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Genetics and Biochemistry of the Peptidoglycan-Associated Proteins b and c of *Escherichia coli* K12

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Summary. To collect information on synthesis and regulation of the peptidoglycan-associated pore-forming outer membrane proteins b and c, mutants resistant to phages Mel and Tula were analyzed. Genetic analysis showed three linkage groups, corresponding with the genes *tolF* (phenotype b^-c^+), *meoA* (phenotype b^+c^-) and *ompB* (phenotypes b^-c^-, b^-c^+, $b^{+\,+}c^-$ and $b^{+\,+}c^{\pm}$). It has recently been described that also a b^+c^- phenotype can occur in the latter linkage group [Chai, T., Foulds, J., J. Bacteriol. 130, 781–786 (1977)]. Among *ompB* (b^-c^+)/*meoA* (b^+c^-) double mutants strains were found with the b^+c^- phenotype, showing that *ompB* is not the structural gene for protein b. Studies on purified proteins b and c showed profound differences between the two proteins with respect to the electrophoretic mobility of fragments obtained by treatment with cyanogen bromide, trypsin and chymotrypsin. The amino acid in position three of the amino-termini of proteins b and c, isolated from isogenic strains, were identified as isoleucine and valine respectively. Both the genetic and biochemical results are consistent with a model recently published [Ichihara, S., Mizushima, S., J. Biochem. (Japan) 83, 1095–1100 (1978)] which predicts that *tolF* and *meoA* are the structural genes for the proteins b and c respectively and that *ompB* is a regulatory gene whose product regulates the levels of both proteins.

Introduction

The outer membrane of *Escherichia coli* contains relatively few protein species (Schnaitman, 1970), some of which occur in an extremely high number of copies per cell namely Braun's lipoprotein (Braun, 1975; Hindennach and Henning, 1975; Inouye et al., 1972), protein III (Henning et al., 1973) and several proteins with apparent molecular weights between about 33,000 and 44,000 daltons (Schnaitman, 1970; Henning et al., 1973; Rosenbusch, 1974; Lugtenberg et al., 1975; Uemura and Mizushima, 1975; Bassford et al., 1977). The nomenclature of Lugtenberg et al. (1975) will be used in this paper. Proteins b and c differ from proteins a and d in that they remain firmly but non-covalently attached to the peptidoglycan in the presence of sodium dodecyl sulphate (SDS) at 60° C (Rosenbusch, 1974; Lugtenberg et al., 1976; Schmitges and Henning, 1976).

Mutants lacking one or both of the proteins b and c have been isolated in various laboratories (Davies and Reeves, 1975; Schnaitman et al., 1975; Foulds, 1976; Schmitges and Henning, 1976; Bassford et al., 1977; Beacham et al., 1977; Chai and Foulds, 1977; Henning et al., 1977; Lutkenhaus, 1977; Verhoef et al., 1977; W. van Alphen et al., 1978a; Bavoil et al., 1978). The mutations from which the position of the gene has been determined, are probably located in three genes, designated as *tolF* (Foulds, 1976), *ompB* (Sarma and Reeves, 1977) and *meoA* (Verhoef et al., 1977), the latter probably being identical to *par* (Bassford et al., 1977). These genes are localized at minutes 21, 73.7 and 48 respectively of the *E. coli* chromosome (Bachmann et al., 1976). Primarily based on similarity of cyanogen bromide fragments, Schmitges and Henning concluded that the proteins b and c are almost identical concerning their primary structure. They suggested that the proteins could either be products of two separate but almost identical genes or that one protein is a posttranslational modification product of the other (Schmitges and Henning, 1976). The structural gene(s) for the proteins b and c is (are) not known, although some authors suggest that *ompB* is the structural gene for both proteins (Bassford et al., 1977; Henning

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et al., 1977). The results obtained with a mutant with a changed electrophoretic mobility of protein c (see accompanying paper of L. van Alphen et al., 1979) suggested to us that the two proteins are coded for by two different structural genes. We therefore decided to carry out an extensive genetic analysis of a series of mutants affected in the appearance of the proteins b and c in the outer membrane. The chemistry of the proteins was studied at the same time. The results of these studies are described in this paper.

Materials and Methods

Strains and Growth Conditions. All relevant *E. coli* K12 strains used in this study are listed in Table 1. The nomenclature of Bachmann et al. (1976) was used except that in this reference *ompA* is indicated as *tolG*. Phages TuIa (Henning et al., 1977), Mel (Verhoef et al., 1977) and K3 (Skurray et al., 1974) were used to select for strains that are deficient for proteins b, c and d respectively. Other phages used are laboratory stocks. The compositions of glucose minimal medium, yeast broth, and brain heart medium have been described previously (Lugtenberg et al., 1976).

Isolation of Mutants. Independent mutants resistant to phages TuIa or Mel were isolated from strain PC0479 as described (Verhoef et al., 1977) with either 10^{10} (for high titer resistant mutants) or 10^6 (for low titer resistant mutants) plaque forming units per plate. One colony was purified from each plate and tested. Conjugation experiments and transduction with P1 lysates were performed as described previously (Verhoef et al., 1977).

Membrane Fractions. Procedures for the isolation of cell envelopes (Lugtenberg et al., 1975) and protein-peptidoglycan complexes (Rosenbusch, 1974; Lugtenberg et al., 1977) and for the determination of protein, lipopolysaccharide (LPS) and phospholipid (L. van Alphen et al., 1977b) have been described previously. The method of Galanos et al. (1969) as modified by Boman and Monner (1975), was used to characterize LPS of various mutants.

Purification of Proteins. The procedure for the isolation of peptidoglycan-associated proteins, which is basically the one described by Rosenbusch (1974) for the isolation of the matrix protein from *E. coli* BE, has been described briefly in a previous communication (Lugtenberg et al., 1977). In contrast to *E. coli* B, wild type *E. coli* K12 contains two peptidoglycan-associated proteins (Lugtenberg et al., 1976; Schmitges and Henning, 1976). The isolation of protein b of *E. coli* K12 was performed by starting from c^- mutants. Protein c was isolated either from wild type cells grown in a batch of yeast broth that resulted in an extremely low b over c ratio or from wild type cells grown in yeast broth supplemented with 0.3 M NaCl (Nakamura and Mizushima 1976; Lugtenberg et al., 1977; W. van Alphen and Lugtenberg, 1977). The problem of contamination of the final preparation by protein d was prevented by starting from a phage K3 resistant, protein d deficient derivative, which usually has the additional advantage that the strain produces higher amounts of protein b and/or c (W. van Alphen et al., 1976). Cell envelopes were isolated from exponentially growing cells. Ultracentrifugation (Lugtenberg et al., 1975) is not required in this case as practically all outer membrane protein can be recovered by centrifugation for 45 min in a refrigerated centrifuge at $16{,}000 \times g$. The method used for the major purification step was essentially the one described by Rosenbusch (1974). It is based on the fact that the two proteins b and c of *E. coli* K12 remain peptidoglycan-associated when cell envelopes are incubated with two percent SDS at 60° C. This protein-peptidoglycan fraction was isolated by centrifugation at 23° C for 30 min at $225{,}000 \times g$, once washed with distilled water, and resuspended in a buffer containing 2% SDS, subsequently heated for 5 min at 98° C to dissociate the protein-peptidoglycan complex and centrifuged at 23° C as described above (Rosenbusch, 1974). The supernatant fluid, containing essentially all peptidoglycan-associated protein, was freeze-dried and washed five times with 90% acetone and at least three times with water (Hindennach and Henning, 1975). The final pellet was freeze-dried, dissolved in 10 mM Tris (hydroxymethyl)-amino-methane-5 mM ethylenediamine tetraacetate-1.5% SDS, pH 7.8, and chromatographed on a Biogel P150 column (Hindennach and Henning, 1975) at 28° C, which resulted in better separations than chromatography on Sepharose 6B (Rosenbusch, 1974). The traces of pilin and of the free form of the lipoprotein which are present in the washed protein-peptidoglycan complex (Lugtenberg et al., 1977) were separated from the major protein in this column chromatography step. The fractions containing the pure protein were lyophilized, washed with acetone and water as described above, and lyophilized again (Hindennach and Henning, 1975).

Amino Acid Composition and N-Terminus. For amino acid analysis aliquots of the purified proteins were hydrolyzed with 6 N HCl *in vacuo* at 110° C for 24 and 72 hrs. Analyses were carried out on a Technicon TSM and a Beckman Unichrom amino acid analyser. Edman degradation (Tarr, 1975) in the presence of SDS (Bailey et al., 1977) was used to identify the N-terminal amino acids. Dansylation was carried out as described by Gray (1972).

Cyanogen Bromide Fragmentation. Conditions which result in good cleavage and only minor artificial bands, as judged from incubations without CNBr, were established for protein b and found to be suitable for the other proteins. Cleavage was carried out either in 70% formic acid or in 70% trifluoroacetic acid (Schroeder et al., 1969; Garten et al., 1975a). Six mg protein was dissolved in 3 ml of 70% formic acid, containing a 1000-fold molar excess of CNBr relative to the methionine residues of the protein. After incubation with occasional shaking for 3 hours in the dark at 37° C, the reaction was stopped by cooling and adding a 10-fold excess of distilled water. After lyophilization the resulting powder was dissolved in water and lyophilized again. Cleavage in trifluoroacetic acid was carried out with 6 mg of protein in 0.17 ml 70% trifluoroacetic acid, containing a 100-fold excess of CNBr relative to methionine. After incubation for 8 hours as described above, one volume of the CNBr solution in 70% trifluoroacetic acid was added. After an additional incubation for 16 hrs the reaction was stopped and the fragments were isolated as described above.

Proteolytic Fragments (Cleveland et al., 1977). Purified protein was solubilized at 1 mg/ml in 0.15% SDS and incubated at 37° C with trypsin or chymotrypsin (dissolved in 1 M Tris-HCl, pH 7.8) in weight ratio's of protein to enzyme of 100:1 to 10:1 for trypsin and of 1000:1 to 200:1 for chymotrypsin. After incubation for 5–120 min samples were withdrawn and immediately inactivated by boiling in sample buffer and the partial cleavage products were analyzed by SDS gel electrophoresis (Lugtenberg et al., 1975).

SDS Polyacrylamide Gel Electrophoresis. The previously described system (Lugtenberg et al., 1975) with 11% acrylamide was used for analysis of the cell envelope protein pattern and for testing fractions obtained during the purification of the proteins. Convex exponential gradient gels containing 10–13.5% acrylamide and 2.7–20% glycerol were used for the analysis of CNBr fragments. In contrast to the former gels (Lugtenberg et al., 1975) highly purified commercial SDS was used for the latter ones. Both gel

systems were used for the analysis of proteolytic fragments. Gels were usually stained with Fast Green FCF (Lugtenberg et al., 1975) but in some cases Coomassie Brilliant Blue was used.

Chemicals. Trypsin, treated with tolysulfonyl phenylalanyl chloromethyl ketone to inactivate chymotrypsin, was purchased from Serva, Heidelberg, GFR. Chymotrypsin A was obtained from Boehringer Mannheim, Amsterdam, The Netherlands. Cyanogen bromide was purchased from Sigma Chemical Co, St. Louis, Mo.

Results

Mutants Resistant Towards Bacteriophage Me1 and TuIa

All phage TuIa resistant mutants were of the b^-c^+ phenotype, irrespective the phage titer used in the selection procedure. All mutants isolated as resistant to a high titer of phage Me1 had no detectable protein c and contained a normal amount of protein b (b^+c^-) (strains CE1104, CE1105, and CE1106). In contrast, mutants isolated as resistant to a low titer of the phage can be devided in four classes with respect to the amounts of the proteins b and c, namely (i) $b^{++}c^{\pm}$ (strain CE1115), (ii) $b^{++}c^-$ (strain CE1110), (iii) b^+c^- (strains CE1111 through CE 1114, CE1116 and CE1117) and (iv) b^-c^- (strain CE1107).

Mutants without detectable protein b and protein c did not adsorb phage TuIa and Me1 respectively whereas strains of the $b^{++}c^{\pm}$ phenotype adsorb phages Me1 with a strongly decreased but significant rate. As could be predicted from previous studies (Ames et al., 1974; Koplow and Goldfine, 1974; Havekes et al., 1976; W. van Alphen et al., 1976) a number of TuIa resistant mutants contained heptose-deficient LPS. Only the TuIa resistant strains which contained wild type LPS (strains CE1121 through CE1126) were studied further.

In addition to the Me1 and TuIa resistant mutants described so far, several other strains with deficiencies in proteins b and/or c are known. A number of representative examples are listed in Table 1. These strains were also included in the studies on the genetic localization of the positions of the genes involved in the appearance of the two proteins in the outer membrane. The results will be described briefly as these genetic studies did not result in the identification of more than the three already mentioned genes *tolF*, *meoA* or *par* and *ompB* at approximately 21, 48 and 74 minutes on the recalibrated linkage map (Bachmann et al., 1976).

It appeared that the presence or absence of proteins b and c always corresponded with sensitivity and resistance to phages TuIa and Me1 respectively. In later stages of this work the presence or absence of these proteins in recombinants was therefore routinely assayed by determination of their sensitivity towards the appropriate phage(s). Determination of the map positions of the mutations was carried out by transduction with phage P1 lysates of the phage resistant mutants, using phage resistance as an unselected marker. Examination of the membrane protein patterns of many transductants showed that the phenotype of each mutation was transduced to the recipient strain without alteration.

In the minute 74 area three-point transductions were carried out with the *aroB*, *malT* strain CE1109 as the recipient and P1 lysates of phage resistant mutants as the donor. The *ompB* mutation of strain P692 was studied as a reference (Sarma and Reeves, 1977). After selection for $aroB^+$ fifty transductants were purified and tested for maltose utilization and phage sensitivity. The results with a P1 lysate of strain P692 as the donor showed that *aroB* and *ompB* were cotransducible for 62 percent whereas a cotransduction percentage of 46 was found for *aroB* and *malT*. These results as well as the frequencies of the four recombinant classes are in close agreement with those of Sarma and Reeves (1977). We performed similar transduction experiments with P1 lysates of the following other protein b and/or protein c deficient mutants with mutations in the minute 74 area: the Me1 resistant strains CE1115 ($b^{++}c^{\pm}$) and CE1110 ($b^{++}c^-$), the *cry* mutant 3-41 (b^-c^-), the TuIa resistant mutants CE1121 through CE1126 and the *cry* mutant 3-4 (all b^-c^+). No significant differences in the localization of the mutations was observed despite the large variety of phenotypes involved. Although these results do not exclude the possibility that these mutations are localized in more than one gene, the simplest explanation for the results is that mutation of the *ompB* gene can cause all mentioned phenotypes and probably also the b^+c^- phenotype described by Chai and Foulds (1977). We also observed that the mutations causing the $b^{++}c^-$ phenotype of strain CE1096 (resulting from a spontaneous reversion of the b^-c^- phenotype) are cotransducible with *aroB* without alteration of the phenotype, suggesting that this $b^{++}c^-$ phenotype is the result from two mutations in the same gene.

Phage P1 lysates of strains JF688 *tolF* and 5-70 (*cry*) were transduced to strains AB2829 (*aroA*) and CE1161 (*pyrD*). We confirmed the result of Foulds (1976) that the *tolF* gene is cotransducible with both *aroA* (50%) and *pyrD* (55%) and found values of 54 and 53 percent respectively for the *cry* gene. As *tolF*, *cmlB* (Foulds, 1976) and *cry* are genetically indistinguishable and as all three types of mutants have the same b^-c^+ phenotype (Chai and Foulds, 1977; Beacham et al., 1977), it is likely that they are mutants in the same gene.

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

Strain	Relevant characteristics	Relevant protein pattern	Source, reference(s)
BE	Derivative *E. coli* B	b^+c^- [b]	J. Rosenbusch (1974)
PC0479	Polyauxotrophic[c]	wild type	Phabagen Collection; Lugtenberg et al. (1976)
AB2826	*aroB*	wild type	Phabagen Collection
CE1109	*malT* derivative of AB2826	wild type	This paper
AB2829	F^+, *aroA*	wild type	Phabagen Collection
CE1161	F^-, *rpsL*, *aroA*$^+$, *pyrD*34 derivative of strain AB2829	wild type	C. Verhoef
Lin221	*glpT*	wild type	E.E.C. Lin
Lin221 *glpT*, *nalA*	*nalA* derivative of Lin221	wild type	This paper
JF688	*tolF*	b^-c^+	J. Foulds (1976)
JF700	d^- derivative of JF688	$b^-c^+d^-$	J. Foulds
P692	Colicin tolerant *ompB* derivative of AB1133	b^-c^-	P. Reeves; Davies and Reeves (1975)
P692 2dI	d^- mutant of P692	$b^-c^-d^-$	U. Henning; Henning and Haller (1975)
CE1096	Spontaneous pseudorevertant of strain P692 2dI	$b^{+\pm}c^-d^-$ [d]	L. van Alphen et al. (1978)
CE1034	d^- derivative of AB1859	$b^+c^+d^-$	Lugtenberg et al. (1976)
CE1036	Derivative of AB1621	b^+c^-	B. Bachmann; Lugtenberg et al. (1976)
CE1041	*tolG* derivative of CE1036	$b^{+\pm}c^-d^-$	J. Foulds; W. van Alphen et al. (1976)
3–4	Cryptic derivative of AB1157-1	b^-c^+	I. Beacham; Beacham et al. (1973)
3–41	Cryptic derivative of AB1157-1	b^-c^-	I. Beacham; Beacham et al. (1977)
5–70	Cryptic derivative of AB1157-1	b^-c^+	I. Beacham; Beacham et al. (1973)
CE1062	*meoA* derivative of PC0479	b^+c^-	Verhoef et al. (1977)
CE1131	d^- derivative of PC0479	$b^+c^+d^-$	This paper
CE1104, CE1105, CE1106	Mel resistant derivatives of PC0479	b^+c^-	This paper
CE1115	Mel resistant derivative of PC0479	$b^{+\pm}c^{\pm}$	This paper
CE1110	Mel resistant derivative of PC0479	$b^{+\pm}c^-$	This paper
CE1111, CE1112, CE1113, CE1114, CE1116, CE1117	Mel resistant derivatives of PC0479	b^+c^-	This paper
CE1107	Mel resistant derivative of PC0479	b^-c^-	This paper
CE1121 through CE1126	Tula resistant derivatives of PC0479	b^-c^+	This paper
CE1143	*meoA* mutant of CE1122	b^+c^-	This paper

[a] Except strain BE all strains are derivatives of *E. coli* K12
[b] *E. coli* B strains usually lack protein c
[c] Genetic markers: *thr, leu, thi, pyrF, codA, thyA, argG, ilvA, his, lacY, tonA, tsx, rpsL, deoC, supE, uvrB*
[d] Contains trace amounts of protein c

All studied mutations near minute 48 (strains CE1036, CE1062, CE1104, CE1105, CE1106, CE1111 through CE1114, CE1116 and CE1117) appeared to involve only protein c. Precise localization, using strain Lin 221 *glpT, nalA* as the recipient and P1 lysates of phage Mel resistant strains as donors, was hampered by the fact that the *glpT* mutation as well as the mutations causing phage Mel resistance (except that of strain CE1036) are unstable. An alternative, namely selection for *nalA* as a donor marker, has the disadvantages that spontaneous mutations leading to nalidixic acid resistance occur at a high frequency and that a relatively long time is needed for phenotypic expression of nalidixic acid resistance. The problems could only partly be overcome by starting from fresh single colonies of both the recipient strain Lin 221 *glpT, nalA* and of the Mel resistant mutants used for the preparation of P1 lysates. After selection for $glpT^+$ the transductants were purified and tested for sensitivity towards nalidixic acid and phage Mel. Cotransduction of Mel resistance with *glpT* was observed for all mutations in the 48 minutes area. However, the cotransduction frequencies differed from experiment to experiment, even for the same Mel resistance mutation, probably as a result of the mentioned high mutation rates of all three markers used.

The position of mutations causing a b^+c^- phenotype has been localized clockwise (*meoA*, Verhoef et al., 1977) as well as anticlockwise (*par*, Bassford et al., 1977) from *nalA*. As the same recipient strain was used for the localization of the gene(s) responsible

for this phenotype (Bassford et all, 1977; this paper) the difference in the localization might be caused by the unstable mutations used. We conclude that the slight discrepancy reported for the map positions of *meoA* and *par* provides no reason to assume that more than one gene in the 48 minutes area is involved in the appearance of protein c in the outer membrane but also that this possibility cannot be excluded.

The simplest interpretation of the genetic data is that three genes are involved in the appearance of the proteins b and c in the outer membrane, namely *tolF* (which affects only the level of protein b), *meoA* or *par* (which affects only the level of protein c) and *ompB* (which can affect the levels of both proteins b and c). Obviously, the simplest interpretation of the data discussed so far is the hypothesis that *tolF* and *meoA* are the structural genes for proteins b and c respectively, whereas *ompB* could be a regulatory gene affecting the levels of both proteins b and c. Such a regulatory gene provides an explanation for the observation that the total amount of proteins b and c is rather constant although the amounts of the individual proteins b and c are very much dependent on the growth medium (Lugtenberg et al., 1976; W. van Alphen and Lugtenberg, 1977).

Isolation and Characterization of Double Mutants

Of the three genes under study *tolF* and *ompB* are the only candidates for the structural gene for protein b as mutations in these genes can lead to the b^-c^+ phenotype. The introduction of a *meoA* mutation in a b^-c^+ mutant which is affected in the structural gene for protein b should never result in a b^+c^- double mutant. However, the latter phenotype might be obtained when the original b^-c^+ phenotype results from a mutation in another than the structural gene, f.i. in a regulatory gene. Therefore the phenotype of a double mutant might indicate which of the two genes is not the structural gene for protein b. Using a high titer of phage Mel, tentative *meoA* mutants were therefore isolated from the two b^-c^+ strains JF688 *tolF* and CE1122 (*ompB*). All resulting double mutants lacked protein c due to a mutation near minute 48 as was shown by cotransduction of Mel resistance with *glpT*. All eight independent Mel resistant derivatives of the *tolF* strain were phage TuIa resistant and had the b^-c^- phenotype. Of the five Mel resistant derivatives of the *ompB* strain CE1122 two were of the b^-c^- phenotype, resistant to phage TuIa and sensitive to 3% SDS. The other three double mutants had the b^+c^- phenotype, were sensitive to phage TuIa and resistant to SDS. P1 lysates of the three b^+c^- double mutants were transduced to strain CE1109 as the acceptor strain. After selection for $aroB^+$ it was shown that all three strains still possessed the original b^-c^+ mutation as 55% of the transductants were phage TuIa resistant and, for twelve transductants tested, also b^-c^+. These results show that the *ompB* mutation in strain CE1122 does not affect the structural gene for protein b. The results therefore exclude *ompB* as the structural gene for protein b and leave *tolF* as the most likely candidate for the structural gene for this protein. In the accompanying paper (L. van Alphen et al., 1979) we will show that *ompB* is not the structural gene for protein c either. One of the b^+c^- double mutants, strains CE1143, was used in order to compare the chemical properties of its protein b with that of other b proteins.

Purification, Amino Acid Composition and Amino-Terminus of Proteins b and c

The proteins b and c, purified as described previously, were practically free of other protein contaminants as judged from SDS gel electrophoresis (Fig. 1). The contamination by LPS was 0.06–0.56% (w/w). Contaminating phospholipid could not be detected.

The relative amino acid composition of proteins b and c, purified from the isogenic strains CE1041 and CE1034 respectively, showed that small but significant differences exist in the relative amounts of

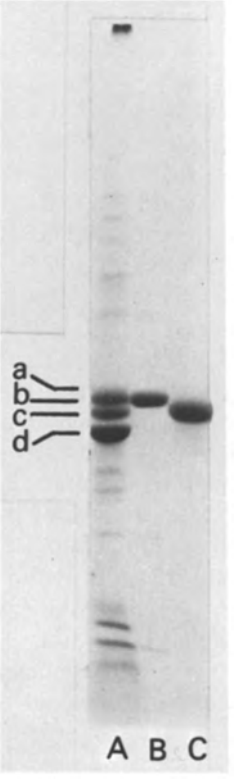

Fig. 1A–C. Polyacrylamide gel electrophoresis of cell envelopes of strain PC0479 (**A**) and purified proteins b (**B**) and c (**C**). 11% gel

glutamic acid and proline. The results are in good agreement with those published recently by Ichihara and Mizushima (1978).

The amino termini of the proteins were identified as follows:

protein b: H_2N – Ala – Glu – Ile

protein c: H_2N – Ala – Glu – Val

The difference in the third amino acid shows that the proteins cannot be products of the same structural gene. Henning et al. (1977) reported the same sequences for the proteins of *E. coli* K12 except that they found that the third amino acid in protein b of one particular K12 strain was valine. In contrast to the latter authors we isolated the proteins from isogenic strains. Recently Ichihara and Mizushima (1978) observed the same difference in the third amino acid as we found. In addition they showed a difference in amino acid number eleven. These authors used one particular *E. coli* K12 strain to isolate both proteins and concluded that the two proteins must be coded for by two structural genes. Our results confirm this conclusion.

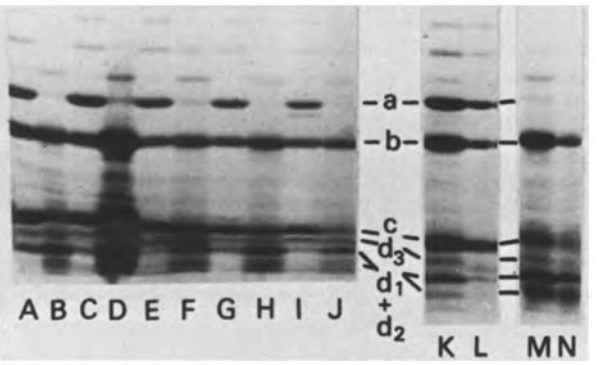

Fig. 2A–N. Cyanogen bromide fragments of protein b obtained by cleavage in formic acid (**A, C, E, G, I, K** and **L**) or trifluoroacetic acid (**B, D, F, H, J, M** and **N**). Protein b was purified from the following strains: BE (**A, B, K, M**), CE1041 (**C, D**), CE1062 (**E, F**), CE1110 (**G, H**), CE1096 (**I, J**) (the latter protein was contaminated with a small amount of protein c) and CE1143 (**L, N**). The designations used by Schmitges and Henning (1976) for the CNBr fragments of protein b are indicated. Gradient gel

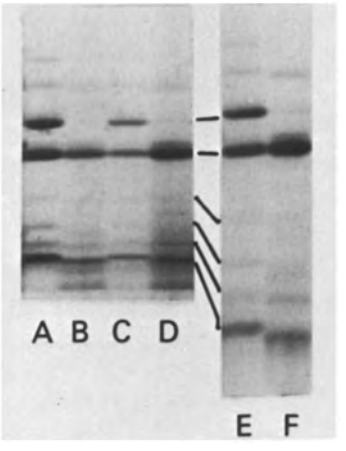

Fig. 3A–F. Cyanogen bromide fragments of protein c obtained by cleavage in formic acid (**A, C, E**) or trifluoroacetic acid (**B, D, F**). Protein c was purified from strain CE1034 (**A, B**), CE1131 (**C, D**) (both after growth in medium with high osmolarity) and from strain CE1034 (**E, F**) (after growth in a batch of yeast broth that resulted in a high c over b ratio). Gradient gel

Cyanogen Bromide Fragmentation

We confirmed the observation of Henning and coworkers (Garten et al., 1975; Schmitges and Henning, 1976) that the relative amounts of the CNBr fragments of protein b depend on the solvent used in that cleavage is much more complete in trifluoroacetic acid than in formic acid. Using this phenomenon, comparison of their (Garten et al., 1975; Schmitges and Henning, 1976) and our (Fig. 2) gels make it likely that the order of the fragments a, b and c is the same, whereas fragment d_3 is the slowest moving d-fragment in our gel system. We are not certain about the order of the two fastest moving fragments d_1 and d_2. The b proteins isolated from *E. coli* BE and from the *E. coli* K12 strains CE1041, CE1062, CE1110, CE1096 and CE1143 yielded indistinguishable CNBr fragments except that fragment d_3 in derivatives of strain PC0479 had a slightly higher electrophoretic mobility than those of strains BE and CE1041. These results show that the c^- phenotype, whether caused by an *ompB* or a *meoA* mutation, has no detectable influence on the structure of protein b. Another important observation is that strain CE 1143, an *ompB*/*meoA* double mutant which is phenotypically b^+c^- and phage TuIa sensitive, produces a b protein which is indistinguishable from other b proteins. From these results and from the genetic data presented earlier, it can definitely be concluded that *ompB* cannot be the structural gene for protein b. The presence of an apparently normal b protein in strain CE1096, a double mutant in the *ompB* gene, supports this conclusion. The CNBr fragments of three preparations of protein c are indistinguishable (Fig. 3) whereas the solvent used plays a similar role as described for protein b.

Profound and reproducible differences were observed when the cyanogen bromide fragments of proteins b and c were compared (Fig. 4). In some gels the resolution of the small fragments was such that all d-fragments of protein b had a higher electrophoretic mobility than those of protein c. Fragment b is the only fragment of protein b for which the electrophoretic mobility corresponds with one of the fragments of protein c. However, it has been reported

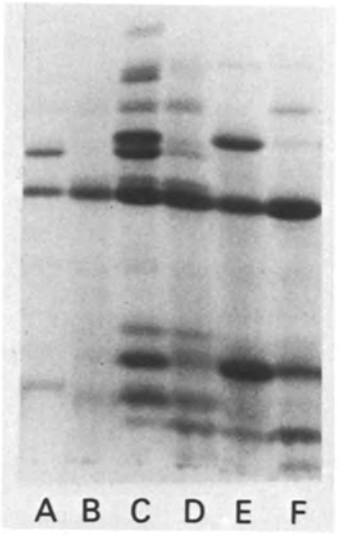

Fig. 4A–F. Comparison of cyanogen bromide fragments of proteins c and b. Purified proteins c (from strain CE1131, grown in medium with high osmolarity) (**A, B**), a mixture of purified proteins b (from strain CE1041) and c (from strain CE1034) (**C, D**) and protein b (from strain CE1062) (**E, F**) were cleaved with cyanogen bromide in formic acid (**A, C, E**) and in trifluoroacetic acid (**B, D, F**). Gradient gel

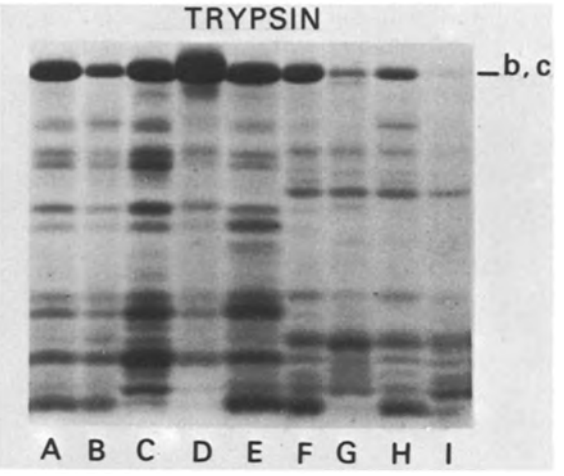

Fig. 5A–I. Tryptic fragments of proteins b from strains CE1110 (**A**), CE1062 (**B**), BE (**C**), CE1041 (**D**) and CE1143 (**E**), proteins b plus c (strain CE1131) (**F**) and proteins c of strains CE1122 (**G**), JF700 (**H**) and CE1131 (grown in medium with high osmolarity) (**I**). Gradient gel

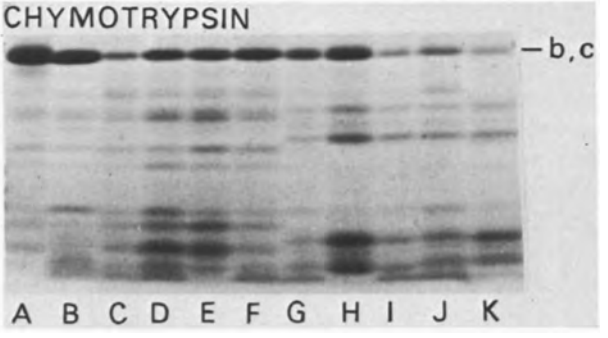

Fig. 6A–K. Chymotryptic fragments of proteins b from strains CE1041 (**A**), CE1096 (contaminated with a small amount of protein c) (**B**), CE1062 (**C** and **F**), CE1110 (**D**), BE (**E**), proteins b plus c from strain CE1131 (**G**), and proteins c from strains CE1131 (**H**), CE1034 (**I**), JF700 (**J**) and CE1122 (**K**). Gradient gel

that also this fragment is different in the two proteins (Schmitges and Henning, 1976). CNBr cleavage, performed on mixtures of both proteins, obtained either from cells with approximately equal amounts of both proteins or after mixing equal amounts of isolated proteins b and c (Fig. 4C and D), showed that the differences in CNBr fragments between the two proteins was not due to an artefact. Therefore all cyanogen bromide fragments of the proteins b and c are different. The same conclusion has recently been reached by Ichihara and Mizushima (1978). It thus must be concluded that the seemingly strong similarity between the CNBr fragments of protein b and those of proteins b plus c observed earlier (Schmitges and Henning, 1976) is accidental and probably caused by the fact that no purified protein c was used (see Ichihara and Mizushima, 1978).

Proteolytic Fragments

A fast means for comparison of proteolytic fragments in various stages of degradation is the method of Cleveland et al. (1977), which is based on proteolysis in a solution of SDS, followed by analysis on SDS-polyacrylamide gels. We used this method because it has the advantage that the tryptic and chymotryptic fragments of the two proteins can directly be compared with each other. The tryptic digests of the various proteins in similar stages of degradation are shown in Fig. 5; the chymotryptic digests are shown in Fig. 6. Control incubations without outer membrane protein showed that none of the observed bands was due to the added enzyme. As expected, incubation of a given purified protein with the two enzymes yielded different sets of peptides. With neither enzyme significant differences could be detected between the various preparations of protein b. Similarly all protein c preparations were mutually indistinguishable. Comparison of peptides of the purified proteins b and c after incubation with the same proteolytic enzyme showed that the proteins differ in almost all fragments in all stages of the degradation. Henning et al. (1977) state that the tryptic peptides of proteins b and c differ from each other but that the general appearance is rather similar. We prefer to interprete our data on tryptic and chymotryptic fragments of these

proteins in terms of considerable differences (Figs. 5 and 6) whereas we cannot exclude some similarities, the latter being either real or accidental. It should be noted that the proteolytic fragments shown in this paper differ from those shown by Henning et al. (1977) in that in our case the fragments are not the final products of proteolysis. However considerable differences between the fragments of proteins b and c were found in all stages of degradation and the differences visible in Figs. 5 and 6 represent real differences between the proteins.

Discussion

At least three genes are specifically involved in the appearance of proteins b and c in the outer membrane, namely (i). *tolF* [identical to *cmlB* (Foulds, 1976; Chai and Foulds, 1977) and to one of the *cry* genes (Beacham et al., 1973; Beacham et al., 1977; this paper)], (ii). *meoA* [also designated as *par* (Bassford et al., 1977] and, (iii). *ompB* [identical to *kmt* (Bavoil et al., 1978; von Meyenburg, 1971) and to the second known *cry* gene (Beacham et al., 1973; Beacham et al., 1977; this paper)]. The map position of these genes and the phenotypes of mutants in these genes are shown in Fig. 7. Although the genetic experiments do not exclude the possibility that the mutants are affected in more than these three genes, this possibility is not very likely as the phenotype of all *meoA* mutants is b^+c^- and those of the *tolF* mutant and the *cry* mutant 5–70 are b^-c^+. Only the assumed *ompB* mutants have many different phenotypes (Fig. 7) which could either be explained by the presence of several genes at minute 74 but also, and much simpler, by assuming that the mutations near minute 74 are affected in only one gene with a regulatory function (see later on).

The finding that several *ompB* (b^-c^+)/*meoA* (b^+c^-) double mutants have the b^+c^- phenotype excludes *ompB* as a candidate for the structural gene for protein b. The observation that neither alterations were detected in the CNBr fragments of protein b of the *ompB* double mutant CE1096 (Fig. 2), nor any structural alterations were found in protein b of a b^+ revertant of an *ompB* mutant (Bavoil et al., 1978), are consistent with this idea.

Our chemical data show that the proteins b and c differ considerably from each other with respect to the fragments obtained after treatment with cyanogen bromide (Fig. 4), trypsin (Fig. 5) and chymotrypsin (Fig. 6). Also their third amino acid of the N-terminus is different. Their amino acid compositions are very similar but not identical. The chemical data of Ichihara and Mizushima (1978) are consistent with ours. The differences observed in the amino termini and overall amino acid composition (Ichihara and Mizushima, 1978; this paper) exclude the possibility that one structural codes for both proteins b and c. The differences observed between the fragment patterns of the two proteins support this conclusion.

The combined genetic and chemical data are consistent with the model of Ichihara and Mizushima (1978) for the synthesis and regulation of proteins b and c in which *tolF* and *meoA* are the structural genes for proteins b and c respectively, whereas *ompB* is involved in the regulation of the levels of both proteins. The only observation which contradicts this hypothesis was published by Bassford et al. (1977) who reported that a *tolF*/*par* double mutant produces protein b. However Foulds and Chai (1978) reported that the latter protein only had the same electrophoretic mobility as protein b but that it is in fact related to protein E. As *meoA* mutants have the b^+c^- phenotype this gene is the most likely candidate for the structural gene for protein c. In the accompanying paper (L. van Alphen et al., 1979) we will show that *meoA* is indeed the structural gene for protein c. Many data indicate that proteins b and c are similar. Both are peptidoglycan-associated *in vivo* (Lugtenberg et al., 1976; Schmitges and Henning, 1976), bind to peptidoglycan in vitro (Yu and Mizushima, 1977), have pore functions (Nakae, 1976; Lutkenhaus, 1977; W. van Alphen et al., 1978a, b) and are also chemically related (Henning et al., 1977; Bassford et al., 1977; Ichihara and Mizushima, 1978; this paper). Evidence for a common regulation mechanism for the proteins b and c came from experiments that showed that the total amount of b plus c is almost constant, whereas growth conditions can cause large variations in the levels of these two individual proteins (Lugtenberg et al., 1976; W. van Alphen et al., 1977). Mutations in *ompB* can lead to a wide variety of phenotypes with respect to the levels of proteins b and/or c (Fig. 7). The phenotypes of at least some *ompB* mutants suggest that *ompB* is involved in regulation in such a way that the *ompB* product is required for the appearance of proteins b and c in the outer membrane. The *ompB* product could either be a positive regulatory protein or a protein acting on the level of the cell envelope and f.i. be involved in recognition or translocation of both proteins b and c or of their unprocessed precursors. Modulation of the *ompB* gene product under the influence of growth conditions could be responsible for the observed differences in b over c ratio's (Lugtenberg et al., 1976; W. van Alphen et al., 1977).

As the two proteins are very similar in overall amino acid composition and N-terminus, and as they might be regulated by a common mechanism, the

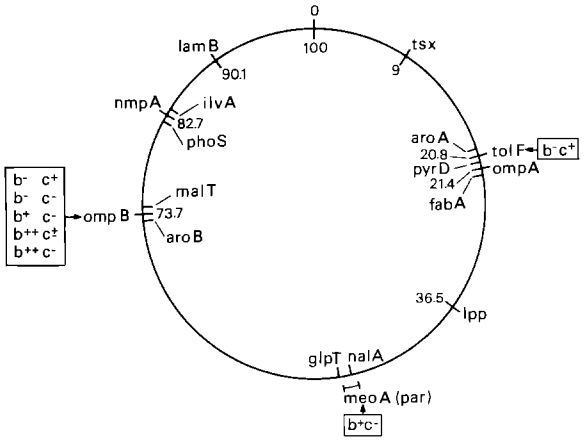

Fig. 7. Map position of genes involved in the appearance of proteins b and c in the outer membrane. The phenotypes are indicated in boxes. Well-known reference genes and a number of genes related to other outer membrane proteins are indicated inside and outside the circle respectively. *nmp* (new membrane protein): mutant form of this gene results in the appearance of protein E (Foulds and Chai, 1978) in the outer membrane (Foulds, personal communication)

two structural genes probably originate from one gene, followed by independent evolutionary changes in the two genes. If this has indeed occurred, the rather drastic differences observed in the fragment patterns (Figs. 4–6) of the proteins are surprizing. However these might not represent real differences in molecular weight as Dietrich et al. (1977) found only slight differences in the CNBr fragment patterns of the two proteins in a gel system that separates the fragments according to their molecular weight. The pronounced differences that we observed therefore might rather be caused by differences in the degree or mechanism of post-translational modification than in primary structure.

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