JOURNAL OF BACTERIOLOGY, Sept. 1978, p. 1118-1129
0021-9193/78/0135-1118$02.00/0
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Vol. 135, No. 3
Printed in U.S.A.

Identification of Three Genes Controlling Production of New Outer Membrane Pore Proteins in *Escherichia coli* K-12

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Received for publication 15 May 1978

Escherichia coli K-12 strains carrying mutations in the *ompB* gene or double mutations in the *tolF* and *par* genes lack the major outer membrane proteins 1a and 1b. These strains are deficient in the transport of small hydrophylic compounds and are multiply colicin resistant. When revertants of these strains were sought, a number of extragenic pseudorevertants were obtained which produced new outer membrane proteins. These new proteins could be divided into three classes by differences in electrophoretic mobility on polyacrylamide gels, by differing specificities for transport of small molecules, and by the identification of three different genetic loci for genes controlling their production. These genetic loci are designated as *nmpA* (at approximately 82.5 min on the *E. coli* K-12 genetic map), *nmpB* (8.6 min), and *nmpC* (12 min). The new proteins produced in strains carrying *nmpA, nmpB,* or *nmpC* mutations did not cross-react with antiserum against a mixture of proteins 1a and 1b, or with antiserum against phage-directed protein 2. Production of the new membrane proteins restored sensitivity to some of the colicins.

The term "porin" has been coined to designate major proteins in the outer membrane of gram-negative bacteria which facilitate the passage of a variety of low-molecular-weight solutes through the outer membrane (17). Evidence for such pore proteins has come both from in vitro studies where these proteins have been shown to confer permeability to solutes to membrane vesicles (17), and from in vivo studies which have shown that mutant strains lacking these proteins are defective in transport of a number of solutes or are resistant to various toxic molecules which enter through these pores (3, 14, 22).

Wild-type strains of *Escherichia coli* K-12 produce two similar, and possibly related, proteins which function as porins (22). We designate these as proteins 1a and 1b (see reference 2 for other nomenclature systems). In addition, strains lysogenic for the lambdoid phage PA-2 produce large amounts of a different protein which we term protein 2 (22), and we have recently shown that this protein, which replaces proteins 1a and 1b in lysogens, is also a porin.

At least three genetic loci determine the production of proteins 1a and 1b. Strains carrying mutations in the *par* (*meo*) locus lack protein 1b (2, 29), and strains carrying mutations in the *tolF* (also termed *cmlB* or *cry*) locus lack protein 1a (4, 8). Strains with mutations in the *ompB* locus fail to produce either protein 1a or 1b, as do strains carrying mutations in both *par* and *tolF* (9, 12).

Strains lacking both protein 1a and 1b (either strains carrying *ompB* or *par* and *tolF* mutations) have as a phenotype multiple transport defects and resistance to several colicins (22). Such strains are at a considerable disadvantage, and revertants rapidly overgrow cultures of these strains. Some of these are true revertants at the original mutant loci. However, several laboratories have now reported phenotypic revertants which are extragenic (not at the *ompB, par,* or *tolF* locus), and such strains produce "new" major outer membrane proteins which have been designated variously as "protein 1c" (12), "E" (9), or "e" (28).

In the present study, we report the examination of a number of phenotypic revertants of strains carrying *ompB* or *par tolF* mutations which produce new membrane proteins. We have found that several genetic loci are involved in the production of these new membrane proteins, and preliminary evidence indicates that these various new membrane proteins are not identical to each other, or to previously described proteins present in wild-type *E. coli* K-12.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. The following convention has been employed in the nomenclature of strains producing new outer membrane proteins. The genetic symbol *nmp* (for new membrane protein) is used. The phenotypic designation Nmp$^+$

indicates a strain producing a new membrane protein, whereas Nmp$^-$ refers to the wild-type phenotype, or to strains which have lost the ability to produce such proteins. In allele designations, p$^+$ indicates production of a new protein, whereas p$^-$ indicates lack of production of that protein. Thus, *nmpA*(p$^+$) indicates a mutation at the *nmpA* locus which results in production of a new protein, whereas *nmpA*(p$^-$) indicates a mutation at the *nmpA* locus which results in inability to produce the new protein.

The strains of *E. coli* K-12 used in this study are listed in Table 1. The *ompA* mutations were obtained by selection for resistance to bacteriophage K3 (15), and the identity of the *ompA* mutations was confirmed by transducing strain CGSC4278 to *pyrD*$^+$ using P1 bacteriophage grown on CS146 and CS148 and demonstrating appropriate cotransduction frequencies (15). Strain PB109 was previously described as TolXIV (2), but was shown by transduction analysis to carry mutations in *tolF* and *par*. The *tolF* mutation of PB109 cotransduced at a frequency of 42% with *aroA*$^+$ into AB2829, and 54% with *pyrD*$^+$ into CGSC4278. The *par* mutation of PB109 cotransduced at 12% with *glpT*$^+$ into Lin221 (2). The phenotype of PB109 was

TABLE 1. *Escherichia coli K-12 strains*

Strain no.	Genotype/phenotype	Source and reference
W1485F$^-$	*thi*	CGSC[a]
PB109	*tolF par-13* of W1485F	P. Bassford (2)
CS146	*ompA154* of PB109	This study
CS374	*nmpB*(p$^+$) of CS146	This study
CS108	hy7 lysogen of PB109	This study
CS197	*ompB151* of W1485F$^-$	(22)
CS383	*nmpA*(p$^+$) of CS197	This study
CS228	hy7 lysogen of CS197	(22)
P1700	*thr leu proA his argE thi trp galK lacY non mtl xyl ara rpsL*	P. Reeves
CS327	*ompB156* of P1700	This study
CS384	*nmpC*(p$^+$) of CS327	This study
CS328	hy7 lysogen of CS327	This study
JF568	*ilvY227 metB65 his-53 aroA357 purE41 proC24 cyc-1 xyl-14 lacY229 rpsL77 tsx-63*	J. Foulds
CS252	hy7 lysogen of JF568	This study
CS493	*pur*$^+$ transductant of JF568 via P1 grown on W1485F$^-$	This study
CS241	*par-12* of JF568	This study
CS490	*pur*$^+$ transductant of CS241 via P1 grown on W1485F$^-$	This study
JF703	*aro*$^+$ *tolF4* transductant of JF568	J. Foulds (8)
CS489	*pur*$^+$ transductant of JF703 via P1 grown on W1485F$^-$	This study
CS209	*par-11* of JF703	This study
CS482	*pur*$^+$ transductant of CS209 via P1 grown on W1485F$^-$	This study
CS492	hy7 lysogen of CS482	This study
CS483	*nmpC*(p$^+$) *pur*$^+$ transductant of CS209 via P1 grown on CS384	This study
CS484	*nmpA*(p$^+$) *ilv*$^+$ transductant of CS482 via P1 grown on CS383	This study
CS485	*nmpB*(p$^+$) *pro*$^+$ transductant of CS482 via P1 grown on CS374	This study
CS457	Δ*cbr-51*[b]-*nmpC*(p$^-$) of CS384	This study
CS458	Δ*cbr-52-nmpC*(p$^-$) of CS384	This study
CS459	*cbr-53* of CS384	This study
CS460	*cbr-54* of CS384	This study
CS461	*cbr-55* of CS384	This study
CS462	*cbr-56* of CS384	This study
CS463	*cbr-57* of CS384	This study
CS486	*nmpA*(p$^+$) *ilv*$^+$ transductant of JF568 via P1 grown on CS383	This study
CS487	*nmpB*(p$^+$) *pro*$^+$ transductant of JF568 via P1 grown on CS374	This study
CS488	*nmpC*(p$^+$) *pur*$^+$ transductant of JF568 via P1 grown on CS384	This study
JF720	*nalA thyA deo ilvY227 metB65 his-53 purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx-63 tolF4 par nmpA*	J. Foulds (9)
RK1041	*ilv argH metB his pyrE60 cysE lac rpsL bglC mtl*	R. Kadner
CS391	*bglC*$^+$ transductant of CS383 via P1 grown on RK1041	This study
CGSC4278	*thi pyrD36 gltA6 galK30 rpsL129 supE44 relA1*	CGSC
AB2829	*aroA354 supE42*	CGSC
AN248	*entA ilvC7 argH tonA*	F. Gibson
AB2847	*aroB351 tsx malA354 supE42*	CGSC
Lin221	*phoA8 glpT6 relA1 tonA22*	CGSC
CGSC4286	*thi his-4 proA2 purB15 lip-9 mtl-1 xyl-5 galK2 lacY1 rpsL35 supE44*	CGSC

[a] CGSC, Coli Genetic Stock Center.

[b] *cbr* is probably identical to *fepA* (30) and *feuB* (10).

indistinguishable from that of CS197 (*ompB*). Strains carrying independently derived *cbr* mutations were obtained by selection for resistance to colicin D-CA23 (21). The auxotrophic strains CS443, CS448, CS449, and CS450 were derived from strain CS146 by nitrosoguanidine mutagenesis followed by ampicillin selection as described by Miller (16). All of these strains were at least partially sensitive to bacteriophage K3, presumably due to reversion at the *ompA* locus during prolonged incubation in minimal medium. Colicinogenic strains and bacteriophage K3, TuIb, TuIa, Mel, PA-2, λc1, and the PA-2 hybrids hy2 and hy7 are as previously described (15, 22). Bacteriophage TC45 was kindly supplied by J. Foulds, and was cultivated on strain CS383 (Nmp^+).

Culture media. Complex media were TY broth (1% tryptone, 0.5% yeast extract, pH 7.2), TYS broth (TY broth + 0.5% NaCl), and TYHS broth (TY broth + 2.5% NaCl). For screening for sugar fermentation, MacConkey agar base (Difco) containing 0.5% of the appropriate carbohydrate was used. Minimal media were as described previously (22).

Genetic techniques. Conjugations and transductions were performed as described by Miller (16) with streptomycin used as the counterselection agent in the conjugation experiments. In all cases a minimum of 250 recombinants were scored for unselected markers. Cotransduction frequencies are expressed as the nearest whole percent.

Colicin and bacteriophage sensitivity. Colicin and bacteriophage sensitivity were measured by cross-streak and spot dilution methods described previously (22) using cultures grown in TYS broth and plated on TYS agar. Sensitivity to chloramphenicol was determined using Difco antibiotics disks (30 μg per disk) as previously described (22) using cells grown in TY, TYS, and TYHS broths and plated on the same media plus agar. Sensitivity was determined by zone size.

Transport assays. Assays for the uptake of labeled solutes were essentially as described previously (22). Cells were grown in minimal salts medium, washed, and suspended in 0.5% glucose-minimal salts solution to a final concentration of 6×10^8 cells per ml. Glucose was omitted from cell suspensions used to measure mannitol and α-methyl glucoside uptake. All assays were performed at 37°C at a final substrate concentration of 5 μM. With the exception of AMP, which was ^{3}H-labeled, ^{14}C-labeled substrates were employed. We were unable to obtain consistent results for sulfate uptake, since the strains employed grew well in low-sulfate medium only when cysteine was added.

Polyacrylamide gel analysis of membrane proteins. Outer membrane was prepared by extraction of the envelope fraction with Triton X-100 as previously described (2), except that the outer membrane was pelleted at 46,000 × *g* for 1 h.

All polyacrylamide gel electrophoresis was carried out in slabs 0.75 mm thick and 30 cm long. Two buffer systems were employed. The first of these was the alkaline phosphate system of Bragg and Hou (25). The separation gel for this system consisted of 11.5% acrylamide, 0.21% bisacrylamide, 0.5 M urea, 0.1% sodium dodecyl sulfate (SDS), 0.032% ammonium persulfate, and 0.081% *N,N,N',N'*-trimethylenediamine (TEMED) prepared in 0.1 M phosphate buffer (pH 7.2). A sample loading gel which contained 4.5% acrylamide, 0.12% bisacrylamide, 0.1% SDS, 0.15% ammonium persulfate, and 0.1% TEMED in the same phosphate buffer was used for sample application. The samples were dissolved and then heated at 100°C for 5 min in 0.1 M phosphate buffer (pH 7.2) containing 12.5% glycerol, 1% SDS, and 1.25% 2-mercaptoethanol. The upper and lower electrophoresis buffers were alkaline and acidic phosphate buffer as previously described (25).

The second electrophoresis buffer system employed was the tris(hydroxymethyl)aminomethane (Tris)-glycine system of Laemmli (13), and with this buffer system four different separation gels were employed. The constituents of these separation gels are listed in Table 2. The separation gels were prepared in 0.375 M Tris adjusted to pH 8.8 with HCl. A stacking gel consisting of 4.5% acrylamide, 0.12% bisacrylamide, 0.1% SDS, 0.05% ammonium persulfate, and 0.05% TEMED prepared in 0.125 M Tris adjusted to pH 6.8 with HCl was used with all four systems. The upper and lower electrophoresis buffers, sample buffer, and sample preparation were as previously described (2). Electrophoresis was carried out at constant voltage at 50 V for 3 h followed by 150 V for 14 h. The slabs were maintained at 25°C throughout the electrophoresis. Gels were stained as previously described (2).

Immune precipitation. The antisera raised against a mixture of proteins 1a and 1b or against protein 2 were those described previously (7). Labeled outer membranes were prepared from cultures grown in minimal salts medium containing 0.1 μCi of [^{3}H]leucine and 10 μg of unlabeled leucine per ml. The outer membrane samples were dissolved in 50 mM Tris-chloride buffer (pH 7.2) containing 2% Triton X-100 and 5 mM ethylenediaminetetraacetic acid, and any insoluble material was removed by centrifugation at 46,000 × *g* for 30 min. Protein in the supernatant was precipitated by the addition of 3 volumes of cold acetone. The precipitated protein was redissolved in Tris-Triton-salt buffer (TTS; 7). Dilutions of antisera in TTS were mixed with outer membrane proteins (0.1 to 0.5 mg of protein) in a final volume of 1 ml of TTS and incubated for 1 h at 37°C. The mixture was then filtered through a 0.45-μm membrane filter (Millipore

TABLE 2. *Separation gels used in the Tris-glycine buffer system*

Gel system	Acrylamide (%)	Bisacrylamide (%)	Ammonium persulfate (%)	TEMED (%)	Urea (M)
A	11.5	0.21	0.015	0.08	0.5
B	15.0	0.21	0.015	0.05	0.5
C	11.5	0.45	0.15	0.05	0.5
D	9.0	0.21	0.006	0.12	8.0

Corp.), and the filter was rinsed with 5 ml of TTS, dried, and counted (19). When the immune precipitate was to be examined by polyacrylamide gel electrophoresis, the precipitate was collected by centrifugation instead of filtration and the pellet was washed twice with TTS. The pellets were then dissolved in electrophoresis sample buffer.

Outer membrane proteins from cells grown in TY broth were also examined for their ability to block the precipitation of proteins 1a and 1b by homologous antiserum. A mixture of proteins 1a and 1b labeled with [^{3}H]leucine was prepared as described previously (7), and the unlabeled proteins from the various strains grown on TY broth were prepared as described above. Preliminary experiments determined the amount of antiserum necessary to precipitate 50% of the labeled protein 1a-1b mixture (excess antigen). Various dilutions in TTS of unlabeled proteins from cells grown in TY broth were then added to this mixture to determine if these resulted in inhibition of precipitation of 1a and 1b by homologous antiserum.

Protein assays. The protein assay was a modification of that described by Schacterle and Pollack (24). Samples were suspended in 2 ml of distilled water or, in cases where the sample contained Triton X-100, in 2 ml of 5% SDS. To this was added 1 ml of a solution containing 2.0% NaOH, 10% Na_2CO_3, 0.10% $KNaC_4H_4O_6 \cdot 4H_2O$ (Rochelle salt), and 0.078% $CuSO_4 \cdot 5H_2O$ followed by 0.25 ml of 2 N Folin-Ciocalteau reagent (Fisher). The tubes were mixed vigorously and incubated for 5 min at 56°C before the optical density was measured at 650 nm. Bovine serum albumin was used as a standard.

RESULTS

Isolation and characterization of Nmp$^+$ strains. The loss of proteins 1a and 1b results in high-level resistance to colicins A, E2, E3, K, L, S4, and X as well as to a number of bacteriophage which use these proteins as receptors (22). We have previously demonstrated that lysogeny of strains missing these proteins with derivatives of bacteriophage PA-2 and the resulting production of protein 2 results in a restoration of colicin E3 sensitivity (22). This observation was used as a basis for screening for revertants of strains carrying *ompB* or *par tolF* mutations. Colicin E3-sensitive phenotypic revertants of strains CS146, CS197, and CS327 appeared readily in cultures which had been incubated either in broth or on agar slants for several days at 37°C. When the colicin and bacteriophage sensitivities of such revertants were examined, these fell into two classes. One class of revertants was fully sensitive to colicins A, E2, E3, K, L, S4, and X (only partial sensitivity to K and L was observed in such revertants obtained from CS146, which also carried an *ompA* mutation), and some of these revertants were also sensitive to bacteriophage TuIa, TuIb, Mel, and hy2. When the outer membrane proteins of these revertants were examined by gel electrophoresis (Tris-glycine system A), these were found to produce both protein 1a and 1b in the case of revertants of CS197 or CS327, or protein 1a in the case of revertants of CS146. Thus, these revertants appear to be the result of reversion at the *ompB* or *tolF* loci.

The second class of phenotypic revertants remained resistant to colicins K, L, S4, and X, and to bacteriophage TuIa, TuIb, Mel, and hy2, but were sensitive or partially resistant to colicins A, E2, and E3. When the outer membranes of these revertant strains were examined by gel electrophoresis using either the Bragg-Hou system (data not shown, see reference 26) or Tris-glycine system A, they were found to produce a new outer membrane protein with an electrophoretic mobility similar to that of protein 2 (Fig. 1A).

When the outer membrane proteins of these Nmp$^+$ strains were examined on Tris-glycine gels in which the ratio of acrylamide to bisacrylamide was changed (systems B and C) or when 8 M urea was added to the separation gel (system D), it was found that these new outer membrane proteins could be distinguished from protein 2 by small, but significant, changes in electrophoretic mobility. Moreover, when a number of such revertants isolated in strains with different genetic backgrounds were compared, it was found that the strains producing new membrane proteins could be grouped into three distinct classes (NmpA$^+$, NmpB$^+$, and NmpC$^+$) based on the electrophoretic mobility of the new membrane proteins which were produced (Fig. 1B, C, and D). The inclusion of high urea in the separation gel had a particularly profound effect on the relative electrophoretic mobilities of proteins 1b, 2, and 3b as well as on the mobility of the new membrane proteins. A single representative strain of each class of revertants (strains CS383, NmpA$^+$; CS374, NmpB$^+$; and CS374, NmpC$^+$) was selected for further study.

Once the loci which determined the production of the new membrane proteins in each of these classes were mapped (see below), it was possible to construct strains carrying mutations at each of these loci in an otherwise isogenic *tolF par* background. When these strains [strain CS484, *nmpA*(p$^+$); strain CS485, *nmpB*(p$^+$); and strain CS483, *nmpC*(p$^+$)] were compared to the original revertant strains, it was found that the new membrane proteins exhibited identical electrophoretic mobility, indicating that the characteristic electrophoretic mobility of each of the three classes of new membrane proteins was not determined by the genetic background of the strains in which the *nmp* mutations were isolated. The availability of strains carrying the

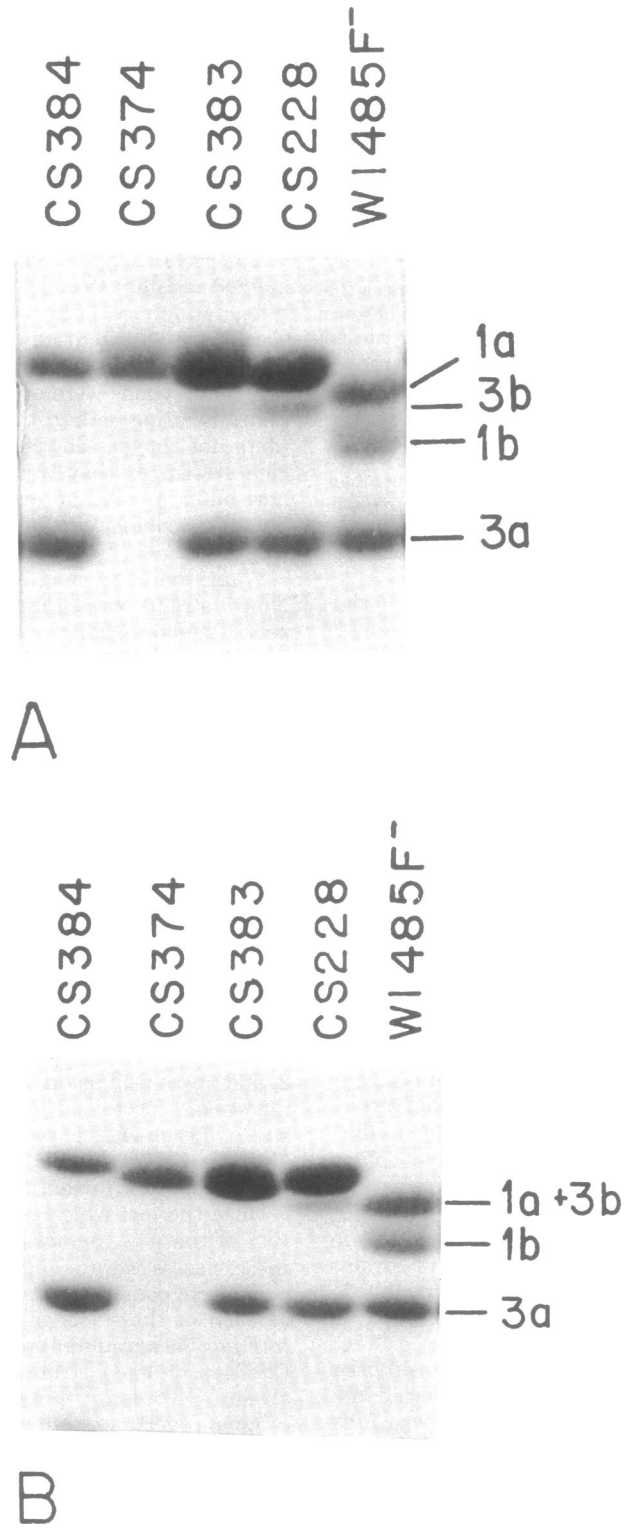
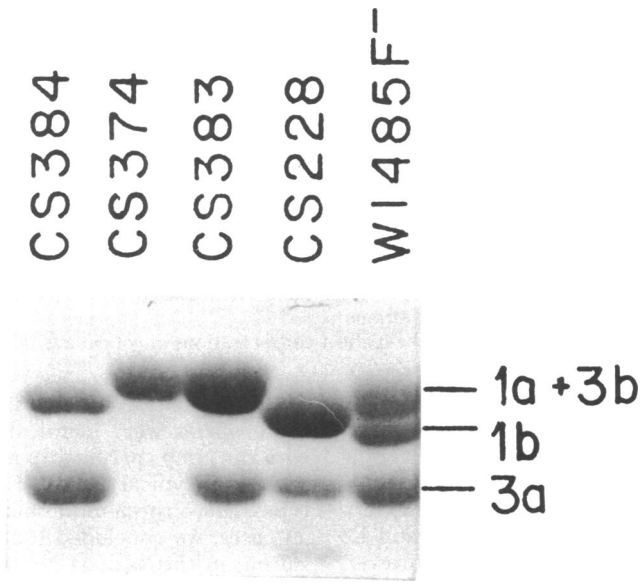


FIG. 1. *Polyacrylamide gel electrophoresis of outer membrane proteins of strains CS384 [ompB nmpC(p⁺)], CS374 [tolF par nmpB(p⁺)], CS383 [ompB nmpA(p⁺)], CS228 (ompB hy7 lysogen), and W1485F⁻ grown in TY broth. Only the region of the gels showing the 33,000- to 40,000-dalton outer membrane proteins are shown. (A–D) Patterns obtained using systems A through D as listed in Table 2. Outer membrane from strain P1700 (the parent strain for CS384) gave an identical pattern to that of W1485F⁻.*


CS384
CS374
CS383
CS228
W1485F⁻
1a +3b
1b
3a
C

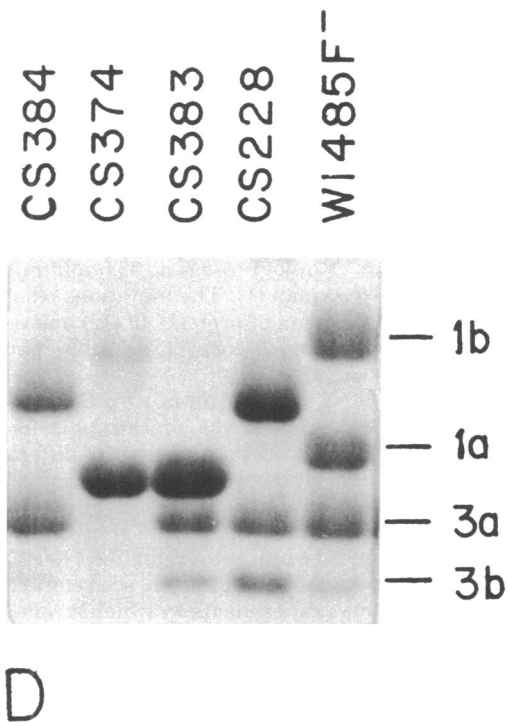

CS384
CS374
CS383
CS228
W1485F⁻
1b
1a
3a
3b
D

FIG. 1. *C, D*

TABLE 3. *Relative colicin resistance of strain CS482 (tolF par), isogenic Nmp⁺ derivatives of this strain, and a PA-2 lysogen of this strain*

Strain and relevant genotype	Relative resistance to colicins[a]		
	A-CA31	E2-CA42	E3-CA38
CS493 (wild type)	1	1	1
CS482 (*tolF par*)	$>2 \times 10^{3}$ [b]	$>3.2 \times 10^{4}$	$>1.6 \times 10^{5}$
CS484 [*tolF par nmpA*(p⁺)]	$>2 \times 10^{3}$	8	8
CS485 [*tolF par nmpB*(p⁺)]	$>2 \times 10^{3}$	8	8
CS483 [*tolF par nmpC*(p⁺)]	32	64	8
CS492 (*tolF par* hy7 lysogen)	$>2 \times 10^{3}$	32	8

[a] The relative resistance is the colicin titer in arbitrary units as measured with the wild-type strain divided by the colicin titer as measured with the mutant strains.

[b] The symbol > denotes full resistance to the highest concentrations of colicin available.

various *nmp*(p⁺) mutations in an otherwise isogenic *tolF par* background allowed us to determine the effect of each of the new membrane proteins on colicin sensitivity. As seen in Table 3, all three classes of Nmp⁺ strains were substantially more sensitive to colicins E2 and E3 than the Nmp⁺ *tolF par* strain CS482. However, only CS483 (NmpC⁺) was measurably more sensitive to colicin A. The Nmp⁺ strains remained fully resistant to colicins K, L, S4, and X, and remained sensitive to colicins B, D, G, H, Ia, Ib, M, S1, and V. We also examined strain JF720, a derivative of the Nmp⁺ strain described by Foulds and Chai (9), and found this strain to be almost fully sensitive to colicins E2 and E3, and therefore similar to our NmpA⁺ and NmpB⁺ mutants.

Chai and Foulds (5) isolated phage TC45, which grew on their Nmp⁺ strains but which did not grow on Nmp⁻ strains. We found that two of the three classes of Nmp⁺ strains (NmpA⁺ and NmpB⁺) are sensitive to this bacteriophage. Bacteriophage TC45 does not grow on NmpC⁺ strains, including NmpC⁺ transductants of CS209, and it does not grow on any of the Nmp⁻ strains of *E. coli* B or *E. coli* K-12 which we have tested. NmpA⁺ and NmpB⁺ transductants of strain CS209 are sensitive to TC45. This bacteriophage also grows on all of the Nmp⁺ strains reported by the laboratories of Lugtenberg et al. (28), Henning et al. (12) and Foulds (9; and personal communication).

Unlike strain JF720 (9), none of our Nmp⁺ strains became resistant to λ or to hy8 (a PA-2 hybrid with λ host range).

Preliminary experiments indicated that the *ompB* mutations in strains CS383 (NmpA⁺) and CS384 (NmpC⁺) could be transduced into strain AB2847 (*aro*⁺ or *mal*⁺ were the selected markers), and both the *tolF* and *par* mutations of strain CS374 (NmpB⁺) could be transduced into strains CGSC4278 and AB2829 (selecting *pyr*⁺ and *aro*⁺) and into strain Lin221 (selecting *glp*⁺). The cotransduction frequencies for all of these alleles were identical to those observed with the Nmp⁻ parent strains, and the transductants had typical OmpB, Par, or TolF phenotypes. Since these mutations were conserved in all cases, we concluded that the mutations resulting in the various Nmp⁺ phenotypes were located elsewhere on the chromosome.

Mapping of *nmpA*. The gene controlling production of the NmpA⁺ phenotype in strain CS383 cotransduces with *ilv* and *bglC* (Table 4) and is thus similar to the locus described by Foulds and Chai (9; and personal communication). The possible identity of these genes was further demonstrated by transducing strain JF720 [*par tolF nmp*(*p*⁺)] to *ilv*⁺ with P1 grown on CS383 [*ompB nmpA*(p⁺)]. All of the 1,600 transductants tested were sensitive to bacteriophage TC45 (i.e., Nmp⁺), indicating that the two genes are very closely linked or identical. Analysis of the frequencies of the various classes of recombinants obtained in the three-factor crosses shown in Table 4 indicated that the gene order is *bglC-nmpA-ilv*, with *nmpA* very closely linked to *bgl* at 82.5 min on the *E. coli* genetic map (1). The mutations resulting in the production of proteins Ic (12) and e (9) also map at the *nmpA* locus (J. Foulds, personal communication).

Mapping of *nmpB*. Since the NmpB⁺ strain CS374 was prototrophic, it was necessary to construct a number of different auxotrophic derivatives to use as recipients in conjugation crosses with various Hfr strains. Results of these crosses indicated that the *nmpB* locus was between the origins of HfrP4X and Hfr Cavalli (data not shown, see reference 1). Transduction experiments in which strain CS374 was used as the donor and strain CS209 was used as the recipient indicated cotransduction of *nmpB*(p⁺) with *proC* and *lacY*. Analysis of the frequencies of recombinant classes obtained in four-factor crosses (Table 4) indicate that the gene order is

lacY-nmpB-proC-tsx, with the *nmpB* locus at approximately 8.6 min on the *E. coli* genetic map (1).

Mapping of *nmpC*. The approximate location of the *nmpC*(p^+) mutation in strain CS384 was identified by gradient transfer conjugation experiments (16) using various Hfr strains as donors. These experiments demonstrated that *nmpC* was closely linked to *cbr*, and this was confirmed by the cotransduction of *cbr* and *nmpC* into strain CS209 with selection for *purE*$^+$.

As a coincidence of the selection of colicin D-resistant (*cbr*) mutants of strain CS384, we obtained two independently selected mutant strains (CS457 and CS458) which, in addition to becoming resistant to colicins B and D, also became resistant to colicins A, E2, and E3 and no longer produced the new outer membrane protein (Nmp^-). This led us to suspect that these strains carried deletions of part or all of the *cbr* and *nmpC* genes, as well as the insertion sequence which is the origin of Hfr Cavalli (1). The evidence for such deletions is that the cotransduction frequency between *cbr* and *purE* is much greater with these strains than is observed with other Cbr^- derivatives of strain CS384 which remained $NmpC^+$ (Table 4). Furthermore, the *cbr* mutations of strains CS457 and CS458 cotransduced with *lip*$^+$ into strain CGSC4286 with approximately the same frequency (6 to 15%) as other *cbr* mutations (18).

Strains CS457 and CS458 became Ent^- as indicated by their inability to secrete entero-

TABLE 4. *Transduction data for the nmpA, nmpB, and nmpC genes*

P1 donor	Recipient	Selected allele	Cotransduction of unselected alleles	
			Allele	%
CS383 [*nmpA*(p^+)]	RK1041 (*ilv bglC*)	*ilv*	*nmpA*(p^+)[a]	46
			bglC	44
CS391 [*nmpA*(p^+) *bglc*]	AN248 (*ilvC*)	*ilvC*	*nmpA*(p^+)[a]	48
			bglC	44
CS391 [*nmpA*(p^+) *bglC*]	CS209 (*ilvY*)	*ilvY*	*nmpA*(p^+)[b]	40
			bglC	33
RK1041 [*ilv bglC*]	CS383 [*nmpA*(p^+)]	*bgl*	*nmpA*(p^+)[c]	96
			ilv	44
CS374 [*nmpB*(p^+)]	CS209 (*proC lacY tsx*)	*proC*	*nmpA*(p^+)[b]	81
			tsx	62
			lacY	10
CS374 [*nmpB*(p^+)]	CS209 (*proC lacY tsx*)	*lacY*	*nmpB*(p^+)[b]	17
			tsx	6
			proC	14
CS457 [Δ*cbr-nmpC*(p^-)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	0
			cbr	72
CS458 [Δ*cbr-nmpC*(p^-)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	0
			cbr	78
CS459 [*cbr-nmpC*(p^+)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	67
			cbr	10
CS460 [*cbr-nmpC*(p^+)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	61
			cbr	22
CS461 [*cbr-nmpC*(p^+)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	61
			cbr	14
CS462 [*cbr-nmpC*(p^+)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	64
			cbr	17
CS463 [*cbr-nmpC*(p^+)]	CS209 (*purE*)	*purE*	*nmpC*(p^+)[d]	65
			cbr	15

[a] Scored as bacteriophage TC45 sensitive.
[b] Scored as bacteriophage TC45 and colicin E3 sensitive.
[c] Scored as bacteriophage TC45 and colicin E3 resistant.
[d] Scored as colicin E3 sensitive.

chelin when grown in minimal medium in the absence of iron and citrate (20). 2,3-Dihydroxybenzoic acid was, however, secreted and this, together with the transduction data (Table 4), indicates that the gene order is *purE-nmpC-[cbr-entDEF-fepB]-entABC-lip*. This places *nmpC* at approximately 12 min on the *E. coli* K-12 map (1).

Isolation of Nmp$^-$ mutants. Nmp$^-$ derivatives of strains CS483 (NmpC$^+$), CS484 (NmpA$^+$), and CS485 (NmpB$^+$) were isolated by selection for resistance to colicin E3 in the presence of 50 μM $AgNO_3$ (22) or for resistance to phage TC45. Seven independently isolated Nmp$^-$ derivatives of each strain were selected for further study. All of the Nmp$^-$ derivatives became resistant to colicins A, E2, and E3 and no longer produced the characteristic new outer membrane proteins.

Bacteriophage P1 grown on each of these Nmp$^-$ derivatives was used to transduce strain CS209 with selection for *ilv*$^+$, *pro*$^+$, and *pur*$^+$ markers. P1 bacteriophage grown on the NmpA$^-$ and NmpB$^-$ strains were unable to transduce strain CS209 to Nmp$^+$, indicating that the mutations in these strains were within the *nmpA* and *nmpB* loci. However, four of the seven NmpC$^-$ mutants were able to function as donors in tranducing strain CS209 to NmpC$^+$, indicating that the mutations resulting in loss of the ability to produce a new outer membrane protein in these strains were not located at the *nmpC* locus. The remaining three mutants were unable to transduce CS209 to NmpC$^+$ and are presumed to be at the *nmpC* locus.

New membrane proteins as pores. Since we previously demonstrated that production of protein 2 as a consequence of phage lysogeny could restore outer membrane permeability to strains of *E. coli* K-12 lacking proteins 1a and 1b (22), it was of interest to determine if the new outer membrane proteins produced by NmpA$^+$, NmpB$^+$, and NmpC$^+$ would function similarly in restoring permeability. This was determined by examining the uptake of various solutes and the chloramphenicol sensitivity of strains carrying various mutations affecting the outer membrane in an otherwise isogenic background.

The results in Table 5 indicate that the new membrane proteins produced by NmpA$^+$, NmpB$^+$, and NmpC$^+$ strains can restore the ability to transport solutes which is lost as a result of the absence of proteins 1a and 1b. However, these results indicate that there may be some specificity of each of these new proteins with respect to the type of compound which can permeate the outer membrane. As noted by others (28), protein 1a permits permeation of the outer membrane by nucleotides, whereas protein 1b does not. Thus, the new proteins produced in NmpA$^+$ and NmpB$^+$ strains resemble protein 1a in that the presence of these proteins allows permeation of AMP, whereas the new protein present in NmpC$^+$ strains and protein 2 present in phage lysogens resemble protein 1b in that these proteins do not appear to restore AMP uptake. This also appears true of chloramphenicol uptake (data not shown). The presence of the proteins produced by NmpA$^+$ and NmpB$^+$ strains restored sensitivity to chloramphenicol to mutants lacking proteins 1a and 1b, whereas protein 2 and the new protein produced by the NmpC$^+$ strain did not.

There is another similarity between these new membrane proteins and other proteins produced by wild-type *E. coli* K-12 which have been shown to function as pores. Proteins 1a, 1b, and the phage λ receptor, all of which are known to function as pores, share the property that these proteins remain associated with the peptidoglycan when the envelope fraction of the cell is extracted under mild conditions with SDS in the

TABLE 5. *Transport specificities of outer membrane pores measured by uptake of solutes*

Strain and relevant genotype	Relative initial uptake rate[a]					
	Serine	Glutamine	AMP[b]	Putrescine	α-Methyl glucoside	Mannitol
CS493 (wild type)	1 (710)	1 (530)	1 (90)	1 (88)	1 (210)	1 (1,300)
CS482 (*tolF par*)	0.32	0.28	0.09	0.35	0.69	0.53
CS484 [*tolF par nmpA*(p$^+$)]	1.14	0.83	1.38	0.54	1.00	0.96
CS485 [*tolF par nmpB*(p$^+$)]	1.15	0.94	1.30	0.91	1.09	0.97
CS483 [*tolF par nmpC*(p$^+$)]	0.91	0.66	0.17	0.97	0.89	0.77
CS492 (*tolF par* hy7 lysogen)	1.09	0.76	0.23	0.86	1.09	0.97
CS490 (*par*)	1.00	0.70	0.94	0.89	1.04	0.97
CS489 (*tolF*)	1.12	1.11	0.13	1.01	1.04	0.99

[a] Results are given as uptake rates relative to the wild-type strain CS493. Figures in parentheses are the actual uptake rates in picomoles per 10^9 cells per min.

[b] All strains were *tsx*, and were therefore defective in at least one other known outer membrane nucleotide transport system (11, 28).

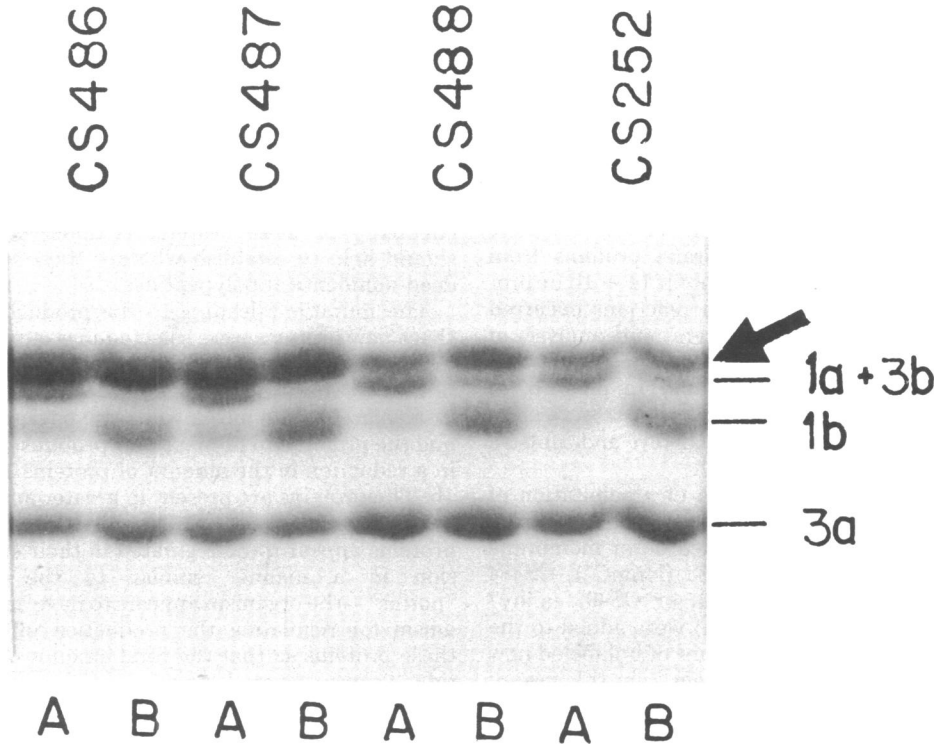


FIG. 2. *Polyacrylamide gel electrophoresis of outer membrane proteins of strains CS486 [nmpA(p⁺)], CS487 [nmpB(p⁺)], CS488 [nmpC(p⁺)], and CS252 (hy7 lysogen) after growth in TY broth (no NaCl; A) or TYHS broth (2.5% NaCl; B). Arrow indicates position of protein 2 or the new membrane proteins produced by the Nmp⁺ strains. The gel system used was system A (Table 2). Only the region of the gel displaying the 33,000- to 40,000-dalton outer membrane proteins is shown.*

presence of Mg (2, 22). The new outer membrane proteins produced by NmpA⁺, NmpB⁺, and NmpC⁺ strains are all similarly peptidoglycan associated.

Expression of the new membrane proteins. Previous studies have demonstrated that the relative amounts of proteins 1a and 1b in the outer membrane of *E. coli* K-12 depend upon the osmolarity of the culture medium (22, 27). Although protein 2 and the new membrane proteins produced by the Nmp⁺ strains resemble either protein 1a or protein 1b in their pore functions, the relative amounts of these proteins were not affected by changes in the osmolarity of the medium (Fig. 2).

As demonstrated in Fig. 2, the new outer membrane proteins characteristic of the various *nmp*(p⁺) mutations are produced in reasonably large amounts in strains which are wild type at the *ompB*, *par*, and *tolF* loci. Just as is observed in strains which produce protein 2, the strains which produce new membrane proteins produce less protein 1a and 1b, so that the total amount of peptidoglycan-associated protein remains constant at between 30 and 40% of the total outer membrane protein. We have observed a similar reduction in proteins 1a and 1b when cells are induced with maltose to produce large amounts of λ receptor (D. Palermo and C. Schnaitman, unpublished results). On the other hand, in an *ompB* or *tolF par* background, the amount of each of the new membrane proteins which is present in the outer membrane is increased, so that it is roughly comparable to the amount of protein 1a + 1b present in OmpB⁺, Par⁺, TolF⁺ Nmp⁻ strains.

Lack of antigenic cross-reactivity between proteins 1a, 1b, and 2 and the new membrane proteins. Foulds and Chai (9) reported that the new outer membrane protein produced by their Nmp⁺ strain cross-reacted with antiserum prepared against "matrix protein" (a mixture of proteins 1a and 1b). Since we had antisera prepared against a highly purified mixture of proteins 1a and 1b, and also against protein 2 (7), we examined the various new membrane proteins to determine if they cross-reacted with these antisera.

We were unable to detect cross-reactivity between either anti-protein 1 or anti-protein 2 sera

and the new proteins produced by NmpA$^+$, NmpB$^+$, or NmpC$^+$ strains. Outer membrane proteins labeled with [^{3}H]leucine prepared from strains CS383 (NmpA$^+$), CS374 (NmpB$^+$), and CS384 (NmpC$^+$) showed only a small amount of nonspecific precipitation with either antiserum, and when analyzed on gels, the small amount of precipitate obtained did not contain the new membrane proteins. When these antisera were reacted with outer membrane proteins from strains producing either protein 1a + 1b or protein 2, strong precipitation reactions occurred with the homologous antisera, and analysis of the precipitates by gel electrophoresis indicated that they consisted of protein 1a plus 1b, or protein 2, respectively. The patterns were similar to those reported by Dankert and Hofstra (6).

In addition, no inhibition of precipitation of labeled protein 1a + 1b by anti-protein 1 serum was observed when unlabeled outer membrane proteins from strains CS483 (NmpC$^+$), CS484 (NmpA$^+$), CS485 (NmpB$^+$), or CS492 (a hy7 lysogen of a *par tolF* strain) were added to the reaction mixture. The amount of unlabeled new membrane protein (or protein 2, in the case of CS492) was several times that of the protein 1a + 1b at the lowest dilutions employed, and the reaction was made more sensitive by the fact that it was carried out at slight antigen excess (see Materials and Methods). From this experiment it was concluded that there was no cross-reactivity between the new membrane proteins and antiserum against protein 1a + 1b. The reason for the discrepancy between our results and those of Foulds and Chai (9) is not known at present.

DISCUSSION

The results presented here indicate that at least three distinct genetic loci control some aspect of the production by *E. coli* K-12 of new major outer membrane proteins which are not normally expressed in wild-type strains. The loci which we have identified (*nmpA, nmpB, nmpC*) are not linked to other identified loci which determine outer membrane protein production. Since this study was not exhaustive, it is likely that additional loci (and proteins produced as a consequence of mutations at these loci) will be found.

The new outer membrane proteins which are expressed as a result of mutations at the three loci we have identified all appear to function as pores permitting passage of solutes across the outer membrane, and all of these proteins are peptidoglycan associated. Thus, these new proteins appear to have the same functional properties as proteins 1a and 1b in wild-type strains. The new proteins all appear to differ from proteins 1a and 1b in antigenic reactivity as well as in migration on gels. The proteins also appear to differ from each other, not only in terms of migration on gels, but also in terms of their pore function and their ability to mediate infection by phage TC45. A more detailed study of the chemistry of these proteins is underway and should help to establish whether these are indeed nonidentical polypeptides.

The mutations leading to the production of these new proteins were selected in strains lacking proteins 1a and 1b. However, the new membrane proteins are produced when the *nmp* mutations are moved into a wild-type background, and the production of these new proteins results in a reduction in the amount of proteins 1a and 1b. The proteins are present in greater amounts in strains lacking proteins 1a and 1b. Thus, these proteins appear to be regulated in their expression in a manner similar to the other "porins"—the organism appears to have a mechanism for regulating the production of all of these proteins so that the total amount of peptidoglycan-associated (or pore) protein remains fairly constant at a given growth condition without regard to which proteins are being expressed.

The most reasonable interpretation of our results is that there are, at various points on the *E. coli* chromosome, a number of "silent" structural genes for major outer membrane proteins which can serve as functional replacements for proteins 1a and 1b. Our studies have not yet shown whether the three *nmp* loci which we have identified are actually structural genes, but the fact that we can isolate Nmp$^-$ derivatives which carry mutations at these three loci is consistent with the hypothesis that these are structural genes. In one sense, it seems unlikely that the *nmp*(p$^+$) mutations which we have studied are mutations in regulatory genes. The mutants exhibit an "all or none" expression of the new membrane proteins. In strains which are wild type for the *nmp* loci, we see no evidence on gels of any protein corresponding to the new membrane proteins, and (in the case of NmpA$^-$ and NmpB$^-$ strains) the strains are fully resistant to phage TC45. We observe the same phenotype in the various *nmp*(p$^-$) strains—mutations at the various *nmp* loci result in a complete loss in the ability to produce the new membrane proteins. However, when strains carry *nmp*(p$^+$) mutations, they express the new membrane proteins at high levels, similar to the level of protein 1a + 1b found in wild-type strains.

It seems likely that it would be advantageous

for *E. coli* to carry a number of "silent" genes for outer membrane pore proteins on its chromosome. Pore proteins are essential for normal outer membrane solute permeability, and thus the organism cannot survive without them. At the same time, these proteins are exposed on the cell surface, where they serve as receptors for phage and mediate the lethal action of colicins. Thus, the ability of the organism to express a new pore protein which did not have the same phage receptor activity or mediate the same response to colicins would be of considerable survival value. It will be of interest to determine the mechanism by which these new membrane protein genes are regulated and the type of genetic or physiological events which are required for the expression of these silent genes.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM-18006 from the National Institute of General Medical Sciences.

We thank John Foulds for communicating results prior to publication.

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