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Genes Affecting the Major Outer Membrane Proteins of *Escherichia coli* K-12: Mutations at *nmpA* and *nmpB*

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Summary. Strains of *Escherichia coli* K-12 carrying mutations at either of two distinct loci (*nmpA* and *nmpB*) produce a new outer membrane pore protein which is not present in wild type cells. Mutations at either of these loci result in sensitivity to phage TC45, which can use this new protein as its receptor, and the new protein (the NmpAB protein) appears to be identical in both NmpA and NmpB mutants. In order to determine whether both of these loci contain structural genes for the NmpAB protein, strains carrying secondary mutations at either of these loci which produced altered proteins were sought by selecting for resistance to phage TC45. Mutants which produced proteins with altered electrophoretic mobility and altered peptide maps were isolated from strains carrying both *nmpA* and *nmpB* mutations, and these secondary mutations mapped at the same sites as the original mutations leading to production of the NmpAB protein. This suggests that both loci contain structural genes. Strains mutant at *nmpB* which can no longer produce the protein can mutate at the *nmpA* locus to produce the new protein, indicating that both genes can exist in the same cell. Since the altered proteins of mutant strains could be distinguished from one another, we attempted to construct strains in which both *nmpA* and *nmpB* were expressed. In all cases only the protein produced by the *nmpB* mutation was produced, indicating some form of cooperative regulation of the two genes.

Introduction

The major factor affecting permeability of the outer membrane of *Escherichia coli* to small hydrophilic solutes is the presence in the membrane of a group of oligomeric proteins which form pores (for review, see DiRienzo, Nakamura and Inouye, 1978). Several different classes of pore proteins have been identified in various derivatives of *E. coli* K-12 (Pugsley and Schnaitman, 1978a, 1978b), and chemical analyses have indicated that these proteins can be one of four different types (Diedrich et al., 1976; Lee et al., 1979; Gamon et al., 1978; Lugtenberg et al., 1978). Production of these proteins is affected by a number of genes on the *E. coli* K-12 chromosome and on the temperate bacteriophage PA-2 (Pugsley and Schnaitman, 1978b; Pugsley et al., 1979). One of the chromosomal genes (*ompB*) affects the production of more than one protein (Sarma and Reeves, 1977) and is thought to have a regulatory function (Palva, 1979). Other strains of *E. coli* exhibit some variability in the types of pore proteins expressed, presumably reflecting small differences in primary sequences of the polypeptides, strain-specific variations in post-translation modification and differences in the expression of genes affecting their production.

Mutants of *E. coli* K-12 which lack the constitutive pore proteins (OmpC and OmpF) revert readily to produce new membrane proteins. We have identified three genes which affect the production of these new membrane proteins (Pugsley and Schnaitman, 1978b). The proteins produced by strains carrying the *nmpA*(p^+) or *nmpB*(p^+) mutations are identical by a number of different criteria (Lee et al., 1979), and this has led us to propose that *nmpA* and *nmpB* are structural genes for the same outer membrane protein. Reeves (1979) has recently argued that at least one of these genes may have a regulatory function. In order to study the roles of the *nmpA* and *nmpB* genes we have isolated a range of mutants which are affected in the production or functions of the *nmpAB* protein. The results of our studies with these mutants are described in this paper.

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Table 1. *E. coli* K-12 strains

Strain number	Genotype/phenotype	Source/reference[a]
CS482	*ilvY227 metB65 his-53 proC24 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompF (tolF4) ompC171* TC45^R	A
CS483	as CS482 but *nmpA104*(p$^+$) *ilv*$^+$ TC45^S	A
CS485	as CS482 but *nmpB105*(p$^+$) *pro*$^+$ TC45^S	A
CS487	as CS485 but *ompF*$^+$ *ompC*$^+$ *aroA pdx*, TC45^S	A
CS809	as CS484 but *nmpA114*(p*) TC45^R	This study
CS810	as CS484 but *nmpA115*(p*) TC45^R	This study
CS812	as CS485 but *nmpB116*(p*) TC45^R	This study
CS649	as CS484 but *nmpA110*(p$^+$) TC45^R	This study
CS652	as CS485 but *nmpB113*(p$^+$) TC45^R	This study
CS513	as CS484 but *nmpA107*(p$^-$) TC45^R	A
CS514	as CS485 but *nmpB108*(p$^-$) TC45^R	A
CS741	as CS485 but *rbs*::Tn10, TC45^S	This study
CS840	Hfr Cavalli *tonA*::Tn10 *lac*::Tn5 *relA1* T2^R, TC45^R	This study
CS745	Hfr P10 *sup-53 malB16 ilv*::Tn5 *tna*::Tn10, TC45^R	This study
CS791	Hfr PK191 *thi relA1 Δlac-proB supE44 ompC*::Tn5, TC45^R	This study
CS819	Hfr KL16 *thi relA1 ompC*::Tn5 *nalA* Tn10^b TC45^R	This study
WP80	*rbs*::Tn10 TC45^R	A. Wright
P400	*thr leu proA argE non rpsL thi galK lacY ara xyl mtl* TC45^R	B
P239	as P400 but *rfa* TC45^R	C
P424	as P400 but *rfa* TC45^R	C
P489	as P400 but *rfa* TC45^R	C

[a] A, Pugsley and Schnaitman, 1978b, B, Manning, Pugsley and Reeves, 1977, C, Hancock and Reeves, 1975, 1976

[b] Transposon Tn10 in strain CS819 is 85% cotransduced with *supD* and 10% cotransduced with *his*

Materials and Methods

Bacterial Strains, Bacteriophages and Culture Media. Strains of *E. coli* K-12 are listed in Table 1. *E. coli* B strain 837 [*met btuB gal hsd* (r_B^-, m_B^-)] and a derivative (CS199) carrying a mutation in the *ompB* gene were as used previously (Pugsley and Schnaitman, 1978a). Smooth *E. coli* strains were O-serotyped clinical isolates provided by Kenneth L. Vosti. Colicinogenic strains were as used previously (Pugsley and Schnaitman, 1978a) except strains producing colicins E4 (316BM), E5 (271BM), E6 (318BM), E7 (245BM) and E? (P33) which were provided by Bruce A.D. Stocker. Colicin sensitivity was determined by the cross-streak method or by spotting dilutions of the colicin onto lawns of the indicator strains (Pugsley and Schnaitman, 1978a). In some cases we used colicin I-resistant derivatives of the test strains to avoid killing by the colicin I which is produced by some of the E-colicinogenic strains.

Bacteriophages TC45, K3, TuIb, Mel and PA-2- hy2 were all as used previously (Manning et al., 1977; Pugsley and Schnaitman, 1978a, b). Bacteriophage SS4 was isolated from local sewage and uses the OmpC protein of *E. coli* K-12 as its receptor (Lee, Pugsley and Schnaitman, manuscript in preparation). Bacteriophage sensitivity was determined by the cross streak method or by spotting dilutions of the phage onto lawns of the test strains (Pugsley and Schnaitman, 1978a).

Culture media were tryptone-yeast extract broth containing 0.5% NaCl (TYS) and minimal medium A supplemented with 0.5% glucose and 0.01% growth factors (Table 1) as used previously (Pugsley and Schnaitman, 1978a).

Genetic Procedures. Generalised transductions with bacteriophage P1 were performed as described by Miller (1972). The temperate phage P1 ts *cml-100* was used for transductions in which smooth *E. coli* strains were used as donors (Miller, 1972). Selections for transfer of transposons conferring antibiotic resistance were performed by incubating the transduced cells for 15 min in TYS broth containing 50mM sodium citrate to allow expression of the antibiotic resistance and then plating on TYS agar containing 10 mM citrate and either 40 μg/ml kanamycin (Tn5) or 15 μg/ml tetracycline (Tn10).

Conjugation experiments with various Hfr strains (Table 1) were performed by mixing donors and recipients at a ratio of 1:5 in TYS broth and incubating for 1–2 h. Cells were then mixed rapidly and plated on TYS agar containing 100 μg/ml streptomycin and either kanamycin or tetracycline as above. Control experiments in which the % transfer of linked markers into smooth *E. coli* was compared with *E. coli* K-12 indicated that transfer of distal markers occurred less frequently with the smooth strains, presumably because the mating aggregates were less stable.

Mutagenesis with ethylmethane sulfonate (EMS) was as precribed by Miller (1972).

SDS Gel Electrophoresis. Procedures for SDS polyacrylamide gel electrophoresis have been described elsewhere (Pugsley and Schnaitman, 1979). The three systems used were the Tris-glycine system, the Tris-glycine system with 8M urea and the phosphate-cacodylate system (Lee et al., 1979; Pugsley and Schnaitman, 1979). Peptides obtained by cleavage with cyanogen bromide were separated in linear gradient gels (8–12% acrylamide at an acrylamide: bisacrylamide ratio of 50:1.33 and 2.7–20% glycerol) using the cacodylate-phosphate buffer system.

Isoelectric Focusing. Isoelectric focusing was performed as described previously (Lee et al., 1979) except that tube gels were used. The final concentration of ampholytes in the gels was 3%, and mixtures of various ampholyte preparations (BioRad, Richmond, Ca) were used to give the desired pH range. After focusing the gels were sliced and counted for radioactivity as previously (Pugsley and Schnaitman, 1979).

Purification of Outer Membranes Outer membranes were prepared by extracting with Triton as previously (Pugsley and Schnaitman, 1979). Triton-insoluble walls were extracted with SDS in the presence of Mg^{++} at 56° C (Rosenbusch, 1974), and the pore proteins in the SDS-Mg^{++}-insoluble extract were further purified by ion exchange chromatography and gel filtration in Sephacryl S300 in the presence of SDS (Lee et al., 1979). Purity of the final preparation was confirmed by SDS gel electrophoresis using the Tris-glycine buffer system.

Peptide Mapping. Cyanogen bromide cleavage was performed under nitrogen in the dark at room temperature using a 1,000-fold molar excess of cyanogen bromide over methionine. Proteins were dissolved in 70% trifluoracetic acid. The reaction was allowed to continue for 14 h after which ten volumes of cold distilled water were added and the mixture frozen and lyophilised. The cleaved preparation was dissolved in SDS sample buffer (Pugsley and Schnaitman, 1979) and examined by SDS gel electrophoresis.

Cleavage with trypsin and chymotrypsin either singly or together was performed as previously (Lee et al., 1979). The peptides were resolved by high pressure chromatography as previously (Lee et al., 1979) or by dissolving the sample in isoelectric focusing buffer (Lee et al., 1979) followed by isoelectric focusing (see above). In the latter case, the samples were centrifuged at $15.000 \times g$ for 30 min to remove any uncleaved protein or insoluble material.

Transport Assays. Transport assays were as described previously (Pugsley and Schnaitman, 1978a) except that substrates were used at 2 μM.

Nomenclature. Outer membrane proteins are identified here by the mnemonics of the genes which affect their production Several lines of evidence indicate that *ompC, ompA, ompF* and *lamB* are structural genes for outer membrane proteins (Reeves, 1979; van Alphen et al., 1979; Sato and Yura, 1979), and evidence presented in this paper suggests that *nmpA* and *nmpB* may also be structural genes. Other names applied to these genes and proteins were listed by Lee, Schnaitman and Pugsley (1979). The trivial name 3b has been retained for the present since the gene(s) affecting its production have not been identified.

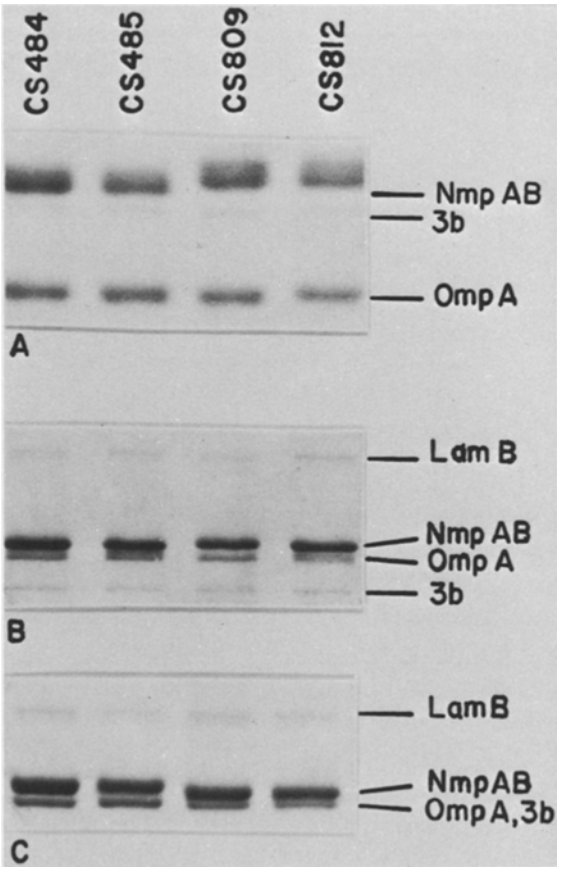

Fig. 1 A–C. Major outer membrane proteins of *E. coli* K-12 strains mutated at the *nmpA* and *nmpB* loci resolved by SDS gel electrophoresis in the Tris-glycine gel system (**A**), the Tris-glycine gel system with 8M urea (**B**) and the cacodylate-phosphate buffer system (**C**). Strains CS809 and CS812 produce altered forms of the NmpAB protein. Only the region of the gels displaying proteins with molecular weights of 33–42.000 are shown

Results

Mutations at nmpA and nmpB

Starting with strains CS484 *[nmpA104*(p^+), *ompC, ompF]* and CS485 *[nmpB105*(p^+), *ompC, ompF]* we selected over 100 independent spontaneous and EMS-induced mutants which were resistant to bacteriophage TC45, which uses the NmpAB protein as part of its receptor (Chai and Foulds, 1978; Pugsley and Schnaitman, 1978b). The mutants were screened for sensitivity to colicins E2 and E3, which require the presence of the NmpAB protein for lethal action (Pugsley and Schnaitman, 1978b), and for the production of SDS-Mg^{++}-insoluble (peptidoglycan-associated) major outer membrane proteins. The mutants obtained were of three types. The first type, which we shall refer to as $NmpAB^-$, were fully resistant to colicins E2 and E3 and were devoid of major SDS-Mg^{++}-insoluble outer membrane proteins; these mutants were therefore similar to those described previously (Pugsley and Schnaitman, 1978b). The second type of mutants ($NmpAB^+$) still produced an outer membrane protein which co-migrated with the NmpAB protein produced by the parent strain in all SDS gel systems tested. These strains remained sensitive to colicins E2 and E3. The third type of mutants (NmpAB*) also remained sensitive to colicins E2 and E3, but produced a peptidoglycan-associated outer membrane protein which differed somewhat from the NmpAB protein with respect to mobility in some SDS gel systems (Fig. 1).

All three types of mutants appeared in both strains at approximately the same frequency. $NmpAB^+$ mutants were obtained at 6 times the frequency of the $NmpAB^-$ mutants, and NmpAB* mutants were only detected at low frequencies in EMS-treated cultures.

In order to map the mutations causing resistance

Table 2. TC45 sensitivity of *E. coli* K-12 strains mutated at the *nmpA* and *nmpB* loci

Strain number	*nmp* genotype/ phenotype	Sensitivity to TC45 and host range mutants[a]				
		TC45	TC45*h5* (CS649)	TC45*h10* (CS809)	TC45*h11* (CS810)	TC45*h12* (CS812)
CS484	*nmpA104*(p$^+$)[b]	1	1	1	1	1
CS485	*nmpB105*(p$^+$)	1.3	0 9	0.9	0.8	1.2
CS482	–	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
CS649	*nmpA104* *nmpA110*(p$^+$)	4×10^{-6}	0.3	0.08	0.09	0.1
CS652	*nmpB105* *nmpB113*(p$^+$)	6×10^{-5}	0.4	0.6	0.7	0.6
CS809	*nmpA104* *nmpA114*(p*)	9×10^{-5}	0.6	0.8	0.3	0.5
CS812	*nmpB105* *nmpB116*(p*)	5×10^{-6}	0.4	0.4	0.2	0.9
CS513	*nmpA104* *nmpA107*(p$^-$)	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$
CS514	*nmpB105* *nmpB108*(p$^-$)	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$	$<10^{-10}$

[a] Sensitivity measured relative to strain CS484

[b] p$^+$, normal or apparently unchanged NmpAB protein produced; p*, altered NmpAB protein produced; p$^-$, NmpAB protein not produced

to TC45 and colicin E3, we used P1 phage grown on several representatives of each mutant type and used these to transduce strain CS482 (*ompC ompF ilvY proC*) to *ilv*$^+$ or *pro*$^+$. Previous studies have indicated that *nmpA* is closely linked to *ilvY* and that *nmpB* is closely linked to *proC* (Pugsley and Schnaitman, 1978b; Foulds and Chai, 1978b). P1 phage grown on each of the NmpAB$^-$ mutants were unable to transduce CS482 to sensitivity to TC45 or colicin E3 at either locus, indicating that these strains carry mutations within the *nmpA* or *nmpB* genes respectively. P1 phage grown on the NmpAB* and NmpAB$^+$ mutants were also unable to transduce CS482 to TC45 sensitivity, but were still able to transduce sensitivity to colicin E3 when *ilv*$^+$ (NmpA* or NmpA$^+$) or *pro*$^+$ (NmpB* or NmpB$^+$) transductants were selected. Furthermore, these recombinants had the same phenotype as the donor cells with respect to bacteriophage and colicin sensitivity and production of peptidoglycan-associated major outer membrane protein as the donor cells. In other experiments, P1 phage grown on strains WP80 and CS741 were used to transduce NmpA$^+$ and NmpA* mutants to tetracycline resistance (*rbs*::Tn10). Among the recombinants, 60–80% were resistant to colicin E3 or sensitive to phage TC45 respectively. These cotransduction frequencies are similar to those recorded for the *nmpA*(p$^+$) and *rbs* markers. These results indicate that the mutations producing the NmpAB*, TC45^R and NmpAB$^+$, TC45^R phenotypes are also located within or close to the *nmpA* and *nmpB* genes.

Representatives of all three classes of TC45-resistant mutants were examined in greater detail. NmpAB$^-$ mutants were fully resistant to bacteriophage TC45 (see Table 2) and to colicins E2 and E3 (resistance relative to parent strains, 4×10^4 and 8×10^5 respectively). These strains were also defective in the accumulation of tyrosine, lysine, glutamine, serine and adenosine monophosphate when compared with the parent strains, and were therefore phenotypically identical to other pore-deficient mutants described previously (Pugsley and Schnaitman, 1978a, b).

NmpAB$^+$ and NmpAB* mutants remained partially sensitive to TC45 (Table 2). Independent host range mutants were isolated from the plaques which appeared when TC45 was plated with these mutants (see Manning et al., 1976). All of these mutant phage formed plaques with increased frequency on the NmpAB$^+$ and NmpAB* mutants irrespective of the strain on which the phage mutants were originally isolated (Table 2). Evidence that these phage were indeed host range mutants was provided by the fact that only NmpAB$^-$ mutants were obtained when they were used to select resistant mutants of CS484 and CS485. Furthermore, these mutants appeared at

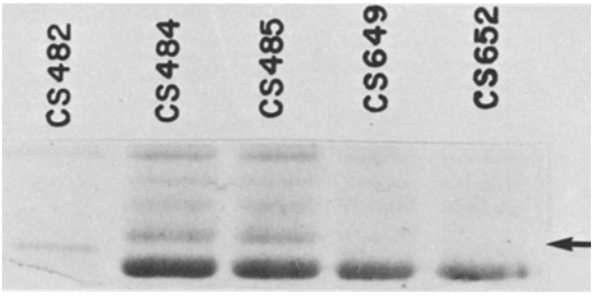

Fig. 2. Oligomeric forms of the NmpAB proteins. Outer membranes were extracted with SDS-Mg^{++} and the insoluble material was dissolved in SDS sample buffer without heating. Proteins were separated in the Tris-glycine gel system. CS482 does not produce major pore proteins, and CS649 and CS652 produce altered forms of the NmpAB protein (note the absence of the band indicated by arrow). Only the region of the gel displaying the NmpAB protein oligomers is shown

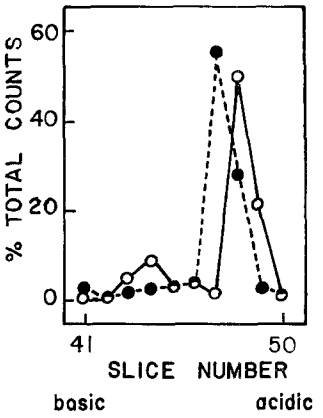

Fig. 3. Isoelectric focusing profiles of NmpAB proteins produced by strains CS484 [*nmpA104*(p$^+$). ^{14}C-leucine, ●] and CS809 [*nmpA104*, *nmpA114*(p*), ^{3}H-leucine, ○]. The pIs of the two proteins were calculated as 5.3 and 5.1 respectively. This system does not resolve these proteins into the two isoelectric species seen previously (Lee, Schnaitman and Pugsley, 1979) Simultaneous isoelectric focusing of the NmpAB proteins produced by strains CS485 [*nmpB105*(p$^+$)] and CS812 [*nmpB105*, *nmpB116*(p*)] produced an identical pattern to that shown

approximately 10–20% of the frequency of mutants resistant to TC45.

NmpAB$^+$ and NmpAB* mutants also remained as sensitive to colicins E2 and E3 as the parent strains (Pugsley and Schnaitman, 1978b). Cross streak tests indicated that these mutants were also unchanged in their sensitivity to colicins A, K, L, S4, E7 and X (resistant); E1, E4 and E? (sensitive) and E5 and E6 (partially sensitive). All of these strains were also able to accumulate serine, glutamine, lysine and tyrosine at rates similar to the parent strains, but NmpAB* mutants exhibited a 30–50% reduced capacity to accumulate adenosine monophosphate. Thus, the only physiological feature which distinguishes the TC45-resistant, NmpAB$^+$ strains from their parent strains is their reduced sensitivity to the phage. NmpAB* mutants can be distinguished from their parent strains by their altered phage sensitivity, by their reduced ability to accumulate adenosine monophosphate and by the production of a pore protein which is apparently different from that produced by the parent strain.

We have also examined the oligomeric form of the pore proteins produced by the TC45-resistant mutants. Previous studies have shown that the oligomeric NmpAB protein produces a characteristic profile upon electrophoresis in SDS gels, although the basis for the unusual patterns obtained remains unclear (Lee et al., 1979). Only two of the 50 mutants tested (one each derived from CS484 and CS485) produced proteins which gave SDS gel profiles which differed from the profiles obtained with proteins produced by the parent strains under these conditions. Both mutants were of the NmpAB$^+$ type (Fig. 2).

Chemical Analyses of the NmpAB Proteins. Further tests were carried out to determine whether the pore proteins produced by the NmpAB$^+$ and NmpAB* mutants were related to those produced by the parent strains. The proteins were purified from the outer membranes of several representatives of each class of mutants grown in the presence of ^{3}H-tyrosine or ^{3}H-leucine and compared with ^{14}C-tyrosine or ^{14}C-leucine-labeled NmpAB proteins purified from the parent strains. Proteins produced by four NmpAB$^+$, TC45^R mutants were indistinguishable from the parent strains proteins on the basis of isoelectric focusing, and peptide mapping. However, differences in some of these properties were observed in comparing proteins from the NmpAB* strains and their parents. Examples of these slight differences in isoelectric focusing profiles of the monomeric proteins and in SDS gel electrophoresis and isoelectric focusing profiles of peptides derived by cleavage with cyanogen bromide or with chymotrypsin are shown in Figs. 3–5. We did not observe differences in isoelectric focusing profiles of leucine-labeled, trypsin cleaved proteins.

Expression of nmpA and nmpB. The results of these chemical analyses suggested that *nmpA* and *nmpB* might indeed be structural genes for the same pore protein. In order to determine whether both genes could be present in the same cell line we obtained revertants of TC45-resistant, NmpAB$^-$ mutants of strains CS484 and CS485. Revertants to sensitivity to phage TC45 and to colicin E3 appeared readily in all cell lines, and sensitivity to both of these agents was invariably mapped at the *nmpA* locus, as shown by cotransduction with *ilv* and with *rbs*::Tn10. This may explain why other groups have only isolated

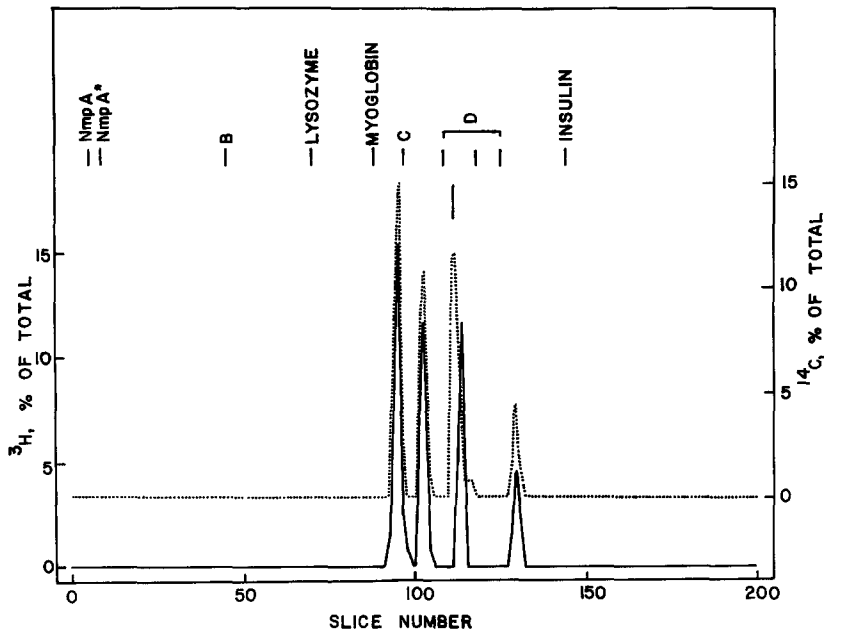

Fig. 4. Cyanogen bromide peptide profiles of the NmpA proteins produced by strains CS484 [*nmpA104*(p$^+$), ^{14}C-tyrosine, ····) and CS809 [*nmpA104 nmpA114*(p*), ^{3}H-tyrosine, ——). Peptides were resolved by SDS gel electrophoresis using slab gels. Markers included in the gels were uncleaved NmpA and NmpA* proteins, lysozyme, myoglobin, insulin monomer (Pugsley and Schnaitman, 1979) and peptides B, C and D1, D2 and D3 obtained by cyanogen bromide cleavage of OmpF protein purified from the outer membrane of *E. coli* B. Only the region of the gel containing the peptides was examined. Simultaneous cleavage and resolution of the NmpB proteins produced by strains CS485 [*nmpB105*(p$^+$)] and CS812 [*nmpB105 nmpB116*(p$^+$)] produced an identical peptide profile

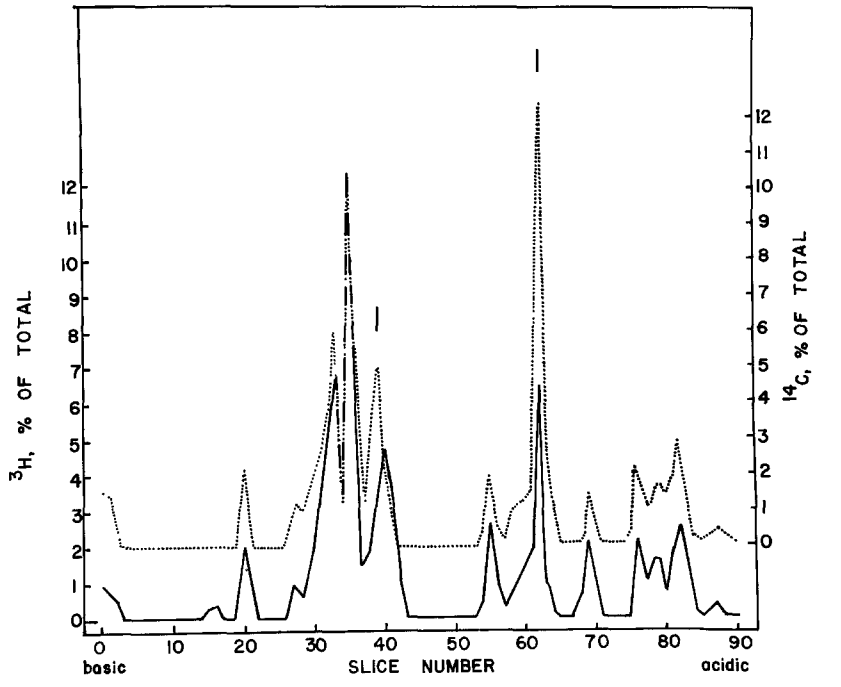

Fig. 5. Chymotryptic peptides of the NmpB proteins produced by strains CS485 [*nmpB105*(p$^+$), ^{14}C-tyrosine, ····] and CS812 [*nmpB105, nmpB116*(p*) ^{3}H-tyrosine, ——]. Peptides were resolved by isoelectric focusing (linear gradient pH 7.8–3.4) Differences between the two peptide profiles are indicated by bars. Simultaneous cleavage and resolution of the NmpA proteins produced by strains CS484 [*nmpA104*(p$^+$)] and CS809 [*nmpA104, nmpA114*(p*)] produced a similar peptide profile except that the differences in recovery from the more acidic of the two peptides which appear different in Fig. 6 were less pronounced

NmpA$^+$ mutants when screening pore-deficient strains for pseudorevertants (Foulds and Chai, 1978a; Henning et al., 1977; Lugtenberg et al., 1978). Indeed, we also find that TC45-sensitive pore$^+$ pseudoreversions appear most frequently at the *nmpA* locus in all pore-deficient strains tested. Furthermore, we find that these NmpA$^+$ revertants can be of two types corresponding to the TC45-sensitive, colicin E3-sensitive types described previously (Pugsley and Schnaitman, 1978b) or to the TC45-resistant, colicin E3-

Table 3. Expression of *nmpA*(p$^+$) and *nmpB*(p$^+$) genes in transductants of *E. coli* K-12 carrying both genes

Donor	Protein produced	Recipient[a]	Protein produced	Number tested	*nmp* transductants[b]	Nmp protein expressed	OmpA protein expressed
CS741	NmpA	CS812	NmpB*	10	6	NmpB*	+
CS484	NmpA	CS812	NmpB*	8	4	NmpB*	+
CS809	NmpA*	CS485	NmpB	10	5	NmpB	+
CS485	NmpB	CS809	NmpA*	10	7	NmpB	−
CS485	NmpB	CS810	NmpA*	8	7	NmpB	−
CS812	NmpB*	CS484	NmpA	10	5	NmpB*	−

[a] Transductants were selected as follows: donor CS741, tetracycline resistance (*rbs*::Tn10, donors CS484 and CS809, *ilv*$^+$; donors CS485 and CS812, *pro*$^+$

[b] Transfer of the *nmp* genes was determined by using P1 grown in the transductants to transduce strain CS482 to *ilv*$^+$, *pro*$^+$ or *rbs*.::Tn10 as appropriate and screening for transfer of colicin E3 sensitivity (=transfer positive)

sensitive mutants described in this paper. However, the finding that strains carring the *nmpB*(p$^-$) mutation can subsequently mutate at the *nmpA* locus to produce the NmpAB protein is strong evidence that both genes can exist in this particular derivative of *E. coli* K-12.

The availability of strains producing altered NmpA or NmpB proteins afforded us the opportunity to study the regulation of the two proteins in strains carrying both the *nmpA*(p$^+$) and *nmpB*(p$^+$) mutations. Strains were constructed by transduction as detailed in Table 3. The transductants were screened for sensitivity to phage TC45 and TC45*h5*, and the outer membranes were examined by SDS gel electrophoresis using the cacodylate-phosphate buffer system. One surprising feature of these results was that none of the transductants produced two Nmp proteins (NmpA* and NmpB or NmpB* and NmpA). The possibility that either of the two *nmp* alleles were being eliminated or mutated in these recipients was ruled out by the fact that P1 phage grown on the transductants could still transduce strain CS482 to sensitivity to colicin E3 when either *ilv*$^+$ or *pro*$^+$ recombinants were selected. Furthermore, only the NmpB or NmpB* proteins were detected irrespective of which strain was used as donor (Table 3). A second surprising feature was that when either the NmpB$^+$ strain CS484 or an NmpB* derivative (CS812) was used as donor, all of the transductants which received the *nmpB* allele were devoid of OmpA protein (Fig. 6).

P1 phage grown on strain CS741 was also used to transfer the *nmpA*(p$^+$) mutation into other *E. coli* strains. The NmpA protein was not detected in outer membranes of *E. coli* B strain 837 or the deep rough derivatives of K-12 strain P400 when tetracycline-resistant recombinants were selected (*rbs*::Tn10). In order to demonstrate that a functional *nmpA*(p$^+$) allele had been transferred to these strains we used

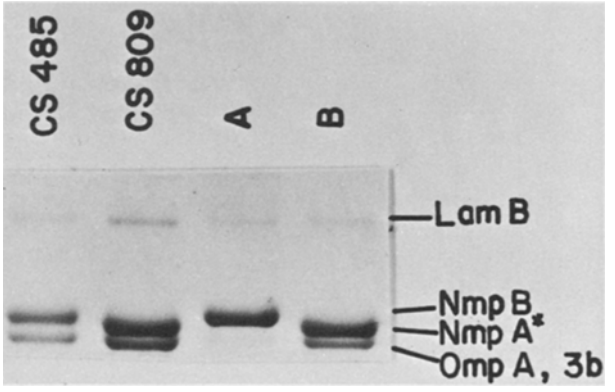

Fig. 6. Major outer membrane proteins of strains carrying the *nmpA*(p*) and *nmpB*(p$^+$) alleles. P1 phage grown on CS485 was used to transduce CS809 to *ilv*$^+$ and transductants were screened for the presence (A) or absence (B) of the *nmpB*(p$^+$) allele as described. Proteins were resolved by SDS gel electrophoresis using the cacodylate-phosphate gel system. Only the region of the gel displaying proteins with molecular weights between 33 and 42,000 is shown

P1 grown on them to transduce wild type K-12 strains to tetracycline resistance (*rbs*::Tn10) and screened the transductants for sensitivity to TC45 (Table 4). Similar results pertaining to the expression of the *nmpA* in deep rough *E. coli* K-12 mutants have also been reported elsewhere (Lugtenberg et al., 1978).

Sensitivity to Phage TC45 in Other E. coli Strains

Twenty six smooth clinical isolates of *E. coli* were screened for sensitivity to phage TC45 and its host range mutants. Six strains were sensitive to all of these phage while the others were fully resistant. Outer membranes prepared from the strains contained 2–4 peptidoglycan-associated major proteins, but none of these comigrated with the NmpAB

Table 4. Expression of *nmpA*(p$^+$) allele in deep rough mutants of *E. coli* K-12

Recipient[a]	LPS neutral sugars[b] (micromoles/mg LPS)			Major OM proteins produced[c] (% of total)				
	glucose	galactose	heptose	OmpA	OmpC	OmpF	3b	NmpA
P400	0.39	0.26	0.58	30	16	20	5	29
P239	0.02	0.01	0.26	46	2	44	8	0
P424	0.09	0	0.27	44	0	46	10	0
P435	0	0	0	45	22	24	9	0
P489	0.02	0.04	0.12	0	96	0	4	0

[a] Transductants were selected as tetracycline-resistant recipients using P1 phage grown on strain CS741 [*nmpA104*(p$^+$)]

[b] Lipopolysaccharide analysis is from Hancock and Reeves (1976) and Manning (personal communication)

[c] All of the deep rough mutants produced lower amounts of the major outer membrane proteins (molecular weights 33–42,000) relative to lipoprotein. The amount of protein present in the outer membranes was determined by scanning Coomassie-blue stained gels in which the proteins were resolved

protein in SDS gels. We therefore attempted to locate the gene(s) conferring TC45 sensitivity in these strains by two methods. P1 phage grown on these strains was used to transduce the K-12 strain CS482 to *ilv*$^+$ and *pro*$^+$. None of the transductants were sensitive to colicin E3 or phage TC45 or produced a new outer membrane protein. This may be either because the smooth *E. coli* strains did not carry *nmpA*(p$^+$) or *nmpB*(p$^+$) alleles or because these genes were not expressed upon transfer into *E. coli* K-12. In order to differentiate between these possibilities, we mated streptomycin-resistant derivatives of the smooth *E. coli* strains with Hfr strains carrying transposons close to the *nmpA* and *nmpB* genes, and screened the transconjugants for resistance to TC45. Only in one case were we able to convert the TC45-sensitive strains to TC45-resistance by this procedure. In this case, TC45-resistant recombinants were obtained after mating with the Hfr strain CS 840, and the frequency of transfer was similar to that into the NmpB$^+$ K-12 strain CS487. However, none of these recombinants showed any change in the protein composition of their outer membranes.

One interesting problem concerning sensitivity to T-even phage such as TC45 is whether there can be some ambiguity relating to receptor specificity, as, for example, in the case of phage TP1 (Wandersman and Schwartz, 1978). Chai and Foulds (1979) have already reported that bacteriophage K3, which normally binds to the OmpA protein in *E. coli* K-12, can also be inactivated by the NmpAB protein. Thus, we used a variety of other Hfr strains to try to locate the genes conferring TC45 sensitivity upon the smooth *E. coli* strains. In two cases, these strains became resistant to TC45 at high frequencies after mating with Hfr strains CS791 and CS819. This change was not due to transfer of the *ompC*::Tn10 marker itself since all of the recombinants became resistant to phage which use the OmpC protein as receptor (PA-2- hy2, TuIb, Mel and SS4). We feel that a more likely explanation may be that resistance to TC45 was conferred upon those transconjugants which received the K-12 *rfb* allele affecting synthesis of the lipopolysaccharide side chain. Transfer of this allele would block synthesis of the side chain in these recipients, and would render the cells resistant to phage which used these determinants as receptors. We also noted that resistance to phage K3 was also transferred at this locus. None of these transconjugants showed any alteration to their major outer membrane proteins other than that which could be attributed to the loss of the OmpC protein.

Discussion

The procedure adopted here for isolating mutants which produce altered pore proteins, namely that of selecting for loss of one function (phage receptor activity) and retention of another (colicin sensitivity) has also been successfully applied to the selection of mutants in another gene (*ompC*) affecting a constitutive major outer membrane pore protein (Lee, Pugsley and Schnaitman, manuscript in preparation). However, we noted that mutants which produced a protein with altered electrophoretic mobility in SDS gels appeared much more frequently among OmpC mutants than among NmpA and NmpB mutants. This may be either because less of the NmpAB protein is exposed on the surface of the cell and therefore the potential for change in the TC45 receptor area is less or because there is a particularly labile group in the *nmpA* and *nmpB* genes such that mutant proteins with altered TC45 receptor activity and apparently unaltered structure appear very readily. A

single amino acid substitution could be sufficient to produce the changes in peptide profiles, isoelectric focusing profiles and SDS gel mobility we observe in the NmpAB* proteins. If the substituted amino acid were of the same charge and hydrophobicity as that which it replaced, it may be very difficult to detect the structural change by the criteria we have applied (Pugsley and Schnaitman, 1979; de Jong et al., 1978).

Van Alphen et al. (1979) have also isolated strains carrying mutations in the *ompC* gene which produce a protein with altered electrophoretic mobility in SDS gels. This can be considered strong evidence that *ompC* is the structural gene for the OmpC protein. By analogy we might also propose that *nmpA* and *nmpB* are structural genes for outer membrane pore proteins, but further evidence is needed to confirm this. One method to do this might be to study the expression of these genes in heterologous strains, as recently reported for the *ompF* locus in *E. coli* K-12 and *Salmonella typhimurium* (Sato and Yura, 1979). It was with this aim in mind that we screened other TC45-sensitive *E. coli* strains for the production of a protein analagous to the K-12 NmpAB protein, but it may be necessary to screen other, TC45-resistant strains.

If the hypothesis that *nmpA* and *nmpB* are duplicate structural genes for the same protein is correct (Lee et al., 1979), there must still be some form of co-operative regulation. Both genes can exist in the same cell line, and it seems likely that their expression is blocked by a mutation within the gene itself rather than in the promoter since we were able to identify two apparently different forms of the proteins (high and low receptor activity for phage TC45) produced by strains mutating to NmpAB$^+$ at the *nmpA* locus. However, attempts to construct strains producing both the NmpA and NmpB proteins were unsuccessful. This may be simply due to a gene dosage effect on the promoter of one of the genes, but this does not explain why there appears to be a preference for the expression of the *nmpB* gene among the recombinants tested or why the production of the OmpA protein should also be affected. It may be important to remember that the original *nmpB105*(p$^+$) mutation was selected in a pore-deficient strain which also lacked the OmpA protein (Pugsley and Schnaitman, 1978b). The possibility that there is a third, regulatory gene affecting production of the NmpAB protein and analogous to the *ompB* gene affecting production of the OmpF and OmpC pore proteins (Palva, 1979) cannot be overlooked, but all of the identified mutations affecting the production of the NmpAB protein have so far been located close to or within the *nmpA* or *nmpB* genes.

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