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# A short helix in the C-terminal region of LolA is important for the specific membrane localization of lipoproteins

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**Abstract The structures of a lipoprotein carrier, LolA, and a lipoprotein receptor, LolB, are similar except for an extra C-terminal loop containing a $3_{10}$ helix and β-strand 12 in LolA. Lipoprotein release was significantly reduced when β-12 was deleted. Deletion of the $3_{10}$ helix also inhibited the lipoprotein release. Furthermore, lipoproteins were non-specifically localized to membranes when LolA lacked the $3_{10}$ helix. Thus, the membrane localization of lipoproteins with the LolA derivative lacking the $3_{10}$ helix was independent of LolB whereas LolB was essential for the outer membrane localization of lipoproteins with the wild-type LolA.**



*Keywords:* Lipoprotein; Molecular chaperone; Outer membrane; Periplasm; LolA; *Escherichia coli*

## 1. Introduction

Lipoproteins are synthesized as precursors in the cytoplasm and then translocated across the inner membrane, followed by sequential processing to the mature forms on the periplasmic leaflet of the inner membrane [1]. Mature lipoproteins are anchored to either the inner or outer membrane through acyl chains attached to the N-terminal Cys [2]. Sorting of lipoproteins to the outer membrane is then catalyzed by the Lol system depending on the lipoprotein sorting signals located at position 2 [3].

Five Lol proteins (A–E) constitute the Lol system [3,4] and are essential for the growth of *Escherichia coli*. The LolCDE complex, an ATP-binding cassette transporter, releases outer membrane-specific lipoproteins from the inner membrane in an ATP-dependent manner [5], leading to the formation of a water-soluble complex comprising one molecule each of a lipoprotein and LolA in the periplasm [6]. LolA then transfers the associated lipoprotein to outer membrane receptor LolB [7]. LolB is itself an outer membrane lipoprotein and catalyzes the membrane anchoring of lipoproteins. Thus, the Lol system mediates efficient and one-way transport of lipoproteins from the inner to the outer membrane.

Although the amino acid sequences of LolA and LolB are dissimilar, their structures are strikingly similar except for an extra C-terminal loop in LolA [8]. This loop contains a short $3_{10}$ helix and β strand 12. We report here that the $3_{10}$ helix is important for prevention of the non-specific membrane localization of lipoproteins.

## 2. Materials and methods

### 2.1. Bacterial strains and growth

*E. coli* K-12 strains, DLP79-36 [9], JE5505 [10], SM704 [11], and TT016 [12] were used. TT016 carries the chromosomal *lolA* gene under the control of the lactose promoter-operator. Expression of this gene was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown on LB broth or LB agar plates (Difco). When required, chloramphenicol was added at 25 μg/ml.

### 2.2. Construction of LolA mutants

C-terminal deletion mutants of LolA were constructed by means of PCR using a QuickChange site-directed mutagenesis kit (Stratagene) with pAM201 carrying *lolA-His* under the control of $P_{BAD}$ [12] as a template and a pair of oligonucleotides (Supplementary Table 1) as primers. Mutations were confirmed by sequencing.

### 2.3. Purification of LolA derivatives

TT016 cells harboring pAM201 or a derivative of it were grown on LB broth supplemented with 0.1 mM IPTG at 37 °C. At the mid-exponential phase of growth, cells were induced with 0.2% arabinose for 2 h, and then converted into spheroplasts as reported [13]. A periplasmic fraction was obtained as a spheroplast supernatant, and then dialyzed against 20 mM Tris–HCl (pH 7.5) containing 300 mM NaCl and 10 mM $MgCl_2$ overnight at 4 °C. Hexahistidine-tagged (His-tag) LolA or a derivative was adsorbed to TALON resin (Clontech), and then eluted with 20 mM Tris–HCl (pH 7.5) containing 300 mM NaCl and 250 mM imidazole. The purified LolA proteins were kept at −80 °C in 20 mM Tris–HCl (pH 7.5) containing 10% glycerol after dialysis.

### 2.4. Release of L10P from spheroplasts

The release of L10P from spheroplasts was performed as described [14]. Briefly, *E. coli* DLP79-36 cells harboring pJYL10P ($P_{BAD}$-L10P) [9] were grown on M63 (0.5% NaCl), 0.2% maltose minimal medium at 37 °C. At $A_{660} = 0.8$, the cells were induced with 0.2% arabinose for 5 min and then converted into spheroplasts. The spheroplast suspension (300 μl) was kept on ice for 2 min in the presence and absence of LolA or one of its derivatives. Labeling was started at 30 °C by the addition of M63 (0.5% NaCl), 0.2% maltose minimal medium (750 μl) supplemented with 250 mM sucrose and 10 μCi of Tran$^{35}$S-label (a mixture of 70% [$^{35}$S]Met and 20% [$^{35}$S]Cys; 1000 Ci/mmol, MP Biochemicals). After 2 min, the labeling was chased for 2 min by the addition of 12 mM non-radioactive Met and Cys. The release reaction was terminated by chilling the reaction mixture on ice, followed by fractionation into spheroplasts and medium by centrifugation at

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*Abbreviations:* IPTG, isopropyl-β-D-thiogalactopyranoside; His-tag, hexahistidine-tag; DDM, *n*-dodecyl-β-D-maltopyranoside

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16 000 × g for 2 min. The spheroplasts and supernatants thus obtained were subjected to trichloroacetic acid precipitation and immunoprecipitation with anti-Lpp antibodies as reported [6]. $^{35}$S-labeled L10P was analyzed by SDS–PAGE and fluorography as reported [6].

### 2.5. In vitro membrane incorporation of L10P

The incorporation of L10P into outer membranes was examined as described previously [7]. Briefly, $^{35}$S-labeled L10P was released from spheroplasts as described above, and the spheroplast supernatant containing [$^{35}$S]L10P complexed with LolA or one of its derivatives was incubated with outer membranes containing or not containing LolB. The reaction mixture 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. Each fraction was subjected to trichloroacetic acid precipitation and immunoprecipitation with anti-Lpp antibodies, and then analyzed by SDS–PAGE and fluorography as reported [7].

### 2.6. Other methods

Outer membranes containing or not containing LolB were prepared from *E. coli* JE5505 cells or SM704 cells as reported [7], respectively. Immunoprecipitation was performed as described [15]. SDS–PAGE was carried out according to Laemmli [16] or, in the case of L10P, Hussain et al. [17]. Densitometric quantification was performed with an ATTO Densitograph.

## 3. Results

### 3.1. Construction of 12 C-terminal deletion mutants

The structures of LolA and LolB are very similar except for the extra C-terminal loop in LolA (Fig. 1A). This loop contains a less-conserved short $3_{10}$ helix and well-conserved β strand 12 among the indicated five γ proteobacteria (Fig. 1B). To determine the role of the C-terminal loop in the outer membrane sorting of lipoproteins, 12 mutants lacking various portions of the C-terminal region were constructed (Fig. 2A). TT016 (*lacPO-lolA*) cells require IPTG for growth, because

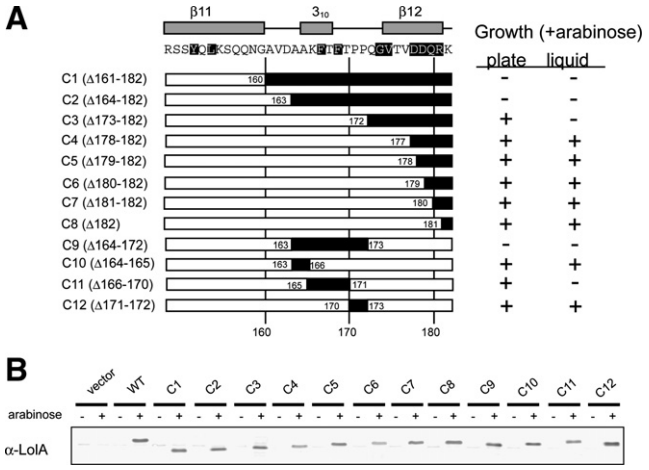


Fig. 2. Construction of C-terminal deletion mutants of LolA. (A) The deleted regions are indicated in black. Growth of TT016 (*lacPO-lolA*) cells expressing one of the indicated derivatives from pAM201 variants was examined on agar plates or liquid medium supplemented with arabinose alone. (B) Cells were grown on LB broth supplemented with 0.1 mM IPTG at 37 °C. The indicated LolA derivatives were then induced for 2 h with 0.2% arabinose. The induced cells ($1 \times 10^7$ cells) were analyzed by SDS–PAGE and immunoblotting with anti-LolA antibodies. Chromosomally encoded LolA was hardly visible under these conditions.

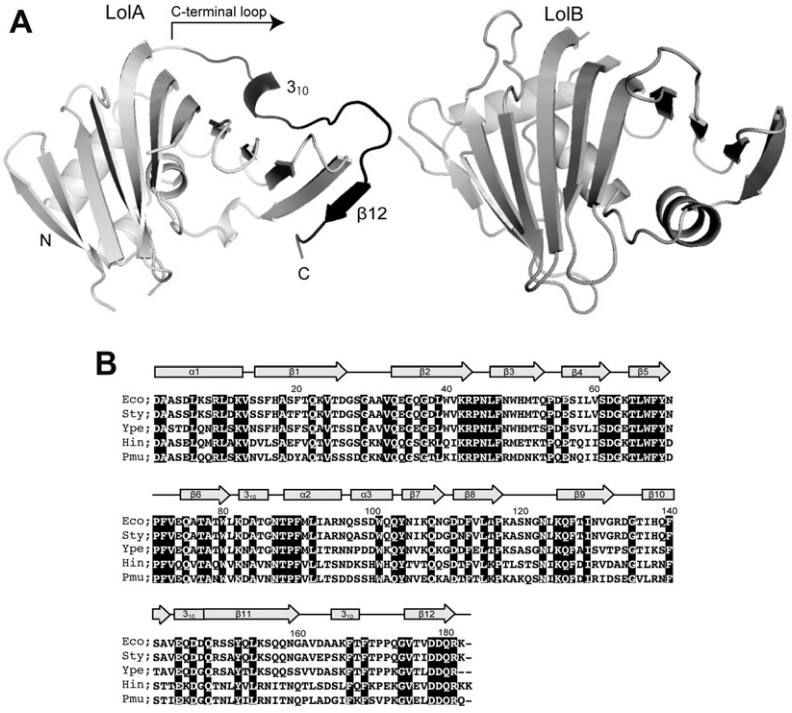


Fig. 1. Structures of LolA and LolB. (A) The structural information on LolA (1IWL, left) and LolB (1IWM, right) was obtained from the RCSB protein data bank (http://pdb.protein.osaka-u.ac.jp/pdb/) and visualized with PyMOL ver.0.98 (http://pymol.sourceforge.net/index.php). The C-terminal loop only present in LolA is indicated in black. β Strand 12 and a $3_{10}$ helix in the C-terminal loop are marked. (B) The sequences of LolA homologues were aligned using ClustalW (http://clustalw.genome.jp). Residues completely conserved among five gram-negative bacteria are highlighted in black. *Eco, E. coli; Sty, Salmonella typhimurium; Ype, Yersinia pestis; Hin, Haemophilus influenzae; Pmu, Pasteurella multocida.*