Deletion of Lipoprotein Gene rlpA Causes Supersensitivity of *Escherichia coli* to Several Antibiotics

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Abstract

The *Escherichia coli* RlpA lipoprotein localizes to the septal ring and binds to peptidoglycan through the SPOR domain. We generated an rlpA deletion mutant. RlpA deletion mutant and double mutants lacking both RlpA and Lpp lipoproteins grew normally without significant changes in cell morphology. However, these mutants are supersensitive to several antibiotics such as moenomycin, macarbomycin, enramycin, vancomycin, and bacitracin.

Introduction

Bacterial growth and morphology are regulated by peptide glycan synthesis complexes and associated membrane proteins [1]. The chromosomal region at 14 min on the *Escherichia coli* chromosome map (the mrd region) contains several genes involved in cell duplication and morphogenesis [2]. These include mrdA [3] (also called pbpA [4]), which codes for the penicillin-binding protein PBP-2; mrdB [3] (also called rodA [5]), which codes for the RodA protein; and dacA [6], which codes for PBP-5. PBP-2 and RodA are involved in determination of the rod shape of the *E. coli* cell, functioning in the biosynthesis of peptidoglycan during cell growth [7]. PBP-5 is a D-alanine carboxypeptidase involved in the maturation of peptidoglycan [6].

Previously, we identified two rare lipoprotein genes, rlpA and rlpB (also called lptE), in the mrd region [8]. RlpA is located between mrdB and dacA and encodes a 36-kDa lipoprotein, while rlpB/lptE is located several kilobases upstream from mrdA/pbp2 and encodes a 19-kDa lipoprotein. These lipoproteins localize to the Outer Membrane (OM), and their maturation is inhibited by globomycin, an inhibitor of signal peptidase for the major *E. coli* Lipoprotein (Lpp) and other lipoproteins [9,10]. Though rlpA and rlpB/lptE appear to be expressed at low levels, their product lipoproteins may still be functionally important for the cell.

Materials and Methods

Recent studies have revealed that the RlpB/LptE lipoprotein forms the LptD- LptE complex, which is responsible for inserting lipopolysaccharide into the outer leaflet of the OM [11-15]. The RlpA lipoprotein localizes to the ring-shaped apparatus called the septal ring, which mediates bacterial cytokinesis by binding to septal peptide glycan through the C-terminal SPOR domain [16-18]. The *E. coli* chromosome encodes four SPOR domain proteins, namely FtsN, DamX, DedD, and RlpA, all of which localize to the septal ring. Mutation studies have revealed that FtsN, DamX, and DedD are cell division proteins and are involved in bacterial cytokinesis [16-19]. However, there is no evidence as yet that RlpA is involved in cell division and morphogenesis. Thus, the physiological role of RlpA remains unknown. To elucidate the physiological function of RlpA, we constructed an RlpA deletion mutant through homologous recombination. Double mutants lacking both the RlpA and Lpp lipoproteins were also constructed,

and the effect of deleting rlpA was determined.

The *E. coli* strains used in this study are listed in Table 1. For the genetic experiments and the antibiotic sensitivity test, modified Lennox-broth, L' broth, were used, supplemented with 10 g/L polypeptone (Daigo Eiyo Chemical Co., Osaka), 5 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose, 20 mg/L thymine, and 0.1 mg/L lipoic acid (L' agar). For the P1 phage transduction experiments, L'-agar plate containing 50 m g/ml kanamycin, and M9 agar plates supplemented with 50 mg/L appropriate amino acids, 1 mg/L thiamine and 0.1 mg/L lipoic acid were used. P1 phage transduction technique was performed as previously described [20].

Strains	Relevant properties*	References
JE1011	F- thr leu trp his thi thy ara lac gal xyl mtl rpsL tonA	M. Ishibash
EC2009	JE1011, DrlpA km^r.dir.	This study
EC3001	JE1011, DrlpA km^r.rev.	This study
EC2001	JE1011, DrlpA km^r.dir. dacA11191	This study
EC3002	JE1011, DrlpA km^r.rev. dacA11191	This study
GRB19	JE1011, lpp	Spratt and Stoker [4]
BC2001	GRB19, DrlpA km^r.dir. lpp	This study
BC3001	GRB19, DrlpA km^r.rev. lpp	This study
BCA2001	GRB19, DrlpA km^r.dir. dacA11191 lpp	This study
BCA3001	GRB19, DrlpA km^r.rev. dacA11191 lpp	This study
AT1325	F-lip-9 thi-1 his-4 purB15 proA2 mlt-1 xyl-5 galK12 lacY1 str35	Taylor and Thoman [26]

*km^r.dir. and km^r.rev. Indicate insertion of the km^r gene in the same direction as the rlpA gene and in the reverse direction, respectively.

Table 1: *E. coli* strains used in this study.

To construct the rlpA deletion mutant (DrlpA), we adopted a homologous recombination method, using a temperature-sensitive plasmid, as reported by Matsuyama et al. [21]. A 15-kb fragment of the *E. coli* dacA11191 [6] chromosome covering the region from leuS to dacA (mrd region) was cloned into the temperature-sensitive plasmid pMAN031. The rlpA gene was then replaced with a kanamycin resistance (km^r) gene. The km^r gene was inserted in each direction, resulting in the two plasmids pHK002 and pHK003, each of which carried km^r in an opposite direction from the other (Figure 1(A)). *E. coli* JE1011 was transformed with these plasmids. Cells were then grown on L' agar plates containing

50 µg/mL kanamycin, and were incubated overnight at 42°C. The colonies were replicated on an L' agar plate containing 50 µg/mL ampicillin and incubated overnight at 30°C. Five out of 10^3 Km^r colonies were also determined to be ampicillin sensitive (Amp^s). These Km^r Amp^s colonies are believed to be mutants lacking the rlpA gene from the chromosome.
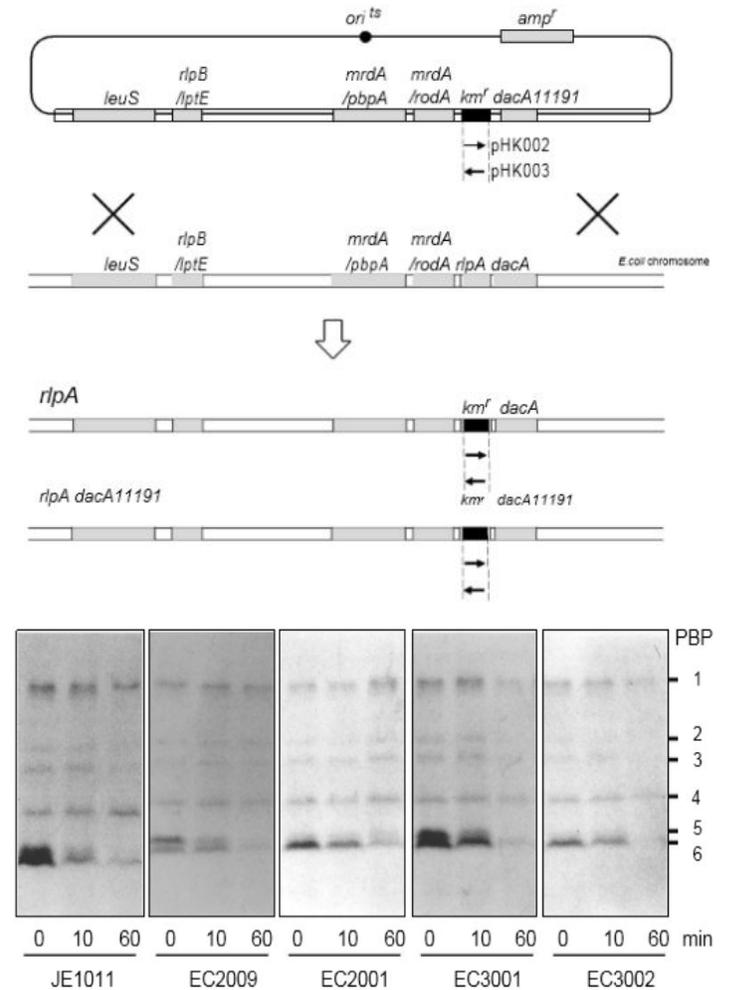

Figure 1: Isolation of DrlpA mutants by homologous recombination. (A) Homologous recombination of chromosome and plasmid led to deletion of the rlpA gene and insertion of the km^r gene into the chromosome. Arrows indicate the direction of km^r gene transcription. Two types of rlpA deletion mutants were obtained from transformants according to the sites of recombination, one deriving from recombination between km^r and dacA and the other deriving from recombination between km^r and dacA11191. (B) Chromosomal DNAs were digested with BamHI (left) and BglII (right), and were subjected to southern blot hybridization with probe 1 (left) and probe 2 (right), respectively.

Since the plasmids pHK002 and pHK003 contained the dacA11191 mutant gene coding for a mutant PBP-5 with no penicillin-releasing activity [22]. We expected to obtain two types

of mutants: DrlpA mutants with wild-type dacA and DrlpA dacA11191 mutants. Previously, we found that the *E. coli* dacA11191 mutant was more sensitive to penicillins than the wild-type dacA strain [3]. To distinguish between wild-type dacA$^+$ strains and dacA11191 mutant strains, we measured the ampicillin sensitivities of the Km^r Amp^s strains using a paper strip method. This allowed us to classify these strains as either ampicillin-sensitive or ampicillin-supersensitive. Two strains, *E. coli* EC2009 and EC3001, exhibited normal ampicillin sensitivity, whereas the other two strains, *E. coli* EC2001 and EC3002, exhibited ampicillin supersensitivity (Table 2). EC2001 and EC2009 were pHK002 transformants, and EC3001 and EC3002 were pHK003 transformants. We performed a penicillin-releasing assay [22] to confirm that the ampicillin-sensitive transformants expressed wild-type PBP-5, while the ampicillin-supersensitive transformants expressed the mutant PBP-5 (Figure 2)

Strains	Relevant properties	Antibiotics								
		Paper strip method								Step dilution method
		Ampicillin	Benzyl Penicillin	Moeno-Mycin	Macar-Mycin	Enra-Mycin	Vanco-Mycin	Baci-Tracin	Globo-Mycin	Moeno-Mycin (MIC)
JE1011		4	4	1.2	0.5	0.5	1	0.5	4	50
EC2009	JE1011, DrlpA km^r.dir.	4	4.5	4.2	2.5	2	2	2	5	25
EC3001	JE1011, DrlpA km^r. rev.	4.5	4.5	4	3	2.5	2.5	2.5	5	25
EC2001	JE1011, DrlpA km^r.dir. dacA11191	6	8	5.2	5	3.5	4	4	4.5	12.5
EC3002	JE1011, DrlpA km^r. rev. dacA11191	7	7	5.5	5.2	4	3.8	3.5	4.8	12.5
GRB19	lpp	4	5.5	6	6.1	5	4	4	0.5	6.25
BC2001	GRB19, DrlpA km^r. dir. lpp	4	5.5	7	7.2	6	6	5	1	3.12
BC3001	GRB19, DrlpA km^r. rev. lpp	4.2	5.5	7.5	7.2	6.4	5	5	1	3.12
BCA2001	GRB19, DrlpA km^r. dir. dacA11191 lpp	6	7	8.5	8	7	6.8	6.5	1	1.56
BCA3001	GRB19, DrlpA km^r. rev. dacA11191 lpp	6.5	7	8.5	8	7.5	6.8	6.8	1	1.56

For antibiotic sensitivity assay using the paper strip method, cell culture (A$_{660}$ = 0.1) was diluted 10-fold with L' broth without NaCl, and 10 ∝L aliquots were streaked onto L' plates without NaCl. Whatman No. 3 MM filter paper strips (0.3 × 7.0 cm) were moistened with solutions of solutions of ampicillin (0.2 mg/ml), penicillin (0.2 mg/ml), moenomycin (2.5 mg/ml), macarbomycin (2.5 mg/ml), enramycin (20 mg/ml), vancomycin (20 mg/ml), bacitracin (20 mg/ml) or globomycin (2 mg/ml), and placed on the agar plates. *E. coli* were incubated for 48 h at 42˚C. Values are expressed as mm length of inhibitory zone. In the step dilution method, cells (10^4/mL) were incubated in L' broth without NaCl containing serially diluted moenomycin for 48 h at 42°C. Numbers show Minimum Inhibitory Concentrations (MIC) of moenomycin (mg/ml).

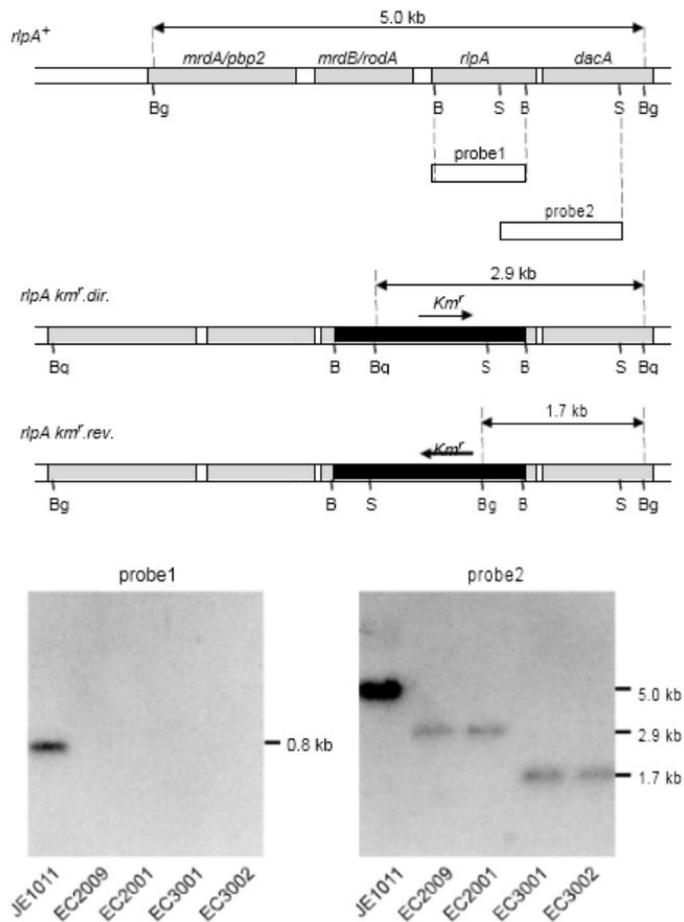**Table 2:** Estimation of antibiotic sensitivities of the *E. coli* rlpA$^+$ and ΔrlpA strains.

Figure 2: Penicillin-releasing assay of DrlpA mutants. Penicillin-binding proteins from the membrane fractions of *E. coli* strains were labeled with [^{14}C] penicillin G, and the release of penicillin was analyzed by addition of unlabeled penicillin G. After various lengths of time, the reaction was stopped, and the samples were subjected to SDS-PAGE and gel fluorograms.

The order of genes on the DrlpA mutant chromosome was determined by mapping of the km^r gene with P1 phage and by Southern blot hybridization analysis. P1 phage transduction technique was performed as previously described [20]. DrlpA mutation was transduced with the km^r gene as the marker using P1 phages grown in the DrlpA transformants into the recipient *E. coli* lip$^-$ AT1325. The co-transduction frequencies of the km^r gene and the lip gene were 79% in EC2009, 80% in EC3001, 78% in EC2001, and 74% in EC3002. These frequencies were similar to the co-transduction frequency (76%) of mrdB and lip in a previous study [3]. Deletion of the rlpA gene from the *E. coli* chromosome was confirmed by Southern hybridization (Figure 1(B)).

Isolated DrlpA mutants grew normally in L' broth, exhibiting the normal rod shape. Omitting NaCl from the medium and growing cells at a higher temperature (42°C) did not affect the growth or morphology of the DrlpA mutants. We detected no sign that the rlpA gene is indispensable for growth and shape-determination of the cell. Double mutant strains lacking both the RlpA and Lpp lipoproteins (BC2001 and BC3001) were isolated by transducing the lpp mutant strain *E. coli* GRB19 with P1 phage grown in one of the *E. coli* DrlpA mutants (EC2009 or EC3002) using the km^r gene as a transduction marker. Triple mutants BCA2001 and BCA3001, carrying DrlpA lpp dacA11191, were isolated by transduction of *E. coli* GRB19 with P1 phage grown in *E. coli* EC2001 or EC3002. All mutants with both lipoprotein deletions exhibited the normal rod shape.

Despite exhibiting normal morphologies, the DrlpA mutants showed increased sensitivity to several antibiotics, as measured by paper strip and step dilution methods (Table 2). Vancomycin and bacitracin were obtained commercially from Sigma (St. Louis, MO, USA); ampicillin, benzylpenicillin, and macarbomycin were obtained from Meiji-Seika Co. (Tokyo, Japan); moenomycin was obtained from Hoechst (Frankfurt, Germany); enramycin was obtained from Takeda Chemical Industries (Osaka, Japan); and globomycin was obtained from Sankyo Co. (Tokyo, Japan). Sensitivity to antibiotics such as moenomycin, macarbomycin, enramycin, vancomycin, and bacitracin increased appreciably when the DrlpA mutation was introduced into the JE1011 strain, and sensitivity to globomycin also increased slightly. In contrast, deletion of rlpA did not greatly affect sensitivity to penicillin, rifampicin, or novobiocin (data not shown). No increases in sensitivity to the above antibiotics have been observed due to introduction of the kanamycin resistance mutation alone (data not shown).

It is well known that SPOR-domain proteins are recruited to the septal ring, which mediates bacterial cytokinesis [1]. Among these proteins, FtsN is essential for cell division [19], and DamX and DedD are genuine division proteins that contribute significantly to the cell constriction process [16-18]. In contrast, the function of the SPOR-domain protein RlpA remains unclear. A recent study by Yahasiri et al. revealed that in *Pseudomonas aeruginosa,* RlpA is a lytic transglycosylase that contributes to rod shape and daughter cell separation [23]. Thus far, however, researchers have been unable to obtain any evidence indicating that the *E. coli* RlpA exhibits lytic transglycosylase activity [16]. We found that *E. coli* rlpA deletion mutants divided normally and did not show any significant morphological changes, consistent with previous findings [16-18].

Conclusion

Nevertheless, it is unlikely that *E. coli* RlpA is not involved in cell division or morphogenesis in some way. It has been reported that a truncated rlpA mutant is a multicopy suppressor of a mutation of the periplasmic protease gene prc, which is involved in cell division [24]. It is probable that double or multiple mutations in different lipoproteins are required to cause such phenotypic changes in the cell. Alternatively, RlpA may be

involved in the membrane integrity of *E. coli*. RlpA localizes not only to the septal ring but also to foci at various sites along the cell cylinder, suggesting that RlpA plays distinct roles in cytokinesis and envelope maturation [17]. Sensitivity to globomycin increased slightly in the DrlpA mutants, while it has been shown to decrease in lpp mutants [25]. As formation of the RlpA lipoprotein and its assembly at the membrane are inhibited by globomycin [8], the increased sensitivity of DrlpA mutants to globomycin may indicate that RlpA is involved in cell proliferation. In this model, RlpA would act as a minor component of membrane lipoproteins in the formation of the *E. coli* cell envelope.

Acknowledgments

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