New Major Outer Membrane Protein Found in an *Escherichia coli tolF* Mutant Resistant to Bacteriophage TuIb

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Cell envelopes prepared from an *Escherichia coli tolF* strain selected as resistant to phage TuIb contained a new major outer membrane protein related to outer membrane proteins Ia and Ib. The strain that produces this protein is a *tolF par* double mutant but contains an additional mutation leading to the production of the new major outer membrane protein. Antibiotic sensitivity lost as a result of the *tolF* mutation is regained in strains that contain the new major outer membrane protein. This indicates that this protein functions to restore the selective permeability of the outer membrane to low-molecular-weight hydrophilic molecules.

The *Escherichia coli* outer membrane serves as the initial permeability barrier of the cell, allowing passage of hydrophilic molecules with a molecular weight less than about 700 (19). This membrane contains a set of three to four major proteins with molecular weights from 35,000 to 40,000, some of which may have a role in maintaining this permeability barrier. Several laboratories have described similar or identical sets of proteins, and a variety of nomenclatures have evolved. These nomenclatures have been summarized by Henning and Haller (10), Lugtenberg et al. (14), and, more recently, by Bassford et al. (2).

Sekizawa et al. (24) have suggested that the major outer membrane proteins are synthesized from precursor molecules. Thus, outer membrane proteins Ia and/or Ib (matrix proteins) may be formed by the action of one or more toluene-sensitive proteases acting on what has been called promatrix protein.

Proteins Ia and Ib are closely related proteins (22) strongly associated with the peptidoglycan of the cell envelope (20). These proteins, together with lipopolysaccharide, can inactivate certain bacteriophages and are apparently part of the specific bacteriophage receptors for bacteriophages TuIa and TuIb (5). Interaction with both peptidoglycan and bacteriophages suggests that these proteins span the outer membrane.

Proteins Ia and Ib may contribute directly to the permeability of the outer membrane to low-molecular-weight hydrophylic substances. Lutkenhaus has shown that *E. coli* B strains missing protein Ia were markedly deficient in their abil-ity to incorporate a variety of substrates when these substrates were present at low concentrations (15). Nakae has demonstrated reconstitution of selective permeability to low-molecular-weight substances using membrane vesicles containing outer membrane proteins similar to proteins Ia and Ib prepared from *Salmonella typhimurium* (17) or *E. coli* (18). Together with the likelihood that proteins Ia and Ib span the outer membrane, these data suggest that proteins Ia and Ib may be involved in the formation of transmembrane channels, or pores.

We have isolated a mutant strain of *E. coli* that contains a new peptidoglycan-associated major outer membrane protein. We call this protein E. The mutant strain containing protein E contains neither major outer membrane proteins Ia nor Ib and, thus, may be deficient in proteins required for the formation of transmembrane channels or pores. We find that a mutation at a locus separate from the loci leading to the loss of proteins Ia or Ib leads to the synthesis of protein E. The presence of protein E in the outer membrane serves to restore the selective permeability or transmembrane channels of the outer membrane.

MATERIALS AND METHODS

Microorganisms and growth media. The microorganisms in this study are described in Table 1. The cells were grown at 37°C usually in PPBE medium (8) containing protease peptone no. 3 (Difco Laboratories, Detroit, Mich.), beef extract (Difco), and NaCl. For propagation of all bacteriophages except phage P1*vir* and testing sensitivity to bacteriophages, tryptone medium was used. Tryptone medium contained 10 g of

Table 1. *Microorganisms*[a]

Strain	Genotype or comment	Source or reference
E. coli K-12 derivatives		
JF568	*aroA357 ilv-277 metB65 his-53 purE41*	4
	cyc-1 xyl-14 lac629 rpsL97 tsx63 proC24	
JF703	*aroA+ tolF4* transductant of strain JF568	4
JF694	JF703 *par*	This paper
Lin221	CGSC4861: *phoA8 glpT6 relA1 tonA22*	2
PB105	*nalA*	2
W1485F⁻·PA2	Phage PA2 lysogen	2
CSH75	*ara leu lacY proC purE gal trp his argG*	Cold Spring Harbor Lab-
	malP rpsL xyl mtl ilv, metB or metA thi	oratory
Bacteriophages		
TuIa	Ia[b]	5
TuIb	Ib	5
PA2c	Ib	2
Hy2	Ib	2
Hy8	Ib	2
TuII*	II*	5

[a] Genetic symbols are described by Bachmann et al.

[b] These bacteriophages do not grow on an *E. coli* strain whose outer membrane is missing the indicated protein.

tryptone (Bacto, Difco) and 5 g of NaCl per liter. LB medium (16) containing 2.5 mM CaCl₂ was used for the growth of bacteriophage P1*vir*. Minimal medium for transductions was that described by Vogel and Bonner (27), supplemented with 0.2% glucose or 0.4% α-glycerol phosphate and growth requirements at appropriate concentrations. All media were solidified with 1.5% agar.

Genetic techniques. Growth of bacteriophage P1*vir* and transduction procedures were accomplished as described by Signer (25). *nalA* transductants were selected as described by Hane and Wood (9).

Sensitivity to bacteriophages and colicins. Bacteriophage sensitivity was tested by cross-streaking after first resuspending a portion of a single colony of the strain to be tested in 0.1 to 0.2 ml of saline or tryptone broth. Sensitivity to colicins was determined by placing about 5-µl portions of each of several serial dilutions of colicin solutions on the surface of a plate containing PPBE agar medium inoculated with about 2 × 10⁷ exponentially growing cells of the strain to be tested. These cells were spread over the surface of a PPBE agar plate by the method of Low (12). After overnight incubation at 37°C, the activity of the colicin was determined as previously described (8) as the reciprocal of the highest dilution that completely inhibited growth. Purified colicin L-JF246 was prepared as previously described (7). Crude preparations of other colicins were prepared from mitomycin C-induced cells (8), and the activity of these preparations was estimated by a spot test as described above.

Bacteriophage induction. Bacteriophage synthesis in lysogenic strains was induced by treatment with either mitomycin C or UV irradiation. Mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added at a final concentration of 2 µg/ml to a freshly growing culture in tryptone broth (approximately 2 × 10⁸ cells per ml). After a 3-h incubation at 37°C with vigorous aeration, a few drops of chloroform were added to kill the cells and promote the release of intracellular bac-

teriophage. Next, cells and cellular debris were removed by centrifugation (5,000 × *g* for 5 min), and the supernatant fluid containing the bacteriophage was stored at 4°C over chloroform. Induction by UV light was accomplished by irradiation of freshly grown cells (2 × 10⁸ cells per ml) that had been collected by centrifugation and resuspended at 2 × 10⁹ cells per ml in saline. After irradiation (survival about 50%), the cells were diluted 10-fold into fresh tryptone broth, and bacteriophage were collected after a 3-h incubation as described above.

Determination of antibiotic sensitivity. Antibiotic sensitivity was determined by using antibiotic susceptibility test disks (Sensi-Discs, Baltimore Biological Laboratory, Cockeysville, Md.) on PPBE agar medium. Plates were inoculated with about 2 × 10⁷ cells by the method of Low (12), and up to 12 disks were placed on the surface of the medium. After overnight incubation at 37°C, the diameter of the clear zones of inhibition was estimated with an accuracy of ±0.5 mm. We scored strains as resistant if the zone of inhibition was reduced more than 6 mm. The diameter of zones of inhibition on all strains scored as sensitive was nearly identical for individual antibiotics.

Preparation of cell envelopes. Cell envelope materials were prepared as previously described (3) by differential centrifugation of cells disrupted in a French pressure cell.

Polyacrylamide gel electrophoresis in SDS. Samples of cell envelope materials were heated at 100°C for 5 min in sodium dodecyl sulfate (SDS)-containing buffer as previously described (3). Slab polyacrylamide gel electrophoresis was performed as described by Lugtenberg et al. (13). Gels were stained as described by Fairbanks et al. (6) with Coomassie brilliant blue G and destained by soaking at 37°C in several changes of 10% acetic acid.

RESULTS

Isolation of *tolF*, phage TuIb-resistant

mutants. Outer membrane materials prepared from strain JF703 *(tolF)* contain protein Ib but no protein Ia as determined by gel electrophoresis (4). Mutants of strain JF703 missing protein Ib as well as Ia were selected by first mixing 10^9 to 10^{10} phage TuIb with 10^8 cells of strain JF703 and plating the mixture on tryptone agar plates. After overnight incubation at 37°C, at least two types of colonies appeared. Large mucoid colonies, accounting for about one-third of the total, were not further analyzed. From the remaining colonies, 192 were picked and purified by single-colony streaks. These were next tested for sensitivity to phages TuIa, TuIb, and TuII*. To infect a host, these phages require the presence of major outer membrane proteins Ia, Ib, and II*, respectively (5). Like the parental strain, all 192 strains were resistant to phage TuIa and sensitive to phage TuII*. About two-thirds (123 of 192) were resistant to phage TuIb. Of these, eight were chosen for further analysis.

Major outer membrane proteins missing in phage TuIb-resistant mutants. SDS-polyacrylamide gel electrophoresis of cell envelope materials prepared from each of these eight strains showed that seven of these eight strains were missing major outer membrane proteins Ia and Ib. These seven strains invariably had increased amounts of a few higher-molecular-weight proteins (80,000 to 100,000). Strain JF693, previously described (4), is an example of this group.

Of the eight *tolF* phage TuIb-resistant mutants studied, cell envelope materials prepared from one mutant, strain JF694, contained no protein Ia or Ib but did contain substantial amounts of a new major outer membrane protein. This protein, which we call protein E, migrated more slowly than protein Ia after SDS-polyacrylamide gel electrophoresis, with an apparent molecular weight of about 40,000 as compared to about 37,500 for protein Ia.

Protein E is tightly associated with the peptidoglycan layer of the cell envelope, for it is not extracted after incubation of cell envelope materials with 2% SDS at 60°C as described by Rosenbusch (20) (data not shown). These and other data that demonstrate conclusively that protein E is associated with the outer membrane fraction of the cell envelope will be presented separately (Chai and Foulds, manuscript in preparation).

Figure 1 shows stained gels after electrophoresis of cell envelope materials prepared from each of several strains. Major outer membrane proteins Ia and Ib were present in materials prepared from strain JF568. Similar preparations from strain JF703 contained protein Ib but no Ia, whereas strain JF694 contained neither
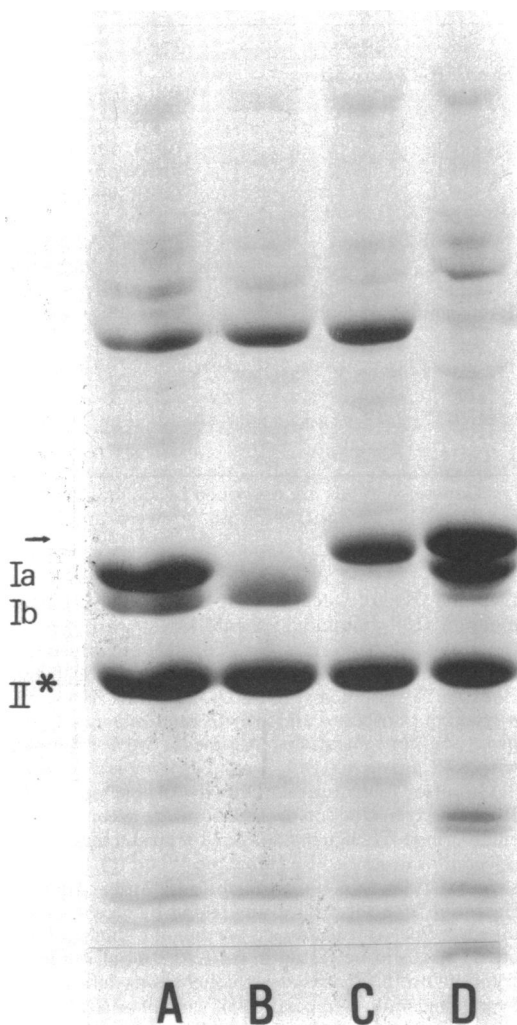

FIG. 1. *Stained SDS-polyacrylamide gel after electrophoresis of cell envelope materials prepared from the following strains: JF568 (A), JF703 (B), JF694 (C), and W1485F⁻·PA2c (D). The positions of proteins Ia, Ib, and II* are indicated. The arrow indicates the position of peptidoglycan-associated protein E. The figure represents only a portion of the gel.*

proteins Ia nor Ib. Strain JF694 contained an additional protein, labeled protein E in Fig. 1.

The electrophoretic mobility of protein E is close to that of protein 2 found by Schnaitman et al. (23) in *E. coli* strains lysogenized by bacteriophage PA2. Our experience indicates that protein E migrates somewhat more rapidly than protein 2. We have tried to show that strain JF694 is not a PA2 lysogen. Strain JF694 is not a normal PA2 lysogen, for no detectable bacteriophage were found after UV or mitomycin C

induction under conditions in which similar treatment of strain W1485 · PA2 yielded lysates containing more than 10^9 bacteriophage per ml. This does not eliminate the possibility that strain JF694 could be lysogenized by a defective bacteriophage. Unfortunately, we could not examine this possibility by using techniques for rescue of a putative bacteriophage PA2 host range gene by superinfection of strain JF694 with phage λvir as described by Bassford et al. (2) because this strain is resistant to λvir (Table 4). We have isolated two new bacteriophages that require the presence of protein E for adsorption (Chai and Foulds, manuscript in preparation). Strain JF694 is sensitive to these bacteriophages, whereas all other strains described in Table 1, including strain W1485F⁻ · PA2, are resistant. These data indicate that the protein E is not identical with protein 2.

Genetic characterization of strain JF694. Strain JF694 still contains the *tolF* allele as indicated by the following results. Bacteriophage P1*vir* grown on this strain was used to transduce strain JF568 (*aroA*). Examination of the *aroA*⁺ transductants showed that 53% were resistant to bacteriophage TuIa and colicin A as expected for *tolF* strains (Table 2, cross 1).

The data in Table 2 demonstrate that strain JF694 apparently carries a mutation leading to the loss of protein Ib located close to *glpT* and *nalA*.

When bacteriophage P1*vir* prepared on strain JF694, was used to transduce *glpT*⁺ into strain Lin221, 10% of these transductants were resistant to phage TuIb (Table 2, cross 2). SDS-gel electrophoretic analysis of cell envelope materials prepared from four of these *glpT*⁺, phage TuIbʳ transductants showed that all four strains were missing outer membrane protein Ib. These preparations did not contain protein E.

When bacteriophage P1*vir* prepared on strain PB105 was used to prepare nalidixic acid-resistant transductants of strain JF694, 43% of these transductants were sensitive to bacteriophage TuIb. SDS-gel electrophoresis analysis of cell envelope materials prepared from five *nalA*, phage TuIb-sensitive transductants showed five

of five contained outer membrane protein Ib. These preparations still contained protein E. The cotransduction frequencies of the mutation in strain JF694 leading to the loss of protein Ib with *nalA* and *glpT* agree well with those described by Bassford et al. for the *par* loci (2) that are involved in the expression of outer membrane protein Ib. Therefore, we believe that strain JF694 contains a *par* mutation. This is supported by the resistance of strain JF694 to bacteriophage PA2c (Table 4), for *par* mutants are resistant to this bacteriophage (2). Strain JF694 does not contain a mutation at the *ompB* locus which affects proteins Ia or Ib, for no *malP* transductants examined were resistant to either bacteriophage TuIa or bacteriophage TuIb (Table 2, cross 4).

These data indicate that the mutation leading to the synthesis of protein E is distinct from both the *tolF* mutation leading to the loss of protein Ia and the *par* mutation leading to the loss of protein Ib.

Using conjugation and transduction with bacteriophage P1*vir*, we have located the mutation leading to the synthesis of protein E on the *E. coli* chromosome. This mutation is located near *ilvE* at about 82.7 min on the linkage map described by Bachmann et al. (1) (Foulds and Chai, manuscript in preparation).

Sensitivity to colicins, antibiotics, and bacteriophages. The data summarized in Table 3 show that strains JF703 and JF694 (both of which carry the *tolF* mutation) have identical patterns of colicin sensitivity. Introduction of the *par* mutation and a spontaneous mutation leading to the production of protein E in strain JF694 does not affect this pattern.

The data in Table 4 show that the bacteriophage sensitivity of strain JF694 has been altered. The resistance of strain JF694 to phages PA2, TuIb, and hy2 can be explained by the loss of outer membrane protein Ib that serves as a receptor for these phages (2, 5). We do not understand why strain JF694 is resistant to phages λvir and hy8. Perhaps insertion of protein E in the outer membrane reduces the amount or interferes with the function of the

TABLE 2. *Genetic analysis by phage P1vir transduction*

Donor no.	Donor	Recipient	Selected marker (no. scored)	Recombinant class	Cotransduction frequency (%)
1	JF694	JF568	*aroA*⁺ (192)	*aroA*⁺ *tolF*ᵃ	53
2	JF694	Lin221	*glpT*⁺ (192)	*glpT*⁺ TuIbʳ	10
3	PB105	JF694	*nalA* (192)	*nalA* TuIbˢ	43
4	JF694	CSH74	*malP*⁺ (192)	*malP*⁺ TuIaʳ	<0.5
				malP⁺ TuIbʳ	<0.5

ᵃ *tolF* recombinants are identified as resistant to 1,000 U of colicin A per ml and/or resistance to bacteriophage TuIa.

TABLE 3. *Colicin[a] sensitivity of strain JF694*

Strain	Colicin (U/ml)									
	A	B	C	D	E1	E2	E3	K	L	M
JF568	20,000	1,280	1,600	160	8,000	2,560	1,024	640	1,000	1,280
JF703	<10	1,280	400	160	8,000	40	<10	<10	40	1,280
JF694	<10	1,280	400	160	8,000	40	<10	<10	<10	1,280

[a] Colicins were prepared as previously described (8).

TABLE 4. *Bacteriophage sensitivity of strain JF694*

Strain	Bacteriophage						
	TuIa	TuIb	TuII*	PA2c	hy2	hy8	λvir
JF568	S[a]	S	S	S	S	R	S
JF703	R	S	S	S	S	S	S
JF694	R	R	S	R	R	R	R

[a] S, Sensitive; R, resistant.

lamB gene product that is responsible both for the adsorption of bacteriophage lambda and transport of maltose (26). All three strains listed in Table 2 were sensitive to phages K3 and BF23.

It would be interesting to examine the outer membrane proteins in a derivative of strain JF694 lysogenic for bacteriophage PA2. Both protein 2 (23) and protein E are major outer membrane proteins closely associated with the peptidoglycan layer of the cell envelope. The presence of one may interfere with the other. However, because strain JF694 is resistant to both bacteriophages PA2 and hy8 (Table 4), we will have to wait until we transduce the mutation leading to the production of protein E into a PA2 lysogen.

Insertion of the protein E in the outer membrane of strain JF694 restores sensitivity to all the antibiotics to which *tolF* strains are resistant (Table 5). This suggests that the protein E can serve to restore the selective permeability barrier of the outer membrane.

DISCUSSION

We have called the peptidoglycan-associated outer membrane protein found in strain JF694 protein E for we believe the structural gene for this protein is distinct from other loci involved in the expression of peptidoglycan-associated proteins Ia, Ib, and 2. We have located this gene on the *E. coli* chromosome and will propose that the locus be called *ompE* (Foulds and Chai, manuscript in preparation). Other properties of this protein suggest that it is related to the matrix protein. We find that the protein E remains associated with the peptidoglycan after incubation of cell envelope preparations in SDS-containing buffer at 60°C as described by Rosenbusch (20), as do proteins Ia and Ib. Purified

preparations of this protein produce a single band of precipitation on Ouchterlony double-diffusion plates by using antibody produced in response to purified matrix protein. This precipitin band is identical to that produced by purified protein Ia. Furthermore, comparison of the amino acid composition of purified protein E with that of proteins Ia and Ib shows all three to be nearly identical. All three proteins have the same N-terminal amino acid (alanine). Details of these data will be reported separately (Chai and Foulds, manuscript in preparation).

Many of the properties of the protein E are also shared by outer membrane protein Ic recently described by Henning et al. (11). Comparison of their electrophoretic mobility shows what may be a discrepancy. In our gel system, protein Ic migrates just ahead of protein Ia, whereas protein E migrates somewhat more slowly than protein Ia.

We do not believe that the protein E is identical with protein a described by Lugtenberg et al. (14). This protein is reportedly present in small amounts in several *E. coli* strains (4, 21) and should render these strains sensitive to the bacteriophages that specifically require the presence of the protein E. These strains are not sensitive to these bacteriophages. In addition, protein a is not associated with the peptidoglycan (20) (Foulds and Chai, unpublished data) as is the protein E.

The promatrix protein described by Sekizawa et al. (24) has many properties in common with protein E. Both are closely associated with peptidoglycan and migrate more slowly than protein Ia in SDS-gel electrophoresis. However, comparison of the electrophoretic mobility of purified, radioactively labeled protein E with radioactively labeled toluene-treated cell envelopes containing promatrix protein demonstrates these proteins are distinct (Sekizawa, personal communication).

A *tolF par* double mutant has been previously isolated and described (2). This strain contained a major outer membrane protein that had an electrophoretic mobility similar to protein Ia. We suggest that this protein is related to protein E or protein Ic rather than to protein Ia. A similar conclusion has been reached by Schnaitman (personal communication).

TABLE 5. *Antibiotic sensitivity of strain JF694*

Strain	Antibiotic							
	Chlortet-racycline	Oxytetracy-cline	Tetracy-cline	Genta-mycin	Kanamy-cin	Cephalo-thin	Chloram-phenicol	Polymyxin B
JF568	S[a]	S	S	S	S	S	S	S
JF703	R	R	R	R	R	R	R	R
JF694	S	S	S	S	S	S	S	S

[a] S, Sensitive; R, resistant.

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