Large-scale preparation of the homogeneous LolA–lipoprotein complex and efficient in vitro transfer of lipoproteins to the outer membrane in a LolB-dependent manner

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Abstract

An ATP-binding cassette transporter LolCDE complex of *Escherichia coli* releases lipoproteins destined to the outer membrane from the inner membrane as a complex with a periplasmic chaperone, LolA. Interaction of the LolA–lipoprotein complex with an outer membrane receptor, LolB, then causes localization of lipoproteins to the outer membrane. As far as examined, formation of the LolA–lipoprotein complex strictly depends on ATP hydrolysis by the LolCDE complex in the presence of LolA. It has been speculated, based on crystallographic and biochemical observations, that LolA undergoes an ATP-dependent conformational change upon lipoprotein binding. Thus, preparation of a large amount of the LolA–lipoprotein complex is difficult. Moreover, lipoproteins bound to LolA are heterogeneous. We report here that the coexpression of LolA and outer membrane-specific lipoprotein Pal from a very efficient plasmid causes the unusual accumulation of the LolA–Pal complex in the periplasm. The complex was purified to homogeneity and shown to be a functional intermediate of the lipoprotein localization pathway. In vitro incorporation of Pal into outer membranes revealed that a single molecule of LolB catalyzes the incorporation of more than 100 molecules of Pal into outer membranes. Moreover, the LolB-dependent incorporation of Pal was not affected by excess-free LolA, indicating that LolB specifically interacts with liganded LolA. Finally, the LolB depletion caused the accumulation of a significant amount of Pal in the periplasm, thereby establishing the conditions for preparation of the homogeneous LolA–lipoprotein complex.

Keywords: bacterial lipoprotein; membrane sorting; LolA; Pal; coexpression; LolB

Escherichia coli has at least 90 species of lipoproteins, most of which are anchored to the periplasmic leaflet of the outer membrane, while some are anchored to that of the inner membrane through acyl chains attached to the N-terminal Cys (Juncker et al. 2003; Brokx et al. 2004; Miyadai et al. 2004; Tokuda et al. 2007). Lipoproteins are synthesized as precursors in the cytoplasm and then translocated across the inner membrane by a Sec apparatus, followed by sequential processing to the mature forms on the periplasmic leaflet of the inner membrane, i.e., attachment of the diacylglyceryl moiety to the Cys residue at the N terminus of the mature region, cleavage of the signal peptide, and transfer of an acyl chain to the N-terminal Cys (Hayashi and Wu 1990; Sankaran and Wu 1994), thereby generating N-acyl-S-sn-1,2-diacylglycerylcysteine at the N terminus (Jackowski et al. 1991; Pugsley 1993).

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Five Lol proteins (A, B, C, D, and E) constitute the Lol system, which catalyzes the sorting and outer membrane localization of lipoproteins (Narita et al. 2004; Tokuda and Matsuyama 2004) and are essential for the growth of *E. coli*. The LolCDE complex, an ABC (ATP-binding cassette) transporter, releases outer membrane-specific lipoproteins from the inner membrane in an ATP-dependent manner (Yakushi et al. 2000), leading to the formation of a water-soluble complex comprising one molecule each of a lipoprotein and LolA in the periplasm (Matsuyama et al. 1995). LolA then transfers the associated lipoprotein to outer membrane receptor LolB (Matsuyama et al. 1997). LolB is itself an outer membrane lipoprotein and catalyzes the membrane anchoring of lipoproteins. The sorting of lipoproteins depends on the residue at position 2 in *E. coli* (Yamaguchi et al. 1998; Seydel et al. 1999). Lipoproteins with Asp at position 2 remain in the inner membrane, because Asp functions as a LolCDE avoidance signal (Masuda et al. 2002; Hara et al. 2003; Miyamoto and Tokuda 2007), whereas other residues direct lipoproteins to the outer membrane (Terada et al. 2001).

The structures of lipoprotein-free LolA and LolB are strikingly similar to each other, whereas their amino acid sequences are dissimilar (Takeda et al. 2003). Both proteins comprise an incomplete β-barrel with an internal hydrophobic cavity, which is presumably the binding site for lipoprotein acyl chains and covered by a lid composed of three α-helices. The lid closes the hydrophobic cavity of LolA, because hydrogen bonds are formed between Arg at position 43 located in the β-barrel and residues in the α-helices. On the other hand, the hydrophobic cavity of LolB is open. Indeed, polyethylene glycol monomethyl ether, which had been used in the crystallization process, was present in the hydrophobic cavity of one crystal form of LolB (PDB accession no. 1IWN). It has been speculated, based on crystallographic and biochemical data, that the LolA lid undergoes opening and closing upon binding and release of lipoproteins, respectively (Taniguchi et al. 2005; Watanabe et al. 2006). A LolA–lipoprotein complex is formed when LolA is added to release lipoproteins from spheroplasts or proteoliposomes reconstituted with LolCDE. On the other hand, the LolA–lipoprotein complex was not formed in vitro even when LolA and lipoproteins were mixed under various conditions; lipoproteins dissolved in detergent were rapidly diluted with a solution containing LolA or dialyzed with LolA against a solution containing no detergent, or LolA was denatured and renatured in the presence of lipoproteins (H. Tokuda, unpubl.). It is therefore speculated that LolCDE utilizes ATP energy for not only membrane detachment of lipoproteins but also opening of the LolA lid. Because of these mechanisms, preparation of a large amount of the LolA–lipoprotein complex was not possible. The lipoprotein transfer from LolA to LolB and then to outer membranes was therefore examined with supernatants obtained after the LolA-dependent release reaction. Since the supernatants contained a small amount of the LolA–lipoprotein complex and excess-free LolA, it remained to be clarified whether or not free LolA affects the transfer reaction. Moreover, the LolA–lipoprotein complex in the supernatants was heterogeneous as to associated lipoproteins, rendering kinetic examination difficult.

We report here the construction of a plasmid from which LolA and outer membrane Pal can be efficiently coexpressed and large-scale preparation of the LolA–lipoprotein complex. Taking advantage of the homogeneous LolA–Pal complex, we examined the effect of free LolA on lipoprotein transfer to LolB. We show that a large amount of Pal can be delivered to the outer membrane through the LolA–LolB pathway.

Results

Accumulation of the LolA–Pal complex in the periplasm

Lipoproteins are highly hydrophobic because of their N-terminal acyl chains and rapidly transferred from the inner to the outer membrane by the Lol system. Therefore, lipoproteins are not found in the periplasm unless LolA (Miyamoto et al. 2001; Taniguchi et al. 2005; Watanabe et al. 2006) or LolB (Tanaka et al. 2001; Wada et al. 2004) is mutated. Since we have frequently examined the interaction of LolA with outer membrane-specific lipoprotein Pal, we tried to find conditions that cause the periplasmic accumulation of a large amount of the LolA–Pal complex. When Pal alone was overexpressed from a high copy vector, pTTQ18, Pal was localized in the outer membrane and not accumulated in the periplasm (Fukuda et al. 2002). We thought that the LolA–Pal complex might be accumulated in the periplasm if LolA and Pal are efficiently coexpressed from the same plasmid. We therefore cloned the genes for LolA and Pal in tandem, and placed them under the control of both the T7 promoter and *lac* operator in a plasmid, pCDF-Duet. A FLAG-tag and hexahistidine (His)-tag were attached to the C termini of LolA and Pal, respectively, to facilitate their purification. The two proteins were induced by the addition of 50 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The induced cells continued to grow for 3–4 h. Subcellular fractionation of *E. coli* cells revealed that Pal-His was present in the periplasm when expressed on the addition of IPTG (Fig. 1A). Although most Pal molecules including the Pal precursor were found in the total membrane fraction, about 4% of them were accumulated in the periplasm. No Pal molecules were detected in the periplasm in the absence of

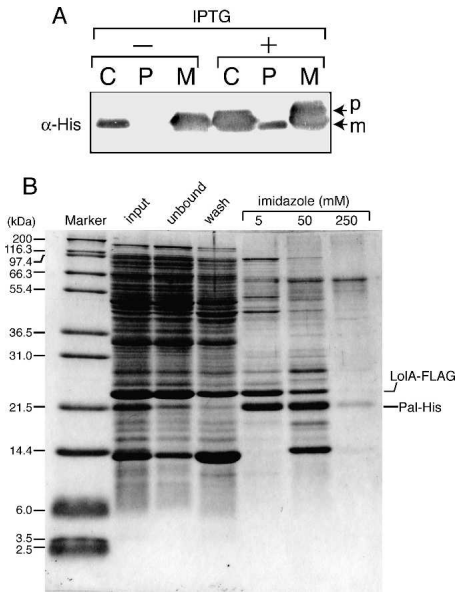

Figure 1. Coexpression of LolA and Pal causes the periplasmic accumulation of Pal. (*A*) *E. coli* BL21(DE3) cells harboring pSS9, which encodes both LolA and Pal, were induced with IPTG for 2 h, and then fractionated into periplasm and total membranes as described under Materials and Methods. SDS-PAGE analysis was performed for whole cells (C) in 1 μL of culture with $OD_{660} = 1$, and 1 μg each of periplasm (P) and total membranes (M). Pal was visualized by Western blotting with anti-His-tag antibodies. As a control, noninduced cells grown in the absence of IPTG were also analyzed. The positions of the precursor (p) and mature (m) forms of Pal are indicated at the *right*. (*B*) The periplasmic fraction mentioned in *A* was dialyzed and then applied to a TALON column. The LolA–Pal complex was eluted as described under Materials and Methods. SDS-PAGE analysis was performed for the initial periplasmic fraction (30 μg), TALON unbound fraction (30 μg), washing fraction (150 μL), and fractions (0.5 mL) eluted with the indicated concentrations of imidazole.

IPTG, although leaky expression of Pal took place. These results suggested that extensive expression of Pal together with LolA caused accumulation of the LolA–Pal complex in the periplasm. Indeed, the periplasmic fraction contained significant amounts of LolA-FLAG and Pal-His (Fig. 1B), the latter of which is normally not detected in the periplasm. We tried to purify the LolA–Pal complex by two-step affinity chromatography, i.e., first on a FLAG-tag column and then on a His-tag column. However, the yield of the LolA–Pal complex was very low (about 100 ng/L of culture), since significant amounts of proteins were lost with the first FLAG-tag column. We therefore applied the periplasmic fraction directly to a His-tag affinity TALON column. LolA was recovered in the fractions eluted with 5 and 50 mM imidazole, which also eluted Pal-His (Fig. 1B). Since both fractions contained impurities, we further purified them by size-exclusion chromatography.

The fraction eluted with 5 mM imidazole was centrifuged and the supernatant was subjected to size-exclusion chromatography. The fraction gave a single peak at a position corresponding to a molecular mass slightly larger than 43 kDa (Fig. 2A). Each fraction obtained on the size-exclusion chromatography was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2B). The peak fraction (elution volume = 11.25 mL) contained one molecule each of LolA and Pal as major components. It was previously reported that LolA (20 kDa) behaves as a 27-kDa protein on size-exclusion chromatography (Matsuyama et al. 1995).

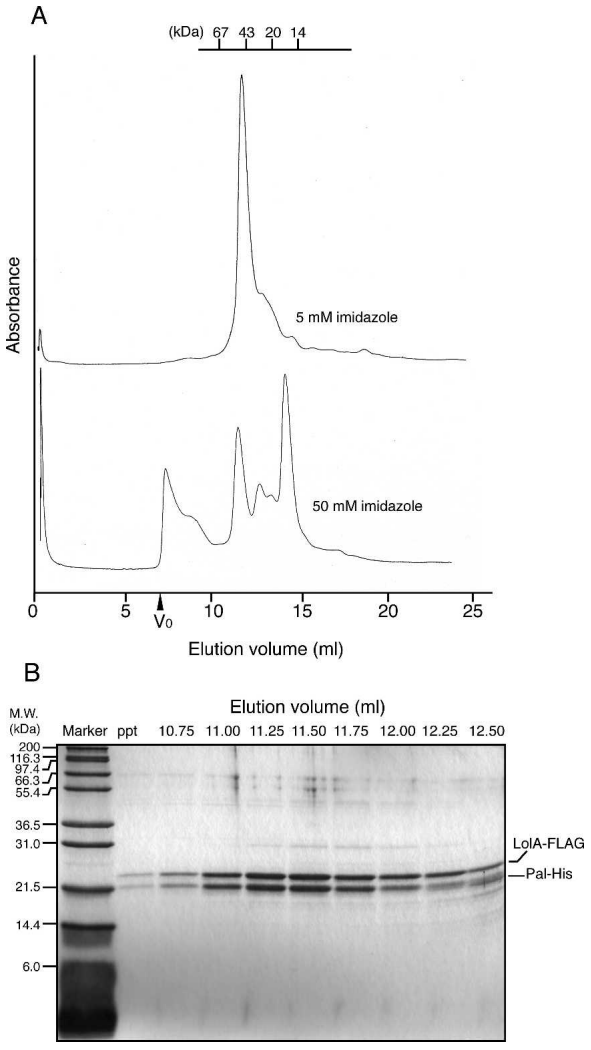

Figure 2. Size exclusion chromatography of the LolA–Pal complex. (*A*) The fractions eluted from a TALON column with 5 (*top*) and 50 (*bottom*) mM imidazole shown in Figure 1B were applied to a Superdex 75 column. V_0 represents the void volume. The elution positions of molecular mass markers are indicated at the *top*. (*B*) Aliquots (25 μL) of the indicated fractions obtained on size-exclusion chromatography of the 5 mM imidazole eluate shown in *A* were analyzed by SDS-PAGE, followed by staining with CBB. (ppt) Pellet obtained on centrifugation of 5 mM imidazole eluate at 100,000*g* for 60 min.

These results indicate that the single peak represents the LolA–Pal complex accumulated in the periplasm of cells coexpressing LolA and Pal (17 kDa). In contrast to the 5-mM imidazole fraction, proteins eluted with 50 mM imidazole were easily aggregated and gave various peaks (Fig. 2A), suggesting that this fraction does not contain LolA–Pal as a major component. Thus, the LolA–Pal complex of more than 90% purity was isolated in a yield of 1∼2 mg/L of culture on size-exclusion chromatography of the 5 mM imidazole eluate from a metal-affinity column. It was also found that the LolA–Pal complex accumulated in the periplasm was stable on overnight dialysis at 4°C and a cycle of freezing–thawing. These properties are important for future crystallographic study of the LolA–Pal complex.

In vitro incorporation of a large amount of Pal into outer membranes

To determine whether or not the purified LolA–Pal complex is a functional intermediate of the lipoprotein transfer reaction, the complex was incubated with outer membranes containing or not containing LolB for 30 min at 30°C, followed by fractionation into pellets and supernatants (Fig. 3). Essentially, all LolA and Pal molecules remained in the supernatant when incubation was performed in the absence of outer membranes (Fig. 3A, lane 2). About 60% of the Pal molecules (0.9 μg, 53 pmol) were incorporated into 40 μg of the Pal-depleted outer membrane (lane 3), which contained 0.3 ∼ 0.6 pmol LolB (Matsuyama et al. 1997), indicating that one molecule of LolB catalyzed the incorporation of about 100 molecules of Pal, although this is a rough estimation. As a result, Pal became a major component of the outer membrane in vitro (Fig. 3A, cf. lanes 3 and 5). When the LolA–Pal complex was incubated with LolB-depleted outer membranes, no Pal molecules were incorporated into outer membranes (lane 7), indicating that the incorporation is strictly dependent on LolB, as previously reported (Matsuyama et al. 1997; Tanaka et al. 2001; Wada et al. 2004). When outer membranes containing an overproduced amount of LolB were used, the incorporation of Pal was stimulated (see Fig. 4). The LolB-depleted outer membranes contained Pal expressed from the chromosome. However, the level of Pal incorporated in vitro (Fig. 3A, lane 3) was significantly higher than this chromosomally encoded level (lanes 7 and 9). It may be noteworthy that the protein profile of Pal-depleted membranes was slightly different from those of others such as LolB-depleted ones because of the *ΔtolB-pal* mutation. Although an unidentified band migrated to almost the same position as that of LolA-FLAG in LolB-depleted membranes, this band did not react with anti-LolA antibodies (data not shown).

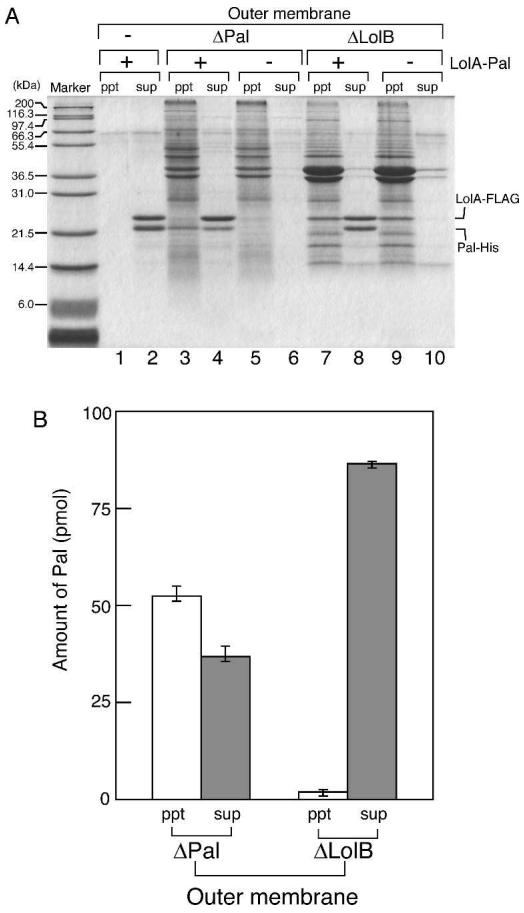

Figure 3. LolB-dependent transfer of a large amount of Pal from the LolA–Pal complex to outer membranes. (*A*) The LolA–Pal complex (3 μg) was incubated at 30°C for 30 min with 40 μg of ΔPal (lanes *3*,*4*) or ΔLolB (lanes *7*,*8*) outer membranes. The reaction mixture was fractionated into a supernatant (sup) and pellet (ppt) by centrifugation, followed by SDS-PAGE analysis and CBB staining. Where specified, the LolA–Pal complex was incubated in the absence of outer membranes (lanes *1*,*2*), or outer membranes were incubated in the absence of the LolA–Pal complex (lanes *5*,*6*,*9*,*10*), and then analyzed by SDS-PAGE as described *above*. (*B*) The amounts of Pal transferred from the LolA–Pal complex to ΔPal and ΔLolB outer membranes were determined three times and the averages are shown with error bars.

Free LolA does not inhibit the outer membrane incorporation of Pal

The LolA–lipoprotein complex was previously obtained only through the lipoprotein release reaction performed with spheroplasts or proteoliposomes in the presence of a saturating amount of LolA. Therefore, the LolB-dependent incorporation of lipoproteins from the LolA–lipoprotein complex into outer membranes was always examined in the presence of excess-free LolA. To determine whether or not free LolA has any effect on the LolB-dependent outer membrane incorporation of Pal, the homogeneous LolA–Pal complex was incubated with

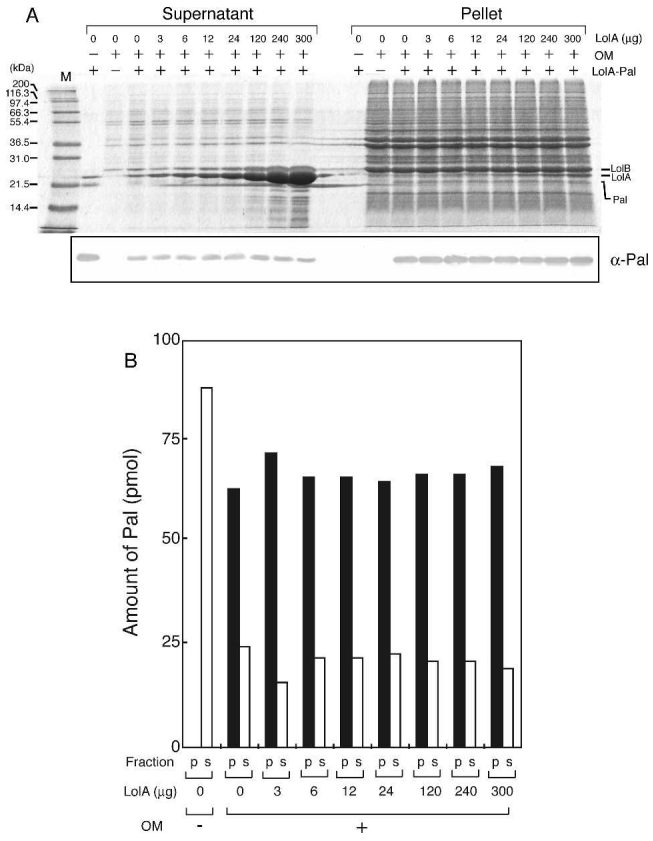

Figure 4. Free LolA does not inhibit the incorporation of Pal. (*A*) The LolA–Pal complex (6 μg) was incubated at 30°C for 30 min with 40 μg of ΔPal outer membranes containing an overproduced amount of LolB in the presence of the indicated amounts of LolA. The reaction mixture was fractionated into a pellet and supernatant, followed by precipitation with trichloroacetic acid. Aliquots (1/2) of each sample were analyzed by SDS-PAGE and CBB staining. The gel was also analyzed by Western blotting with anti-Pal antibodies (*lower* panel). (*B*) The amounts of Pal were quantified from the Western blotting shown in *A*.

Pal-depleted outer membranes in the presence of various amounts of free LolA (Fig. 4). The incorporation of Pal into outer membranes was examined by Coomassie Brilliant Blue (CBB) staining (upper panel) and Western blotting with anti-Pal antibodies (lower panel). The outer membranes used here were prepared from cells overproducing LolB. Free LolA did not affect the incorporation of Pal even when 0.16 nmol of the LolA–Pal complex was incubated in the presence of 300 μg (15 nmol) of free LolA. These results strongly indicate that LolB specifically interacts with liganded LolA and that the interaction between free LolA and LolB, if any, is very weak.

LolB depletion increases the amount of periplasmic Pal

The number of molecules of the major outer membrane lipoprotein, Lpp, in a single cell is extremely high (about 10^5–10^6) (Braun 1975). Because of this, the growth of *E. coli* cells expressing Lpp is immediately arrested when the Lol system is impaired (Miyamoto et al. 2001; Tanaka et al. 2001). The unusual periplasmic accumulation of the LolA–Pal complex in BL21(DE3) cells expressing both LolB and Lpp strongly suggested that the amount of the complex exceeded the capacity of LolB when LolA and Pal were efficiently coexpressed from pSS9. We therefore expected that the lack of both LolB and Lpp may cause further accumulation of Pal in the periplasm, since LolB-depletion has been found to cause the periplasmic accumulation of lipoproteins (Tanaka et al. 2001), and since Lpp$^-$ cells are known to be more resistant to LolB depletion (Wada et al. 2004). Thus, the KT5 (*ΔlolB::kan lpp*) strain harboring pKT021, which carries the temperature-sensitive replication origin and *lolB*, was transformed with pSS9 and then grown at 42°C to delete pKT021. Growth of cells (generation time = ~70 min) was inhibited by LolB depletion after about seven generations (data not shown). Coexpression of LolA and Pal was induced at various time points and the levels of LolB and Pal were determined (Fig. 5). The level of LolB became undetectable after about five generations in both the presence and absence of ITPG. The level of Pal in the periplasm increased for about four generations, whereas that in membranes remained nearly constant. As a result, more than half of the Pal expressed was accumulated in the periplasm after four generations. Moreover, the Pal precursor was not detected in the membrane fraction, presumably because of the absence of Lpp. Pal detected in the membrane fraction most likely represents the mature form localized in the outer membrane, and that detected in the absence of IPTG was expressed from the chromosome. The levels of Pal expressed from pSS9 were similar in BL21 (Fig. 1) and KT5 (Fig. 5) (data not shown). These results indicate that co-overexpression of LolA and Pal in cells depleted of both Lpp and LolB results in the accumulation of a significant amount of the LolA–lipoprotein complex in the periplasm.

The LolA–Pal complex was purified from the periplasm of KT5 cells harboring pSS9 by means of TALON affinity column and size exclusion column as described before. The purified complex contained only Pal and LolA on CBB-stained SDS-PAGE (Fig. 6A). Furthermore, outer membrane-specific lipoproteins, YcfM, YfgL, and YfiO, were not detected in the purified LolA–Pal complex even after immunoblotting, whereas these were detected in the periplasm (Fig. 6B). The yield of this highly purified LolA–Pal complex was two- to threefold better than that obtained from BL21(DE3)/pSS9.

Discussion

The number of molecules of Lpp in a single cell is about 10^5–10^6, while those of LolA and LolB expressed from

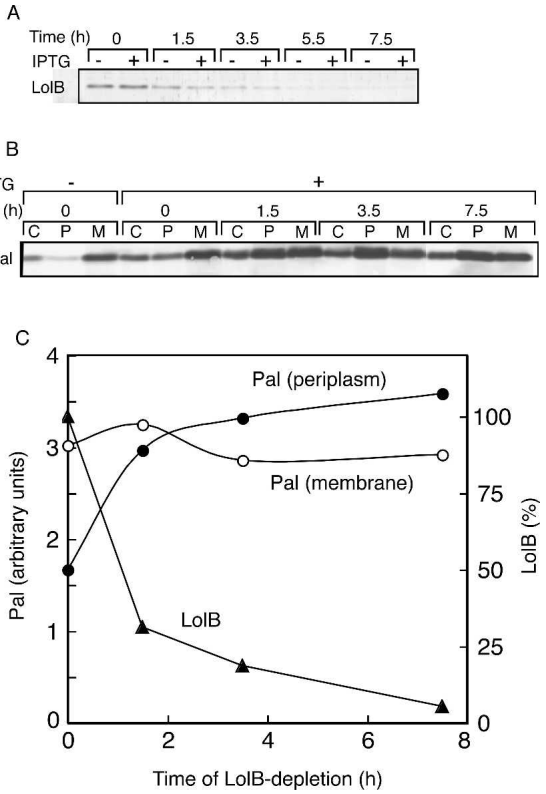

Figure 5. Periplasmic accumulation of Pal in cells depleted of both Lpp and LolB. KT5 (*ΔlolB::kan lpp*) cells harboring pKT021, which carry a temperature-sensitive replicon and *lolB*, were transformed with pSS9 and then grown at 42°C to delete pKT021 for about 12 h by inoculating a portion of a culture into fresh medium. LolA and Pal were coexpressed from pSS9 for 1.5 h by the addition of IPTG at the indicated times after the start of LolB-depletion. (*A*) The levels of LolB in induced and uninduced cells were determined by SDS-PAGE and Western blotting with anti-LolB antibodies. (*B*) The levels of Pal in whole cells, the periplasm, and total membranes were determined by SDS-PAGE and Western blotting with anti-His-tag antibodies. As a control, the level of Pal in uninduced cells was also determined at 0 time. Whole cells (C) derived from 2.5 μL of culture with $OD_{660} = 1$ were analyzed. The periplasmic (P) and total membrane (M) fractions analyzed were each derived from 150 μL of culture with $OD_{660} = 1$. (*C*) The levels of LolB and Pal in *A* and *B* were determined and plotted as a function of time after the start of LolB depletion.

the chromosome are only about 400 each (Matsuyama et al. 1997). It was therefore speculated that the activity of the Lol system is very high. Indeed, overexpression of lipoproteins did not cause their periplasmic accumulation. The accumulation of lipoproteins in the periplasm was previously observed only when LolA (Miyamoto et al. 2001; Taniguchi et al. 2005; Watanabe et al. 2006) or LolB (Tanaka et al. 2001; Wada et al. 2004) was mutated. On the other hand, when LolA and Pal were coexpressed from an efficient vector, pCDF-Duet, about 4% of the overexpressed Pal was accumulated as a complex with LolA in the periplasm (Fig. 1A). The accumulated LolA–Pal complex was purified to homogeneity (Fig. 2) and found to be an intermediate of the lipoprotein transfer to the outer membrane (Fig. 3). Co-overexpression of LolA and Pal also caused accumulation of the precursor form of Pal in the membrane fraction (Fig. 1A), suggesting that the amount of overexpressed Pal exceeds the lipoprotein releasing capacity of LolCDE in cells expressing Lpp. However, since the LolA–Pal complex was accumulated in the periplasm, the rate of the reaction catalyzed by the LolCDE complex seems to be faster than that catalyzed by LolB. Therefore, the reaction catalyzed by LolB is a rate-limiting step under these conditions. Indeed, KT5 cells lacking both Lpp and LolB accumulated a strikingly higher amount of Pal in the periplasm (Fig. 5). Unlike the BL21 cells possessing Lpp and LolB, the precursor form of Pal was not accumulated in the KT5 cells lacking both Lpp and LolB. Thus, these cells are expected to be very useful for the large-scale preparation of the homogenous LolA–lipoprotein complex. In addition to Pal, outer membrane-specific lipoproteins RlpB and YcfM were also accumulated in the periplasm of BL21 cells when overexpressed with LolA (S. Watanabe and H. Tokuda, unpubl.).

The outer membrane incorporation of lipoproteins was previously examined with a spheroplast supernatant

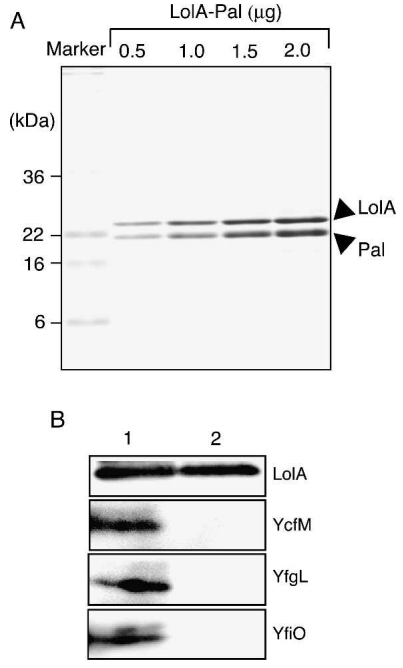

Figure 6. Purification of the homogenous LolA–Pal complex. The LolA–Pal complex was purified from the periplasmic fraction of KT5/pSS9 by TALON and size exclusion columns as mentioned in the legends to Figures 1 and 2. (*A*) Indicated amounts of the LolA–Pal complex were analyzed by SDS-PAGE and stained with CBB. (*B*) The periplasmic fraction (1) and the LolA–Pal complex (2) containing 150 ng of LolA were analyzed by SDS-PAGE and immunoblotting with antibodies raised against specified lipoproteins as reported (Ito et al. 2006).

containing a small amount of the LolA–lipoprotein complex and excess-free LolA. Furthermore, the LolA–lipoprotein complex contained various lipoproteins expressed in spheroplasts. As shown here, isolation of the homogeneous LolA–Pal complex enabled quantitative examination of the LolB-dependent incorporation of lipoproteins into the outer membrane (Fig. 3). As speculated from the number of lipoprotein molecules and that of Lol proteins in a single cell, LolB was found to catalyze the incorporation of more than 100 molecules of Pal into outer membranes. Moreover, free LolA was found to have no effect on this incorporation reaction (Fig. 4), indicating that LolB specifically interacts with lipoprotein-associated LolA. It is therefore strongly suggested that LolA undergoes a conformational change upon the binding of a lipoprotein. The crystal structures of free LolA and LolB are similar to each other (Takeda et al. 2003). Both proteins comprise an incomplete β-barrel having an internal hydrophobic cavity covered by an α-helical lid. Since the transfer of lipoproteins from LolA to LolB efficiently occurs in the absence of any energy input (Matsuyama et al. 1997), the conformational change upon lipoprotein binding seems to be critical for the efficient one-way transfer reaction. It would be of great interest to determine the structure of liganded LolA. Our findings here are important for resolution of the structure of liganded LolA.

Materials and Methods

Materials

TALON Co^{2+} affinity resin (Clontech) was used to purify His-tagged proteins. Size exclusion chromatography and ion-exchange chromatography were carried out with Superdex 75 and MonoQ (GE Healthcare), respectively. Plasmid pCDF-Duet was purchased from Novagen. Restriction enzymes were from TaKaRa Bio, Inc. and New England Biolabs. IPTG and spectinomycin were from Sigma. Antibodies against Pal (Tajima et al. 1998) and His-tag (Ikegami et al. 2005) were raised in rabbits as described.

Bacterial stains and media

E. coli B strain BL21(DE3) (F^- *ompT* $hsdS_B[r_B^- m_B^-]$ *gal* [λ*c*I 857 *ind*1 *Sam*7 *nin*5 *lacUV*5-T7*gene*1] *dcm* [DE3]) (Sambrook et al. 1989) was used for protein overexpression. K-12 strain DH5α (F^- ϕ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17($r_K^- m_K^+$) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1) (Sambrook et al. 1989) was used for DNA manipulation. JC7752 (*supE, hsdS, met, gal, lacY, fhuA, ΔtolB-pal*) (Mizuno 1981) was used to prepare Pal-depleted outer membranes. KT6 (F^- *ΔlolB::kan pps his proA argE thi gal lac xyl mtl tsx* λ^-) harboring pKT021 carrying a temperature-sensitive replication origin and the gene for LolB (Tanaka et al. 2001) was used for preparation of LolB-depleted outer membranes. KT5 (KT6 *lpp*) harboring pKT021 (Tanaka et al. 2001) was used for the coexpression of LolA and Pal after transformation with pSS9. DH5α was grown in LB broth (containing per liter, 10 g Bacto trypton, 5 g Bacto yeast extract, and 5 g NaCl). BL21(DE3) was grown in 2xYT broth (containing per liter, 16 g Bacto trypton, 10 g Bacto yeast extract, and 5 g NaCl) (Sambrook et al. 1989). When required, spectinomycin or ampicillin was added at a concentration of 50 μg/mL.

Construction of a plasmid coexpressing LolA and Pal

The gene for Pal-His was amplified by PCR using the genome of *E. coli* HMS174 (DE3) (F^-, *relA*1 *hsdR gal* [λ*c*I 857 *ind*1 *Sam*7 *nin*5 *lacUV*5-T7*gene*1] [DE3]) (Sambrook et al. 1989) as a template and a pair of primers, 5′-GGAATTCCATATGcaactg aacaaagtgctgaaaggg-3′ and 5′-GGTTAATTA*ATGATGATGATG ATGATG*gtaaaccagtaccgcacgacggtt-3′, in which the uppercase letters represent nonnative sequences, restriction sites are underlined, and the His-tag is italicized. The PCR fragment was digested with NdeI and PacI, and then cloned into the same sites of pCDF-Duet to construct pSS4.

The gene for *lolA-FLAG* was amplified by PCR using pSW77 (Watanabe et al. 2006) as a template and a pair of primers, 5′-CATGCCATGGCAaaaaaaattgccatcacctgtgca-3′ and 5′-CGGAA TTCCG*TTACTTGTCGTCATCGTCTTTGTAGTCCTC*-3′, in which the uppercase letters represent nonnative sequences, restriction sites are underlined, and the FLAG-tag is italicized. After treatment with DpnI, the PCR fragment was digested with NcoI and EcoRI, and then cloned into the same sites of pSS4. The Ala to Met mutation generated at position 2 of LolA by this cloning was then corrected. The plasmid pSS9 thus constructed encodes LolA-FLAG and Pal-His, each under the control of both the T7 promoter and *lac* operator.

Subcellular fractionation

E. coli BL21 (DE3) cells harboring pSS9 were grown at 37°C in 2xYT medium supplemented with 50 μg/mL spectinomycin. When the culture OD at 660 nm reached 0.8, the cells were induced by the addition of 50 μM IPTG for 2 h and then converted to spheroplasts as described (Osborn et al. 1972). A periplasmic fraction was obtained as a spheroplast supernatant on centrifugation at 100,000*g* for 30 min. Spheroplasts were subjected to a cycle of freezing–thawing three times, and then resuspended in 50 mM sodium phosphate (pH 7.2) containing 5 mM $MgCl_2$ and 100 μg/mL DNase. This solution was incubated on ice for 30 min and then centrifuged at 10,000*g* for 10 min to remove unbroken cells. The supernatant was further centrifuged at 100,000*g* for 30 min to obtain the total membrane fraction as a pellet.

Purification of the LolA–Pal complex

A periplasmic fraction (300 mL) containing overproduced LolA and Pal was prepared from 12 L of culture as described above, dialyzed overnight at 4°C against 50 mM sodium phosphate (pH 7.2) containing 300 mM NaCl and 10 mM $MgCl_2$, and then applied to a TALON column (1 mL). The column was washed with 15 mL of 50 mM sodium phosphate (pH 7.2) containing 300 mM NaCl, and then eluted with 5 mL of 50 mM sodium phosphate (pH 7.2) containing 300 mM NaCl and a specified concentration of imidazole. The eluates were dialyzed against 50 mM sodium phosphate (pH 7.2) for 12 h at 4°C and then concentrated with an Amicon Ultra (Millipore).

The LolA–Pal complex eluted from the TALON column was centrifuged at 100,000*g* for 60 min to remove aggregated proteins. The supernatant was further purified with Superdex 75, which had been equilibrated with 50 mM sodium phosphate (pH 7.2). The column was developed with 50 mM sodium phosphate (pH 7.2) at the flow rate of 0.25 mL/min. Fractions (0.25 mL) were collected and analyzed by SDS-PAGE and CBB staining. Fractions containing the LolA–Pal complex were concentrated with Amicon Ultra.

Efficient in vitro outer membrane incorporation of Pal

The reported method (Matsuyama et al. 1997) was slightly modified to examine the incorporation of a large amount of Pal. Briefly, the purified LolA–Pal complex (3 μg) was centrifuged at 100,000*g* for 30 min to remove any insoluble materials. The supernatant was then incubated at 30°C for 30 min in the presence and absence of 40 μg of outer membranes prepared from JC7752 (ΔPal) or KT6 (ΔLolB) cells. The reaction mixture (100 μL) was chilled in ice water to terminate the reaction, and then fractionated into a supernatant and pellet by centrifugation at 100,000*g* for 30 min. The fractions were then analyzed by SDS-PAGE after precipitation with trichloroacetic acid, followed by CBB staining.

Preparation of LolA-His

To construct highly efficient LolA-producing plasmid pSS2, the *lolA-His* gene was amplified by PCR using pAM201 (Miyamoto et al. 2001) as a template and a pair of primers, 5′-CATG CCATGGCAaaaaaaattgccatcacctgtgca-3′ and 5′-CGGAATTC CG*TTAATGATGATGATGATGATGCTC*-3′, in which the uppercase letters represent nonnative sequences, restriction sites are underlined, and the His-tag is italicized. After treatment with DpnI, the PCR fragment was digested with NcoI and EcoRI, and then cloned into the same sites of pCDF-Duet. The Ala to Met mutation generated at position 2 of LolA on this cloning was then corrected. *E. coli* BL21 (DE3) cells harboring pSS2 were grown in 2xYT medium supplemented with 50 μg/mL spectinomycin at 37°C. When the culture OD reached 0.8, the cells were induced with 1 mM IPTG for 2 h, and then converted to spheroplasts. The periplasmic fraction was dialyzed overnight at 4°C against 50 mM sodium phosphate (pH 7.2) containing 300 mM NaCl and 10 mM $MgCl_2$, and then applied to a TALON column (1 mL). The column was washed with 15 mL of 50 mM sodium phosphate (pH 7.2) containing 300 mM NaCl. His-tagged LolA was eluted with 250 mM imidazole, and then dialyzed against 20 mM Tris-HCl (pH 8.0) overnight at 4°C. LolA-His was further purified on an anion exchanger MonoQ column, which had been equilibrated with 20 mM Tris-HCl (pH 8.0), with a linear gradient of NaCl (0–1.0 M) as reported (Matsuyama et al. 1995).

Effect of free LolA on outer membrane incorporation of Pal

The LolA–Pal complex (6 μg) was centrifuged at 100,000*g* for 30 min to eliminate any aggregated materials. The resultant supernatant and 40 μg of outer membranes prepared from JC7752 (ΔPal) cells harboring pYKT100 (Yokota et al. 1999), which carry *lolB* under P_{BAD}, were incubated at 30°C for 60 min in the presence of various amounts of LolA-His (0–300 μg). The outer membranes contained an overproduced amount of LolB. The reaction mixture (100 μL) was chilled in ice water to terminate the reaction, and then fractionated into a supernatant and pellet by centrifugation at 100,000*g* for 30 min. These fractions were analyzed by SDS-PAGE after TCA precipitation, with detection by CBB staining or Western blotting using anti-Pal antibodies.

Effect of LolB depletion on the amount of Pal accumulated in the periplasm

KT5 cells harboring pKT021 were transformed with pSS9 and then grown at 42°C, where replication of pKT021 is restricted, for about 12 h by inoculating a portion of a culture into fresh medium. Coexpression of LolA-FLAG and Pal-His was induced for 1.5 h at the specified time points during cultivation. As a control, cells were also grown without induction at 42°C. The levels of LolB in cells induced and not induced were determined by SDS-PAGE and Western blotting with anti-LolB antibodies. The levels of Pal-His in the periplasm and total membranes were determined by SDS-PAGE and Western blotting with anti-His-tag antibodies as described for the subcellular fractionation of BL21 (DE3) cells.

Other methods

Outer membranes were prepared by sucrose density gradient (25%–55%) centrifugation as reported (Matsuyama et al. 1995) and confirmed by the presence of OmpA and absence of SecG. SDS-PAGE analysis was according to Laemmli (1970). Densitometric quantification was performed with an ATTO Densitograph.

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