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Uncoupling of Osmoregulation of the *Escherichia coli* K-12 *ompF* Gene from *ompB*-Dependent Transcription

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The expression of the genes for the *Escherichia coli* K-12 outer membrane proteins, *ompF* and *ompC*, is subject to osmoregulation and responds to changes in the osmolarity of the growth medium. The transcription of these genes is dependent on the products of the regulatory locus *ompB* (comprising the genes *ompR* and *envZ*). The native promoter of *ompF* was replaced with an inducible *lpp* promoter to eliminate this transcriptional dependence of *ompF* expression on *ompB*. As a result, it was possible for the OmpF protein to be produced in an *ompB* mutant strain that does not normally express *ompF*. Surprisingly, the expression of *ompF* under the *lpp* promoter was still osmoregulated not only in the *ompB*$^+$ strain but also in two *ompB* strains tested. These results indicate the involvement of a factor(s) besides the *ompR* and *envZ* gene products in the osmoregulation of OmpF production. This factor may interact with a sequence downstream of the *ompF* promoter. In addition, we show that the expression of *ompF* under the *lpp* promoter has no direct effect on *ompC* expression.

The porin proteins OmpF and OmpC of *Escherichia coli* K-12 are major outer membrane proteins that form diffusion pores for small hydrophilic molecules across the membrane (14, 23). The genes encoding these proteins are located at different positions on the *E. coli* chromosome (*ompF* at 21 min and *ompC* at 47 min) (4, 5, 29, 30), and their expression has been shown to respond to changes in the osmolarity of the growth medium in a reciprocal manner (12, 28). This phenomenon of osmoregulation of the porins, whereby *ompF* is preferentially expressed in low osmolarity and *ompC* is preferentially expressed in high osmolarity, has been shown to occur primarily at the level of transcription (7, 8).

A third locus, the *ompB* operon, at 74 min on the *E. coli* chromosome, has been identified as a regulatory region for both *ompF* and *ompC* (26, 31). This locus has been cloned and shown to encode two genes, *ompR* and *envZ* (8, 21, 34). An analysis of mutations in the *ompB* locus has indicated that both genes of the operon are necessary for transcription of the porin genes. Thus, mutations in these genes affect the expression of either *ompF* or *ompC* or both, with a simultaneous alteration in the mode of osmoregulation. The model proposed to explain the regulation of the porin genes by the *ompB* gene products postulates that EnvZ acts as an osmosensor in the membrane (8). Information from EnvZ is transduced to modulate the functional state of OmpR in the cytoplasm so that it can selectively potentiate the transcription of either *ompF* or *ompC*. The sequence analysis of *envZ* (22) supports the idea that the EnvZ protein, with a long stretch rich in hydrophobic residues, is membrane associated. The sequence analysis of *ompR* suggests that OmpR is a basic protein and could interact with EnvZ, as well as with the promoter regions of the porin genes (33). The model thus envisages a specific osmolarity-linked modulation of transcription of the porin genes by OmpR.

An additional gene, *micF*, involved in the regulation of the expression of *ompF*, has been identified (18, 19). This gene, located upstream of *ompC*, encodes an RNA with a high degree of complementarity to the 5′ end of the *ompF* mRNA. The expression of this gene results in the inhibition of OmpF production, apparently at the translational level. *ompB* mutations affect *ompC* and *micF* expression similarily. Evidence from chromosomal deletions of the *micF* gene and the *ompC* gene lends further support to the role of *micF* in the suppression of *ompF* in high osmolarity (27). Thus, *micF* is also implicated in the osmoregulation of *ompF*.

By analysis of the expression of *ompF* and *ompC* after a mutual exchange of the DNA sequences at the 5′ ends of the two genes, Matsuyama et al. (16) showed that *ompB*-mediated regulation acts on this interchanged region of the porin genes. Their results indicated that the region upstream of the *Bgl*II site in the mature porin-coding sequence (see Fig. 1) is responsible for the *ompB*-mediated osmoregulation of the porins.

Mutants with *ompF* that are phenotypically OmpF$^-$ are known to constitutively produce OmpC, suggesting that the expression of the *ompF* gene is directly involved in the osmoregulation of OmpC levels by suppressing *ompC* expression in low osmolarity (25).

In the present report, we further analyzed the role of OmpR and EnvZ in mediating the osmoresponse. In particular, we examined the role of the *ompF* promoter in osmoregulation. We replaced the *ompF* promoter with an inducible *lpp* promoter and analyzed the expression of *ompF* for any dependence on *ompB*. We found that this *ompF* could now be expressed even in an *ompB* mutant that does not allow the expression of the native *ompF*. Furthermore, we found that *ompF* expression under the inducible *lpp* promoter was still subject to osmoregulation in both the *ompB*$^+$ strain and the *ompB* mutant strains. These results suggest a mechanism for the osmoregulation of *ompF* that is independent of *ompB*-mediated transcription. The induction of *ompF* expression under the *lpp* promoter did not suppress *ompC* expression, indicating that the production of OmpF does not directly suppress OmpC production.

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TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype	Source or reference
MC4100	F⁻ Δ*lacU169 araD139 rpsL relA thiA flbB*	T. J. Silhavy (1,7)
MH1160	MC4100 *ompR101* (*ompR1*)[a]	T. J. Silhavy (7)
MH760	MC4100 *ompR472* (*ompR2*)[a]	T. J. Silhavy (8)
MH1461	MC4100 *envZ11*	T. J. Silhavy (8)
MC4105	MC4100 *ompF*::Tn*5*	D. Oliver
T19 *recA*	F⁻ *kmt-7 tsx-354 recA*	34
JA221/F′ *recA*	*hsdR leuB6 lacY thi recA* Δ*trpE5*/F′ *lacI*^{q} *proAB lacZYA*	11

[a] *ompR1* and *ompR2* represent classes of *ompR* mutations initially identified as affecting genetically separable functions.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains used in this work were derivatives of K-12 strain MC4100 (1) (Table 1). All plasmids were pBR322 derivatives and were maintained in either the T19 *recA* strain (34) or the JA221/F′ *recA* strain (11). Plasmid pINIII-A2 has been previously described (15).

Media and culture conditions. Nutrient broth (Difco Laboratories) was used for the growth of cells in low osmolarity. For high-osmolarity conditions, nutrient broth was supplemented with 20% sucrose. Cells were grown overnight in M9 minimal medium supplemented with 0.2% Casamino Acids, 0.2% glucose, and 2 μg of thiamine per ml before being transferred into rich medium of a different osmolarity. Isopropyl β-D-thiogalactoside (IPTG; Sigma Chemical Co.) at a 2 mM concentration was used for the induction of *ompF* expression under pINIII *lppp lacZpo* control. Ampicillin was used in the media at a concentration of 50 μg/ml.

Preparation and analysis of outer membranes. Overnight cultures in minimal media were inoculated into 10 ml of the appropriate culture medium at a 1:20 dilution. Cells were grown at 37°C, and growth was monitored with a Klett-Summerson colorimeter with a red filter. Cells carrying the pINIII-derived plasmid pGR202 were induced with IPTG at a Klett reading of 30 and harvested after 3 h of induction. Outer membranes were prepared essentially as described by Coleman et al. (2). Cells were washed once in 20 mM sodium phosphate buffer (pH 7.1), resuspended in 0.5 ml of the same buffer, and disrupted by sonication. The lysates were incubated at room temperature for 30 min in 0.5% *N*-lauroylsarcosine (sodium salt; Sigma) to solubilize the inner membrane (3). The outer membranes were recovered by centrifugation at 100,000 × *g* in a Beckman 50 Ti rotor for 1 h at 4°C. The membranes were suspended in 20 mM phosphate buffer and solubilized by being boiled in the sample buffer described by Laemmli (13). The membrane preparations were normalized according to cell density at harvest. Outer membrane proteins were analyzed by electrophoresis on 8 M urea-sodium dodecyl sulfate-polyacrylamide gels as described by Mizuno and Kageyama (20). Gels were stained with Coomassie brilliant blue.

DNA manipulations. Plasmids pGR201, pGR202, and pGR203 were constructed as described in the text and in Fig. 1. Restriction enzymes, T4 DNA ligase, and Klenow enzyme (large fragment of DNA polymerase I) were purchased from Bethesda Research Laboratories. Enzymes were used in accordance with instructions provided by the manufacturer. *Xba*I linkers d(CTCTAGAG) were purchased from New England BioLabs, Inc.

RESULTS

Construction of plasmids. The *ompF* gene has been cloned and sequenced (10). Based on the published restriction map and the characterization of the *ompF* gene, we were able to construct plasmid pMY222 carrying a 6.15-kilobase (kb) *Eco*RI- and *Hin*dIII-digested chromosomal fragment in pBR322. The initiation of transcription of *ompF* has been mapped to a *Pst*I site 110 bases upstream from the initiation codon (9) (indicated by the arrowhead in Fig. 1). By partial *Pst*I digestion of pMY222 followed by Klenow enzyme treatment and *Xba*I linker ligation, the *Pst*I site at the start of transcription was replaced with an *Xba*I site. From the resultant plasmid pGR201, the 4.9-kb *Xba*I-*Bam*HI fragment carrying the structural *ompF* gene was cloned into pINIII-A2 digested with *Xba*I and *Bam*HI (Fig. 1). The plasmid thus constructed, pGR202, carries all of the transcribed region of *ompF* under IPTG-inducible pINIII *lppp lacZpo* control. Plasmid pGR203 was constructed by deletion of a 4.6-kb *Ava*I fragment from pMY222 (Fig. 1). pGR203 carries all of the *ompF* gene and the upstream region. Plasmids pGR201 and pGR203 were found to be identical to plasmid pMY222 in the expression of *ompF* when transformed into various *ompB*$^+$ and *ompB* mutant strains (data not shown).

Expression in an *ompF*::Tn*5* strain. Strain MC4105 carries a Tn*5* insertion in the chromosomal *ompF* gene and is therefore unable to express the OmpF protein. Figure 2, lanes 1 and 2, show the outer membrane pattern of MC4105 transformed with pBR322. The absence of OmpF and the constitutive production of OmpC in low osmolarity and high osmolarity were observed. MC4105 transformed with pGR203 produced OmpF in low osmolarity; the levels were significantly reduced in high osmolarity (Fig. 2, lanes 3 and 4). A simultaneous fluctuation in the levels of the OmpC protein was observed, with a reduced level in low osmolarity as compared with high osmolarity. OmpF and OmpC levels were examined in pGR202-transformed MC4105 grown in the absence or presence of an inducer under different osmotic conditions (Fig. 2, lanes 5 through 8). Lanes 5 and 6 correspond to cells grown in low osmolarity, and lanes 7 and 8 represent cells grown in high osmolarity. A significant increase in the levels of OmpF after IPTG induction was observed. The production of OmpF in the absence of an inducer (lanes 5 and 7) was probably due to an incomplete repression of the strong *lpp* promoter and was also observed in minimal medium (data not shown). After full induction, cells grown in nutrient broth with sucrose showed a fourfold decrease in the levels of OmpF as compared with cells grown in nutrient broth without sucrose. This ratio is comparable to the ratio of OmpF levels in MC4105 carrying the *ompF* gene with its native promoter (plasmid pGR203, lanes 3 and 4) and indicates that the expression of *ompF* under the *lpp* promoter and the *lac* promoter-operator is also subject to osmoregulation. We confirmed that the vector pINIII-A2 itself does not alter porin levels in *ompB*$^+$ strains in the presence or absence of an inducer (data not shown). We also determined that β-galactosidase produced from a pINIII *lppp lacZpo-lacZ* fusion shows no significant fluctuation in activity in media of different osmolarities (data not shown), thus confirming that the *lppp lacZpo* activity in itself is not subject to osmoregulation. Figure 2, lanes 5 through 8, also show that although OmpC production was increased in high osmolarity, no obvious change in the levels was effected by the induction of *ompF* expression with IPTG. These results

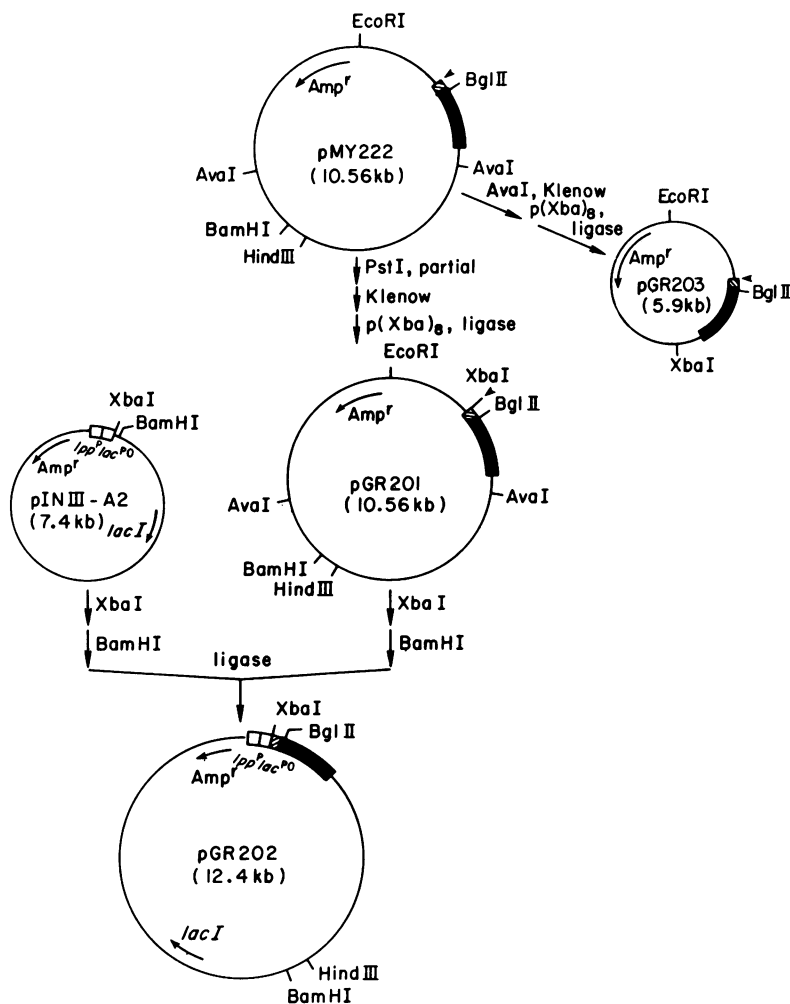

FIG. 1. Construction of plasmids. The hatched bars represent the region encoding the untranslated leader of the *ompF* mRNA. The solid bars represent the translated region, including the signal peptide and the mature protein-coding sequence. The start of transcription of *ompF* is indicated by the arrowheads and is at a *PstI* restriction site. The open bars denote lpp^P in the pINIII-A2 vector as the lipoprotein promoter (*lppp*), and lac^{PO} as the lactose promoter-operator (*lacZpo*). Amp^r is the ampicillin resistance marker. Relevant restriction enzyme sites on the plasmids are indicated. $p(Xba)_8$ represents the *XbaI* 8-mer linker used in the construction of pGR201.

indicate that the level of expression of *ompF* does not directly affect *ompC* expression, as was previously suggested (25).

Expression in *ompB* strains. The dependence of the expression of the *ompF* gene on *ompB* was evident from an analysis

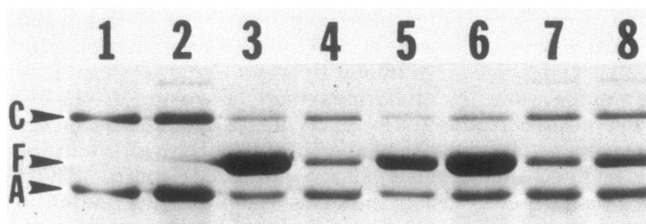

FIG. 2. Expression of *ompF* in an *ompF*::Tn*5* strain. Outer membranes of strain MC4105 harboring various plasmids were analyzed on polyacrylamide gels after growth in low osmolarity (lanes 1, 3, 5, and 6) and high osmolarity (lanes 2, 4, 7, and 8). Lanes: 1 and 2, pBR322; 3 and 4, pGR203; and 5 through 8, pGR202. Lanes 5 and 7 represent growth in the absence of IPTG; lanes 6 and 8 represent IPTG-induced cultures. Only the relevant portion of the outer membrane pattern is presented, and the positions of OmpC (C), OmpF (F), and OmpA (A) are indicated.

of OmpF production in various *ompB* mutants. Figure 3A represents the patterns of OmpF and OmpC production in the pBR322-transformed $ompB^+$ strain MC4100 and in three *ompB* derivatives in low and high osmolarities. Lanes 1 and 2 represent MC4100; the pattern of reciprocal fluctuation in the levels of OmpF and OmpC after an osmolarity change is obvious. The *ompR101* (*ompR1*) mutant did not produce either porin (lanes 3 and 4). The *ompR472* (*ompR2*) mutant had an $OmpF^+$ $OmpC^-$ phenotype, and the repression of OmpF production in high osmolarity was incomplete (lanes 5 and 6). The *envZ11* mutant did not express the chromosomal *ompF* gene, and OmpC levels showed a reverse fluctuation in low and high osmolarities (lanes 7 and 8).

The expression of *ompF* carried on a plasmid (pGR203) in the various *ompB* strains is shown in Fig. 3B. Lanes 1 and 2 represent *ompF* expression in the $ompB^+$ strain. With

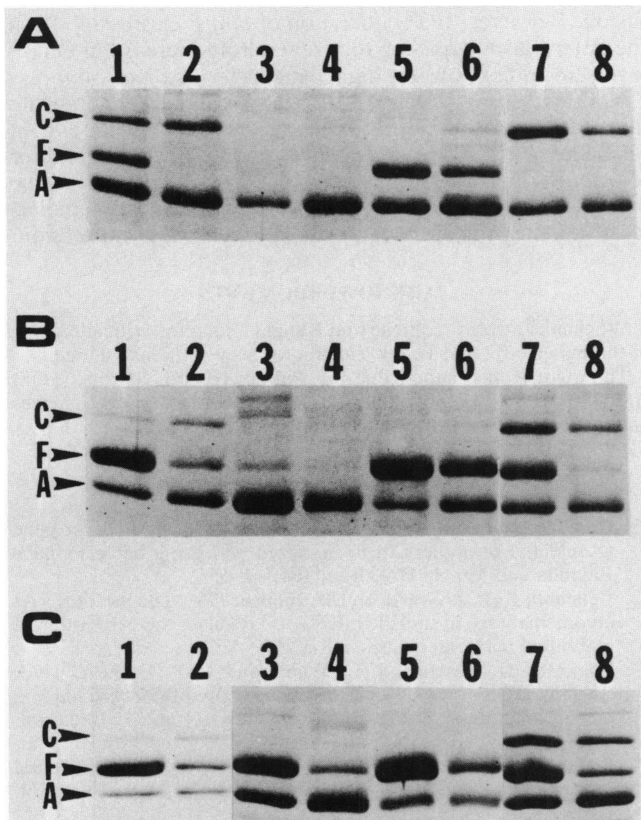

FIG. 3. Expression of *ompF* in *ompB*$^+$ and *ompB* strains. Outer membranes of strains MC4100 (lanes 1 and 2), MH1160 (*ompR1*, lanes 3 and 4), MH760 (*ompR2*, lanes 5 and 6), and MH1461 (*envZ11*, lanes 7 and 8) were analyzed in low osmolarity (1, 3, 5, and 7) and high osmolarity (2, 4, 6, and 8) after transformation with various plasmids. (A) pBR322; (B) pGR203; (C) pGR202 after induction with IPTG. C, OmpC; F, OmpF; A, OmpA.

OmpA levels as the control, OmpF levels were seen to be increased over the chromosomal *ompF* expression seen in Fig. 3A, lanes 1 and 2. A clear osmoregulation of OmpF and OmpC levels was evident. Matsuyama et al. (16) showed that the expression of *ompF* carried on a plasmid in an *ompB*$^+$ strain that has mutations in both *ompF* and *ompC* is constitutive; the additional presence of *ompC* on the plasmid rendered the expression osmoresponsive. In the *ompR1* strain transformed with pGR203, the *ompF* gene was virtually silent (lanes 3 and 4). As expected, the *ompR2* strain showed significant OmpF production, and although a reduction was observed in high osmolarity, the repression was partial (lanes 5 and 6). In low osmolarity, the *envZ11* strain transformed with pGR203 was able to express the *ompF* gene on the plasmid to levels almost comparable to those in *ompB*$^+$ strain, and a similar osmoregulation was evident (lanes 7 and 8). *ompC* expression, however, was still reversely osmoregulated, independent of OmpF production.

Figure 3C represents porin production after IPTG induction in the four strains transformed with pGR202. Like the *ompF*::Tn*5* strain MC4105, the *ompB*$^+$ strain MC4100 showed a clear osmoregulation of *ompF* expression (Fig. 3C, lanes 1 and 2). The levels of *ompF* expression from the induced *lpp* promoter were comparable to the levels of *ompF* expression directed by the native promoter (Fig. 3B, lanes 1 and 2). The relative levels in low and high osmolarities were also similar. The *ompR1* mutant, which was unable to express the native *ompF* gene, was able to express *ompF* under *lpp* promoter control to levels like those in the *ompB*$^+$ strain (Fig. 3C, lanes 3 and 4); the osmoregulation of *ompF* expression was also evident. The *ompR2* and *envZ11* mutants expressed *ompF* on pGR202 in a manner similar to that on pGR203 (Fig. 3C, lanes 5 through 8).

These experiments indicated that the removal of the transcriptional dependence on *ompB* did not disturb the potential for osmoregulation of porin production in cells. Further, the expression of *ompF* did not directly affect the expression of *ompC*.

DISCUSSION

Previous studies on the osmoregulation of the *ompF* and *ompC* genes have always used native promoters of these genes, so that the transcriptional requirement for OmpR and EnvZ has dominated the osmotic response. Studies with deletions upstream of the *ompF* gene have suggested that the target site for regulation by OmpR lies between −18 and −110 bases from the start of transcription and that the region upstream of the *ompF* promoter is also important for EnvZ-mediated functions (9). Replacement of all of the sequences upstream of the start of transcription of *ompF* with an inducible *lpp* promoter should eliminate any requirement of *ompB* function for the transcription of *ompF*. We have shown in this report that under the control of the *lpp* promoter, OmpF can be produced at levels like those in an *ompB*$^+$ strain even in an *ompR1* strain (phenotypically OmpF$^-$ OmpC$^-$), although the native *ompF* gene carried on a multicopy plasmid is not expressed in this strain (Fig. 3B, lanes 3 and 4). It is clear, therefore, that the OmpF protein can be expressed in the *ompR1* mutant once the transcriptional dependence of *ompF* on *ompR* is removed. From our observation that this expression is osmoregulated, it may be concluded that the mechanism of this osmoregulation is independent of transcriptional mediation by OmpR. This does not rule out the possibility that OmpR or EnvZ or both also interact with the region downstream of the *ompF* promoter (transcribed region) to enhance or repress OmpF production.

In the *ompR2* strain (phenotypically OmpF$^+$ OmpC$^-$), the repression of *ompF* expression is such that the level of OmpF in high osmolarity is higher than that in the *ompB*$^+$ strain (Fig. 3A, lanes 5 and 6). This incomplete repression of OmpF levels in high osmolarity indicates that although the *ompR2* mutant is capable of activating *ompF* transcription, the osmoregulatory mechanism is impaired. A mutation in *ompR* (*res ompR20*) that causes a reverse osmoregulation of OmpF has been reported (24). This observation taken in conjunction with our findings on osmoregulation in the *ompR1* and *ompR2* mutants indicates that OmpR acts bifunctionally to regulate *ompF* expression, positively as a transcriptional factor and negatively as a mediator of osmoregulation.

Expression of the chromosomal *ompF* gene was highly repressed in the *envZ11* mutant (Fig. 3A, lanes 7 and 8). Matsuyama et al. (16) observed the osmoregulated expression of *ompF* carried on a plasmid in this strain. In agreement with this are our findings that *ompF* expression under the control of both the native promoter and the inducible *lpp* promoter is osmoregulated in the *envZ11* mutant. The involvement of *envZ* in the osmoregulation of chromosomal *ompF* has been demonstrated by Villarejo and Case (32). They showed that the low level of *ompF* expression in an *envZ22*(Am) mutant (6) is independent of the osmolarity of the medium. The *envZ22*(Am) mutant then has lost osmo-

regulatory function for *ompF*, but the *envZ11* mutant has not. This implies that *envZ* is involved bifunctionally in the activation and osmoregulation of *ompF*.

To reconcile our results with published observations, it must be concluded that there is another factor(s) besides OmpR and EnvZ involved in the osmoregulation of OmpF. By interchanging the DNA sequences upstream of the region coding for the mature *ompF* and *ompC* (the site of exchange is indicated by the *Bgl*II site at +210 bases within the transcribed region of *ompF* in Fig. 1), Matsuyama et al. (16) showed that all the information required for osmoregulation of the porin genes lies in the interchanged stretch of DNA. Plasmid pGR202 in our experiment has lost all of the region upstream of the start of transcription and is still a target for osmoregulation. The common sequence of *ompF* in our constructs is the stretch of 210 bases from the start of transcription. This stretch includes the 110-base untranslated leader region, the region coding for the 22-amino-acid signal peptide important for transport across the inner membrane, and 12 amino acid residues at the amino terminus of the mature *ompF*-coding sequence. It is likely that the factor responsible for osmoregulation interacts with some part of this 210-base stretch. We have shown that the transcribed region of *ompC* includes a 32-base sequence that is highly homologous to the stretch +56 to +88 bases in the *ompF* gene (17). This sequence may act as the site for the osmoregulation of both genes.

The involvement of the *micF* gene in *ompF* regulation has been demonstrated previously (18, 19). The high complementarity of the *micF* RNA to the 5′ end of the *ompF* RNA suggests the formation of a hybrid, thus blocking translation and lowering the levels of OmpF synthesized. An analysis of the *micF-lacZ* fusion indicated that *micF* expression is regulated by *ompB* in a manner similar to *ompC* (18). Schnaitman and McDonald (27) reported increased levels of OmpF in cells with chromosomal deletions that included the *micF* gene. *micF*-mediated repression was more effective in high osmolarity. These results indicate that *micF* is coregulated with *ompC* so that it can act to repress *ompF* expression under conditions favorable for *ompC* expression. Thus, *micF* may contribute to the osmoregulation of *ompF*. The 72-base stretch of *ompF* mRNA involved in hybrid formation with *micF* RNA is contained in the 210-base stretch described above. However, certain lines of evidence indicate that *micF* is unlikely to be the major mediator of the osmoregulation of *ompF*. As shown in this study, the expression of *ompF* in multiple copies was osmoregulated in the absence of a simultaneous increase in *micF* gene dosage. Furthermore, *ompF* expression under *lpp* promoter control was osmoregulated in the *ompR1* mutant, which poorly expresses *micF*, as judged from *micF-lacZ* fusions (18).

Ozawa and Mizushima (25) have previously reported that the *ompF* gene regulates the expression of *ompC*. They suggested that the primary target of osmoregulation is the *ompF* gene and that *ompF* expression results in the suppression of *ompC*. The *envZ11* mutant has a high level of OmpC in low osmolarity and a reduced level in high osmolarity (Fig. 3A, lanes 7 and 8). When *ompF* is expressed to significant levels in this strain, no reduction is seen in OmpC levels (Fig. 3B and C, lanes 7 and 8). Consistent with the observations of Ozawa and Mizushima (25), we observed that the introduction of a functional *ompF* gene into an *ompF*::Tn*5* mutant relieves constitutive OmpC production, i.e., *ompC* expression is now subject to osmoregulation. This effect was seen with both the plasmid carrying the native *ompF* gene and the one with the *lppp lacZpo-ompF* fusion. However, IPTG induction of *ompF* expression from the latter plasmid leading to a severalfold increase in OmpF levels in either low or high osmolarity did not suppress OmpC levels (Fig. 2, lanes 5 through 8). Our results indicate that the osmoregulation of *ompC* is not directly dependent on the level of *ompF* expression. Instead, a regulatory factor for *ompC* is implicated whose activity, while under the influence of *ompF* expression, is determined by the osmolarity of the medium and not by the level of *ompF* expression.

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