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Function of *micF* as an Antisense RNA in Osmoregulatory Expression of the *ompF* Gene in *Escherichia coli*

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To analyze the function of *micF* as an antisense RNA in the osmoregulatory expression of the *ompF* gene in *Escherichia coli*, we performed two experiments. In the first experiment, two strains were constructed in which the transcription initiation site of the *ompF* gene and the transcription termination site of the *micF* gene were separated by 186 and 4,100 base pairs, respectively, on the chromosome. These two strains showed almost the same profile of *ompF* expression as the wild-type strain in which the two genes are separated by 10^6 base pairs. When a high-copy-number plasmid carrying the *micF* gene was introduced into these strains, *ompF* expression was completely repressed, whereas no repression was observed with a low-copy-number plasmid carrying the *micF* gene. These results indicate that the distance between the two genes on the chromosome is not critical for the function of *micF*. In the second experiment, expression of the *ompF* gene was examined by pulse-labeling in both the *micF*$^+$ and the *micF* deletion strains. Upon a shift from a low- to a high-osmolarity medium, suppression of OmpF protein synthesis occurred more quickly and more extensively in the *micF*$^+$ strain than in the *micF* deletion strain. The steady-state synthesis of the OmpF protein was also completely suppressed in the *micF*$^+$ strain in the high-osmolarity medium, whereas the suppression was incomplete in the *micF* deletion strain. From these results we conclude that (i) the *micF* gene contributes to the fast and complete response of the OmpF synthesis to the medium osmolarity, and that (ii) the distance between the *micF* and *ompF* genes on the chromosome is not critical for the function of the *micF* gene. The results suggest, rather, that the ratio of the copy numbers of the two genes is critical for the function of the *micF* gene.

Evidence has accumulated recently suggesting that RNAs play important roles in the regulation of gene expression. Interesting examples are antisense RNAs which appear to regulate gene expression at the translational level. In *Escherichia coli* cells, replication of the ColE1 plasmid is regulated by a short antisense transcript that is believed to form an RNA-RNA duplex with the replication primer to inhibit replication (30). For Tn*10*, a transposable element, expression of the gene coding for the transposase involved is suppressed by an RNA molecule, a transcript of the antisense strand of the 5′ end of the transposase gene, possibly through the antisense RNA-mRNA interaction (28).

A similar mechanism has been proposed to explain the reciprocal osmoregulation of the *ompF* and *ompC* genes coding for major outer membrane proteins OmpF and OmpC, respectively, of *E. coli*. Expression of the two genes is regulated in opposite directions by the osmolarity of the growth medium (16, 31). As the osmolarity increases, OmpF synthesis is depressed and OmpC synthesis is enhanced. Two regulatory proteins, OmpR and EnvZ, have been found to participate in the osmoregulation (12, 13, 23, 24, 29). The OmpR protein serves as a positive regulator that binds to the upstream region of the *ompF* and *ompC* genes to trigger transcription (8, 14, 22), whereas the EnvZ protein may serve as an osmosensor (13) in its interaction with the OmpR protein (18). The *micF* gene, which codes for an RNA that is complementary to the 5′ end of the *ompF* mRNA, was proposed (20) as a third regulatory gene for osmoregulation. The *micF* gene is located to the right upstream of the *ompC* gene, and the direction of its transcription is opposite to that of the *ompC* gene. Expression of the *micF* gene is under the control of the OmpR and EnvZ proteins, in the same manner as the *ompC* gene is. When the *micF* gene is cloned into a high-copy-number plasmid, its transcript inhibits the translation of the *ompF* mRNA. Based on these facts, the *micF* RNA was proposed to inhibit the translation of the *ompF* mRNA by hybridizing with it and to play a critical role in the osmoregulation of OmpF and OmpC synthesis.

For a critical study on the role of the *micF* RNA, Matsuyama et al. (19) constructed a *micF* deletion mutant. Expression of the *ompF* gene in the mutant was osmoregulated normally and was not enhanced, suggesting that one copy of the *micF* gene does not play a central role in osmoregulation.

The *micF* RNA is peculiar as an antisense RNA in that the gene coding for it is located 27 min apart from the *ompF* gene, the proposed target gene, whereas other antisense RNAs originate from the 5′ ends of the target genes. It was therefore suggested that the distance between the two genes had something to do with the function of the antisense RNAs. The present work was undertaken to study further the function of the *micF* RNA, with emphasis on the following three questions. (i) Is the distance between the *micF* gene and the target gene (*ompF*) on the chromosome critical? (ii) Is the ratio of the copy number of the *micF* and *ompF* genes critical? (iii) Is the one copy of the *micF* gene on the chromosome not involved in the osmoregulatory expression of the *ompF* and *ompC* genes at all?

MATERIALS AND METHODS

Bacteria, bacteriophages, and plasmids. The strains of *E. coli* K-12, bacteriophages, and plasmids used in this work are listed in Table 1. Plasmid pIA600, which carries the minimal functional region of the *ompF* gene, was constructed from the *Eco*RI-*Bgl*II small fragment of pHF129 and the *Eco*RI-*Bgl*II large fragment of pMAN007. Plasmid pMAN035 was constructed from the *Pst*I-*Hin*dIII large

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TABLE 1. Bacteria, bacteriophages, and plasmids

Strain	Relevant properties[a]	Reference or source
Escherichia coli K-12		
MC4100	F⁻ Δ*lacU169 araD rpsL relA thi flbB*	6
SM3001	MC4100 Δ*micF1*	19
JF568	*aroA ilv277 metB65 his53 purE41 proC24 cyc-1 xyl-14 lacY29 rpsL tsx63*	11
JF703	JF568 *ompF254 aroA*⁺	7
YO170	*gyrA ompC* conjugant of HO201 *ompC*; donor, KL98 *nalA*	17
YO181	*mal*⁺ *envZ*⁺ transductant of YO180; donor, JF568	15
HAI0001	Km^r *ompF*⁺ recombinant of MC4100; *ompF* relocated (see text)	This study
HAI0003	Km^r *ompF*⁺ recombinant of MC4100; *ompF* relocated (see text)	This study
HAI0005	Km^r *ompF*⁺ transductant of JF703; donor, HAI0001	This study
HAI0006	Km^r *ompF*⁺ transductant of JF703; donor, HAI0003	This study
Bacteriophages		
Tula	Receptor; OmpF and lipopolysaccharide	9
Tulb	Receptor; OmpC and lipopolysaccharide	9
P1 *kc*	Used for generalized transduction	Our laboratory stock
Plasmids		
pMAN006	Ap^r; vector, pKEN403; cloned gene, *ompC micF*	17
pMAN043	Ap^r; replication origin derived from pSC101	24
pMAN023	Km^r	19
pMAN035	Ap^r, temperature-sensitive replicon; cloned gene, *lpp* promoter-controlled *lacZ-lacY* operon	This study
pMAN055	Ap^r; vector, pBR322; cloned gene, *micF*	19
pMAN056	Ap^r; vector, pKEN403; cloned gene, *micF*	19
pBR322	Ap^r Tc^r	3
pKEN403	Ap^r Km^r; replication origin derived from pSC101	K. Nakamura
pIA600	Ap^r; vector, pKEN403; cloned gene, *ompF*	This study
pMAN007	Ap^r; vector, pKEN403; cloned gene, *ompF*	17
pHF129	Ap^r Tc^r	14
pMAN032	Ap^r; vector, pBR322; cloned gene, *lpp* promoter-controlled *lacZ-lacY* operon	19
pMAN031	Ap^r; temperature-sensitive replicon	19
pHAI014	Ap^r; vector, pBR322; cloned gene, *micF*	This study
pHAI015	Ap^r; vector, pBR322, cloned gene, *micF*	This study
pTUN9-CF	Ap^r; vector, pKEN403; cloned gene, *ompF ompC*	Mizuno et al., in press
pHAI056	Ap^r; vector, pBR322; cloned gene, *micF ompF*	This study

[a] Ap, Ampicillin; Km, kanamycin; Tc, tetracycline.

fragment of pMAN032 and the *Pst*I-*Hin*dIII large fragment of pMAN031. Plasmids pHAI014 and pHAI015 were constructed by insertion of the *micF* gene-carrying *Bam*HI-*Bgl*II fragment (about 600 base pairs) from the chromosomes of HAI0005 and HAI0006, respectively, into the *Bam*HI site of the pBR322. Plasmid pHAI056 was constructed from the *Hin*dIII-*Sal*I large fragment of pMAN055 and the *ompF* gene-carrying *Hin*dIII-*Xho*I fragment of pTUN9-CF.

Media. Expression of the *ompF* and *ompC* genes was studied with medium A supplemented with different concentrations of sucrose as described previously (16). Transduction and transformation experiments were done in L broth. Lactose-MacConkey plates were used to examine lactose-utilizing ability. When required, ampicillin, kanamycin, and nalidixic acid were added at concentrations of 50, 30, and 10 μg/ml, respectively. For cultivation on a solid surface the medium was supplemented with 1.5% agar.

Preparation of outer membrane protein fractions and polyacrylamide gel electrophoresis. The outer membrane protein fraction was prepared by extracting cell envelopes with sodium *N*-lauroyl sarcosinate as described previously (25). Urea-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the outer membrane proteins was performed as described previously (21).

DNA techniques. Restriction endonucleases, bacteriophage T4 ligase, Klenow fragment, and *Bam*HI linker (dCGGATCCG) were obtained from Takara Shuzo Co. Plasmid DNA and chromosomal DNA were prepared as described by Birnboim and Doly (2).

Pulse-labeling experiment. Minimal medium A (10) supplemented with all the L-amino acids except methionine and cysteine (50 μg/ml each), vitamin B_1 (10 μg/ml), and yeast extract (50 μg/ml) was used; however, glucose was replaced by 1% glycerol in the present study. Cells were cultured in 30 ml of the medium at 37°C. At the logarithmic growth phase, 10 ml of fresh medium containing 60% (wt/vol) sucrose was added. A 1-ml sample of the culture was taken periodically and labeled for 1 min by the addition of 10 μCi of [^{35}S]methionine (1,420 Ci/mmol). Cultures were immediately cooled in ice-water, and the cells recovered by 10 min of centrifugation at 4°C were subjected to sonication at 0°C to prepare the cell envelope fraction. Outer membrane protein fractions were prepared and then analyzed on urea-SDS-polyacrylamide gels followed by processing for fluorography as described previously (4). The radioactivities of individual protein bands were quantitated by densitometric scanning of the fluorograms. A Shimadzu CS-930 chromatoscanner was used. The amounts of membrane preparations were normalized by cell density at the sampling time.

RESULTS

Construction of plasmids pHAI005 and pHAI009 carrying the *micF* and *ompF* genes at different distances apart. The

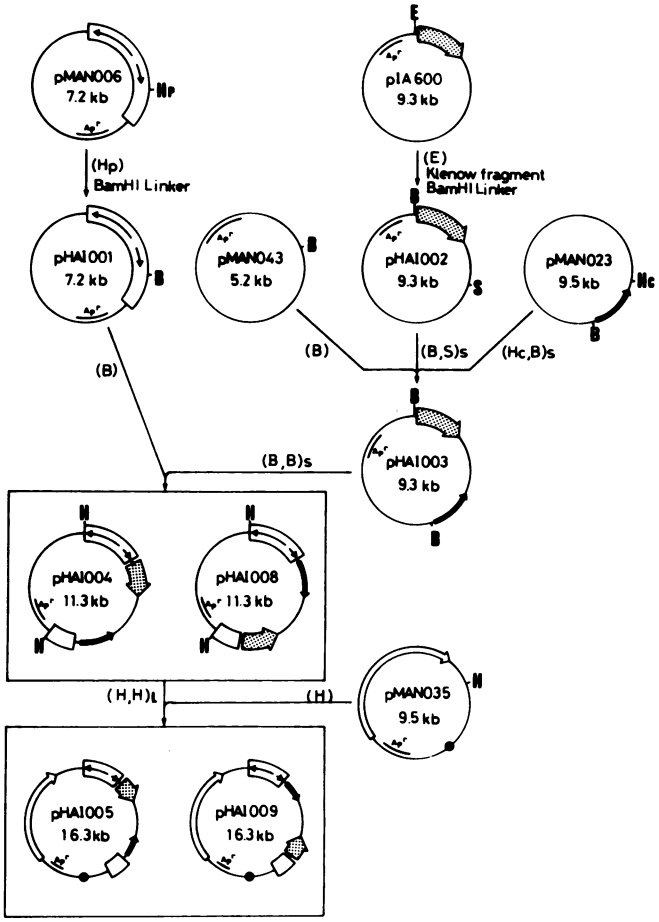

FIG. 1. Construction of plasmids. The open boxes represent the chromosomal DNA of the *micF-ompC* region. The short and long arrows in the open boxes denote the coding region and the direction of the transcription of the *micF* and *ompC* genes, respectively. ➡, ▷, and ➧ represent the coding regions and the directions of transcription of the *ompF*, *lacZ-lacY* and Km^r genes, respectively. Other symbols: ——, vector plasmid DNA; ●, temperature-sensitive replicon. The restriction endonucleases used are shown in parentheses with the following abbreviations: B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; S, *Stu*I; Hc, *Hin*cII; Hp, *Hpa*I. The cleavage sites are also shown, using the same abbreviations. S and L outside the parentheses denote small and large fragments, respectively, formed as a result of digestion with the respective endonucleases. The antibiotic resistance genes and plasmid sizes are also indicated. kb, Kilobases.

procedure for plasmid construction is shown in detail in Fig. 1. The structures of the individual plasmids shown in Fig. 1 were confirmed by DNA restriction analysis. The *ompF* gene was derived from pIA600, in which an *Eco*RI site is located 115 base pairs upstream of the *ompF* mRNA start site. The *Eco*RI site was created at the 5′-terminal limit of the functional *ompF* promoter (14). The *ompF* gene, together with the kanamycin resistance (Km^r) gene, was inserted into the *Bam*HI site of pHAI001 carrying the chromosomal region covering the *micF* and *ompC* genes to construct pHAI004 and pHAI008. In pHAI004, the *micF* gene is located to the right upstream of the *ompF* gene in the same orientation, and the distance between the transcription termination site of the *micF* gene and the *Eco*RI site (the 5′ end of the *ompF* promoter) was estimated to be only about 70 base pairs (for the estimation, see references 14 and 20). On the other hand, the distance between these two sites was estimated to be 4.1 kilobases (kb) in pHAI008, in which the two genes are arranged in opposite orientations with the Km^r gene between them. Finally, *Hin*dIII fragments carrying the Km^r, *ompF*, *micF*, and *ompC* genes were isolated from pHAI004 and pHAI008 and inserted into the *Hin*dIII site of pMAN035 carrying a temperature-sensitive replicon derived from pSC101 (1) and the *lpp* promoter-controlled *lacZ-lacY* operon.

Construction of two chromosomal mutants with different distances between the *ompF* and *micF* genes. *E. coli* MC4100 (Δ*lac*) was transformed with pHAI005 or pHAI009 and then grown at 30°C for 2 h. It was further incubated on lactose-MacConkey plates containing kanamycin (30 μg/ml) at 42°C overnight. All the transformants appeared as red colonies (Km^r Lac$^+$), indicating that the plasmids had been integrated into the chromosome through a single crossover at either region A or B (Fig. 2). After prolonged cultivation at room temperature, white regions appeared in some of the red

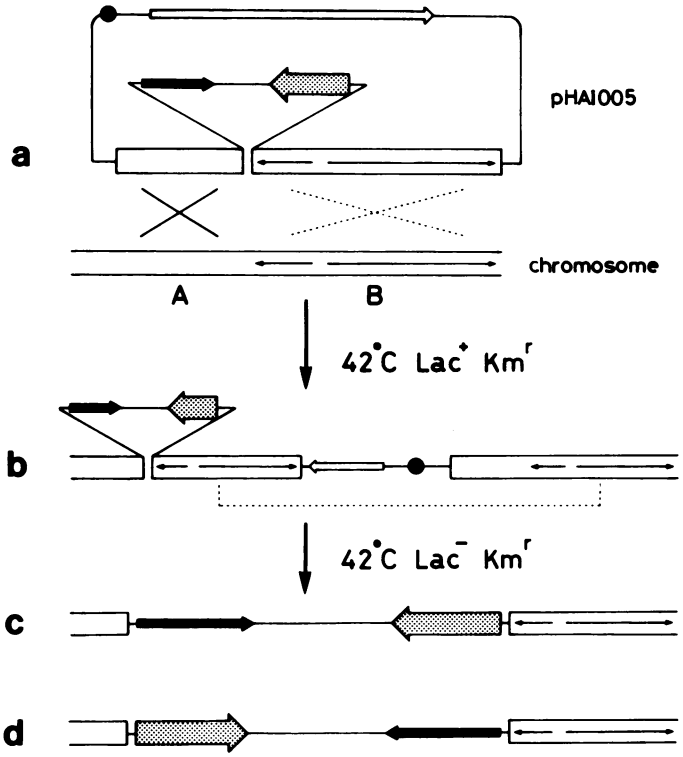

FIG. 2. Replacement of the *micF-ompC* region of the chromosome with the Km^r-*ompF*-inserted corresponding region of pHAI005. MC4100, a Δ*lac* strain, was transformed with pHAI005 and then incubated at 42°C on lactose-MacConkey plates containing kanamycin. Plasmid integration into the chromosome through homologous recombination takes place at either region A or region B (a). Transformants carrying the plasmid on the chromosome are Km^r Lac$^+$. In the case of recombination at region A, if subsequent plasmid segregation from the chromosome through homologous recombination takes place at the region indicated by the dotted line in panel b, the chromosomal structure shown in panel c should result. Since the segregated plasmid is lost from in the cells because of the inability to replicate autonomously at 42°C, this segregant is detected as a white colony on a lactose-MacConkey plate containing kanamycin at 42°C. When MC4100 is transformed with pHAI009 instead of pHAI005, the chromosomal structure shown in panel d should result. The symbols used are the same as those described in the legend to Fig. 1.

colonies. These regions were transferred to a fresh plate at 42°C. Provided that the events shown in Fig. 2 did take place in the transformants, they are most probably candidates for chromosomal mutants having the gene arrangement shown in Fig. 2c and d. We designated the one derived from pHAI005 as HAI0001 and that from pHAI009 as HAI0003. Finally, the chromosomal regions in question were transferred from HAI0001 and HAI0003 into JF703 (*ompF*) by P1 transduction with Km^r as the selection marker to obtain HAI0005 and HAI0006, respectively.

Determination of genomic structures of HAI0005 and HAI0006. To confirm the genomic structures of HAI0005, HAI0006, HAI0001, and HAI0003, we performed P1 transduction using one of these strains as the donor and YO170 (Km^s *ompC gyrA*) as the recipient. All the Km^r transductants were $OmpC^+$, and about 20% of them were nalidixic acid sensitive ($gyrA^+$). When strain YO180 (*ompF* Km^s) was used as the recipient, nearly 100% cotransduction was observed between the Km^r and *ompF* genes. These results indicate that the Km^r, *ompF*, and *ompC* genes are very closely linked to each other on the chromosome and that their loci are close to the *gyrA* locus as well.

For further confirmation of the genomic structures, we isolated the 6.8-kilobase *Hin*dIII chromosomal fragments carrying the gene cluster in question from HAI0005 and HAI0006 and cloned them into pKEN403, using the Km^r gene as the marker. Restriction analysis of the cloned fragments with *Pst*I, *Hin*dIII, *Bam*HI, and *Hin*cII supported the gene arrangement shown in Fig. 2.

Effect of the distance between *micF* and *ompF* genes on *ompF* expression. The *ompF* expression of the three isogenic strains HAI0005, HAI0006, and JF568 was examined. In HAI0005, the *micF* gene is located to the right upstream of the *ompF* promoter, and in HAI0006 the distance between the *micF* gene and the *ompF* promoter is about 4.1 kilobases. JF568 is wild type with respect to the *micF* and *ompF* genes, the distance between the two genes being 27 min (about 1,000 kilobases). No significant difference was observed in the osmoregulatory profiles of *ompF* expression among these strains, regardless of the distance between the two genes (Fig. 3). In the wild-type cells, *ompF* expression was even weaker. This may be due to the fact that the deletion of the region far upstream of the *ompF* promoter slightly enhanced *ompF* expression (Y.-L. Jo, T. Mizuno, and S. Mizushima, unpublished observation).

Two critical control experiments were performed relating to the experiments shown in Fig. 3. In one experiment,

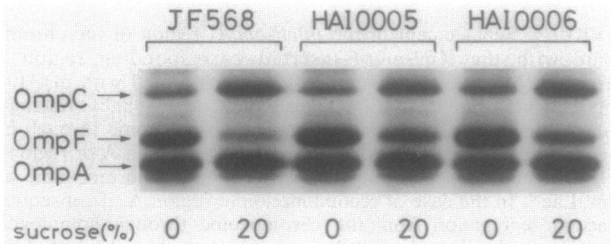

FIG. 3. Effect of the distance between the *micF* and *ompF* genes on *ompF* expression. The strains indicated were grown with the indicated concentrations (wt/vol) of sucrose. Sodium *N*-lauroyl sarcosinate-insoluble fractions were prepared from cell envelopes and then analyzed on urea-SDS-polyacrylamide gels. The amounts of the samples were normalized by cell density at the times of harvesting. The positions of OmpC, OmpF, and OmpA are indicated.

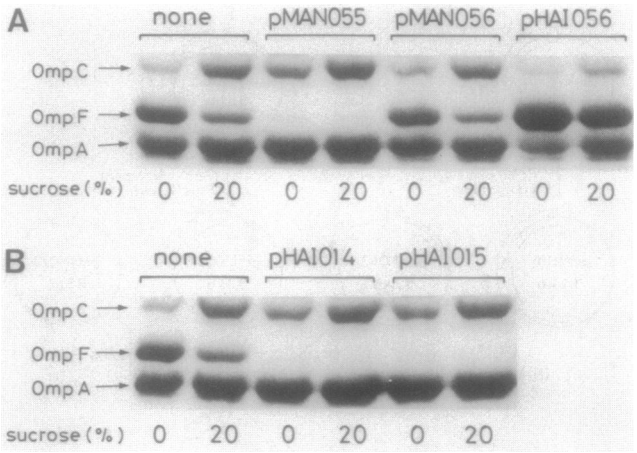

FIG. 4. Functioning of the *micF* and *ompF* genes on the chromosome and plasmids and the gene dosage effect. Strain HAI0005 harboring the indicated plasmids was grown with the indicated concentrations (wt/vol) of sucrose. Outer membrane protein fractions were prepared from cell envelopes and then analyzed on urea-SDS-polyacrylamide gels. The amounts of the samples were normalized by cell density at the times of harvesting. The positions of OmpC, OmpF, and OmpA are indicated.

ompF expression was completely repressed when pMAN055, a high-copy-number plasmid carrying the *micF* gene, was introduced into these strains (an example with HAI0005 is shown in Fig. 4A). This result indicates that the *ompF* gene in these strains is accessible to the *micF* RNA. On the other hand, when pMAN056, a low-copy-number plasmid carrying the *micF* gene, was introduced, no repression was observed, consistent with the previous observation (19) (Fig. 4A). The ratio of the copy number of pMAN055 to that of pMAN056 in strain HAI0005 was about 5 to 1 (data not shown), and the copy number per genome equivalent of pMAN056 was assumed to be about six, since the vector domain of the plasmid was derived from pSC101 (5). In the second control experiment, the region covering the *micF* gene was isolated from HAI0005 and HAI0006 and then recloned into pBR322. The plasmids thus constructed, pHAI014 and pHAI015, repressed *ompF* expression when they were transferred into a host strain (HAI0005), indicating that the *micF* gene in both HAI0005 and HAI0006 is capable of inhibiting *ompF* expression when it exists in multiple copies (Fig. 4B). It should also be noted that the inhibition was not observed when the *ompF* gene was cloned together with the *micF* gene into pBR322 (Fig. 4A).

Taking all these results together, we conclude that the inhibitory effect of a single copy of the *micF* gene is not appreciable even when the *micF* RNA is synthesized directly upstream the *ompF* promoter, the target of this RNA molecule. The results also suggest that the copy number of the *micF* gene, i.e., the number of *micF* RNA molecules, is critically important for repression of *ompF* expression.

Role of *micF* gene on the chromosome in *ompF* expression. Matsuyama et al. (19) found that deletion of the *micF* gene did not lead to a significant difference in the osmoregulation profile of *ompF* expression. In the present work, we reexamined in more detail the expression profile of the *ompF* gene in both the $micF^+$ and *micF* deletion ($\Delta micF$) strains.

After being transferred from a low- to a high-osmolarity medium, cells were pulse-labeled for 1 min with [^{35}S]methionine periodically to examine the level of *ompF* expression. With the $micF^+$ strain (MC4100), synthesis of the OmpF

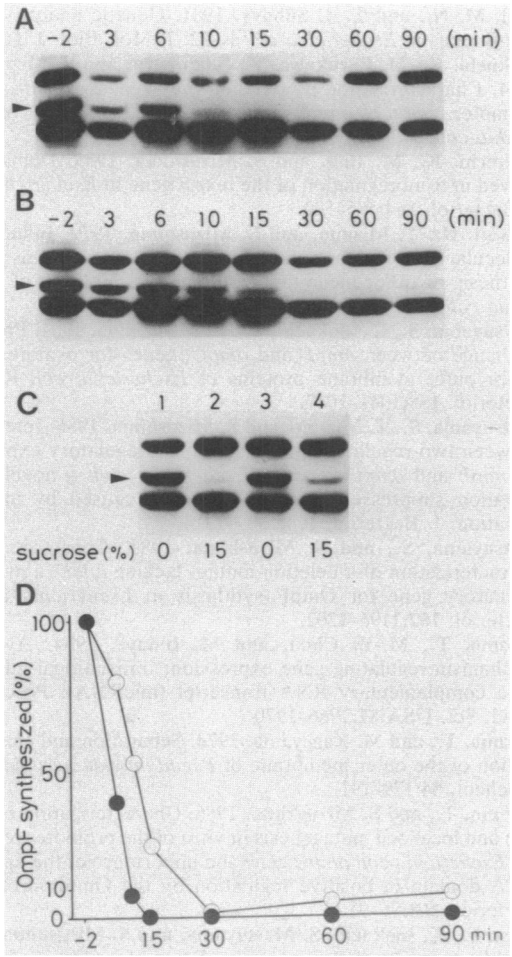

FIG. 5. Pulse-labeling experiment. MC4100 (A) and SM3001 (B) were cultured at 37°C. At the logarithmic growth phase, sucrose was added to a final concentration of 15% (wt/vol), and then cells were labeled for 1 min at the indicated times with 10 μCi of [^{35}S]methionine. As a control, cells were labeled before the addition of sucrose. Outer membrane protein fractions were prepared from the labeled cells and analyzed on urea-SDS-polyacrylamide gels. The gels were then processed for fluorography. (C) MC4100 (lanes 1 and 2) and SM3001 (lanes 3 and 4) which had been cultured with the indicated concentrations of sucrose (wt/vol) were pulse-labeled with [^{35}S]methionine and then analyzed for outer membrane proteins as described for panels A and B. The arrowheads indicate the position of the OmpF protein. (D) The fluorograms shown in panels A and B were subjected to densitometric scanning to quantitate the amounts of the OmpF protein synthesized in 1 min at the indicated times. Symbols: ●, MC4100 (wild type); ○, SM3001 (Δ*micF*).

protein was almost completely suppressed 10 min after the transfer (Fig. 5A and D). Although the suppression was also observed with the isogenic Δ*micF* strain (SM3001), a longer time was required to reach the maximum suppression, and the suppression was rather incomplete (Fig. 5B and D). These results indicate that although the *micF* gene does not play a central role in the osmoregulation, as previously indicated (19), it most likely contributes to the sharp and complete response of OmpF synthesis to the osmolarity of the medium. Since MC4100 produced a considerable amount of OmpC protein even in the low-osmolarity medium (19), the effect of the *micF* deletion on OmpC synthesis was not clearly demonstrated. The pulse-labeling experiment was also performed after growth reached the steady state in the high-osmolarity medium (Fig. 5C). OmpF synthesis, which was almost completely suppressed in MC4100 (*micF*$^+$), was enhanced in the Δ*micF* background.

The experiments shown in Fig. 5A and B were also performed with two strains in which the arrangements of the Km^r, *ompF*, *micF*, and *ompC* genes are the same as in HAI0005 and HAI0006, respectively. No differences in the expression profiles were observed (data not shown).

DISCUSSION

In a previous paper (19), we suggested that *micF* RNA does not play a critical role as an antisense RNA in the osmoregulation of *ompF* expression. Since the *micF* gene is located 27 min apart from the *ompF* gene on the chromosome, we assumed that the nascent *ompF* mRNA cannot be inactivated by the *micF* RNA originating from such a distance. Although the tertiary structure of RNA molecules has not been well elucidated, their structure is generally assumed to be less compact than that of soluble globular proteins. We also assumed that this feature makes the intracellular diffusion of RNA molecules much slower than that of proteins.

In the present work, the *ompF* gene was relocated on the chromosome to the right downstream of the *micF* gene in the same orientation, so that the transcribed *micF* RNA could readily interact with the 5′ terminus of the emerging *ompF* mRNA. However, the relocation caused no change in *ompF* expression, indicating that the distance effect is not critical in the inhibition by the antisense RNA. It should be noted, however, that the 3′ terminus of the *micF* gene and the 5′-terminal portion of the *ompF* gene coding for the target domain of the *micF* RNA are still separated by the 236 bases of the long *ompF* promoter sequence. In contrast, so-called antisense RNAs and target mRNAs are transcribed from different DNA strands of the same locus.

The gene dosage effect, on the other hand, is seemingly significant for the *micF* function. The *micF* gene carried by a high-copy-number plasmid strongly suppressed *ompF* expression, whereas *micF* on a low-copy-number plasmid as well as *micF* on the chromosome did not (Fig. 4A) (19). With the high-copy-number plasmid, however, inhibition was not observed when the *ompF* gene was cloned together with the *micF* gene (Fig. 4A). These results suggest that the ratio of the molecules of *micF* RNA to the molecules of *ompF* mRNA in a cell determines the degree of inhibition.

Although the osmoregulation profile of *ompF* expression in the Δ*micF* strain was roughly the same as that in the *micF*$^+$ strain, as previously reported (19), precise experiments revealed that the suppression upon a shift from a low- to a high-osmolarity medium was sharper and completer in the *micF*$^+$ background. The most probable explanation for this is as follows. *ompF* expression is mainly controlled at the transcriptional level through a mechanism mediated by the OmpR and EnvZ proteins (12, 13, 17, 23, 24, 29). A change in the medium osmolarity, therefore, should result in a decrease in the total number of *ompF* mRNA molecules, and the fewer the *ompF* mRNA molecules, the greater the inhibition by the *micF* RNA.

Thus far the following three regulatory mechanisms have been proposed to be involved in the osmoregulatory expression of the *ompF* and *ompC* genes: OmpR-EnvZ-mediated and *micF* RNA-mediated mechanisms, and one in which protein-coding regions are involved (15, 27). The first mechanism functions at the transcriptional level. The results of genetic and biochemical analyses strongly support the pos-

sibility that it plays a primary role in the regulation. The second mechanism could function at the translational level (20), and the present work suggests that it participates in the regulation as a secondary tuner to make the regulation sharper and completer. The importance of the third mechanism in the osmoregulation remains unclear, although it is unlikely to play a primary role (15). In any case, the evidence suggests that *E. coli* cells make a great effort to regulate the synthesis of the OmpF and OmpC proteins. Our latest study on the promoters of the *ompF* and *ompC* genes also revealed that *E. coli* cells chose a nucleotide sequence in the Pribnow box region to achieve sharp and complete osmoregulation (26). Although the physiological importance of this osmoregulatory mechanism remains unknown, the evidence suggests that *E. coli* cells need such strict control under certain circumstances which probably occur in a natural environment.

ACKNOWLEDGMENTS

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