which are complementary to each other, possess *Kpn*I and *Pst*I sites at each end and encode the signal peptide of OmpF. The plasmid, pYKT102, thus constructed encodes mLolB with the OmpF signal peptide plus the N-terminal alanine of the mature region of OmpF and the product of *orf2* under the control of the *tac* promoter.

In vitro membrane incorporation of Lpp

Spheroplast medium containing the [^{35}S]Lpp–LolA complex was prepared as described previously (Matsuyama *et al.*, 1995). Briefly, a spheroplast suspension containing 1.6×10^9/ml spheroplasts of the MC4100 strain was mixed with purified LolA (final concentration 9.5 μg/ml) on ice. Labeling of the spheroplasts was initiated by the addition of M63 medium (2.4 vols) containing 0.25 M sucrose and 10 μCi/ml Tran^{35}S-label (1000 Ci/mmol, ICN) at 30°C. After 3 min, the labeling was chased for 3 min with 12 mM non-radioactive methionine and cysteine. The labeled culture was chilled and then centrifuged at 16 000 *g* for 2 min to obtain the spheroplast medium containing the [^{35}S]Lpp–LolA complex. Membrane incorporation of Lpp was examined by incubating the spheroplast medium with 0.4 mg/ml total membranes, 0.2 mg/ml outer membrane or a specified amount of proteoliposomes. The incorporation of Lpp into the membrane was determined by SDS–PAGE and fluorography after centrifugation.

Reconstitution of proteoliposomes possessing Lpp incorporation activity

Proteoliposomes were reconstituted by the octylglucoside dilution method, which has been used for reconstitution of the protein translocation machinery (Akimaru *et al.*, 1991). The outer membrane (1 mg protein/ml) was solubilized with 2% octylglucoside in 50 mM K phosphate (pH 7.5), 1 mM EDTA at 4°C for 10 min. After centrifugation at 100 000 *g* for 30 min, 100 μl of the supernatant was mixed with 12.5 μl of 100 mg/ml *E.coli* phospholipids in 2% octylglucoside, and then kept on ice for 10 min. The mixture was diluted rapidly with 40 vols of 50 mM K phosphate (pH 7.5), and then stirred at room temperature for 5 min. Proteoliposomes collected by centrifugation at 160 000 *g* for 2 h were resuspended in 50 μl of 50 mM K phosphate (pH 7.5), frozen at –80°C, thawed at room temperature, and then sonicated briefly. The proteoliposomes thus reconstituted were assayed for the *in vitro* incorporation of Lpp.

Purification and amino acid sequencing of LolB

MC4100 cells (wet weight, 40 g) were resuspended in 200 ml of 50 mM K phosphate (pH 7.5) and then disrupted by passage through an Aminco French pressure cell twice at 1000 kg/cm^2. The total membrane fraction was recovered by centrifugation at 100 000 *g* for 1 h, and then subjected to sucrose density gradient centrifugation. The outer membrane fraction (300 mg of protein) was collected and solubilized with 30 ml of 2% octylglucoside, 25 mM Tris–HCl (pH 8.8), 1 mM EDTA at 4°C for 10 min. After centrifugation at 100 000 *g* for 30 min, the supernatant (6 ml) was applied to a column of an anion exchanger, MonoQ (1×10 cm, Pharmacia) which had been equilibrated with 2% octylglucoside, 25 mM Tris–HCl (pH 8.8). The column was then eluted at the flow rate of 4 ml/min with a linear gradient of NaCl (0–0.5 M). Aliquots of each fraction were reconstituted into proteoliposomes and assayed for Lpp incorporation. The chromatography was repeated five times, and the flow-through fractions containing most of the activity were combined, dialyzed against 25 mM Na acetate (pH 5.0), 2% octylglucoside, and fractionated further by cation-exchange chromatography on a column of MonoS (1×10 cm, Pharmacia) which had been equilibrated with 25 mM Na acetate (pH 5.0), 2% octylglucoside. The column was eluted with the same buffer at the flow rate of 4 ml/min with a linear gradient of NaCl (0–0.5 M). The Lpp incorporation activity was examined after reconstitution of proteoliposomes with each fraction.

The fraction exhibiting incorporation activity was analyzed by SDS–PAGE, followed by staining with Coomassie brilliant blue. A 23 kDa protein band exhibiting the activity was cut out from the gel, and the protein was extracted from the crushed gel by incubation with 50 mM Tris–HCl (pH 7.5), 0.5% SDS at room temperature for 12 h. After removal of the crushed gel, acetone was added to the solution. The precipitate containing 20 μg of the 23 kDa protein, LolB, was collected by centrifugation, dissolved in 30 μl of the SDS–PAGE sample buffer containing 0.8 μg of V8 protease, and then immediately analyzed by SDS–PAGE on a 19% acrylamide gel. Proteolytic peptides in the gel were transferred to a PVDF membrane (Millipore) and then stained with Coomassie brilliant blue. The peptide bands migrating to positions corresponding to molecular masses of 10 and 6 kDa were cut out and then subjected to amino acid sequencing with a Beckman protein sequencing system LF3600D.

Overproduction and purification of LolB

An outer membrane fraction (300 mg protein) was prepared from 40 g (wet weight) of JM83 harboring pMAN650, which was grown in the presence of 1 mM IPTG for 2 h to overproduce LolB. The outer membrane fraction containing the overproduced LolB was solubilized with 25 mM Tris–HCl (pH 8.8), 2% octylglucoside. LolB was purified from the solubilized fraction by means of ion-exchange column chromatography on MonoQ and MonoS, as described above. The purified LolB (1.5 mg) was stored at –80°C until use.

Preparation of an anti-LolB antibody

Although LolB purified from the LolB-overproducing strain by column chromatography did not exhibit impurities on SDS–PAGE, an anti-LolB antibody was raised in rabbits against further purified LolB, which was electroeluted from the SDS gel.

Construction of a lac–lolB mutant

A 8.0 kb *Kpn*I fragment carrying the *lac–lolB* gene, the *kan* gene and the 5′- and 3′-flanking regions of *lolB* was isolated from pMAN654. FS1576, a *recD* mutant, was transformed with the *Kpn*I fragment and then kanamycin-resistant transformants were selected on L-broth plates containing 1 mM IPTG as described (Stahl *et al.*, 1986; Ogura *et al.*, 1989). The three transformants thus isolated required IPTG for both growth and LolB synthesis. One of them, SM602, was used in this study.

Partial purification of a soluble LolB derivative (mLolB)

A periplasmic fraction was prepared from 8.7 g (wet weight) of MC4100 harboring pYKT102, which was grown in the presence of 1 mM IPTG, as described previously (Matsuyama *et al.*, 1995). The periplasmic fraction (180 ml) was concentrated with an Amicon concentrator, and then dialyzed against 25 mM Na acetate (pH 5.0). The concentrated fraction (50 ml) was applied to a MonoS column (1×10 cm, Pharmacia), which had been equilibrated with 25 mM Na acetate (pH 5.0). The column was then eluted at the flow rate of 4 ml/min with a linear gradient of NaCl (0–0.4 M). The amount of mLolB in each fraction was determined by densitometric scanning of an SDS gel stained with Coomassie brilliant blue. The fraction which contained mLolB at the highest purity (60%) was used.

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