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Escherichia coli K-12 *tolF* Mutants: Alterations in Protein Composition of the Outer Membrane

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Outer membrane materials prepared from three independently isolated spontaneous *Escherichia coli tolF* mutants contained no detectable protein Ia. The loss of this protein was nearly completely compensated for by an increase in other major outer membrane proteins, Ib and II*. Thus, the major outer membrane proteins accounted for 40% of the total cell envelope protein in both *tol*$^+$ and *tolF* strains. No changes were found in the levels of inner membrane proteins prepared from *tolF* strains when compared with similar preparations from the *tol*$^+$ strain. Phage-resistant mutants were selected starting with a *tolF* strain by using either phage TuIb or phage PA2. These phage-resistant *tolF* strains contained neither protein Ia nor protein Ib. The mutation leading to the loss of protein Ib in these strains is independent of the *tolF* mutation and is located near *malP* on the *E. coli* genetic map.

The *Escherichia coli* cell envelope is composed of three distinct layers. There are two membranes separated by a layer of peptidoglycan. The two membranes can be separated, and they differ markedly in both composition and function (16). Cellular functions such as active transport and energy metabolism are associated with the inner, or cytoplasmic, membrane. The outer membrane serves as the initial permeability barrier of the cell. Receptors for colicins, bacteriophages, and vitamins are also associated with this structure. Like the inner membrane, the outer membrane is composed primarily of phospholipid and protein but contains lipopolysaccharide as well (2). Depending on growth conditions, as much as 70% of the total protein in the outer membrane can be accounted for by a few distinct polypeptides, the so-called "major" outer membrane proteins (11, 21). These include several proteins with molecular weights of about 30,000 to 35,000 and the lipoprotein described by Braun and Hantke (2) with a molecular weight of about 7,500. In this paper, the proteins described as "major outer membrane proteins" do not include the lipoprotein, but only the outer membrane proteins with molecular weights near 30,000 to 35,000, i.e., proteins Ia, Ib, and II* (nomenclature of Henning and Haller, who have summarized the nomenclature used by several authors [10; see Table 1]).

The major outer membrane proteins have been studied in some detail (e.g., 2, 12, 18, 19, 21), and mutants isolated that are deficient in one or more of them have been described (3, 10, 23). Surprisingly, a mutant missing all major outer membrane proteins (10) grows well and does not exhibit a markedly altered sensitivity to dyes, detergents, or antibiotics. Mutants with altered levels of lipoprotein have also been isolated. For example, Hirota, Suzuki, Nishimura, and Yasuda (submitted for publication) have recently described a strain that contains no detectable free or bound lipoprotein. This strain grows well but exhibits some hypersensitivity to ethylenediaminetetraacetic acid and is unable to prevent the loss of periplasmic enzymes such as ribonuclease (Hirota, personal communication).

Thus, it appears that one or several proteins present in the outer membrane of *E. coli* can be lost by mutation without affecting the vital functions of the cell.

Certain *E. coli* mutants tolerant to colicins (2, 6) or resistant to bacteriophages (7, 19) have altered levels of the major outer membrane proteins. Thus, *ompA* (*tolG*) mutants often have no protein II* (3, 10, 23). *tolIV* and *tolXIV* mutants described by Davies and Reeves (5) are evidently missing proteins Ia and Ib (6), and phage-resistant mutants deficient in both of these proteins or just in protein Ib have been described (19). In addition, Davies (Ph.D. thesis, University of Adelaide, Adelaide, Australia, 1976) has described several classes of tolerant mutants that have decreased levels of protein I. (Proteins Ia and Ib were not distinguished by Davies.) These classes include *tolIIb*, *tolIb*, and *tolX*.

The mutation in each of these classes is lo-

cated near *gal* (Davies, Ph.D. thesis) on the *E. coli* chromosome, close to the mutations in *tolF* strains that are located between *aroA* and *pyrD* on the *E. coli* genetic map (8, 9).

MATERIALS AND METHODS

Microorganisms. The microorganisms used in this study are described in Table 1. Cells were grown at 37°C in a proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.)-beef extract (Difco) medium (PPBE) already described (9) to a concentration of about 3×10^8 cells/ml. Phage lysates were prepared in PPBE medium at 37°C, using strain JF568 as the host.

Preparation of colicins. Purified colicin L-JF246 was prepared as previously described (7). Crude colicin A was prepared from mitomycin C-induced cells (9). The activity of these preparations was estimated by a spot test (9) where the activity, in arbitrary units, was the reciprocal of the highest dilution that completely inhibited the growth of the indicator strain on PPBE agar medium.

Preparation of cell membranes. Cell envelopes (total cell membranes) were prepared as previously described (3) by differential centrifugation of cells after disruption in a French pressure cell. Inner and outer membrane fractions were prepared according to Osborn et al. (16). To determine the purity of inner and outer membrane preparations, L-lactate dehydrogenase (localized in the inner membrane) and lipopolysaccharide (localized in the outer membrane) were determined as described by Osborn et al. (16). By these criteria, our preparations of inner and outer membranes were at least 90 and 95% pure, respectively. Membrane protein was determined by the method of Lowry et al. (13), using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Tube and slab polyacrylamide gel electrophoresis to separate cell envelope proteins was performed, using either the gel system described by Neville (15) or that described by Lugtenberg et al. (14).

Quantitative estimation of proteins on polyacrylamide gels. Qualitative comparisons of protein patterns after electrophoresis on polyacrylamide tube or slab gels were made visually by using gels stained with Coomassie brilliant blue G. For quantitative estimation of proteins, the tube gels were stained overnight with 0.1% Fast green in 10% acetic acid and 50% methanol. After destaining in 10% acetic acid, the gels were soaked in 45% methanol to reduce their size. The gels were scanned at 637 nm by using a Gilford spectrophotometer equipped with a linear transport apparatus and a recorder. The relative amount of each major outer membrane protein was determined by the area under the peak of the recorder tracing. These values were always obtained in duplicate, and the variability was less than 5%. To estimate the variability of the entire procedure, eight separate cultures of strain JF568 were used to prepare eight cell envelope preparations. Estimation of the fraction of the individual major outer membrane proteins in each preparation showed a variation of less than 9% (see Table 2).

RESULTS

Colicin-tolerant cells adsorb colicin but are not killed by colicin concentrations that would be lethal to wild-type colicin-sensitive cells. *tolF* cells are tolerant to colicins A, E2, E3, L, and S4 and sensitive to colicins E1 and X (9).

TABLE 1. *Microorganisms*[a]

Strain	Genotype and comments	Source or reference
E. coli K-12 derivatives		
JF404	HfrH *thyA*	9
JF688	JF404-4a; spontaneous *tolF* mutant	9
CSH75	*ara leu lacY proC purE gal trp his argG malP rpsL xyl mtl ilv, metB* or *MetA thi*	Cold Spring Harbor Laboratory
JF568	*aroA357 ilv-277 metB65 his-53 purE41 proC24 cyc-l xyl-14 lacY29 rpsL177 tsx-63*	8
JF703	*aroA*$^+$ *tolF4*; transductant of strain JF568 from strain JF688	8
JF704	*aroA*$^+$ *tolF11*; transductant of strain JF568 from strain JF404-11a	8
JF705	*aroA*$^+$ *tolF1*[b]; transductant of strain JF568 from strain RE107	8
JF693	JF568 *tolF4* TulbR	This paper
RE107	*proA trp his lacY ton rpsL tolF1*[b]	17
Colicinogenic strains		
JF246	*Serratia marcescens trp* (colicin L-JF246)	8
JF385	*E. coli* O6:H16.23 (colicin A)	S. Luria
Bacteriophages		
TuIb	Grows only on strains that contain outer membrane protein Ib	U. Henning (19)
PA2		C. Schnaitman

[a] Genetic symbols are described by Bachmann et al. (1).

[b] *tolF* and *cmlB* mutations are genetically and phenotypically identical (8). Therefore, for simplicity we will use *tolF1* to refer to the allele originally described as *cmlB* (17).

TABLE 2. *Relative levels of major outer membrane proteins Ia, Ib, and II**

Strain	Relevant genotype	No. of prepn	Relative amt of protein[a]			
			Ia	Ib	II*	Sum[b]
JF568	*tol*$^+$	8	12.1 ± 0.9	5.9 ± 0.2	21.0 ± 0.5	39
JF703	*tolF4*	2	0	12.9 ± 0.6	28.6 ± 3.8	42
JF705	*tolF1*	2	0	20.3 ± 1.4	21.4 ± 1.4	42

[a] Expressed as percentage of total cell envelope protein. The relative level of each preparation was determined in duplicate.

[b] Sum of relative levels of proteins Ia, Ib, and II*.

Strain JF688 was described as sensitive to 1,000 arbitrary units per ml of colicins E2 and E3 (9). However, this strain, as well as other *tolF* mutants, was tolerant to 200 arbitrary units per ml of these colicins (8). They are also sensitive to the group B colicins described by Davies and Reeves (5).

After polyacrylamide gel electrophoresis of cell envelope materials prepared from *tol*$^+$ or *tolF* cells, comparison of the stained gels showed that the *tolF* strains contained no detectable protein Ia and an apparently increased level of protein Ib or proteins Ib and II*. A photograph of a typical stained gel is presented in Fig. 1, where the absence of protein Ia in cell envelope materials prepared from three independently isolated *tolF* strains is demonstrated. To quantitate the relative levels of the major outer membrane proteins in these preparations, densitometer scans of tube gels were obtained. Typical densitometer tracings are presented in Fig. 2, and the relative levels of the major outer membrane proteins (Ia, Ib, and II*), obtained from several cell envelope preparations, are summarized in Table 2. The three major outer membrane proteins accounted for about 40% of the total protein in the cell envelope fraction prepared from the *tol*$^+$ strain JF568. In cell envelope materials prepared from *tolF* strains, protein Ia was absent. The level of protein Ib or proteins Ib and II* was increased such that, in the *tolF* strains as well, the major outer membrane proteins accounted for 40% of the total protein in the cell envelope fraction.

When inner and outer membranes were separated, proteins Ia, Ib, and II* were, as expected, associated with the outer membrane fraction. Comparison of the inner membrane fraction (prepared from *tol*$^+$ or *tolF* cells) showed that the patterns of protein bands on the stained polyacrylamide gels were identical (data not shown). However, the level of resolution in this gel system (14) does not allow us to conclude that there are no alterations in protein composition of the inner membrane.

Phage TuIb is related to phage T4 (19), whereas phage PA2 is related to phage lambda (C. Schnaitman, personal communication). The

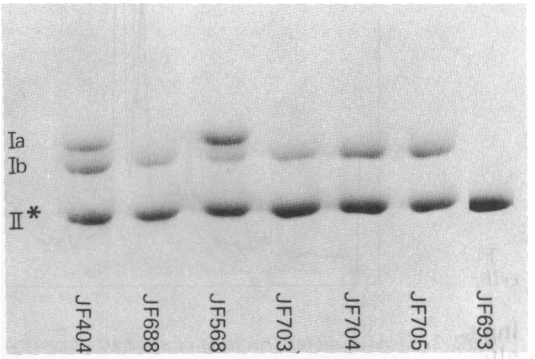

FIG. 1. *Stained polyacrylamide gels (14) after electrophoresis of cell envelope materials prepared from the following strains: JF404 (tol*$^+$*), JF688 (tolF4), JF568 (tol*$^+$*), JF703 (tolF4), JF704 (tolF11), JF705 (tolF1), and JF693 (tolF4 TuIb*^{r}*).*

presence of protein Ib in the outer membrane confers sensitivity to both of these phages, and this protein may be part of their receptor. Mutants selected as resistant to either of these phages often are missing outer membrane protein Ib (19; C. Schnaitman, personal communication).

tolF strains contain protein Ib, and they are as sensitive as the *tol*$^+$ strain to phages TuIb and PA2. A lysate of phage TuIb gives an equivalent efficiency of plating with either a *tol*$^+$ or a *tolF* strain as the host, and both types of strains give similar numbers of phage progeny after a single round of infection.

It is possible that the *tolF* mutation results in the synthesis of an altered protein with an electrophoretic mobility similar to that of protein Ib. Thus, the protein peak assumed to be protein Ib in cell envelope materials prepared from a *tolF* strain may be a mixture of protein Ib and altered protein Ia. To examine this possibility, we first isolated spontaneous mutants resistant to either phage TuIb or phage PA2 from strain JF703 (*tolF*) and then examined the cell envelope materials prepared from these mutants for the presence of a protein with an electrophoretic mobility similar to that of protein Ib. Presumably, most of these phage-resistant mutants would have lost protein Ib without chang-

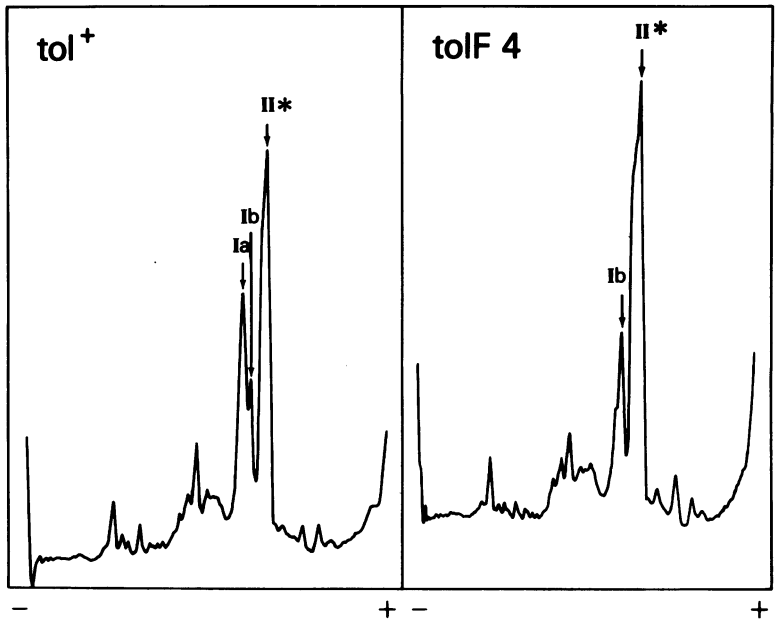

FIG. 2. *Densitometer tracings of stained polyacrylamide tube gels (Neville system) after electrophoresis of cell envelope materials prepared from strain JF568 (tol+) or strain JF703 (tolF4).*

ing the electrophoretic mobility of the putative altered protein Ia.

Cell envelope materials were prepared from four of these *tolF4*, phage-resistant mutants, and the proteins were examined by gel electrophoresis. Three contained no detectable major outer membrane protein Ia or Ib. Figure 1 shows the absence of proteins Ia and Ib after polyacrylamide gel electrophoresis of cell envelope materials prepared from a typical *tolF*, phage TuIb-resistant mutant (strain JF693). The level of protein II* in cell envelope materials prepared from strain JF693 was markedly increased above that found in its phage-sensitive parent, but in this strain there is a substantial increase in the level of two cell envelope proteins with apparent molecular weights of about 65,000 and 50,000. The other phage-resistant mutant isolated from strain JF703 was missing proteins Ia and Ib and had an additional major outer membrane protein (altered Ib?) that migrated on polyacrylamide gels (14) more slowly than protein Ia. This strain will be reported on separately.

Figure 3 is a densitometer tracing of a stained tube gel following electrophoresis of cell envelope materials prepared from strain JF693. In addition to the absence of proteins Ia and Ib, this tracing shows the increased levels of the higher-molecular-weight proteins. One or both of these proteins may represent a precursor of outer membrane protein Ia or Ib.

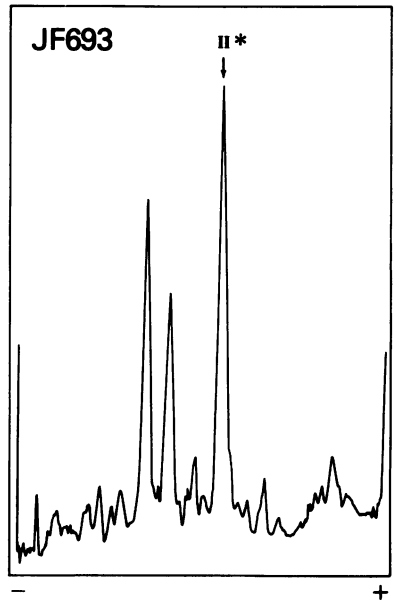

FIG. 3. *Densitometer tracings of stained polyacrylamide tube gel (Neville system) after electrophoresis of cell envelope materials prepared from strain JF693 (tolF4 TuIbr).*

The mutation in strain JF693 leading to resistance of phage TuIb and loss of protein Ib is located close to *malP* on the *E. coli* genetic map. Table 3 shows the cotransduction fre-

TABLE 3. *Cotransduction of a mutation leading to the loss of protein Ib with malP*

No. of *malP*$^+$ transductants[a]	No. of transductants resistant to phage TuIb[b] (%)
138	84 (61)
77	43 (56)
102	61 (60)

[a] Transduction was done as originally described (8), using strain CSH75 as a recipient. Each experiment was done with an independently isolated phage TuIb-resistant variant of strain JF703 as donor.

[b] *malP*$^+$ transductants were picked and tested for sensitivity to phage TuIb on minimal maltose plates supplemented with the appropriate growth requirements.

quency of this mutation with *malP* in three independently isolated phage TuIb-resistant strains derived from strain JF703. At present we have not proposed a gene symbol for this locus, for the mutation may be located in the *ompB* locus described by Sarma and Reeves (personal communication).

We were able to show by genetic manipulation that the mutation leading to the loss of protein Ib in strain JF693 was independent of the *tolF* mutation. Using phage P1 grown on strain JF693, *malP*$^+$ transductants were isolated from strain CSH75. Cell envelope materials were prepared from two *malP*$^+$ transductants that also received the locus leading to phage TuIb resistance. There was protein Ia but no protein Ib in these preparations (data not shown). Conjugation experiments using HfrH strain JF404 (donor) and strain JF693 (recipient) produced *proC*$^+$ recombinants, some of which (25%) were also *tolF*$^+$. These mating experiments were interrupted after 30 min. All the *proC*$^+$ and *tolF*$^+$ recombinants were resistant to phage TuIb and contained protein Ia but no protein Ib in their cell envelopes (data not shown).

DISCUSSION

Schnaitman et al. (20) showed that *E. coli* strains lysogenized by phage PA2 contained a new major outer membrane protein and much less proteins Ia and Ib. In a separate study, Schnaitman observed that the amount of protein II* in strain AB1621, missing proteins Ia and Ib, was about equivalent to the sum of proteins Ia, Ib, and II* present in the parental strain, AB1859 (22). We have obtained similar results where the relative levels of the major outer membrane proteins of *tol*$^+$ and *tolF* strains were compared. The most dramatic alteration in the protein composition of the cell envelope materials prepared from *tolF* strains was the decreased level of protein Ia and the concomitant increase in proteins Ib and II*.

Apparently, in certain mutant strains the decrease in major outer membrane protein resulting from the loss of protein Ia or Ib (or both) may be compensated for by an increased level of other outer membrane proteins. This indicates that the cell has the capacity to maintain the total amount of the major outer membrane proteins. We have observed that F'106 merodiploid strains that carry either one or two copies of the *tolF*$^+$ and *ompA*$^+$ genes do not have markedly different levels of protein Ia or II*, indicating that there is an upper limit to the level of the major outer membrane proteins and that this limit is not altered in cells containing two copies of the *tolF*$^+$ and *ompA*$^+$ genes (Chai and Foulds, unpublished observations). Recently, Datta et al. (4) have examined the level of protein II* in strains diploid for the *ompA*$^+$ gene, and they did not find a gene dosage effect.

The mutation in strain JF693 that leads to a loss of the major outer membrane protein Ib is located close to *malP* (60% cotransduction) on the *E. coli* genetic map, at a site distinct from the *tolF* locus. Examination of recombinants arising after transduction or conjugation indicates that these two loci act independently. Thus, where *tolF*$^+$ was introduced into strain JF693 by conjugation, the cell envelope of the recombinant contained protein Ia but not protein Ib. Mutants P530 and P692 described by Davies and Reeves (6) have been shown to lack proteins Ia and Ib (11; Davies and Reeves, personal communication). The mutation in these strains is located at the *ompB* locus, close to *malP* (Sarma and Reeves, Biochem. Biophys. Res. Commun., in press). Strain AB1621, described by Schnaitman (21), apparently has lost both protein Ia and protein Ib as a result of a combination of *tfr* and *tsx* mutations. Thus, the loss of these proteins may be accomplished by either one or two mutational events.

Primarily on the basis of similarity of cyanogen bromide peptides, Schmitges and Henning concluded that proteins Ia and Ib are similar (19). They speculated either that the proteins were products of separate closely related genes or that one protein may represent a modification of the other. Alternatively, Ia and Ib may arise after distinct modifications of a single, common precursor.

The data presented demonstrate that the *tolF* mutation does not affect the structural gene for protein Ib or the modification of a putative precursor of protein Ib. The absence of protein Ia in *tolF* mutants and proteins Ia and Ib in *tolF*, phage-resistant mutants is consistent with the notion that these two proteins are

distinct and that their electrophoretic separation on polyacrylamide gels is not an artifact of preparation.

It is likely that protein Ia plays an important role in sensitivity to some colicins for certain novobiocin-sensitive mutants because they do not contain protein Ia in their cell envelopes and exhibit patterns of colicin sensitivity similar to those described for *tolF* strains (Coleman and Leive, personal communication). Specifically, protein Ia may be the receptor for colicin A and also have a function in helping other colicins, such as colicin L, breach the outer membrane barrier.

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