Genetic analyses of the in vivo function of LolA, a periplasmic chaperone involved in the outer membrane localization of *Escherichia coli* lipoproteins

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Abstract The major outer membrane lipoprotein (Lpp) of *Escherichia coli* is released from the cytoplasmic membrane into the periplasm as a complex with LolA, a periplasmic chaperone, prior to the localization in the outer membrane. To determine whether or not LolA is generally involved in the outer membrane localization of lipoproteins in vivo, the chromosomal *lolA* gene was manipulated so as to be controlled by the *lac* promoter-operator. Depletion of LolA caused a severe growth defect, and impaired the outer membrane localization of Lpp and Pal, another outer membrane lipoprotein. Although LolA depletion did not immediately arrest the growth of cells lacking Lpp, disruption of the chromosomal *lolA* gene was lethal to the *lpp*$^-$ strain, indicating that LolA is generally required for the outer membrane localization of lipoproteins, and therefore essential irrespective of the presence or absence of Lpp.

Key words: Lipoprotein; Outer membrane; LolA; Chaperone; Periplasm; *Escherichia coli*

1. Introduction

Many species of lipoproteins have been predicted to be present in various microorganisms from the results of complete genome sequencing. All lipoproteins are thought to be synthesized with a signal peptide at the N-terminus and then to be translocated across the inner (cytoplasmic) membrane in a Sec machinery-dependent manner [1,2]. Cys residues at the N-termini of mature lipoproteins are modified with lipids, which function as an anchor to the membrane. Lipid modification and processing to mature lipoproteins in *Escherichia coli* have been shown to take place in the inner membrane, followed by their localization to either the inner or outer membrane. The amino acid residue next to the lipid-modified Cys functions as a sorting signal [3]. An Asp residue at this position makes lipoproteins specific to the inner membrane whereas other residues direct lipoproteins to the outer membrane.

The release of Lpp from the inner membrane of spheroplasts was found to require a periplasmic fraction, which led to the identification of LolA as an Lpp releasing factor [4]. Inner membrane-specific lipoproteins were found to remain anchored to the inner membrane even in the presence of LolA. These results indicate that LolA is a molecular chaperone specific to the outer membrane lipoproteins. However, it remains to be determined whether LolA alone is the lipoprotein-specific chaperone or another chaperone exists for other species of lipoproteins. To address this, we constructed an *E. coli* mutant in which the chromosomal *lolA* gene is placed under a controllable promoter. We report here that LolA is a general chaperone for most, if not all, lipoproteins in *E. coli*.

2. Materials and methods

2.1. Materials

Restriction enzymes were purchased from Takara Shuzo. Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Sigma. Anti-LolA antiserum was prepared as described [4]. Anti-Pal antiserum was a gift from T. Mizuno, Nagoya University. Tran^{35}S label was obtained from ICN.

2.2. Bacterial strains

E. coli FS1576 [5] and JE5505 [6] were used.

2.3. Construction of a plasmid carrying lolA under the control of the lac promoter-operator

A 5.1 kb *Pst*I fragment containing the *lolA* locus was isolated from pMAN994 [4] and then cloned into the *Pst*I site of pBR322 to construct pTAN10. A synthetic DNA linker containing *Xho*I, *Bgl*II, *Sma*I and *Xba*I sites was inserted into pTAN10 at the *Pvu*I site, which is located immediately upstream of the ribosome binding site of *lolA*, to construct pTAN11. A 1.4 kb *Xho*I-*Bam*HI fragment of pSY343 [7] containing the kanamycin resistance gene (*kan*) was inserted into the *Xho*I-*Bgl*II sites of pTAN11 to construct pTAN12. A 205 bp *Pvu*II-*Xba*I fragment of pUC19 [8] carrying the *lac* promoter-operator (*lacPO*) was inserted into the *Sma*I-*Xba*I sites of pTAN12. The plasmid thus constructed, pTAN15, carries the *lolA* gene under the control of *lacPO* (*lac-lolA*), the *kan* gene located upstream of *lac-lolA* in the opposite direction, and the 5′- and 3′- chromosomal flanking regions of *lolA* (Fig. 1A).

2.4. Construction of pTAN20

A DNA fragment containing the *pal* gene was amplified by polymerase chain reaction with a pair of primers (5′-TTACCAGTCGACGAATTCAATAGTAAAGGAATCATTGAAATGCAACTG-3′ and 5′-TCTCGACCCGGGCAATTCTCTTAGTAAACCAGTACCGCACGA-3′) and the chromosomal DNA from MC4100, followed by digestion with *Sal*I and *Sma*I. The resultant fragment was inserted into the *Sal*I-*Sma*I site of pMAN885 [9] to construct pTAN20 carrying *pal* under the control of the arabinose promoter.

2.5. Membrane localization of lipoproteins

The preparation of an envelope fraction, sucrose density gradient centrifugation, SDS-polyacrylamide gel electrophoresis and fluorography were carried out as described [4]. The immunoprecipitation of Pal was performed according to the method described previously [10].

3. Results and discussion

3.1. Construction of an E. coli mutant carrying the chromosomal lolA gene under the control of lacPO

To replace the chromosomal *lolA* gene with the *lac-lolA* gene by homologous recombination, FS1576 (*recD*) was transformed with pTAN15, which carries *lac-lolA* and which had

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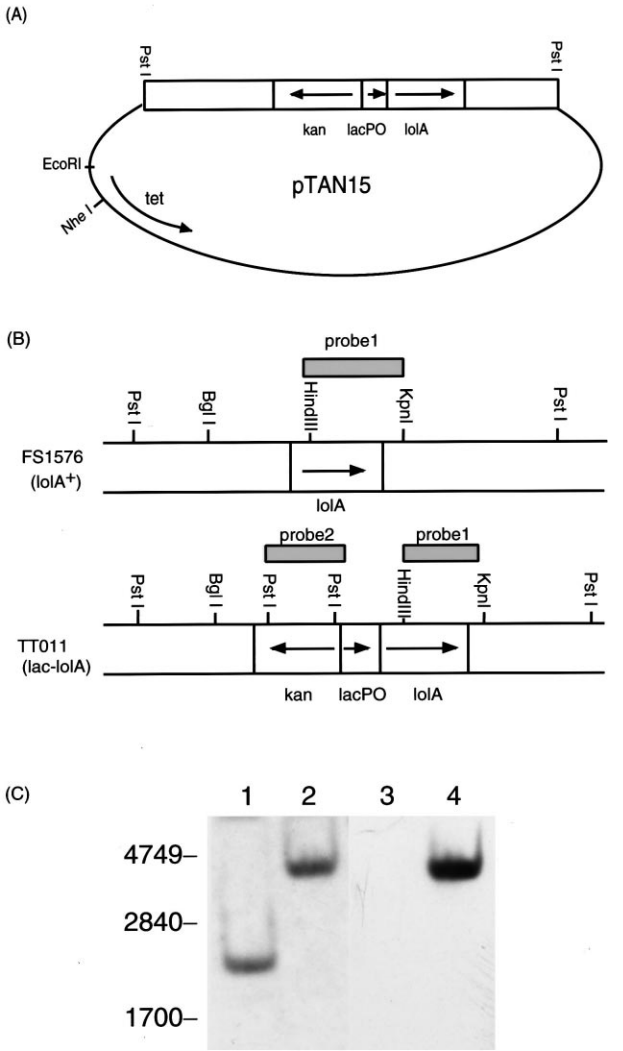

Fig. 1. Construction of *lac-lolA* mutant TT011. A: The structure of pTAN15 used for the construction of TT011 is shown. Arrows indicate the direction of the transcription. B: Restriction maps around the chromosomal *lolA* locus of FS1576 (*lolA*$^+$) and TT011 (*lac-lolA*) are shown. The 0.76 kb *Hin*dIII-*Kpn*I fragment (probe 1) and 0.96 kb *Pst*I fragment (probe 2) used for hybridization are also indicated. C: The genomic structure containing the *lolA* locus of FS1576 (lanes 1 and 3) and TT011 (lanes 2 and 4) was analyzed by Southern hybridization using probes 1 and 2. Chromosomal DNAs were digested with *Bgl*I and *Kpn*I, and then analyzed on a 0.8% agarose gel. Probe 1 (lanes 1 and 2) and probe 2 (lanes 3 and 4) were labeled with biotin using an Amersham biotin-labeling kit. The migration positions of DNA size markers are shown at the left.

been linearized by digestion with *Eco*RI and *Nhe*I (Fig. 1A). Transformants were selected for resistance to 25 μg/ml kanamycin on L-broth plates supplemented with 1 mM IPTG. One of five kanamycin-resistant transformants, TT011, was used for further analysis.

To confirm the genomic structure of the relevant region of TT011, the chromosomal DNAs of FS1576 and TT011 were digested with *Bgl*I and *Kpn*I, and then subjected to Southern hybridization analyses using a 0.76 kb *Hin*dIII-*Kpn*I fragment (probe 1) carrying most of the *lolA* gene (Fig. 1B). This probe hybridized with a 2.2 kb fragment derived from FS1576 DNA and a 3.7 kb fragment derived from TT011 DNA (Fig. 1C). A TT011 DNA fragment of the same size hybridized with a 0.96 kb *Pst*I fragment of the *kan* gene (probe 2), whereas no DNA fragment derived from FS1576 hybridized with probe 2. These results are consistent with the restriction map shown in Fig. 1B, suggesting that the *lolA* gene on the TT011 chromosome was replaced by the *lac-lolA* gene together with *kan*.

3.2. Growth inhibition by LolA depletion in lpp$^+$ cells

To determine whether or not the expression of *lolA* in TT011 (*lpp*$^+$) is controlled by *lacPO*, the amounts of LolA in TT011 cells grown in the presence and absence of 1 mM

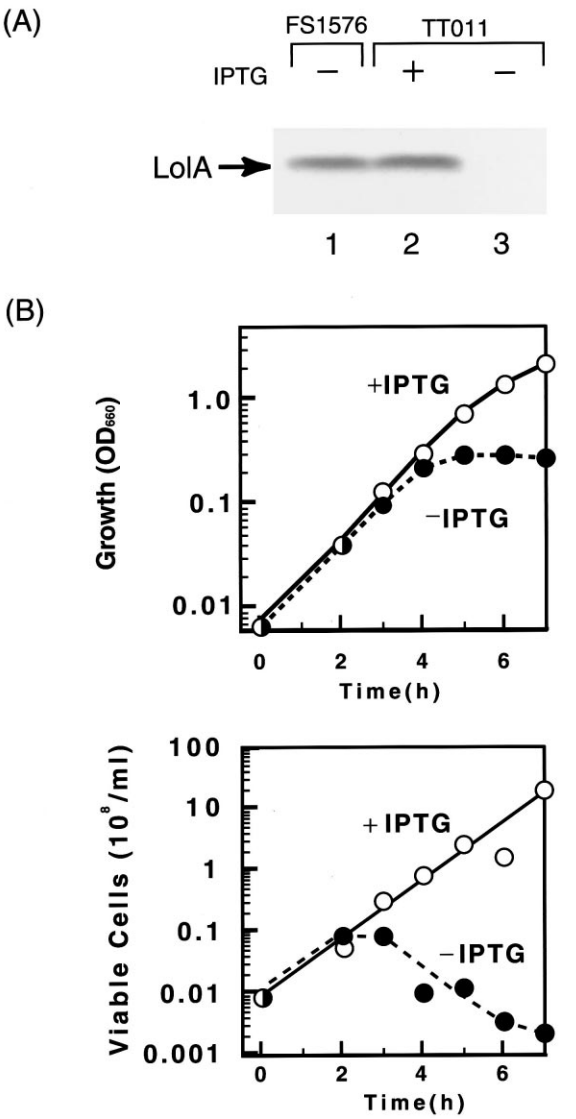

Fig. 2. Effect of LolA depletion on the growth of TT011. A: FS1576 (lane 1) and TT011 (lanes 2 and 3) were grown on L-broth containing 1 mM IPTG at 30°C. The cells were harvested, washed three times with fresh L-broth containing no IPTG, and then grown in L-broth with (lane 2) or without (lanes 1 and 3) 1 mM IPTG at 30°C for 5 h. Cells were harvested and treated with 1% SDS at 100°C for 5 min. Cellular proteins (80 μg) were analyzed by SDS-PAGE and immunoblotting with the anti-LolA antibody. B: TT011 was grown on L-broth with (open circles) or without (closed circles) IPTG as described in A. Growth was followed by monitoring the OD at 660 nm. The number of viable cells was determined on an L-broth plate containing 1 mM IPTG.

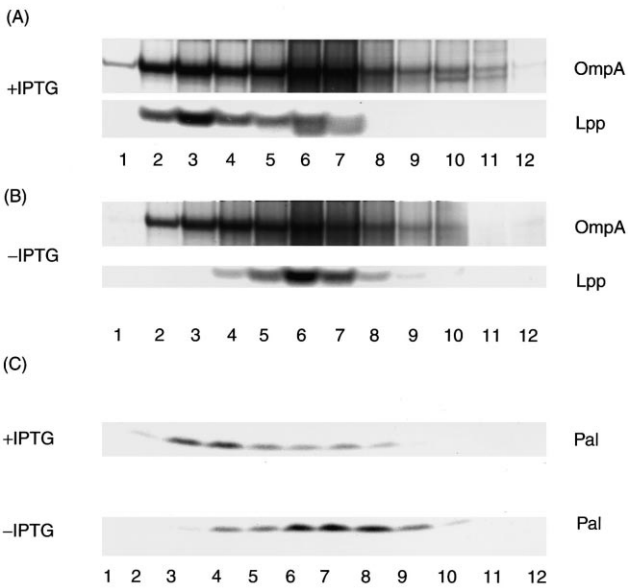

Fig. 3. Effect of LolA depletion on the in vivo localization of Lpp and Pal. TT015 harboring pJY811 (A and B) or pTAN20 (C) was grown in M63 minimal medium [13] with or without 1 mM IPTG at 30°C for 3 h. The synthesis of Lpp and Pal was induced for 5 min by the addition of 0.1% arabinose. Lpp and Pal were labeled in 1 ml of the culture with Tran^{35}S label (10 μCi) for 1 min and then chased by the addition of 12 mM each of methionine and cysteine for 30 s. The envelope fraction was prepared and then subjected to sucrose density gradient (25–55%, w/w) centrifugation, followed by fractionation into fractions 1–12, from the bottom to the top of the gradient. Each fraction was analyzed by SDS-PAGE and fluorography. Pal was analyzed after immunoprecipitation with an anti-Pal antibody.

IPTG were determined by means of Western blotting with the anti-LolA antibody (Fig. 2A). In the presence of IPTG, TT011 cells produced a comparable amount of LolA to FS1576 cells (lanes 1 and 2). In contrast, the cultivation of TT011 cells for 5 h in the absence of IPTG reduced the amount of LolA to an undetectable level (lane 3), indicating that the *lolA* gene of TT011 is under the control of *lacPO*. The depletion of LolA caused growth arrest and a decrease in the number of viable cells (Fig. 2B), indicating that the *lolA* gene is indispensable for the TT011 strain possessing Lpp.

3.3. Effects of LolA depletion on the in vivo localization of Lpp and Pal

To examine the in vivo role of LolA in the localization of outer membrane lipoproteins, TT011 cells were grown in the presence and absence of IPTG for 3 h, and then labeled with Tran^{35}S label for 1 min. An envelope fraction was prepared from these cells and then subjected to fractionation by sucrose density gradient centrifugation. The fraction prepared from the cells grown in the absence of IPTG was not separated into the inner and outer membranes. This was most likely caused by the formation of a covalent linkage between Lpp accumulated in the inner membrane and the peptidoglycan, since an Lpp derivative, LppDK, possessing the inner membrane sorting signal covalently attaches to the peptidoglycan and thus prevents the membrane separation [9]. When the synthesis of LppDK was induced for a short time, the membrane separation was significantly improved as the association of LppDK with the peptidoglycan was not significant [9]. We therefore constructed the TT015 strain (*lac-lolA lpp*$^-$) harboring pJY811 [9], which carries P_{BAD}-*lpp*, by introducing the *lac-lolA* gene into JE5505 (*lpp*$^-$)/pJY811 by P1 transduction.

The TT015 strain harboring pJY811 was grown in the presence and absence of IPTG for 3 h. The cells were induced to express *lpp* for 5 min by the addition of 0.1% arabinose and then labeled with Tran^{35}S label for 1 min, followed by a chase with non-radioactive methionine and cysteine for 30 s. The cell envelope fraction was prepared and then subjected to sucrose density gradient centrifugation to fractionate the membranes. Lpp was exclusively found in the outer membrane fraction containing OmpA when TT015/pJY811 was grown in the presence of IPTG (Fig. 3A). On the other hand, Lpp was recovered in the inner membrane fraction of the LolA-depleted cells (Fig. 3B), indicating that only LolA among all periplasmic proteins is involved in the in vivo release of Lpp. We also examined the localization of Pal, another outer membrane lipoprotein, in TT015 cells harboring pTAN20 carrying P_{BAD}-*pal*. The depletion of LolA also caused the inner membrane localization of Pal (Fig. 3C). However, this was only observed when Pal was labeled for a short time. When expressed for a longer period, a substantial portion of Pal was found in the outer membrane (data not shown), suggesting that either the outer membrane localization of Pal takes place at a slow rate due to incomplete depletion of LolA or a factor substituting for the LolA function exists.

3.4. Disruption of lolA in an lpp$^-$ strain

TT011 (*lac-lolA lpp*$^+$) exhibited a severe growth defect in the absence of IPTG (Fig. 2B), whereas strains such as TT015 (*lac-lolA lpp*$^-$) lacking Lpp were found to grow normally even in the absence of IPTG (data not shown). Furthermore, the inner membrane accumulation of Pal in TT015 cells was observed only when the cells were labeled for a short period. The number of Lpp molecules in a single cell is several orders of magnitude higher than those of other lipoproteins [11]. These results therefore suggest that either *lolA* is essential for *E. coli* only when *lpp* is expressed or an uninduced level of LolA is sufficient for the release of other outer membrane lipoproteins. To address this, we attempted to construct an *lpp* null mutant in the *lpp*$^-$ background.

The *kan* gene was inserted into the *Nde*I site within the *lolA* gene on pMAN994 to construct pTAN1001. FS1576 harboring pMAN995, which carries P_{BAD}-*lolA* and an ampicillin resistance gene, was transformed with pTAN1001 linearized with *Eco*RI and *Apa*LI. Kanamycin-resistant transformants were selected on L-broth plates supplemented with arabinose. One of them, TT020, was found to harbor intact pMAN995. Southern blot analysis of the chromosomal DNA from this strain confirmed that the *lolA* gene was disrupted. The *lolA::kan* gene was then introduced into JE5505 (*lpp*$^-$) by P1 transduction. Although many tiny colonies appeared on L-broth plates containing kanamycin, none of them could continue to grow.

Taking all the results presented here together, we conclude that the lipoprotein localization system involving LolA and LolB [12] is essential for *E. coli* irrespective of the presence or absence of Lpp. We therefore assume that an uninduced level of LolA in TT015 is sufficient for the release of lipoproteins including Pal since the cell lacks Lpp.

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