

Outer Membrane of *Escherichia coli* K-12: Differentiation of Proteins 3A and 3B on Acrylamide Gels and Further Characterization of *con* (*tolG*) Mutants

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Two classes of mutants, *con* and *tolG*, that appeared to be very similar in a number of respects have been shown to be identical and cotransducible with *pyrD*. By diethylaminoethyl-cellulose chromatography of the outer membranes, we have shown that the mutants are missing only protein 3A and retain protein 3B. Using *con* mutants, we were thus able to identify protein 3B on the pH 7.2 gel system of Maizel where it runs separately from protein 3A if unheated samples are used. *tolG* mutants were shown to be identical to *con* mutants in being conjugation defective with most F-like plasmid donors but not with I-like plasmid donors, and in their resistance pattern to bacteriophages and colicins. During the course of this study, it was observed that the bacteriocin produced by *Serratia marcescens* JF246 was identical in its activity spectrum to colicin L-398 and is now considered to be a colicin of type L.

Recent studies of bacteriophage- and colicin-resistant mutants of *Escherichia coli* have led to the identification of outer membrane proteins able to be used as receptors for these bacteriophages and colicins, and also to characterization of the biological role of the proteins.

Sabet and Schnaitman (30) showed that *bfe* mutants, resistant to colicins E1, E2, E3, and A and to bacteriophage BF23, have lost the receptor for at least some of these lethal agents and that this receptor is a protein involved in the transport of vitamin B12 (7). Bacteriophages T1, T5, and ϕ 80 and colicin M also share a receptor, which in this case has been shown to be the binding site for the uptake of ferri-chrome-bound iron (2, 16, 22, 41). The receptor for bacteriophage λ has been identified as an outer membrane protein involved in the chemotaxis and high-affinity binding of maltose (18, 19, 28, 38). Each of these receptor proteins is only a relatively minor component of the outer membrane.

con mutants have lost the receptor for bacteriophage K3 (34; P. A. Manning and P. Reeves, manuscript in preparation), and analysis of the proteins of the outer membrane by polyacrylamide gel electrophoresis has shown them to be virtually lacking peak 3 (nomenclature of Schnaitman [33]), using the pH 11.4 buffer system of Bragg and Hou (1), and peak C, using unheated samples on the pH 7.2 buffer system of Maizel (24). *con* mutants have been isolated

as resistant to phage K3 (15, 34) or as tolerant to colicins K and L (4, 6).

tolG mutants isolated by Foulds and Barrett (10) as tolerant to bacteriocin JF246 have also been shown to be missing a major outer membrane protein (3).

In this study we show that the previously described defect in the outer membranes of both *con* and *tolG* mutants is identical and consists of the absence of protein 3A only. We are able to distinguish proteins 3A and 3B by their behavior upon polyacrylamide gel electrophoresis.

We also extend the known phenotypes of both *con* and *tolG* mutants to show that *tolG* mutants exhibit properties previously described for *con* mutants and vice versa, indicating that the two are identical.

We also present evidence that bacteriocin JF246 is a colicin of type L.

MATERIALS AND METHODS

Media and culture conditions. Nutrient broth, nutrient agar, and minimal agar, supplemented with the appropriate growth factors and carbon source, were as described previously (35, 36). All cultures were incubated at 37°C unless stated otherwise.

Bacterial strains. The colicinogenic strains were described elsewhere (4, 8, 11, 17). All other strains were derivatives of *E. coli* K-12 and are listed in Table 1. The Col-factors and R-factors used, the colicins and antibiotic resistances for which they code, and those used for selection in the matings are

summarized in Table 2. All Col-factors and R-factors were maintained in strain JC6256.

Bacteriophages. All bacteriophages are from stocks maintained in this laboratory and described previously (15).

The 64 bacteriophages used were: T1, T2, T3, T4, T5, T6, T7, BF23, A, B, C, D, F, G, J, E4, E7, E11, E15, E21, E25, H1, H3, H8, K2, K3, K4, K5, K6, K8, K9, K10, K11, K12, K15, K16, K17, K18, K19, K20, K21, K22, K25, K26, K27, K29, K30, K31, O_x1, O_x2, O_x4, O_x5, M1, M3, Ac3, Ac4, H⁺, V, ØI, ØII-T, H, F27, and W31.

Colicin sensitivity. Sensitivity was measured in

two ways. Initially this was done by the conventional cross-streaking plate test, as described previously (4), using colicins A, B, D, E1, E2, E3, G, H, Ia, Ib, K, L, M, N, Q, S1, S4, V, and X.

In the second method, preparations of colicins K-235, L-398, and L-JF246 were titrated on the strain being tested, and the titer was compared with that on the wild type. This was done by taking log-phase bacteria diluted to a density of 2×10^8 cells per ml. To 0.1 ml of cell suspension was added 0.1 ml of a dilution of colicin. This was then incubated at 37°C with gentle shaking for 30 min, 4 ml of 0.7% nutrient agar was added, and the entire 4.2 ml was poured as

TABLE 1. *Bacterial strains*

Strain	Characteristics	Source/reference
AB1133	F ⁻ /thi argE proA thr leu his mtl xyl ara galK lacY strA supE λ ⁻	A. L. Taylor
P212	con mutant of AB1133	5
P400	non his ⁺ transductant of AB1133	34
P460	con mutant of P400	34
JF404	HfrH/thyA	J. Foulds (10)
JF404-2a	tolG mutant of JF404	J. Foulds (10)
P1635	F ⁻ /thi argE mtl xyl ara str non λ ⁻	^a
P1636	F ⁻ /thi argE mtl xyl ara str non λ ⁻ tolG	^a
AB259	HfrH/thi rel λ ⁻	B. Bachmann
AB257	HfrC/metB rel λ ⁻	B. Bachmann
KLF11/JC1553	F' 111/argG metB his leu recA mtl xyl malA gal lacY str tonA tsx supE λ ⁺ λ ⁻	B. Bachmann
CSH23(E5014)	F' lac ⁺ proA ⁺ , B ⁺ /Δ(lac pro)	Cold Spring Harbor
JC6256	F ⁻ /trp lac Δ	N. Willetts
W620	F ⁻ /thi pyrD gltA galK str rel λ ⁻	B. Bachmann

^a Strains P1635 and P1636 were obtained by mating JF404-2a with P400 for 32 min at 37°C, interrupting the mating, and selecting for gal⁺ str^r recombinants. The growth factor requirements of the two strains were identical as was the suppressor status (Su⁻ or supE⁺), measured by using a series of T4 amber, ochre, and opal phage kindly supplied by A. J. Clark.

TABLE 2. *List of plasmids^a*

Plasmid	Incompatibility type ^b	Antibiotic resistance ^c					Colicin
		Tc ₂₀	Str ₁₀	Str ₁₀₀	Cm ₅₀	Kan ₅₀	
F' lac pro	FI	S	S	S	S	S	
F' 111	FI	S	S	S	S	S	
R1d _{rd} 19	FII	R	R	S	S	S	
R100-1	FII	R	R	S	R	R	
R136f _{in} ⁻	FII	R	S	S	S	S	
R386	FI	R	S	S	S	S	
R538F _{rd} 1	FII	S	R	S	R	S	
ColV2	FI	S	S	S	S	S	V
ColVB _{trp}	FI	S	S	S	S	S	V, B
R64-11	Iα	R	R	S	S	S	
R144d _{rd} 1	Iα	S	S	S	S	R	
R163d _{rd} 1	Iα	R	R	S	S	R	I
R538I _{rd} 2	Iα	R	R	S	S	S	

^a Strains bearing the plasmids were kindly supplied by N. Willetts and E. Meynell.

^b Incompatibility types were obtained from reference 27.

^c R, Resistant; S, sensitive. Tc, Tetracycline; Str, streptomycin; Cm, chloramphenicol; Kan, kanamycin. The subscripts indicate the level of resistance used in micrograms per milliliter. The antibiotic resistances and colicins italicized are those used for selection of transfer, and 1,000 μg of streptomycin per ml was used as contraselection in all cases.

an overlay on a nutrient agar plate, prior to overnight incubation and scoring for colony-forming units. The titer of the colicin was taken to be the reciprocal of the dilution that gave 50% bacterial survival.

The conventional plate test was used for the initial screening of mutants, and the second method of titrating the colicins on the mutants was used to quantitate the extent of the resistance detected.

Bacteriophage sensitivity and adsorption. Bacteriophage sensitivity was scored by using the multiple-syringe phage applicator used previously (15), with phage at a density of 10^5 to 10^6 plaque-forming units per ml.

Bacteriophage adsorption was measured as follows. The bacterial strain being tested was grown to a density of 2×10^8 cells per ml, and bacteriophages were added to a portion at a multiplicity of 1.0. Immediately after the addition of the phage suspension, the culture was returned to incubate, and at 1.5-min intervals samples were taken and diluted 10^{-4} in prewarmed nutrient broth containing $20 \mu\text{g}$ of chloramphenicol per ml. A 0.1-ml volume was added to 0.1 ml of a culture of strain JC6256/R538F Δ rd1 at a density of 2×10^8 cells per ml; the mixture was allowed to stand for 15 min at 37°C and then was poured as an overlay with 4 ml of 0.7% nutrient agar containing chloramphenicol ($20 \mu\text{g}/\text{ml}$) onto a nutrient agar plate. Plates were then incubated overnight and scored for plaque-forming units.

Mating procedures. Matings were performed as described previously (25) with the exception of Hfr matings, which were incubated for 60 min. For the transfer of colicin-producing plasmids, the donor cultures were preincubated at 37°C for 5 min in the presence of 5 mg of trypsin per ml to destroy any colicin present. Transfer was then detected as described previously (34).

Preparation of outer membranes, column chromatography, and polyacrylamide gel electrophoresis. Outer membranes were prepared by using Triton X-100 as described by Schnaitman (33), although cells were broken in an X-Press (LKB Instruments, Bromma, Sweden). For chromatography on diethylaminoethyl (DEAE)-cellulose (Whatman DE-52), the outer membranes were solubilized in Triton X-100 plus ethylenediaminetetraacetate (EDTA) and chromatographed as described previously (33).

Samples for electrophoresis were prepared by the method of Schnaitman (32, 33) and run under his conditions using both the pH 7.2 buffer system of Maizel (24) and the pH 11.4 buffer system of Bragg and Hou (1).

Gels were stained in Coomassie brilliant blue (37), and densitometer tracings were obtained with a Quick Scan Jr. gel scanner (Helena Laboratories Corp., Beaumont, Tex.).

Protein estimation. Two methods were employed for estimating the concentration of protein in samples. For protein solutions in water or non-Triton-containing buffers, the method of Schacterle and Pollack (31) was used. For samples containing Triton X-100, the method of Wang and Smith (40) was used. Bovine serum albumin was used as a standard in both cases.

Transduction. P1 phage stocks were prepared as described by Miller (26), using heat-inducible P1 carrying chloramphenicol resistance derived from the R-factor R100.

Colicin preparations. Colicins K-235, L-398, and L-JF246 were prepared by induction; a culture of the colicinogenic strain at a density of 4×10^8 cells per ml was induced by the addition of 50 ng of mitomycin C per ml. The culture was allowed to continue growing, with vigorous aeration, for 2.5 h, centrifuged at $5,000 \times g$ for 20 min, and sterilized by the addition of 1% chloroform. Samples of this crude colicin were then stored frozen until required.

RESULTS

***con* mutants lack only protein 3A.** The outer membrane from *con* mutants was shown previously to have a greatly reduced amount of peak C, using the pH 7.2 gel buffer system of Maizel (24), and of peak 3, using the pH 11.4 buffer system of Bragg and Hou (1). This has been confirmed and shown also to apply to the *tolG* mutants isolated by Foulds and Barrett (10) (Fig. 1 and 2). Originally, it was thought that both proteins 3A and 3B were absent in *con* mutants. Schnaitman has since shown (33) that these proteins can be separated by ion-exchange chromatography using DEAE-cellulose (Whatman DE52), and this technique has enabled us to reexamine the defect in outer membrane proteins of *con* mutants. It was shown previously (33) that proteins 3A and 3B have quite different cyanogen bromide peptide profiles, indicating that these two proteins are distinct polypeptides. When the outer membrane of strain P460 was examined by DE-52 chromatography, it was observed that only protein 3A was absent and that the level of protein 3B was unaltered (Fig. 3 and 4). Protein 3A occurs (if present) in fractions I, II, III, and perhaps IV, whereas protein 3B occurs in fractions IV and V.

A *tolG* mutant, strain JF404-2a, gave a result identical to that for the *con* mutant P460 (unpublished data).

Differentiating proteins 3A and 3B upon polyacrylamide gel electrophoresis. Schnaitman (33) showed that, with heated samples of outer membrane, proteins 3A and 3B run together as peak 3 on Bragg-Hou pH 11.4 gels (1) and as part of peak B on Maizel pH 7.2 gels (24).

Our results confirm these observations but show that the two proteins can be differentiated by using unheated samples on Maizel gels. If we look at the fractions from the DE-52 columns that are enriched in protein 3B and contain no 3A, we find that the small peak which runs slightly faster than peak C is correspondingly enriched, that this peak (and peak A) disappears upon heating, and that peak B appears in their place (Fig. 5).

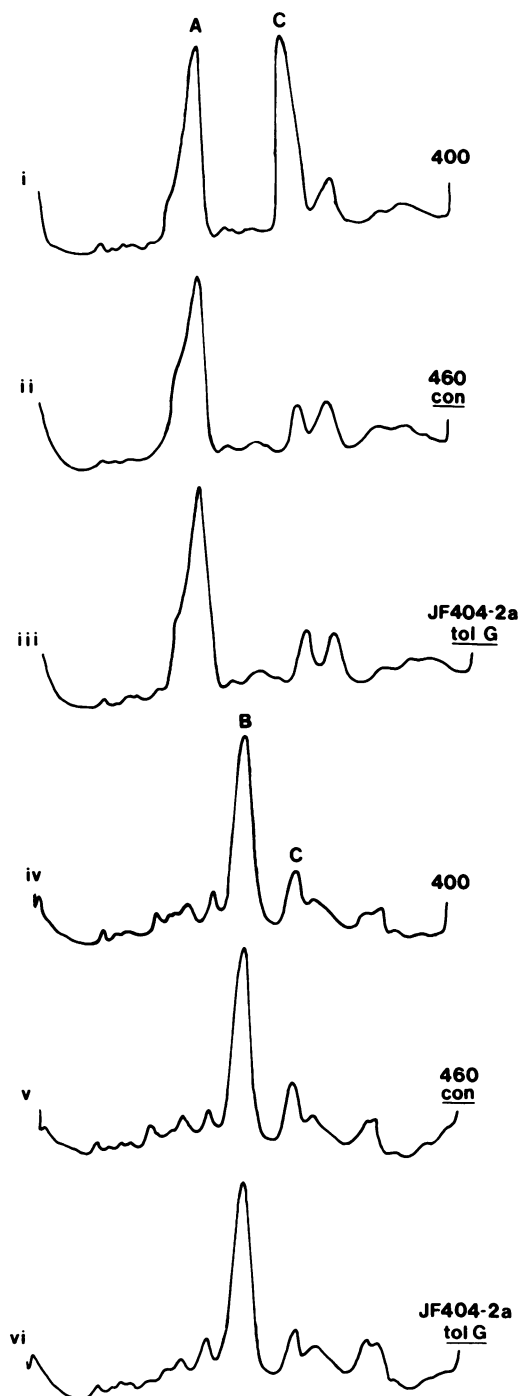


FIG. 1. Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (con), and JF404-2a (tolG) run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 7.2 buffer system of Maizel (24) with unheated (i, ii, and iii) and heated (iv, v, and vi) samples. Peaks are labeled by the method of Schnaitman (33).

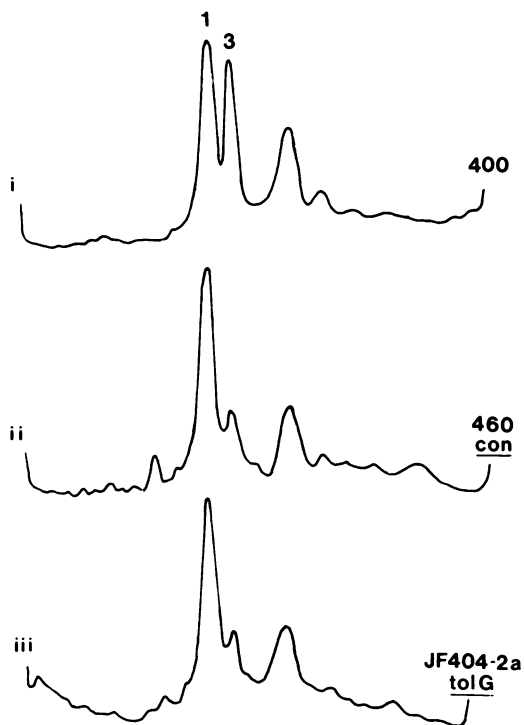


FIG. 2. Comparison, by densitometry, of the outer membrane proteins of strains P400, P460 (con), and JF404-2a (tolG) run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1) with heated samples. Peaks are labeled by the method of Schnaitman (33).

In Fig. 4 the amount of protein 3B relative to protein 1 in the Bragg-Hou gels of fractions IV and V seems higher than expected since this is also the only fractions that contain protein 1. This may be due to some of the protein 1 selectively remaining bound to the column.

Other properties of con and tolG mutants. We examined both con and tolG mutants for properties previously reported as being affected by one or both of the mutations. We found that both con and tolG mutants were identical as far as can be determined.

The mutants resemble the parent strains in being resistant to (per milliliter) 500 μ g of sulfafurazole, 10 μ g of oleandomycin, 5 U of penicillin G, 10 μ g of methicillin, 10 μ g of fusidic acid, 5 μ g of novobiocin, and 5 μ g of cloxacillin and sensitive to 50 μ g of colistin methane sulfonate, 5 μ g of kanamycin, 10 μ g of neomycin, and 2 μ g of ampicillin as shown previously for con mutants (34). However, the mutants had become sensitive to 30 μ g of novobiocin, whereas both parent strains are resistant, as shown previously for tolG mutants (10).

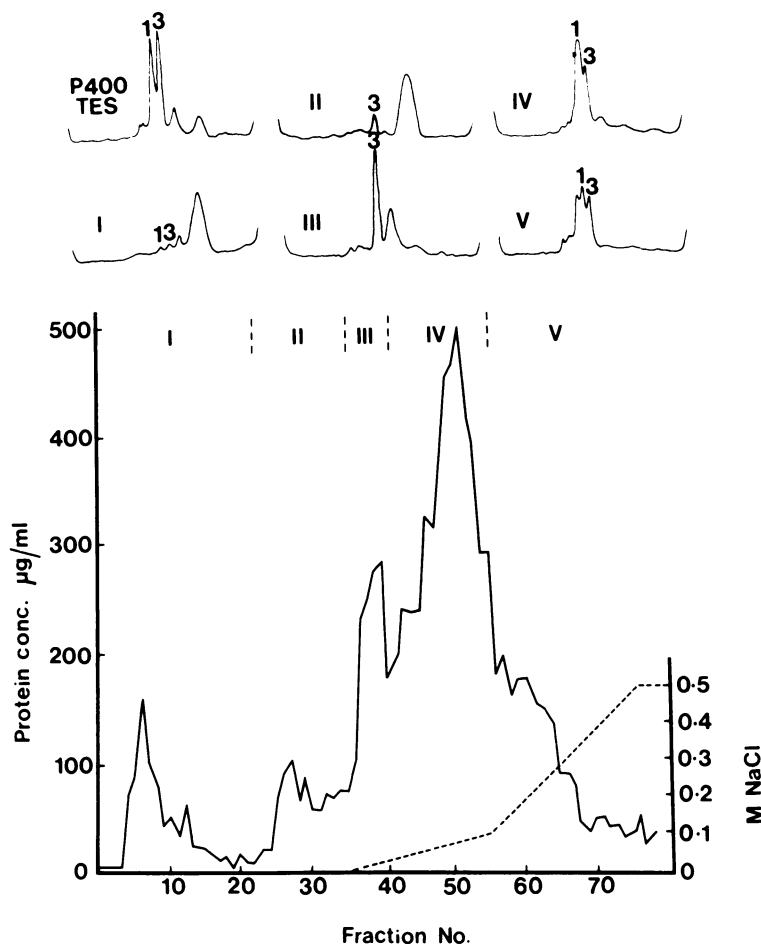


FIG. 3. DEAE-cellulose chromatography of the Triton-plus-EDTA-soluble (TES) outer membrane proteins of strain P400. The various pooled fractions were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1). Densitometer traces of the stained gels are labeled by the method of Schnaitman (33).

The mutants were resistant to a group of nine K3-like phage, were unable to adsorb K3 (Fig. 6), were tolerant only to colicins K and L (Table 3), and were defective as recipients in conjugation with Hfr strains and most F-like plasmid donors (Table 4), as described previously for *con* mutants (10, 15, 34). *tolG* mutants had been reported as being sensitive to colicin K (10), but it has now been shown that, like *con* mutants, they are partially resistant (Table 3).

Map position of *con* and *tolG* mutants. It was reported previously that *tolG* mutants are cotransducible with *pyrD* (9) at about 21.5 min on the *E. coli* K-12 linkage map (39) and that *con* mutants are cotransducible with *lip* at 14.5 min (6). We therefore repeated both transductions because of the similarity of the pheno-

types of the mutants. We showed that both of the *con* mutants, P460 and P212, as well as the *tolG* mutant, JF404-2a, are cotransducible with *pyrD* (Table 5). We have been unable to repeat the result previously obtained in our laboratory (6) demonstrating cotransduction with *lip* and suggest that the earlier result may be due, in part, to the difficulty we encountered using the strain HfrH *lip*-22.

Identity of colicins produced by *E. coli* 398 and *Serratia marcescens* JF246. Because it had been possible to select *con* (*tolG*) mutants using either colicin L-398 or bacteriocin JF246, we suspected they may be similar colicins.

The colicin produced by strain JF246 had not been typed previously, and, when it was tested against all of the type mutants isolated by Dav-

ies and Reeves (4, 5) (except for strains P516 and P653, which have been lost), it was observed that the patterns of resistance to L-398 and JF246 were identical. We also isolated 80 spontaneous mutants of strain P400 resistant to bacteriocin JF246 and examined their pattern of colicin resistance; all were also resistant to colicin L-398 and could be included in the classification of Davies and Reeves for mutants resistant to the A group of colicins (4). We suggest, therefore, that according to the nomenclature of Fredericq (12) and with the agreement of Foulds (J. Foulds, personal communication) bacteriocin JF246 is a colicin of type L and that it be called L-JF246.

DISCUSSION

We have confirmed that the properties previously ascribed to either *con* or *tolG* mutants apply to both. Thus, *tolG* mutants resemble *con* mutants in being resistant to only one group of 9 phages out of the 64 phages tested, in being tolerant to only colicins K and L of the set of 19 colicins used in this laboratory, and in being defective as recipients in conjugation with most F-like plasmid donors. Conversely, *con* mutants resemble *tolG* mutants in tolerance to the colicin of strain JF246; indeed, we showed that this colicin activity is indistinguishable from colicin L and it has been renamed as colicin L-

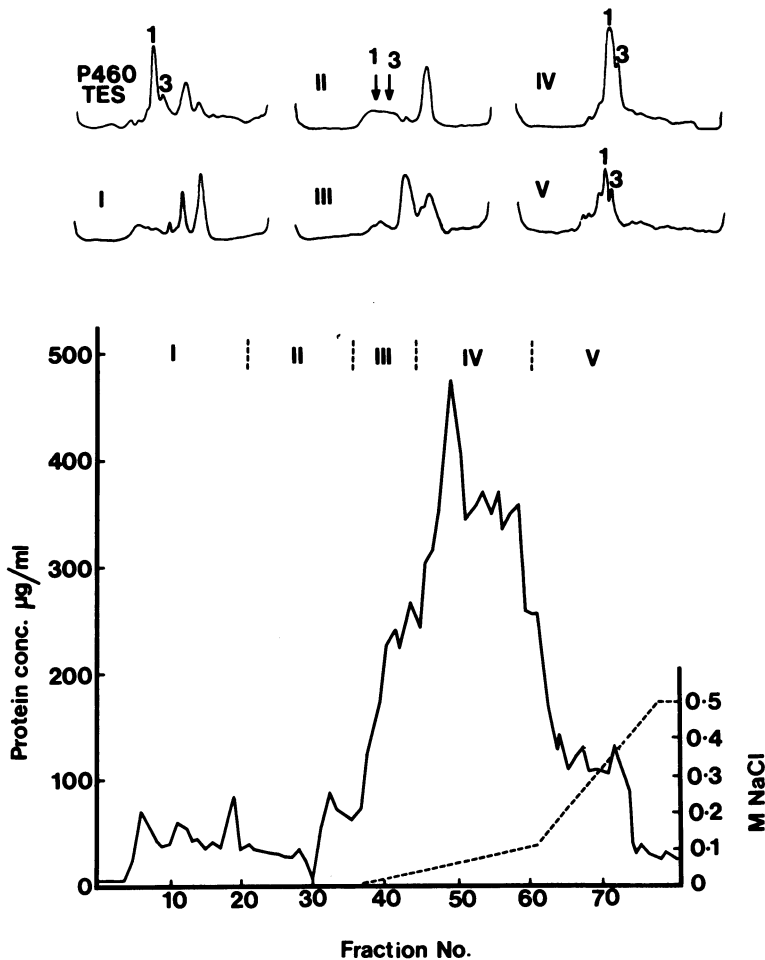


FIG. 4. DEAE-cellulose chromatography of the Triton-plus-EDTA-soluble (TES) outer membrane proteins of strain P460 (*con*). The various pooled fractions were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 11.4 buffer system of Bragg and Hou (1). Densitometer traces of the stained gels are labeled by the method of Schnaitman (33).

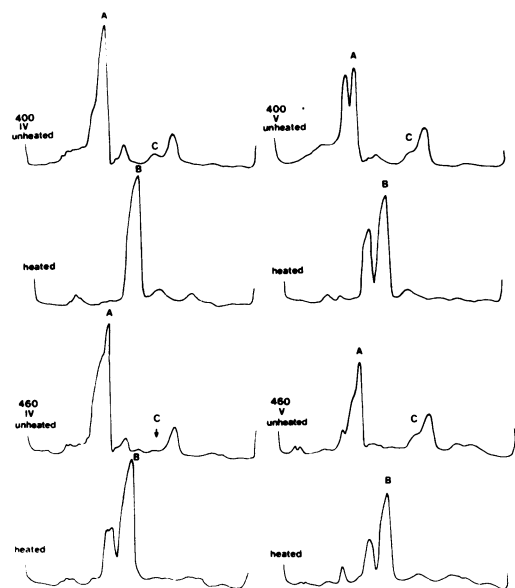


FIG. 5. Comparison, by densitometry, of polyacrylamide gels of the protein composition of pools IV and V from the DEAE-cellulose columns of strains P400 and P460. The samples were run on sodium dodecyl sulfate-polyacrylamide gels, using the pH 7.2 buffer system of Maizel (24) with unheated and heated samples. The peaks are labeled by the method of Schnaitman (33).

JF246. Both types of mutation are cotransducible with *pyrD* and have a defect in their outer membrane composition, which is shown in both cases to be the loss of protein 3A only.

From Fig. 1 it can be seen that peak C is reduced in the *con* (and *tolG*) mutant; if samples are heated, this peak remains the same size in the *con* (and *tolG*) mutant but in P400 is reduced to the smaller size observed in the mutant. This suggests that it is only the portion of peak C that moves into peak B upon heating which is missing in *con* (and *tolG*) mutants. The residual peak C in P460 corresponds to another protein (the *tsx* protein), which has been shown to be absent in *tsx* mutants and functions as the receptor for phage T6 and colicin K (P. A. Manning, M. Lavoie, and P. Reeves, manuscript in preparation).

The small peak of protein running slightly faster than peak C also appears to decrease upon heating (Fig. 1), and this position corresponds to that of the heat-modifiable protein enriched in the column fractions containing protein 3B (Fig. 5). We believe that this peak, in fact, contains protein 3B. Thus, the positions of the various outer membrane proteins can be summarized diagrammatically as in Fig. 7.

The heat-modifiable protein B* of Reithmeier

and Bragg (29), which Henning has shown to be identical with his protein II* (13, 14, 20, 21) and to be missing in *tolG* mutants (21), must therefore be protein 3A. This confirms the recent suggestion of Lugtenberg and his co-workers (23).

Phage TuII*, isolated by Henning as being unable to plaque on *tolG* mutants (20), thus fits into the group of K3-like phage as defined by Hancock and Reeves (15).

The loss of the ability of *con* mutants to plaque phage K3 had been assumed to be due to

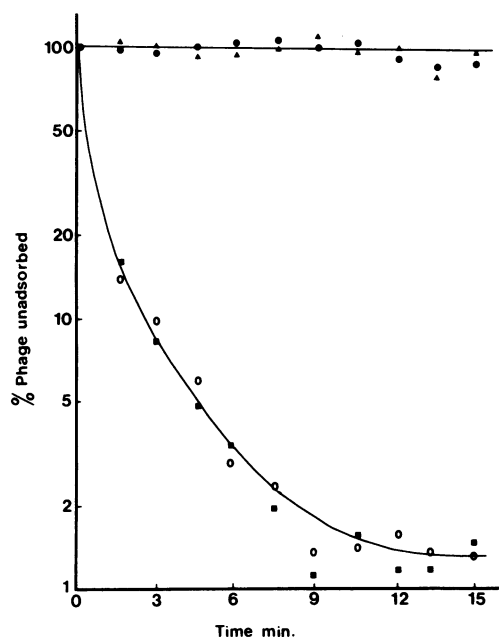


FIG. 6. Adsorption of bacteriophage K3 to whole cells of strain P400 (○); its *con* mutants, P460 (●) and JF404 (■); and its *tolG* mutant, JF404-2a (▲). Cells were at a density of 2×10^8 /ml, and phage were added to a multiplicity of 1.0.

TABLE 3. Colicin titers on the mutants^a

Strain	Titer of colicin			Mutation
	L-398	L-JF246	K-235	
P400	256	512	4,028	
P460	1	1	4	<i>con</i>
AB1133	256	512	4,028	
P212	1	2	8	<i>con</i>
JF404	256	512	256	
JF404-2a	1	1	2	<i>tolG</i>
P1635	256	512	4,028	
P1636	1	1	2	<i>tolG</i>

^a The titers of the colicins were measured by using method two as described in the text. The mutants and their parents were fully sensitive to all other colicins.

TABLE 4. Recipient abilities with the different donors

Donor	% Transfer ^a to:				
	P400	P460 (con)	P212 (con)	P1635	P1636 (tolG)
HfrH	1.8	<0.01	0.13		
HfrC	1.5	<0.01		2.0	<0.01
F' 111	12.5	<0.01		13.0	<0.01
F' lac pro	25.5	0.013	0.73		
R1drd19	16.9	1.31		12.3	0.74
R100-1	13.8	14.8	9.3	19.4	18.9
R136fn	14.9	14.0		15.7	18.2
R386	13.0	0.02		14.5	0.04
R538Fdrd1	11.2	0.07	0.30	13.2	0.03
ColV2	5.3	<0.01		5.0	<0.01
ColVBtrp	1.2	<0.01		1.2	<0.01
R64-11	1.1	1.2	0.72	0.7	0.7
R144drd3	8.0	7.2		6.5	10.5
R163drd1	6.4	5.8		8.7	9.8
R538ldrd2	2.4	2.0	1.8	2.3	1.9

^a Transfer was measured as a percentage of input donor cells. Each result is the mean of at least three experiments.

TABLE 5. Cotransduction of con and tolG with pyrD^a

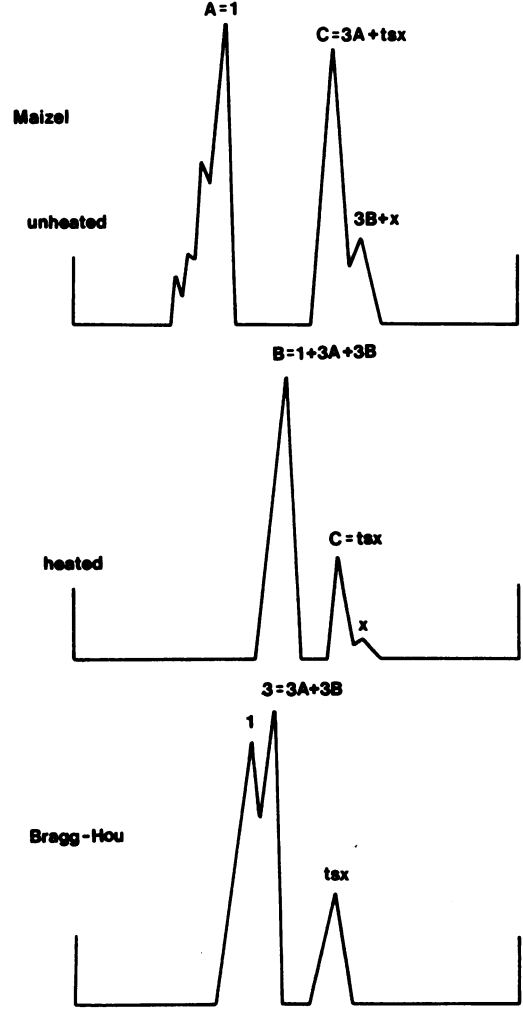
Strain	Mutation	Resistance of pyrD ⁺ transductants to phage K3 and colicins K-235, L-398, and L-JF246	% Cotransduction
P212	con	33/152	21.7
P460	con	35/152	23
JF404-2a	tolG	10/56	17.6

^a Resistances to phage K3 and colicins K-235, L-398, and L-JF246 were all transduced together. All transductants were *glt*, *gal*, and *str* and sensitive to colicins E2, E3, and A.

the loss of the receptor (34). Protein 3A can be shown to be the receptor for phage K3 and also for some of the other K3-like phage able to be neutralized by cell fractions (Manning and Reeves, manuscript in preparation).

Our studies with *con* mutants are continuing. We are investigating the role of protein 3A in conjugation and also in phage K3 sensitivity. We understand that its role in sensitivity to colicin L-JF246 is also being investigated (Foulds, personal communication).

FIG. 7. Diagrammatic representation of the positions into which the major outer membrane proteins of *E. coli* K-12 move upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using the pH 7.2 buffer system of Maizel (24) and the pH 11.4 buffer system of Bragg and Hou (1). Peaks are labeled by the method of Schnaitman (33) with the exception of *tsx*, which represents the protein now shown to be the receptor for bacteriophage T6 and colicin K (Manning, Lavoie, and Reeves, manuscript in preparation).



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ADDENDUM IN PROOF

We have shown in this paper that our *con* mutants and the *tolG* mutants described by Foulds and Barrett (J. Bacteriol. 116:885-892, 1973) are identical. These mutants are also identical to the *tut* mutants recently described by Henning et al. (FEBS Lett. 61:46-48, 1976). It has now been resolved that these three designations, *con*, *tolG*, and *tut*, be renamed *ompA* (outer membrane protein) since this is the site of the structural gene for protein 3A (Manning et al., J. Bacteriol. 127:1080-1084, 1976; Henning et al., FEBS Lett. 61:46-48, 1976). Any reference to this mutation should now use the *ompA* designation.

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