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Overexpression of LolCDE Allows Deletion of the *Escherichia coli* Gene Encoding Apolipoprotein *N*-Acyltransferase▽

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Bacterial lipoproteins represent a subset of membrane-associated proteins that are covalently modified with lipids at the N-terminal cysteine. The final step of lipoprotein modification, N-acylation of apolipoproteins, is mediated by apolipoprotein *N*-acyltransferase (Lnt). Examinations with reconstituted proteoliposomes and a conditional mutant previously indicated that N-acylation of lipoproteins is required for their efficient release from the inner membrane catalyzed by LolA and LolCDE, the lipoprotein-specific chaperone and ABC transporter, respectively. Because Lnt is essential for *Escherichia coli*, a mutant lacking Lnt activity has not been isolated. However, we report here that *lnt*-null strains can be constructed when LolCDE is overproduced in strains lacking either the major outer membrane lipoprotein Lpp or transpeptidases that cross-link Lpp with peptidoglycan. Lipoproteins purified from the *lnt*-null strain exhibited increased mobility on SDS-PAGE compared to those from wild-type cells and could be sequenced by Edman degradation, indicating that lipoproteins in this mutant exist as apolipoproteins that lack N-acylation. Overexpression of Lpp in the *lnt*-null strain resulted in the accumulation of apoLpp in the inner membrane and caused growth arrest. In contrast to the release of mature Lpp in the presence of LolA and LolCDE, that of apoLpp from the inner membrane was significantly retarded. Furthermore, the amount of lipoproteins copurified with LolCDE was significantly reduced in the *lnt*-null strain. These results indicate that the affinity of LolCDE for apolipoprotein is very low, and therefore, overexpression of LolCDE is required for its release and sorting to the outer membrane.

Bacterial lipoproteins are synthesized in the cytoplasm as precursors with N-terminal signal peptides. Modification of lipoproteins occurs after lipoprotein precursors have been translocated from the cytosol to the periplasmic side of the inner membrane through the action of Sec translocase. The modification reaction comprises three steps in Gram-negative bacteria: (i) formation of a thioether linkage between a cysteine in the N-terminal region and diacylglycerol by phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt); (ii) cleavage of a signal peptide by signal peptidase II (LspA), which turns the S-lipidated cysteine into the N-terminal residue of the mature protein; and (iii) N-acylation of this cysteine by apolipoprotein *N*-acyltransferase (Lnt) (Fig. 1) (30). Thus, lipoproteins have an N-terminal glycerylcysteine containing two ester-linked acyl chains and one amide-linked acyl chain. Gram-positive bacteria, except for actinomycetes, lack a homologous gene for Lnt. It is therefore considered that lipoproteins exist as diacylated forms without amide-linked acyl chains in most Gram-positive bacteria, though exceptions have been recently reported for several Gram-positive bacteria (1, 18, 47, 49). In Gram-negative bacteria, mature lipoproteins are further targeted to the outer membrane by the Lol system, which comprises the inner membrane ABC transporter LolCDE, the periplasmic lipoprotein carrier protein LolA, and the outer membrane receptor LolB (30). LolCDE binds a lipoprotein in the inner membrane and transfers it to LolA to form a soluble lipoprotein-LolA complex that traverses the periplasm to the outer membrane, where LolB accepts the lipoprotein and incorporates it into the lipid layer of the outer membrane. The residue next to the lipid-modified Cys functions as the sorting signal for lipoproteins in enterobacteria (20, 39, 45, 53). When Asp is present at this position (position 2), the lipoprotein remains in the inner membrane. Asp at position 2 is considered to make the N-terminal structure of lipoproteins distinct from those with other residues, thereby functioning as a "LolCDE avoidance signal" (10, 22).

The importance of triacylation for the outer membrane targeting of lipoproteins was first demonstrated by an *in vitro* study. It was shown that apoPal, an immediate precedent of mature Pal, was not released from proteoliposomes even in the presence of LolCDE and LolA (7). Because it is essential, only conditional-lethal mutants of *lnt* have been isolated. A temperature-sensitive mutant of *Salmonella enterica* that accumulated apolipoproteins at nonpermissive temperature was shown to have a mutation in the *lnt* gene (9). More recently, Robichon et al. (35) constructed a conditional mutant of *E. coli* in which the chromosomal *lnt* gene was placed under the arabinose-inducible promoter and showed that apolipoproteins synthesized in the absence of arabinose remained in the inner membrane. The functions of Lnt mutants have been studied by using a conditional *lnt* strain to identify essential residues, including the catalytic triad E267-K335-C387 (48). It has also been recently established that Lnt forms a thioester acyl-enzyme intermediate between C387 and an acyl group derived from phospholipids (3). On the other hand, it remained unclear how the lack of Lnt activity affects the functions of Lol proteins. In this study, we report that *lnt*-null

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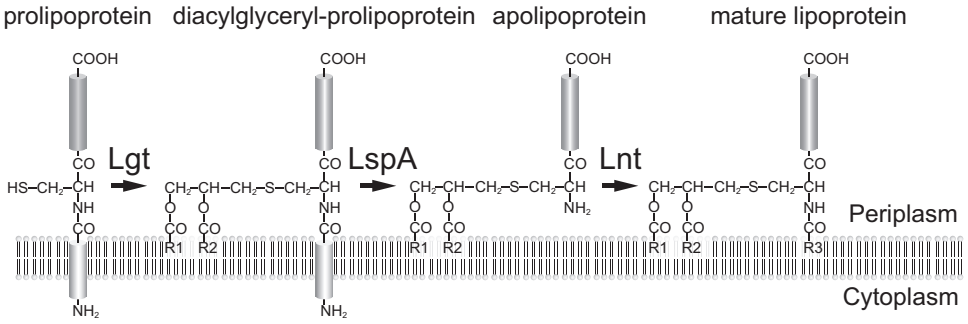

FIG. 1. Biosynthetic pathway for lipoproteins. After being translocated across the inner membrane by Sec translocase, prolipoproteins are processed into mature lipoproteins through the sequential actions of three inner membrane enzymes, phosphatidylglycerol-prolipoprotein diacylglyceryl transferase (Lgt), signal peptidase II (LspA), and apolipoprotein *N*-acyltransferase (Lnt).

mutants of *E. coli* that grow despite the absence of N-acylated lipoproteins can be constructed under specific conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strains and plasmids used in this study are listed in Table 1. Plasmid pKD46 (5) was obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT). Plasmid pCP20 (4) and the Keio collection (2) were from the NBRP (National Institute of Genetics, Mishima, Japan).

Construction of plasmids. pBLH631, encoding Lnt-His, was constructed by subcloning the EcoRI-HindIII fragment derived from pTLH631 (7) into the same sites of pMAN885EH (50). pNASCDEH2, which constitutively overexpresses LolCDE-His, was constructed as follows. A DNA fragment encoding LolC, LolD, and LolE-His was amplified by PCR with pKM001 (52) as a template and a pair of primers, LolC-3 and LolE-4 (Table 2). The amplified DNA fragment was digested with EcoRI and HindIII and then cloned into the same sites of pNAS885, a derivative of pMAN885EH in which the *araC* gene was deleted by removing a 0.5-kb EcoRV-EcoRV fragment inside *araC*, and *araI*(Con) *araX*(Con) mutations (12) were introduced into the *araBAD* promoter by site-directed mutagenesis with a pair of complementary primers, pBAD-f and pBAD-r (Table 2).

Construction of *lnt*-null strains. The chromosomal *lnt* gene was replaced with a kanamycin resistance cassette flanked by FLP recognition target (FRT) sites as described by Datsenko and Wanner (5). PCR was performed by using the chromosomal DNA of JW1105 (Δ*nagK*::FRT-*kan*-FRT) (2) as a template and a pair of primers, lnt-1 and lnt-2 (Table 1), that contained 50 nucleotides homologous to each side of the *lnt* gene and 20 nucleotides for priming upstream and downstream of the FRT sites. BW25113 carrying pKD46 and pBLH631 was grown in LB medium supplemented with 0.02% arabinose and electroporated with the PCR fragment, and then the cells were spread onto LB agar containing kanamycin and incubated overnight at 37°C. A kanamycin-resistant colony was selected, and chromosomal deletion of the *lnt* gene was verified by PCR with primers lnt-2 and lnt-3 (Table 2). The resultant *lnt*-null strain was named LNT0654. The Δ*lnt*::FRT-*kan*-FRT allele was transduced by P1-mediated transduction at 30°C (25) into DLP79-36 harboring either the LolCDE-overexpressing plasmid pKM001 or pNASCDEH2, yielding LNT6541 and LNT6542, respectively. A Δ*ybiS* Δ*erfK* Δ*ycfS* triple mutant, SN495, was constructed by successively transducing the kanamycin resistance cassette from one corresponding clone in the Keio collection (2) into BW25113, followed by transformation with pCP20 to excise the kanamycin resistance cassette (4) and transduction of another kanamycin resistance cassette. The Δ*lnt*::FRT-*kan*-FRT allele was transduced into SN495 harboring pNASCDEH2, yielding LNT6543.

Partial purification and Edman degradation of lipoproteins. Lpp was purified as previously described (11). DLP79-36 or LNT6541 cells harboring pKM001 and pJY811 (50) were cultivated in LB medium at 30°C. When the culture optical density (OD) reached 0.2, expression of Lpp was induced for 3 h by the addition of 0.2% L-arabinose. Cells were passed through a French press twice at 10,000

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
E. coli strains		
BW25113	*lacI*^{q} *rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	5
DLP79-22	HfrC *pps man*	14
DLP79-36	DLP79-22 *lpp*	45
SN495	BW25113 Δ*ybiS* Δ*erfK* Δ*ycfS*	This study
LNT0654	BW25113 Δ*lnt*::FRT-*kan*-FRT	This study
LNT6541	DLP79-36 Δ*lnt*::FRT-*kan*-FRT, constructed in the presence of pKM001	This study
LNT6542	DLP79-36 Δ*lnt*::FRT-*kan*-FRT, constructed in the presence of pNASCDEH2	This study
LNT6543	SN495 Δ*lnt*::FRT-*kan*-FRT, constructed in the presence of pNASCDEH2	This study
Plasmids		
pKD46	pSC101 derivative; *Rep*(Ts) *bla* P$_{BAD}$-*gam-bet-exo*	5
pCP20	pSC101 derivative; *Rep*(Ts) *bla cat* λcI857 λP_R *FLP*$^+$	4
pTLH631	pTTQ18 derivative; P$_{tac}$-*lnt-his*$_6$ *bla*	7
pBLH631	pMAN885EH derivative; P$_{BAD}$-*lnt-his*$_6$ *cat*	This study
pKM001	pUC19 derivative; P$_{lac}$-*lolCDE bla*	52
pNASCDEH2	pNAS885 derivative; P$_{BAD}$ (Δ*araC araI*^{C}*X*^{C})-*lolCDE-his*$_6$ *cat*	This study
pAM201	pMAN885EH derivative; P$_{BAD}$-*lolA-his*$_6$ *cat*	26
pJY811	pMAN885EH derivative; P$_{BAD}$-*lpp cat*	50
pJY851	pMAN885EH derivative; P$_{BAD}$-*lppSR cat*	50
pJY856	pMAN885EH derivative; P$_{BAD}$-*lppDR cat*	50
pLMALE2-His	pUCP20 derivative; P$_{lac}$-*lipomalE*(*S*) *bla*	29
pLMALE2(S2D)-His	pUCP20 derivative; P$_{lac}$-*lipomalE*(*D*) *bla*	29

TABLE 2. Primers used in this work

Primer	Sequence
LolC-3	5′-GATGAATTCGGAGGTTTAAATTTATGTACCAACCTGTCGCTCTATTTA-3′
LolE-4	5′-CAATTCAAGCTTAATGATGATGATGATGATGCTCCAGCTGGCCGCTAAGGACTCGCGCAG-3′
pBAD-f	5′-AGCGGATCCTACCTGGCGCTTTTTATCGCAACTCTCTACTATTTCTCCATACCCGT-3′
pBAD-r	5′-ACGGGTATGGAGAAATAGTAGAGAGTTGCGATAAAAAGCGCCAGGTAGGATCCGCT-3′
lnt-1	5′-CCCAGCCGAAGCTGGATGAATAAAACCGAAACTGGATAGATAACTACATGATTCCGGGGATCCGTCGACC-3′
lnt-2	5′-GGGATGTATTCCGGCACGATAAGAAGGGATTATTTACGTCGCTGACGCAGTGTAGGCTGGAGCTGCTTCG-3′
lnt-3	5′-TCCCGGATGACTCACCCCAG-3′

lb/in^2. After removal of unbroken cells by centrifugation at 10,000 × *g* for 10 min, membranes were recovered by centrifugation at 100,000 × *g* for 1 h and then solubilized with 10 mM Tris-HCl, pH 7.5, containing 5 mM $MgCl_2$ and 1% Triton X-100 at 4°C for 10 min, followed by centrifugation at 100,000 × *g* for 30 min. The precipitate was solubilized with 10 mM Tris-HCl, pH 7.5, containing 1% SDS and then boiled for 5 min, followed by centrifugation at 100,000 × *g* for 30 min. Trichloroacetic acid (TCA) was added to the supernatant to a final concentration of 10%. Lpp was obtained in the TCA-soluble fraction after centrifugation at 5,000 × *g* for 10 min and then precipitated with 9 volumes of acetone overnight at −20°C. Pal was purified from DLP79-36 or LNT6541 cells harboring pKM001 as reported previously (27). Partially purified Pal and Lpp were subjected to SDS-PAGE, followed by transfer to a polyvinylidene difluoride membrane and Coomassie brilliant blue (CBB) staining. Bands were excised from the membrane, and N-terminal amino acid sequences were determined by Edman degradation using a pulsed-liquid sequencer (477A/120A; Applied Biosystems).

Separation of inner and outer membranes. The membrane localization of LppSR, a derivative of Lpp devoid of the C-terminal Lys, or its derivative LppDR, with a Ser-to-Asp substitution at position 2, was determined as follows. DLP79-36 or LNT6541 cells harboring pKM001 were transformed with pJY851 or pJY856, encoding LppSR or LppDR, respectively, and then grown on LB medium at 30°C. When the culture OD reached 0.6, expression of LppSR or LppDR was induced for 30 min by the addition of 0.1% L-arabinose. To determine the membrane localization of lipoMalE, DLP79-36 or LNT6542 cells harboring pNASCDEH2 were transformed with pLMALE2-His or pLMALE2(S2D)-His (29) encoding the model lipoprotein lipoMalE(S) or lipoMalE(D), respectively, and then grown on LB medium at 37°C. Cells were harvested and then converted into spheroplasts as described previously (23). The spheroplasts were disrupted by a single passage through a French pressure cell at 10,000 lb/in^2. A total membrane fraction was recovered by centrifugation at 100,000 × *g* for 1 h at 4°C, suspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and then layered on a 30 to 55% (wt/wt) sucrose gradient. After centrifugation at 60,000 × *g* for 12 h at 4°C, fractions were collected from the gradient with a Piston Gradient Fractionator (BioComp Instruments, Fredericton, Canada) and then analyzed by SDS-PAGE and immunoblotting.

Assay for lipoprotein release from spheroplasts. DLP79-36 or LNT6541 cells harboring pKM001 and pJY851 were grown in M63 medium (50) at 30°C. When the culture OD reached 0.8, expression of LppSR was induced for 5 min by the addition of 0.2% L-arabinose. Cells were harvested and converted into spheroplasts as previously described (23). Aliquots (300 μl) of suspensions containing 5×10^8 spheroplasts were incubated with or without LolA (10 μg) at 30°C for 1 min. M63 medium (750 μl) containing 0.25 M sucrose and 370 kBq of Tran^{35}S-label (MP Biomedicals, Solon, OH) was then added to label LppSR for 2 min, followed by the addition of nonradioactive Met and Cys (each at 12 mM) to chase the labeling. Aliquots were withdrawn after 2, 5, or 10 min and chilled on ice to terminate the release of LppSR. Spheroplasts and medium were separated by centrifugation at 16,000 × *g* for 2 min. Each fraction was immunoprecipitated with anti-Lpp antibodies, and then the radiolabeled LppSR was analyzed by SDS-PAGE and phosphorimaging with a Storm 820 (GE Healthcare).

Isolation of liganded LolCDE. DLP79-36 or LNT6542 cells harboring pNASCDEH2 were grown on LB medium at 30°C until the culture OD reached 1.5. A total membrane fraction (10 mg protein) was prepared as described above and then solubilized with 10 ml of 20 mM Tris-HCl (pH 7.5) containing 1% DDM (*n*-dodecyl-β-*o*-maltopyranoside), 5 mM $MgCl_2$, and 10% glycerol for 30 min on ice. A supernatant was obtained by centrifugation at 100,000 × *g* for 30 min and then applied on a 0.25-ml Talon column preequilibrated with buffer A (20 mM Tris-HCl, pH 7.5, containing 300 mM NaCl, 0.01% DDM, and 10% glycerol). After washing with 10 ml of buffer A, the protein was eluted using a stepwise imidazole gradient comprising 10-ml each of buffer A containing 1, 2, 5, 10, 20, and 50 mM imidazole. Where specified, 2 mM ATP was included in all purification steps.

RESULTS

Overproduction of LolCDE allowed the construction of an *lnt*-null strain. Although Lnt is essential for the growth of both *S. enterica* (9) and *E. coli* (35), the lack of the major outer membrane lipoprotein Lpp renders these strains less dependent on Lnt activity. This is most likely caused by the fact that an extraordinarily large number of Lpp molecules are present in a single cell and the fact that mislocalized Lpp in the inner membrane forms a lethal covalent linkage with peptidoglycans, resulting in cell lysis (50). It was shown by both *in vivo* and *in vitro* examinations that lipoproteins lacking N-acylation were scarcely released from the inner membrane (7, 35), which is dependent on both LolCDE (52) and LolA (23). Based on these observations, we attempted to disrupt the *lnt* gene in an *E. coli* mutant that lacks Lpp and overexpresses LolCDE or LolA, or both.

We first replaced the chromosomal *lnt* gene with a kanamycin resistance gene (*kan*) in the presence of Lnt expressed from a plasmid, pBLH631. The Δ*lnt*::*kan* allele was then transferred by P1 transduction from the Δ*lnt* strain to either an *lpp*$^+$ or *lpp* mutant strain, which harbored either pKM001 encoding LolCDE or pUC19, the empty vector. DLP79-22 (*lpp*$^+$) cells generated a small number of kanamycin-resistant colonies, but they were nonviable irrespective of the plasmid species. In contrast, more than 1,000 kanamycin-resistant colonies were derived from DLP79-36 (*lpp* mutant) cells harboring either plasmid. However, colonies derived from DLP79-36/pUC19 were nonviable on new plates. In marked contrast, colonies derived from DLP79-36 cells harboring pKM001 were viable even after purification. When DLP79-36 cells were grown in the absence of ampicillin at 37°C, they were readily cured of pKM001. On the other hand, the plasmid could not be deleted from the Δ*lnt*::*kan* transductant LNT6541. These results are partly consistent with the observation that the absence of Lpp lowers the level of Lnt necessary for growth, though it remains essential (35). Furthermore, the results mentioned above strongly indicate that Lnt activity is dispensable in the absence of Lpp when LolCD is overexpressed. It should be noted that pKM001 does not carry any suppressor mutations because pKM001 isolated from LNT6541 did not affect the frequency of the Δ*lnt*::*kan* transduction when transformed into DLP79-36.

Growth phenotype of the *lnt*-null strain. Growth of LNT6541 (Δ*lnt*) cells harboring pKM001 was slower than that of the parental strain harboring the same plasmid at both 30°C and 37°C and severely defective at 42°C (data not shown). Since the growth of the DLP79-36 strain was also retarded when the strain harbored pKM001, it seemed likely that the

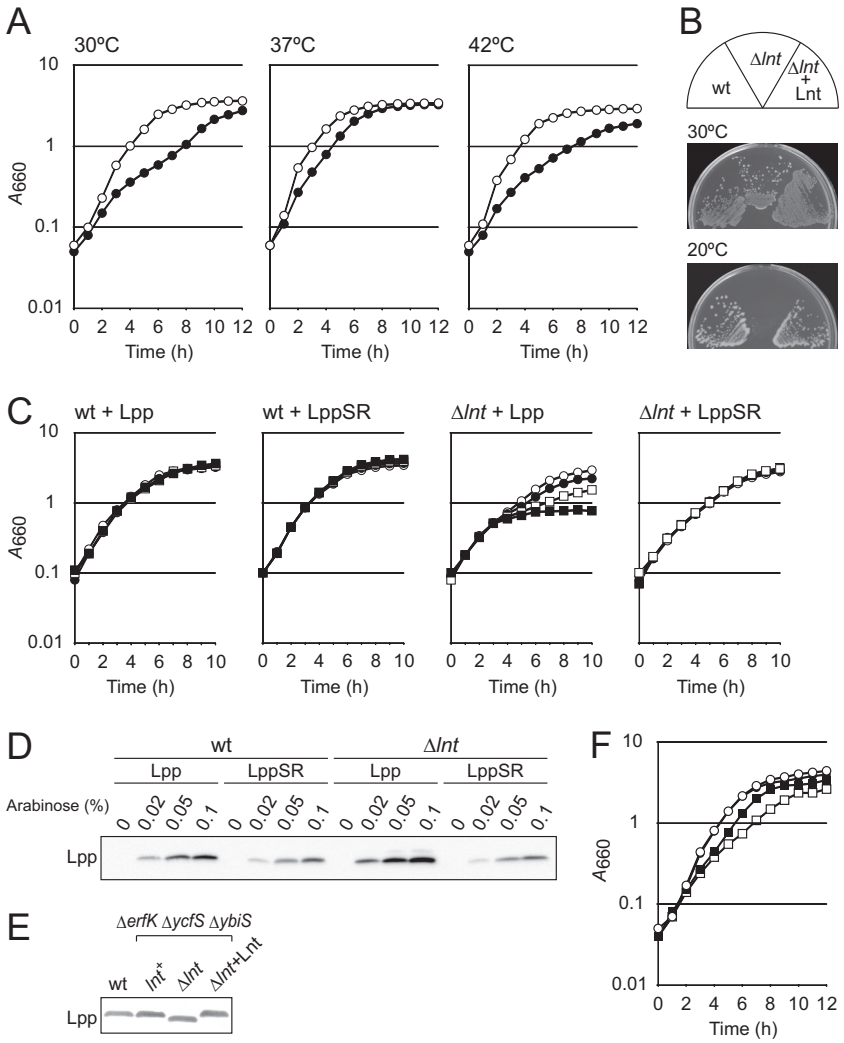

FIG. 2. Growth of the *lnt*-null strain. (A) DLP79-36 (open circles) and its Δ*lnt*::*kan* derivative, LNT6542 (closed circles), both harboring pNASCDEH2, were grown in LB medium at the indicated temperatures. (B) DLP79-36 (wild type [wt]), LNT6542 harboring pNASCDEH2 (Δ*lnt*), and LNT6542 harboring both pNASCDEH2 and pTLH631 encoding Lnt-His (Δ*lnt* + Lnt) were grown on LB agar at the indicated temperatures. (C) DLP79-36 (wt) and LNT6541 (Δ*lnt*), both harboring pKM001, were transformed with a plasmid encoding Lpp or LppSR. Growth without arabinose (open circles) or with arabinose concentrations of 0.02% (closed circles), 0.05% (open squares), and 0.1% (closed squares) was examined. (D) Aliquots in panel C were withdrawn at 4 h, and expression of Lpp was examined by SDS-PAGE (13) and immunoblotting. (E) Cell extracts of BW25113 (wt), SN495 (BW25113 Δ*ybiS* Δ*erfK* Δ*ycfS*) (*lnt*$^+$), LNT6543 (SN495 Δ*lnt*) (Δ*lnt*), and LNT6543 harboring pTLH631 (Δ*lnt* + Lnt), all of which contained pNASCDEH2, were subjected to 16% acrylamide Tricine-SDS-PAGE (38), followed by immunoblotting with anti-Lpp antibodies. (F) DLP79-36 and LNT6541, both harboring pKM001, were transformed with pAM201 encoding LolA-His. Growth in the absence or presence of 0.2% arabinose was examined. Closed and open circles, DLP79-36 with and without arabinose, respectively; closed and open squares, LNT6541 with and without arabinose, respectively.

growth defect of LNT6541 at high temperature is caused by the toxic effect of LolCDE overexpression from the pUC-derived high-copy-number plasmid. To address these issues, another Δ*lnt*::*kan* transductant, LNT6542, was constructed from DLP79-36 cells harboring pNASCDEH2, a low-copy-number plasmid that carries a p15A replicon and constitutively overexpresses LolCDE-His. Transformation of DLP79-36 with pNASCDEH2 caused no obvious growth defect at 42°C (Fig. 2A). The growth of LNT6542 harboring pNASCDEH2 was significantly slower than that of DLP79-36/pNASCDEH2. However, LNT6542/pNASCDEH2 continued to grow at 30°C, 37°C, and 42°C, indicating that the Δ*lnt* mutation can be suppressed by the overproduction of LolCDE even at high temperature. On the other hand, the *lnt*-null strain did not form colonies at 20°C on LB agar even after incubation for 3 days (Fig. 2B). This cold-sensitive growth was corrected by plasmid pTLH631 encoding Lnt, suggesting that the Lnt activity is more critical at lower temperature. Lnt has been inferred to be important for the copper tolerance of *E. coli* (9, 36). Indeed, LNT6541 harboring pKM001 failed to form colonies on LB agar containing 2 mM $CuSO_4$, while DLP79-36 harboring pKM001 and LNT6541 harboring both pKM001 and pBLH631, encoding Lnt, formed colonies (data not shown). We also found that *lnt*-null strains are more sensitive than the parental strain to several structurally and functionally unrelated antibiotics, such as vancomycin and erythromycin, or the

dye ethidium bromide (data not shown), suggesting that the integrity of the outer membrane is perturbed by the Δ*lnt* mutation, which makes cells sensitive to copper.

The C-terminal Lys of Lpp covalently cross-links with a peptidoglycan. This cross-linking contributes to the integrity of the outer membrane when Lpp is present in it. However, the cross-linking becomes lethal to *E. coli* when Lpp is mislocalized to the inner membrane (50). It has been reported that deletion of the C-terminal Lys makes *E. coli* cells resistant to both mislocalization and globomycin, an inhibitor of lipoprotein-specific signal peptidase (50), and less dependent on Lnt activity (35). We examined whether the C-terminal Lys of Lpp is important for growth inhibition by the *lnt*-null mutation. Lpp or its mutant LppSR, lacking the C-terminal Lys, was expressed in DLP79-36 and LNT6541 from plasmids carrying the respective genes under the control of the *araBAD* promoter (Fig. 2C). Growth of LNT6541 was increasingly inhibited when Lpp, but not LppSR, was expressed on the addition of arabinose. The expression level of LppSR, as judged on Western blotting with anti-Lpp antiserum, was lower than that of Lpp (Fig. 2D), presumably because the absence of the C-terminal Lys decreased the reactivity with antibodies.

Three different L,D-transpeptidases, encoded by *ybiS*, *erfK*, and *ycfS*, are capable of catalyzing cross-linking between Lpp and peptidoglycans, while YbiS is the main transpeptidase whose inactivation almost completely suppresses the covalent anchoring of Lpp to the peptidoglycans (21). The Δ*lnt*::*kan* allele could be transduced by P1 into a triple mutant of *ybiS*, *erfK*, and *ycfS*, in which LolCDE-His was overexpressed. Immunoblotting with anti-Lpp antibodies revealed that Lpp in the resultant *lnt*-null strain was as abundant as in wild-type cells and migrated faster on Tricine-SDS-PAGE (Fig. 2E), presumably because it lacked N-acylation. These results support the above prediction that the formation of a covalent linkage between mislocalized Lpp and peptidoglycans is the main cause of toxicity upon Lnt inactivation.

The release of lipoproteins from the inner membrane requires both LolCDE and LolA. It was shown previously that LolA did not cause the release of apolipoproteins from proteoliposomes reconstituted with LolCDE (7). We next examined whether overexpression of LolA also allows the construction of an *lnt*-null strain. The Δ*lnt*::*kan* allele could be transferred to DLP79-36 cells harboring pAM201 encoding LolA-His (26). However, the purified transductants formed no colony after overnight incubation even when LolA-His was induced (data not shown). On the other hand, introduction of pAM201 into the LNT6541 strain slightly improved its growth (Fig. 2F). These results indicate that overexpression of LolA does not support but improves the growth of the *lnt*-null strain.

Lipoproteins in the *lnt*-null strain lack N-acylation. Pal and transiently expressed Lpp were partially purified from the *lnt*-null strain as described in Materials and Methods. Both Pal and Lpp derived from the *lnt*-null strain migrated slightly faster than those from wild-type cells (Fig. 3). These bands were excised and subjected to N-terminal sequence analysis by Edman degradation. Both Pal and Lpp prepared from wild-type cells gave no signal because their N termini are blocked. In contrast, Pal and Lpp prepared from the *lnt*-null strain possessed modified residues at the N terminus that were followed by the sequence Ser-Ser-Asn-Lys and Ser-Ser-Asn-Ala, respectively, which coincided with the N-terminal second to fifth residues of the respective lipoproteins. These results indicate that lipoproteins in the *lnt*-null strain are present as apolipoproteins.

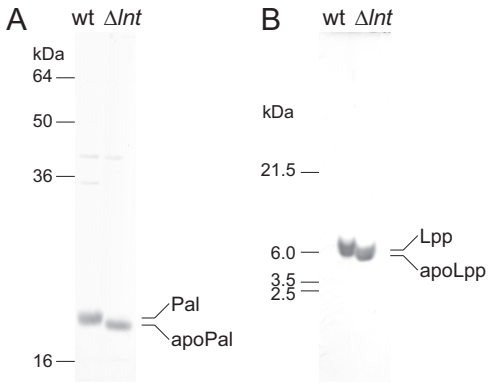

FIG. 3. SDS-PAGE analysis of lipoproteins purified from the *lnt*-null strain. (A) Pal was purified from DLP79-36 (wt) and LNT6541 (Δ*lnt*), both of which harbored pKM001, and then subjected to 17.5% acrylamide SDS-PAGE (19), followed by staining with CBB. (B) Lpp was expressed from a plasmid, pJY811, in the strains described for panel A, purified, and analyzed by SDS-PAGE (13) and CBB staining. The indicated bands were excised, and the N-terminal amino acid sequences were determined by Edman degradation.

Sorting of apolipoproteins to the outer membrane. N-acylation is essential for the release of lipoproteins from proteoliposomes reconstituted with LolCDE (7). Moreover, lipoproteins expressed under Lnt-depleted conditions remained in the inner membrane (35). Based on these observations, we examined the membrane localization of apolipoproteins in the *lnt*-null strain overexpressing LolCDE. DLP79-36 and LNT6541, both harboring pKM001, were transformed with pJY851 encoding LppSR. Membranes were prepared from these cells after 30-min expression of LppSR induced by the addition of arabinose. An outer membrane protein, OmpA, and an inner membrane protein, LolC, were localized in the correct membrane fractions of not only the wild-type, but also the *lnt*-null strain (Fig. 4). Pal and LppSR were localized in the outer membranes of wild-type cells (Fig. 4A). ApoPal was also found in the outer membrane of the *lnt*-null strain, indicating that apoPal can be correctly sorted to the outer membrane when the Δ*lnt* mutation is suppressed by the overexpression of LolCDE. However, most apoLppSR molecules remained in the inner membrane of the *lnt*-null strain (Fig. 4A), presumably because the amount of apoLppSR exceeds the capacity of LolCDE. Alternatively, because Lpp loses its release competence soon after maturation on the outer leaflet of the inner membrane, presumably due to trimerization, while Pal retains its release competence for at least 15 min after maturation (51), decreased release activity would have preferentially caused accumulation of release-incompetent Lpp trimers in the inner membrane of the *lnt*-null strain. In any case, these results reveal why the Δ*lnt*::*kan* allele could not be transferred to wild-type cells expressing Lpp. Accumulation of apolipoproteins in the inner membrane was also observed for lipoMalE(S), a model lipoprotein that has a lipoprotein-specific signal peptide fused to the mature region of maltose-binding protein (29). LipoMalE(S), which has Ser at position 2,

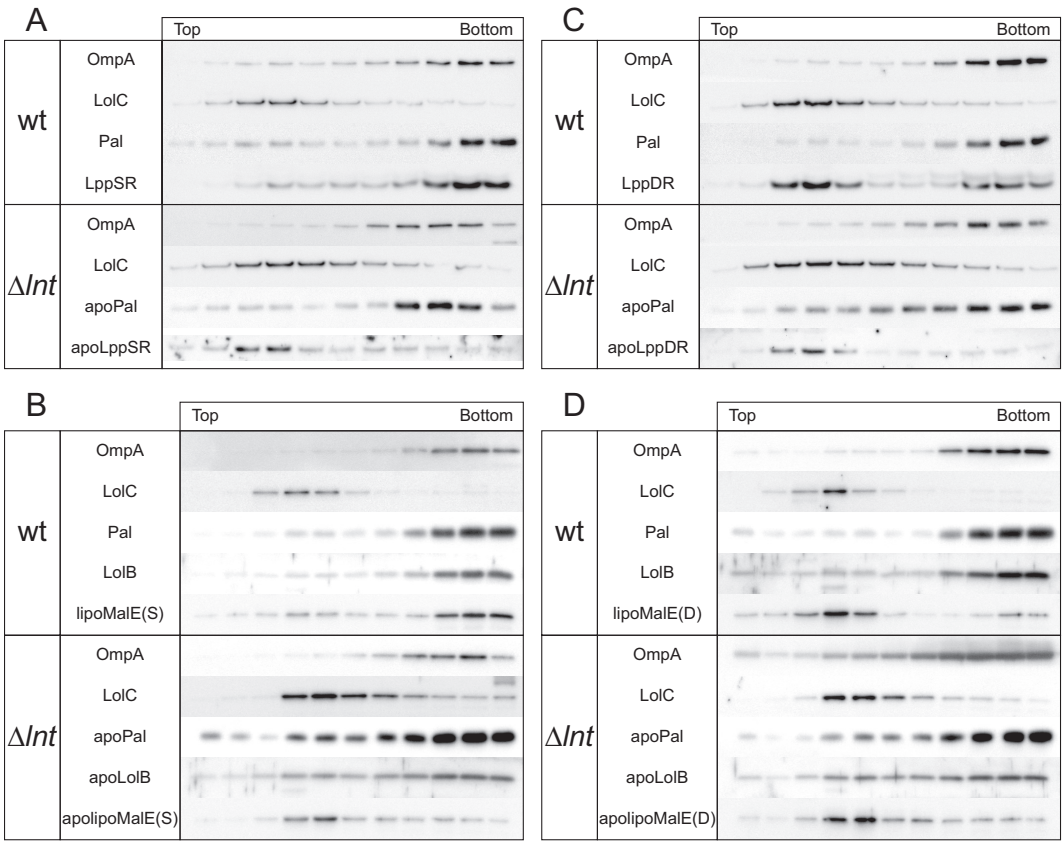

FIG. 4. Membrane localization of lipoproteins in the *lnt*-null strain. The wild type (wt) and the *lnt*-null strain (Δ*lnt*) were transformed with a plasmid encoding LppSR (A), lipoMalE(S) (B), or their derivatives with Asp at position 2 (C and D). Expression of LppSR and LppDR was induced by the addition of arabinose for 30 min. Membranes were separated with a sucrose density gradient into inner and outer membranes. Proteins were detected by immunoblotting using antibodies raised against the respective proteins. LipoMalE was expressed without induction and was detected with anti-maltose-binding protein.

was sorted to the outer membrane of wild-type cells as previously observed (29), whereas it remained in the inner membrane when expressed in the *lnt*-null strain (Fig. 4B). While apoPal was correctly sorted to the outer membrane, sorting of another authentic lipoprotein, LolB, was less efficient in the *lnt*-null strain (Fig. 4B). Thus, the efficiency of outer membrane targeting of each apolipoprotein varies depending on the nature of its protein moiety. It is assumed that a considerable amount of apolipoproteins could be located in the inner membrane of the *lnt*-null strain, despite their outer membrane-targeting signals.

Asp at position 2 of lipoproteins functions as an inner membrane retention signal because it prevents the recognition of lipoproteins by LolCDE (10, 22). The membrane localization of LppDR, which has a Ser-to-Asp substitution at position 2 while the C-terminal Lys has been deleted to avoid cross-linking with peptidoglycans, was determined. Most LppDR molecules with Asp in place of Ser at position 2 accumulated in the inner membranes of wild-type cells (Fig. 4C), although an appreciable amount of LppDR was also found in the outer membranes of wild-type cells, presumably because of the overproduction of LolCDE, as observed previously (28). LipoMalE(D), a derivative of lipoMalE(S) with Asp at position 2, was mainly accumulated in the inner membranes of wild-type cells (Fig. 4D). In the *lnt*-null strains, both apoLppDR and apolipoMalE(S) were exclusively detected in the inner membrane fraction (Fig. 4C and D). Because the sorting of these apolipoproteins was inefficient, it is unclear whether Asp at position 2 functions as a LolCDE avoidance signal without N-acylation.

LolA-dependent release of LppSR and apoLppSR was examined in spheroplasts prepared from DLP79-36 and LNT6541 cells, both of which were overproducing LolCDE. LppSR was efficiently released from spheroplasts prepared from wild-type cells in a LolA-dependent manner (Fig. 5). In contrast, only a marginal amount of apoLppSR was released from spheroplasts of LNT6541 cells in the presence of LolA, indicating that the efficiency of apoLppSR that was released from the inner membrane was much lower than that of mature LppSR.

When LolCDE was purified in the absence of ATP after solubilization of membranes with a detergent, outer-membrane-specific, but not inner-membrane-specific, lipoproteins were copurified (15). This LolCDE-lipoprotein complex represents the intermediate of the LolCDE-mediated release reaction (15, 44). We examined complex formation between LolCDE and apoPal. Membrane fractions were prepared from the wild-type and *lnt*-null strains, both overexpressing LolCDE

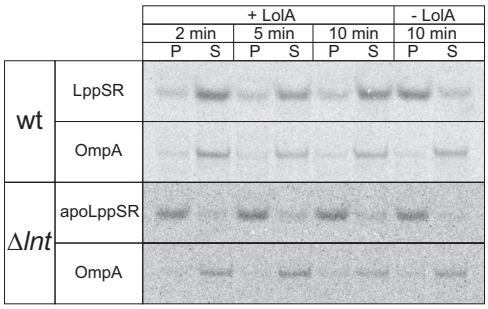

FIG. 5. Release of lipoproteins from spheroplasts of the *lnt*-null strain. DLP79-36 (wt) and LNT6541 (Δ*lnt*), both harboring pKM001 and pJY851, were converted into spheroplasts. Release of ^{35}S-labeled LppSR from the spheroplasts was examined in the presence and absence of LolA. The spheroplasts were subjected to labeling for 2 min, followed by chasing for the indicated periods. LppSR and OmpA in the spheroplast (P) and supernatant (S) fractions were immunoprecipitated and analyzed by SDS-PAGE, followed by phosphorimaging.

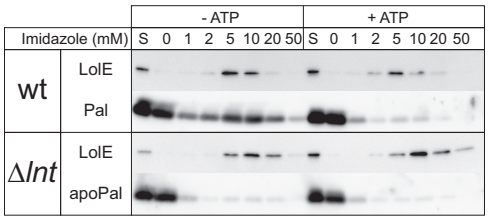

FIG. 6. N-acylation is required for formation of the lipoprotein-LolCDE complex. LolCDE was solubilized with DDM from membranes of DLP79-36 and LNT6542, both harboring pNASCDEH2. LolCDE-His was adsorbed to a metal affinity resin and eluted with the indicated concentrations of imidazole. Each fraction was analyzed by SDS-PAGE and immunoblotting. S, 1/10 the amount of solubilized membrane proteins.

with a hexahistidine tag at the C terminus of LolE, and solubilized with DDM, followed by immobilized metal affinity chromatography in the presence and absence of ATP. LolE-His was always eluted from the metal affinity resin with a buffer containing 5 to 20 mM imidazole, indicating that LolCDE is eluted in these fractions (Fig. 6). Immunoblotting with anti-Pal antibodies showed that Pal was copurified with LolCDE-His that was purified from wild-type membranes in the absence of ATP. In contrast, apoPal was hardly copurified with LolCDE-His solubilized from membranes of the *lnt*-null strain (Fig. 6). Because it was recently shown that Pal derivatives with Asp at position 2 were not copurified with LolCDE (37), it is unlikely that mature Pal exhibits intrinsic affinity for the metal affinity resin. Rather, these results indicate that the affinity of LolCDE for apoPal is low in regard to the formation of a stable complex.

DISCUSSION

It is known that a lack of Lpp decreases the requirement for the activities of both lipoprotein-processing enzymes (8, 9) and the Lol system (41, 43, 52). Lpp is the most abundant protein in *E. coli*, and its mislocalization to the inner membrane causes the formation of lethal cross-linking with peptidoglycans (50). As demonstrated here, the *lnt* gene could be deleted when LolCDE was overexpressed in cells lacking Lpp or L,D-transpeptidases that cross-link Lpp with peptidoglycans. Unless LolCDE was overexpressed, the *lnt* gene was still required even in the absence of Lpp. This might indicate that N-acylation is important to ensure the efficient sorting of lipoproteins, especially ones such as LolB, BamD, and LptE, which are involved in outer membrane biogenesis. Alternatively, some other lipoproteins, like Pal and LolB, might be very deleterious when they remain in the inner membrane (54). In any case, we concluded that by means of overexpessed LolCDE, non-N-acylated lipoproteins can be released from the inner membrane, transported across the periplasm by LolA, and then incorporated into the outer membrane by LolB.

Suppression of the *lnt*-null mutation by LolCDE overexpression resembles the suppression of the *htrB*-null mutation by the overexpression of MsbA (16). The *htrB* (later renamed *lpxL*) gene encodes an enzyme that transfers an acyl chain to the tetra-acylated precursor of di[3-deoxy-D-*manno*-octulosonyl]-lipid A (Kdo_2-lipid A), a core component of essential lipopolysaccharides (LPS) (40). The *htrB*-null strain accumulates the tetra-acylated LPS precursor in the inner membrane under nonpermissive conditions (32, 55). Overexpression of MsbA translocates the tetra-acylated LPS precursor to the outer membrane and allows the growth of the *htrB*-null strain. The tetra-acylated form of Kdo_2-lipid A is a poor substrate for MsbA and is sorted to the outer membrane when a large amount of MsbA is present. Although Kdo is required to stimulate HtrB activity, it was recently reported that overexpression of MsbA also compensates for a Kdo deficiency (24, 34).

In contrast to Pal, affinity purification of LolCDE in the absence of ATP revealed that apoPal was not copurified with LolCDE (Fig. 6), indicating that N-acylation is important for the intimate interaction between lipoproteins and LolCDE. It is not known at present whether N-acylation of lipoproteins also affects the interaction with LolA and LolB. However, since apolipoproteins are localized to the outer membrane of the *lnt*-null strain, both Lol proteins should be able to recognize apolipoproteins. Indeed, overproduction of LolA improved the growth of the *lnt*-null strain, but LolCDE overexpression remained essential (Fig. 2E). Taken together, these results indicate that the lipoprotein release step catalyzed by LolCDE is the most critical step of the lipoprotein-sorting pathway and therefore requires ATP energy.

The crystal structures of LolA and LolB revealed that these proteins comprise similar hydrophobic cavities that are most likely the binding sites for the acyl chains of lipoproteins (42). However, the size of the cavities is not large enough to accommodate all three acyl chains. The LolA homolog of *Pseudomonas aeruginosa* was recently reported to possess hydrophobic surface patches that might accommodate two acyl chains (33). On the other hand, LprG is a lipoprotein of *Mycobacterium tuberculosis* and has a LolA/LolB-like hydrophobic cavity, whose size (~1,500 Å^3) is large enough to bind three acyl chains (6). LprG was speculated to carry triacylated lipoproteins to Toll-like receptor 2 (TLR2), a member of the TLR family that recognizes various microbial molecules and plays a crucial role in innate immunity (17). It was recently found that the LolA cavity undergoes opening and closing upon the bind-

ing and release of lipoproteins, respectively (31). The cavity size of the open form of LolA was speculated to be ~1,700 Å^3 (Y. Oguchi and H. Tokuda, unpublished data). Therefore, both LolA and LolB might be able to accommodate all three acyl chains in their cavities, although the crystal structure of LolA or LolB bound to a lipoprotein has not been solved yet.

The *S. enterica* mutant with a point mutation of *lnt* was temperature sensitive (9). Amino acid substitutions in Lnt of *E. coli* that result in temperature-sensitive complementation of a conditional *lnt* mutant have been also reported (48). In contrast, the *lnt*-null strain exhibited a cold-sensitive phenotype, indicating that the absence of N-acylation itself has a more deleterious effect on the growth of *E. coli* at lower temperatures. This might indicate that the activity of LolCDE is low in the cold, where the membrane fluidity decreases. On the other hand, the cold sensitivity might be due to the impaired function of the outer membrane. The *E. coli* chromosome encodes at least 80 species of outer membrane lipoproteins (46). In the *lnt*-null strain, they are expected to exist as diacylated lipoproteins in the outer membrane. The way in which diacylated lipoproteins affect the integrity of the outer membrane is of great interest.

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