JOURNAL OF BACTERIOLOGY, Oct. 1986, p. 86–95
0021-9193/86/100086-10$02.00/0

Vol. 168, No. 1

Characterization by Deletion and Localized Mutagenesis In Vitro of the Promoter Region of the *Escherichia coli ompC* Gene and Importance of the Upstream DNA Domain in Positive Regulation by the OmpR Protein

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Received 12 March 1986/Accepted 17 June 1986

The *ompC* gene codes for a major outer membrane protein whose expression is regulated by the *ompR* and *envZ* genes. Two sets of promoter deletion mutants, with upstream and downstream deletions, were constructed on a plasmid in vitro, and their promoter activity was studied by connecting them with the *lacZ* gene. The DNA sequence for the *ompC* promoter, including the −35 and −10 regions and the mRNA start site, was defined at the region about 100 base pairs upstream from the ATG initiation codon for the pro-OmpC protein. An additional 61-base-pair sequence extending upstream from the −35 region was required for the *ompC* promoter to function fully. After targeting the upstream region of the *ompC* promoter fused to the *lacZ* gene on a plasmid, in vitro-localized mutagenesis was performed to isolate *cis*-dominant mutations that affect *ompC* transcription. Four mutant groups, each of which had common phenotypes for expression and regulation of the gene, were identified. The individual groups also had common base substitutions. In two of the groups, the common base substitutions were localized in the upstream region of the *ompC* promoter, whereas in the other two they were localized in the −35 region. From these results, the upstream region of the *ompC* promoter was considered to be the domain responsible for activation by the *ompR* gene product.

The major outer membrane proteins of *Escherichia coli*, OmpF and OmpC, are encoded by the *ompF* and *ompC* genes, respectively (26). Expression of these genes is regulated by the osmolarity of the culture medium (13, 32). The osmoregulation of the *ompF* and *ompC* genes is controlled by two genes, *ompR* and *envZ* (4–7). The nucleotide sequences of these two genes have also been determined (19, 25, 33), and their gene products have been characterized (18, 19, 25, 29). Many *ompR* mutants exhibiting different osmoregulation phenotypes have been isolated (4–7, 24, 25, 30) and characterized at the molecular level (25). The results of these studies strongly indicated that the OmpR protein is directly involved in osmoregulation.

The *ompC* and *ompF* genes have been cloned, and their total nucleotide sequences have been determined (11, 20, 21). The extensive homology between them in the coding DNA sequences and in the primary amino acid sequences is obvious (21). In spite of the high homology in their coding regions, their 5′-end noncoding sequences, including the possible promoter regions, seemed to differ to some extent. It is not yet fully understood how the expression of these two genes is regulated in a reciprocal manner by their common regulatory factors, the OmpR and EnvZ proteins. Recently, the promoter region for the *ompF* gene has been characterized in detail by means of mutagenesis in vivo and in vitro (3, 9, 31). On the other hand, detailed studies on the *ompC* promoter region have not yet been performed.

In the present work, to define and characterize the functional region for the *ompC* promoter in comparison with that of the *ompF* gene, deletions upstream and downstream of the *ompC* promoter were generated. It was found that about 60 base pairs (bp) upstream from the −35 region was required for the *ompC* promoter to function fully. In vitro-localized mutagenesis of the upstream regulatory region of the *ompC* promoter was also carried out by using the *ompC-lacZ* fusion gene. In the mutants thus isolated, most of the base substitutions were in the regulatory region. Based on the results, the structure and function of the *ompC* promoter are discussed in relation to regulation by the *ompR* and *envZ* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The following strains derived from *E. coli* K-12 were used: MC4100 [F⁻ Δ(*argF-lac*)*U169 araD139 rpsL150 flbB5301 relA1 ptsF25*], MH1160 (*ompR101*; a mutant of MC4100), MH760 (*ompR472*; a mutant of MC4100), MH1461 (*envZ11*; a mutant of MC4100) (2, 5), SB4288 [F⁻ *recA thi-7 relA mal-24 spc-12 supE20* Δ(*proB-lac*)] (22, 23), and K58 (*ung*; a uracil-*N*-glycosidase mutant; kindly provided by M. Takanami, Kyoto University). The last strain was used as a host for localized mutagenesis (1). Plasmid pMY150, carrying the entire *ompC* gene (22, 23), and pKM005, carrying the promoter-deleted *lacZ* gene (15), were used.

Media, enzymes, and chemicals. Unless otherwise specified, bacteria were grown in L-broth. For the β-galactosidase assay on plates, lactose-MacConkey solid medium (Difco Laboratories) was used. When required, ampicillin was added at a concentration of 100 μg/ml. Restriction endonucleases, bacteriophage T4 ligase, exonuclease *Bal*31, the Klenow fragment of DNA polymerase I, synthetic *Xba*I linker (dCTCTAGAG), *Hin*dIII linker (dCAAGCTTG), and a dideoxy DNA sequencing kit were obtained from Takara

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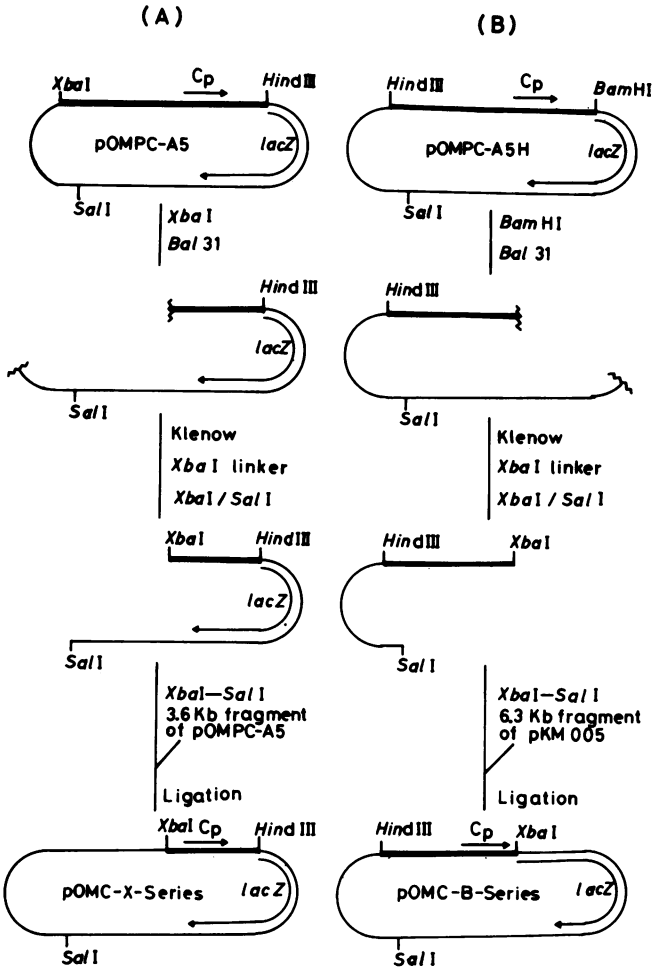

FIG. 1. Structure of plasmid pOMPC-A5, carrying the *ompC-lacZ* fusion gene, and construction of a series of plasmids with upstream deletions (A) and downstream deletions (B) of the *ompC* promoter region. Solid bars represent *ompC* DNA, and the approximate location of the *ompC* promoter region (Cp) is also indicated. The series of plasmids with upstream deletions and downstream deletions are designated the pOMPC-X series and pOMPC-B series, respectively.

Shuzo Co. ONTG (*O*-nitrophenyl-β-D-galactopyranoside) was obtained from Sigma Chemical Co. [α-^{32}P]dCTP (3,000 Ci/mmol) was purchased from Amersham International. Sodium bisulfite and hydroquinone were obtained from Wako Chemical Co.

DNA techniques. The conditions used for restriction endonuclease reactions, exonuclease *Bal*31 digestion, and Klenow fragment treatment were those suggested by the suppliers. Other recombinant DNA techniques were carried out by the conventional methods described by Maniatis et al. (14). DNA sequences were determined by the dideoxy chain termination method of Sanger et al. (27), using phages M13mp10 and M13mp11 and strain JM103 [Δ(*lac-pro*) *thi rpsL supE endA sbcB15 hsdR4* F'::*traD36 proAB lacI*^{q} *lacZM15*] as the host (16).

Assay of β-galactosidase activity. β-Galactosidase was assayed as described by Miller (17) with slight modifications. Cells were cultivated until the mid-logarithmic phase in medium A (13) in the absence or presence of 15% (wt/vol) sucrose. The cells were then collected and suspended in the same volume of 250 mM sodium phosphate buffer (pH 7.2) to accurately determine the cell density in the absence of sucrose. A portion of the cell suspension was subjected to the β-galactosidase activity assay. Cells were permeabilized with toluene before the assay.

Localized mutagenesis of plasmid DNA with sodium bisulfite. Localized mutagenesis of single-stranded DNA on a plasmid was carried out as described by Kalderon et al. (12) and Shortle et al. (28) with slight modifications. Plasmids pOMPC-X338 and pOMPC-X14 were linearized with *Eco*RI and *Sal*I, respectively. Each linear plasmid (5 μg) was mixed together in 23 μl of H_2O. Then 1 M NaOH (4.8 μl) was added, and the mixture was left at room temperature for 10 min. Renaturation was accomplished by sequential additions of 280 μl of H_2O, 40 μl of 0.5 M Tris hydrochloride (pH 8.0), and 48 μl of 0.1 N HCl prior to incubation at 63°C for 2 h. The annealed products were precipitated in ethanol, suspended in a small volume of H_2O, and then incubated in freshly prepared 3 M sodium bisulfite (pH 6.0)–2 mM hydroquinone at 37°C for 4 h in the dark under liquid paraffin. Sequential dialyses of the sample against buffers were carried out as described previously (28). The mutagenized plasmids were finally precipitated in ethanol and then transferred into *E. coli* K58 (*ung*).

RESULTS

Construction of the *ompC-lacZ* fusion gene on a plasmid. For the rapid and quantitative characterization of the *ompC* promoter function, the *ompC* promoter region was fused to the *lacZ* coding sequence on a promoter-proving vector, pKM005 (15). The resultant plasmid (pOMPC-A5) carries the 501-bp fragment encompassing the putative *ompC* promoter region and the translational initiation codon (ATG) of the *ompC* gene (Fig. 1A). Another plasmid (pOMPC-A5H) was also constructed, by converting the *Xba*I site of pOMPC-A5 to an *Hin*dIII site (Fig. 1B). The nucleotide sequence coding for the four amino acids at the amino terminus is derived from the *ompC* gene. The sequence is followed by a short linker sequence providing unique restriction sites (*Hin*dIII and *Bam*HI) and the *lacZ* coding sequence in the correct reading frame. The nucleotide sequence of the junction region was confirmed by DNA sequencing (data not shown).

Construction of plasmids with deletions upstream and downstream of the *ompC* promoter region. The strategy for construction of plasmids with deletions upstream and downstream of the *ompC* promoter region fused to the *lacZ* gene is shown in Fig. 1A and B, respectively. For construction of plasmids with upstream deletions, plasmid pOMPC-A5 linearized with *Xba*I was digested with exonuclease *Bal*31 to produce the *ompC* promoter region deleted to various extents. After treatment with Klenow fragment, the resultant blunt-ended fragments were ligated with *Xba*I linker. The ligated products were digested with *Xba*I and *Sal*I. The *Xba*I-*Sal*I fragments, which were assumed to include the *ompC* promoter region with different upstream deletions, were ligated back into the *Xba*I-*Sal*I sites of pOMPC-A5. The strategy for generation of the plasmids with downstream deletions was similar. Plasmid pOMPC-A5H was linearized with *Bam*HI and then digested with *Bal*31. After treatment with Klenow fragment, the resultant blunt-ended fragments were ligated with *Xba*I linker. The ligated products were digested with *Xba*I and *Sal*I, and then the *Xba*I-*Sal*I fragments, which were assumed to include the *ompC* promoter region with different downstream deletions, were ligated into

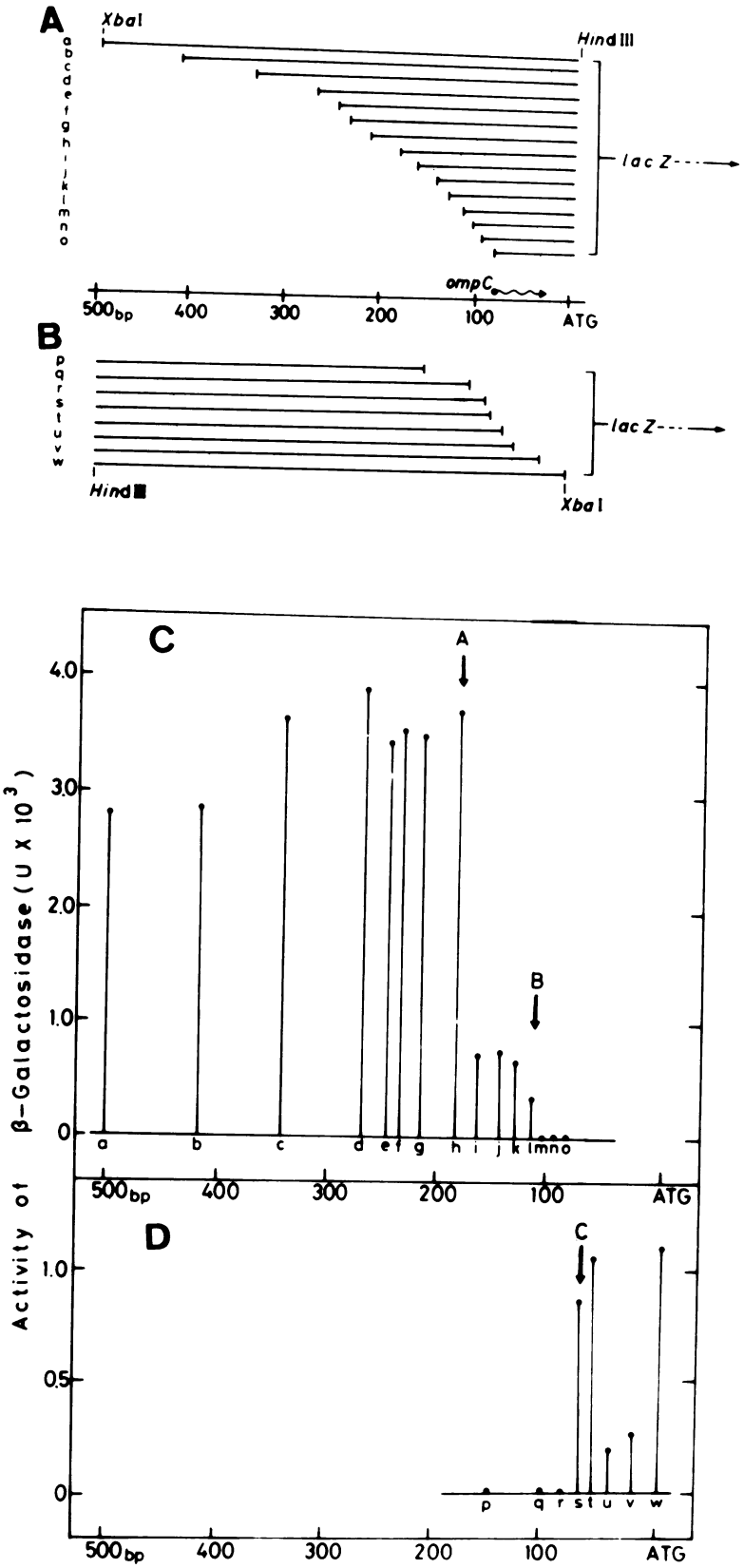

FIG. 2. Series of plasmids with upstream deletions and downstream deletions of the *ompC-lacZ* fusion gene, and β-galactosidase activity expressed by the series of deletion plasmids in wild-type cells. (A and B) Solid lines represent the promoter region remaining in the upstream deletions (A, pOMPC-X series) and the downstream deletions (B, pOMPC-B series). For the base pair scale, the zero point is the initiation codon (ATG) of the *ompC-lacZ* gene. The location of the *ompC* mRNA start site (●) is also indicated. The letters a through w represent the series of deletion plasmids: (a) pOMPC-A5 (original plasmid); pOMPC-X series plasmids (b) X79, (c) X41, (d) X153, (e) X322, (f) X105, (g)

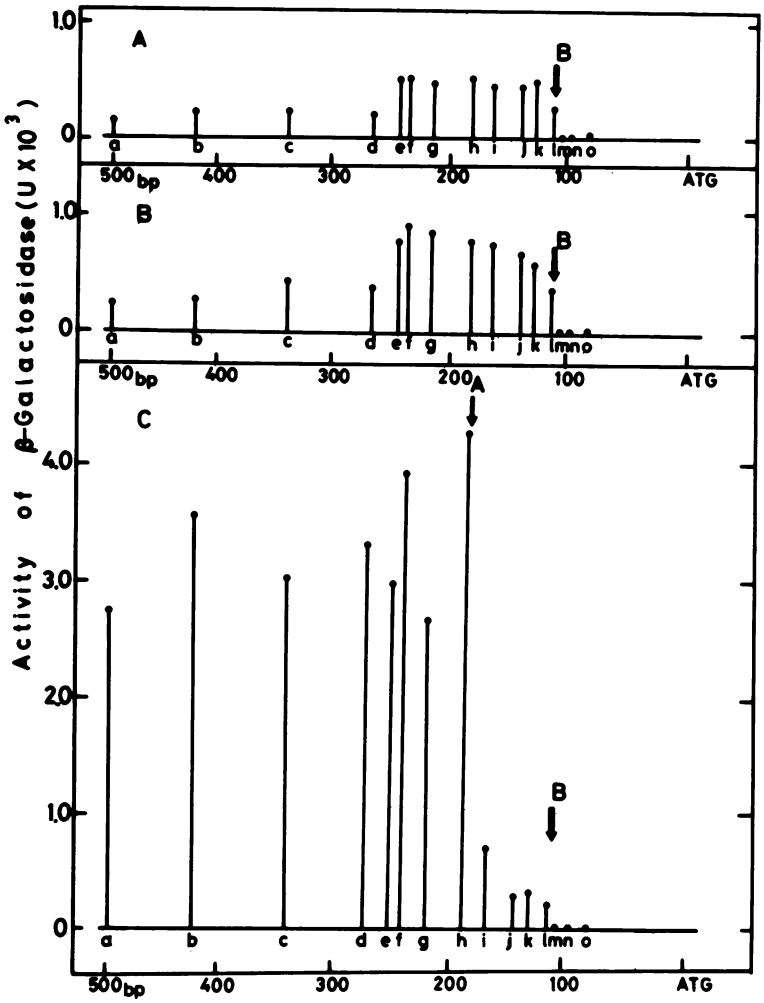

FIG. 3. β-Galactosidase activity expressed by upstream deletion plasmids in *ompR* and *envZ* mutant cells. The upstream deletion plasmids (a through o, see Fig. 2) were transferred into (A) MH1160 (*ompR101*), (B) MH760 (*ompR472*), and (C) MH1461 (*envZ11*). The cells were grown in medium A in the presence of 15% sucrose and then subjected to the β-galactosidase assay. The activity expressed by each plasmid is indicated at the position reached by the deletion in each *ompC* promoter (see Fig. 2). The two critical points for β-galactosidase activity are indicated by arrows A and B.

the 6.3-kilobase (kb) *Xba*I-*Sal*I fragment of pKM005. Plasmid pKM005 carries a promoter-deleted *lacZ* gene (15). Over 100 deletion plasmids were thus constructed. As shown in Fig. 2A and B, 15 upstream deletion plasmids (pOMPC-X series) and 8 downstream deletion plasmids (pOMPC-B series) were selected and subjected to further characterization.

Characterization of a series of upstream and downstream deletions to define the functional *ompC* promoter region. Deletion plasmids were transferred into MC4100 (*ompR*$^+$ *envZ*$^+$), and then the β-galactosidase activities were examined. As shown in Fig. 2C, there were two critical points (indicated by arrows A and B) in the upstream deletions with respect to the level of expression. One was about 170 bp upstream from the ATG initiation codon (point A), and the other was about 110 bp upstream from the ATG initiation codon (point B). When the deletion proceeded beyond point A, the level of expression was significantly decreased. β-Galactosidase activity was completely abolished by deletion beyond point B. The results of β-galactosidase assays for the downstream deletions revealed the presence of a critical point (indicated by arrow C in Fig. 2D), which resided about 80 bp upstream of the ATG initiation codon. The expression of β-galactosidase was completely lost by further deletion upstream from point C. These results indicate that the *ompC* promoter resides between 110 and 80 bp

X338, (h) X215, (i) X49, (j) X250, (k) X293, (l) X101, (m) X14, (n) X57, and (o) X251; pOMPC-B series plasmids (p) B71, (q) B98, (r) B83, (s) B110, (t) B76, (u) B7, (v) B64, and (w) B114. (C and D) β-Galactosidase activity of upstream deletion plasmids (C, a through o) and downstream deletion plasmids (D, p through w) were measured in MC4100 (*ompR*$^+$ *envZ*$^+$). The cells were grown in medium A in the presence of 15% sucrose and then subjected to the β-galactosidase assay as described in the text. The activity expressed by each plasmid is indicated at the position reached by the deletion in each *ompC* promoter. The three critical points for β-galactosidase activity are indicated by arrows A, B, and C.

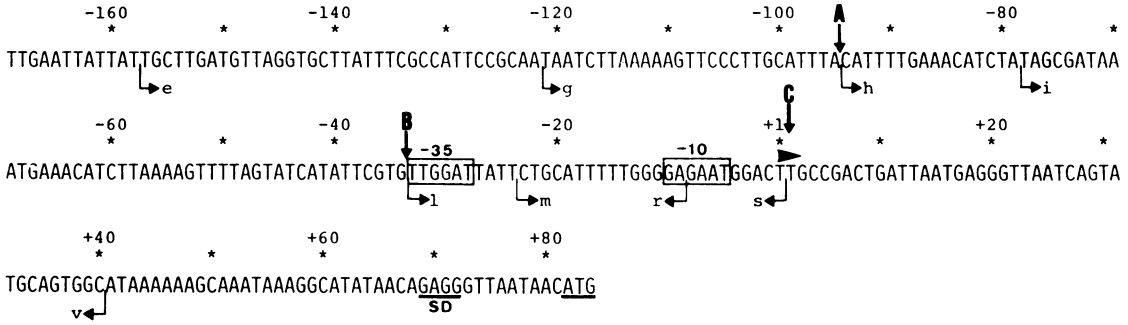

FIG. 4. Nucleotide sequence around the *ompC* promoter region and the deletion endpoints in individual plasmids. The deletion endpoints of upstream deletions [(e) pOMPC-X322, (g) X338, (h) X215, (i) X49, (e) X101, and (m) X14] and those of downstream deletions [(r) pOMPC-B83, (s) B110, and (v) B64] were determined by DNA sequencing and are indicated by arrows with lowercase letters that correspond to those shown in Fig. 2. The −35 region (TTGGAT) and the −10 region (GAGAAT) of the *ompC* promoter are boxed. The *ompC* mRNA start site (▶) is numbered +1. The Shine-Dalgarno (SD) sequence and the initiation codon (ATG) are underlined. The three critical points indicated by arrows A, B, and C are the same as those in Fig. 2.

upstream from the ATG initiation codon (i.e., the region between points B and C). It was also revealed that a region consisting of about 60 bp (i.e., the region between points A and B) was required for full expression of the *ompC* promoter.

To investigate the roles of the upstream region, the deletion plasmids were further studied in the *ompR* background (Fig. 3A and B). A critical point, corresponding to point B in the *ompR*$^+$ background, was observed. However, enhancement of expression by the upstream region was not observed. These results indicate that the nucleotide sequence between points A and B is responsible for activation of the *ompC* promoter by the *ompR* gene product. The profile of β-galactosidase activity in the *envZ11* mutant was essentially the same as that in the wild-type strain (Fig. 3C).

The results of β-galactosidase assay (Fig. 2 and 3) were obtained with a series of promoter deletion mutants carried on a multicopy plasmid (pBR322). Some of the deletion mutation genes were transferred onto a low-copy number plasmid, pSC101. The profile of β-galactosidase activity of the low-copy-number plasmids was essentially the same as that of the multicopy plasmids (data not shown).

Localization of the *ompC* promoter on the nucleotide sequences. To determine the exact location of the functional *ompC* promoter, the extents of deletions were determined by DNA sequencing (Fig. 4). The mRNA start site of the *ompC* gene has been determined to be the TT residue, 82 and 81 bp upstream from the ATG initiation codon (23). Based on this, the −35 and −10 regions have been tentatively shown as TTGGAT and GAGAAT, respectively, in Fig. 4. The results of nucleotide sequencing analyses of the deletion mutants clearly showed that the region between points B and C contained the putative −35 and −10 regions and the *ompC* mRNA start site, confirming that the region indeed contains the promoter region for the *ompC* gene. It is also clear that at least 61 bp upstream from the −35 region are essential for full expression of the *ompC* promoter.

Localized mutagenesis of the *ompC* promoter region. Two

TABLE 1. β-Galactosidase activity of mutant plasmids carrying the *ompC-lacZ* fusion gene

Group	Plasmid or mutant	β-Galactosidase activity (U)[a]		
		MC4100 (*ompR*$^+$ *envZ*$^+$)	MH1160 (*ompR101*)	MH1461 (*envZ11*)
	pOMPC-X338	3,854	472	5,112
	pOMPC-X14	60	64	71
Down-I	116W	255	230	3,813
	109W	308	305	3,681
	120W	293	149	3,541
	231W	306	378	2,893
Down-II	161W	711	167	1,449
	169W	828	178	1,408
	178W	766	219	1,314
	179W	721	146	1,309
Down-III	125W	327	156	487
	185W	235	319	499
	122W	239	483	348
	213W	262	686	297
Up-I	127R	4,669	2,782	6,139
	102R	5,453	3,250	7,731
	109R	4,417	2,652	6,208
	105R	3,965	2,228	8,016

[a] β-Galactosidase activity was measured as described in the text.

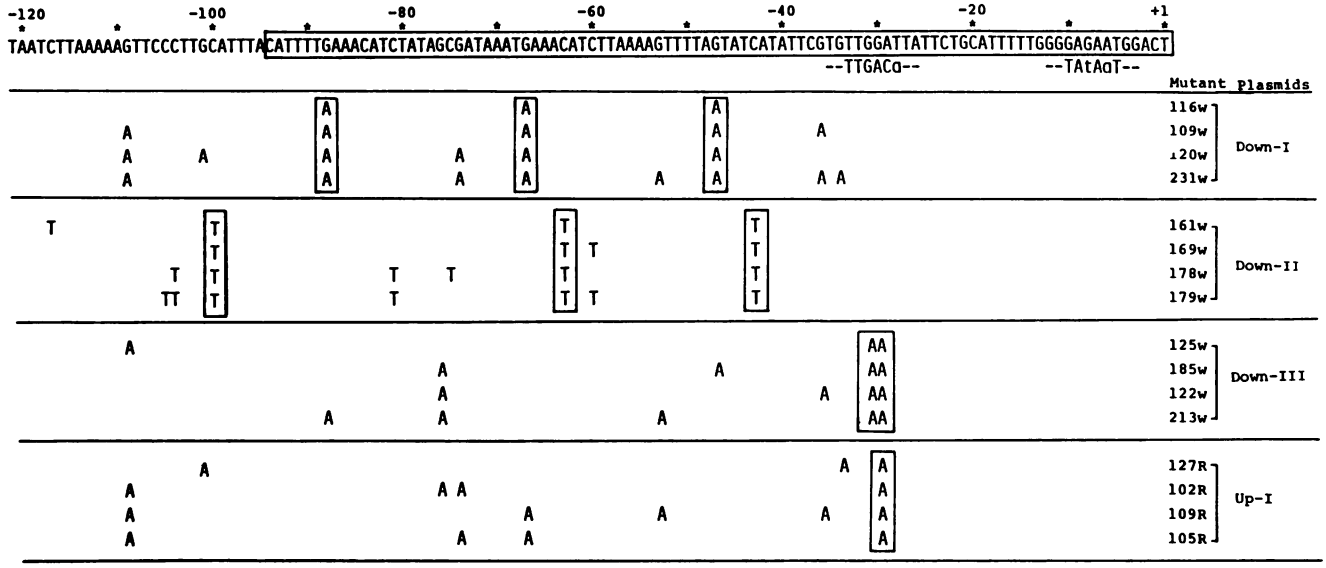

FIG. 5. Base substitutions observed in the mutant plasmids. The original DNA sequence of the *ompC* promoter region is shown at the top. The sequence that is indispensable for the *ompC* promoter function is boxed. The consensus sequences of the −35 to −10 regions of *E. coli* are indicated beneath the corresponding sequence of the *ompC* promoter. The *ompC* mRNA start site is numbered +1. The base substitutions in each mutant plasmid are indicated, and the common base substitutions in each group are boxed.

of the upstream deletion mutants were used. One (pOMPC-X338) has 121 nucleotides upstream of the mRNA start site, and the other (pOMPC-X14) has only 23 nucleotides from the mRNA start site, as shown in Fig. 4. Thus, pOMPC-X338 has a 98-bp-longer sequence that encompasses the −35 region and its upstream region. Plasmids pOMPC-X338 and pOMPC-X14 were linearized with *Eco*RI and *Sal*I, respectively. Heteroduplex molecules formed after denaturation and annealing of the linearized plasmids contained the 98-bp single-stranded region, which was susceptible to the single-strand-specific chemical mutagen sodium bisulfite. The modified heteroduplexes were then transferred directly into *E. coli* K58, which lacks the uracil repair enzyme uracil-*N*-glucosidase (1). We thus isolated about 10^4 transformants which were assumed to contain the mutant *ompC* promoter.

Selection of mutants that exhibit decreased expression of the *ompC-lacZ* fusion gene. Transformants isolated as described above were combined by sixties, and plasmid mixtures were prepared from more than 50 such combined transformants. Plasmids were transferred into strain SB4288 [Δ(*proB-lac*)] and plated on lactose-MacConkey indicator agar. Cells harboring pOMPC-X338 were expected to be deep red, while cells harboring pOMPC-X14 were white. We looked for pink colonies, which were expected to harbor plasmids carrying the mutant *ompC-lacZ* gene showing a decreased expression level. We picked up only one colony from any one plate to avoid selecting the same mutant plasmid.

Selection of mutants that exhibit OmpR-independent expression of the *ompC-lacZ* fusion gene. The expression of the *ompC-lacZ* gene on pOMPC-X338 was OmpR-dependent (Fig. 2 and 3). To isolate mutant plasmids exhibiting OmpR-independent expression of the *ompC-lacZ* fusion gene, the mixtures of mutagenized plasmids described above were transferred into strain MH1160 (*ompR101*). This host cell appeared pale pink on lactose-MaConkey indicator agar when it was transformed with pOMPC-X338, the original plasmid. Deep red colonies, which were expected to carry OmpR-independent promoter mutations, were selected.

Classification and characterization of the mutant plasmids. We measured the β-galactosidase activity of strains MC4100 (*ompR*$^+$ *envZ*$^+$), MH1160 (*ompR101*), and MH1461 (*envZ11*), which carried the mutant plasmids. The plasmids carrying the *ompC* promoter mutation and exhibiting a low level of β-galactosidase activity (down mutants) were classified into three groups based on the enzyme level in the three strains (Table 1). Mutant plasmids classified as Down-I exhibited a significant reduction of β-galactosidase activity in strain MC4100 (*ompR*$^+$ *envZ*$^+$). In this group, however, about 70% of the original activity was maintained in the *envZ* mutant, which means that the activity was much higher in the *envZ11* mutant than in the *envZ*$^+$ background. Mutant plasmids classified as Down-II exhibited about 20% of the original β-galactosidase activity in both the wild type and the *envZ* mutant. The mutant plasmids classified as Down-III exhibited the same degree of reduction of β-galactosidase activity in MC4100 (*ompR*$^+$ *envZ*$^+$) as in the Down-I group. This group, however, was clearly distinguishable from the Down-I group with respect to the activity in the *envZ11* background. Although the β-galactosidase activity expressed by these mutant plasmids was significantly low in the *ompR101* background, it was comparable to that expressed by the wild-type plasmid. Mutant plasmids exhibiting OmpR-independent expression of the *ompC-lacZ* fusion gene exhibited increased levels of β-galactosidase activity in the *ompR101* background, and the enzyme levels were comparable to that in the wild type plasmid in the wild-type background (Table 1). These mutants were classified as Up-I. We isolated four independent mutant plasmids for each group and subjected them to further characterization.

Sequence alterations in individual mutant *ompC* promoters. To confirm that the mutations on the plasmids resided only in the *ompC* promoter region, the *Xba*I-*Hin*dIII fragments encompassing the *ompC* promoter region were isolated from

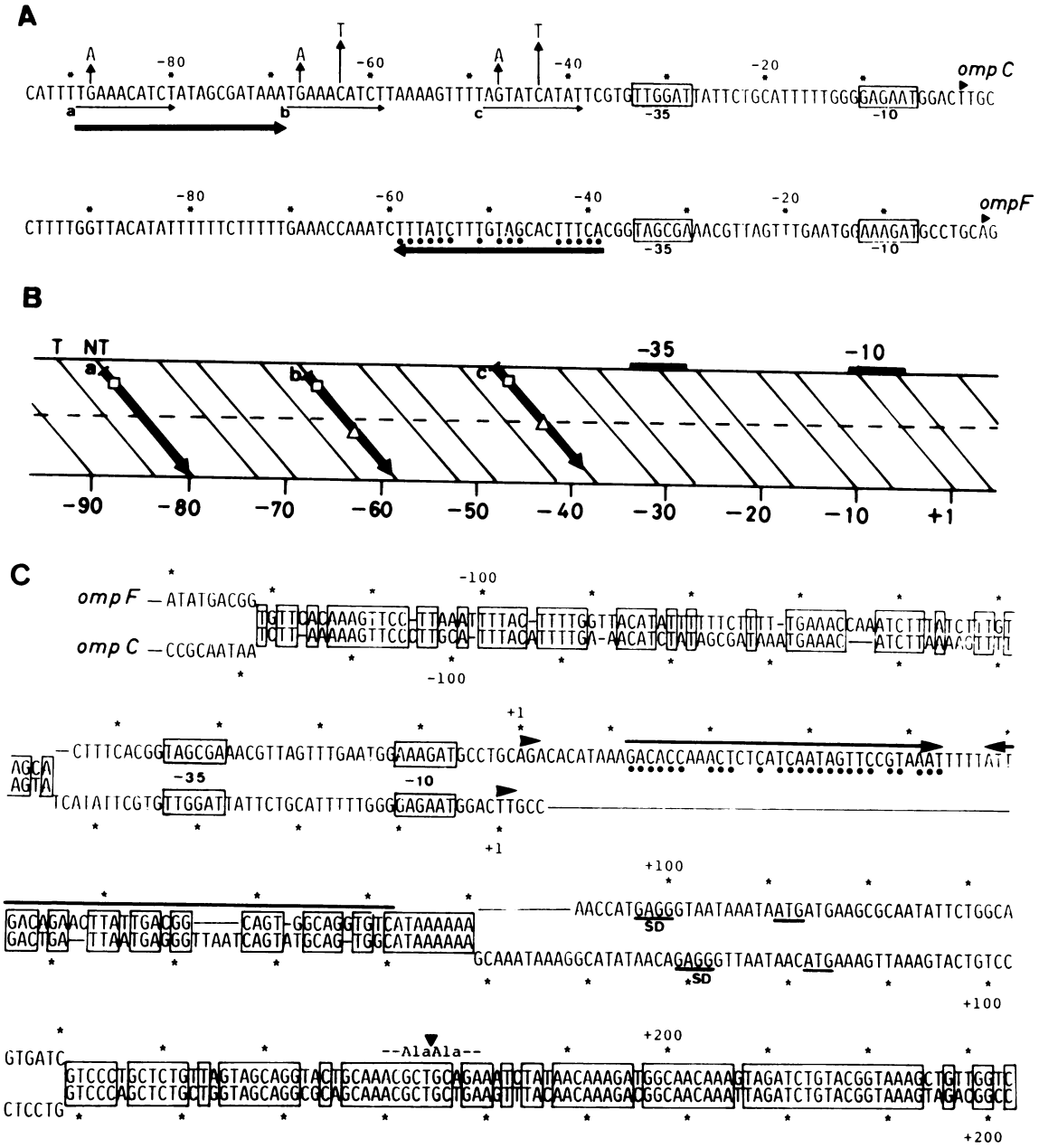

FIG. 6. DNA structures of the *ompC* and *ompF* promoter regions, and DNA sequence comparison between the *ompC* and *ompF* genes. (A) The upper and lower sequences represent the *ompC* promoter region and the *ompF* promoter region, respectively. The positions of the common base substitutions in the *ompC* promoter mutants are indicated by vertical arrows (short arrows for Down-I and long arrows for Down-II; see Fig. 5). The three horizontal arrows with lowercase letters (a through c) represent 10-bp repetitive sequences. Heavy horizontal arrows represent 21-bp highly complementary sequences found in the *ompC* and *ompF* promoter regions. Matched bases are indicated by dots beneath the *ompF* DNA sequence. (B) Structure of the *ompC* promoter region depicted on a cylindrical projection of B form DNA with 10.5 bp per turn. T, Template strand; TN, nontemplate strand. The topological positions of the three 10-bp repetitive sequences are indicated by arrows with lowercase letters (a through c). The positions of the common base substitutions observed in the Down-I (□) and Down-II (△) groups are also indicated. (C) DNA sequences of the *ompF* and *ompC* genes. The DNA sequences are numbered with the *ompF* and *ompC* mRNA start sites taken as +1. The Shine-Dalgarno (SD) sequences and the initiation codons (ATG) are underlined. The −35 and −10 regions of the *ompF* and *ompC* genes are boxed separately. Homologous nucleotides in regions showing significant homology are boxed together. The horizontal inverted arrows above the *ompF* sequence indicate inverted sequences. Matched nucleotides are dotted.

the mutant plasmids and ligated back into the same sites of nonmutagenized parental plasmid pOMPC-X338 (see Fig. 1). These reconstructed plasmids exhibited the phenotypes typical of each mutant group. We concluded, therefore, that the mutations resided only in the *ompC* promoter region. The DNA sequences of the *Xba*I-*Hin*dIII region were determined for all 16 mutant plasmids (Fig. 5). Base substitutions were found only within the region targeted by sodium bisulfite. In all cases, the G:C to A:T substitution, which is typical of sodium bisulfite mutagenesis, was the main event. Although

base substitutions were found at multiple sites and some of them differed from one mutant to another, mutations in each group had common base substitutions (Fig. 5). It was found that two of the mutant groups (Down-I and Down-II) had base substitutions only in the upstream region of the *ompC* promoter, whereas the other two (Down-III and Up-I) had base substitutions in the −35 region.

DISCUSSION

Mizuno et al. determined the total nucleotide sequence of the *ompC* gene (21) and tentatively assigned the promoter region of the gene (23). In the present work, the region that stretches from positions −33 to +1 was found to contain a canonical promoter of *E. coli*, including the mRNA start site (position +1), the −10 region (GAGAAT) at position −10 to −5, and the −35 region (TTGGAT) at position −33 to −28 (Fig. 4). The 34-bp sequence encompassing the −10 and −35 regions, however, was insufficient for full expression of *ompC* transcription. At least an additional 61-bp sequence residing upstream from the −35 region (positions −94 to −34) was required for full activation of the *ompC* promoter. This upstream region could enhance *ompC* expression by one order of magnitude in the *ompR*$^+$ background. Enhancement was not observed in the *ompR101* and *ompR472* mutants, which are phenotypically OmpC$^-$. From these results, we conclude that the 61-bp region is responsible for activation by the *ompR* gene product of the *ompC* promoter. Although the *ompC* gene is one of the most efficiently expressed in *E. coli* cells, the promoter sequence appeared to show a low level of homology to the consensus sequences for *E. coli* promoters (8). The OmpR protein most likely interacts with the upstream 61-bp region, which facilitates the binding of RNA polymerase to the poorly conserved *ompC* promoter.

Although many *trans*-dominant mutations that affect the transcription of the *ompC* gene have been isolated and characterized (7, 25, 31), the *cis*-dominant mutations have not yet been characterized. We demonstrated here that the 61-bp upstream region extending to −94 from the −35 region of the *ompC* promoter is responsible for the OmpR-mediated activation of *ompC* transcription. Therefore, some of the mutations in this region are expected to be *cis*-dominant for *ompC* expression. After targeting the upstream region, we could isolate such mutants by localized mutagenesis of the *ompC-lacZ* fusion gene. In two mutant groups (Down-I and Down-II) that exhibited decreased levels of *lacZ* expression, base substitutions were localized in the region upstream from the −35 region. The base substitutions that were common to the Down-I group were at positions −88, −67, and −47 with the A-to-G transition, whereas those of the Down-II group were at positions −101, −63, and −43 with the C-to-T transition. It should be emphasized in this respect that mutant 116W in the Down-I group did not have any additional base substitutions, indicating that these common base substitutions are sufficient for exhibition of the mutant phenotype. Therefore, we suspect that these base substitutions hinder the functional recognition of the *ompC* promoter by an activator protein, the OmpR protein. It is interesting that the Down-I mutation was phenotypically suppressed by a mutation in another regulatory factor, the EnvZ protein. This may suggest either that the EnvZ protein also recognizes the upstream region of the *ompC* promoter or that the EnvZ protein functionally interacts with OmpR protein.

The common base substitution in the Up-I group, which rendered the *ompC* promoter independent of the OmpR protein, was localized at position −30 in the putative −35 region with the G-to-A transition. Comparison of *E. coli* promoter DNA sequences has revealed that the −35 consensus sequence is TTGACa (the bases that are strongly conserved are capitalized) (8). Considering these facts, the Up-I group can be regarded as carrying a mutation that makes the fourth base of the −35 region match that of the consensus sequence. In previous studies (3), we showed that the A-to-T substitutions at the first position of *ompF* allow the promoter to function independently of the OmpR protein. The substitution also increases the degree of homology with the consensus sequence of the −10 region of the *ompF* gene. In the Down-III group, common base substitutions were found at positions −31 and −30 with the GG-to-AA transition. Although the G-to-A substitution at position −30 was an up-promoter mutation, the Down-III mutation drastically lowered promoter function. It is highly probable that the G-to-A substitution at the third position of the −35 region almost completely abolishes *ompC* promoter activity, being dominant over the G-to-A substitution at the fourth position. Consistent with this view, G at the third position of the −35 region is one of the most strongly conserved bases in the *E. coli* promoter sequences (8). The results discussed here confirmed our assignment of a TTGGAT sequence to the −35 region of the *ompC* promoter.

To further characterize the upstream region of the −35 region of the *ompC* promoter (positions −94 to −34), we plotted the base substitutions common to the Down-I and Down-II groups on the nucleotide sequence (Fig. 6A). In this region, two perfectly matched 10-bp sequences (TGAAACATCT, at positions −89 to −80 and −68 to −59) and another similar sequence (aGtAtCATaT, at positions −48 to −39; the bases matching those in the former sequences are capitalized) were found with an 11- or 10-bp interval. The three G-to-A substitutions of the Down-I group took place at the common second G's of the three repetitive sequences. The two C-to-T transitions of the Down-II group were localized at the common sixth C's in the two downstream repetitive sequences. These characteristic features became clearer when the data were depicted on a cylindrical projection of B form DNA with 10.5 bp per turn (Fig. 6B). The three repetitive sequences constitute three topologically corresponding DNA helices. Each base substitution of the Down-I and Down-II groups is lined up on one face of the DNA duplex. It is also noteworthy that the 21-bp sequence of *ompC*, which contains one of the repetitive sequences, was found to be highly complementary to a 21-bp sequence residing upstream of the −35 region of *ompF*, as shown in Fig. 6A. These features of the DNA sequences in the upstream regions of the *ompC* and *ompF* promoters may be important for reciprocal recognition by the common regulatory factor.

Finally, we compared the nucleotide sequences of the *ompF* and *ompC* genes. Several characteristic features were found (Fig. 6C). (i) Significant homology was found in the regions upstream from the −35 regions of the *ompF* and *ompC* promoters. Since these regions are suggested to be responsible for OmpR-mediated activation of both the *ompF* and *ompC* genes, the homology may be important for recognition by the common activator, the OmpR protein. (ii) The DNA sequences encompassing the canonical promoter regions for the two genes showed no significant homology. (iii) Homology was also found in the region between the nRNA start site and the initiation ATG codon. The *ompF* genes, however, had an extra 50-bp sequence. This extra sequence was found to be a part of the long inverted repeats,

as indicated in Fig. 6C. The *ompF* mRNA is therefore able to form a possible stable stem and loop structure at its 5′ end (ΔG was calculated to be −14.7 kcal). This particular feature, which is not seen in the *ompC* gene, might be involved in the regulation of expression of the *ompF* gene in a specific manner, although the importance of the region in regulation is not clear yet (10). The high degree of homology in the coding sequences for the mature OmpF and OmpC proteins and a part of the signal sequences was discussed previously (21). The homology and divergence found in the regulatory regions should provide us with information that will allow better understanding of the complex mechanism of osmoregulation.

Here we have provided genetic evidence for the importance of the upstream region of the *ompC* promoter in positive regulation by the *ompR* promoter. This should be further proved by biochemical evidence, for example, by a footprinting analysis and a direct DNA-binding assay with the purified OmpR protein. Recently, we purified the OmpR protein (Y. Jo, H. Nara, S. Ichihara, T. Mizuno, and S. Mizushima, J. Biochem., in press), and these experiments are now in progress in our laboratory.

ACKNOWLEDGMENTS

We thank M. Hasegawa and H. Kokubo for their technical assistance and S. Teranishi for her secretarial assistance.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan, the Science Technology Agency of Japan, and the Mishima-Kaiun Memorial Foundation.

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