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Deletion of internal twenty-one amino acid residues of *Escherichia coli* prolipoprotein does not affect the formation of the murein-bound lipoprotein

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Mutation *pgsA* affecting the phosphatidylglycerol phosphate synthesis is lethal for all but certain *E. coli* strains such as strains deleted for the *lpp* gene or strains containing unmodifiable prolipoprotein like *lpp*D14. Strain SD312 *pgsA3* is tolerant to *pgsA* mutation, which suggests the *lpp* alleles in strain SD312 *pgsA3* and its parental strain SD12 may be defective. DNA sequence analysis of the *lpp* genes in *Escherichia coli* strains SD12 and SD312 *pgsA* using asymmetric polymerase chain reaction showed that the *lpp* alleles in these two strains contained a 63 base pair deletion corresponding to the 37th to 57th codons of the wild-type *lpp* gene. [^{3}H]Palmitate labeling of strains SD12 and SDS312 showed that the mutant lipoprotein in SD12 strain was modified with lipid, while the prolipoprotein in SD312 was not modified. The shortened mature lipoprotein in SD12 and the lipid-modified prolipoprotein in globomycin-treated SD12 were found to be covalently attached to the peptidoglycan, while the unmodified prolipoprotein in SD312 did not form significant amounts of murein-bound lipoprotein.

lpp deletion mutant; Prolipoprotein modification and processing; Bound-form lipoprotein

1. INTRODUCTION

The *E. coli* murein lipoprotein is first synthesized as a precursor, the prolipoprotein, which contains a twenty amino acid residue signal peptide [1]. The prolipoprotein undergoes a series of modification and processing reactions to become the mature free-form lipoprotein, one third of which is covalently attached to the peptidoglycan [2]. Lipid modification of the prolipoprotein is a multi-step process; the first step is the transfer of the glyceryl moiety from phosphatidylglycerol to the cysteine residue in the unmodified prolipoprotein by prolipoprotein glyceryl transferase [3]. *O*-acylation of glyceryl-modified prolipoprotein by *O*-acyl transferase(s) utilizes the major glycerophosphatides (phosphatidylglycerol, phosphatidylethanolamine or cardiolipin) as the acyl donor [4]. Asai and coworkers observed that the *pgsA3* allele encoding a defective phosphatidylglycerophosphate synthase is lethal for all but certain *E. coli* strains such as SD strains [5]. *pgsA3* mutation is tolerated in the latter strains as well as in *E. coli* mutants deleted for the *lpp* gene and those containing unmodifiable mutant prolipoprotein such as *lpp*D14 [5]. These results suggest that the lipoprotein gene in SD strains may be defective as well.

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In this paper, we report the cloning and DNA sequence determination of the *lpp* alleles in strains SD12 and SD312 *pgsA3* using asymmetric polymerase chain reaction. Our results show that the *lpp* gene in these two strains contains a 63 base pair deletion corresponding to the 37th to 57th codons of the wild-type *lpp* gene. In spite of this extensive alteration in the prolipoprotein structure, the mutant prolipoprotein is modified, processed and covalently attached to peptidoglycan in strain SD12.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and medium*

E. coli K-12 strain SD12 (F$^-$, *lpp-12 galK35 glpD3 glpK glpR2 his68 phoA8 pyrD34 rspL118*) and its acidic phospholipid-deficient derivative, SD312 (SD12 *cls-1 pgsA3*) were gifts from I. Shibuya [5]. Media used in this study included L broth, M9 glucose minimal medium supplemented with uracil (25 μg/ml), thiamine (5 μg/ml) and histidine (50 μg/ml).

2.2. *Enzymes and chemicals*

Taq polymerase was from Perkin Elmer-Cetus Co. (Norwalk, CT). DNA sequenase kit was from United States Biochemical Corp. (Cleveland, OH). [^{3}H]Palmitic acid (60 Ci/mmol) and [α-^{35}S]dATP (1200 Ci/mmol) were from Amersham Corp. (Arlington Heights, IL). [^{35}S]Methionine (1000 Ci/mmol) was from ICN Biomedical Inc. (Irvin, CA). Oligonucleotide primers M1 (GTGTAATACTTGTAACGC, Fig. 1) and M2 (GTAGCGGTAAACGGCAGAC, Fig. 1) were purchased from the Oligonucleotide Synthesis Facility at the Uniformed Services University of the Health Sciences. Globomycin was obtained from M. Arai, Sankyo Co. (Tokyo, Japan).

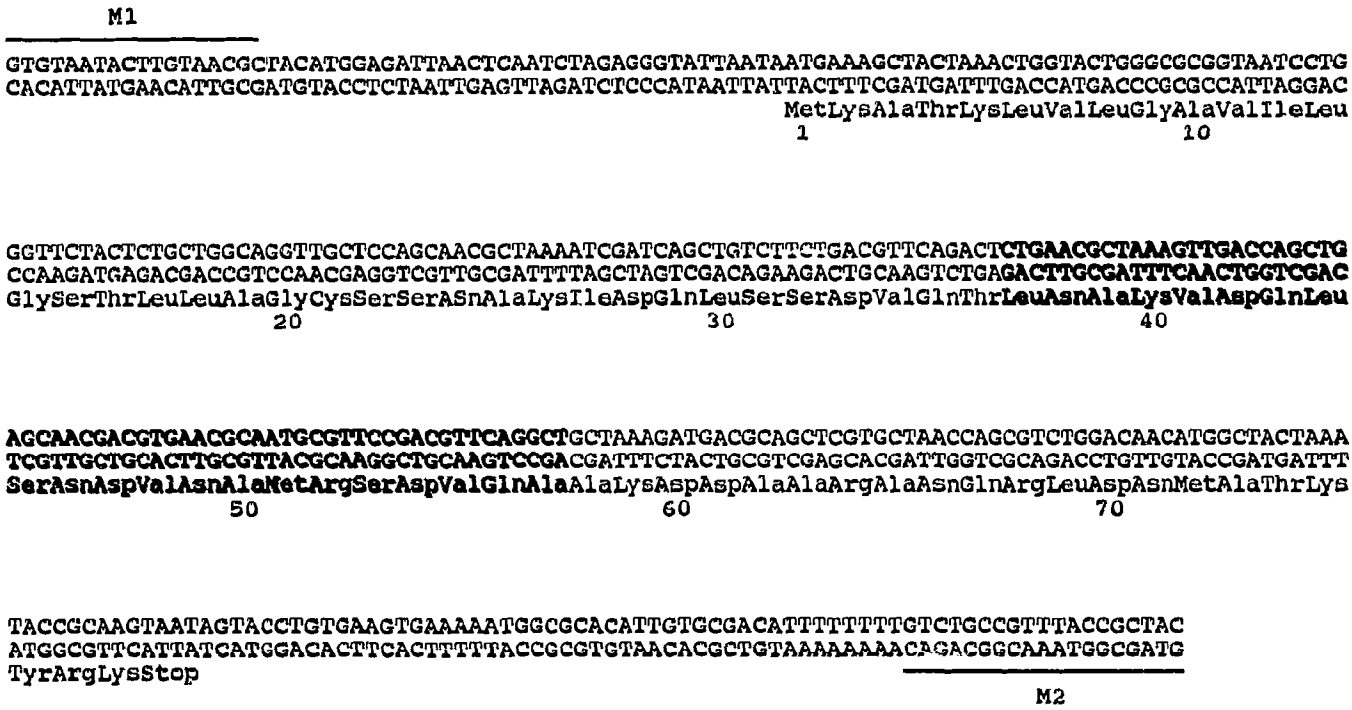

Fig. 1. PCR amplification and DNA sequence determination of the *lpp* alleles from strains SD312 and SD12. The nucleotide and amino acid sequences in bold-face are deleted in *lppΔ*37–57 mutant strains SD12 and SD312. M1 and M2 are oligonucleotide primers used in PCR and DNA sequence analysis.

2.3. *PCR amplification of the* lpp *gene and sequence analysis of the PCR products*

Bacterial genomic DNA was isolated by the methods described by Silhavy et al. [6]. PCR reactions were performed according to Erlich [7] in 100 μl reaction mixture containing 100 ng genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.2 mM each of dNTP. Taq polymerase (2.5 units) was added to the reaction mixture last. For asymmetric PCR, 1 pmol limiting primer (M1) and 100 pmol excess primer (M2) were added to the reaction mixture, and the amplification was performed for 35 cycles with the addition of 1 unit Taq polymerase to the reaction mixture during the last 10 cycles. In each cycle, the reaction mixture was sequentially incubated at 94°C for 1 min, 55°C for 2 min and 72°C for 1 min. The products of asymmetric PCR were purified using microconcentrator Centricon 30 and used for sequencing. DNA sequencing was carried out using a sequencing kit and a protocol from the United States Biochemical Corp. The products of asymmetric PCR were treated with 1 N NaOH before annealing with the sequencing primer, which was the limiting primer (M1) in the asymmetric PCR reaction.

2.4. *Labeling and analysis of the free- and bound-form lipoproteins*

Strains SD12 and SD312 were labeled with [^{3}H]palmitic acid or [^{35}S]methionine; when indicated, globomycin (80 μg/ml) was added 20 min prior to the labeling. Strains SD12 and SD312 were grown to A_{600nm} = 0.3 in 5 ml M9 medium supplemented with glucose, required amino acids and growth factors, and labeled with 50 μCi [^{35}S]methionine until A_{600nm} = 1.0. To study lipid modification of lipoprotein, strains SD12 and SD312 in 5 ml LB medium were labeled with 200 μCi [^{3}H]palmitic acid for 1 h. The preparation of the cell envelope and the isolation of the free- and bound-form lipoproteins were performed as described [8]. The radioactivities in the free- and bound-form lipoprotein were determined by liquid scintillation counting, and analyzed by Tricine-SDS polyacrylamide gel electrophoresis using a 16.5% T and 6% C separating gel [9].

3. RESULTS

3.1. *PCR amplification and sequence determination of the* lpp *alleles in strains SD12 and SD312*

The *lpp* alleles in strains SD12 and SD312 were amplified by an asymmetric PCR, and the single-stranded PCR products were used directly in DNA sequencing. DNA sequence analysis using M1 oligonucleotide as the sequencing primer revealed that the *lpp* alleles in both strain SD12 and SD312 were identical, and contained a deletion of 63 base pair corresponding to the 37th to 57th codons of the wild-type *lpp* gene of *E. coli* (Fig. 1). In addition, we used two primers, 5′GGCTCTGCAGAGCAATCTGG3′ and 5′CAGCAGAGTAGAACCCAGG3′, flanking the *lpp* promoter in asymmetric PCR followed by DNA sequencing reactions. The DNA sequence from −320 bp to 14 bp (based on the *lpp* mRNA start site) in strains SD12 and SD312 were found to be identical to that reported for the wild-type *lpp* gene (data not shown).

3.2. *Lipid-modification and processing of the prolipoproteins with a internal deletion in strains SD12 and SD312*

Ouchterlony test of the crude extract of strains SD12 and SD312 revealed that strain SD12 produced significant, albeit reduced, amounts of lipoprotein while SD312 contained negligible amounts of lipoprotein

(data not shown). [^{3}H]Palmitic acid labeling of strain SD12 showed that the shortened lipoprotein and prolipoprotein in control and globomycin-treated SD12 cells were modified with lipid (Fig. 2, lanes 2 and 5). SDS-PAGE analysis of the [^{35}S]methionine-labeled lipoproteins from globomycin-treated SD12 cells revealed that the lipid-modified prolipoprotein SD12 was processed by the signal peptidase II (Fig. 3, lanes 2, 5). Strain SD312 is defective in phosphatidylglycerol phosphate synthase; consequently, the prolipoprotein in SD312 was not modified with lipid (Fig. 2, lane 6). Because of the lack in lipid modification, the unmodified prolipoprotein in mutant SD312 was not processed (Fig. 3, lanes 3, 6).

3.3. *Formation of murein-bound lipoprotein in* lpp *internal deletion mutants*

SD12 and SD312 were checked for the phenotypes of leakiness of periplasmic RNase and sensitivity to sodium dodecyl sulfate (SDS) as described previously [10,11]. Strain SD312 exhibited increased sensitivity toward SDS and leakiness of periplasmic RNase, while strain SD12 was SDS-resistant and RNase-nonleaky. These results suggest that SD312 mutant might be defective in the attachment of murein-bound lipoprotein. The shortened free-form lipoprotein in strain SD12 was covalently attached to the peptidoglycan (Table I and Fig. 3, lane 8). The lipid-modified prolipoprotein in globomycin-treated SD12 cells was also covalently attached to the peptidoglycan (Table I and Fig. 3, lane 11). These results indicate that the deletion of internal twenty-one amino acid residues of prolipoprotein does not affect the formation of murein-bound lipoprotein. In contrast, the unmodified prolipoprotein in strain SD312 did not form murein-bound lipoprotein (Table I and Fig. 3, lane 9 and 12). This defect in the assembly of bound-form lipoprotein in SD312 probably results from a defective translocation of prolipoprotein in SD312 mutant cells.

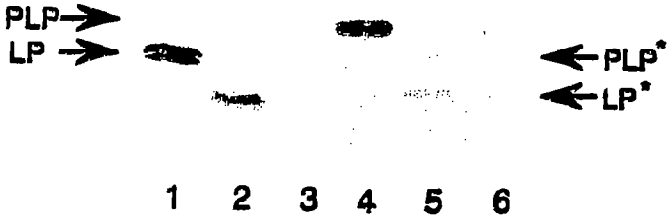

Fig. 2. SDS-polyacrylamide gel electrophoresis of [^{3}H]palmitic acid-labeled lipoprotein and prolipoprotein from the wild-type strain E613 and *lpp* internal deletion mutant strains SD12 and SD312. Five ml cultures of these strains were labeled with 200 μCi [^{3}H]palmitic acid at A_{600nm} = 0.3 for 1 h. Globomycin (80 μg/ml) was added 20 min prior to the labeling. Trichloroacetic acid (TCA) was added to a final concentration 10% to stop the labeling reaction. The TCA precipitation was collected by centrifugation at 5,000 × *g* for 10 min at 4°C, washed with 3 ml cold acetone twice, dried in air and extracted with 100 μl 1% SDS (pH 7.0) at 100°C for 20 min, and the free-form lipoprotein was isolated from 1% SDS soluble fraction by a modified immunoprecipitation procedure described previously [8]. Ten μl of the lipoprotein and prolipoprotein samples were loaded in each lane of a Tricine-SDS gel. Lanes 1–3 represent the free-form lipoproteins from E613, SD12 and SD312, respectively, and lanes 4–6 represent the prolipoproteins from the globomycin-treated E613, SD12 and SD312, respectively. PLP, prolipoprotein; LP, lipoprotein; PLP*, mutant prolipoprotein; LP*, mutant lipoprotein.

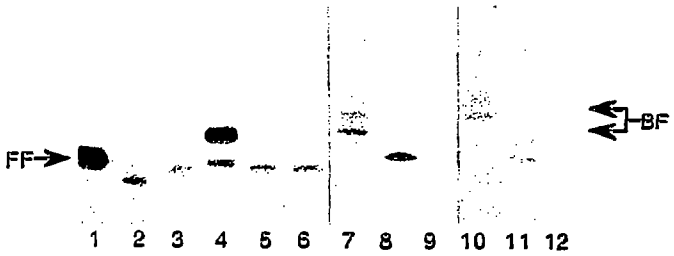

Fig. 3. SDS-polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled free- and bound-form lipoproteins from strains E613, SD12 and SD312 with or without globomycin-treatment. The labeled cells were harvested, disrupted in a sonicator; the cell envelope was isolated by centrifugation at 200,000 × *g*, for 1 h at 4°C, and extracted with 1% SDS at 100°C for 20 min. The free-form lipoprotein was isolated from the 1% SDS soluble fraction by immunoprecipitation. The 1% SDS insoluble pellet was extracted with 4% SDS at 100°C for 30 min; and the murein sacculus containing bound-form lipoprotein was isolated by centrifugation at 200,000 × *g* for 90 min, washed with 6 ml water three times, resuspended in 150 μl 10 mM sodium phosphate buffer and digested with 200 μg/ml lysozyme at 37°C overnight. Ten μl of the free- and bound-form lipoprotein were loaded in each lane of a Tricine-SDS gel. (Lanes 1–3) Free-form lipoprotein from strain E613, SD12 and SD312, respectively; (lanes 4–6) prolipoproteins from globomycin-treated E613, SD12 and SD312, respectively; (lanes 7–9) bound form lipoprotein from E613, SD12 and SD312, respectively; (lanes 10–12) murein-bound prolipoproteins from globomycin-treated E613, SD12 and SD312, respectively. FF, mature free-form lipoprotein; BF, wild-type bound-form (pro)lipoprotein.

4. DISCUSSION

Asymmetric PCR has been successfully used for the amplification and DNA sequence analysis of the *lpp* alleles in strains SD12 and SD312. The lipoprotein in strains SD12 and SD312 was found to contain a deletion of twenty-one amino acid residues internal to lipoprotein. The deleted internal sequence of the prolipoprotein is not essential for the lipid modification and processing of the prolipoprotein. This is consistent with the observation that an Lpp-Bla hybrid prolipoprotein containing the Lpp signal sequence plus nine amino acid residues at the amino-terminal region of the mature lipoprotein followed by the β-lactamase sequence is lipid-modified and processed like the wild-type lipoprotein [12].

It is not surprising that the deletion of internal twenty-one amino acid residues does not affect the formation of murein-bound lipoprotein. Analysis of the amino acid sequence of prolipoproteins from *E. coli* and four other enterobacteriaceae species including *Proteus mirabilis*, *Morganella morganii*, *Erwinia amylovora*, and

Table I

Murein-bound lipoprotein contents in *lpp* internal deletion mutants

Strain	Murein-bound lipoprotein (% of wild-type strain)
E613 (wild-type)	100
SD12	82
SD312	8
Globomycin-treated E613	47
Globomycin-treated SD12	36
Globomycin-treated SD312	6

Serratia marcescens [13] reveals that the signal sequence of prolipoprotein, the amino-terminal sequence (C21–L30) and the carboxy-terminal sequence (A62–K78) of the mature lipoprotein are highly conserved, with a sequence homology of 65%, 70%, and 71%, respectively. In contrast, the internal sequence of the prolipoprotein (S31–D61) has a low (32%) sequence homology. Our results indicate that the less conserved internal sequence of the prolipoprotein is not essential for the formation of murein-bound lipoprotein.

The defect in the lipid-modification of the mutant prolipoprotein in strain SD312 *pgsA3* is most likely due to the defective synthesis of phosphatidylglycerol phosphate, which results in a deficiency of phosphatidylglycerol in the mutant cell envelope. However, the defect in the attachment of the unmodified prolipoprotein to the peptidoglycan in strain SD312 is not due to the lack of lipid-modification and processing of the mutant prolipoprotein. We have shown recently that neither the lipid-modification nor the processing of prolipoprotein is a prerequisite for the formation of murein-bound lipoprotein [14]. Most likely, the defect in the formation of bound-form lipoprotein in mutant strain SD312 results from a defective translocation of the prolipoprotein across the cytoplasmic membrane. It has been reported that acidic phospholipids are required for the translocation of precursor proteins in *E. coli*, both in vivo and in vitro [15]. As a result of the defective translocation of prolipoprotein in strain SD312, the unmodified prolipoprotein is not attached to the peptidoglycan in a reaction presumably occurring in the periplasm.

The biochemical basis for the survival of strain SD312 containing the *pgsA3* allele remains unknown. It is not clear how the internal deletion in the *lpp* gene might contribute to this tolerance to the *pgsA3* allele, since the *lppΔ37–57* deletion does not appear to affect the modification and processing of mutant prolipoprotein in strain SD12. The tolerance of strain SD312 to the *pgsA3* mutation may be related to the low level of (pro)lipoprotein in this mutant strain; Ouchterlony test revealed that SD312 strain contains greatly reduced amount of (pro)lipoprotein as compared to strain SD12. The molecular basis for the reduced steady-state level of lipoprotein in this mutant remains to be determined.

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