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# Role of *micF* in the *tolC*-Mediated Regulation of OmpF, a Major Outer Membrane Protein of *Escherichia coli* K-12

R. MISRA† AND P. R. REEVES*

*Department of Microbiology, The University of Sydney, Sydney, New South Wales 2006, Australia*

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**Mutation in the *tolC* locus greatly reduces normal synthesis of OmpF, a major porin protein of *Escherichia coli* K-12. Experiments that use *ompF-ompC* chimeric genes demonstrate that a *tolC* mutation exerts its effect at either the promoter or the amino-terminal end of the *ompF* gene. Direct analysis of *ompF* mRNA from *tolC*$^+$ and *tolC* strains showed that the amount of *ompF* transcript in the latter was greatly reduced. We have also observed that, in addition to reducing the amount of OmpF, a *tolC* mutation increases the level of OmpC protein to a much greater extent than occurs in an *OmpF* mutant and also increases *micF* RNA synthesis as shown by increased β-galactosidase synthesis in a *micF-lacZ* fusion strain. Based on these observations, we suggest that an increased expression of the *micF* gene in a *tolC* mutant results in the reduced expression of *ompF* and that a major effect of the *tolC* mutation may be to push the porin-regulating system to favor *ompC* and *micF* to a greater extent than under high-osmolarity conditions.**

The two proteins, OmpF and OmpC, of *Escherichia coli* K-12 facilitate diffusion of small hydrophilic molecules through the outer membrane. The amounts of OmpF and OmpC in the membrane vary under different growth conditions, with osmotic pressure having a substantial influence: in media of low osmolarity OmpF is synthesized preferentially, whereas in high-osmolarity media synthesis of OmpC is preferred (15, 37). The synthesis of these proteins is regulated such that a decrease in the amount of one protein is compensated by an increase in the amount of the other, with the combined amount of the two proteins remaining nearly constant (37).

Apart from mutation in the structural genes for OmpF and OmpC, mutations in several other genes can affect the expression of these proteins. Mutation in one such gene, *ompB*, first characterized by Sarma and Reeves (32), resulted in a loss of both OmpF and OmpC proteins. It was later shown that mutation in the *ompB* locus could result in any of three phenotypes: OmpF$^-$ OmpC$^-$, OmpF$^-$ OmpC$^+$, or OmpF$^+$ OmpC$^-$ (32, 38). The *ompB* locus was further studied by Hall and Silhavy (11, 12), who revealed the presence of at least two genes at this locus: *envZ* and *ompR*. OmpR is a cytoplasmic protein and postulated to function as a positive regulatory element (13, 28); the role of EnvZ is less clear. The *envZ* and *ompR* genes have been cloned (24) and sequenced (6), but the molecular nature of their role in *ompF* and *ompC* gene expression is not clearly understood. Recently a third regulatory locus, *micF*, was located upstream from the *ompC* gene (23). The 3′ end of the 173-base-long *micF* RNA is complementary to the 5′ end of *ompF* mRNA and would be expected to interfere with *ompF* mRNA translation by forming a stable RNA-RNA hybrid: strains harboring high-copy-number *micF*$^+$ plasmids in fact lack both OmpF protein and *ompF* mRNA (23).

Mutations in the *tolC* locus elicit several phenotypic changes, and under certain circumstances *tolC* mutants lack detectable levels of OmpF protein (26). Regulation of this protein by the *tolC* locus appeared to be independent of that exerted by the *ompB* locus, because *tolC* mutants had a similar effect on two other proteins (NmpC and Lc) (26) which are not under *ompB*-positive control (30). Experiments with an *ompF-lacZ* operon fusion strain indicated that the *tolC*-mediated effect on the expression of *ompF* is at some stage after transcription (26).

In this communication we report on studies of the manner in which mutation in the *tolC* locus affects the expression of the *ompF* gene. We present data on the strength of the *tolC*-mediated regulation of OmpF synthesis and compare it with that of the previously characterized *ompB* regulation. We also studied the effect of *tolC* on OmpF in *micF* deletion strains.

Our results suggest that the effect of a *tolC* mutation on OmpF is indirect and involves activation of *micF*, and this was confirmed by the use of *micF-lacZ* fusion strains and by use of clones of *micF*. We have also looked at the interaction of *tolC* and *ompR* mutations to further understand the regulation of *ompF* and *ompC* expression.

## MATERIALS AND METHODS

**Bacterial strains, media, and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Cultures were grown in our standard nutrient broth medium, being 16 g of nutrient broth (0003; Difco Laboratories) plus 5 g of NaCl per liter. This is sometimes referred to as high-osmolarity medium. Low-osmolarity medium was 8 g of nutrient broth (0003; Difco) per liter. When required, the following antibiotics were added: ampicillin (25 μg/ml) chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), and tetracycline (16 μg/ml).

RNA was labeled in a phosphate-limiting medium which contained 20 mM KCl, 85 mM NaCl, 100 mM Tris, 20 mM $NH_4Cl$, 1 mM $MgCl_2$, 0.1 mM $KH_2PO_4$, 1 μg of thiamine per ml, 1% Casamino Acids (dephosphorylated), and 5 mg of glucose per ml. Whole-cell envelopes were prepared from cultures grown to late log phase and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-

* Corresponding author.
† Present address: Molecular Biology Department, Princeton University, Princeton, NJ 08544.

TABLE 1. Bacterial strains and plasmids

| Bacterial strain/ plasmid | Characteristics | Source or reference |
|---|---|---|
| Bacterial strain | | |
| 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 |
| CS1253 | W1485 F⁻ *ompC178 zei-198*::Tn*10* | 33 |
| FN101 | W4626 Phe⁻ *ompR20* | 27 |
| MC4100 | F⁻ λ⁻ *araD139* Δ*(argF-lac)205 rpsL150 relA1 flbB5301 deoC1 ptsF25* λ⁻ | 4 |
| MH513 | MC4100 *araD*⁺ Φ(*ompF'-lacZ*⁺)*16-13* | 11 |
| MH610 | MC4100 *araD*⁺ Φ(*ompF'-lacZ*⁺)*16-10*(Hyb) | 11 |
| MH760 | MC4100 *ompR472* (*ompR2*) | 12 |
| P210 | AB1133 *ompF* | 7 |
| P530 | AB1133 *ompR101* | 7 |
| P602 | AB1133 *tolC203* | 7 |
| P1533 | AB1133 *ompC* | P. R. Reeves |
| P2731 | W1485F⁻ *tolC210*::Tn*10-48* | R. Morona |
| P2770 | P602 *ompC* | 5 |
| P3011 | MC4100 *tolC* | This study |
| P3183 | P1533 *ompF* | This study |
| P3224 | P210(pMAN007) | This study |
| P3225 | P210(pMAN009) | This study |
| P3226 | P602(pMAN007) | This study |
| P3227 | P602(pMAN009) | This study |
| P3228 | 2770(pMAN006) | This study |
| P3229 | 2770(pMAN007) | This study |
| P3230 | 2770(pMAN009) | This study |
| P3231 | 2770(pMAN010) | This study |
| P3283 | 3183(pMAN006) | This study |
| P3284 | 3183(pMAN007) | This study |
| P3285 | 3183(pMAN009) | This study |
| P3286 | 3183(pMAN010) | This study |
| P3289 | MH610 *tolC* | This study |
| P3393 | FN101 *tolC* | This study |
| P3394 | MH760 *tolC* | This study |
| P3396 | W4626 Phe⁻ *tolC* | This study |
| P3398 | CS1253 *tolC* | This study |
| P3418 | CS1253(pMAN006) | This study |
| P3419 | P3398(pMAN006) | This study |
| P3423 | CS1253(pPR426) | This study |
| P3424 | P3398(pPR426) | This study |
| P3427 | P3183(pPR426) | This study |
| P3493 | SM3001 *tolC* | This study |
| P3501 | MC4100(pmicB21) | This study |
| P3502 | P3011(pmicB21) | This study |
| P3503 | MH760(pmicB21) | This study |
| P3504 | P3394(pmicB21) | This study |
| P3625 | MH513(pBR322) | This study |
| P3626 | MH513(pCX28) | This study |
| P3627 | MH610(pBR322) | This study |
| P3628 | MH610(pCX28) | This study |
| P3685 | MC4100(pPR569) | This study |
| P3686 | P3011(pPR569) | This study |
| P3687 | MH760(pPR569) | This study |
| P3688 | P3394(pPR569) | This study |
| SM3001 | MC4100 Δ*micF1* | 20 |
| W1485F⁻ | | C. Schnaitman |
| W4626 Phe⁻ | F⁻ *purE pheA trp lac-85 galK2 malA mtl xyl-2 ara rpsL* (λ) | 27 |
| Plasmid | | |
| pBR322 | Ap$^r$ Tc$^r$ | 3 |
| pCX28 | Ap$^r$; vector, pBR322; cloned gene, *micF* | 23 |
| pDF41 | *trpE*⁺; mini-F replicon | 14 |
| pJP33 | Cm$^r$; vector, pACYC184; cloned gene, *ompF* | 36 |
| pLF11 | Ap$^r$; vector, pBR322; cloned gene, 5′ end of *ompF* | 18 |
| pLG339 | Km$^r$ Tc$^r$; pSC101 replicon | 34 |
| pMAN006 | Ap$^r$; vector, pKEN403; cloned genes, *ompC micF* | 18 |
| pMAN007 | Ap$^r$; vector, pKEN403; cloned gene, *ompF* | 18 |
| pMAN009 | Ap$^r$; vector, pKEN403; cloned gene, Φ(*ompC'-ompF*⁺)(Hyb) (*ompC* promoter with *ompF* gene) | 18 |
| pMAN010 | Ap$^r$; vector, pKEN 403; cloned gene, Φ(*ompC'-ompC*⁺)(Hyb) (*ompF* promoter with *ompF* gene) | 18 |
| pMC1403 | Ap$^r$; vector, pBR322; cloned genes, *lacZ, lacY, lacA* | 5 |
| pmicB21 | Ap$^r$; vector, pKEN403; cloned genes, Φ(*micF'-lacZ*⁺) (*micF* promoter with *lacZ* gene) | 23 |
| pPR268 | Ap$^r$ Tc$^r$; vector, pBR322; cloned gene, *ompF* | This study |
| pPR272 | Km$^r$; pSC101 replicon; cloned gene, *ompF* | This study |
| pPR274 | Cm$^r$, *trpE*; mini-F replicon | This study |
| pPR275 | Cm$^r$; mini-F replicon; cloned gene, *ompF* | This study |
| pPR426 | *micF* deletion of pMAN006 | This study |
| pPR569 | Φ(*ompC'-lacZ*⁺)(Hyb) in pPR274 | This study |

*Continued in next column*

PAGE) as described previously (16, 26). All cultures were grown at 37°C.

**DNA techniques.** Plasmid DNA was purified by the two-step CsCl step gradient method of Garger et al. (8). Digestion of plasmid DNA with restriction enzymes, ligation, and transformation were all performed by standard techniques. DNA fragments were analyzed by electrophoresis in 0.6% agarose gels as described by Maniatis et al. (17). *Eco*RI-generated fragments of bacteriophage SPPI were used as molecular weight markers. Nick translation of plasmid DNA fragments extracted from low-melting-point agarose gel was performed by the method of Rigby et al. (31).

Digestion of plasmid DNA with restriction enzymes or *Bal*31, end filling with Klenow fragment, and ligation were performed as described by Maniatis et al. (17).

A diagrammatic illustration of the subcloning of the *ompF* gene into different copy number vector plasmids is shown in Fig. 1: pJP33, pPR272, and pPR275 carry *ompF* in p15A, pSC101, and mini-F replicons, respectively.

Plasmid pMAN006 contains both *micF* and *ompC* genes, and pPR426 (*micF*⁻ *ompC*⁺) was derived from this plasmid by *Bal*31. Briefly, pMAN006 was cut at a unique *Sal*I site (located approximately 700 base pairs from the start of the *micF* gene) and then digested with *Bal*31: samples were taken at various times and the reaction was stopped by the addition of 5 mM EGTA [ethylene glycol-bis(β-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]. The DNA was incubated with Klenow fragment in the presence of all four deoxyribonucleotides (dCTP, dATP, dGTP, and TTP) and ligated in the presence of phosphorylated *Bam*HI linker (8-mer; pdCGGATCCG). To determine the deletion endpoint, a 571-base pair *Bam*HI-*Eco*RI fragment from pPR426

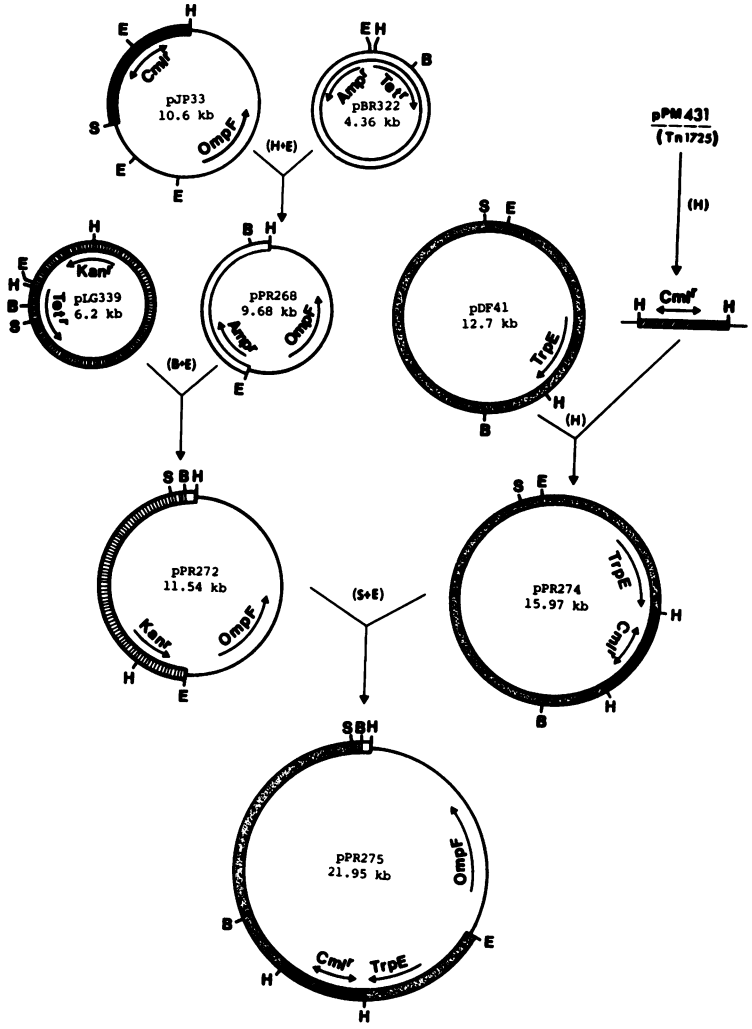


FIG. 1. Subcloning of the *ompF* gene into different copy number vector plasmids. The *Eco*RI-*Hin*dIII fragment from pJP33 (*ompF* in pACYC184 [36]) that carries the *ompF* gene was inserted between the *Eco*RI and *Hin*dIII sites of pBR322, resulting in pPR268. The *Eco*RI-*Bam*HI fragment from pPR268 was inserted between the *Eco*RI and *Bam*HI sites of a six-copy-number plasmid, pLG339, resulting in pPR272. To obtain a single-copy *ompF* plasmid, the chloramphenicol resistance gene of Tn*1725* (39) was inserted into the *Hin*dIII site of pDF41, resulting in pPR274, and then the *Eco*RI-*Sal*I fragment from pPR272 was inserted between the *Eco*RI and *Sal*I sites of pPR274, resulting in pPR275. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; kb, kilobases.

was subcloned and sequenced from the *Bam*HI linker into *ompC*.

**RNA techniques.** RNA was purified from exponentially growing bacterial cultures by the method of Aiba et al. (1) and was further purified by centrifugation in a CsCl gradient (10). Northern transfer of glyoxal-denatured RNA samples and hybridization with $^{32}$P-labeled DNA probe was performed essentially as described by Thomas (35). The method of Parnes et al. (29) was used when a more sensitive assay of *ompF* mRNA was required. Briefly, about 10 μg of purified *ompF* DNA (the 513-base pair *Pst*I-*Pvu*II piece from pLF11) was spotted onto a nitrocellulose disk, baked, and hybridized with approximately $5 \times 10^6$ cpm of in vivo labeled [$^{32}$P]RNA (bacteria were grown with [$^{32}$P]phosphoric acid in phosphate-limiting medium). Hybridized RNA was eluted from nitrocellulose filters and, after two phenol extractions, precipitated by ethanol and suspended in 0.1 mM EDTA. The $^{32}$P-labeled RNA was then electrophoresed through 5% acrylamide–8 M urea gels which were autoradiographed at −70°C for 10 to 16 h.

**β-Galactosidase assay.** The level of β-galactosidase in freshly grown cultures was assayed as described by Miller (21).

## RESULTS

**Analysis of the *ompF* transcript.** To determine the level at which a mutation in the *tolC* locus exerts its effect on *ompF* expression, we studied transcription of the *ompF* gene by directly analyzing *ompF* mRNA from *tolC*, *ompR101*, and *ompF* mutants. RNA was purified from two different parent strains and their mutant derivatives. These RNA preparations were electrophoresed in an agarose gel and subjected to Northern transfer to nitrocellulose filters, hybridized with a $^{32}$P-labeled DNA fragment of the *ompF* gene, and autoradiographed. The *ompF* transcript was present in the parent

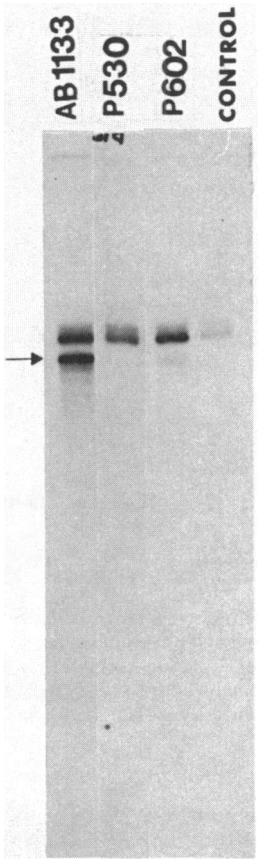


FIG. 2. Assay of *ompF* mRNA from wild-type (AB1133), *tolC* (P602), and *ompR101* (P530) strains by the method of Parnes et al. (29). $^{32}$P-labeled RNA that hybridized with *ompF* DNA on filters was eluted and electrophoresed on a 5% acrylamide–8 M urea gel and autoradiographed. As a control, the radioactive RNA isolated from AB1133 was hybridized to a filter with no DNA. Arrow indicates the position of *ompF* mRNA. A band running above *ompF* mRNA is the contaminating chromosomal DNA extracted with the crude labeled RNA preparations.

strains (AB1133 and W1485F$^-$) but was not detected in the *tolC*, *ompF*, or *ompR101* mutant (data not shown). When a more sensitive RNA-DNA hybridization method (see Materials and Methods) was used, the *tolC* mutants were shown to have 50-fold less *ompF* transcript than was present in the parent strain, and none was detected in the *ompR101* mutant (Fig. 2).

**Use of the *ompF-ompC* chimeric genes to determine the region of the *ompF* gene affected by the *tolC* mutation.** The results presented above showed that a mutation in the *tolC* locus drastically reduces the amount of *ompF* transcript and presumably affects the promoter function of the *ompF* gene. To confirm this, we used chimeric plasmids in which the *ompF* structural gene was placed under *ompC* promoter control or vice versa (18). These and control *ompF* and *ompC* plasmids were transformed into strains with *ompF* or *tolC* mutations either alone or in combination with an *ompC* mutation. Whole cell envelopes of these strains were prepared and analyzed by SDS-PAGE (Fig. 3). Compare P3224 and P3226 to see the effect of *tolC* mutation on *ompF* under its own *ompF* promoter control, and compare P3286 and P3231 to see the effect on *ompC* under the same control. When a gene was under *ompC* promoter control, the *tolC* mutation had no effect (compare P3225 and P3227 for the effect on *ompF* and P3285 and P3230 for the effect on *ompC* expression). Only when a gene was under *ompF* promoter control was its product reduced by *tolC* mutation, with the greater effect being on *ompC* under *ompF* promoter control. These results show that the effect of the *tolC* mutation is exerted at a point upstream of the chimera junction at amino acid 11 of the mature OmpF protein, on a region which includes the promoter and the *micF* RNA interaction sites.

It should be noted that the *tolC* effect on OmpF, in strains carrying chimeric genes, is not as strong as observed in a strain carrying a single copy of the chromosomal *ompF* gene (e.g., P602). This reduced *tolC* effect is due to the increase in *ompF* gene dosage, and this aspect is further illustrated in an experiment described below. At this stage it is not clear why the *tolC* effect on OmpC is greater than on OmpF when these proteins were synthesized under *ompF* promoter control.

**Synthesis of OmpF protein in strains carrying *ompF*$^+$ plasmids of varying copy number.** The effect of *tolC* and *ompR101* mutations on *ompF* expression was studied in mutant (*tolC* or *ompR*) strains carrying *ompF*$^+$ plasmids derived from a mini-F, pSC101, or p15A replicon, which has an approximate copy number of 1, 6, or 50, respectively. Whole cell envelopes of these strains were prepared and analyzed by SDS-PAGE (Fig. 4). When OmpF was synthesized from the single-copy chromosomal gene, either mutation (*tolC* or *ompR101*) reduced the amount of OmpF protein below the level which could be detected in whole cell envelopes. However, if the copy number of the *ompF* gene was increased, the effect of the *ompR101* mutation remained essentially the same, whereas the *tolC* mutation was increasingly unable to affect the level of OmpF. Thus for 2, 7, and 51 copies of *ompF*, the *tolC* mutation produced a 20-fold, 4- to 5-fold, and negligible reduction, respectively, in OmpF level.

**Effect of *tolC* mutation on OmpF synthesis in *micF-ompC* and *micF* deletion mutants.** A strain (CS1253) in which the *ompC* and *micF* genes are deleted was kindly given to us by Schnaitman and McDonald (33). A *tolC* mutation did not have the usual dramatic effect on OmpF in this deletion mutant, giving only a two- to threefold reduction in the level of OmpF when the strains were grown in high-osmolarity medium and no reduction in strains grown in low-osmolarity medium (Fig. 5). This experiment indicated that the *tolC* effect on OmpF is largely mediated via the *micF* or *ompC* gene or both, but as the absence of the OmpC protein itself

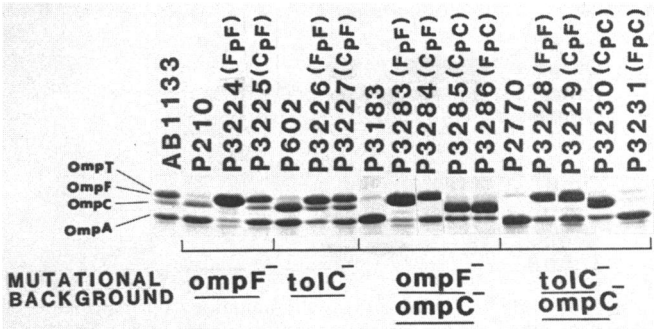


FIG. 3. Determination of the region of the *ompF* gene affected by the *tolC* mutation. Strains, with the mutational background indicated, were transformed with pMAN007 (FpF), pMAN009 (CpF), pMAN006 (CpC), and pMAN010 (FpC), and whole cell envelopes prepared from these strains were analyzed by SDS-PAGE. Only the relevant part of the gel is shown. CpF indicates *ompC* promoter with *ompF* gene, etc.

does not interfere with the *tolC* effect on OmpF (see, for example, P2770 [Fig. 3]), it is the *micF* gene which is implicated in the suppression of *ompF* expression observed in *tolC* mutants.

To test this hypothesis, we constructed a *micF*$^-$ *ompC*$^+$ plasmid, pPR426, from pMAN006 (see Materials and Methods) with a deletion which removed the entire *micF* gene and ended 61 base pairs upstream of the putative $^-$35 region of the *ompC* gene (the *Bam*HI linker was followed by the sequence TACATTTT [23]), leaving the *ompC* gene intact: an equal amount of the OmpC protein was produced by strain P3183 (*ompF ompC* double mutant) carrying pMAN006 or pPR426 (data not shown). Both of these plasmids were transformed into the *micF-ompC* deletion strain CS1253 and a *tolC* derivative, P3398, to give strains effectively *micF*$^+$ *ompC*$^+$ (P3418 and P3419) or *micF*$^-$ *ompC*$^+$ (P3423 and P3424).

A comparison of the outer membrane protein profiles of strains P3418 and P3419, grown in low-osmolarity medium, showed that the *tolC* mutation in P3419 has the same major effect on OmpF level when *ompC* and *micF* are encoded on the plasmid (pMAN006) as it has when they are on the chromosome. In contrast, comparison of the outer membrane protein profiles of strains P3423 and P3424, which carry pPR426, showed that in the *micF*$^-$ background the *tolC* mutation has a negligible effect on OmpF synthesis (data not shown). In high-osmolarity medium the level of OmpF in P3418 is reduced more than usual relative to low-osmolarity medium, presumably due to the additional copies of *ompC*, but in the *micF* strain P3423 the reduction is only two- to threefold, with a further four- to fivefold reduction in P3424, the *tolC* derivative.

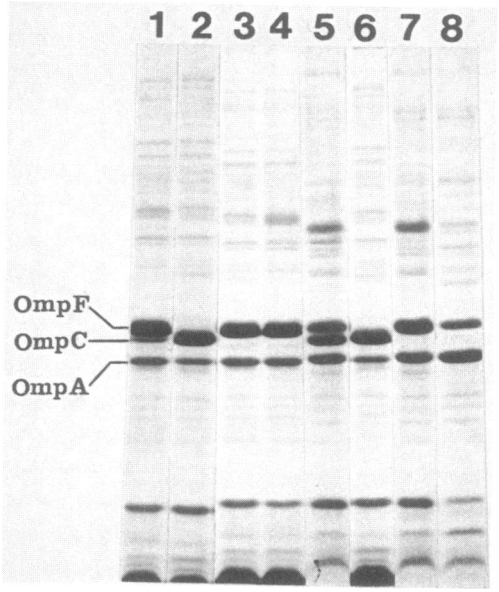


FIG. 5. Effect of *tolC* on OmpF in *micF*$^+$ *ompC*$^+$ and *micF*$^-$ *ompC*$^-$ strains. Whole cell envelopes from strains W1485F$^-$ (lanes 1 and 5), P2731 (lanes 2 and 6), CS1253 (lanes 3 and 7), and P3398 (lanes 4 and 8) were analyzed by SDS-PAGE. Strains were grown in a low-osmolarity (lanes 1 to 4) or high-osmolarity (lanes 5 to 8) medium.

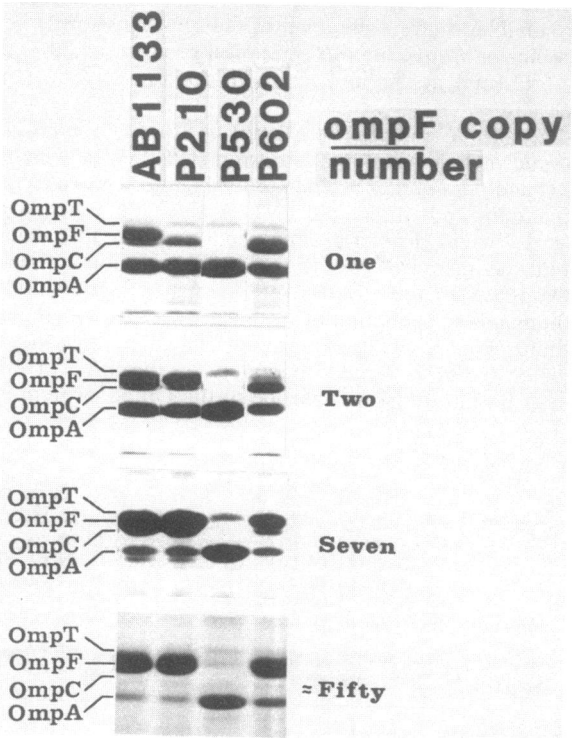


FIG. 4. Effect of *tolC* and *ompR* mutations on expression of OmpF, synthesized by varying copy number plasmids. Whole cell envelopes from wild-type and mutant strains either without plasmid or harboring *ompF*$^+$ plasmid pPR275, pPR272, or pJP33 were analyzed by SDS-PAGE. Only the revelant part of gels is shown. It should be noted that strains carrying multicopy *ompF*$^+$ plasmid overproduce OmpF protein and the amount of OmpC protein is consequently reduced; therefore, it is difficult to resolve the residual OmpC protein from OmpF.

After completion of these experiments, we learned of a strain (SM3001) constructed by Matsuyama and Mizushima (20) in which a deletion specifically removed the entire chromosomal *micF* gene while leaving the *ompC* gene intact. We are grateful to them for sending us their strain. A *tolC* mutation in SM3001 caused a four- to fivefold reduction in the level of OmpF when cultures were grown in a high-osmolarity medium, and in a low-osmolarity medium *tolC* produced a two- to threefold reduction in the level of OmpF (Fig. 6). A *tolC* mutation in the parent *micF*$^+$ strain (MC4100) resulted in almost a total loss of OmpF under similar growth conditions (Fig. 6). It should be noted that the deletion of *micF* itself reduces the OmpF level and increases the OmpC level, which somewhat obscures the *tolC* effect on OmpF.

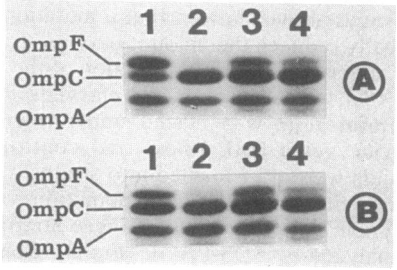


FIG. 6. Effect of *tolC* on OmpF in the presence or absence of the chromosomal *micF* gene. Whole cell envelopes from MC4100 (lane 1), P3011 (lane 2), SM3001 (lane 3), and P3493 (lane 4) were analyzed by SDS-PAGE. Strains were grown in a low-osmolarity (A) or high-osmolarity (B) medium.

TABLE 2. β-Galactosidase activities of strains carrying Φ(*ompC'-lacZ*$^+$) or Φ(*micF'-lacZ*$^+$) plasmids

| Strain | Mutation | Plasmid | Enzyme units |
|---|---|---|---|
| MC4100 | | | 0 |
| P3501 | | Φ(*micF'-lacZ*$^+$) | 169 |
| P3502 | *tolC* | Φ(*micF'-lacZ*$^+$) | 1,378 |
| P3503 | *ompR472* | Φ(*micF'-lacZ*$^+$) | 18 |
| P3504 | *ompR472 tolC* | Φ(*micF'-lacZ*$^+$) | 1,121 |
| P3685 | | Φ(*ompC'-lacZ*$^+$) | 151 |
| P3686 | *tolC* | Φ(*ompC'-lacZ*$^+$) | 459 |
| P3687 | *ompR472* | Φ(*ompC'-lacZ*$^+$) | 0 |
| P3688 | *ompR472 tolC* | Φ(*ompC'-lacZ*$^+$) | 110 |

Thus, whereas in low-osmolarity medium *tolC* had very little or no effect on OmpF in *micF* deletion strains SM3001 and CS1253, in a high-osmolarity medium the *tolC* effect on OmpF was always detectable, but much less than that observed in a corresponding *micF*$^+$ strain.

**Effect of *tolC* on expression of *ompC* and *micF*.** The results of the experiments described above suggest that the *tolC* effect on *ompF* is in large part mediated by *micF*. It is possible that *tolC* exerts its effect on *ompF* by varying *micF* expression. To study this, a *micF-lacZ* fusion plasmid (pmicB21) was transformed into wild-type and *tolC* strains. The expression of *micF* was assayed by measuring β-galactosidase activity (Table 2). The *tolC* mutant had eightfold more β-galactosidase activity than the wild-type strain. The tenfold reduction in *micF* expression in an *ompR* mutant confirms the results obtained by Mizuno et al. (23). These observations clearly show that a mutation in the *tolC* locus enhances the expression of *micF*.

We find that a *tolC* mutation, in addition to lowering the OmpF level, increases the amount of OmpC to a greater extent than is observed in an *ompF* mutant, suggesting that *tolC* directly affects *ompC* expression. It has been proposed that the *micF* and *ompC* genes are coregulated (23) and we have already shown that a *tolC* mutation increases *micF* expression. To provide more direct evidence for the effect of a *tolC* mutation on *ompC* expression, we constructed an *ompC-lacZ* fusion plasmid in which the expression of *lacZ* was placed under *ompC* promoter control (Fig. 7). This fusion consisted of the *ompC* promoter, the coding region for the signal peptide and the first 11 amino acid residues of the mature OmpC protein, and the coding region for the LacZ protein from residue 8. This fusion was constructed in a multicopy plasmid (pMB1 replicon) to give pPR522 but, due to the poor or zero growth of strains carrying it, was subsequently moved into a very low-copy-number plasmid (mini-F replicon) to give pPR569, which was transformed into wild-type and *tolC* strains. The expression of *ompC* was assayed by measuring β-galactosidase activity (Table 2). The *tolC* mutant had threefold more β-galactosidase activity present than the wild-type strain: the *ompR* mutant, as expected, had no detectable β-galactosidase activity. These results agree well with the data to be presented below and confirm that a *tolC* mutation increases *ompC* expression.

**Interaction of *tolC* and *ompR* mutations.** Mutations in the *ompR* gene affect synthesis of one or the other of the OmpF and OmpC proteins. The original *ompR101* mutation results in the absence of both OmpF and OmpC proteins; *ompR472* and *ompR20* mutations result in a greatly reduced level of OmpC, with OmpF synthesized at high levels regardless of osmolarity in the *ompR472* mutant (12), and at a reduced level with reversed osmolarity effect in the *ompR20* mutant (27). By comparison, mutation in the *tolC* locus results in a greatly reduced level of OmpF with constitutive synthesis of OmpC.

If our *ompC-lacZ* fusion plasmid, pPR569, is put into an *ompR472* strain and its *tolC* derivative, then the *ompR472* strain has greatly reduced the β-galactosidase level as expected, but in the *tolC ompR472* strains the level is almost as high as in the *ompR*$^+$ *tolC*$^-$ strain (Table 2): the *tolC* mutation appears to have almost overridden the *ompR472* effect, and the increase due to *tolC* in this background was about 100-fold.

The results presented above indicate that *tolC* interferes with the normal effect of *ompR* on OmpC. However, the interpretation was difficult due to the presence of the *ompC-lacZ* fusion product which interferes with the physiology of the cell, and to study the interaction of *tolC* and *ompR* under normal physiological conditions, we used nonfusion strains. The addition of a *tolC* mutation to two different *ompR* mutants (MH760, *ompR472*; FN101, *ompR20*) leads to a substantial increase in the level of OmpC and a decrease in the OmpF level (Fig. 8). These results confirm our observation that a *tolC* mutation increases *ompC* expression.

**Effect of *tolC* in *ompF-lacZ* fusion strains.** We showed

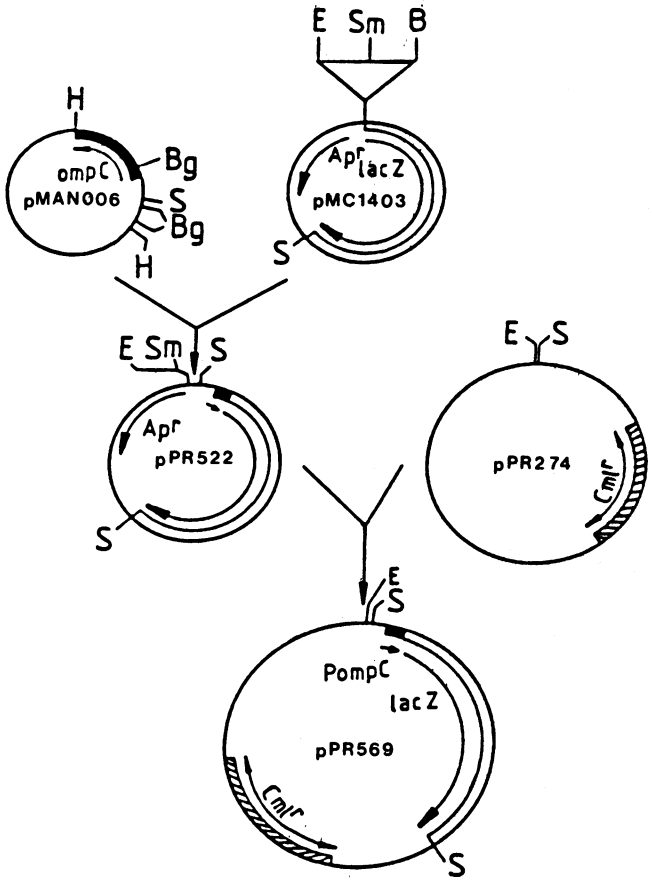


FIG. 7. Construction of the *ompC-lacZ* fusion. The *Bgl*II fragment from pMAN006, which includes the *ompC* promoter and a unique *Sal*I site, was subcloned into the *Bam*HI site of pMC1403, resulting in pPR522. This resulted in a unique fusion of the *ompC* promoter to the *lacZ* gene (at codon 8 of the truncated LacZ protein). The *Sal*I fragment from pPR522 carrying the *ompC-lacZ* fusion was subcloned into the unique *Sal*I site of pPR274, resulting in pPR569. Abbreviations: B, *Bam*HI; Bg, *Bgl*II, E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; Sm, *Sma*I.

previously that an *ompF-lacZ* operon fusion was less sensitive to a *tolC* mutation than the wild-type *ompF* gene: a *tolC* mutation reduces the amount of OmpF below detectable levels, whereas it reduces *ompF* expression in the fusion only by two- to threefold (26). In this study we used an *ompF-lacZ* protein fusion strain in which the *lacZ* gene was placed under *ompF* transcription and translation controls (2). The hybrid OmpF-LacZ protein contained the first 35 amino acid residues of the amino terminus of OmpF and most of the LacZ protein. The *tolC* mutation reduced both β-galactosidase activity (data not shown) and the amount of the hybrid protein by 10-fold in the *micF*$^+$ strain (Fig. 9, tracks 1 and 2) but not in the *micF*$^-$ strain (Fig. 9). The *ompR101* mutation reduced *ompF* expression by >50-fold (data not shown).

The protein fusion contains the *ompF* promoter and the first 35 amino acid residues of OmpF and hence presumably the whole of the control region, including the *micF* binding site. In the case of the operon fusion, the fused operon is known to carry the *ompF* promoter, but may have none or only part of the *micF* binding site. The greater effect of *tolC* on the protein fusion compared to the operon fusion could then be due to differences in response to *micF* RNA.

To test this possibility, we transformed a multicopy *micF*$^+$ plasmid into *ompF-lacZ* operon and protein fusion strains and assayed *ompF* expression by measuring the β-galactosidase activity. The *micF* plasmid had no effect in the operon fusion strain (Table 3), whereas it reduced *ompF* expression dramatically in the protein fusion strain. Thus, both *tolC* and high levels of *micF* produce a significant effect only in the protein fusion strain, both confirming our hypothesis that *tolC* acts via *micF* and explaining the low effect of *tolC* on the operon fusion studied previously. However, it should be noted that whereas high levels of *micF* produce no effect in the operon fusion strain, a *tolC* mutation reduces

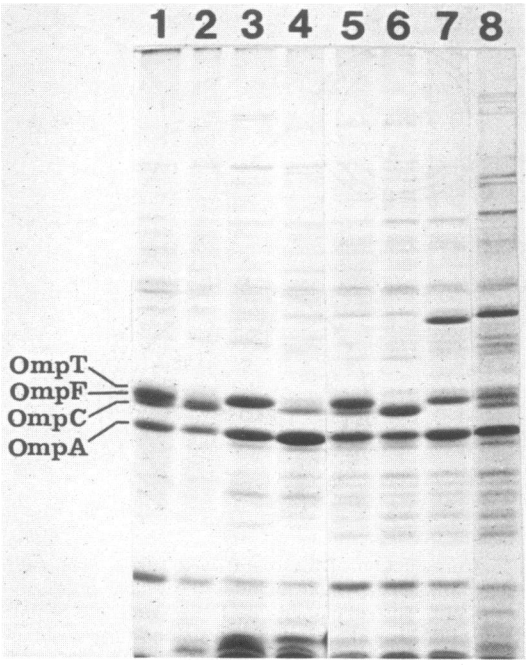


FIG. 8. Effect of *tolC* on OmpF and OmpC in *ompR* and *ompR*$^+$ strains. Whole cell envelopes from MC4100 (lane 1), P3011 (lane 2), MH760 (lane 3), P3394 (lane 4), W4626 Phe$^-$ (lane 5), P3396 (lane 6), FN101 (lane 7), and P3393 (lane 8) were analyzed by SDS-PAGE.

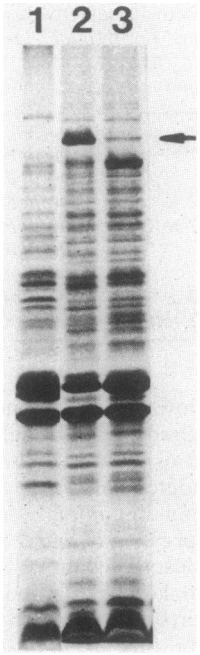


FIG. 9. Effect of the *tolC* mutation on hybrid OmpF-LacZ fusion protein. Whole cell extracts from MC4100 (lane 1), MH610 (lane 2), and P3289 (MC4100 *tolC*, lane 3) were analyzed by SDS-PAGE. Arrow indicates the position of hybrid OmpF-LacZ protein.

*ompF* expression by two- to threefold (see above). We believe that this difference reflects a limited *tolC* effect on *ompF* which is independent of *micF*. A similar limited effect of *tolC* on *ompF* was observed in the *micF* deletion strains (Fig. 5 and 6).

## DISCUSSION

TolC is a minor outer membrane protein which, in addition to affecting colicin E1 tolerance and sensitivity to detergents and dyes, has a major effect on OmpF, since in a *tolC* mutant the amount of OmpF protein present in the outer membrane is dramatically reduced (26). In this paper we show that this effect of *tolC* mutation on OmpF is due to activation of *micF*, which then inhibits translation of *ompF* mRNA. The activation of *micF* may well be mediated by OmpR protein.

When we compare the effect of a *tolC* mutation on strains which carry different copy numbers of the *ompF* gene, we find that in our standard nutrient broth (which includes 0.5% NaCl), a *tolC* mutation reduces the amount of OmpF protein in *E. coli* K-12 to a level not detectable on stained polyacrylamide gels, whereas it has no effect on OmpF level if there are 50 copies of the *ompF* gene present: there was an

TABLE 3. Effect of a multicopy *micF* plasmid (pCX28) on β-galactosidase activity in operon and protein fusion strains

| Strain | Fusion | Plasmid | Enzyme units |
|---|---|---|---|
| MC4100 | | | 0 |
| MH513 | Operon | | 418 |
| P3625 | Operon | pBR322 | 382 |
| P3626 | Operon | pCX28 | 394 |
| MH610 | Protein | | 1,520 |
| P3627 | Protein | pR322 | 1,426 |
| P3628 | Protein | pCX28 | 10 |

intermediate reduction if 7 or 2 copies were present. It is clear that TolC protein is not essential for OmpF synthesis, and this conclusion can also be drawn from our earlier observation (26) that *tolC* mutants do produce OmpF protein in a low-salt medium.

Hybrid *ompF-ompC* genes, constructed by Matsuyama et al. (18) by in vitro recombination at the common *Bgl*II site, enabled us to show that a *tolC* mutation exerts its effect at the promoter or at the amino-terminal end of the *ompF* gene. This is comparable to the effects exerted via *ompR*, which acts on the promoter.

We have also observed that, in addition to apparently reducing the amount of OmpF protein, a *tolC* mutation increases the level of OmpC protein present in the membrane. This increase is greater than occurs in an *ompF* mutant (compare, for example, AB1133, P210, and P602 in Fig. 3) and hence is not a simple compensation for the lack of OmpF protein, but is presumably an effect of *tolC* mutation at the *ompC* locus itself. Since we first reported that the *tolC* gene affected expression of the *ompF* gene, it has been shown (23) that a locus, *micF*, which maps upstream of and very close to *ompC* and is probably coregulated with *ompC* has a strong negative regulatory effect on *ompF* expression, at least when present on a multicopy plasmid (23). Schnaitman and McDonald (33) also proposed a regulatory element upstream of, and coregulated with, *ompC*, with a product which inhibited OmpF synthesis.

We therefore examined the possibility that the effect of *tolC* mutation is primarily on the *ompC* and *micF* genes and only secondarily on the *ompF* gene.

We found that in a *micF* deletion background a *tolC* mutation has virtually no effect on the level of OmpF when cultures are grown in low-osmolarity medium, i.e., nutrient broth without NaCl: the effect in a high-osmolarity medium (nutrient broth plus NaCl) is difficult to interpret since *tolC* mutants are sensitive to high salt levels (unpublished observations), as they are to many other environmental factors, but certainly the effect of *tolC* is much less in a *micF* mutant than in a *micF*$^+$ strain.

Furthermore, *tolC* mutation is shown to have a differential effect on an *ompF-lacZ* operon fusion and protein fusions which correlates with differential sensitivity to a multicopy *micF* plasmid. The operon fusion may well lack the *micF* binding site and is barely affected by *tolC* mutation, again supporting the role of *micF* RNA in the *tolC* effect on OmpF.

It appeared likely, then, that the effect of *tolC* on OmpF is mediated by activation of the *micF* gene, which has an RNA product known to inhibit expression of *ompF* (23). This hypothesis is supported by our finding that *tolC* mutation substantially increases the transcription of *micF* in a *micF-lacZ* fusion strain and by our earlier finding (13) that the Stc$^-$ mutation, which partially reverses the effect of a *tolC* mutation on OmpF protein level (25), is in effect a deletion of *micF* (22). That the major effect of *tolC* mutation on *ompF* is at the posttranscription level is quite compatible with it being mediated by *micF* RNA, as *micF* at high copy number has been shown to block translation of *ompF* mRNA (23).

The effect of *tolC* on *ompC* was confirmed by use of an *ompC-lacZ* fusion. A *tolC* mutation increases the expression of *ompC*, and in particular we show that a *tolC* mutation increases *ompC* expression when it has been reduced by *ompR* mutations. This effect is very clear in the two *ompR* mutants used in this study, MH760 (*ompR472*) and FN101 (*ompR20*), but was also evident, although not noted at the time, in a strain (P530) carrying a typical *ompR* mutation (*ompR101*) which normally lacks OmpF and OmpC (32).

Our data confirm that the expression of *micF* and *ompC* is coregulated. The results we report support the hypothesis that the *tolC* effect on OmpF is brought about by an effect on this regulatory system, i.e., by affecting expression of the *ompC* and *micF* genes, with the latter then affecting the *ompF* gene.

*tolC* mutants are pleiotropic and are extremely sensitive to detergents and dyes, indicating that they have a membrane defect: our major results could be explained if this membrane defect leads to a modification of the osmolarity detection and response system (which involves *ompR* and *envZ* gene products) of the cell such that the OmpF/OmpC ratio is pushed even further in favor of OmpC (and *micF*) than is the case for a normal strain grown in the high-osmolarity medium.

While this would account for the major effect of *tolC* mutation on *micF*, *ompC*, and *ompF*, we cannot yet account for the residual low-level effects on *ompF* discussed above or the previously described effects on NmpC and Lc proteins (26) which do not require OmpR as a positive control element (30).

Too little is known as yet of the molecular mechanisms involved in osmoregulation or of the role of *ompR* and *envZ* (9, 19) for us to speculate on the primary effect of TolC at the molecular level.

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