ALTERATIONS IN THE OUTER MEMBRANE PROTEINS OF *ESCHERICHIA COLI* B/r ASSOCIATED WITH THE PRESENCE OF THE R PLASMID rRM98

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Received 28 September 1977

1. Introduction

The conjugative antibiotic resistance plasmid rRM98 belongs to the N incompatibility group of plasmids which specifies sensitivity to the filamentous bacteriophage IKe when present in *Escherichia coli* and some other gram-negative bacterial hosts [1,2]. Attempts to identify a rRM98-specified pilus in an *E. coli* K12 host harbouring this plasmid by electron microscopy have failed to reveal such an appendage on the cell surface [3]. We have therefore examined this isolate and a strain of *E. coli* B/r which has received the plasmid from it, for changes in integral membrane proteins in the hope that this would lead to the identification of any membrane proteins associated with plasmid transfer and/or the provision of IKe attachment sites. On the contrary, as described here, the presence of the plasmid is associated with major alterations to a host envelope protein. The evidence presented indicates that the presence of rRM98 or other group N plasmids in the B/r host is associated with a variable decrease in the level of a major outer membrane protein mol. wt 36 500 (36.5 K protein, designated as protein b [4] or Ia [5]) including its virtual elimination in several isolates. These changes do not prevent the expression of the plasmid functions of phage sensitivity or gene transfer [6]. The 36.5 K protein which has been reported to provide an important channel through the envelope for the uptake of several metabolites [7–9] is nevertheless not affected when the plasmid is present in *E. coli* K12 strains [10].

2. Materials and methods

2.1. *Strains*

Bacterial strains (table 1) were maintained on nutrient L agar supplemented as required with ampicillin (Ap) or tetracycline (Tc) at 30 μg/ml [3].

2.2. *Preparation of membranes*

Envelopes or membranes were obtained from logarithmic phase cultures following sonication of whole cells or spheroplasted cells, respectively. Membranes from spheroplasted cells were fractionated on discontinuous sucrose gradients [6,12]. Whereas 4 fractions of densities 1.14, 1.17 (inner membranes), 1.2 and 1.24 (outer membranes) are obtained from the LEB18 host, in replicate experiments, the most band in the case of LEB500 (rRM100) carrying a derivative of rRM98 (see table 1 for derivation of all strains) is either missing or considerably diminished and the 1.17 density fraction is inflated. This effect is similar to that reported in the case of lipopolysaccharide-deficient mutants of *Salmonella typhimurium* and *E. coli* [13–15] and in *E. coli* strains which specifically lack one or more major outer

Table 1
Escherichia coli strains

Strain No.	Relevant Markers		Derivation[b]	Selection	Source	
	Host	Plasmid			Strain	Plasmid
B/r strain LEB18	*str lacz*				P. Meacock	
LEB500 (rRM100)[a]	*str lacz*	Ap Tra$^-$ IKe^S	JE2571 (rRM98) X LEB18	Ap^r		R. Iyer
LEB501 (rRM98)	*str lacz*	Ap Sm Sp Tc Tra$^+$ IKe^S	JE2571 (rRM98) X LEB18	Ap^r		
LEB502 (rRM98)	*str lacz*	Ap Sm Sp Tc Tra$^+$ IKe^S	JE2571 (rRM98) X LEB18	Ap^r		
LEB503 (rRM98)	*str lacz*	Ap Sm Sp Tc Tra$^+$ IKe^S	JE2571 (rRM98) X LEB18	Ap^r		
LEB18 (rRM98)[c]	*str lacz*	Ap Sm Sp Tra$^+$ IKe^S	JE2571 (rRM98) X LEB18	Ap^r		
LEB505 (rR48)	*str lacz*	Pen Sm Tc Su Tra$^+$ IKe^S	J5 (rR48) X LEB18	Tc^r		N. Datta
LEB506 (rR269)	*str lacz*	Ap Km Sm Tc Tra$^+$ IKe^S	J5 (rR269) X LEB18	Tc^r		S. Dennison
LEB18 (r64.11)	*str lacz*	Su Sm Tc Tra$^+$	JE2571 (r64drd11) X LEB18	Tc^r		G. G. Meynell
LEB18 (pF'lac)	*str lacz*	Lac$^+$		–	P. Meacock	
K12 strain JE2571	*thr leu pil fla*			–	Y. Nishimura	
K12 strain J5	*proA met* λ^+			–	S. Dennison	
B/r strain LEB16	*thy str lacz*			–	P. Meacock	
LEB507 (rRM98)	*thy str lacz*	Ap Sm Sp Tc Tra$^+$ IKe^S	JE2571 (rRM98) X LEB16	Ap^r		

[a]Strains LEB500–503, LEB505–506, and LEB507 are plasmid transconjugants of LEB18 or LEB16 which lack or have reduced levels of the outer membrane 36.5 K protein

[b]Logarithmic phase cultures of the donor and recipient were mixed 1:4 on sterile millipore filters and the medium removed by application of vacuum. 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 L broth and plating for transconjugants on minimal salts agar containing the appropriate antibiotic

[c]This strain is Tc^S although it can transfer this marker to a suitable recipient

Abbreviations: Ap, Ampicillin; Pen, Penicillin; Sm, Streptomycin; Sp, Spectinomycin; Su, Sulfonamide; Tc, Tetracycline; tra, conjugal transfer; IKe^S, sensitivity to phage IKe; *str*, (chromosomal) Streptomycin resistance; *pil*, synthesis of common pili; *fla*, synthesis of flagella

Designation of plasmid bearing strains conforms to the recommendations [11]. In plasmid transconjugants, plasmid serial numbers change when plasmid characters appear to be affected whilst host serial numbers are changed when alterations to the host (e.g., the envelope) are involved

membrane proteins [16]. The identity of inner and outer membrane fractions in this study was confirmed by analysis of their polypeptide content on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis [17] and agrees well with previous studies [6,10,12,14].

3. Results

Gel analysis of total envelopes from *E. coli* B/r LEB18 and 5 independently isolated rRM98-bearing transconjugants obtained by ampicillin (Ap) selection, using an *E. coli* K12 plasmid donor, are compared in fig.1. The 36.5 K protein, a major constituent of the outer membrane fraction [13,14,16,20] and suggested to constitute hydrophilic channels through the outer membrane [4,8] is virtually absent in the envelopes of LEB500 (rRM100), LEB501 (rRM98) and LEB502 (rRM98); it is considerably diminished in LEB503 (rRM98) and present in wild type amounts in LEB18 (rRM98). In contrast, no changes to the 36.5 K protein have detected in *E. coli* K12 hosts bearing rRM98 [10]. In the case of LEB500 (rRM100) at least, no alterations in the inner membrane proteins were detected [6] and moreover we failed to detect significant levels of the 36.5 K protein in the cytoplasmic fraction of this strain. A slight enhancement of one or more proteins corresponding to an app. mol. wt 76 K is seen only in LEB500 (rRM100). This may reflect either a non-specific increase in a host envelope protein or an effect related to the loss of several plasmid-determined characters, including transfer functions, observed in this particular isolate (see table 1). In another transconjugant LEB501 (rRM98), a band corresponding to an apparent molecular weight of 37.5 K is evident. This protein is absent in the plasmidless host and most likely represents a modified form of the 36.5 K protein.

When total envelopes from *E. coli* B/r bearing the I and F plasmids R64drd11 and F'lac respectively are analysed, no detectable alterations in envelope proteins, including the 36.5 K protein (fig.1b slots iii and iv) are observed although two other N plasmids rR48 and rR269 also give rise to clones lacking the protein [6]. Thus, the loss of the protein associated with the presence of rRM98 in *E. coli* B/r may be specific to this and related plasmids.

The properties of strain LEB500 (rRM100) indicate that diffusible products from the Tc and conjugal transfer (Tra) regions of the plasmid cannot be

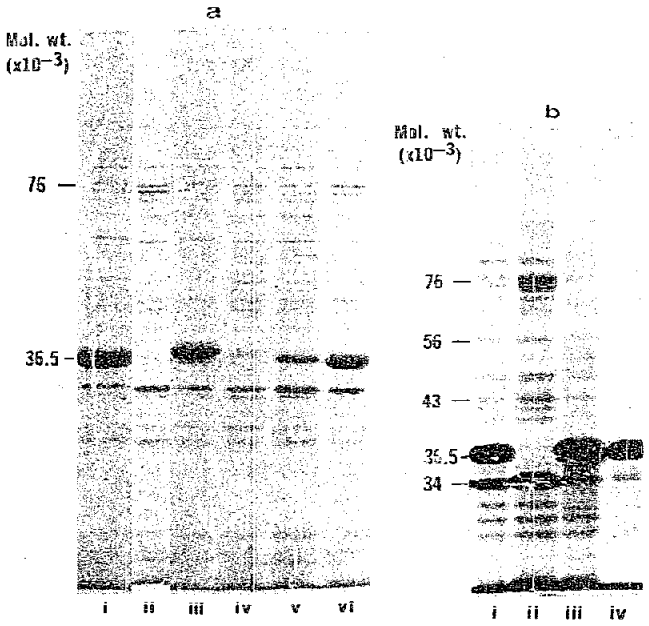

Fig. 1(a,b). SDS–PAGE analysis of total envelopes from *E. coli* B/r LEB18 and different transconjugants carrying the plasmid rRM98. Bacteria were grown as described [6]. Protein concentrations were estimated from A_{260} and A_{280} values [17]. The electrophoresis procedure, gel constituents and buffers were as described [18]. A Bio Rad slab-gel system (Model 220) was operated at 100 V for 4–5 h. Gel slabs were 1.5 mm thick; the stacking and separating gels were 1.6 cm and 9.5 cm high, respectively. Gels were stained with Coomassie brilliant blue and destained by a modification of the method [19]. Envelope samples containing 50 μg protein in about 20 μl sample buffer were boiled for 5 min before use. Protein standards (2 μg each) were similarly treated. The polyacrylamide concentration of the separating gel was 9% (w/v).
Left (a): (i) LEB18 (plasmid free wild type); (ii) LEB500 (rRM100), transconjugant of LEB18 lacking the 36.5 K protein and carrying a transfer defective rRM98; (iii–v) LEB510 (rRM98), LEB502 (rRM98), LEB503 (rRM98), plasmid bearing derivatives of LEB18 with various levels of the 36.5 K protein; (vi) LEB18 (rRM98), a transconjugant of LEB18 with no detectable change in the 36.5 K protein.
Right (b): (i) LEB18; (ii) LEB500 (rRM100); (iii) LEB18 (r64.11), a derivative of LEB18 carrying the R-factor r64; (iv) LEB18 (pF'*lac*) a derivative of LEB18 carrying the F-prime factor F'*lac*.

responsible for the observed envelope defects since these are apparently absent or not expressed in this transconjugant (see table 1). The absence of these genes is in fact confirmed when plasmid DNA isolated (as in [21]) from LEB500 (rRM100) is used to transform *E. coli* K12 strain JE2571 for Ap resistance (as in [22]). These transformants are Tc-sensitive, fail to transfer Ap by conjugation, and have normal levels of the 36.5 K outer membrane protein, demonstrating further the specific nature of the host—plasmid interaction which occurs in *E. coli* B/r. In addition, when a Tc-sensitive segregant of rRM98 is transferred from a K12 donor to LEB18, most of the transconjugants are still found to lack the 36.5 K protein; the evidence therefore rules out a role for the Tc region in affecting this protein.

If the elimination of the 36.5 K protein is directly due to a plasmid gene product, strains 'cured' of the plasmid would be expected to have wild type levels of this component. The isolation of strains cured of rRM98, rR48 or rR269 and the results of gel analysis of their total envelopes are described in table 2. The protein is fully or partially restored in some cured isolates of the strains bearing rR48 and rR269, respectively, but not in any of 4 strains cured of rRM98. These contradictory results are difficult to interpret but, in the latter case, they indicate some permanent change to the host's chromosome.

The effects on the 36.5 K protein in different plasmid transconjugants from the cross between K12 (rRM98) and *E. coli* B/r, although varied, appear to be quite stable. Therefore attempts were made to examine the inheritability of the varied protein changes observed in different rRM98 bearing transconjugants by secondary transfer of the plasmids to the phage T6-resistant derivative of LEB18. Transconjugants were selected from mating mixtures for inheritance of Ap resistance. Transconjugants were obtained at a frequency of about 1/1000 recipients. The results in fig.2 show that in 7 of 12 secondary transconjugants examined from the four crosses, the 36.5 K protein is reduced or eliminated whilst wild type levels are observed in the remainder. Strikingly, however, there is no particular correlation between donor and transconjugant with regard to the status of the 36.5 K protein (see fig.1a for donor envelope profiles). This experiment, which involves transfer between *E. coli* B/r strains, rules out any role for the host's DNA restrictive system in modification of the incoming plasmid which might have affected the initial crosses between *E. coli* K12 and B/r.

Table 2
Levels of 36.5 K membrane protein in plasmid-'cured' derivatives

Strain[a]	Curing procedure	'Cured' isolates tested	36.5 K protein
LEB500 (rRM100)	Maintenance on drugless medium	1	absent
LEB502 (rRM98)	Chloropromazine 10 μg/ml [23]	1	absent
LEB507 (rRM98)	5-Bromodeoxyuridine [24]	2	absent
LEB505 (rR48)[b]	Maintenance on drugless medium	1	fully restored
LEB506 (rR269)[b]	Maintenance on drugless medium	1	absent
LEB506 (rR269)[b]	Chloropromazine 10 μg/ml [23]	2	slightly increased

[a] All strains listed in this column lack the 36.5 K outer membrane protein [6]
[b] rR48 and rR269 are plasmids of the N incompatibility group from 2 different sources (see table 1)

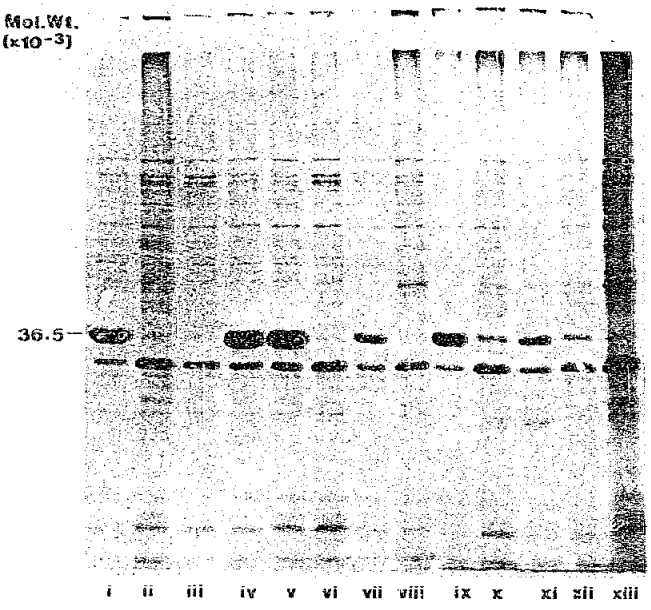

Fig.2. Analyses of total envelopes of LEB18 (phage T6-resistant) and secondary transconjugants from crosses with the plasmid-bearing LEB18 donors analysed in fig.1. (i) LEB18 [T6^r], plasmid-free host; (ii–vi) 5 transconjugant derivatives of LEB18 after crossing with LEB501 (rRM98), a strain in which the 36.5 K protein has a reduced mobility; (vii,viii) 2 LEB18 transconjugants after crossing with LEB502 (rRM98) which lacks the 36.5 K protein; (ix,x) 2 LEB18 transconjugants after a cross with LEB503 (rRM98) which has reduced levels of the 36.5 K protein; (xi–xiii) 3 transconjugants from a cross with LEB18 (rRM98) a donor strain with normal levels of 36.5 K protein.

4. Discussion

In order to explain the apparent effect of the rRM98 plasmid on the 36.5 K protein in *E. coli* B/r we have considered three alternative hypotheses:

1. That rare (mutant) recipient cells with altered levels of the 36.5 K protein are able to receive or to maintain the plasmid more efficiently than are wild type bacteria.

2. A diffusible product determined by the plasmid directly affects the synthesis and/or the assembly of the protein into the outer membrane.

3. That integration of the whole plasmid or the transposition of a specific region of the plasmid (i.e., a transposon) into the host chromosome might block the normal incorporation of the protein into the envelope.

The continued absence of the 36.5 K protein in at least some strains cured of the plasmid appears to rule out the action of a diffusible plasmid product. The direct demonstration of the presence of plasmid DNA in at least some strains lacking the protein appears to rule out integration of the plasmid into a chromosomal gene controlling the appearance of the protein in the envelope. The insertion of a specific small fragment of DNA or transposon [25] derived from the plasmid into various sites within a specific chromosomal gene could explain many of the observations. However, the results of the secondary transfer experiments described in fig.2 could then only be explained if it were further postulated that a copy of the transposon sequence was inserted, leaving the original sequence in the plasmid molecule. The most economical hypothesis is that rare variants within the recipient population are best able to receive and to replicate the plasmid and are therefore either selected initially or eventually outgrow any clones with normal levels of the 36.5 K protein. In support of this possibility we find (unpublished data) that when a B/r strain, which lacks the 36.5 K protein (after infection with followed by curing of the rRM98 plasmid), is used in crosses with an rRM98 donor, the number of rRM98 transconjugants obtained increases 20–50-fold compared to a wild type recipient which has normal levels of the 36.5 K protein.

In order to distinguish further between these three hypotheses, the genetic basis of the loss of this outer membrane protein is being studied in strains of *E. coli* B/r cured of the plasmid.

Acknowledgement

This work was supported in part by an operating grant (MA2839) from the Medical Research Council of Canada to R.I. The assistance of Winnie Leong during part of this work is appreciatively acknowledged.

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