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Regulation of Outer Membrane Protein Synthesis in *Escherichia coli* K-12: Deletion of *ompC* Affects Expression of the OmpF Protein

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A chromosomal deletion beginning at a Tn*10* located ca. 8 kilobases upstream from the *ompC* structural gene and extending through the 2.6-kilobase *Hin*dIII fragment carrying the *ompC* was isolated. The 2.6-kilobase *ompC* fragment was cloned into λ540 to obtain phage λ540C1. When the deletion mutant was lysogenized with λ540C1, the resulting strain produced normal levels of OmpC protein, and expression of this protein was regulated by osmolarity, carbon source, and the *lc* gene of phage PA-2, indicating that the cloned fragment contained all of the information required for regulated expression of *ompC*. The strain carrying the deletion was partially constitutive for expression of OmpF protein, whereas the λ540C1 lysogen of this strain and other strains with mutations in *ompC* repressed OmpF synthesis under conditions which lead to high-level expression of OmpC protein. Strains which are diploid or triploid for *ompC* show strong inhibition of synthesis of OmpF protein. We conclude that a regulatory element located upstream from the *ompC* coding sequence inhibits translation of OmpF protein under conditions which favor OmpC expression. Since *ompF* is known to repress transcription of *ompC*, we propose that these two genes constitute a closed regulatory loop which acts to amplify regulatory signals which control expression of these proteins.

Escherichia coli K-12 is known to produce five distinct outer membrane proteins which have the capability of forming solute permeation channels. These channel-forming proteins, also called porins, are the products of the *ompC*, *ompF*, *phoE*, *lamB*, and *nmpC* genes (1) and are named after their structural genes. The first four genes listed above have been sequenced (3, 12, 17, 22). In addition, the lambdoid bacteriophage PA-2 encodes a channel-forming protein which is expressed in lysogens (27). This protein is encoded by the *lc* locus (24) of the phage and is referred to as the Lc protein (formerly protein 2). The Lc and NmpC proteins are very similar (14), and it has recently been determined that the NmpC protein is encoded by a defective prophage which shares homology with both PA-2 and λ (P. Highfield, Y. Chang, W. Marcotte, Jr., and C. Schnaitman, manuscript in preparation) and which is located at 12 min on the *E. coli* map (13).

There is no known regulatory system which regulates all of these proteins as a group. Instead, the data presently available suggest a kind of pairwise regulation. The OmpC and OmpF proteins, which are the major channel-forming proteins found in cells grown under ordinary laboratory conditions, are regulated in a coordinate fashion by the osmolarity of the culture medium (30) and the nature of the carbon sources (2). This regulation is mediated by the *ompR* and *envZ* genes, which together comprise a single operon located at 75 min on the *E. coli* map (7, 19). The *ompR* gene product is required for expression of both *ompC* and *ompF*, and experiments with *lac* operon fusions indicate that *ompR* encodes a positive regulator of transcription of these genes (8, 9). The role of *envZ* is less clear. The phenotype of some of the first mutants studied was lack of OmpF protein and constitutive (osmolarity or carbon source-independent) expression of OmpC protein. This led to a model proposed by Hall and Silhavy (9), which suggested that *envZ* is involved in environmental sensing; in the absence of such sensing *ompC* was expressed constitutively. More recently, analysis of chain-terminating mutations in *envZ* indicates that the gene product is required for expression of both *ompC* and *ompG* (7).

The regulation of *ompC* and *ompF* is not solely accomplished by *ompR* and *envZ*. Ozawa and Mizushima have shown that a mutation in *ompF* resulted in constitutive expression of OmpC protein in the outer membrane and constitutive expression of an *ompC-lac* operon fusion, indicating that an element at the *ompF* locus regulates transcription of *ompC* (23). It is not clear whether this kind of cross-gene signaling is reciprocal; Ozawa and Mizushima reported than an *ompC* mutation had no effect on OmpF expression, whereas Morona and Reeves (20) reported that OmpF was expressed constitutively in an *ompC* background.

The expression of *ompC* and *ompF* is reduced when other channel-forming proteins are expressed. The relative amounts of OmpC and OmpF proteins decrease in mutants which express PhoE or NmpC proteins (26), but these effects have not been examined in detail. Synthesis of Lc protein in strains lysogenic for PA-2 results in a dramatic reduction in the amount of OmpC and OmpF proteins (6), which is not observed in strains lysogenic for lc^- mutants of PA-2 (24). Hall and Silhavy (10) showed that this down regulation of OmpC and OmpF proteins was not accompanied by a decrease in transcription of *ompC-lac* and *ompF-lac* operon fusions. The relationship between the mass amounts of Lc, OmpC, and OmpF proteins in the outer membrane has been examined by Fralick and Diedrich (6), who used both *lc* diploidy and temperature to modulate the amount of Lc protein. They found that the sum of OmpC protein plus OmpF protein in the outer membrane decreased in direct proportion to the increase in Lc protein and that the amount of Lc protein was proportional to *lc* gene dosage at temperatures which were intermediate for expression of this gene.

Induction of *lamB* also leads to a reduction of the levels of OmpC and OmpF proteins (5), and this effect appears different from that of Lc. At least part of the reduction appears to be due to reduced transcription, as monitored by an *ompC-lac* operon fusion.

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Our approach to understanding this complex regulatory system has been to attempt to isolate well-defined chromosomal *ompC* mutations and to examine the phenotype when we complement these mutations with a chromosomal *ompC* fragment cloned into an integration-proficient λ vector. In the present report, we describe both the isolation and characterization of strains deleted for the chromosomal *ompC* locus and the expression of cloned *ompC* sequences in this strain background.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial and bacteriophage strains used in this study are summarized in Table 1. Cultures were grown in Luria broth (LB) medium which contained (per liter) 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract (Difco), and 5 g of NaCl and which was adjusted to pH 7.0 with KOH before sterilization. Low-salt and high-salt LB contained, respectively, no NaCl and 2.0% NaCl. As noted, some cultures were grown in Difco nutrient broth or Difco tryptic soy broth. In these experiments, overnight cultures were grown in LB and diluted with 100 volumes of the final culture medium. All cultures were harvested near the end of log-phase growth.

Genetic techniques. Transduction with bacteriophage P1 was as described by Miller (16). Drug-resistant transductants were selected or screened on LB agar plates containing 1.25 mM sodium PP_i and tetracycline or kanamycin at 25 μg/ml or nalidixic acid at 50 μg/ml. Phage sensitivity was screened by cross streaking on LB agar plates. Lysogens were prepared by spotting phage lysates (titer, ca. 10^9 phage per ml) on LB agar plates with LB soft agar overlays seeded with overnight LB cultures. After incubation overnight, lysogens were isolated by streaking from within the spots to obtain single colonies, which were then screened by cross streaking against phage λW248 (for imm^{λ}), λNM508 (for imm^{21}), Hy8 (for $imm^{PA\text{-}2}$), or SS-4 (for λ540C1 in *ompC* backgrounds). To select a Tn*10* insertion near *ompC*, we infected strain CS197 (*ompR ompC*) with phage λNK370 and plated on LB-tetracycline plates as above. Approximately 10,000 colonies were pooled to give a zoo and phage p1 grown on this zoo was used to transduce strain CS1147. Transductants were plated on MacConkey agar containing 1.25 mM sodium PP_i and 25 μg of tetracycline per ml. Lac^- colonies were selected and examined for linkage of Tn*10* and *ompC* by transduction into strain CS109. Tetracycline-sensitive derivatives of strains carrying Tn*10* were selected by the procedure of Maloy and Nunn (15).

Recombinant DNA techniques. Procedures for isolation of DNA from bacteria or phage, for restriction enzyme digestion and analysis of agarose gels, and for ligation, transfection, and transformation were essentially those of Davis et al. (4). Southern blot hybridization was done by blot transfer procedure 21 of Davis et al. (4), with the exceptions that GeneScreen (New England Nuclear Corp., Boston, Mass.) membrane was used instead of nitrocellulose and that hybridization of nick-translated probe was done by procedure 46 of Silhavy et al. (T. Silhavy, M. Berman, and L. Enquist, *Experiments with Gene Fusions*, in press). The porin plaque assay used to identify recombinant phage carrying cloned genes encoding porin functions employed basal plates containing 12.5 ml of LB containing 1.5% agar and an overlay consisting of 2.5 ml of LB containing 0.6 to 0.7% agar. Dilutions of phage were added to 1 drop of an overnight culture of the indicator strain (CS146) grown on LB containing 0.2% maltose; this was used to seed the overlay. The plates were incubated overnight at 37°C and in some cases stored for several days at room temperature to enhance the development of $porin^+$ (very turbid) plaques.

TABLE 1. Bacterial and bacteriophage strains

Strain or bacteriophage	Relevant genotype and source
E. coli K-12	
MH150	*ompC*::Tn*5* in MC4100 (T. Silhavy)
MH225	MC4100 φ(*ompC'-lacZY*$^+$) 10-25 (S. Garrett)
CS109	W1485F$^-$ prototrophic (24)
CS138	*gyrA* of CS109 (2); formerly PB105
CS139	*ompC161 gyrA* of CS109 (2); formerly PB106
CS146	*ompA ompC ompF* of CS109 (25)
CS197	*ompC ompR* of CS109 (24); this strain was originally described as *ompB151*. Strain carries a transdominant *ompC* mutation and a polar *ompR* mutation.
CS218	*gyrA ompC163*(ParII) of CS109 (2); formerly PB107
CS444	*gyrA ompC166*(Am) of MX364; this mutation selected as OmpC(Ts) in a strain which was *supD*(Ts); P. Bassford
CS1112	As CS109 except *supD*
CS1146	Δ*lac-5* F$^-$; CGSC3677 cured of F' *lac* by growth at 42°C
CS1147	As CS1146 except lysogenic for λφ(*ompC'-lacZY*$^+$) 10-21 of T. Silhavy
CS1148	As CS1146 except lysogenic for λφ(*ompF'-lacZY*$^+$) 16-13 of T. Silhavy
CS1228	As CS109 except *zei-298*::Tn*10*; this study; *ompC161* and *gyrA261* derivatives of CS109 carrying this Tn*10* insertion can be obtained from Coli Genetic Stock Center (Yale University) as CGSC6600 and CGSC6601
CS1252	As CS138 except *gyrA*$^+$ Δ*ompC177 zei-298*::Tn*10*; this study
CS1253	As CS138 except *gyrA*$^+$ Δ*ompC178 zei-198*::Tn*10*; this study
CS1254	Tet^s derivative of CS1252; this study
CS1255	Tet^s derivative of CS1253; this study
CS1291	As CS138 except *rpsL* by p1 on MC4100 and *ompC*::Tn*5* by p1 on MH150; this study
CS1292	As CS1291
Bacteriophage	
SS-4	OmpC-specific phage from local sewage
Hy7	PA-2*h*λ
Hy8	PA-2*c h*λ
λW248	Δ(*B2-int*) *c h*80 (Cold Spring Harbor Laboratory)
λNM508	imm^{21} *c h*80 att^{λ} (M. Smith)
λNK370	Tn*10* donor phage (N. Kleckner)
λp1081.1	*lacZ*::Kan^r *int*am *c*I857 (T. Silhavy)
λ540	att^{λ} imm^{21} *c*I$^+$ *int*$^+$ Δ(*Hin*dIII 2-3) *nin5* (20) (N. Murray)
λ540C1	As λ540 except carrying the 2.6-kb *ompC Hin*dIII fragment from *E. coli*; this study

Protein analysis. A simplified outer membrane isolation procedure was employed. Cultures (20 ml each) were harvested, washed once with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4), and suspended to 12.5 ml in the same buffer. The cells were broken by passage through a French press and centrifuged at 5,000 rpm in a Sorvall SS-34 rotor for 5 min to remove whole cells, and the outer membrane was pelleted by centrifugation for 1 h at 20,000 rpm in a Beckman 50 Ti rotor. The outer membrane protein was analyzed on Tris-glycine-sodium dodecyl sulfate gels as previously described (26). Gels were loaded so that each well (1 cm by 0.5 mm) contained 20 μg of

protein. Gels were stained with Coomassie brilliant blue (26), and in some cases gels were scanned after destaining with an LKB 2202 Ultroscan laser densitometer fitted with a Hewlett-Packard 3390A integrator. Each track on the gel was scanned three times, and the average of these scans was used to compute the relative density of each band on the gel. All tracks on a single gel were scanned without resetting the base line so that intensity of bands in different samples could be compared directly.

RESULTS

Isolation of *ompC* deletions. To isolate strains deleted for the chromosomal *ompC* gene, we used a modification of the procedure devised by Silhavy et al. (Silhavy et al., in press) (Fig. 1). Strain MH225, which carries the λp1 (209) *ompC'-lacZY*$^+$ operon fusion 10-25, was transduced to Tet^r with p1 grown on strain CS1228 (*zei-298*::Tn*10*). A broth culture of the resulting strain was infected with phage λp1081.1 and spread on a kanamycin (Kan)-MacConkey plate to select for lysogens. The majority of the lysogens were Lac$^+$, but a small percentage were Lac$^-$, indicating that λp1081.1 had integrated by recombination between the *lac* sequence of the resident operon fusion phage and the *lac* sequence in λp1081.1 lying 5′ to the kanamycin insertion as shown in Fig. 1. The resulting strains are thermoresistant, owing to the *c*I$^+$ allele contributed by the resident operon fusion phage. These strains are unstable and will segregate out one copy of λ carrying the *c*I$^+$ allele as well as *att int* as shown in Fig. 1. Several Lac$^-$ thermoresistant strains were streaked on kanamycin-MacConkey plates and grown at 30°C. The resulting

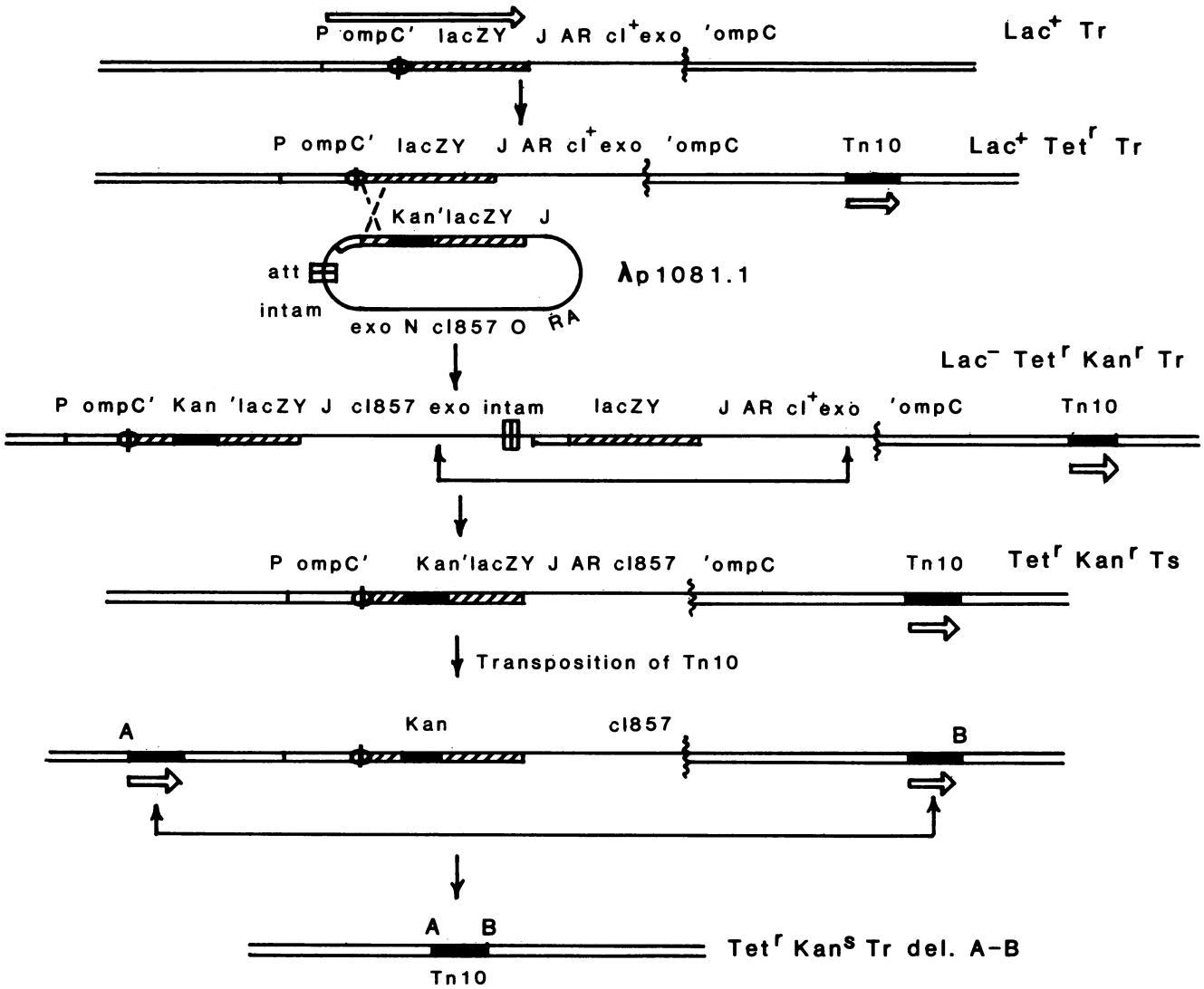

FIG. 1. Isolation of *ompC* deletions from a strain carrying an *ompC-lacZY* operon fusion. Line 1 shows the parental strain (MH225), and line 2 shows a derivative carrying a closely linked Tn*10* insertion. Lines 2 and 3 show the integration of phage λp1081.1. This phage is *c*Its857 *int*am and carries *lacZY*, which has no promoter and into which the kanamycin resistance (Kan) determinant of Tn*5* has been inserted. Lysogens formed by integration as indicated by the dotted lines will be Lac$^-$ Kan^r Tet^r and will remain thermoresistant (Tr). Lines 3 and 4 show the segregation of one copy of λ including the *c*I$^+$ allele of the original fusion phage, resulting in a strain which is thermosensitive (Ts). Lines 5 and 6 show one possible mechanism whereby deletions 100% linked to Tn*10* could be generated, involving transposition of Tn*10* in the same orientation to a site on the opposite side of the fusion, followed by segregation of the material between the two copies of Tn*10*. The direction of the arrows indicating the orientation of Tn*10* and the orientation of the fusion with respect to Tn*10* were chosen arbitrarily for purposes of illustration.

colonies were tested for growth at 30 and 42°C. Thermosensitive segregants were obtained at a frequency of about 1%.

Cultures of several such thermosensitive strains were grown in LB at 30°C and plated on LB-tetracycline (Tet) plates incubated at 42°C. Thermoresistant survivors were obtained at a frequency of about 1 per 10^9 cells plated. We initially tested 14 such thermoresistant survivors for *c*I by cross streaking against phage λW248 and for sensitivity to the OmpC-specific phage SS-4 and to kanamycin. One of these was cI^+ Kan^r, and one was cI^- Kan^r, indicating thermoresistance due to deletion or mutation within the prophage. The remaining 12 were cI^- Kan^s, and all were $OmpC^-$.

To screen for large deletions, we grew p1 lysates on 4 of these 12 strains and used these to transduce strain CS138 (*ompC*$^+$ *gyrA*) to Tet^r. These were scored for phage SS-4 resistance to determine linkage to *ompC* and for nalidixic acid (Nal) resistance to determine linkage to *gyrA*. Three of these showed 100% linkage to *ompC* and a significant (greater than 10%) increase in cotransduction of *gyrA*.

Of the three strains carrying *ompC* 100% linked to Tn*10*, we chose the two showing the highest *gyrA*-Tn*10* cotransduction frequency (Δ*ompC177*, 43%; Δ*ompC178*, 50%) for analysis by Southern blot hybridization. As probe we used pAT153 into which was subcloned the 2.6-kilobase (kb) *Hin*dIII fragment carrying *ompC*, which is described later in this report. DNA cut with either *Eco*RI or *Hin*dIII was analyzed from strains CS1252 and CS1253, which are Tet^r recombinants carrying these deletions, from strains CS1254 and CS1255, which are Tet^s derivatives of these strains, and from the parental *omp*$^+$ strain CS138. In strain CS138 cut with *Hin*dIII, a single, 2.6-kb band was detected, whereas in *Eco*RI digests of this strain, fragments of 2.0 and 8.5 kb were detected. No hybridization was detected in DNA from any of the deletion strains cut with either enzyme. As a control, filters carrying DNA from the parent and the deletion strains were stripped of probe and reprobed with pBR322 carrying a 1-kb insert cloned from the *tonB* region of *E. coli* K-12 (kindly provided by B. Mann). With both enzymes, appropriate chromosomal bands of equal intensity were detected in DNA from all of the strains. Since we were unable to detect hybridization to either of the deletions and since Δ*ompC178* is significantly larger than Δ*ompC177* on the basis of cotransduction of Tn*10* with *gyrA*, we conclude that, at least in Δ*ompC178*, the deletion must include the entire chromosomal *Hin*dIII fragment carrying *ompC* and extend into flanking DNA on the *gyrA* side of the gene.

Physical and genetic mapping of the *ompC* region. The location of the Tn*10* was determined as follows: p1 lysates grown on strain CS1228 (*omp*$^+$ *gyr*$^+$ *zei-198*::Tn*10*) were used to transduce strain CS139 (*gyrA ompC*) to Tet^r. Recombinants were scored for resistance to nalidixic acid and phage SS-4. From analysis of single and double recombinant classes, the gene order was found to be *gyrA-ompC*-Tn*10*. This was confirmed by a reciprocal cross in which p1 grown on strain CS139 was used to transduce strain CS1228 to Nal^r, and recombinants were scored for resistance to tetracycline and phage SS-4.

Southern blots of chromosomal DNA probed with pAT153 carrying the cloned 2.6-kb *ompC Hin*dIII fragment were used to construct a partial physical map of the *ompC* region and to determine the direction of transcription of *ompC* (Fig. 2). As noted above, this probe hybridizes to 2.0- and 8.5-kb *Eco*RI fragments from wild-type DNA. To orient these flanking *Eco*RI sites with respect to the *Hin*dII fragment, we examined DNA from strain MH150 (*ompC*::Tn*5*) and from two derivatives of strain CS109 which had been transduced to Kan^r *ompC* with p1 grown on strain MH150. Blots of DNA from these strains cut with *Hin*dIII yielded a doublet migrating slightly faster than the 2.6-kb fragment from wild-type DNA, indicating that the Tn*5* insertion was roughly in the middle of the *ompC Hin*dIII fragment. Analysis of DNA from these strains cut with *Bgl*II and *Pvu*II indicated that Tn*5* was within the 1-kb internal *Bgl*II fragment of the probe and was located ca. 100 base pairs 5′ to the first amino acid codon of the *ompC* signal sequence. When DNA from these strains cut with *Eco*RI was analyzed, the 2.0-kb *Eco*RI fragment was conserved and the 8.5-kb fragment was altered, indicating that the 8.5-kb fragment was 5′ to *ompC*. When DNA from strain CS1228 cut with *Eco*RI was analyzed, the 2.0-kb fragment was retained, whereas the 8.5-kb fragment increased to 11.5 kb, indicating that Tn*10* was inserted just within the *Eco*RI site 5′ to *ompC* and was oriented with the single asymmetric *Eco*RI site in Tn*10* towards the *ompC* gene as shown in Fig. 2. This indicates that the direction of transcription of *ompC* is towards *gyrA*.

Cloning of *ompC*. To clone *ompC*, we took advantage of a porin plaque assay developed for genetic analysis of the *lc* gene in phage PA-2 (T. Gregg, unpublished data). The basis of this assay is as follows: porin-deficient strains such as *ompR* or *ompC-ompF* mutants have a growth disadvantage in complex media such as LB, particularly at high culture density, when the supply of readily utilized substrates becomes growth limiting. Wild-type λ forms clear plaques when plated on a lawn of a porin-deficient indicator strain, because the lysogens which form within the plaque during late stages of growth are unable to compete for nutrients with the surrounding lawn. However, if an integration-proficient phage carries a functional porin gene or a regulatory gene such as *ompR* which complements the defect in the host, it will form very turbid plaques, since the lysogens enjoy a growth advantage over the bacteria in the lawn. We found that phage PA-2, which gives strongly $porin^+$ (very turbid) plaques on a variety of *ompR* mutant strains, gave only weakly $porin^+$ plaques on *ompC-ompF* indicator strains. PA-2 gave very strongly $porin^+$ plaques on strain CS146, which contains in addition a somewhat leaky *ompA* mutation. We cloned the 2.6-kb *Hin*dIII fragment carrying *ompC* by cutting *E. coli* chromosomal DNA with *Hin*dIII and ligating into *Hin*dIII-cleaved phage λ540 (21) DNA. After ligation, this DNA was transfected into a suitable recipient to generate a library of clones, and this library was screened on strain CS146 for $porin^+$ plaques. Very turbid plaques were obtained at a frequency of about 1 in 10^3 plaques. One of these plaques yielded phage λ540C1, which carried a 2.6-kb insert containing the *ompC* gene. This insert is oriented in the single *Hin*dIII site of phage λ540 so that the direction of transcription of *ompC* is the same as transcription from the phage λp_L promoter, as determined by Southern blots on lysogens with the probe described in the previous section. Thus, the *ompC* gene is located downstream from the *tI* (*sib*) terminator of phage λ; in a lysogen it is unlikely that there is any transcription of *ompC* originating from a phage promoter or from chromosomal DNA adjacent to λ *att* (11). The 2.6-kb *Hin*dIII insert from phage λ540C1 was subcloned into the *Hin*dIII sites of plasmids pBR322 and pAT153, and the restriction sites of the insert were mapped. The restriction map which we obtained is identical to that of Mizuno et al. (17).

We wish to emphasize the ease and utility of the porin plaque assay for cloning porin genes and porin-related functions. Using the same strategy with different phage

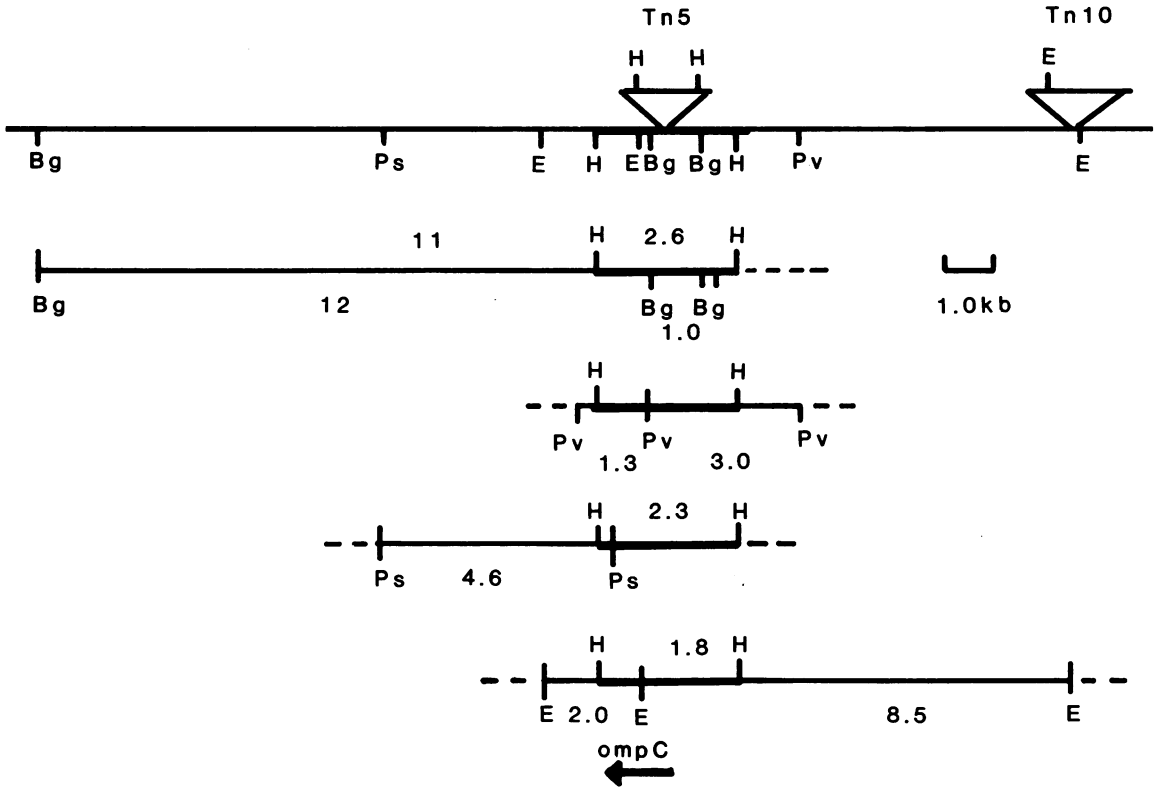

FIG. 2. Partial physical map of the *ompC* region. The double-heavy line indicates the 2.6-kb *Hin*dIII fragment used as probe, and the dashed lines indicate regions where cuts could not be detected with this probe. Bg, *Bgl*II; Ps, *Pst*I; E, *Eco*RI; H, *Hin*dIII; Pv, *Pvu*II. The sites of insertion of the Tn*5* in strain MH150 and of *zei-198*::Tn*10* are shown in line 1. Some of the restriction sites in line 1 have been omitted for clarity; a complete map is shown in reference 17.

vectors and with an *ompR* indicator strain, we were successful in cloning *Eco*RI and *Bam* fragments carrying the *ompR* and *ompR-envZ* genes. These experiments are not described here, since the fragments we obtained are identical to those described by others (19, 29). This strategy was also used recently for cloning the porin K gene from a pathogenic *E. coli* strain (28) in our laboratory.

Phenotypic complementation of Δ*ompC* by phage λ540C1. In this series of experiments, we examined the phenotype of strains deleted for *ompC* and the complementation of this phenotype by phage λ540C1. Figure 3 shows polyacrylamide gels of outer membrane from strains grown on LB (0.5% NaCl). OmpC protein was absent in the strain carrying Δ*ompC178* (lane B), and lysogeny of this strain by λ540C1 resulted in the restoration of a wild-type level of OmpC protein (lane C). When this strain was made doubly lysogenic for phage λ540C1 and Hy7 (PA-2*h*λ), the outer membrane profile was identical to that of a wild-type strain lysogenic for PA-2 (lanes E and F). Thus, the cloned *ompC* fragment carried by λ540C1 provides all of the information necessary for expression of a wild-type level of OmpC protein and for the regulation of this expression by the *lc* gene of phage PA-2.

Figure 4 demonstrates the effect of the carbon source (Fig. 4A) and osmolarity (Fig. 4B) on the outer membrane proteins of a strain carrying Δ*ompC178* and of the same strain lysogenic for phage λ540C1. A comparison of lanes A and B with lanes E and F shows that the cloned *ompC* fragment in λ540C1 also carries all of the information required for regulation of *ompC* by osmolarity and medium composition. We examined strains lysogenic for the vector λ540 as controls. This phage had no effect in either OmpC$^+$ or OmpC$^-$ strains (data not shown). We have also repeated this experiment with the shorter deletion (Δ*ompC177*), with identical results.

In this experiment, we also compared the phenotype of the *ompC* deletion mutant to strain CS139, which carries an *ompC* mutation of the ParI phenotype (2) selected for resistance to an OmpC-specific phage. We observed one significant difference between these mutant strains. Under conditions which favor maximum OmpC expression (high-

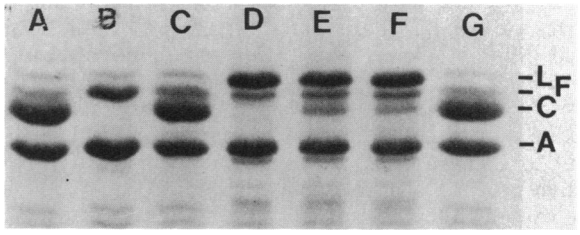

FIG. 3. Effect of the PA-2 *lc* gene on expression of OmpC protein from phage λ540C1. The lines to the right of the gel profile indicate Lc, OmpF, OmpC, and OmpA proteins. Lanes A and G, strains CS138 (*omp*$^+$); lane B, strain CS1255 (Δ*ompC178*); lane C, strain CS1255 lysogenic for phage λ540C1; lane D, strain CS1255 lysogenic for phage Hy7; lane E, strain CS1255 lysogenic for both phage λ540C1 and Hy7; lane F, strain CS109 (*omp*$^+$) lysogenic for phage PA-2. The cultures were grown on LB (0.5% NaCl).

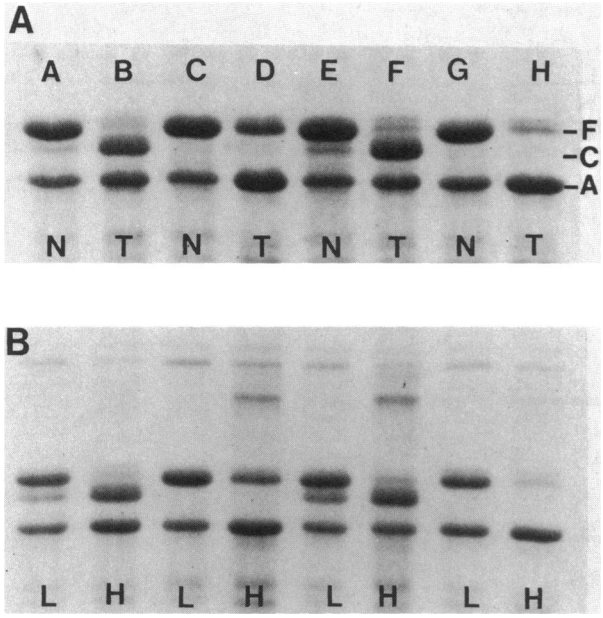

FIG. 4. Effect of carbon source and osmolarity on the expression of OmpF and OmpC proteins. Figure 4A shows the effect of nutrient broth (N) and tryptic soy broth (T), and Fig. 4B shows the effect of high (H)- and low (L)-salt LB. Lanes A and B, strain CS109 (*omp*$^+$); lanes C and D, strain CS1255 (Δ*ompC178*); lanes E and F, strain CS1255 lysogenic for phage λ540C1; lanes G and H, strain CS139 (*ompC161*).

salt LB or tryptic soy broth), strain CS139 produces almost no OmpF protein, as does the wild-type parent. Under these conditions, the deletion mutant produces a substantial amount of OmpF protein (Fig. 4, lane D). When the deletion mutant was made lysogenic for phage λ540C1 (lane F), the amount of OmpF protein was reduced to the level seen in the wild type (Fig. 4). This indicated that the deletion had removed a regulatory element. This regulatory element, which is present in the wild type, in strain CS139, and in the cloned fragment in λ540C1, inhibits OmpF expression under conditions which favor high OmpC expression.

To define the location and properties of this regulatory element, we have examined other *ompC* mutations in our collection. One such experiment is shown in Fig. 5. All of the cultures shown in this figure were grown on high-salt LB. Lane A shows a strain carrying *ompC163*, which is a spontaneous mutation of the ParII (very slightly leaky) phenotype, and lane B shows a strain which is *sup*$^+$ and carries the *ompC166*(Am) mutation; lanes C, D, and E show three different clones obtained when *ompC166*(Am) was transduced into a *supD* background (Fig. 5). All of these strains showed very low levels of OmpF protein, and partial suppression of the amber mutations had no effect on the level of OmpF. Lane F shows a control of the deletion mutant grown under the same conditions (Fig. 5). We have also examined the phenotype of derivatives of strain CS109 carrying the *ompC*::Tn*5* insertion from strain MH150. The outer membrane profiles of strains CS1291 and CS1292 which carry this mutation were identical to those shown in Fig. 5, lanes A and B (data not shown).

Strains carrying two different phenotypic classes of *ompC* mutations selected on the basis of phage resistance, an amber mutation which appears on the basis of its suppression pattern to be located within the *ompC* coding sequence,

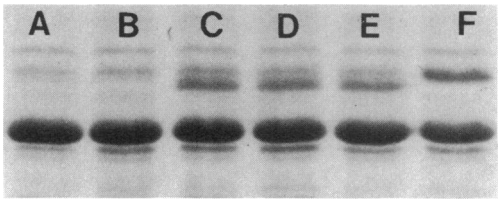

FIG. 5. Effect of various *ompC* mutations on OmpF expression. The cultures shown in this experiment were grown on high-salt LB. Lane A, strain CS218 (*ompC163*, ParII phenotype); lane B, derivative of strain CS109 carrying the *ompC166*(Am) mutations; lanes C, D, and E, derivatives of strain CS1112 (*supD*) carrying the *ompC166*(Am) mutation; lane F, strain CS1255 (Δ*ompC178*).

and a Tn*5* insertion located 100 base pairs upstream from the *ompC* coding sequence have all retained the ability to regulate synthesis of OmpF protein. We conclude that the regulatory element at *ompC* which inhibits expression of OmpF must be located upstream from the *ompC* coding region and does not depend upon synthesis of OmpC protein for its action.

To determine whether the regulation of OmpF by *ompC* is at the level of transcription from the *ompF* promoter, we transduced Δ*ompC178* into strain CS1148, which carries an *ompF-lacZ* operon fusion, and examined the production of β-galactosidase (Table 2). Although there was a slight increase in the expression of β-galactosidase in the deletion strain grown at low salt, there was no difference in the expression at high salt. As an additional control, we transduced an *ompR* mutation into strain CS1148. This resulted in a reduction of β-galactosidase to less than 1% of that exhibited by the *ompR*$^+$ parent, indicating that the operon fusion is monitoring transcription from the *ompF* promoter. This set of experiments indicates that the regulation of OmpF expression by *ompC* is not at the level of transcription from the *ompF* promoter.

Effect of *ompC* copy number on expression of OmpC and OmpF proteins. We predicted that increasing the copy number of the *ompC* gene should have a pronounced effect on both OmpC and OmpF expression, since this should increase the activity of both the structural gene and the element which regulates OmpF expression.

We increased the *ompC* copy number by isolating λ540C1 lysogens of *ompC*$^+$ strains. A number of the lysogens which we isolated carried two or more prophage integrated in tandem at *attλ*; these were readily identified since they overproduced OmpC protein and exhibited very little regulation of OmpC protein by the *lc* gene of phage PA-2. These multiple lysogens were quite stable. One trilysogen (three copies of the prophage in tandem) which we analyzed in detail segregated one or more copies of the prophage at a

TABLE 2. Effect of deletion of *ompC* on the expression of an *ompF-lacZ* operon fusion

Strain	β-Galactosidase (U/mg of protein)[a]	
	Low-salt LB	High-salt LB
omp$^+$	550	320
Δ*ompC178*	750	320

[a] Cells grown to the mid-log phase on low- or high-salt LB were broken with the French press as described in the text and centrifuged at 20,000 × *g* for 10 min to remove unbroken cells and large debris. The supernatants were assayed by the method of Miller (16).

frequency of less than once per 1,000 generations. The stability of such lysogens may reflect a lack of χ sites to facilitate recombinational excision. Since such multiple lysogens were obtained at reasonably high frequency, it was necessary to verify the number of copies of the prophage in each strain. This was done by Southern blots with the *Hin*dIII *ompC* fragment in pAT153 as probe. Digests of DNA from monolysogens yielded leftward 1.0-kb *Pst*I and 2.8-kb *Eco*RI fragments which end in λ sequence and rightward 7.7-kb *Pst*I and 8.5-kb *Eco*RI fragments which cross *attR* and end in *E. coli* sequence. Tandem multiple lysogens yield additional 6.8-kb *Pst*I and 6.0-kb *Eco*RI fragments which extend across *attP* into the adjacent prophage. The number of copies of the prophage was estimated by comparing the relative intensities of these bands.

Figure 6 shows the effect of *ompC* copy number on the relative amounts of OmpC and OmpF proteins and the effect of copy number of *ompC* on the regulation of OmpC expression by the *lc* gene of phage PA-2. The first three lanes of Fig. 6 show strains which are haploid, diploid, and triploid for *ompC*. This set of strains was grown on low-salt LB, a condition which in a wild-type strain slightly favors OmpF over OmpC. The last three lanes show phage Hy7 lysogens of these strains which were grown on LB with normal salt, a condition which favors low levels of OmpC and OmpF proteins in a phage PA-2 lysogen. Table 3 summarizes the results of quantitative densitometry of the gel shown in Fig. 6. In both sets of experiments, the total amount of protein contributed by the major proteins (OmpA, OmpF, OmpC, and Lc) remained reasonably constant, indicating that the sum of these proteins was a constant proportion of the total amount of outer membrane protein. Thus, the relative amount of protein in each band provides at least a rough measure of the expression of each protein. In the three Lc^- cultures grown in low-salt LB, the amount of OmpC protein increased in proportion to the gene dosage. The amount of OmpF protein decreased more than the amount of OmpC protein increased in proportion to gene dosage, whereas the decrease in OmpA protein was less than the increase in OmpC protein. This result is not due to competition for a limited amount of *ompR* product. In other experiments, we have examined omp^+ strains carrying the 2.6-kb *ompC* fragment on a multicopy plasmid and have found that these strains vastly overproduce OmpC protein; thus *ompR* product cannot be limiting for expression. These results will be described in detail elsewhere (G. McDonald, manuscript in preparation). These results are as would be predicted if increasing the copy number of *ompC* not only increased transcription of the *ompC* structural gene but also increased the amount of a regulatory substance which inhibits translation of OmpF protein.

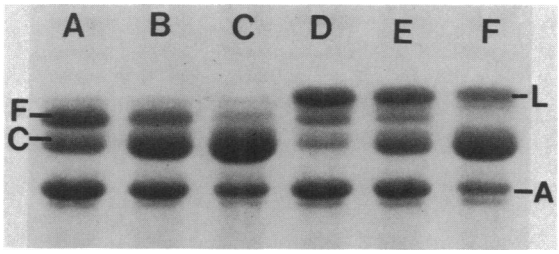

FIG. 6. Effect of *ompC* copy number on OmpF and Lc regulation. Lane A, strain CS109 (haploid for *ompC*); lane B, phage λ540C1 monolysogen of strain CS109 (diploid for *ompC*); lane C, phage λ540C1 tanden dilysogen of strain CS109 (triploid for *ompC*); lanes D, E, and F, same haploid, diploid, and triploid strains lysogenic for phage Hy7. Lanes A, B, and C show cultures grown on low-salt LB, and lanes D, E, and F show cultures grown on LB.

The ability of the phage PA-2 *lc* gene to down regulate OmpC expression is strongly dependent upon *ompC* copy number (Fig. 6 and Table 3). As the *ompC* copy number is increased, there is a disproportionate increase in the amount of OmpC protein produced, as though the PA-2 lysogen were able to regulate only one copy of *ompC*. The increase in *OmpC* protein is accompanied by a decrease in both OmpA and Lc protein.

DISCUSSION

The availability of chromosomal deletions which include the entire *ompC* region and a phage carrying the *ompC* gene on a small restriction fragment have allowed us to define a portion of the pathway involved in the regulation of expression of the OmpC and OmpF proteins. Our findings can be summarized as follows. The 2.6-kb *Hin*dIII fragment carrying *ompC* contains all of the information necessary for normal regulation of OmpC protein. Since this fragment contains very little DNA downstream from the coding region and since this downstream region includes a typical termination signal (17), it is unlikely that a retroregulation system for determining mRNA stability plays a major role in regulation of OmpC expression. This fragment contains ca. 1.5 kb of DNA upstream from the *ompC* coding sequence; this must contain binding sites for *ompR* or other regulatory elements, as well as any other genetic elements necessary for the regulation of OmpC and for regulation of other genes which may be controlled by *ompC*. This sequence contains a genetic element which is involved in the translational (or at least post-transcriptional) control of OmpF expression. This element lies upstream from the coding region, since it is present in strains carrying a variety of mutations (including an amber mutation) which have an $OmpC^-$ phenotype and in a strain carrying a Tn*5* insertion 100 base pairs 5′ to the start of the *ompC* coding sequence. Our results would be most consistent with a regulatory element (protein or regulatory RNA) coregulated with the *ompC* promoter, since the effect of this element on OmpF expression is most pronounced under conditions (high-salt tryptic soy broth) which favor high levels of *ompC* transcription.

Very recently, Mizuno et al. (18) described a unique regulatory transcript encoded upstream from the *ompC* coding sequence which satisfies these criteria. This 174-base transcript, termed micRNA, inhibits OmpF protein production. The transcript is transcribed in the opposite direction from the OmpC protein transcript and originates ca. 253 bases upstream from the beginning of the OmpC signal sequence. Like the transcription of the coding sequence, transcription of micRNA requires a functional *ompR* operon. These authors have observed significant homology between micRNA and the translational initiation region of *ompF* mRNA and have proposed that micRNA inhibits translation of OmpF protein. Our results are entirely consistent with these observations.

Ozawa and Mizushima (23) have shown that an *ompF* mutation resulted in derepression of transcription of *ompC*, indicating that the OmpF protein, or some regulatory element which senses the expression of *ompF*, negatively regulates *ompC*. If this is combined with our observation and those of Mizuno et al. (18) that a regulatory element linked to *ompC* negatively regulates translation of OmpF protein, it is apparent that regulation of both proteins is coupled in a

TABLE 3. Estimation of the amount of the major outer membrane proteins in strains haploid, diploid, and triploid for *ompC*

Strain[a]	Integrated band density (in arbitrary units)[b]			
	OmpA	OmpC	OmpF	Lc
Haploid (lane A)	1.29 (1.0)	0.48 (1.0)	0.59 (1.0)	
Diploid (lane B)	1.00 (0.78)	1.10 (2.3)	0.23 (0.39)	
Triploid (lane C)	0.56 (0.43)	1.61 (3.3)	0.02 (0.03)	
Haploid, Hy7 (lane D)	1.25 (1.0)	0.17 (1.0)	0.12 (1.0)	0.57 (1.0)
Diploid, Hy7 (lane E)	1.01 (0.81)	0.66 (3.8)	0.06 (0.50)	0.47 (0.82)
Triploid, Hy7 (lane F)	0.55 (0.44)	1.38 (8.1)		0.43 (0.75)

[a] Lanes listed within parentheses refer to the lanes in Fig. 6.
[b] Numbers within parentheses are arbitrary units relative to haploid strains. Values are from laser densitometry of the gel shown in Fig. 6.

cloned regulatory loop as shown in Fig. 7. Such a closed loop acts to amplify small signals, since expression of either gene will lead to repression of the other gene, which in turn leads to more expression of the first gene. The magnitude of this amplification effect can be seen by comparing Table 2 and Fig. 4B. A twofold change in transcription has much more than a twofold effect on the relative amounts of OmpC and OmpF proteins. Similarly, tripling the gene dosage for *ompC* results in an almost complete shutdown of OmpF protein synthesis (Fig. 6 and Table 3). The effects of gene dosage are much greater than can be explained simply by the competition between OmpC and OmpF polysomes for secretion sites or secretion machinery.

One consequence of this type of regulation is that the genes encoding these proteins must be placed in similar orientation with respect to the origin and terminus of DNA replication in order that the regulation of the genes be independent of growth rate or position in the cell cycle. This criterion is satisfied by the *ompF* and *ompC* genes, which are located at, respectively, 77 and 69% of the distance between the origin and terminus of DNA replication (1). Thus there should be only a slight bias in favor of OmpC protein at faster growth rates when the copy number of genes near the origin is higher.

The regulation of OmpC and OmpF proteins by the *lc* gene of phage PA-2 appears to be different from the effects which *ompC* and *ompF* exert on each other. First, regulation is not reciprocal; a single copy of *lc* can reduce expression of OmpC protein by more than 90%, whereas triploidy for *ompC* reduced the Lc protein expression by only 25%. Second, there seems to be a strict stoichiometry between Lc, OmpC, and OmpF proteins. This is seen both in the data of Fralick and Diedrich (6) and in the data in Table 3. In the latter case, there is a disproportionate increase in the OmpC-Lc ratio as a function of *ompC* gene dosage. This ratio changes by a factor of 10 in response to a tripling of the gene dosage. These results suggest that phage PA-2 encodes a regulatory element which acts to inhibit translation of both

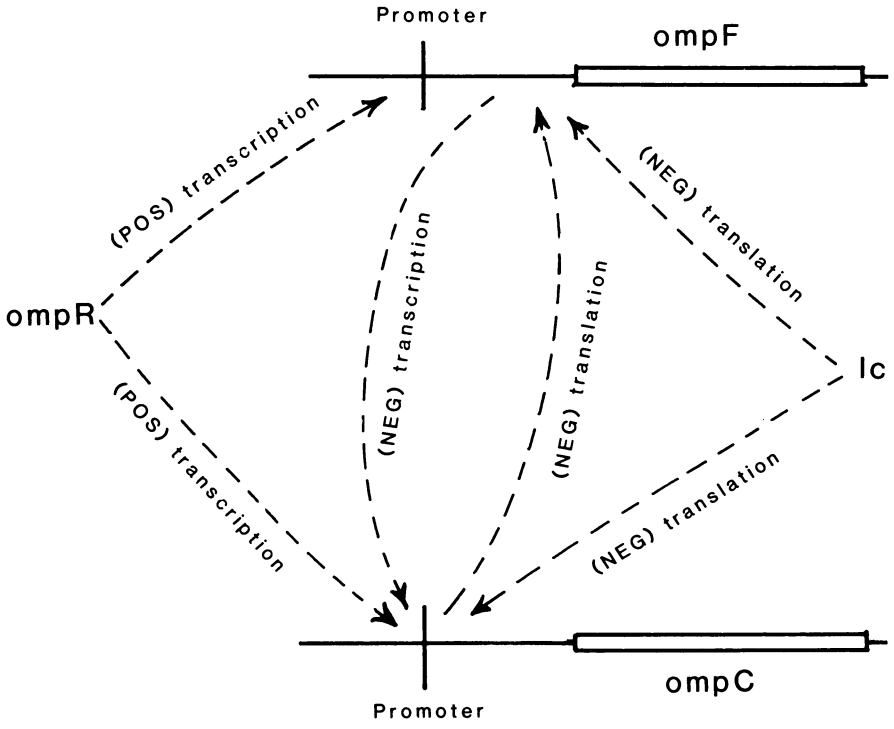

FIG. 7. Scheme illustrating the closed-loop regulation of *ompC* and *ompF* together with other positive and negative regulatory effects.

OmpC and OmpF proteins in a limited way; only enough regulatory substance is produced to shut down an amount of OmpC protein plus OmpF protein which is equivalent to the amount of Lc protein produced. When the *ompC* gene dosage is increased, the additional OmpC expression is unregulated by *lc*. The effect of *lc* is dominant over the mechanisms which regulate *ompC* and *ompF*. As seen in Fig. 3, deletion of *ompC* does not increase the amount of OmpF protein produced in a PA-2 lysogen. There is a small effect of osmolarity on the relative amounts of OmpC and OmpF proteins produced in PA-2 lysogens, but this effect is much smaller in magnitude than it is in a nonlysogen. Thus it appears that *lc* can override the regulatory scheme shown in Fig. 7. It is possible that the *lc* transcriptional unit of phage PA-2 encodes a regulatory molecule which binds to upstream regulatory regions of *ompC* and *ompF*, inhibiting both expression and the normal interaction between these genes. We are presently studying the *lc* region of phage PA-2 in the hope of identifying such a molecule.

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