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PLASMID MEDIATED ALTERATIONS IN COMPOSITION AND STRUCTURE OF ENVELOPES OF *ESCHERICHIA COLI* B/r

RAJUL IYER *
With freeze-fracture electron microscopy by PATRICIA C. ADSHEAD

* *Department of Microbiology and Immunology, University of Ottawa, K1N 9A9 (Canada)*

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Summary

Seven transmissible (Tra^+), antibiotic resistance (r) plasmids, which confer sensitivity to the filamentous bacteriophage IKe on an *Escherichia coli* B/r strain harboring them, have been examined for the changes they evoke in the host cell membranes. The plasmids rR48 and rR269, like rRM98 [1], cause a significant reduction in the density of the outer membrane and the virtual elimination of its 36 500 dalton protein. These 2 properties do not appear to be altered when rR45, rR199 or rR205 are the resident plasmids. No changes in the inner membrane proteins are seen in any of these strains. In the case of the rR46-bearing strain, the density of the outer membrane is increased and the level of the 36 500 dalton protein is unaltered; in addition, several changes in both inner and outer membrane proteins are seen. Spontaneous IKe resistant mutants isolated from strains lacking the 36 500 dalton protein are either Tra^+ or Tra^-. Since most of them also lack this protein, the latter is not important to the expression of inter-bacterial gene transfer and IKe sensitivity.

On freeze fracture, strains lacking the 36 500 dalton protein cleave almost exclusively within the outer membrane. The plasmidless host and the remaining plasmid-bearing strains show a strong fracture plane within the cytoplasmic membrane. Despite the fact that most of the plasmid-bearing strains used are proficient in effecting interbacterial plasmid transfer, no morphological differences which can be correlated with this function have been observed between their etched cell surfaces and that of the plasmidless host.

Introduction

The conjugative antibiotic resistance plasmid rRM98 (Ap Sm Sp Tc Tra^+) confers on *Escherichia coli* and *Salmonella typhimurium* hosts harboring it, specific sensitivity to the filamentous bacteriophage IKe [2,3]. An exhaustive

search for cell surface-associated, sex pilus-type appendages on an rRM98$^+$ *E. coli* K-12 strain has not revealed such structures in negatively stained cells and the phage appears to attach directly to the cell [4]. In the case of F and I plasmids, specific sex pili provide receptor sites for the morphologically similar phages M13 and If [5,6] respectively and these appendages are also implicated in plasmid transfer [7]. We have examined the rRM98-bearing *E. coli* K-12 strain used earlier [4] by freeze fracture electron microscopy and confirmed the absence of plasmid-specific structures on the etched cell surface. The latter, as well as large areas of the fracture face of the exposed cytoplasmic membrane of this strain are morphologically identical to those of the plasmidless control. Cell-free membrane preparations from sonicated whole cells or from lysozyme spheroplasts as well as purified lipopolysaccharide (LPS) and its component fragments from this rRM98$^+$ strain do not possess phage binding activity (Iyer, R., unpublished).

Fractionated envelopes of rRM98-bearing *E. coli* strains K-12 and B/r have been analysed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. In the K-12 host, the appearance of an extra 31 000 dalton envelope protein appears to be specific to plasmid-mediated gene transfer [8]. Since this function and that of IKe sensitivity are closely linked in and coordinately lost from several IKe resistant mutants of this strain we have examined, it is possible that this protein may be implicated somehow in the expression of phage specificity. Analysis of envelopes from several rRM98-bearing *E. coli* B/r transconjugants obtained from a single cross shows that the plasmid exerts variable effects principally on the level of the 36 500 dalton outer membrane protein. In 3 of 5 such isolates, the virtual absence of this protein does not prevent the expression of plasmid transferability and/or phage sensitivity (Iyer, R., Leong, W., Darby, V. and Holland, I.B., submitted to Nature).

We have examined here the generality of the changes in envelope proteins of the *E. coli* B/r strain LEB18 following the construction of derivatives containing each of 7 IKe-specific R plasmids. Alterations in envelope architecture have been monitored by freeze fracture electron micrography since this technique permits visualization of both the etched cell surface as well as the fracture face of inner membranes along which cleavage occurs [9,10].

Materials and Methods

Chemicals. Dithiothreitol, bovine serum albumin (M_r 65 000), lysozyme (14 700), fibrinogen (65 000, 56 000 and 48 000), and β-lactoglobulin (17 000) were obtained from Sigma Chemicals. Figures in brackets represent molecular weights of the respective standards. All components for SDS polyacrylamide gels were from Bio. Rad Company except *N,N,N',N'*-tetramethylethylenediamine which was from Canalco Industrial Corp., Rockville, Md. Ampicillin trihydrate, kanamycin and tetracycline were gifts from Bristol Laboratories of Canada.

Bacterial strains. All bacterial strains were maintained as described earlier [3] on L agar supplemented as required with Ap or Tc at 30 μg/ml. The strains used, their relevant properties, and sources are listed in Table I.

Preparation of fractionated envelopes. Inocula in L broth were prepared by

TABLE I

ESCHERICHIA COLI STRAINS

Strain No. *	Plasmid	Relevant markers **	Selection	Source of plasmid
LEB18	—	F⁻ *str lacz*		...
LEB500(rRM100)	rRM98	Ap Tra⁻ IKe^S	Ap	This laboratory
LEB508(rR45)	rR45	Ap Tc Tra$^+$ IKe^S	Tc	N. Datta
LEB509(rR46)	rR46	Ap Tc Tra$^+$ IKe^S	Tc	N. Datta
LEB505(rR48)	rR48	Sm Tc Tra$^+$ IKe^S	Tc	N. Datta
LEB510(rR199)	rR199	Tc Tra$^+$ IKe^S	Tc	N. Datta
LEB511(rR205)	rR205	Tc Tra$^+$ IKe^S	Tc	N. Datta
LEB506(rR269)	rR269	Ap Km Sm Tc Tra$^+$ IKe^S	Tc	S. Dennison

* Plasmid-bearing derivatives are transconjugants from crosses with *E. coli* K12 donors bearing the respective plasmids. Strains have been designated as described in Iyer et al., submitted to Nature. LEB18 was kindly provided by P. Meacock.

** Abbreviations: Ap, ampicillin; Tc, tetracycline; Sm, streptomycin; Km, kanamycin; Tra, plasmid transfer; IKe^S, sensitivity to IKe; *str*, chromosomal streptomycin resistance.

suspending growth from 24 h-old plate cultures to an absorbance of 0.2 units at 650 nm. These were diluted 200-fold in fresh medium and grown at 37°C in a water bath shaker at 150 strokes/min to an absorbance of 0.4 units (3—5 · 10^8 cells/ml). Envelopes were isolated from cells by a method essentially similar to that described by Osborn et al. [11]. Lysozyme spheroplasts held in a mixture of crushed ice and water were sonicated for 4 × 30-s pulses in a Bronwill Biosonik sonic disintegrator.

For fractionating envelopes into inner and outer membranes, washed membrane pellets were resuspended and centrifuged on discontinuous sucrose gradients [11] and fractions collected from the top. Samples were diluted to a refractive index of 1.25 with 10 mM $NaPO_4$ buffer (pH 7.2), and pelleted in polycarbonate tubes (Beckman) at 45 000 rev./min for 90 min at 4°C in the Type 50 rotor in a Beckman L3-50 ultracentrifuge. The use of these tubes permits a 50—60% recovery of the membrane fractions.

Total and fractionated envelopes were resuspended in a minimal volume of sample $NaPO_4$ buffer and protein concentrations estimated from absorbance measurements at 260 and 280 nm [13] in a Beckman spectrophotometer model DU. Samples containing 2—10 mg protein/ml were stored at —20°C.

SDS polyacrylamide gel electrophoresis. The basic electrophoretic procedure, gel constituents and buffers including sample buffer were those descibed by Laemmli [12]. Electrophoresis was carried out using a water-cooled Bio. Rad slab gel system (Model 220), at a constant 100 V for 4.5 h. Up to 40 samples were analysed simultaneously in twin 1.5 mm thick, vertical slabs containing a 5% stacking gel (1.6 cm high) over a separating gel (9.5 cm high) of the acrylamide concentration indicated in the text. The indicator dye used was 0.1% Bromophenol Blue in 30% sucrose. Gels were stained with shaking at room temperature overnight in 300 ml of 0.05% Coomassie Brilliant Blue dissolved in 10% acetic acid and 25% isopropyl alcohol. Destaining was carried out by a modification of the method of Fairbanks et al. [14]; gels were shaken for 3 h in each of 2 changes of 300 ml of 10% acetic acid/10% isopropyl alcohol

and finally overnight in 300 ml of 10% acetic acid. Using an orange filter, gels were photographed on to 4 × 5 inch negatives; these were then scanned where necessary on an Ansco Automatic recording microdensitometer Model 4.

Envelope samples in sample buffer were normally boiled for 5 min just before loading on to gels. Samples contained 50 μg protein in a volume not exceeding 50 μl. Standard proteins (2 μg each) were applied similarly after boiling.

Freeze fracture and freeze etching. Cultures were grown in L broth at 37°C in a water bath shaker to a density of $2–4 \cdot 10^8$ cells/ml. Cells were harvested by centrifugation at 2800 × *g* at 4°C. Portions of the pellet were resuspended in a drop of the supernatant medium; unfixed cells [15] were rapidly frozen in Freon 22 held in liquid nitrogen. Cells were not cryo-protected prior to freeze fracture in order to preserve the subunit structure of the cell wall and maximize the detection of any surface appendages [16]. All cultures were processed under identical conditions of growth and temperature. Samples were fractured at −100°C under a vacuum of $2 \cdot 10^{-6}$ torr and etched for 3 min in a Balzers freeze etch unit (BA300F). The etched samples were shadowed with platinum-carbon for 8 s prior to reinforcement with a carbon backing for 10 s. Replicas were floated off in distilled water and cleaned in 70% H_2SO_4 for 30 min in the well of a ceramic tile. After rinsing in water, they were washed in 5.25% sodium hypochlorite (commercial Javex bleach) for 30 min. They were passed through 4 changes of distilled water; the final rinse was in 2 ml of water containing approximately 20–25 μl of Photoflo (Eastman Kodak) [17]. Replicas were picked up on acetone-cleaned 200 mesh copper grids. Since micrographs of replicas have not been reversed, shadows appear white. For microscopy, a Philips 300 electron microscope operating at 60 kV was used.

Results

Densities of envelope fractions from plasmid-bearing E. coli B/r strain LEB18

Envelopes were isolated from lysozyme spheroplasts of exponential phase cultures and fractionated on discontinuous sucrose gradients. Envelope fractions were identified on the basis of gel electropherogram profiles and their densities [8] are compared in Table II. The H1 fractions from the strains bearing rRM98, rR48 and rR269 are considerably lighter compared to those from the corresponding fraction of the plasmidless host. In the case of the rR46$^+$ strain, an unseparated membrane fraction (M) is seen and the H1 and H2 fractions are much denser than those of LEB18. The densities of membrane fractions from LEB508(rR45), LEB510(rR199) and LEB511(rR205) are not significantly altered.

SDS polyacrylamide gel electrophoretic analysis of fractionated membranes. In Fig. 1, SDS polyacrylamide gel electrophoresis profiles of the inner membranes from all the strains are compared. With the exception of inner membranes from LEB509(rR46) (Fig. 1, d and l), those from all the other strains appear to be generally similar in protein content to those from the plasmidless LEB18 (Fig. 1, b and j). A comparison of the profiles of H2 and H1 in Fig. 2 reveals alterations in the relative amounts of the 36 500 and 34 000 dalton pro-

TABLE II

PLASMID MEDIATED EFFECTS ON *ESCHERICHIA COLI* B/r STRAIN LEB18

Strain no.	Densities of fractionated membranes [a]					Plasmid transfer	36 500 dalton membrane protein	Freeze fracture exposes [b]	Frequency of Tc transfer to B/r $T6^r$ per 100 donors [c]
	L_1	L_2	M	H_2	H_1				
LEB18	1.1480	1.1720		1.2015	1.2290		+	PF	
LEB508(rR45)	1.1440	1.1710		1.2005	1.2315	+	+	PF	$1.5 \cdot 10^{-2}$
LEB509(rR46)	1.1480	1.1720	1.1990	1.2260	1.2485	+	+	PF	$0.75 \cdot 10^{-2}$
LEB510(rR199)	1.1485	1.1715		1.1985	1.2240	+	+	n.d.	$5.0 \cdot 10^{-2}$
LEB511(rR205)	1.1480	1.1720		1.2000	1.2315	+	+		$3.3 \cdot 10^{-2}$
LEB500(rRM100)	1.1410	1.1720		1.1970	1.2215	—	—	EF	$<1 \cdot 10^{-5}$
LEB500(rRM101)	1.1476	1.1730		1.1990	1.2350	—	+	PF	$<1 \cdot 10^{-5}$
LEB500(rRM102)	1.1470	1.1735		1.1980	1.2225	—	—	EF	$<1 \cdot 10^{-5}$
LEB505(rR48)	n.o.	1.1730		1.1990	1.2200	+	—	EF	$6 \cdot 10^{-2}$
LEB505(rR484)	n.o.	1.1705		1.2015	1.2250	—	—	EF	$<1.2 \cdot 10^{-5}$
LEB505(rR488)	n.o.	1.1735		1.1990	n.o.	+	—	EF	$1.4 \cdot 10^{-2}$
LEB506(rR269)	1.1530	1.1730		1.1795	1.1815	+	—	EF	$2 \cdot 10^{-2}$
LEB506(rR26922)	n.o.	1.1750		1.1980	1.2200	—	—	EF	$<1.1 \cdot 10^{-5}$
LEB506(rR26926)	n.o.	1.1750		1.2000	1.2220	+	—	EF	$1.7 \cdot 10^{-2}$ [d]

[a] H1 and H2, outer and inner cell wall layers respectively; M, unseparated mixture of inner membranes and cell wall; L2 and L1, outer and inner cytoplasmic membrane layers respectively; n.o., (band) not observed.

[b] Nomenclature as in ref. 22. PF, fracture face of cytoplasmic membrane; EF, fracture face of cell wall; ES, etched cell surface; n.d., not done.

[c] Logarithmic phase cultures of the donor and recipient were mixed 1 : 4 on sterile millipore filters and the medium removed by application of vaccum. The filters were set on top of freshly poured L agar plates and incubated for 1 h at 37°C before resuspending cells in 1 ml of T6 lysate ($8 \cdot 10^{10}$) plaque forming units/ml) containing 20 μg/ml tryptophan. Incubation was for 30 min at 37°C; mating mixtures were plated on L agar supplemented with the appropriate antibiotic (30 μg/ml).

[d] Frequency of transfer of Sm (not Tc) resistance.

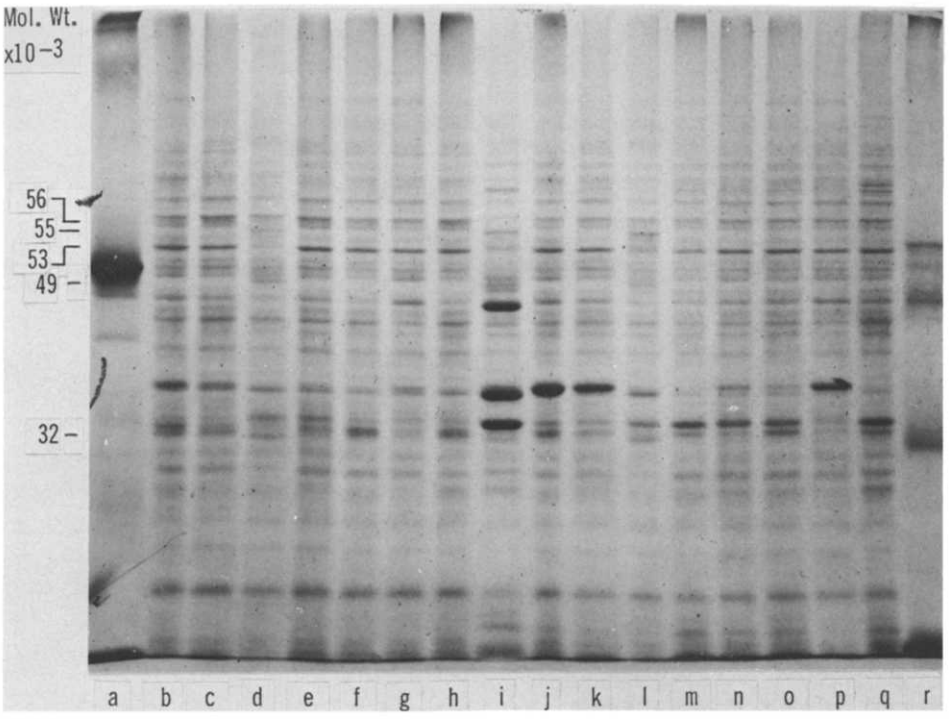


Fig. 1. SDS polyacrylamide gel electrophoresis of inner membrane fractions from *E. coli* LEB18 and its plasmid-bearing strains. Membranes were isolated from spheroplasted cultures grown to exponential phase and fractionated on discontinuous sucrose gradients as described in Materials and Methods. Samples contained 50 μg protein in a maximum volume of 25 μl of sample buffer. The polyacrylamide concentration of the separating gel was 9% (w/v). b—h are L1 fractions; j—q are L2 fractions. a, lysozyme and bovine serum albumin; b, LEB18; c, LEB508(rR45); d, LEB509(rR46); e, LEB510(rR199); f, LEB506(rR269); g, LEB511(rR205); h, LEB500(rRM100); i, fraction M (unseparated membrane) from LEB509(rR46); j, LEB18; k, LEB508(rR45); l, LEB509(rR46); m, LEB505(rR48); m, LEB510(rR199); o, LEB506(rR269); p, LEB511(rR205); q, LEB500(rRM100); r, fibrinogen and β-lactoglobulin.

teins. In the profiles of the rR45$^+$ (c, l) and rR205$^+$ (h, q) strains, these proteins appear to be present in concentrations similar to those seen in the plasmidless host (b, k). The profiles of outer membranes from LEB509(rR46) (d, m) and LEB510(rR199) (f, o) show an increase in the level of the 34 000-dalton protein and the 36 500-dalton protein is not appreciably altered. In the rR48$^+$ (e, n), rR269$^+$ (g, p) and rRM98$^+$ (i, r) strains, the 36 500-dalton protein is virtually absent or greatly diminished and the level of the 34 000-dalton protein is unaltered. The significance of the changes with respect to some plasmid-mediated properties is these 3 strains has been examined below.

The gels of the membranes of LEB509(rR46) were scanned on a microdensitometer. In the inner membrane profiles (Fig. 1, d and l), 3 new proteins of molecular weight 32 000, 49 000 and 55 500 are observed; at the same time, the 53 000 and 56 000 dalton proteins are somewhat decreased. The outer membranes of this strain (Fig. 2, d and m) clearly show decreased amounts of the 26 500, 42 500, 54 000 and 69 000 dalton proteins; the 34 000- and 63 500-dalton proteins are increased and a new protein of apparent molecular

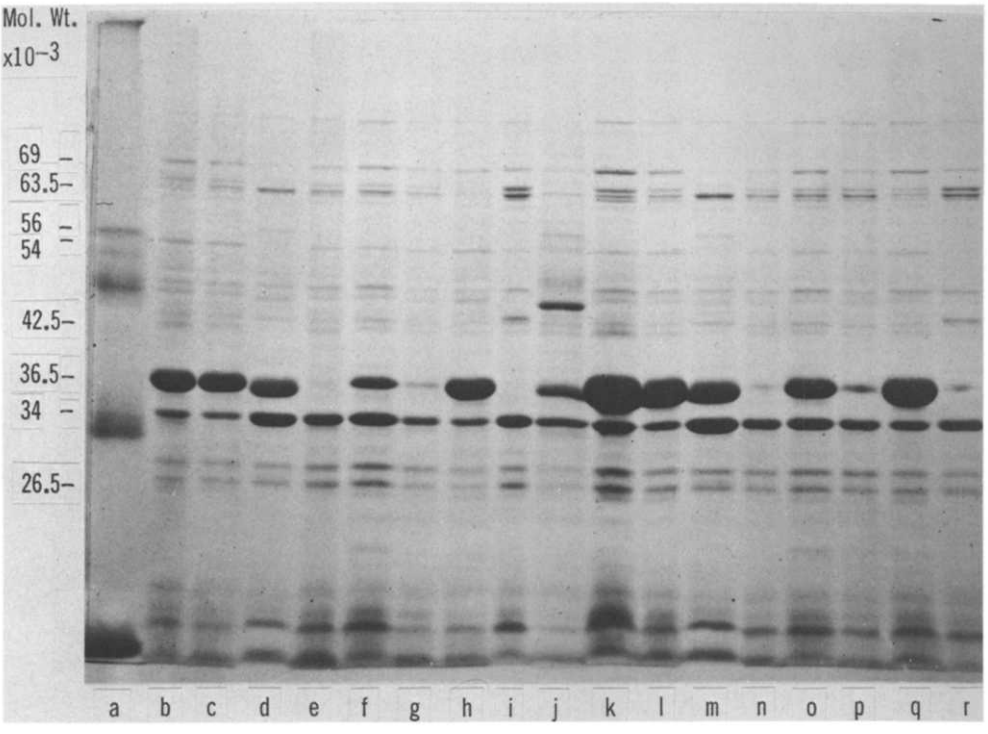


Fig. 2. SDS polyacrylamide gel electrophoretic analysis of outer membrane fractions from *E. coli* LEB18 and its plasmid-bearing strains. Details as in Fig. 1. b—i represent H_2 fractions; k—r represent H_1 fractions. a, fibrinogen and β-lactoglobulin; b, LEB18; c, LEB508(rR45); d, LEB509(rR46); e, LEB505(rR48); f, LEB510(rR199); g, LEB506(rR269); h, LEB511(rR205); i, LEB500(rRM100); j, M fraction from LEB509(rR46); k, LEB18; l, LEB509(rR45); m, LEB509(rR46); n, LEB505(rR48); o, LEB510(rR199); p, LEB506(rR269); q, LEB511(rR205); r, LEB500(rRM100).

weight 56 000 is evident. Whether or not any of these changes are significant to the plasmid transfer and/or IKe sensitivity functions remains to be examined.

Evaluation of freeze etch replicas. Freeze fracture of the plasmidless host strain LEB18 reveals an envelope architecture which is very similar to that described in several other studies with wild-type strains of *E. coli* [18—21]. The etched surface of the outer membrane (ES) (nomenclature as in ref. 22) is slightly wavy and smooth and no fine structure subunits are detected. The layered outer membrane is thick and appears in high relief on the exposed fracture face of the cytoplasmic membrane (PF). Occasionally, the fracture reveals a cell in which a narrow area of the inner layer of the cell wall (EF) (Fig. 3, inset) is visible. In all cells of replicate preparations, the fracture exposes large areas of the PF which is studded with randomly distributed, 8.0—10.0 nm diameter particles interspersed with particle-free (bare) patches.

In LEB508(rR45), fracture occurs in an inner layer of the cell envelope on which the smooth cell wall layers stand out in high relief (Fig. 4). The exposed fracture face appears to be somewhat bumpy and apparently lacks the 8—10 nm particles normally associated with this surface. All cells in replicate preparations made on 2 separate occasions showed the same characteristics. M.E. Bayer

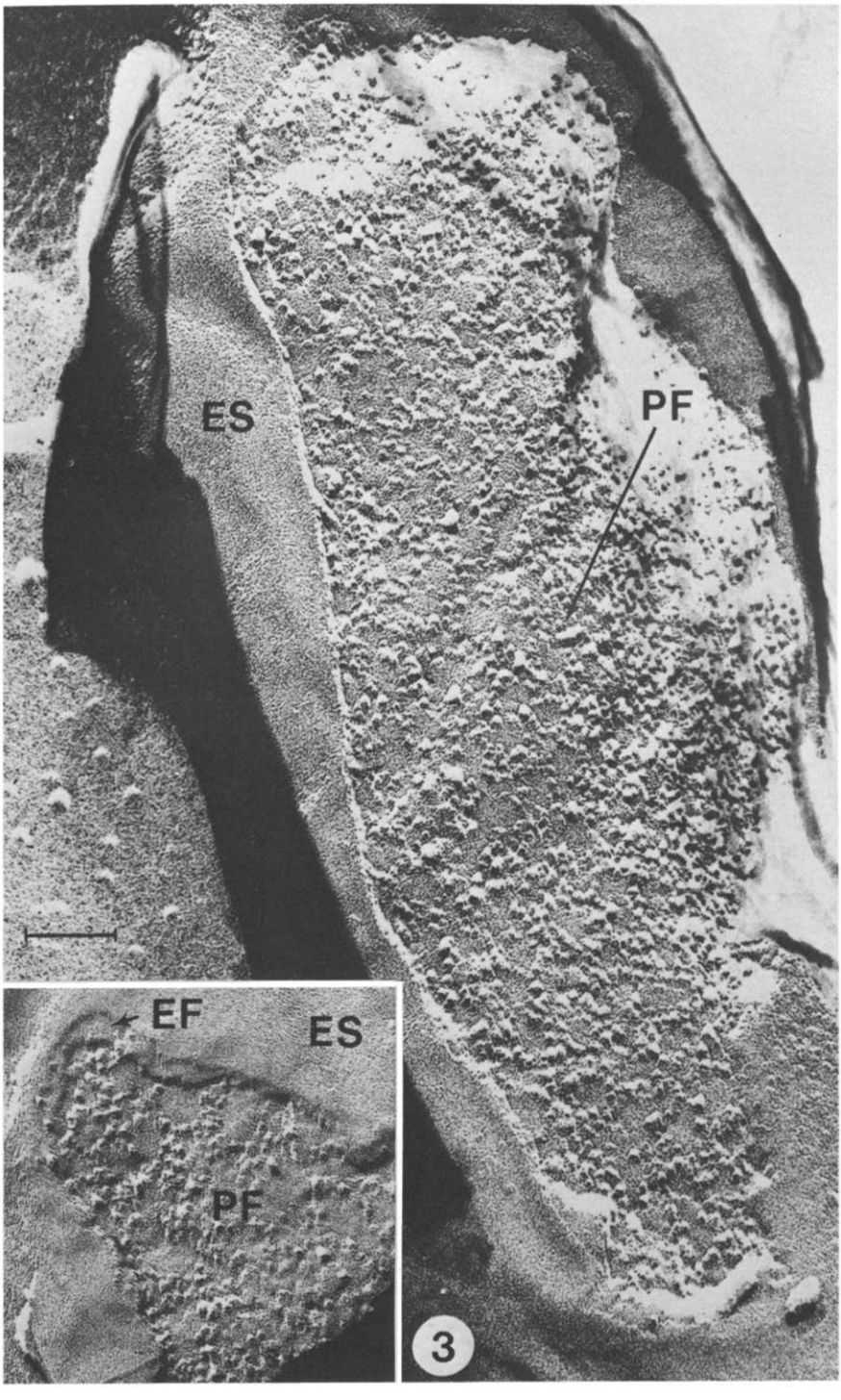


Fig. 3. Freeze fracture of *E. coli* LEB18. A strong fracture plane within the cytoplasmic membrane exposes its particulate fracture face (PF). The bilayered outer membrane is exposed only in narrow areas (EF) in the inset. The bar represents 0.1 μm in all the micrographs. ES, etched cell surface.

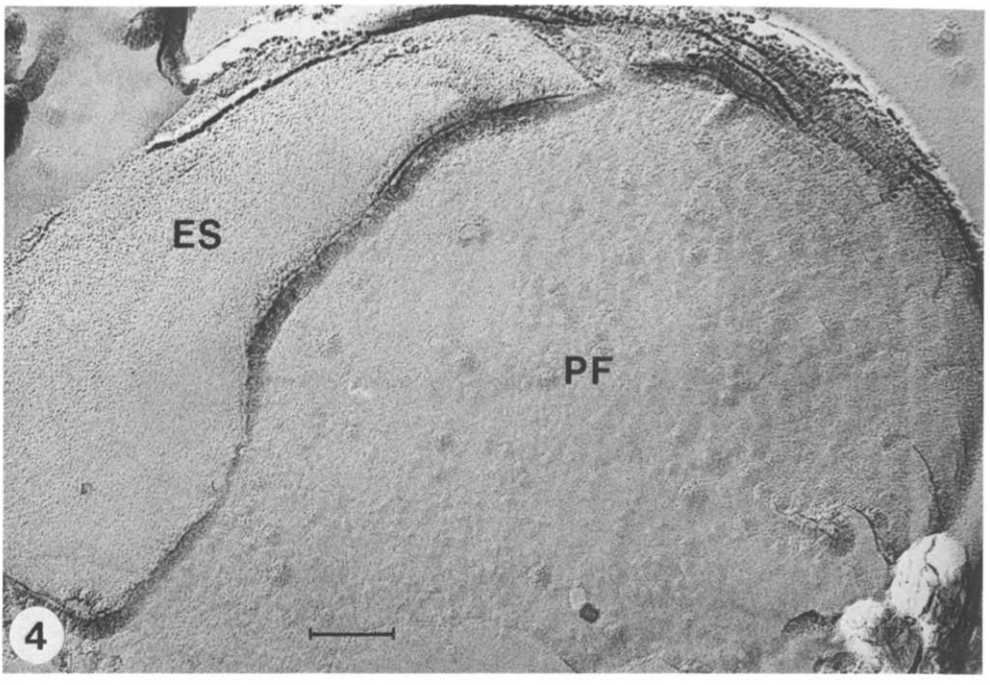


Fig. 4. The fracture of LEB508(rR45) exposes the PF on which the outer cell wall layers form a high relief.

has found that a few strains of *E. coli* he has examined also expose a fracture face with similar characteristics; however, this resolves as the PF when additional metal is cast on the replica (Bayer, M.E., personal communication). We have not verified this with LEB508(rR45).

Replicas of LEB509(rR46) predominantly show the PF (Fig. 5A); this surface is similar to that of the host strain. Rarely, the fracture shows small areas of the EF (Fig. 5B) which are particle-free and similar in texture to the ES.

Freeze fracture of strains bearing rR48, rRM98, and rR269, all of which virtually lack the 36 500-dalton protein, consistently show a strong, superficial fracture plane within the cell wall and large areas of the EF are almost always exposed (Figs. 6—8). A few particles are seen on the EF of LEB505(rR48) and LEB500(rRM100). A rare cell in the LEB500(rRM100) replica (Fig. 7, inset) illustrates the difference in particle density between the PF and EF. In Fig. 8A, the PF of LEB506(rR269) is bumpy and somewhat similar to that seen in Fig. 4 above. The same strain seen in Fig. 8B shows a large area of the exposed EF adjacent to a deeper, highly textured surface, possibly the PF.

Flagella and common pili are occasionally seen in the replicas of the strains examined. A careful examination of the etched cell surfaces in several replicas of each of the strains has shown that they are identical in texture and morphology to those of the plasmidless host.

Analyses of some spontaneous IKe resistant mutants. The phenotypic properties of some spontaneous IKe resistant mutants of the rR48-, rR269- and

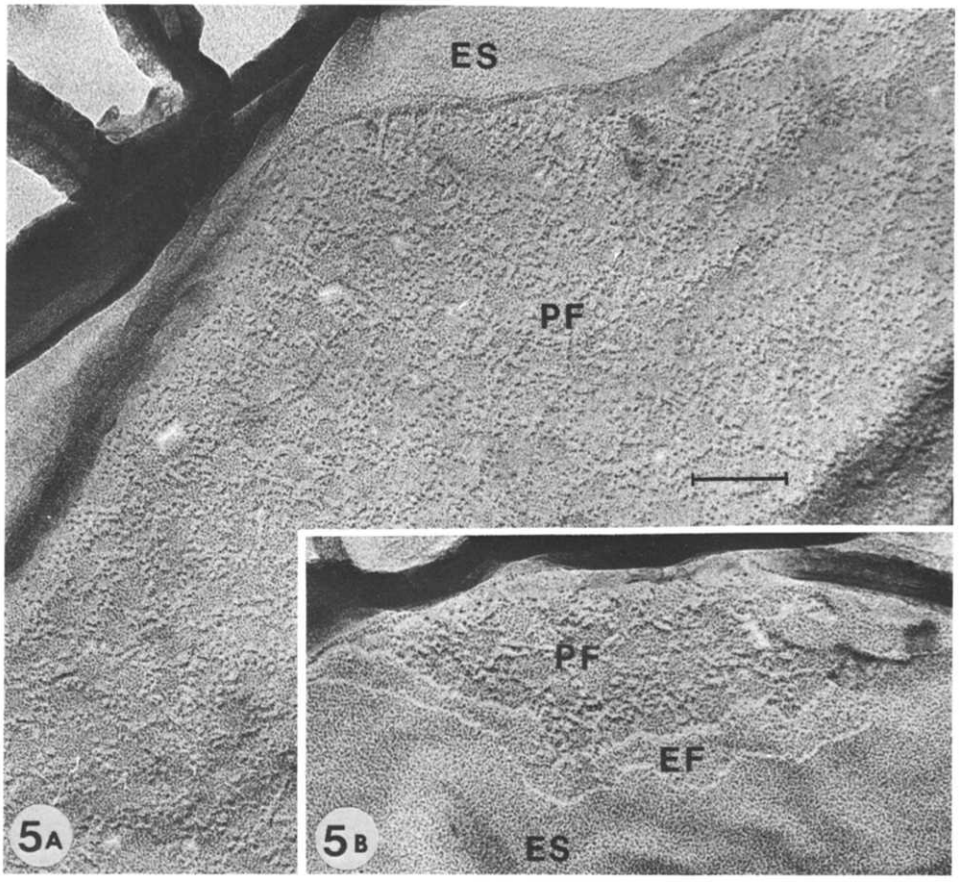
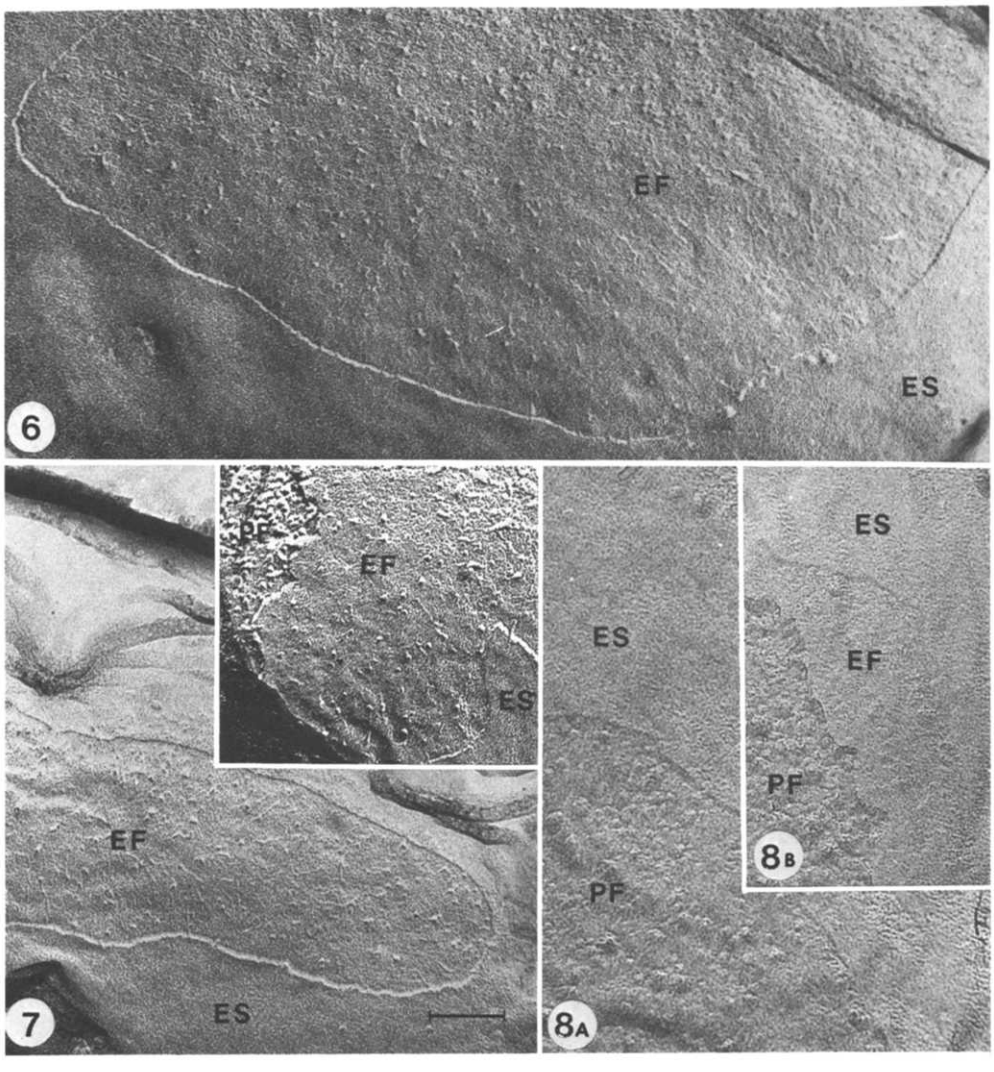


Fig. 5A and 5B. In LEB509(rR46), the fracture plane exposes the PF. In some cells, a non-particulate EF was observed as a rather narrow margin.

TABLE III

SOME PROPERTIES OF SPONTANEOUS IKe RESISTANT MUTANTS LEB500(rRM100), LEB505-(rR48) AND LEB506(rR269)

Strain no.	Ap	Sp	Sm	Tc	Km	IKe	Tra
LEB500(rRM100)	r	s	s	s		s	—
LEB500(rRM101)	r	s	s	s		r	—
LEB500(rRM102)	r	s	s	s		r	—
LEB505(rR48)			r	r		s	+
LEB505(rR484)			s	s		r	—
LEB505(rR488)			s	r		r	+
LEB506(rR269)	r		r	r	r	s	+
LEB506(rR26922)	s		s	s	s	r	—
LEB506(rR26926)	r		r	s	r	r	+



Figs. 6—8. The strains LEB505(rR48), LEB500(rRM100) and LEB506(rR269) respectively show a superficial fracture plane within the cell wall. Large areas of the EF are exposed in all strains. The PF is rarely seen and is illustrated in the inset of Fig. 7. The PF of LEB506(rR269) is bumpy (8A) and lies adjacent to the EF illustrated in 8B.

rRM98-bearing strains are described in Table III. The decreased densities of the H1 fractions from these mutants, with the exception of LEB500(rRM101) are comparable to those of the respective, IKe-sensitive, parental strains (Table II). Since these mutants are either Tra^+ or Tra^- (Table III), it appears that the functions of phage sensitivity and plasmid transfer are not affected by the alterations in composition which result in an outer membrane of reduced density.

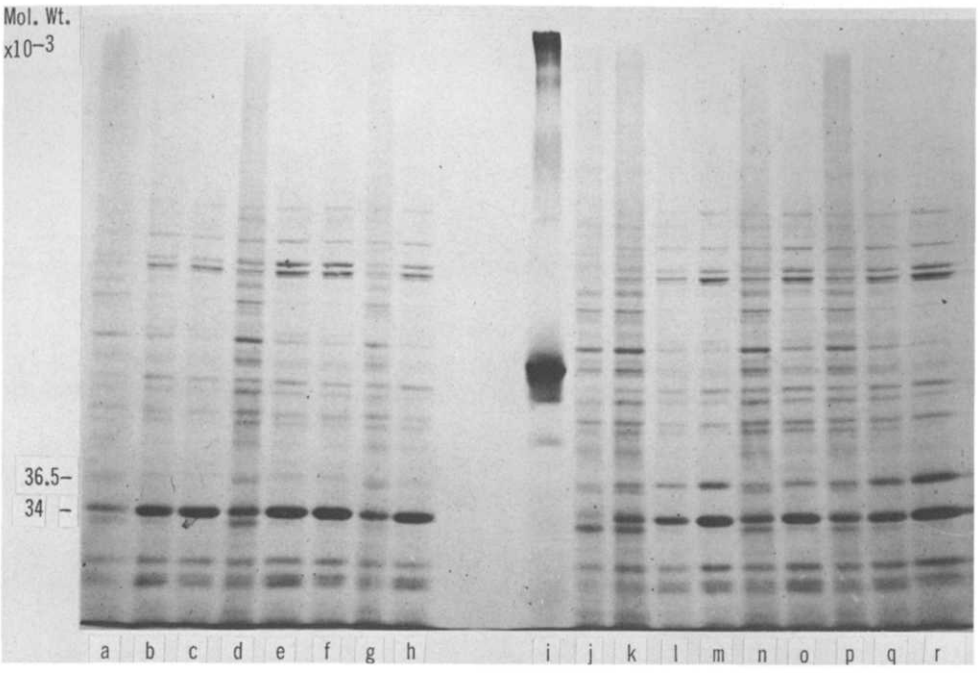


Fig. 9. Comparative SDS polyacrylamide gel electrophoretic analyses of membrane fractions from LEB505(rR48) and LEB506(rR269) and their spontaneous IKe resistant mutants. Details as in Fig. 1. The polyacrylamide concentration of the separating gel was 9% (w/v). a—c, L_2, H_2 and H_1 from LEB505(rR48); d—f, L_2, H_2 and H_1 from LEB505(rR484); g, h, L_2 and H_2 from LEB505(rR488); i, bovine serum albumin; j—m, L_1, L_2, H_2 and H_1 from LEB506(rR269); n, o, L_2 and H_2 from LEB506(rR26922); p—r, L_2, H_2 and H_1 from LEB506(rR26922).

Gel profiles of fractionated envelopes from the rR48$^+$ and rR269$^+$ strains are compared in Fig. 9 and those from the rRM98$^+$ strains in Fig. 10. The protein composition of the envelopes from the mutant and parental strains are identical in all cases except in LEB500(rRM101) (Fig. 10, h and i). In this strain wild type levels of the 36 500 dalton protein are obvious. Since all the mutant strains used are IKe resistant and Tra$^+$ or Tra$^-$, the reduced level of this protein is not important for these plasmid functions.

The replicas of the mutant strains LEB505(rR484) and LEB505(rR488), LEB506(rR26922) and LEB506(rR26926), and LEB500(rRM102) are indistinguishable from those of the respective parental strains; LEB500(rRM101) resembles LEB18 in that the cleavage plane exposes the PF (data not shown). This may be a consequence of the restored level of the 36 500 dalton protein in LEB500(rRM101) (Fig. 10, h and i). As is the case with the strains bearing the wild plasmids, no differences in the etched cell surfaces of the mutant strains and the LEB18 host have been observed.

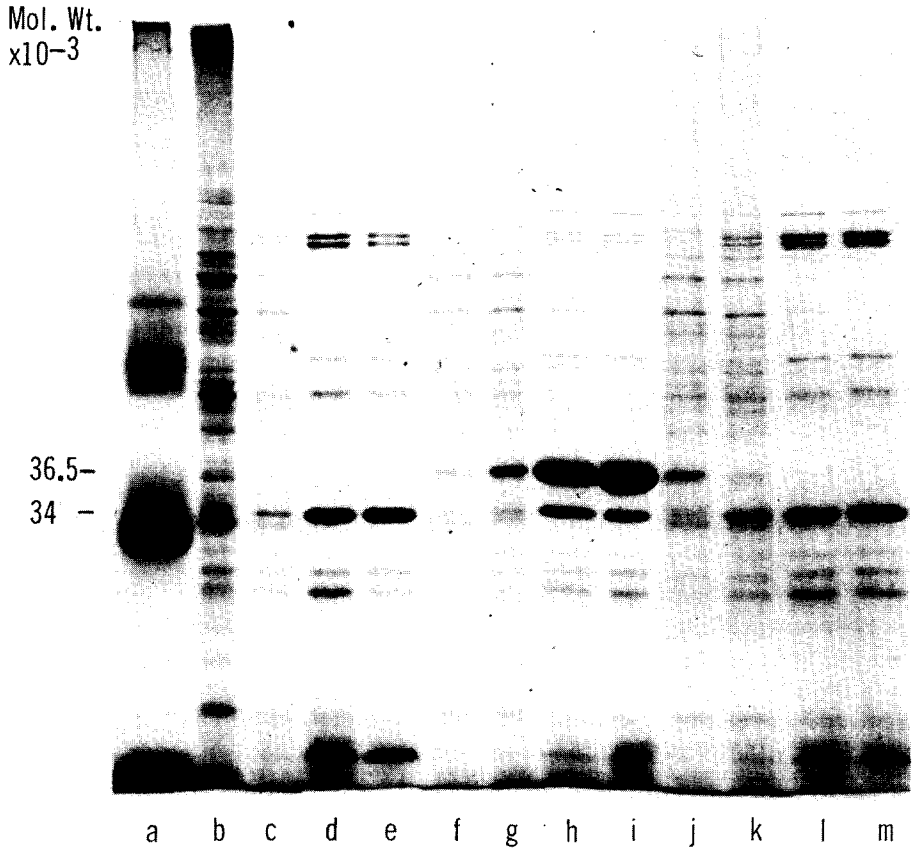


Fig. 10. Comparative SDS polyacrylamide gel electrophoretic analysis of fractionated membranes from LEB500(rRM100) and its spontaneous IKe resistant mutants LEB500(rRM101) and LEB500(rRM102). Details as in Fig. 1. a, fibrinogen and β-lactoglobulin; b—e, L_1, L_2, H_2 and H_1 from LEB500(rRM100); f—i, L_1, L_2, H_2 and H_1 from LEB500(rRM101); j—m, L_1, L_2, H_2 and H_1 from LEB500(rRM102).

Discussion

The 36 500 dalton protein is one of the 4 major outer membrane proteins which is widely distributed in *E. coli*, *S. typhimurium* and some Gram negative bacteria. Although this and the 34 000 dalton protein, each of which occurs in about 10^5 copies/cell, have been implicated in several activities of the cell (see discussion, ref. 23), mutants which lack both these proteins and which do not exhibit any obvious defects have also been described [24]. Thus it is not yet clear how indispensable these proteins are to the cell, nor are their precise roles with respect to specific physiological and other functions of the cell unambiguously established.

In certain LPS mutants of both *E. coli* [25] and *S. typhimurium* [26,27], the level of the 36 500 dalton protein and the density of the outer membranes are considerably diminished; the loci determining the altered LPS are chromosomal in origin [28]. Changes probably of a similar nature, due to the IKe-

specific plasmid rRM98 [1] and some derepressed I-type plasmids [29] in *E. coli* and *S. typhimurium* hosts respectively, have also been reported. However, the underlying mechanism(s) responsible for them are not yet understood and losses of membrane proteins have been attributed to nonsense or deletion mutations or mutations affecting a regulatory site [26,30]. The effects we have observed in the case of rR48, rR269 and rRM98 may be either a direct consequence of a plasmid gene (or its product) or due to an interaction of the latter with a chromosomal gene (or gene product). In either case, these alterations ought to be reversible in plasmid-cured derivatives, and this has been confirmed in the case of LEB505(rR48) and LEB506(rR269). Alternatively, should they result from the insertion of certain plasmid gene(s) at chromosomal sites such as those which control the synthesis of the LPS and/or the 36 500 dalton protein, the loss of the plasmid will not be accompanied by a reversal of the membrane defects. It may be significant that rRM98-mediated membrane changes are fully restored in the IKe resistant derivative LEB500(rRM101), in which plasmid DNA is still evident (Iyer, R. et al., submitted to Nature).

Studies with some phage IKe-specific plasmids in *E. coli* K-12 hosts have permitted the recognition of a transfer-specific envelope protein of molecular weight 31 000 [8]. Although 6 of 7 plasmids used here are Tra^+, no evidence for a protein which coincides with the presence of this function in the *E. coli* B/r host has been found. It is plausible that should such a protein(s) be synthesized in this host, it is masked by a protein(s) having a similar electrophoretic mobility.

LEB505(rR48) and LEB506(rR269) are similar to LEB500(rRM100) in that they show a drastic reduction in the level of the 36 500-dalton protein [1]. Plasmid transfer from all these strains (except the $rRM98^+$ strain used here) is efficient and comparable to that from LEB18 bearing the other 4 plasmids (see Table II). Since all these strains plaque IKe, the expression of the functions of phage sensitivity and gene transfer are not dependent on the presence of the 36 500 dalton protein. The analysis of the spontaneous IKe-resistant mutants LEB505(rR48) and LEB506(rR269) which are either Tra^+ or Tra^- (Table III) and also lack this protein, supports this conclusion.

rR45, rR199 and rR205 comprise a group of plasmids which do not evoke any observable alterations in envelope proteins. On the other hand, several changes, both in inner and outer membrane proteins of LEB509(rR46) have been noted; their role with respect to certain plasmid mediated properties remains to be determined.

The alterations in membrane protein composition described above possibly reflect further differences among plasmids comprising the N incompatibility group. The significance of these observations and the possibility that certain N plasmids effect alterations in membrane LPS and/or phospholipids are being examined.

The freeze fracture studies indicate that strain LEB18 as well as its plasmid-bearing derivatives in which the level of the 36 500 dalton protein is either unaltered or slightly reduced, cleavage always occurs within the cytoplasmic membrane. In some other N plasmid-bearing strains in which this protein is considerably reduced, the fracture plane exposes large areas of the EF, an effect similar to the one described by Bayer et al. [18] and Smit et al. [31] in LPS-deficient

mutants of *E. coli* and *S. typhimurium* respectively. However, the ES of all these strains is similar to that of LEB18. Unfortunately, a careful search for the presence of any appendages, protrusions or other morphological features on the etched or fracture surfaces of the plasmid-bearing strains examined has failed to reveal any characteristics which might suggest a possible role in interbacterial gene transfer. This negative observation is nevertheless significant in that it confirms the existence of sex pilus-independent mechanism(s) of N plasmid transfer among bacteria [see ref. 4].

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