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Escherichia coli Mutants Altered in Murein Lipoprotein

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Mutants with alterations in the structure, biosynthesis, or assembly of murein lipoprotein were selected by a procedure based on radiation suicide of wild-type organisms by [^{3}H]arginine under conditions where the radioactive arginine was preferentially incorporated into lipoprotein. Further screening for the potential mutants among the survivors of [^{3}H]arginine suicide was carried out by using a sensitive immunodiffusion test, followed by radioactive double-labeling experiments. Three mutants were obtained and partially characterized.

The cell envelopes of *Escherichia coli* and other gram-negative bacteria contain a so-called rigid layer that is in essence a giant macromolecule composed of murein and a covalently attached lipoprotein (2, 4). This lipoprotein is present in two forms, free and bound, the latter being attached to the *meso*-diaminopimelic acid residues in the murein sacculus through the ϵ-NH_2 group of the C-terminal lysine of the lipoprotein (2, 3, 5, 14). The primary structure of this unique lipoprotein has been determined by Braun and his co-workers (3, 7). The biochemical mechanism for the synthesis and assembly of lipoprotein in vivo remains to be elucidated (4, 15). Further, the physiological functions of lipoprotein are yet to be ascertained. It has been suggested that one possible function of the murein lipoprotein is to connect the outer membrane with the murein sacculus, thereby allowing the murein lipoproteins to serve as anchored cores of outer membrane components (2). A more specific model for the arrangement and function of lipoprotein in the cell envelope has been postulated (12). It is proposed that a complex of six lipoprotein molecules in the outer membrane provides a tubular hydrophilic channel through the outer membrane, thus constituting a passive diffusion pore.

To facilitate studies on the biosynthesis and assembly of murein lipoprotein and to ascertain the function of lipoprotein, we have attempted to isolate mutants of *E. coli* with alterations in the structure, biosynthesis, or assembly of murein lipoprotein. This paper describes a procedure for the selection and screening of putative lipoprotein mutants as well as the preliminary characterization of these mutants.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in the present study are listed in Table 1. Media used for the present study included L broth, proteose peptone beef extract broth, and M9 minimal medium (19, 20). Cells were grown aerobically at a given temperature with vigorous shaking in Erlenmeyer flasks with capacity at least four times the volume of the culture. Growth was monitored by measuring the optical density at 600 nm in a Zeiss spectrophotometer.

Mutagenesis. Cells were mutagenized with ethyl methane sulfonate by the method of Lin et al. (16), or with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1).

Labeling experiments. M9 minimal medium supplemented with 0.4% glucose and 20 μg of each of required amino acids per ml was used for the growth of cells and their subsequent labeling with radioactive amino acids. A 25-ml culture was grown in a 125-ml Erlenmeyer flask at 30 C with vigorous shaking. At the early logarithmic phase of growth (cell density about 1.5×10^8 ml), the culture was shifted to 42 C for 1 h. Radioactive arginine (12.5 to 25 μCi of [^{3}H]arginine or 5 to 10 μCi of [^{14}C]arginine) was added to a final concentration of 10 μg of arginine per ml of culture, and the labeling was continued for 1 h at 42 C. The labeled cells were harvested by centrifugation at 7,000 $\times$ *g* for 10 min, washed twice with saline, and processed for the isolation of the cell envelope and subsequent fractionations by the procedures described below.

Preparation of cell envelope and isolation of murein lipoprotein from the cell envelope. Washed cells were suspended in 5 ml of 0.01 M sodium phosphate buffer (pH 7) and sonicated for 1 to 3 min with Branson Sonifier with intermittent cooling at 0 C. The crude extract was then centrifuged at 250,000 $\times$ *g* for 2 h. The pellet (cell envelope fraction) was washed once with 5 ml of the same buffer and centrifuged again at 250,000 $\times$ *g* for 2 h.

Murein sacculi were isolated by two extractions of the washed cell envelopes with 1 ml of 4% sodium dodecyl sulfate (SDS) (pH 7.0) at 100 C for 20 min. After each extraction, the SDS-insoluble sacculi were collected by centrifugation at 250,000 $\times$ *g* for 2 h at room temperature. The murein sacculi (or murein lipoprotein complex) were washed three times with 10 ml of either water or 80% acetone in water for the removal of the detergent. To release the

TABLE 1. *Characteristics of bacterial strains*

Strain	Mating type	Genotype	Source including mutagenesis and selection
AB1157	K-12 F⁻	*thr-1, ara-14, leu-6, proA2, lacY1, tsx-33, gal K2, his-4, str-31, xyl-5, mtl-1, argE-3, thi-1, λ⁻, sup-37*	E. A. Adelberg
Mutant 1	K-12 F⁻	Same as AB1157	NTG,[a] [³H]arginine suicide, this paper
Mutant 2	K-12 F⁻	Same as AB1157	NTG, [³H]arginine suicide, this paper
MB204	K-12 F⁻	ara_{am} $lac125_{am}$ $galU4$Kinase$_{am}$ $galE^-$ trp_{am} suIIIA81 (ts *sup*)	J. D. Smith (17) through W. D. Donachie
E600	K-12 F⁻	Same as MB204; in addition, his⁻ pro⁻	EMS,[b] penicillin selection, this paper
Mutant 3	K-12 F⁻	Same as E600	NTG, [³H]arginine suicide, this paper

[a] NTG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.
[b] EMS, Ethyl methane sulfonate.

bound form of lipoprotein, the washed murein sacculi were digested with 50 to 200 μg of egg white lysozyme or T4 phage lysozyme per ml of 0.01 M sodium phosphate (pH 7.0) for at least 2 h at 37 C. The free form of lipoprotein, labeled with [³H]- or [¹⁴C]arginine, was isolated from the SDS-soluble supernatant of the cell envelope by radioimmunoprecipitation as described below.

To obtain lipoprotein-free murein, murein lipoprotein complexes were digested with Pronase (murein/Pronase = 50:1, wt/wt) in 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 0.5 mM $CaCl_2$ at 37 C for 2 days. Murein was reisolated from the incubation mixture by centrifugation at 200,000 × *g* for 2 h after two extractions with 4% SDS at 100 C. The murein was washed extensively with water.

Immunological assay of murein lipoprotein. Rabbits were immunized with the highly purified bound form of lipoprotein isolated from *E. coli* K-12 strain AB1157. An aqueous suspension of murein lipoprotein (2.1 mg/ml) was mixed with an equal volume of Freund complete adjuvant (Difco) and injected subcutaneously into all four footpads as well as along the back. A series of booster injections was administered 4 weeks after the initial challenge. Antiserum with good titer against purified lipoprotein was collected 2 weeks after administration of the booster injections and thereafter at regular intervals. Antisera were also prepared against the purified free form of lipoprotein isolated from various strains of *E. coli* K-12 as well as from *Salmonella typhimurium* These anti-lipoprotein antisera were shown to be specific towards lipoprotein by Ouchterlony double-diffusion techniques, by quantitative precipitation tests, and by immunoelectrophoresis. They did not react with purified lipopolysaccharide, SDS, lysozyme, phospholipids, mucopeptide monomer and dimers, and other membrane proteins (Lin and Wu, unpublished data). Ouchterlony double-diffusion tests were carried out on glass slides coated with 1% agar containing 0.05% SDS in 50 mM sodium barbital buffer at pH 8.6. A 15-μl aliquot of 1% SDS solution of cell envelope proteins containing lipoprotein was added into each well, and 15 μl of undiluted antiserum was placed in the center well. Antigen-antibody complexes were readily visible as precipitating lines, after incubation of the slides for 6 to 12 h at room temperature in a moist chamber. This procedure allowed the detection of as little as 0.5 μg of lipoprotein. For rough strains of *E. coli*, an agglutination test was also carried out. A 0.1-ml aliquot of an overnight culture was added to a tube containing 1 ml of 1% antiserum in L broth. The tubes were incubated at various temperatures for 4 to 6 h. Control tubes (minus antiserum) were incubated under identical conditions.

The assay of lipoprotein by radioimmunoprecipitation was carried out according to Hirashima et al. (10). The optimal concentrations of antigen and antiserum were determined by quantitative precipitation tests to be 150 μg of purified lipoprotein and 0.1 ml of antiserum, respectively. Purified lipoprotein was added as carrier for radioimmunoprecipitation accordingly.

Other analytical techniques. Polyacrylamide gel electrophoresis was carried out in 0.5% SDS as described by Inouye and Guthrie (13). Gels containing 7.5% acrylamide were routinely used. Occasionally, we used gels containing 10%, 12.5%, and 15% acrylamide to assure better separation of lipoproteins from other envelope proteins. Dansylated internal molecular weight standards (11) were the generous gift of M. Inouye of State University of New York at Stony Brook. After electrophoresis, the gels were sectioned into 1- or 2-mm slices, each of which was dissolved in 1 ml of 1 N NH_4OH and counted. Measurements of radioactivities were made in a Beckman scintillation counter with a toluene-based scintillation solution containing 13% Biosolv BBS-3 (Beckman).

To achieve better resoluion of proteins in the molecular weight range of 8,000 to 15,000, polyacrylamide gel electrophoresis was also carried out in the presence of both 1% SDS and 8 M urea (18). Gels containing 12.5% acrylamide with 1:10 (bisacrylamide) cross-linkage were used routinely.

Chemical analyses of the purified murein sacculus were performed as follows. Acid hydrolyses were carried out in a vacuum-sealed ampoules. For amino acid and fatty acid analyses, the murein lipoprotein complex was suspended in 6 N HCl and hydrolyzed at 105 C for 20 h. For determination of total hexosamine, hydrolyses were carried out in 4 N HCl at 100 C for 15 h. Amino acid analyses were performed on a Beckman automatic amino acid analyzer, using

a special program in order to separate muramic acid from glutamic acid and methionine from *meso*-diaminopimelic acid. The total hexosamine content in hydrolysates of murein sacculi was determined by the Elson-Morgan assay as described previously (20). The fatty acids of the murein lipoprotein complex were quantitatively determined by gas-liquid chromatography. The conversion of fatty acids to their methyl esters and their subsequent chromatography using Hewlett-Packard gas-liquid chromatograph were as previously described (9).

To determine the degree of peptide cross-linking in the murein, the amino acid composition of purified murein was determined both before and after dinitrophenylation with fluorodinitrobenzene (6).

Chemicals and radiochemicals. All chemicals were of reagent or analytical grade and were purchased from commercial sources. Radioactive chemicals used in the present study included L-[3-^{3}H(N)]arginine (27 Ci/mmol), L-[U-^{14}C]arginine (309 mCi/mmol) or L-[guanido-^{14}C]arginine (50 mCi/mmol), L-[3-^{3}H]tyrosine (28 Ci/mmol), and L-[U-^{14}C]tyrosine (460 mCi/mmol). These radiochemicals were purchased from either New England Nuclear Corp. or Schwarz/Mann.

RESULTS

Selection of mutants defective in the structure, biosynthesis, or assembly of murein lipoprotein. The rationale for the selection of lipoprotein-deficient mutants is as follows. A unique structural feature of the lipoprotein is the conspicuous absence of certain amino acids, including his, pro, trp, phe, and gly (3). Furthermore, the biosynthesis of this protein is extremely resistant to both puromycin and rifampin (8). We therefore reasoned that mutants deficient in the synthesis and/or assembly of lipoprotein will be spared from radiation damage caused by the incorporation into the lipoprotein of a radioactive amino acid such as arginine under specific conditions, namely, in the presence of puromycin and/or rifampin and in the absence of his, pro, trp, phe, and gly, which favors preferential if not exclusive incorporation of this amino acid into lipoprotein.

Wild-type strains AB1157 and MB204 *his*$^{-}$*pro*$^{-}$(E600) were mutagenized with nitrosoguanidine, and the washed mutagenized cells were grown overnight at 30 C in M9 minimal medium supplemented with 0.4% glucose and 20 μg of each of required amino acids per ml.

The overnight culture was then diluted 10-fold with the same medium to give a 25-ml culture, and growth was continued at 30 C for 3 h. The cells were then harvested, washed, and resuspended in 10 ml of M9 glucose medium supplemented with all required amino acids except histidine and proline. The cells were starved for histidine and proline for 1 h at 30 C and then shifted to 42 C in the same medium for an additional hour. Puromycin was then added to a final concentration of 600 μg/ml. The culture was then split into two equal aliquots. To one of the cultures was added [^{3}H]arginine to a final concentration of 1 to 2 mCi per 5 ml of culture, with specific activity of approximately 30 Ci/mmol. In the case of AB1157 cells, nonradioactive arginine was omitted from the medium during labeling. In a few instances rifampin, as well as puromycin, was added at a final concentration of 200 μg/ml 10 min before the addition of [^{3}H]arginine to further reduce the incorporation of [^{3}H]arginine into other proteins than lipoprotein. Approximately 30% of total [^{3}H]arginine incorporated under these conditions was found in the free form of lipoprotein of the cell envelope, as identified by SDS-polyacrylamide gel electrophoresis. After incorporation of [^{3}H]arginine into one of the two cultures for 30 to 60 min at 42 C, both cultures were harvested by centrifugation at 8,000 $\times$ *g* for 10 min. The cells were washed twice with M9 minimal media and twice with distilled water and then resuspended in 5 ml of M9 minimal media. The washed cells were kept at 4 C, and viable cells were measured at regular intervals of time by plating serial dilutions on LC plates incubated at 30 C. A typical experiment illustrating the radiation damage caused by [^{3}H]-arginine is shown in Fig. 1.

When the loss in viability of ^{3}H-labeled cells reached more than three orders of magnitude, single colonies were obtained at 30 C and grown up in 5 ml of LB cultures at both 30 and 42 C. After growth, the cells were harvested, resuspended in 0.5 ml of 1% SDS, and incubated at 70 C for 20 min in order to solubilize the free form of lipoprotein from the whole cells. The amounts of lipoprotein antigens in the SDS suspensions of various cultures were compared by the double-diffusion techniques. Those cultures showing weaker Ouchterlony precipitin lines than the control or wild-type cultures were saved for further studies.

Screening of potential lipoprotein mutants by double-labeling technique. Each of the potential mutants was grown at 30 C in 25 ml of M9 glucose medium supplemented with the required amino acids, shifted to 42 C during the early logarithmic phase of growth, and then labeled with 10 to 25 μCi of [^{3}H]arginine for 1 h. The wild-type parental strain was grown under identical conditions and subsequently labeled with 5 to 10 μCi of [^{14}C]arginine at 42 C. Cell envelope fractions were prepared from each strain as described in Materials and Methods. The cell envelope of each mutant strain (^{3}H

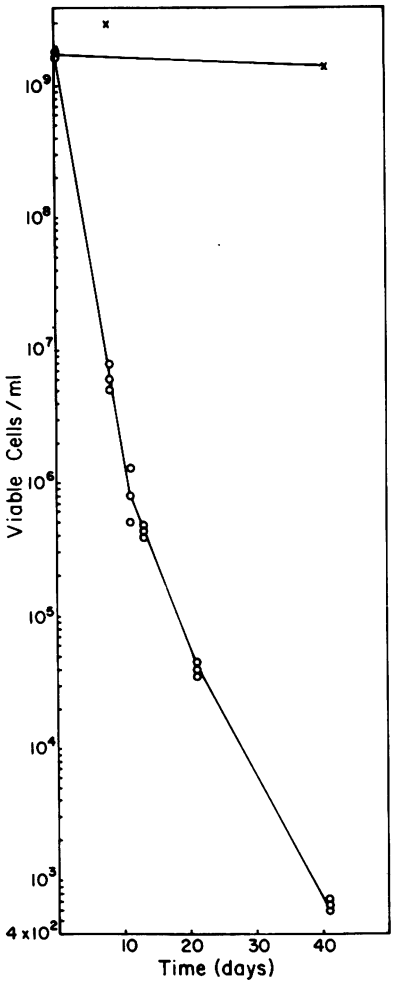

FIG. 1. *Loss of viability of strain E600 (MB204his⁻pro⁻) cells due to incorporation of [³H]arginine. Details are given in the text. In this particular selection, a culture of strain E600 was mutagenized with 300 μg of nitrosoguanidine per ml for 15 min, washed, and grown as described. The cells were then incubated with 1 mCi of [³H]arg (30 Ci/mmol) per 3 ml of culture at 42 C for 1 h in the absence of his and pro. The washed cells containing 0.1 dpm of [³H]arginine per cell were stored at 4 C and subsequently plated for survivors at 30 C at regular intervals. Symbols: ×, Unlabeled control; ○, labeled cells.*

labeled) was then mixed with approximately equal counts of the ^{14}C-labeled wild-type cell envelope. The mixed cell envelope preparation was then processed for the isolaton of murein sacculi. Aliquots of the first SDS supernatant obtained from the cell envelope by SDS extraction at 100 C were subjected to radioimmunoprecipitation. The $^{3}H/^{14}C$ ratios of various fractions (total cell envelope, SDS supernatant, immunoprecipitation supernatant, immunoprecipitate, and murein sacculi) were determined. Those mutants showing significantly lower $^{3}H/^{14}C$ ratios in the murein sacculi as compared with that of the total cell envelope were further studied by analysis of total membrane proteins, murein lipoprotein, and immunoprecipitates by SDS-polyacrylamide gel electrophoresis.

We have thus far obtained three potential mutants altered in the structure, biosynthesis, and/or assembly of murein lipoproteins. Preliminary studies leading to this tentative conclusion are described below.

Mutant 1. Mutant 1 was isolated from strain AB1157 after nitrosoguanidine mutagenesis. All the biochemical studies indicated a decreased amount of the bound form of lipoprotein in this strain as compared with the parental strain. The data presented in Table 2 show a lower $^{3}H/^{14}C$ ratio of murein lipoprotein (0.65) than that of the total cell envelope (1.0). Data in Fig. 2A and 2B were also consistent with this conclusion. Chemical analysis of the purified murein sacculi from both the wild-type and the mutant strains revealed a twofold reduction in the amount of the bound form of lipoprotein in this mutant as compared with that of the wild-type strain (Table 3). Fatty acid compositions of the murein lipoproteins from the wild type and mutant 1 appeared similar (data not shown).

Mutant 2. Mutant 2 was isolated from strain AB1157 by [³H]arginine suicide in a separate experiment. A double-labeling experiment using [³H]arginine (mutant) and [^{14}C]arginine (wild type) indicated that the cumulative rate of arginine incorporation into both the free and bound forms of lipoprotein was about 50 to 60% of that observed in the wild-type strain (Table 4). SDS-polyacrylamide gel electrophoresis of total cell envelope proteins, immunoprecipitates, and murein lipoprotein provided further evidence for a defective synthesis of lipoprotein in this mutant (Fig. 3).

Mutant 3. This mutant was isolated from a

TABLE 2. *Relative rate of appearance of murein lipoprotein in mutant 1 as compared with the wild-type strain (AB1157)*

Fraction	$^{3}H/^{14}C$ ratio	
	Mutant ([³H]arg)/ parent ([^{14}C]arg)	Parent ([³H]arg)/ mutant ([^{14}C]arg)
Total cell envelope	1	1
SDS-soluble envelope protein	1	1
Free lipoprotein (by gel)	0.65	1.35
SDS-insoluble murein sacculus	0.65	1.35

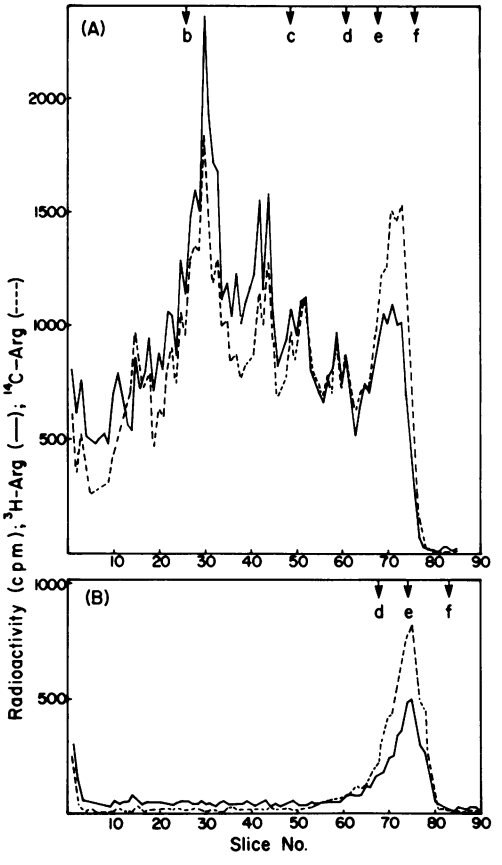

FIG. 2. *SDS-gel electrophoresis of the cell envelope fraction and murein lipoprotein isolated from mixed cell envelopes of [³H]arginine-labeled mutant 1 and [¹⁴C]arginine-labeled wild type (strain AB1157). Details of the growth, labeling and isolation of murein lipoproteins are given in the text. (A) SDS-soluble proteins from the mixed cell envelope; (B) murein lipoprotein isolated by lysozyme treatment of murein sacculi. Arrows with letters in both panels indicate the positions of the internal molecular weight standards (11); b, monomer of dansyl bovine serum albumin (66,000 daltons); c, dimer of dansyl egg white lysozyme (33,000 daltons); d, monomer of dansyl egg white lysozyme (19,000 daltons); e, cytochrome (14,000 daltons); f, dansyl insulin (6,600 daltons).*

TABLE 3. *Chemical composition of murein sacculi from wild-type (AB1157) and mutant 1*

Organism	Molar ratio	
	Murein lipoprotein/murein disaccharide[a]	Palmitate/hexosamine
Parent strain ...	0.13	0.33
Mutant 1	0.06	0.15

[a] The ratio of murein lipoprotein/murein disaccharide was obtained by amino acid analysis of the total acid hydrolysate (4 N HCl, 100 C, 15 h) of the murein sacculi.

TABLE 4. *Relative rate of appearance of murein lipoprotein in mutant 2 as compared with the wild-type strain (AB1157)*

Fraction	Mutant ([³H]arg)/parent ([¹⁴C]arg)
Cell envelope	0.7
SDS-soluble proteins	0.83
Immunoprecipitate	0.35
SDS-insoluble murein sacculus	0.45

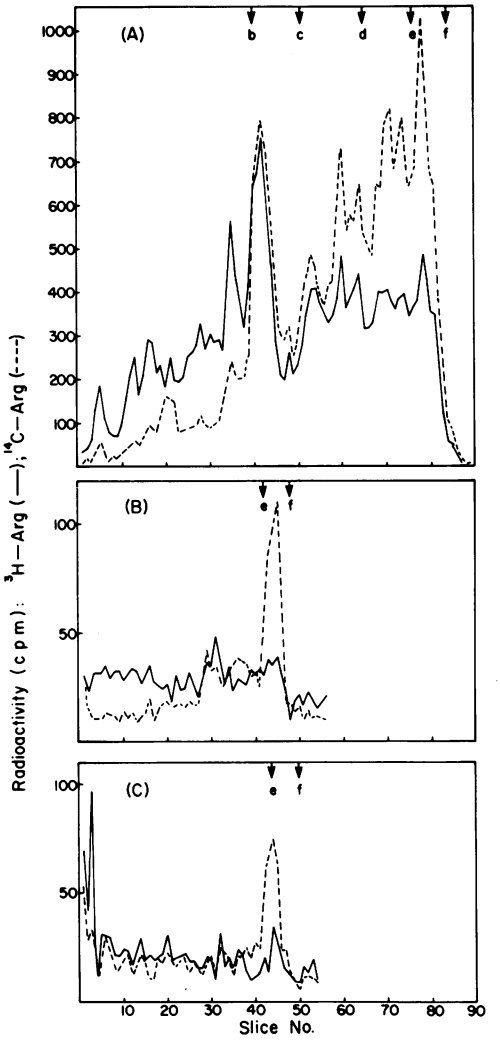

FIG. 3. *SDS-gel electrophoresis of cell envelope fraction, immunoprecipitates, and murein lipoprotein isolated from mixed cell envelopes of [³H]arginine-labeled mutant 2 and [¹⁴C]arginine-labeled wild type (strain AB1157). (A) SDS-soluble proteins from the mixed cell envelope; (B) immunoprecipitates (free form); (C) bound form of lipoprotein. Other details are the same as in the legend to Fig. 2. In panels B and C, slices 2 mm thick were obtained from each gel.*

his⁻pro⁻ derivative of strain MB204 after [^{3}H]arginine suicide as illustrated in Fig. 1. We chose strain MB204 as the parental strain due to the following considerations. The selection procedure based on radiation suicide would, in theory at least, spare only those mutants defective in the synthesis of the murein lipoprotein, not those affecting the structure (thereby activity or function) of this molecule. Thus mutants harboring missense point mutations in the structural gene of lipoprotein will be likewise killed by [^{3}H]arginine suicide. Since mutants defective in the synthesis of lipoprotein (e.g., amber mutants) are likely to be nonviable, one would have to take the precaution of using strains carrying temperature-sensitive suppressors as the parental wild-type strain (17). (We are grateful to D. F. Silbert for this suggestion.)

The murein lipoprotein synthesized by mutant 3 differed in several interesting aspects from that synthesized by the wild-type strain. The data presented in Table 5 indicated a 5- to 10-fold reduction in the cumulative rate of appearance of arginine-labeled bound form of lipoprotein in the murein sacculus as compared with the parental strain. This is supported by the results shown in Fig. 4 and 5, as well as by direct chemical analysis of the purified murein sacculi from the wild-type and mutant strains (Table 6). On the other hand, the cumulative rate of arginine incorporation into the free form of lipoprotein in mutant 3 was nearly normal as compared with that of the parental strain (Table 5).

TABLE 5. *Relative rate of appearance of murein lipoprotein in mutant 3 as compared with the wild-type strain (E600)*

Fraction	^{3}H/^{14}C ratio		
	Mutant ([^{3}H]arg)/parent ([^{14}C]arg)		Parent ([^{3}H]arg)/mutant ([^{14}C]arg) (expt 3)
	Expt 1	Expt 2	
Crude extract	0.35	0.46	10.27
Soluble fraction	0.38	0.46	11.48
Washed cell envelope	0.28	0.44	7.80
SDS-soluble envelope proteins	0.33	0.50	7.35
Immunoprecipitation supernatant	0.31	0.57	7.30
Immunoprecipitate	0.24	0.30	7.91
SDS-insoluble murein sacculi	0.08	0.04	29.32

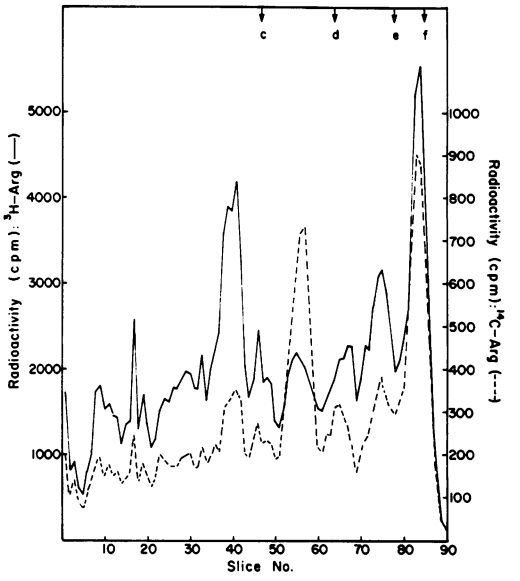

FIG. 4. *SDS-gel electrophoresis of cell envelope fraction prepared from mixed cultures of [^{3}H]arginine-labeled wild type (strain E600) and [^{14}C]arginine-labeled mutant 3. SDS-soluble proteins of the cell envelope (experiment 3 of Table 5) were analyzed by SDS-gel (7.5%) electrophoresis.*

It is generally assumed that the insertion of lipoprotein into the murein sacculi proceeds by a transpeptidation reaction with a concomitant release of D-ala or D-ala-D-ala from the peptide side chain. An alteration in the chemical structure of the peptide chains in murein sacculi, i.e., in the degree of cross-linking as well as the amount of D-ala residues in the peptide chains, may affect the assembly of lipoprotein into the murein sacculi.

The amino acid composition of the lipoprotein-free murein was determined both before and after dinitrophenylation as described in Materials and Methods. There was no difference in the ratios of alanine/glutamic acid between the wild-type and the mutant mureins (Table 7). However, murein isolated from mutant 3 appeared to be less cross-linked than the murein in the parental strain. The significance of the difference in the degree of cross-linking between the mutant and the wild type remains to be ascertained.

It is conceivable that mutant 3 carries a nonsense mutation in the structural gene of lipoprotein. This would account for the drastic reduction in the amount of bound-form lipoprotein since the lipoprotein is linked to the murein sacculus through the ϵ-NH_2 group of the C-terminal lysine (3). However, the data shown

in Table 8 did not support this interesting possibility. There is only one tyrosine residue near the C-terminus of the lipoprotein (3). Were this mutant defective in the completion of lipoprotein peptide chain, one might expect to find a reduction in the tyr/arg ratio of the mutant lipoprotein as compared with that of the wild-type strain, provided that incomplete lipoprotein would cross-react with the lipoprotein antisera. This was not observed in the double-labeling experiment using radioactive tyrosine and arginine; i.e., there was no evidence of incomplete immunologically reactive lipoprotein in mutant 3 (Table 8). Second, although this mutant carries a temperature-sensitive amber suppressor (ts SuIII) (17), the defect in the assembly of murein lipoprotein in mutant 3 was not affected by this amber suppressor activity. Both the parental strain and mutant 3 were temperature sensitive in tryptophan requirement as well as in plating efficiencies of T4 amber mutants (data not shown). The data shown in Table 9 indicated that this mutant is deficient in the bound-form lipoprotein to a similar extent at 25, 37, and 42 C, respectively, as compared with the wild-type strain.

It remains possible that there is a structural alteration in the mutant lipoprotein in such a way that it interferes with the normal assembly into the murein sacculus. Three preliminary observations support this interesting possibility: (i) a small but significant fraction of immunologically reactive lipoprotein of mutant 3 is found in the 200,000 × *g* supernatant fraction, i.e., not membrane bound; (ii) the mutant lipoprotein behaves differently from that of the

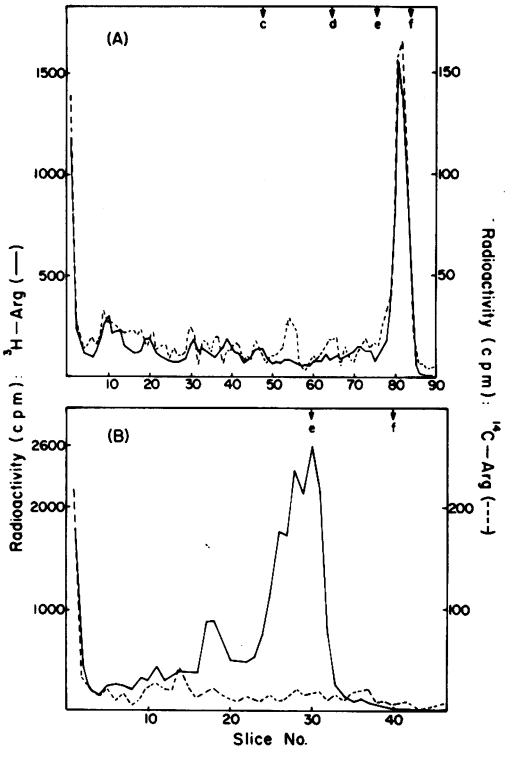

FIG. 5. *SDS-gel electrophoresis of immunoprecipitates and murein lipoprotein prepared from the mixed cultures of [^{3}H]arginine-labeled wild type (strain E600) and [^{14}C]arginine-labeled mutant 3. The same sample of mixed envelope shown in Fig. 4 (experiment 3 of Table 5) was used for radioimmunoprecipitation and for isolation of murein lipoprotein complex. (A) Immunoprecipitates (free form); (B) bound form of lipoprotein. The gel shown in panel B was sectioned into 2-mm slices.*

TABLE 6. *Chemical composition of murein lipoprotein complex isolated from parental strain and mutant 3*

Organism	Molar ratio	
	Murein lipoprotein/murein disaccharide	Fatty acids/total hexosamine
Parent strain ...	0.15	0.23
Mutant 3	0.05	0.07

TABLE 7. *Chemical composition of pronase-digested murein lipoprotein complex isolated from parental strain and mutant 3*

Amino acid or amino sugar	Molar ratio			
	Before dinitrophenylation		After dinitrophenylation	
	Wild type	Mutant	Wild type	Mutant
Muramic acid	0.86	0.92	0.93	0.91
Glucosamine	0.82	0.78	0.84	0.74
Alanine	1.61	1.56	1.56	1.67
Glutamic acid	1	1	1	1
meso-Diaminopimelic acid	1.02	1.05	0.63	0.45
Mono-dinitrophenyl-*meso*-diaminopimelic acid.....			0.40	0.60

TABLE 8. *Relative rate of appearance of murein lipoprotein in mutant 3 as compared with the wild-type strain (E600), using either arginine or tyrosine as the radioactive precrusor*[a]

Fraction	$^3H/^{14}C$ ratio			
	[^{3}H]arg parent/ [^{14}C]arg mutant	[^{3}H]tyr mutant/ [^{14}C]tyr parent	[^{3}H]arg parent/ [^{14}C]tyr parent	[^{3}H]tyr mutant/ [^{14}C]arg mutant
Cell envelope[b]	1	1	1	1
SDS-soluble envelope protein	0.71	0.98	1	1.09
Immunoprecipitation supernatant	0.72	1.08	1.19	1.07
Immunoprecipitate	0.65	0.97	1.15	0.78
Free-form lipoprotein (by gel)	0.46	1.39		0.59
Murein lipoprotein complex	18	0.08	2.15	0.51
Bound-form lipoprotein (by gel)	56.7	0.02		

[a] Cultures were grown and labeled at 30 C.
[b] The $^3H/^{14}C$ ratio of the total cell envelope was arbitrarily set at 1.00.

TABLE 9. *Relative rate of appearance of murein lipoprotein in mutant 3 as compared with the wild-type strain (E600) at various temperatures*

Fraction	Mutant ([^{3}H]arg)/parent ([^{14}C]arg)		
	25 C[a]	37 C	42 C
Cell envelope[b]	1	1	1
SDS-soluble envelope proteins	0.98	1.08	1.25
Immunoprecipitation supernatant	0.85	1.03	1.08
Immunoprecipitate	1.20	1.54	1.17
Murein lipoprotein complex	0.05	0.01	0.04
Bound-form lipoprotein (by gel)	0.06	0.02	0.02

[a] The temperatures at which these cultures were labeled for 1 h.
[b] The $^3H/^{14}C$ ratio of the total cell envelope was arbitrarily set at 1.00.

wild-type molecule during purification with a procedure kindly made available to us by M. Inouye. (S. Inouye et al., manuscript in preparation); (iii) analytical gel electrophoresis in the presence of 8 M urea and 1% SDS revealed that the mutant lipoprotein (free form) migrated more slowly than that of the parental strain (Fig. 6).

Purification of lipoproteins from this mutant and the parental strain and their chemical characterizations are in progress.

It should be noted, however, that in the cases of both mutant 2 and mutant 3, there were other envelope proteins in which the $^3H/^{14}C$ ratios were altered significantly from the overall ratios of the total cell envelope (Fig. 3 and 4). It is not possible at the present time to ascertain the significance of these changes. They could result from additional mutations in these strains. On the other hand, these differences may be related in some direct or indirect way to the alteration in the structure, biosynthesis, or assembly of lipoprotein in these mutants.

DISCUSSION

The biosynthesis and assembly of murein-bound lipoprotein involves at least four steps: (i) synthesis of the apoprotein or its precursor; (ii) *N*-acylation of the α-amino group of the N-terminal cysteine; (iii) attachment of diglyceride to the sulfhydryl group of the N-terminal cysteine by a thioether linkage; and (iv) insertion of the free form of lipoprotein into the murein sacculus, presumably by a transpeptidase reaction. These events may occur in vivo in a sequence other than that presented here.

Using a semi-brute force procedure, we have isolated and partially characterized three potential mutants altered in the structure, biosynthesis, and/or assembly of murein lipoprotein. These mutants should prove valuable in the elucidation of detailed biochemical mechanisms of synthesis, modification, and assembly of murein lipoprotein. Preliminary studies on mutant 3 have yielded encouraging results suggestive of the presence of a structurally altered lipoprotein in this mutant. At least two possible explanations may be offered to account for the slower migration of the mutant lipoprotein as compared with that of the wild-type strain. The mutant lipoprotein may be deficient in covalently linked lipid, thereby, binding less SDS than the wild-type lipoprotein. Alternatively, the mutant lipoprotein may actually contain extra amino acids at either the amino or the carboxy terminus. These two possibilities are not mutually exclusive. Whether this mutant form of lipoprotein represents a normal intermediate in the biogenesis of murein lipoprotein remains to be seen. The purification and chemical characterization of lipoprotein from this mutant should provide invaluable in-

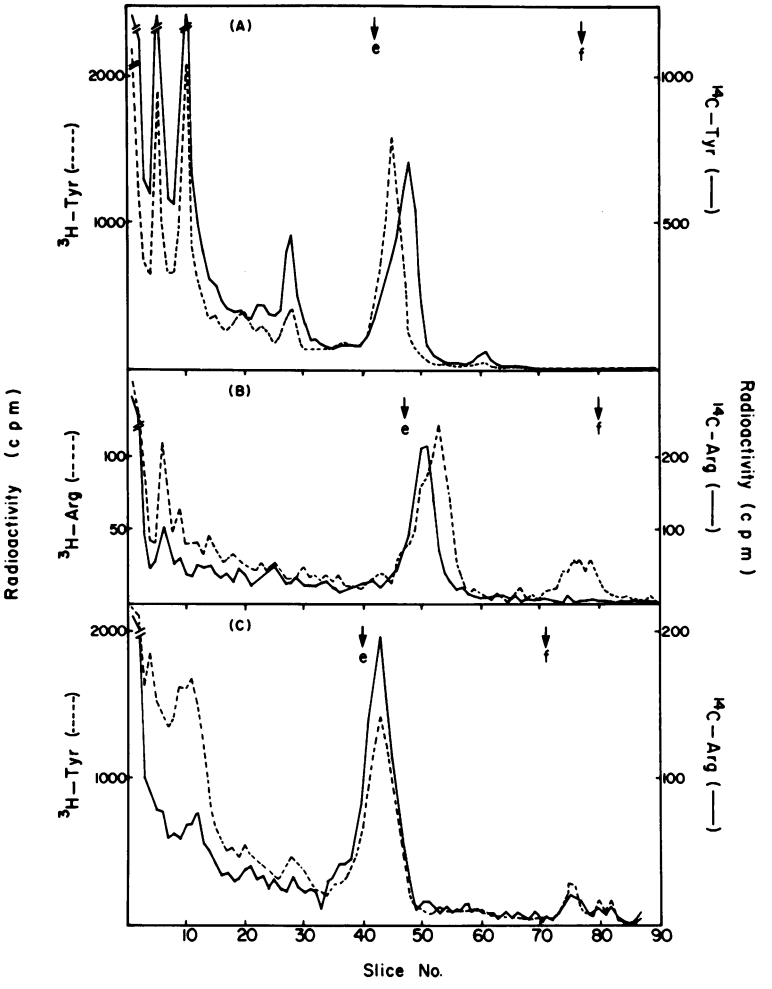

FIG. 6. *SDS-urea-gel electrophoresis of immunoprecipitates prepared from the mixed cell envelopes of (A) [^{3}H]tyrosine-labeled mutant 3 and [^{14}C]tyrosine-labeled wild type (strain E600); (B) [^{3}H]arginine-labeled wild type (strain E600) and [^{14}C]arginine-labeled mutant 3; (C) [^{3}H]tyrosine-labeled mutant 3 and [^{14}C]arginine-labeled mutant 3. Immunoprecipitates containing the free form of lipoprotein were analyzed by SDS-urea-gel electrophoresis (12.5% acrylamide, 1:10 cross-linkage) (18).*

sight into the biochemical basis of this genetic defect.

Although all three mutants so far studied showed temperature sensitivity in growth and division, as well as abnormal cellular morphology at the nonpermissive temperature, it is premature to speculate on the function of murein lipoprotein until we have constructed isogenic strains by transduction or reversion. These genetic studies are in progress.

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LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitroso-guanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. 18:788-795.
2. **Braun, V.** 1973. Molecular organization of the rigid layer and the cell wall of *Escherichia coli*. J. Infect. Dis. 128:(Suppl):S9-S16.
3. **Braun, V., and V. Bosch.** 1972. Sequence of the murein lipoprotein and the attachment site of the lipid. Eur. J. Biochem. 28:51-69.
4. **Braun, V., and V. Bosch.** 1973. *In vivo* biosynthesis of murein lipoprotein of the outer membrane of *E. coli*. FEBS Lett. 34:302-306.
5. **Braun, V., and U. Sieglin.** 1970. The covalent murein-

lipoprotein structure of the *Escherichia coli* cell wall: the attachment site of the lipoprotein on the murein. Eur. J. Biochem. 13:336-346.

6. **Ghuysen, J. M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls, p. 685-699. *In* E. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
7. **Hantke, K., and V. Braun.** 1973. Covalent binding of lipid to protein. Diglyceride and amide-linked fatty acid at the N-terminal end of the murein lipoprotein of the *Escherichia coli* outer membrane. Eur. J. Biochem. 34:284-296.
8. **Hirashima, A., G. Childs, and M. Inouye.** 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. 79:373-389.
9. **Hirashima, A., H. C. Wu, P. S. Venkateswaran, and M. Inouye.** 1973. Two forms of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 248:5654-5659.
10. **Hirashima, A., S. Wang, and M. Inouye.** 1974. Cell-free synthesis of a specific lipoprotein of the *Escherichia coli* outer membrane directed by purified messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 71:4149-4153.
11. **Inouye, M.** 1971. Internal standards for molecular weight determinations of proteins by polyacrylamide gel electrophoresis. J. Biol. Chem. 246:4834-4838.
12. **Inouye, M.** 1974. A three-dimensional molecular assembly model of a lipoprotein from the *Escherichia coli* outer membrane. Proc. Natl. Acad. Sci. U.S.A. 71:2396-2400.
13. **Inouye, M., and J. P. Guthrie.** 1964. A mutation which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. 64:957-961.
14. **Inouye, M., J. Shaw, and C. Shen.** 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. 247:8154-8159.
15. **Lee, N., and M. Inouye.** 1974. Outer membrane proteins of *Escherichia coli*: biosynthesis and assembly. FEBS Lett. 39:167-170.
16. **Lin, E. C. C., S. A. Lerner, and S. E. Jorgensen.** 1962. A method for isolating constitutive mutants for carbohydrate-catabolizing enzymes. Biochim. Biophys. Acta 60:422-424.
17. **Smith, J. D., L. Barnett, S. Brenner, and R. L. Russell.** 1970. More mutant tyrosine transfer ribonucleic acids. J. Mol. Biol. 54:1-14.
18. **Swank, R. T., and K. D. Munkres.** 1971. Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. Anal. Biochem. 39:462-477.
19. **Venkateswaran, P. S., and H. C. Wu.** 1972. Isolation and characterization of a phosphonomycin-resistant mutant of *Escherichia coli* K-12. J. Bacteriol. 110:935-944.
20. **Wu, H. C., and T. C. Wu.** 1971. Isolation and characterization of a glucosamine-requiring mutant of *Escherichia coli* K-12 defective in glucosamine-6-phosphate synthetase. J. Bacteriol. 105:455-466.