

Defective Lipoprotein Sorting Induces *lola* Expression through the Rcs Stress Response Phosphorelay System

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The *Escherichia coli* LolA protein is a lipoprotein-specific chaperone that carries lipoproteins from the inner to the outer membrane. A dominant negative LolA mutant, LolA(I93C/F140C), in which both ⁹³Ile and ¹⁴⁰Phe are replaced by Cys, binds tightly to the lipoprotein-dedicated ABC transporter LolCDE complex on the inner membrane and therefore inhibits the detachment of outer membrane-specific lipoproteins from the inner membrane. We found that the expression of this mutant strongly induced *lola* gene transcription. The depletion of the LolA or LolB protein also triggered *lola* gene transcription, indicating that the inhibition of outer membrane lipoprotein transport triggers *lola* transcription. To elucidate the mechanism, we isolated mutants that are unable to induce *lola* transcription using the *lacZ* gene fused to the *lola* promoter as a reporter and found that the Rcs phosphorelay system directly regulates *lola* transcription. An outer membrane lipoprotein, RcsF, was essential for this activation, while the coactivator RcsA was dispensable. Taking the observation that an RcsF mutant localized in the inner membrane constitutively activated the Rcs phosphorelay system into consideration, the results shown here strongly suggest that correct lipoprotein sorting to the outer membrane is monitored by RcsF, which plays a key role in the Rcs stress response system.

In the envelope of *Escherichia coli*, there are over 90 different species of lipoproteins, most of which are assumed to be localized to the inner leaflet of the outer membrane (41). Their N-terminal Cys residues are modified by lipid moieties, which anchor lipoproteins to membranes. The Lol system, composed of five Lol proteins, is responsible for the sorting and transport of lipoproteins to the outer membrane (31). After translocation across the inner membrane by the Sec translocon, lipoprotein precursors with signal peptides sequentially undergo modification with lipids and cleavage by enzymes on the periplasmic surface of the inner membrane. Mature lipoproteins destined for the outer membrane are then detached from the inner membrane through the action of LolCDE, an ATP-binding-cassette transporter, and bind to LolA, a lipoprotein-specific chaperone in the periplasm. A water-soluble LolA-lipoprotein complex travels through the periplasm to the outer membrane, where lipoproteins are transferred from LolA to the lipoprotein-specific acceptor LolB anchored to the periplasmic surface of the outer membrane. Finally, LolB embeds lipoproteins in the inner leaflet of the outer membrane.

LolA is composed of 11 antiparallel β -strands and three α helices, which form an incomplete β -barrel structure with a cavity inside (37). The cavity is covered by a lid comprising short α helices. The inside of the cavity is hydrophobic and binds to the acyl chains of lipoproteins (30). To study the importance of the opening and closing of the lid covering the cavity, we constructed a LolA mutant protein having two mutations, I93C and F140C (43). This mutant is fixed in the closed-lid conformation by the disulfide bridge formed between the two Cys residues in the absence of a reductant. LolA(I93C/F140C) revealed that the opening and closing of the lid are essential for continuous lipoprotein transfer from LolCDE to LolA (29, 43). This mutant stably bound to LolCDE on the inner membrane and therefore severely inhibited the growth of cells even in the presence of wild-type LolA. Furthermore, this mutant triggered the envelope stress response via the Cpx system (40), which is one of the five envelope stress response systems in *E. coli* (4).

During the study of this Cpx envelope stress response, we found that the expression of LolA(I93C/F140C) increased the expression of LolA. We show here that the transcription of the *lola* gene is induced by the expression of LolA(I93C/F140C) in a manner dependent on the Rcs phosphorelay system, another stress response system comprised of RcsA, RcsB, RcsC, RcsD, and RcsF (21). These *rcs* genes were initially identified as activators of capsular polysaccharide synthesis in *E. coli* (15). Later, they were shown to regulate the transcriptions of genes involved in various cellular processes, mostly cell surface-related functions (4, 17, 21). RcsC and RcsD are inner membrane kinases involved in the phosphorylation of the cytoplasmic response regulator RcsB. Under certain growth conditions, the conserved His residue of the sensor kinase RcsC is autophosphorylated, followed by the transfer of the phosphate to the Asp residue in the C-terminal receiver domain of RcsC. The phosphate is then further transferred to the conserved His residue in the phosphotransfer domain of RcsD and finally to the Asp residue in the receiver domain of RcsB. Phosphorylated RcsB induces the transcription of target genes. A subset of target promoters requires the RcsA coactivator protein for transcriptional activation. RcsF, an outer membrane lipoprotein, is considered to function as a sensor and to transfer a signal to the RcsC sensor kinase under certain environmental conditions (7, 22).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. MY201 is an *ara*⁺ revertant of MC4100 (40) and was used as the parental strain. Strains carrying the

Received 3 April 2012 Accepted 26 April 2012

Published ahead of print 4 May 2012

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doi:10.1128/JB.00553-12

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Description	Reference
<i>E. coli</i> strains		
MC4100	$\Delta(\text{argF-lac})U169 \text{ araD139 rpsL150 thiA1 relA1 flbB5301 deoC1 ptsF25 rbsR}$	6
MY201	<i>ara</i> ⁺ revertant of MC4100	40
MY301	MY201 <i>lolA-lacZ</i>	This study
MY302	MY201 <i>lolB-lacZ</i>	This study
MY303	MY201 <i>lolC-lacZ</i>	This study
MY304	MY301 <i>cpxA::spc</i>	This study
MY418	MY201 <i>rprA-lacZ</i>	This study
MY419	MY201 <i>wza-lacZ</i>	This study
MY3011	MY301 <i>rscF1::mini-Tn10</i>	This study
MY3012	MY301 <i>rscC2::miniTn10</i>	This study
MY3018	MY301 <i>rscF8::mini-Tn10</i>	This study
MY412	MY301 $\Delta rcsA$	This study
MY413	MY301 $\Delta rcsB$	This study
MY414	MY301 $\Delta rcsC$	This study
MY415	MY301 $\Delta rcsD$	This study
MY416	MY301 $\Delta rcsF$	This study
MY425	MY301 P _{bad} - <i>lolA</i> 6×His <i>lolA::Cm</i>	This study
MY427	MY301 P _{bad} - <i>lolB lolB::Cm</i>	This study
Plasmids		
pRS551	<i>lacZ</i> operon fusion vector; Ap ^r Km ^r	35
pCP20	Thermoinducible FLP recombinase; Ap ^r Cam ^r	10
pKD32	Template plasmid for Cm ^r cassette amplification	10
pKD119	<i>oriR101 repA101</i> (Ts) P _{bad} - <i>gam-bet-exo</i> Tet ^r	10
pBAD18	P _{bad} expression vector; Ap ^r	16
pAM201	<i>lolA</i> 6×His	27
pSW77	P _{bad} - <i>lolA</i> Cm ^r	43
pSWIF	P _{bad} - <i>lolA</i> (I93C/F140C) Cm ^r	43
pMY114	pBAD18 P _{bad} - <i>lolA</i> 6×His	This study
pMY117	pBAD18 P _{bad} - <i>lolB</i>	This study

specified *rsc* gene, which had been disrupted by a Km^r insertion, were obtained from the Keio Collection single-gene-deletion library (1). When required, the Km^r cassette sequence was removed by transforming the cells with pCP20 (10). pSW77 and pSWIF encode wild-type *LolA* tagged with FLAG at the C terminus and *LolA*(I93C/F140C), respectively (43). A strain carrying the *lolA-lacZ* fusion gene was constructed as follows. A DNA fragment encompassing the promoter region of the *lolA* gene (between bases -346 and +4 with respect to the translational initiation codon) was amplified by PCR using chromosomal DNA of MC4100 as a template and a pair of primers, lolAp5 and lolAp3 (Table 2). The amplified fragment was digested with EcoRI and BamHI and cloned into the same sites of a *lac* operon fusion vector, pRS551 (35). The resulting fusion plasmid was linearized with SalI and then transformed into TE2680 (12), which carries a DNA sequence homologous to the fusion vector. This allowed recombination between the transfected plasmid and the chromosome, generating a stable single-copy *lolA-lacZ* fusion gene at the *trp* locus on the chromosome. The single-copy fusion gene thus constructed on the chromosome of TE2680 was transduced into wild-type strain MY201 by P1 transduction, generating MY301. Strains carrying other *lacZ* promoter fusions were constructed similarly.

Cells were routinely grown in LB medium (26) at 37°C with aeration. MacConkey lactose plates were used to screen for mutants defective in *lolA* transcription activation. Chloramphenicol and kanamycin were each added to a final concentration of 25 µg/ml, when required. Ampicillin was added to 100 µg/ml.

Depletion of Lol proteins. A DNA fragment carrying the *lolA* gene tagged with 6×His at its C terminus was amplified by PCR using pAM201

TABLE 2 Oligonucleotides used in this study

Primer	Sequence
lolAp5	TAGAATTCGTTGTTTCGATCAGGCCG
lolAp3	CTGGATCCTCATTATTCCTCAAATTACGTC
lolBp5	TAGAATTCCTTCTCGCAGCGATACAGGTG
lolBp3	CTGGATCCAGTGATGACAAGTCCTTGAGA
lolCp5	TAGAATTCGGATAAAAAGGTAATGACCGAG
lolCp3	CTGGATCCGTCTGGTTGCTGTAGCAAAG
wzap5	TAGAATTCATTAAAGAAATATCGCATGTGG
wzap3	ATGGATCCTTTCCAATAATGCAGGAGGAAG
rprAp5	TAGAATTCAGCTGGTAGTACCTGTCTG
rprAp3	ATGGATCCACCGTGAGCTAAATAGTAGGC
lolAEco	TAGAATTCCTTGAGGAATAATGATGAA
lolA6His-rv	ATGGTACCTTAATGATGATGATGATGATGC
lolB-Ec	GCGAATTCCTAGCATTAAGGTTATAACTG
lolB-Sp	TTGCATGCACTAACCGCTTCTATCTGGCC
lolA-f	GGAACCTCCGATCGGGAGTGACGTAATTTG AGGAATAATGGTGTAGCTGGAGCTG
lolA-r	GTTGAAAAGTATTATCCGAAAAATCGAGCGAC AGATTGCTCATATGAATATCCTCCT
lolB-f	GGGTATAACTGCAACGTATCTCAAGGACTT GTCATCACTGTGTAGGCTGGAGCTGCTTC
lolB-r	AAAACAGATTAAGTTTTGCCGGAGAGGGCCA CTGTGTCCGCATATGAATATCCTCCTTA
lolARv	GCCCAAACGCTGCTTGCTACTAAGCTTGAGAG

(27) as a template and a pair of primers, lolAEco and lolAHis-rv (Table 2). The amplified fragment was digested with EcoRI and KpnI and then cloned into the same sites of pBAD18 (16). The resulting plasmid, pMY114, was placed onto the chromosome of MY301 by using the λ InCh recombination system, as described previously (3), yielding strain MY422, which carries *lolA* under the control of the *araBAD* promoter. A chloramphenicol resistance cassette was amplified by PCR using pKD32 as a template and a pair of primers, lolA-f and lolA-r, having sequences homologous to those flanking the *lolA* gene and 20-nucleotide priming sequences for pKD32 (10). To disrupt the authentic *lolA* gene, the amplified DNA fragment was electroporated into MY422 cells harboring pKD119 (10). Cells were plated onto LB agar containing chloramphenicol and then incubated at 37°C. Several chloramphenicol-resistant colonies were selected and examined for a disruption of the *lolA* gene by PCR using primers specific for the inserted chloramphenicol-resistant cassette and the sequences surrounding *lolA*. One of the clones with a correct chromosomal *lolA* deletion was chosen and named MY425. The *LolB*-depleting strain was constructed in a similar manner, except that the untagged *LolB* protein was expressed from the *araBAD* promoter. In brief, the coding region of *lolB* was amplified by PCR using MC4100 chromosomal DNA as a template and a pair of primers, lolB-Ec and lolB-Sp, followed by cloning into the EcoRI-SphI sites of pBAD18. The resulting plasmid was placed onto the chromosome of MY301 as described above, generating MY426. The coding region of the authentic *lolB* gene in MY426 was replaced by the chloramphenicol-resistant cassette as described above, resulting in the construction of MY427.

Cells carrying the *lol* genes under the control of the *araBAD* promoter were grown overnight in the presence of 0.2% arabinose. Cells were harvested by centrifugation and resuspended in the original volume of LB medium without arabinose. Cells were then diluted 100-fold with fresh LB medium containing 0.2% arabinose or 0.2% glucose and grown for 3 h. After the 100-fold dilution was repeated once more, cells were grown for a further 3 h. The cells in the presence of glucose ceased to grow by the end of this period. The β -galactosidase activity expressed from the *lolA-lacZ* fusion gene was measured as described previously (26). The depletion of the *LolA* and *LolB* proteins was confirmed by Western blotting. Antisera against *LolA* and *LolB* were described previously (24, 25).

Screening of mutants defective in *lolA* transcription activation. MY301 was randomly mutagenized with mini-Tn10 using λ 1098 as described previously (26). Mutagenized cells were transformed with pSWIF and plated onto MacConkey lactose plates containing 0.02% arabinose to moderately express LolA(I93C/F140C). Colonies exhibiting reduced color development were purified on the same plates. The inability to induce *lolA* transcription upon LolA(I93C/F140C) expression was examined in cells grown in liquid medium. The linkage between transposon insertions and phenotypes was confirmed after the transfer of the mini-Tn10 insertions into MY301 by P1 transduction. Clones with mutations of the *lolA-lacZ* reporter itself were discarded by examining the link between the Km^r (linked to *lolA-lacZ*) marker and the phenotype. The chromosomal locations of the mini-Tn10 insertions were determined by the sequencing of the DNA fragments of the junctions between the ends of mini-Tn10 and the chromosome, which were amplified by inverse PCR as described previously (19).

Primer extension analysis of *lolA* transcripts. Total RNA was prepared from cells expressing or lacking LolB with RNAprotect bacterial reagent and an RNeasy minikit (Qiagen). Ten micrograms of total RNA was hybridized to a ^{32}P -labeled primer specific for *lolA* (*lolA*-Rv [1 pmol]), followed by extension with Primescript reverse transcriptase (Takara Shuzo). Extension products were analyzed on a 6% denaturing polyacrylamide gel, along with the sequencing reaction products with the same primer. A DNA fragment containing the *lolA* promoter region was amplified by PCR with a pair of primers (*lolAp5* and *lolARv*) and MC4100 chromosomal DNA as a template and then used as a template for the sequencing reaction. Gels were dried, exposed to an imaging plate, and visualized with an FLA9000 image analyzer (Fujifilm).

RESULTS

LolA(I93C/F140C) induces *lolA* expression. In our previous study (40), we noticed that the amount of LolA increased upon the overexpression of LolA(I93C/F140C). To quantitatively assess the expressions of *lol* genes, we fused the promoter regions of the respective *lol* genes to the coding region of *lacZ*, and each resulting fusion gene was then placed onto the chromosome as a single copy. The strains carrying the *lol* promoter-*lacZ* fusion gene were transformed with a plasmid encoding wild-type LolA or LolA(I93C/F140C) under the control of the *araBAD* promoter. Cells were grown to the early logarithmic phase of growth, and the expressions of the wild type and LolA(I93C/F140C) were induced with arabinose (0.2%).

The expression of LolA(I93C/F140C) markedly increased the activity of β -galactosidase encoded by the *lolA-lacZ* fusion gene (Fig. 1). The increase in β -galactosidase activity upon the induction of wild-type LolA was small but reproducible and seemed to be important, as discussed below (see Discussion). We previously reported that LolA(I93C/F140C) induced the Cpx-dependent envelope stress response (40). To determine whether or not the triggering of *lolA* expression is dependent on the Cpx system, we introduced the *cpxR::spc* mutation into a strain carrying *lolA-lacZ* and measured β -galactosidase activity. The *cpx* mutation did not decrease the *lolA* expression level induced by the expression of wild-type LolA or LolA(I93C/F140C) (Fig. 1C), indicating that the triggering of *lolA* transcription observed here is not dependent on the Cpx envelope stress response.

The expressions of *lolB* and *lolC* were examined in a similar manner with strains carrying the *lolB-lacZ* and *lolC-lacZ* fusion genes, respectively. In contrast to that of *lolA* gene expression, the triggering of *lolB* or *lolC* gene expression by LolA(I93C/F140C) was only marginal, and wild-type LolA had no effect on the ex-

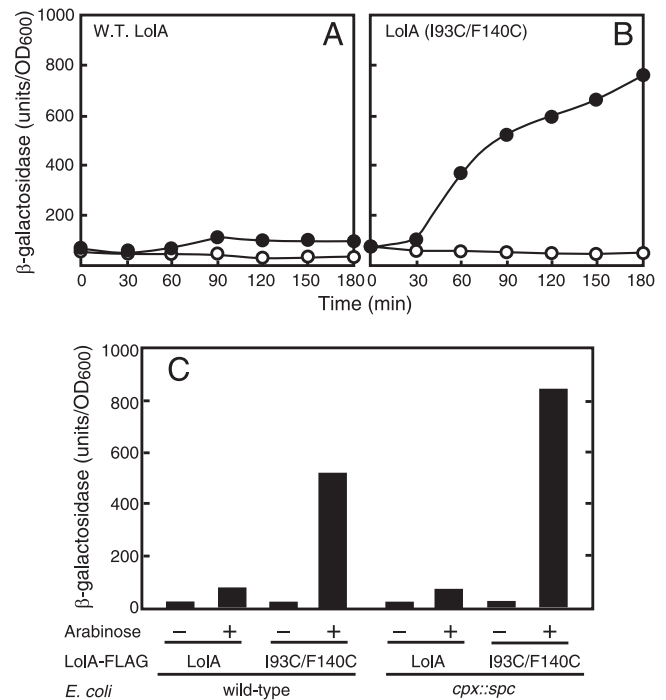


FIG 1 Induction of *lolA* expression by LolA(I93C/F140C). (A and B) MY301 cells harboring either pSW77 (A) or pSWIF (B) were grown to the early logarithmic phase of growth (optical density at 600 nm [OD₆₀₀] of 0.2), and the expressions of LolA and LolA(I93C/F140C) were then induced with 0.2% arabinose at time zero (closed circles) or not induced (open circles). Samples were taken periodically to measure the β -galactosidase activity expressed from the *lolA-lacZ* fusion gene. (C) MY301 (wild-type [W.T.]) and MY304 (*cpx::spc*) cells harboring either pSW77 or pSWIF were grown as described above, and the expression of the plasmid-encoded LolA protein was induced with 0.2% arabinose. β -Galactosidase activity was measured after 2 h of cultivation.

pressions of *lolB* and *lolC* (data not shown). Therefore, we focused on the transcriptional regulation of *lolA* here.

Malfunction of the outer membrane transport of lipoproteins induces *lolA* transcription. We previously showed that LolA(I93C/F140C) formed a stable complex with LolCDE on the inner membrane (40) and therefore severely inhibited both lipoprotein transfer from LolCDE to LolA and cellular growth. To determine whether *lolA* transcription is triggered by the inhibition of lipoprotein transport to the outer membrane or by the aberrant structure of oxidized LolA(I93C/F140C), we depleted LolA or LolB.

Both LolA and LolB are essential for *E. coli* growth (36, 38). The depletion of LolA and LolB causes the accumulation of outer membrane-specific lipoproteins in the inner membrane (36) and in both the periplasm and inner membrane (38), respectively. We constructed mutants carrying the respective *lol* genes under the tightly controlled *araBAD* promoter on the chromosome. The chromosomal *lolA* gene was disrupted by homologous recombination to construct MY425. A *lolB*-disrupted strain, MY427, was constructed in a similar manner. These strains did not grow on LB plates in the absence of arabinose, while they grew normally in the presence of 0.2% arabinose (data not shown). In liquid cultures, these strains grew continuously in the presence of 0.2% arabinose, whereas they stopped growing after about 6 h in medium containing glucose instead of arabinose (Fig. 2A and B). The levels of both

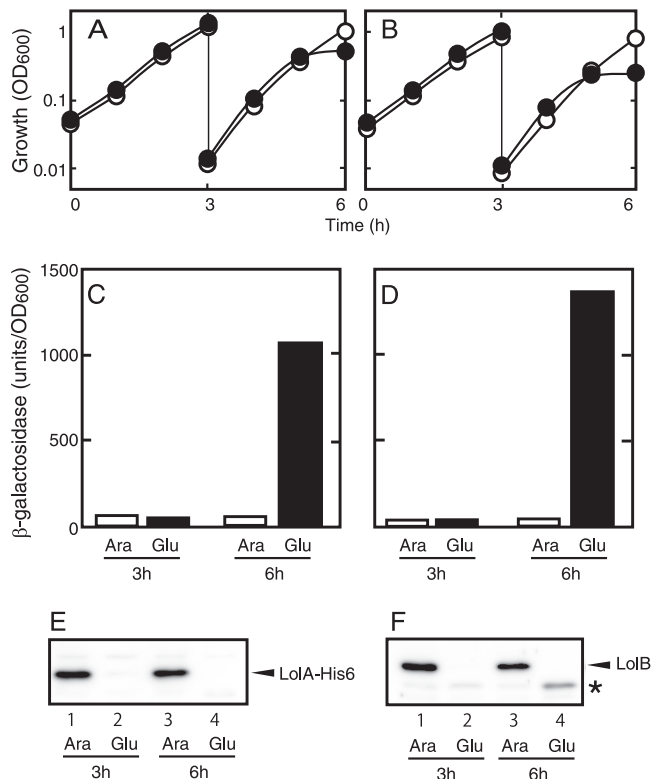


FIG 2 Induction of *lolA* expression by depletion of LolA or LolB. MY425 (A, C, and E) and MY427 (B, D, and F) were grown overnight in LB medium containing 0.2% arabinose and then diluted 100-fold with fresh medium containing 0.2% arabinose (open circles and bars) or 0.2% glucose (closed circles and bars). After 3 h, the cultures were again diluted 100-fold with the same medium and grown for an additional 3 h. At 3 and 6 h after the start of cultivation, samples were taken for measurements of β -galactosidase activity (C and D) and for Western blot analysis (E and F). Cellular growth was monitored by measuring the OD_{600} of the cultures (A and B). Judging from the OD_{600} of the cultures, equivalent amounts of protein were loaded onto each lane for Western blot analysis. The asterisk in panel F indicates an uncharacterized material that seemed to be induced by LolB depletion and to cross-react with the anti-LolB antiserum.

LolA and LolB were below the detection limit at the end of the first 3 h of growth in the absence of arabinose (Fig. 2E and F). The β -galactosidase activity was not increased at either 3 or 6 h in the presence of arabinose, whereas it was strongly induced at 6 h in the absence of arabinose. The level of β -galactosidase activity at 3 h in the absence of arabinose did not increase, even though the amount of LolA or LolB was greatly reduced, indicating that small amounts of LolA and LolB can support cellular growth, as described previously (36, 38). Taken together, these results indicate that the triggering of *lolA* transcription occurs when outer membrane lipoprotein transport malfunctions.

Isolation of mutants unable to induce *lolA* transcription. To elucidate the molecular mechanism underlying the triggering of *lolA* transcription, we isolated mutants defective in the LolA(I93C/F140C)-dependent triggering of *lolA* transcription using the *lolA-lacZ* fusion as a reporter gene. The wild-type strain carrying the *lolA-lacZ* fusion gene was randomly mutagenized with the mini-Tn10 transposon. A plasmid encoding LolA(I93C/F140C) was transformed into the pool of mutagenized cells, followed by cultivation on MacConkey lactose plates containing

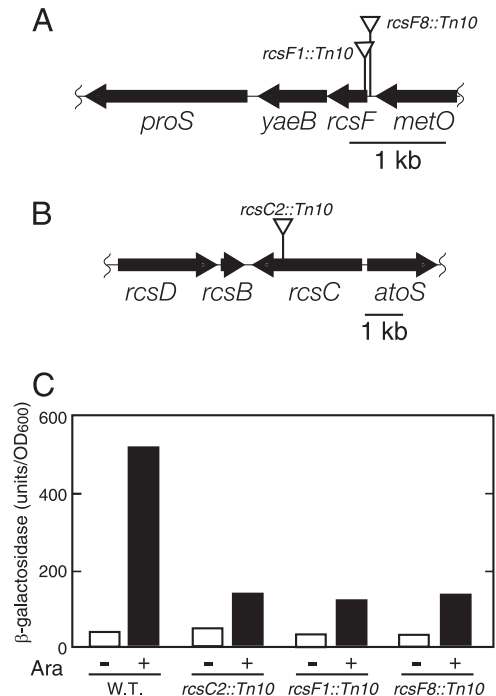


FIG 3 Locations of mini-Tn10 insertions and phenotypes of the mutants. (A and B) The locations of the mini-Tn10 insertions are indicated by triangles above the maps. Cells with the indicated mini-Tn10 insertions were tested for the induction of β -galactosidase activity expressed from the *lolA-lacZ* fusion gene upon the induction of LolA(I93C/F140C). Exponentially growing cells (OD_{600} of 0.2) were induced for LolA(I93C/F140C) expression by 0.2% arabinose. (C) After 2 h, β -galactosidase activity was measured.

0.02% arabinose. At this arabinose concentration, LolA(I93C/F140C) was moderately expressed and caused a slight growth inhibition with a concomitant weak induction of *lolA-lacZ* transcription. Under these conditions, wild-type cells harboring pSWIF formed slightly smaller colonies than those formed in the absence of arabinose, and the center of the colonies was red, whereas colonies in the absence of arabinose were entirely white. We then subjected cells to transposon insertion mutagenesis and sought colonies that exhibited reduced color development in the presence of 0.02% arabinose.

Out of approximately 20,000 mutagenized colonies, we obtained six candidates that exhibited decreased color development on MacConkey lactose plates containing arabinose. They formed colonies with a similar color on MacConkey lactose plates containing 0.02% arabinose and exhibited reduced levels of β -galactosidase activity when LolA(I93C/F140C) was expressed in liquid culture (Fig. 3C).

To determine the locations of the transposon insertions in these mutants, DNA fragments around the inserted transposon were amplified by PCR and sequenced. Three different mutations were found in six mutants: two mutations were located around the *rcsF* coding region, and the third one was found in the coding region of the *rcsC* gene (Fig. 3A and B). One mutation, found in two mutants, was mapped at 5 nucleotides upstream of the *rcsF* initiation codon. Three mutants had an insertion mutation 34 nucleotides downstream of the initiation codon of *rcsF* that disrupted codon 12. The former mutation is likely to affect the expression of *rcsF* upon insertion into the immediate upstream re-

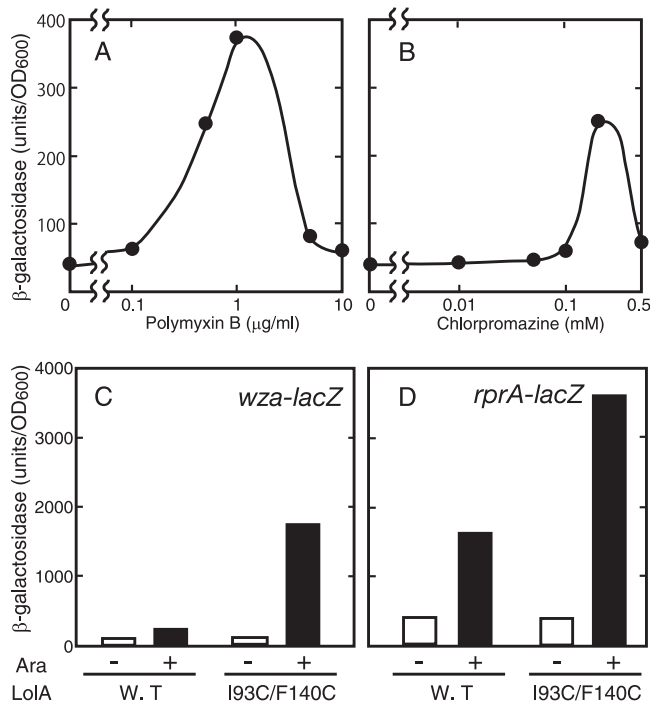


FIG 4 Induction of *lolA* by Rcs-activating compounds, and activation of Rcs regulon genes by LolA(I93C/F140C). (A and B) MY301 cells were grown to the early logarithmic phase, and chlorpromazine (A) and polymyxin B (B) were added to the culture at the indicated concentrations. (C and D) MY419 (*wza-lacZ*) (C) and MY418 (*rprA-lacZ*) (D) cells harboring either pSW77 or pSWIF were grown to the early logarithmic phase, and the expression of LolA(I93C/F140C) was induced with 0.2% arabinose. After a further 2 h of growth, β -galactosidase activity was measured.

gion. The insertion into *rscC* was 2,438 nucleotides from the initiation codon and disrupted codon 813. Taken together, these results suggest that the Rcs phosphorelay system (21) is responsible for the induction of *lolA* transcription in the absence of lipoprotein transport.

Activation of the Rcs stress response system increases *lolA* transcription. Chlorpromazine is a cationic amphipathic drug that intercalates into the membrane lipid bilayer and changes the membrane properties (32). Polymyxin B is a cationic amphipathic antimicrobial peptide that binds to outer membrane lipopolysaccharides, which leads to membrane perturbation (42). Both compounds were shown previously to activate the Rcs phosphorelay system (9, 13, 14). If *lolA* is under the control of the Rcs system, these compounds may induce the transcription of *lolA*. Indeed, chlorpromazine and polymyxin B triggered *lolA* transcription depending on their concentrations (Fig. 4A and B). The highest level of induction was observed at concentrations where growth inhibition became nearly maximum.

We then performed the converse experiment; i.e., we examined whether or not the expression of LolA(I93C/F140C) activates the Rcs regulon genes. Promoters of the *wza* and *rprA* genes were chosen as representative Rcs regulons (21). When LolA(I93C/F140C) was expressed in a strain carrying the *wza-lacZ* or *rprA-lacZ* fusion, the β -galactosidase activity increased about 20-fold or 10-fold, respectively, whereas wild-type LolA was activated about 3-fold in both strains (Fig. 4C and D). Taken together, these results indicate that LolA(I93C/F140C) activates the Rcs phospho-

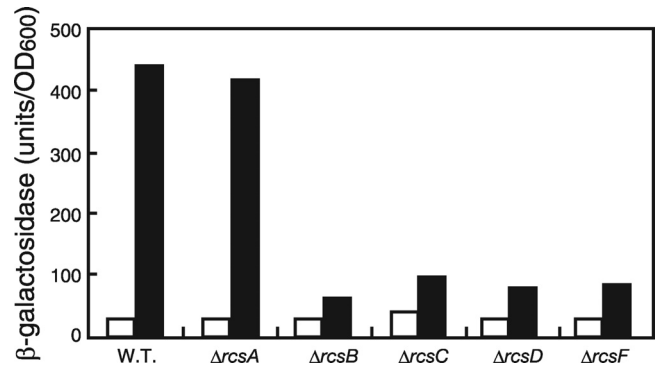


FIG 5 Induction of *lolA* expression by LolA(I93C/F140C) in the *rsc* mutants. Cells harboring pSWIF were grown to the early logarithmic phase of growth (OD₆₀₀ of 0.2), and the expression of LolA(I93C/F140C) was induced with 0.2% arabinose (closed bars) or not induced (open bars). After a 2-h incubation, β -galactosidase activity was measured.

relay system and that the *lolA* gene is a member of the Rcs regulon genes.

Identification of the Rcs-regulated *lolA* promoter. We next examined whether or not all the *rsc* genes are required for the triggering of *lolA* transcription in response to LolA(I93C/F140C) expression in mutants lacking various *rsc* genes (Fig. 5). As expected, neither the $\Delta rcsF$ nor the $\Delta rcsC$ mutant triggered *lolA* transcription. Likewise, the $\Delta rcsB$ and $\Delta rcsD$ mutants were unable to trigger *lolA* transcription in response to LolA(I93C/F140C). In contrast, the *lolA* transcription level induced in the $\Delta rcsA$ mutant was similar to that in the wild-type strain, indicating that the *lolA* promoter belongs to a group which does not require RcsA as a coactivator protein. Taken together, these results establish that *lolA* expression is regulated by the Rcs phosphorelay system.

To locate the Rcs-regulated *lolA* promoter, we determined the transcription start site by means of primer extension experiments. Total RNA was isolated from cells expressing or lacking LolB and then subjected to primer extension reactions with a primer specific to *lolA*, followed by analysis on a denaturing polyacrylamide gel. A transcript induced under LolB-depleting conditions was observed and was mapped to the A residue 41 nucleotides upstream of the translational start site of *lolA* (Fig. 6A). Putative promoter -35 and -10 sequences were found for this transcript (Fig. 6B). Immediately upstream of the -35 element, a nucleotide sequence similar to the RcsB-binding consensus sequence (2, 5, 11, 23) was present (Fig. 6C). This is consistent with previous findings that the RcsB homodimer binds upstream of the -35 sequence of RcsA-independent promoters, whereas the RcsA-RcsB heterodimer binds far upstream of the transcription start sites of RcsA-dependent promoters (21). Overall, we concluded that *lolA* expression is directly regulated by the Rcs phosphorelay system rather than indirectly controlled via Rcs-regulated transcriptional regulators.

DISCUSSION

The molecular mechanisms by which lipoproteins are sorted to the outer membrane by the Lol system have been extensively studied in *E. coli* (31). However, little is known about the regulation of Lol protein expression, although lipopolysaccharide-deficient *Acinetobacter baumannii* was very recently reported to exhibit increased expression levels of genes involved in lipoprotein trans-

the accumulation of the LolA-lipoprotein complexes in the periplasm is not toxic, as observed previously in the case of the LolA(R43L) mutant, in which lipoproteins cannot be transferred to LolB (39). This seems to be why *lolA* gene expression is induced exclusively by a defect in the Lol system.

The function of RcsF, which monitors lipoprotein transport by the Lol system, resembles that of SecM, which is itself a secretory protein and monitors the proper operation of Sec-dependent protein secretion across the inner membrane. When the secretion of SecM is impaired, the expression of SecA, a critical secretion motor protein, is triggered (28).

We have identified a DNA sequence similar to the RcsB-binding consensus sequence immediately upstream of the promoter –35 element of *lolA*. However, it is still possible that the transcriptional activation of *lolA* is mediated by another transcription factor induced by RcsB under these conditions. Further studies are required to conclude definitely whether *lolA* is controlled directly or indirectly by RcsB.

Consistent with data from a previous report (8), the overproduction of wild-type LolA slightly induced *lolA* gene transcription. This was dependent on RcsF (our unpublished observations). It may be possible that the level of the periplasmic LolA-RcsF complex increased upon the overproduction of LolA, causing the interaction with RcsC.

ACKNOWLEDGMENTS

We thank NBRP (NIG, Japan) for providing the *rcs* gene disruptants.

This work was supported by grants to H.T. from the Ministry of Education, Science, Sports, and Culture of Japan.

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