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meoA is the Structural Gene for Outer Membrane Protein c of *Escherichia coli* K12

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Summary. The isolation and characterization of two mutants of *Escherichia coli* K12 with an altered outer membrane protein c is described. The first mutant, strain CE1151, was isolated as a bacteriophage Mel resistant strain which contains normal levels of protein c. Mutant cells adsorbed the phage with a strongly decreased rate. Complexes of purified non-heat modified wild type protein c and wild type lipopolysaccharide inactivated phage Mel, indicating that these components are required for receptor activity for phage Mel. When wild type protein c was replaced by protein c of strain CE1151, the receptor-complex was far less active, showing that protein c of strain CE1151 is altered. The second mutant produces a protein c with a decreased electrophoretic mobility, designated as protein c*. An altered apparent molecular weight was also observed for one or more fragments obtained after fragmentation of the mutant protein with cyanogen bromide, trypsin and chymotrypsin. Alteration of protein c was not accompanied by a detectable alteration in protein b or its fragments. Both mutations are located at minute 48 of the *Escherichia coli* K12 linkage map. The results strongly suggest that *meoA* is the structural gene for protein c.

Introduction

Outer membrane proteins b and c of *Escherichia coli* K12 are firmly but non-covalently associated with peptidoglycan (Rosenbusch, 1974; W. van Alphen et al., 1976; Hasegawa et al., 1976; Schmitges and Henning, 1976). They are involved in the formation of hydrophilic pores, through which nutrients and other solutes with a molecular weight of up to about 700 daltons can pass the outer membrane (Nakae, 1976; Beacham et al., 1977; Lutkenhaus, 1977; W. van Alphen et al., 1978a, b; Bavoil et al., 1978; Yagil et al., 1978).

At least three genes are involved in the appearance of proteins b and c in the outer membrane namely *tolF* (Foulds, 1976; Chai and Foulds, 1977), *ompB* (Sarma and Reeves, 1977) and *meoA* (Verhoef et al., 1977; Verhoef et al., 1979). The latter gene is probably identical to *par* (Bassford et al., 1977). Although the hypothesis that *ompB* is the structural gene for both proteins has been favoured (Bassford et al., 1977; Henning et al., 1977), more recent studies have shown that *ompB* cannot be the structural gene for protein b (Verhoef et al., 1979) and that the two proteins must be coded for by two structural genes (Ichihara and Mizushima, 1978; Verhoef et al., 1979). Based on these data and on the phenotypes of the mutants, the hypothesis has been proposed that *tolF* and *meoA* are the structural genes for the proteins b and c respectively whereas *ompB* is a regulatory gene (Ichihara and Mizushima, 1978; Verhoef et al., 1979).

In the present paper we describe the isolation and characterization of two mutants with an altered protein c in an attempt to identify the structural gene for this protein. The first mutant contains a protein which, unlike the protein c of its parent strain, is unable to function as the protein part of the receptor of phage Mel. The second mutant contains a protein c with an altered electrophoretic mobility. Both mutations are localized at minute 48 of the *E. coli* linkage map. The results show that *meoA* is indeed the structural gene for protein c and they therefore support the hypothesis of Ichihara et al. (1978) and Verhoef et al. (1979).

Materials and Methods

Strains and Growth Conditions. Only *E. coli* K12 derivatives were used. Their relevant properties are listed in Table 1. Spontaneous mutants lacking proteins b, c and d were isolated as described (Verhoef et al., 1979). The composition of the media has been

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Table 1. *E. coli* K12 strains and relevant characteristics[a]

Strain	Relevant characteristics	
PC0479	Multiauxotrophic	A. Rörsch; Verhoef et al. (1979)
CE1062	Spontaneous phage Mel resistant *meoA* (b^+c^-) derivative of strain PC0479	Verhoef et al. (1977)
CE1107	Spontaneous phage Mel resistant *ompB* (b^-c^-) derivative of strain PC0479	Verhoef et al. (1979)
CE1122	Spontaneous phage Tula resistant *ompB* (b^-c^+) derivative of strain PC0479	Verhoef et al. (1979)
CE1151	Spontaneous phage Mel resistant protein c proficient derivative of strain CE1122	This paper
KN126	*trpE* 9829 (am), *tyr* (am), *ilvA*, *supD*126ts	P. Andreoli; Nagata and Horiuchi (1973)
CE1098	Rifampicin resistant derivative of strain KN126	This paper
CE1081	EMS induced derivative of strain CE1098 selected as resistant to phage K3 after growth at 42° C. The strain is K3 sensitive after growth at 30° C and contains protein c*	This paper
CE1082	Spontaneous nalidixic acid resistant derivative of CE1081	This paper
CE1083	Protein c^{*+} derivative of strain CE1062 obtained by transduction with a Pl lysate of strain CE1082	This paper
PC0205	Multiauxotrophic strain used as a source for *E. coli* K12 wild type LPS	Lugtenberg et al. (1976)

[a] Genotype descriptions follow the recommendations of Bachmann et al. (1976)

described (Lugtenberg et al., 1976). If indicated, 0.3 M NaCl was added to the growth medium in order to obtain cells lacking protein b and enriched in protein c (Nakamura and Mizushima, 1976; Lugtenberg et al., 1977; W. van Alphen and Lugtenberg, 1977).

Genetic Techniques. Mutagenization with ethyl methane sulfonate (EMS) (Miller, 1972) and transduction with lysates of phage P1 *vir* (Lennox, 1955) was carried out as described.

Isolation of Membrane Fractions and Purification of Individual Membrane Components. Procedures for the isolation of cell envelopes, protein-peptidoglycan complexes, biologically inactive proteins b and c, phospholipid and lipopolysaccharide (LPS) were carried out as described previously (Verhoef et al., 1979). For the isolation of biologically active protein c, the protein was removed from protein-peptidoglycan complexes using the procedure described by Nakamura and Mizushima (1976) with some modifications. Protein-peptidoglycan complexes were extracted for one h at 25° C in a buffer containing 10 mM Tris (hydroxymethyl)-aminomethane – 0.7 M 2-mercaptoethanol – 2% SDS – 0.5 M NaCl. The final pH was 7.3. After centrifugation at 23° C for 30 minutes at 225.000 × g the supernatant was dialyzed for 24 h at 22° C against 2 mM $NaHCO_3$ – 0.1% SDS, pH 7.5. The protein was precipitated from the solution with 90% acetone (Hindennach and Henning, 1975) for 1 h at 4° C, washed three times with 90% acetone and twice with distilled water, and lyophilized. All purifications were carried out with SDS cat. no. 30176 of BDH, Poole, U.K.

Reconstitution of the Receptor of Phage Mel. Complexes of biologically active protein c und LPS (of strain PC0205), sometimes supplemented with phospholipids (of strain PC0479), were prepared by cosonication of 0.5 mg protein c, 8 mg LPS and 5 mg phospholipids per ml, followed by annealing (Rothfield and Horne, 1967) as described previously for the preparation of protein d-LPS-phospholipid complexes (L. van Alphen et al., 1977a) except that NaCl was omitted.

Rates of Adsorption of Phage Mel to Whole Cells and of Inactivation of Mel by Reconstituted Receptor. The rate of adsorption to whole cells was determined as follows. Cells growing exponentially in yeast broth were harvested and resuspended in yeast broth supplemented with chloramphenicol (100 µg/ml) and KCN (2.5 mM). The final cell concentrations were 5×10^7 per ml and 4×10^9 per ml for sensitive and resistant cells respectively. After prewarming at 37° C, 2×10^7 plaque forming units of phage Mel were added per ml suspension. Samples were taken at various time intervals during a period of 30 min, diluted if necessary, and filtered immediately through a membrane filter (0.45 µm pore size; Millipore Corp., Bedford, Mass. USA). The filtrate was diluted 10.000 times and the number of plaque forming units was determined. Adsorption was irreversible as the same results were obtained when the suspension was diluted prior to filtration. The rate of phage inactivation by reconstituted receptor complexes was determined as described for inactivation by whole cells except that whole cells were replaced by receptor complexes (final concentration 20–100 µg protein c per ml incubation mixture) and that the filtration step was omitted. Both the rate of adsorption to whole cells and the rate of inactivation by receptor complexes followed pseudo first-order kinetics. The adsorption constant k_a was calculated according to the formula $\log P_t/P_o = k_a \cdot N \cdot t$ in which P_t and P_o represent the number of plaque forming units at times t and o respectively, and N is the number of bacteria per ml. For phage inactivation by reconstituted receptor complexes the inactivation constant k_i was calculated with the same formula except that N then represents the final concentration of protein c in µg/ml.

Analytical Methods. Protein was determined according to Lowry et al. (1951) except that the amounts of purified proteins were determined by scanning of stained gel electropherograms (Lugtenberg et al., 1975) with highly purified proteins (Lugtenberg et al., 1977; L. van Alphen et al., 1977a) as references. 2-keto-3-deoxyoctonate (KDO) was determined by the thiobarbituric acid method (Osborn, 1963). The measured values for KDO were corrected for the fact that only two of the three KDO residues are measured (Dröge et al., 1970). The amount of LPS was calculated using the value of 11 weight percent of KDO in LPS isolated from wild type *E. coli* K12 (L. van Alphen et al., 1977b). The amount of phospholipid was measured by determination of total phosphate (Ames and Dubin, 1960) after lipid extraction. Methods for the determination of the amino acid composition, the identification of N-terminal amino acids and cleavage of purified proteins with cyanogen bromide and proteolytic enzymes have been described (Verhoef et al., 1979). The previously described SDS-polyacrylamide gel electrophoresis system (Lugtenberg et al., 1975) with 11% polyacrylamide was used for analysis of membrane fractions and for testing fractions obtained during the purification of proteins. Gradient gels (10–13.5%) (Verhoef et al., 1979) were used for the

analysis of CNBr fragments. Both gel systems were used for the analysis of proteolytic fragments. ^{32}P-labelled LPS of parent and mutant strains was isolated and analyzed as described by Boman and Monner (1975).

Results

Rationale for the Use of a Phage Mel Resistant Protein c Proficient Mutant for the Localization of the Structural Gene for Protein c

As most phage Mel resistant mutants lack protein c, we have suggested that protein c is (part of) the receptor of phage Mel (Verhoef et al., 1977). Phage Mel resistant protein c proficient mutants can also be found (Verhoef et al., 1977). Among such strains mutants with a missense mutation in the structural gene for protein c or with a protein c with an altered electrophoretic mobility can be expected. If it can be demonstrated that wild type protein c is indeed (the protein part of) the receptor of phage Mel, it can be predicted that protein c of such mutants is inactive as the (protein part of the) receptor. Subsequent localization of the gene which determines resistance to phage Mel then indicates the map position of the structural gene for protein c.

Complexes of Protein c and LPS are the Receptor of Phage Mel

Purified protein c, obtained after treatment of protein-peptidoglycan complexes with SDS at 98° C, was inactive in inactivating phage Mel. Activity could not be restored by cosonication with LPS (Table 2). As cell envelopes and protein-peptidoglycan complexes were active, we removed protein c from the peptidoglycan by a method which yields a protein that is able to reassociate with peptidoglycan (Yu and Mizushima, 1977), namely treatment of protein-peptidoglycan complexes at a relatively low temperature with 0.5 M NaCl in the presence of SDS (Nakamura and Mizushima, 1976). Table 2 shows that the protein thus obtained was active in inactivating phage Mel irreversibly, provided that it had been cosonicated with LPS in the absence or presence of phospholipids. Protein c, LPS or phospholipids alone were inactive. Triton X-100 (Datta et al., 1977) or phospholipids were unable to replace LPS. The addition of these components to protein c and LPS even seems to decrease the receptor activity. Boiling of protein c-LPS complexes followed by sonication and annealing did not result in loss of receptor activity. Inactivation of Mel by protein-LPS complexes was specific in that proteins b and d were unable to replace protein c (Table 2). Storage of a suspension of protein c in 1 mM HEPES-10 mM $MgCl_2$, pH 7.4, for two weeks at 4° C or at −20° C resulted in a loss of 80 percent of the activity. Protein c-LPS complexes, stored at 4° C for the same period, were completely stable. The results show that the addition of sufficient LPS to protein c protects the protein from conversion to an inactive form. It should be noted that the protein fraction released from protein-peptidoglycan complexes still contains 3–8% of the cellular LPS, which apparently is not sufficient for phage Mel receptor activity (Table 2), a result consistent with observations reported by Datta et al. (1977) who showed that the addition of extra LPS is required for receptor activity of proteins b and c for phages TuIa and TuIb respectively.

After SDS-polyacrylamide gel electrophoresis it was observed that, under all conditions tested, inactive protein c, incubated in sample buffer at 37° C, corresponded with a protein band on 11% SDS-polyacrylamide gels with an apparent molecular weight of about 37,000 daltons, its monomeric form (Nakamura and Mizushima, 1976). Protein c, active in the receptor assay and tested under the same conditions, resulted in a double protein band (apparent molecular weight 72,000 daltons) whereas substantial amounts of the protein did not enter the running gel

Table 2. Inactivation of bacteriophage Mel by reconstituted receptor[a]

Inactivator	Rate constant k_i ($ml \times min^{-1} \times \mu g$ purified $protein^{-1}$)
None	$\leqq 0.1 \times 10^{-4}$
c (inactivated by SDS at 98° C)	$\leqq 0.1 \times 10^{-4}$
c (inactivated by SDS at 98° C) +LPS	$\leqq 0.1 \times 10^{-4}$
c	$\leqq 0.1 \times 10^{-4}$
c+LPS	24×10^{-4}
c+LPS+phospholipids	12×10^{-4}
LPS[b]	$\leqq 0.1 \times 10^{-4}$
Phospholipids[b]	$\leqq 0.1 \times 10^{-4}$
c (in 0.05% Triton X-100)	$\leqq 0.1 \times 10^{-4}$
c+phospholipids	$\leqq 0.1 \times 10^{-4}$
c+LPS (in 0.05% Triton X-100)	10×10^{-4}
c+LPS (after 5′ 100° C, followed by sonication and annealing)	26×10^{-4}
b+LPS	$\leqq 0.1 \times 10^{-4}$
d+LPS	$\leqq 0.1 \times 10^{-4}$

[a] Experiments were performed with protein c of strain CE1122, released from protein-peptidoglycan complexes with 0.5 M NaCl unless otherwise indicated. If combinations of protein, LPS and/or phospholipids were used, membrane-like structures were prepared by sonication and annealing. The numbers of plaque forming units of phage Mel were determined with cells of strain CE1061 (Verhoef et al., 1971) as the indicator strain

[b] An apparent k_i was calculated using a value for N as if protein c was present

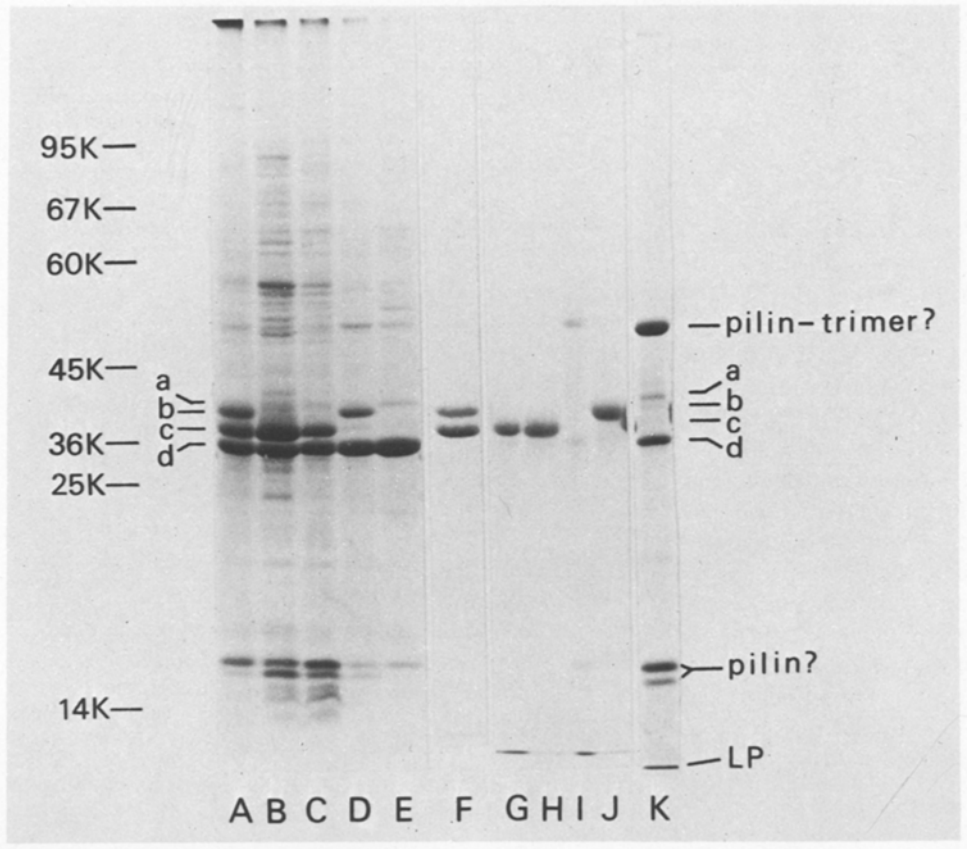

Fig. 1 A–K. SDS-polyacrylamide gel electrophoresis patterns of cell envelopes (**A–E**) and peptidoglycan-associated proteins (**F–K**) of strains PC0479 (b^+c^+) (**A, F**), CE1122 (b^-c^+) (**B, G**), CE1151 (b^-c^+) (**C, H**), CE1062 (b^+c^-) (**D, J**) and CE1107 (b^-c^-) (**E, I, K**). Lane **K** contains the same sample as lane **I**, but was overloaded in order to detect protein c (which is barely visible on this photograph). The positions of molecular weight standards are indicated on the left. *LP*, lipoprotein. 11% gel

(apparent molecular weight higher than 200,000 daltons) or even the stacking gel. Thus it seems that active protein c has a higher apparent molecular weight under not fully denaturing conditions, possibly corresponding with multimers.

Inactivation of Phage Mel by Whole Cells of Wild Type and Mutant Strains and by Isolated Proteins Complexed with LPS

One phage Mel resistant protein c proficient mutant, strain CE1151, was found among ten strains resistant to Mel (10^4 phages per plate). The electrophoretic mobility of protein c of strain CE1151 did not differ from that of wild type protein c (Figs. 1G and 1H). The rate of phage Mel adsorption to cells of wild type and mutant strains (see Fig. 1 for cell envelope protein profiles) was tested. The results (Table 3) show that the phage does not adsorb to protein c deficient (c^-) mutants. The protein c proficient strains PC0479 (wild type) and CE1122 (b^-c^+) adsorbed the phage with about the same rate. The Mel resistant c^+ strain CE1151 was the only c^+ strain that did not adsorb the phage (Table 3). Peptidoglycan-associated proteins of these strains were isolated after treatment of their protein-peptidoglycan complexes with 0.5 M NaCl. Gel electropherograms (Fig. 1) showed that, whereas the method yielded practically pure proteins b plus c for the parent strain PC0479, the proteins b or c from the mutants were contaminated with small amounts of the free form of the lipoprotein, protein d, a 50,000 dalton protein related to type 1 pili (Lugtenberg et al., 1976) and a double band in the position of protein III. McMichael and Ou (1977) recently suggested that the latter two bands both represent type 1 pilin and that the 50,000 dalton protein is a trimer of pilin formed artificially during the preparation of the sample for gel electrophoresis. All protein fractions, which contained 3–6% (w/w) LPS, were tested for their ability to inactivate phage Mel after cosonication of the protein with LPS and subsequent annealing. Table 3 shows that complexes of LPS and protein c of strain CE1151 were about

Table 3. Adsorption of bacteriophage Mel to whole cells and inactivation of bacteriophage Mel by protein-LPS complexes[a]

Strain	Mutation influencing the levels of protein(s) b and/or c	Phenotype	Whole cells Rate constant k_a (ml min^{-1} $bacterium^{-1}$)	Protein-LPS complexes[b]	
				Proteins present	Rate constant k_i $ml \times min^{-1} \times \mu g$ protein c^{-1})
PC0479	None	b^+c^+	8×10^{-10}		Not determined
CE1122	*ompB*	b^-c^+	13×10^{-10}	c	24×10^{-4}
CE1151	*ompB, meoA*	b^-c^+	5×10^{-12}	c^R	0.27×10^{-4}
CE1062	*meoA*	b^+c^-	$\leqq 1 \times 10^{-13}$	b	$\leqq 0.1 \times 10^{-4}$[c]
CE1107	*ompB*	b^-c^-	$\leqq 1 \times 10^{-13}$	c[d]	$(10\text{–}50) \times 10^{-4}$

[a] Gel electropherograms of the corresponding cell envelope proteins and purified proteins are shown in Fig. 1

[b] Experiments were performed with protein released from protein-peptidoglycan complexes with 0.5 M NaCl. Complexes of protein and LPS were prepared by cosonication, followed by annealing

[c] Calculated per μg protein b

[d] Only in this case the indicated protein constituted a minor component: 0.35–1.4 percent of the total protein (see Figs. 1I and 1K) present in this fraction. Consequently a range is given for the k_i value

ninety-fold less active than those of LPS and wild type protein c of strain CE1122. The decreased ability of cells of strain CE1151 to adsorb phage Mel is not due to an altered LPS as the chromatographic mobility of ^{32}P-labeled LPS of strains CE1151 and CE1122 was indistinguishable. Moreover, wild type LPS was used in all in vitro experiments. Also none of the minor protein contaminants observed in the preparations of some isolated proteins (Fig. 1G, H and J) can be the protein part of the phage receptor as these are present in some preparations which are inactive (Figs. 1H and 1J). Therefore the inability of cells of strain CE1151 to adsorb phage Mel must be due an altered protein c, which we designate as c^R. Preparations of protein c^R, incubated at 37° C prior to gel electrophoresis, resulted in the same band pattern as described for wild type protein c, suggesting that the lack of receptor activity is not due to its capacity to form multimers. The results strongly suggest that the alteration of protein c is due to a mutation in the structural gene. As we assume this gene to correspond with *meoA* (Verhoef et al., 1979), P1 lysates of strain CE1151 and its *nalA* derivative were transduced with $glpT^-$ and $nalA^+$ acceptor strains respectively. $glpT^+$ and *nalA* transductants respectively were selected, purified and tested for sensitivity towards phage Mel. As expected, Mel resistance was cotransducible with both *glpT* and *nalA*.

According to our model on the regulation of the synthesis of proteins b and c (Verhoef et al., 1979), *ompB* is a regulatory gene. The model predicts that, if a phage Mel resistant *ompB* mutant produces protein c, the protein has wild type activity. A trace amount of protein c was detected in the proteins isolated from the protein-peptidoglycan complex of the phage Mel resistant *ompB* mutant CE1107 (Figs. 1I and 1K). Although accurate determination of the amount of this protein was difficult (see legend d of Table 3) we were able to show that this protein is approximately equally active as wild type protein c produced by strain CE1122 and far more active than protein c^R of strain CE1151 (Table 3). This result is consistent with our model.

Isolation and Partial Characterization of a Mutant Possessing an Electrophoretically Altered Protein c

Among several Mel resistant protein c proficient mutants isolated no mutants with an electrophoretically altered protein c were detected. However, a mutant with an electrophoretically altered protein c was obtained accidentally during the isolation of a temperature dependent bacteriophage K3 resistant mutant from strain CE1098, which contains a temperature sensitive amber suppressor (Table 1). After mutagenesis of the latter strain with EMS, strain CE1081 was one of several strains which had the required temperature dependent sensitivity for phage K3. Unexpectedly, inspectation of the cell envelope protein pattern of this strain showed that protein c was altered or absent (Fig. 2). After growth at 30° C cells of the mutant strain differ from those of the parent strain in that they lack the two proteins c and d but contain two additional proteins with slightly lower electrophoretic mobilities than the lacking proteins (Figs. 2A and 2B). The fastest moving protein was neither detected after growth of the mutant strain at 42° C (Fig. 2D) nor in phage K3 resistant derivatives of strain CE1081 after growth at 30° C, showing that it represents an altered protein d. The other new

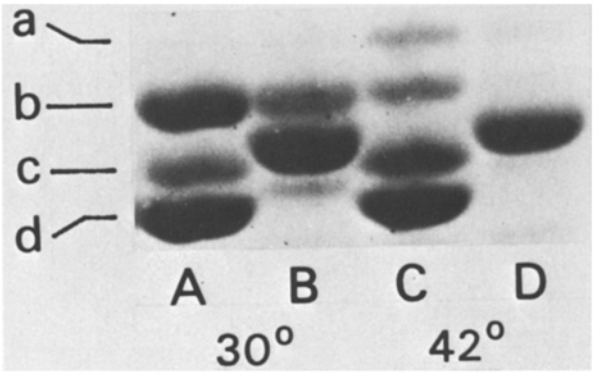

Fig. 2A–D. Polyacrylamide gel electrophoresis of cell envelopes of strains CE1098 (parent) (**A, C**) and mutant CE1081 (**B, D**) isolated from cells grown in yeast broth at 30° C (**A, B**) and 42° C (**C, D**) respectively. 11% gel. Only the relevant part of the gel is shown. Strain CE1081 contains a changed protein c whereas its d protein is both changed as well as temperature sensitive in its synthesis. The amounts of proteins a, b and c are influenced by the growth temperature as described earlier (Lugtenberg et al., 1976)

protein in strain CE1081 was the only major protein in cells grown at 42° C (Fig. 2D). As it is peptidoglycan-associated, absent in four out of five phage Mel resistant derivatives of strain CE1081, and as the b^- derivative of strain CE1081 contains active pores for cephaloridine, a property caused by proteins b and c (W. van Alphen et al., 1978b), we concluded that this protein represents an altered form of protein c, which was designated as protein c*. The fact that protein c* is present in high amounts in cells of strain CE1081 grown at 42° C (Fig. 2D) strongly suggests that the alteration is unrelated to the temperature sensitive amber suppressor which is present in this strain. This idea was confirmed by showing that, after transduction, the mutation causing protein c* is also expressed in strains lacking this suppressor. As it seemed likely that protein c* is caused by a mutation in the *meoA* gene, P1 lysates of strain CE1081 and its *nalA* derivative were transduced with *glpT, meoA* and *meoA* acceptor strains respectively. After selection for $glpT^+$ and *nalA* transductants respectively, it was shown that Mel sensitivity was cotransducible with both *glpT* and *nalA*. The results indicate that the mutation causing protein c* is located in (or close by) the *meoA* gene.

*Comparison of Purified Proteins c and c**

Proteins c and c* were isolated from strains with a CE1098 background (b^-d^- derivatives of the pair CE1098 and CE1081) and also from strains with a PC0479 background (b^-d^- derivatives of the pair CE1122 and CE1083). As the genetic background had

Table 4. Amino acid composition of proteins c and c* (mole%)[a]

Amino acid	Protein c (strain CE1098)	Protein c* (strain CE1081)
Lys	5.19	5.17
His	0.60	0.83
Arg	5.68	5.18
Half Cys	0.15	0.16
Asp	13.29	12.94
Thr	6.47	6.64
Ser	5.33	5.17
Glu	8.92	8.95
Pro	0.66	0.57
Gly	11.68	12.13
Ala	7.36	7.43
Val	6.09	6.27
Met	1.11	0.98
Ile	3.37	3.43
Leu	7.66	7.63
Tyr	9.67	9.93
Phe	6.77	6.59

[a] Proteins c and c* were isolated from phage K3 resistant, protein d deficient derivatives of strains CE1098 and CE1081 respectively after growth of the cells at 37° C in Brain Heart medium, supplemented with 0.3 M NaCl in order to minimize the production of protein b. Neither the latter protein nor other protein contaminants were detected in the final preparation. The data are averages of at least two determinations after hydrolysis for 24 h. Tryptophan was not determined. The values for serine and threonine have been corrected for losses of 10 and 5% respectively during hydrolysis

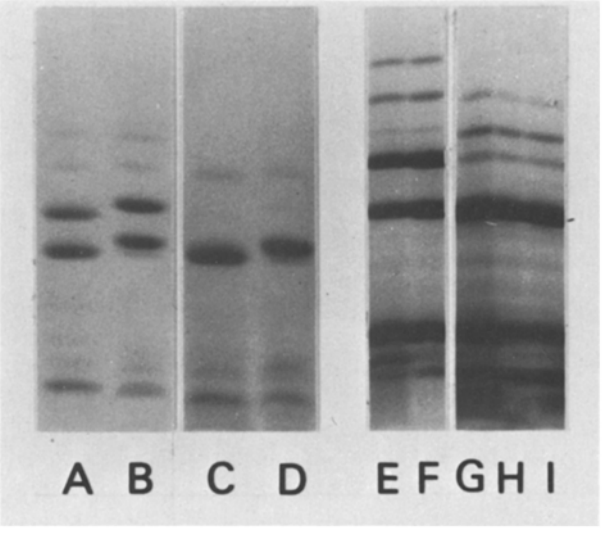

Fig. 3A–I. Cyanogen bromide fragments of protein c of a d^- derivative of strain CE1083 (**B** and **D**), protein b of a d^- derivative of strain CE1062 (**E** and **G**) and protein b of a c^{*-} d^- derivative of strain CE1083 (**F, H** and **I**). Cleavage was performed in formic acid (**A, B, E** and **F**) or in trifluoroacetic acid (**C, D, G, H** and **I**). Gradient gel

no detectable influence on the differences observed between the two proteins, the description of background details will be limited to the legends of the figures. Proteins c and c*, isolated from protein-peptidoglycan complexes by treatment at 98° C in SDS and further purified by column chromatography, were free of other detectable protein contaminants, as judged after SDS-polyacrylamide gel electrophoresis, and contained less than 0.1 percent LPS (w/w). Phospholipid was not detected. The amino acid composition of protein c* was very similar to that of protein c (Table 4), suggesting that no gross alterations are present in its primary structure. The amino terminus of both proteins is H_2N-Ala-Glu. Protein c* is not the unprocessed precursor of protein c as (i) the amino terminus of the precursor of protein I is methionine, and (ii) this precursor is, in contrast to protein c*, not peptidoglycan-associated (Sekizawa et al., 1977).

Cleavage of proteins c and c* with CNBr was less complete in formic acid (Figs. 3A and 3B) than in trifluoroacetic acid (Figs. 3C and 3D) as reported earlier for proteins b and c (Schmitges and Henning, 1976; Verhoef et al., 1979). After complete cleavage in trifluoroacetic acid the largest fragment of protein c* had a lower electrophoretic mobility than that of protein c (Figs. 3C and 3D). Partial cleavage of the two proteins with trypsin (Figs. 4A and 4B) and chymotrypsin (Figs. 4E and 4F) showed that most of the fragments were indistinguishable for proteins c and c* whereas a few fragments of protein c* had a slightly higher apparent molecular weight than the presumably corresponding fragments of protein c. These results show that protein c*, like protein c^R, is a mutant form of protein c. The mutation is probably localized in the structural gene for protein c although localization in a hypothetical modification gene cannot be excluded (but is unlikely, see discussion). Whatever the nature of the mutated gene is, the mutant with protein c* provides an excellent tool to see whether the product of the altered gene also plays a role in the synthesis or modification of protein b. If so, one would expect that protein b, isolated from a strain with the information for protein c*, differs from wild type protein b. If not, the two b proteins should be identical. Proteins b were isolated from c^-d^- derivatives of strains with a wild type gene for protein c and with a gene that codes for protein c*. Fragments obtained from the two proteins b with CNBr (Figs. 3E–3I), trypsin (Figs.4C and 4D) and chymotrypsin (Figs. 4G and 4H) were indistinguishable. These results show that the gene that is altered in the mutant with the changed protein c* does not play a role in the synthesis or modification of protein b.

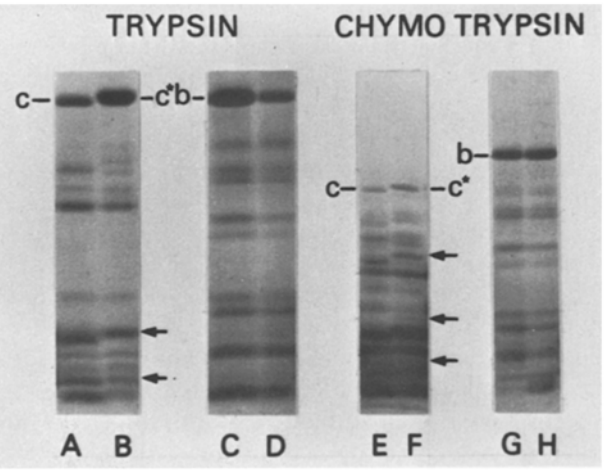

Fig. 4A–H. Tryptic (**A–D**) and chymotryptic (**E–H**) fragments of protein c of a d^- derivative of strain CE1122 (**A** and **E**) compared with those of protein c* of a b^-d^- derivative of strain CE1083 (**B** and **F**). Arrows indicate positions of fragments of protein c* that have not been observed in preparations of fragments of protein c. This figure also shows proteolytic fragments of protein b of a d^- derivative of strain CE1062 (**C** and **G**) and of protein b of a $c^{*-}d^-$ derivative of strain CE1083 (**D** and **H**)

Discussion

Recent investigations on the genetics (Verhoef et al., 1979) and biochemistry (Ichihara and Mizushima, 1978; Verhoef et al., 1979) of proteins b and c have lead to the hypothesis that *tolF* and *meoA* are the structural genes for proteins b and c respectively, whereas a regulatory function of the *ompB* gene product on the levels of both proteins was proposed. In this paper we describe a first attempt to test this hypothesis by the isolation and characterization of mutants with an altered protein c. The phage Me1 resistant protein c proficient strain CE1151 contains a protein, c^R, which is practically inactive both in vivo and in vitro as the receptor protein of phage Me1 (Table 3). The electrophoretic mobility of protein c^R is not significantly altered (Fig. 1). Strain CE1081 contains a c protein with an altered electrophoretic mobility (Fig. 2), designated as protein c*. No significant differences were observed between proteins c and c* with respect to their overall amino acid composition (Table 4), amino terminus, rate of in vivo binding or in vitro inactivation of phage Me1 (not shown) and activity of pores for cephaloridine. Comparison of fragments of proteins c and c* obtained with CNBr (Fig. 3) and proteolytic enzymes (Fig. 4) show that the proteins are very similar but significantly different. Genetic localization of the mutations which lead to the altered proteins c^R and c* showed that the changed genes are, like *meoA*, co-

transducible with *nalA* and *glpT* at minute 48. Although a more precise localization was not possible (see Verhoef et al., 1979) the most likely explanation is that both mutations are located in the *meoA* gene and that *meoA* is the structural gene for protein c. Schmitges and Henning (1976) mentioned the possibility that protein I is subject to posttranslational modification. It is possible that our mutants are not altered in the structural gene for protein c but are altered in such a (hypothetical) posttranslational modification process. However, as the properties of the two proteins c^R and c* differ strongly, this idea would implicate that the mutations are located in two different modification genes, both located at minute 48. As long as proof for posttranslational modification is lacking, the best explanation for the three phenotypes c^-, c^R and c* is that they are all caused by a mutation in the structural gene for protein c, the *meoA* gene.

Attempts to indicate the structural gene for protein b, for which *tolF* is the most likely candidate, by a similar approach have failed so far as no TuIa resistant protein b proficient mutant was obtained.

Our data support the hypothesis on the synthesis and regulation of proteins b and c in two more respects. (i). The finding that the trace amount of protein c isolated from the phage Me1 resistant *ompB* mutant CE1107 is as active as wild type protein c (Table 3) is consistent with the model. (ii). The observation that protein b of a strain containing a mutation leading to protein c^R is apparently wild type shows that the altered (probably structural) gene is not involved in the biosynthesis or modification of protein b.

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