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The *tolC* Locus of *Escherichia coli* Affects the Expression of Three Major Outer Membrane Proteins

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tolC mutants, which are resistant to colicin E1 and also highly sensitive to detergents and dyes, were shown to lack the OmpF outer membrane protein. There was little effect on transcription as judged by the use of an *ompF-lac* operon fusion strain, and the *tolC* effect was probably due to a post-transcriptional effect. The NmpC protein and protein 2 were also *tolC* dependent.

The outer membrane of *Escherichia coli* K-12 is composed of proteins, lipopolysaccharide, and phospholipids, and the protein composition of the outer membrane can be varied by changing the growth conditions (3, 15, 31) or by mutation (28).

Two of the *E. coli* K-12 outer membrane proteins, OmpF and OmpC, act as general porins (24), as these proteins allow the passage of low-molecular-weight, soluble molecules across the outer membrane. *ompF* and *ompC* are the structural genes for these two porins (10, 21), and a third locus, *ompB* (29, 34), acts as a positive regulatory gene at the transcriptional level (10, 11). Mutation at *ompF* or *ompC* results in the loss of the respective protein; both proteins are lost if the mutation is at *ompB* (27). Other mutations are known to affect the expression of *ompF*, namely, *perA* (36) and *tpo* (35), which are close to *ompB* on the chromosome. These appear to be pleiotropic in that they affect the expression of other outer membrane proteins and periplasmic proteins. Lipopolysaccharide and phospholipid mutations also affect the outer membrane protein composition (8, 14, 18), as does lysogenic conversion (26).

Mutants of the *tolC* locus were first described by Clowes (6), and mutants of this type have been isolated many times since (7, 22, 23, 37). The locus is at 66 min on the genetic map and is linked to *metC* (2, 37). The mutants have a pleiotropic phenotype in that they are resistant specifically to colicin E1 and are hypersensitive to dyes, detergents, and antibiotics (7). In this paper we show that the *tolC* mutation prevents the expression of three porin proteins (OmpF, OmpC, and protein 2) and that most of this effect is likely to be at a post-transcriptional control level.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used in this work are listed in Table 1. P1700 was made from P400: (i) it was made *his*$^-$ by transduction from AB1133 while retaining the *non* mutation; (ii) the *supE* mutation was removed by transferring in F' *gal*, selecting a nonsuppressor derivative, and curing the strain of F'*gal*; and (iii) an F'*trp* carrying the *trp* amber mutation of CSH4 was transferred in, a *trp*$^-$ derivative was selected, and the strain was cured of F'*trp*.

Media. The regular nutrient medium used in this laboratory is nutrient broth (Difco) made up double strength (16 g/liter) with added sodium chloride (5 g/liter); in this paper it is referred to as NaCl NB to distinguish it from nutrient broth made up as directed, which is referred to as NB. NaCl NB was used unless otherwise specified. TSB is tryptic soy broth (Difco or GIBCO). Nutrient agar (NA) is Difco blood base agar without the addition of blood. All incubations were done at 37°C unless otherwise specified.

Chemicals. All chemicals were of the highest grade available. Oxytetracycline (Gist Brocade) or rolitetracycline (Hoechst) were used at 16 μg/ml in NA plates. Sodium deoxycholate was used at 0.05% (wt/vol) in NA plates.

Phage sensitivity. Phage sensitivity was tested by streaking the bacterial culture at right angles across a streak of a phage suspension on an NA plate and then incubating the plate at 37°C overnight.

Colicin sensitivity. Colicin sensitivity testing was performed by cross-streaking the test cultures against a streak of the colicinogenic strain which had been grown on NA overnight, chloroformed, and overlaid with 10 ml of NA (7). A colicin E1 preparation was also used for testing cultures; this was prepared by sonicating a UV-irradiated culture of the colicin E1 producer, centrifuging it to remove unbroken cells, and filter sterilizing the supernatant, which was then stored at −20°C.

P1 transduction. Transductions were done with P1 *cml clr*100 or P1 *kc* as described by Miller (20), except that citrate was omitted.

Isolation of *tolC* mutants. The *tolC* mutants were isolated by selection for resistance to colicin E1: the *tolC* mutants were distinguished by their colicin resistance pattern (7). All *tolC* mutations showed linkage to *metC* (37). P2495 was isolated from a P1700::Tn*10* randomly integrated culture, generated with λNK561 (16): the Tn*10* insertion is 100% linked to the colicin E1 resistance gene and is also linked to *metC*. All of the

TABLE 1. *E. coli* K-12 strains used[a]

Strain	Characteristics	Reference, source, or construction
AB1133	F⁻ *thr-1 leu-6 proA2 lacY1 supE44*(?) *galK2 his-4 rpsL31 xyl-5 mtl-1 argE3 thi-1 ara-14*	A. L. Taylor
P602	AB1133 *tolC203*	7
P400	AB1133 *his*⁺ *non-9*	32
P456	P400 *ompF680*	12
P1533	P400 *ompC*	Laboratory collection
C600	F⁻ *thr-1 leu-6 tonA21 lacY1 supE44 thi-1*	W. Arber
A586	F⁻ *thr-1 leu-6 tonA21 supE44 thi-1 pro-43 tolC3*	S. E. Luria
W1485 F⁻	F⁻ prototroph	C. Schnaitman
P2731	W1485 F⁻ *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ W1485F⁻, Tc^r
P1700	F⁻ *thr-1 leu-6 proA2 lacY1 galK2 trp his-4 non-9 rpsL31 xyl-5 mtl-1 argE3 thi-1 ara-14*	This paper
P2495	P1700 *tolC210*::Tn*10-48*	This paper
SA500	F⁻ *his rpsL*	S. Gottesman
P2727	SA500 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ SA500, Tc^r
SA1500	F⁻ Δ*lon his rpsL*	S. Gottesman (9)
P2728	SA1500 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ SA1500, Tc^r
C5 (CGSC 4934)	HfrPO2 *tonA22 phoR17 relA1* T2^r	B. Bachmann
P2724	C5 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ C5, Tc^r
P1927	W1485 F⁻ *gyrA lam*hy7 lysogen	C. Schnaitman
P2725	P1927 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ P1927, Tc^r
CS483	*lacY229 proC24 tsx nmpC*(p⁺) *ompF4 his-53 ompC11 rpsL77 xyl-14 metB65 cycA1 ilv*	C. Schnaitman
P2726	CS483 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ CS483, Tc^r
P530	AB1133 *ompB101*	7
MH70	*araD139* Δ*lacU169 relA rpsL malQ7 thiA flbB*	M. Hall (11)
P2712	MH70 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ MH70, Tc^r
MH513.1	*araD139* Δ*lacU169 relA rpsL malQ7 thiA flbB* Φ(*ompF-lacZ*⁺)*16-13*	M. Hall (11)
P2713	MH513.1 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ MH513.1, Tc^r
P2748	MH513.1 *mal*⁺ *ompB101*	P530 $\xrightarrow{P1}$ MH513.1, mal⁺
MH221.1	*araD139* Δ*lacU169 relA rpsL malQ7 thiA flbB* Φ(*ompC-lacZ*⁺)*10-21*	M. Hall (11)
P2714	MH221.1 *tolC210*::Tn*10-48*	P2495 $\xrightarrow{P1}$ MH221.1, Tc^r
P2749	MH221.1 *mal*⁺ *ompB101*	P530 $\xrightarrow{P1}$ MH221.1, mal⁺
AT2446 (CGSC 4504)	HfrPO1 *relA metC69 thi*	B. Bachmann

[a] Abbreviations: $\xrightarrow{P1}$, P1 transduction, from donor to recipient; Tc^r, selected for tetracyline resistance; mal⁺, selected on maltose as sole carbon source.

tolC mutants are sensitive to 0.05% (wt/vol) sodium deoxycholate in NA plates (37).

Small-scale cell envelope preparation. Envelope preparation was performed by a modification of the method of Ito et al. (14): 10 ml of culture (optical density at 625 nm, 0.5 to 1.0) was centrifuged (Sorvall SM24) at 10,000 rpm for 10 min and then washed in 10 ml of 30 mM Tris-hydrochloride (pH 8.1). The pellet was suspended (sometimes after storage at −20°C) in 0.2 ml of 20% (wt/vol) sucrose in 30 mM Tris-hydrochloride (pH 8.1). A 0.02 ml portion of lysozyme (freshly made at 10 mg/ml in 0.1 M EDTA, pH 7.3) was added, and after incubation for 30 min on ice, 3 ml of 3 mM EDTA (pH 7.3) was added. This mixture was sonicated for 20 s and centrifuged at 15,000 rpm for 60 min. The membrane pellet was suspended in 0.2 ml of Lugtenberg sample-solubilizing buffer (17). The supernatant was precipitated with 5% (wt/vol) trichloroacetic acid and centrifuged; the pellet was washed with acetone, dried, and suspended as described for the membrane pellet. This is the soluble protein fraction.

One-liter-scale membrane preparation. Membrane preparations were made essentially as described by Schnaitman (30). Whole membrane is the cell envelope derived by high-speed centrifugation; outer membrane is the Triton X-100-insoluble material; inner membrane is the ethanol precipitate of the Triton X-100-soluble material.

Sodium dodecyl sulfate-PAGE. Polyacrylamide gel electrophoresis (PAGE) was performed as described

by Lugtenberg et al. (17) with modifications. A mixture of BDH 30175 and BDH 30176 or BDH 44244 sodium dodecyl sulfate was used (19). Samples were also run on 11 to 20% polyacrylamide gradients as previously described (1) and on gels as described above but with 4 M urea in the running gel. Samples were heated at 100°C for 3 min before loading; the gels were stained as previously described (1). The amounts of the major outer membrane proteins present were estimated by scanning the stained gel in a Quick Scan densitometer (Helena Labs, Beaumont, Tex.) and measuring the area under the peaks.

β-galactosidase assay. The assay for β-galactosidase was performed as described by Miller (20).

RESULTS

Several workers in this laboratory, over a period of years, have noted that *tolC* mutations have interesting effects on colicin and bacteriophage sensitivity and on membrane proteins.

Effect on cell envelope proteins. Cell envelopes, prepared as described above, of independently isolated *tolC* mutants derived from three *E. coli* K-12 parental strains are shown in Fig. 1. There were several alterations to the protein pattern in the *tolC* mutants compared with the wild type. The *tolC* mutants are missing the OmpF protein except for P2069, which has reduced levels of OmpF protein. The *tolC* mutants appear to have slightly reduced levels of OmpA protein and increased levels of proteins in the 35,000- to 45,000-dalton region (Fig. 1).

Mutants derived from W1485 F⁻ had additional minor cell envelope proteins which were not seen in *tolC* mutants of P1700. The nature of these proteins is unknown and has not been studied further.

Figure 2 shows the Triton X-100-insoluble envelope (outer membrane) from AB1133 and P602. The OmpF protein was absent in P602 and was not detected even when excess sample was loaded. We did not detect the OmpF protein by PAGE in the soluble protein fraction or culture supernatants of P602.

Effect on other porin proteins. The *tolC210*::Tn*10-48* mutation was transduced into strains producing the PhoE protein (the outer membrane protein over-produced in *nmpA* and *nmpB* mutants; B. Lugtenberg, personal communication), NmpC protein, or protein 2 (25, 26).

Cell envelopes were prepared and examined in three gel systems: 11% linear, 11% linear with 4 M urea, and 11 to 20% gradient (Fig. 3). These gels showed that the *tolC* derivatives of P1927 and CS483 did not have protein 2 or NmpC protein, respectively. The *tolC* derivative of strain C5 expressed PhoE protein and is sensitive to phage TC45 (5). *tolC* mutants are sensitive to lambda phage and produce the LamB protein (data not shown).

The *tolC* mutation prevented the expression of three porin proteins, OmpF, OmpC, and

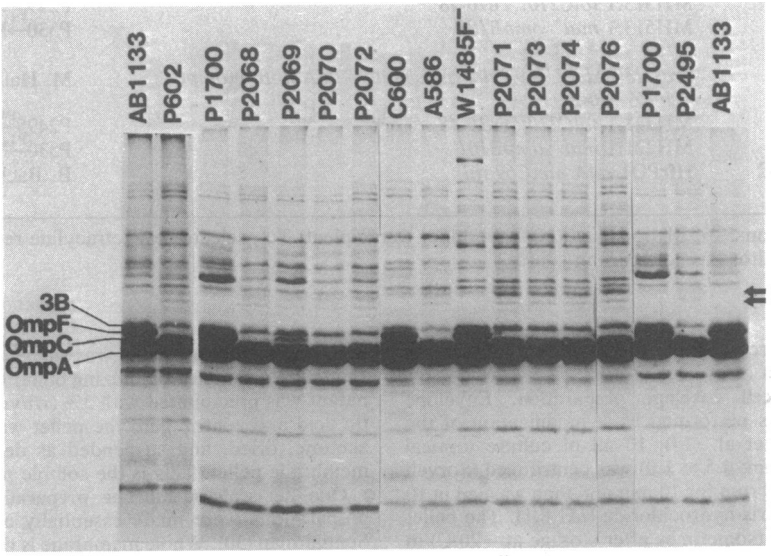

FIG. 1. Sodium dodecyl sulfate-PAGE of cell envelopes of *tolC* mutants. Cell envelopes, prepared by the small-scale method, were isolated from the indicated strains, and samples were run on an 11% linear polyacrylamide gel. P602 is a *tolC* mutant of AB1133; P2068, P2069, P2070, and P2072 are *tolC* mutants of P1700; P2495 is a *tolC*::Tn*10* mutant of P1700; P2071, P2073, P2074, and P2076 are *tolC* mutants of W1485 F⁻; A586 is a *tolC* mutant with a background similar to that of C600. All *tolC* mutants were independently derived. The absence of OmpF protein in the *tolC* mutants can readily be seen. The arrows indicate the region of the gel where *tolC* mutants have increased levels of some proteins.

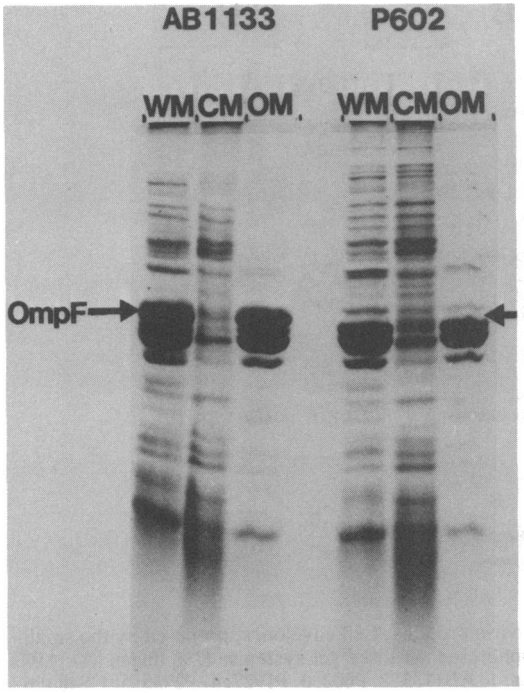

FIG. 2. Sodium dodecyl sulfate-PAGE analysis of the cell envelope fraction prepared from strains AB1133 (*tolC*$^+$) and P602 (*tolC*). Samples of whole cell envelope (WM), cytoplasmic membrane (CM), and outer membrane (OM) from AB1133 and P602, prepared by the 1-liter-scale method, were electrophoresed on an 11% polyacrylamide gel. The arrow indicates the absence of OmpF protein in the outer membrane of P602. The strains were grown in NaCl NB.

protein 2, and this suggests that these three proteins are related in some aspect which is *tolC* dependent.

Effect of growth medium. AB1133 and P602 were grown in three types of media: TSB, NB, and NaCl NB. The different media were used to determine whether growing the *tolC* mutant in media known to influence the level of OmpF protein in a wild-type background (11, 15, 33) would affect the expression of this protein in a *tolC* background. W1485 F$^-$ and P2731 were also used, as AB1133 produces more OmpF protein than OmpC protein in most media. Cell envelopes were prepared and run on PAGE (Table 2 summarizes the results).

When P602 and P2731 were grown under low-salt conditions, in which the respective parent strains, AB1133 and W1485 F$^-$, make higher levels of OmpF protein, OmpF protein was detected in the cell envelope fraction (Table 2). In these mutants, the amount of OmpF protein was much less than in their parent strains; however, they continued to produce large amounts of OmpC protein, which is repressed in the parental strains under low-salt conditions. Likewise, in an *ompF* mutant, P456, the level of OmpC protein remained high in all media (Fig. 4); however, no OmpF protein was seen. An *ompC* mutant, P1533, behaved in a corresponding manner; it still produced OmpF protein when grown in TSB, whereas the parent strain produced lowered amounts (Fig. 4).

Thus, medium-dependent variations in the amount of OmpF or OmpC protein do not occur in the absence of the other protein. The fact that *tolC* mutants produced some OmpF protein in certain media shows that there is no absolute block to the production of this protein.

Effect on transcription of *ompF*. The *tolC* mutation may affect the expression of the OmpF protein at any of the steps involved in the biosynthesis and assembly of this protein into the outer membrane. To study the effect of a *tolC* mutation on transcription of the *ompF* gene, we used the *ompF-lac* fusion strain described by Hall and Silhavy (11). The *tolC210*::Tn*10-48* mutation was transduced into this strain to give P2713. The strains were grown in NaCl NB, and the level of β-galactosidase was measured. The average results of several assays are shown in Table 3. Control derivatives of MH513.1 with Tn*10* inserted in *tsx* or near *ilv* were also constructed and did not differ from the parent in β-galactosidase levels.

Strain P2713 [Φ(*ompF-lac*) *tolC*::Tn*10*] produced two- to threefold less β-galactosidase than did MH513.1 [Φ(*ompF-lac*)] (Table 3). The introduction of an *ompB* mutation into MH513.1 caused a large decrease in the level of the enzyme (Table 3), as described by Hall and Silhavy (10, 11). The *tolC* mutation had little effect on the transcription of the *ompC* gene when put into an *ompC-lac* fusion (Table 3, strain P2714). Thus, the *tolC* mutation specifically produced a two- to threefold reduction of the transcription of the *ompF-lac* fusion; however, the reduction in the amount of OmpF protein in the cell envelopes of *tolC* mutants was far greater than could be accounted for by this decrease in transcription (Table 2). This was in contrast to the results of Hall and Silhavy (10, 11), who found that varying the growth medium results in a four- to fivefold variation in transcription of *ompF* or *ompC*, which correlates with a similar (two- to sixfold) variation in protein level (33; Table 3). Furthermore, *ompB* mutations cause a large reduction (70-fold) in transcription (10, 11; Table 3), and these mutants lack detectable levels of OmpF and OmpC protein (29).

Effect of *lon*. As there is a possibility that some OmpF protein may be made but then degraded, the effect of *lon* (*deg*) on the *tolC*-mediated loss of OmpF protein from the outer membrane was

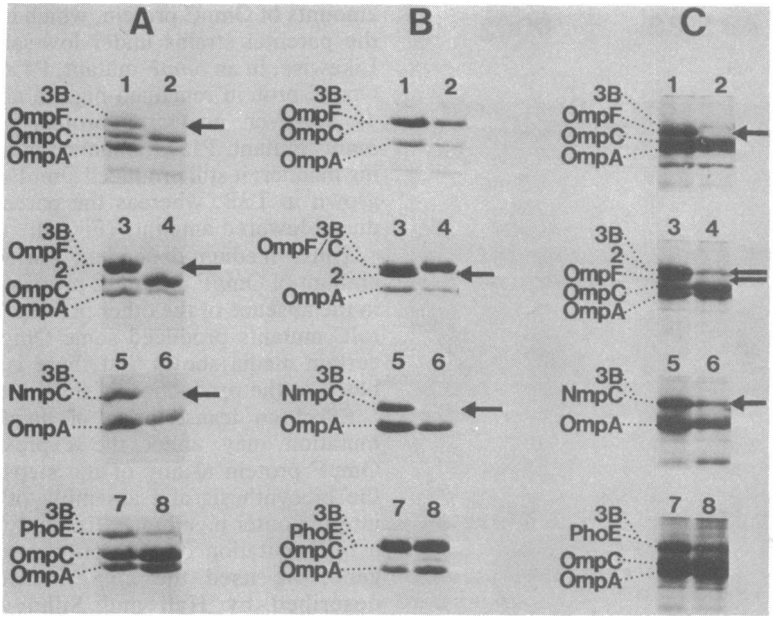

FIG. 3. Effect of a *tolC* mutation on the expression of porin proteins. Cell envelopes, prepared by the small-scale method from the strains listed below, were electrophoresed on three gel systems: 11% linear (A), 11% linear with 4 M urea (B), and 11 to 20% gradient (C). Strains: 1, AB1133; 2, P602; 3, P1927; 4, P2725; 5, CS483; 6, P2726; 7, C5; and 8, P2724. The arrows indicate the absence in the *tolC* mutant of one of the porin proteins (OmpF, NmpC, or protein 2), which can be readily seen when the mutant is compared with the parental strain.

examined. Cell envelopes and soluble proteins from strains SA500, P2727, SA1500, and P2728 were examined on 11% sodium dodecyl sulfate gels (data not shown). P2728 (*lon tolC*) had no detectable OmpF protein or obvious fragment. Hence, the *lon* (*deg*) system (9) is not implicated in the loss of the OmpF protein, although it has been shown to affect amber fragments of the *lamB* protein (4). The possibility that other degradation systems are involved cannot be discounted.

DISCUSSION

Previously, Rolfe and Onodera (28) showed that a *tolC* deletion mutant was missing a membrane protein. We have shown that a range of independently isolated *tolC* mutants lacked detectable OmpF protein (as determined by PAGE) and had altered levels of some other proteins in the cell envelope. The data of Hancock et al. (12) showed that P602 (*tolC*) has a bacteriophage resistance pattern similar to that found for the TolIa (P218) and TolIb (P210) mutants (27), both of which are now believed to be *ompF* mutants. The phage which we use to test for the presence or absence of this protein is K2 (12), which behaves like TuIa (13) in terms of host range but is easier to score. P218 and P210 were completely resistant to K2, whereas P602 and other *tolC* mutants were substantially resistant, showing growth inhibition in cross-streak tests. This incomplete resistance suggests that *tolC* mutants produce very small amounts of OmpF protein, and indeed under some growth conditions the level is sufficient to be detected on polyacrylamide gels.

P602 is resistant to colicin E1 and partially resistant to colicin A, whereas P210 and P218 are resistant to colicins A, L, and S4 (12). The resistance of *ompF* mutants to colicins A, L, and S4 is presumably due to the absence of the OmpF protein. P602, although apparently missing the OmpF protein, is still sensitive to these colicins. It may be that the residual levels of OmpF protein in the *tolC* mutant are sufficient for the action of these colicins, to which *ompF* mutants are resistant. Double mutants of the type *tolC btuB* and *tolC tsx* are still resistant to colicins E2 and E3 and to colicin K, respectively (unpublished data), which indicates that the penetration of the outer membrane of the *tolC* mutant by these colicins still requires the specific colicin outer membrane receptor to be present; thus, the sensitivity of P602 to colicins L and S4 is probably not due to nonspecific penetration of the outer membrane by colicins.

The effect of *tolC* mutations on several cell envelope proteins is in keeping with the known pleiotropic nature of *tolC* mutations which confer tolerance to colicin E1 and sensitivity to

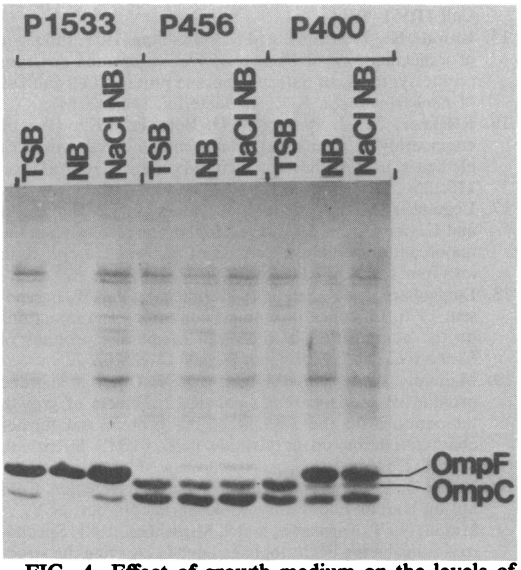

FIG. 4. Effect of growth medium on the levels of OmpF and OmpC proteins in an *ompF* and an *ompC* mutant. Cell envelopes were prepared by the small-scale method from P400, P456, and P1533 grown in the indicated medium. Samples were run on an 11% polyacrylamide gel.

certain dyes, detergents, and antibiotics. These effects are probably not due to the almost total lack of OmpF protein, as mutations in the structural gene *ompF* do not confer these properties;

TABLE 2. Estimation of outer membrane protein levels

Strain	Growth medium	Protein (% of total)[a]			
		3B	OmpF	OmpC	OmpA
AB1133	NaCl NB	8	29	19	44
	TSB	8	6	39	47
	NB	6	38	20	36
P602	NaCl NB	11	0	49	40
	TSB	10	0	41	49
	NB	10	13	34	43
W1485 F⁻	NaCl NB	8	31	22	39
	TSB	8	9	45	38
	NB	6	49	12	33
P2731	NaCl NB	8	0	49	43
	TSB	8	0	47	45
	NB	6	12	46	36
MH70[b]	NaCl NB	9	28	20	43
P2712[c]	NaCl NB	12	0	45	43

[a] Results are expressed as the percentage of total protein (3B + OmpF + OmpC + OmpA), determined as described in the text.
[b] MH70 is the parent strain of MH513.1 and MH221.1.
[c] P2712 is MH70 *tolC210*::Tn*10-48*.

TABLE 3. β-Galactosidase activity of *ompF-lac* and *ompC-lac* fusion strains

Strain[a]	Characteristics[b]				β-Galactosidase activity[c] (U)
	tolC	Φ(*ompC-lacZ*⁺)	Φ(*ompF-lacZ*⁺)	*ompB*	
MH513.1	+		√	+	422
P2713	−[d]		√	+	158
P2748	+		√	−[e]	10
MH221.1	+	√		+	335
P2714	−[d]	√		+	408
P2749	+	√		−[e]	18

[a] All strains were grown in NaCl NB.
[b] Symbols: +, wild type; −, mutant; √, fusion present.
[c] Units as described by Miller (20).
[d] Mutation is *tolC210*::Tn*10-48*.
[e] Mutation is *ompB101*.

however, they may be due to alterations in the levels of some minor proteins.

The *ompB* gene also affects the level of the OmpF protein (29) and is thought to act as a positive control element by acting on transcription of both *ompC* and *ompF* (10, 11). The effect of *tolC* was clearly different, as it had no effect on the OmpC protein but resulted in the loss of NmpC protein or protein 2 (based on PAGE) in strains in which these proteins are otherwise a major component of the outer membrane; *ompB* had no effect on these two proteins. The other known porins, the PhoE and LamB proteins, were not affected to any degree by either *ompB* or *tolC*. A second major distinction between *ompB* and *tolC* is that whereas the *ompB* mutation had a major effect on both transcription and protein levels, the *tolC* mutation had only a two- to threefold effect on transcription of an *ompF-lac* fusion. Thus, whereas the *ompB* gene product directly affected transcription of *ompF*, this did not seem to be true for the *tolC* gene product. Furthermore, we found no evidence for unprocessed OmpF protein in either the cytoplasm or membrane; it is possible that OmpF protein, once formed, is degraded in a *tolC* mutant, but if this is so then the well-documented *lon* (*deg*) system is not involved.

One explanation for the data is that the *tolC* gene product affects synthesis of the OmpF protein at a post-transcriptional stage. It also appears that the NmpC protein and protein 2 are affected in the same way, as neither could be detected in *tolC* mutants. We suggest that *tolC* greatly increases the efficiency of some step common to the biosynthesis and assembly into the outer membrane of the OmpF and NmpC proteins and of protein 2.

In the course of these studies we also observed that in *tolC*, *ompF*, and *ompC* mutants

which effectively make only one of the major porins, the other porin is not only increased in amount to compensate, but the amount present is not subject to medium-dependent variation. It appears that the effect of a medium is on the ratio of the two proteins, but if one cannot be made because of a structural gene mutation or is made in very small amounts because of a *tolC* mutation, then all of the available sites are occupied by the other major porin under all growth conditions.

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