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micF Antisense RNA Has a Major Role in Osmoregulation of OmpF in *Escherichia coli*

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***micF* RNA, produced from a multicopy plasmid, was originally shown to be a major factor in negative osmoregulation of the OmpF outer membrane protein in *Escherichia coli*. However, subsequent experiments with a *micF* deletion strain suggested that chromosomal *micF* RNA was not a key component in this process. We report here that *micF* RNA is essential for the reduction in OmpF levels in cells grown in media of low-to-intermediate levels of osmolarity. Under these conditions, the amount of OmpF was reduced up to 60% in the parent strain while OmpF levels were not altered in the *micF* deletion mutant. In medium of higher osmolarity, OmpF synthesis was strongly inhibited in both strains. RNA measurements showed that *micF* RNA levels rose rapidly in cells grown in low-to-intermediate levels of osmolarity concomitant with the reduction in OmpF protein, while *ompF* mRNA decreased strongly only during high-osmolarity conditions. Taken together, these results strongly suggest that the negative osmoregulation of OmpF at low-to-intermediate osmolarity levels requires *micF* RNA and that this is masked at higher osmolarity by the known strong inhibition of OmpF transcription by OmpR. Results consistent with this model were also obtained by using procaine, a compound reported to inhibit *ompF* expression by a mechanism very similar to that involved in osmoregulation.**

Escherichia coli contains two major outer membrane porin proteins, OmpC and OmpF, that permit the passive diffusion of small, hydrophilic molecules into the periplasm (22). These proteins are encoded by the unlinked *ompC* and *ompF* genes (42, 50). While the structural and functional properties of OmpC and OmpF are similar (22), their expression is regulated in opposite directions by a large number of environmental factors including medium osmolarity (51), temperature (24), pH (18), and the presence of compounds, such as procaine, that act as membrane perturbants (15, 35, 47). In the most thoroughly analyzed of these processes, the reciprocal osmoregulation of OmpC and OmpF is thought to primarily involve transcriptional control mediated by OmpR and EnvZ, the protein products of the *ompB* operon (16, 17). In this two-component regulatory system (39), OmpR alters transcription of *ompC* and *ompF* by binding to specific sequences upstream of the promoters in these operons (31, 32, 49). EnvZ is a transmembrane protein (12) that acts as an osmosensor and phosphorylates and dephosphorylates OmpR (2, 3, 13, 20, 21). Increased osmolarity is believed to stimulate the kinase activity of EnvZ, leading to high levels of phosphorylated OmpR (13, 46, 48) and the subsequent inhibition of *ompF* and activation of *ompC* (13, 46, 48). At low osmolarity, the phosphatase activity of EnvZ is thought to predominate, resulting in low levels of the phosphorylated form of OmpR and activation of *ompF* and reduced expression of *ompC* (14, 41).

In addition to *ompB*, a second locus, *micF*, was found to function in osmoregulation (29). The *micF* gene is located just upstream from *ompC*, and it produces a divergently transcribed small RNA whose expression is controlled by OmpR in response to osmolarity in a manner very similar to that of *ompC* (11, 29). *micF* RNA is highly complementary to the 5′ end of *ompF* mRNA, and when *micF* was cloned in a high-copy-number plasmid, it strongly blocked the production of OmpF (29). These observations led to the proposal that *micF* antisense RNA plays a major role in osmoregulation by hybridizing to the 5′ end of *ompF* mRNA and inhibiting its translation (29). Subsequent in vitro experiments showed strong binding of *micF* RNA to the initial 150 nucleotides of *ompF* mRNA (5). Additional evidence suggesting that *micF* RNA was a significant factor in osmoregulation of OmpF was results obtained with a large chromosomal deletion mutant lacking the *micF-ompC* region (44). This mutant was partially constitutive for OmpF when grown in Luria broth with 2% NaCl (44). However, in experiments specifically designed to test the importance of *micF* RNA in osmoregulation, steady-state OmpF levels during growth in high-osmolarity medium were reduced to extents similar to those in a *micF* deletion strain and its parent (25). Further analysis showed a small decrease in the mutant in the rate at which OmpF levels were reduced when cells were transferred from low- to high-osmolarity medium (1). From these results, these investigators and others have concluded that chromosomal *micF* RNA probably plays only a minor role in osmoregulation, acting as a fine tuner for the major transcriptional osmocontrol of *ompF* mediated by OmpR and EnvZ (1, 33).

In the present report, we analyze the role of chromosomal *micF* RNA in osmoregulation under conditions of low and medium as well as high osmolarity. We show that *micF* RNA is essential for osmoregulation of OmpF at low and medium levels of osmolarity (0 to 6% sucrose). At higher osmolarity, this essential role of *micF* RNA is masked by the strong transcriptional osmoregulation exerted by OmpR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used were the wild type, MC4100 (F⁻ Δ*lacU169 araD rpsL relA thi flbB*) and the *micF* deletion mutant SM3001, which was constructed from MC4100 by Matsuyama and Mizushima (25).

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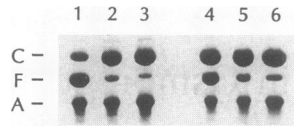

FIG. 1. Effect of high osmolarity on the expression of OmpF in *micF* deletion mutant. The parent strain, MC4100 (lanes 1 to 3) and the *micF* deletion mutant, SM3001, (lanes 4 to 6) were grown in Luria broth supplemented with the following amounts of sucrose: 0%, lanes 1 and 4; 10%, lanes 2 and 5; and 20%, lanes 3 and 6. C, F, and A indicate the positions of OmpC, OmpF, and OmpA, respectively. As was noted previously (25), *ompC* expression appears to be enhanced in the *micF* deletion strain. We believe this is due to the removal of the *ompC* integration host factor-binding site (19) during the construction of the *micF* deletion strain (25). The integration host factor has been shown to bind to this site and negatively effect *ompC* expression (19).

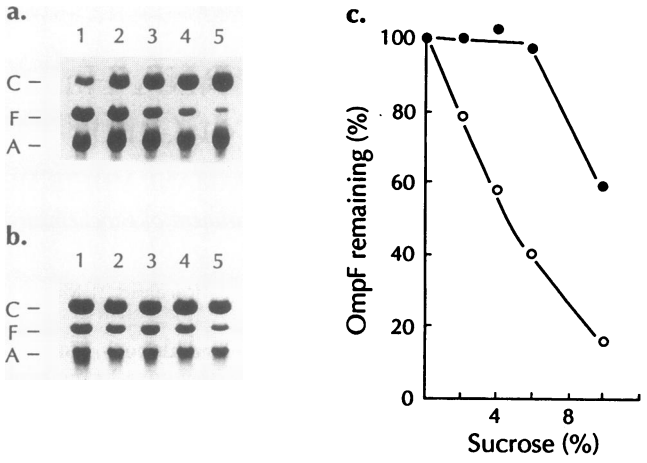

FIG. 2. Effect of increasing osmolarity on the expression of OmpF in *micF* deletion mutant. MC4100 (a) and SM3001 (b) were grown in Luria broth with the following amounts of sucrose: 0%, lane 1; 2%, lane 2; 4%, lane 3; 6%, lane 4; and 10%, lane 5. C, F, and A indicate the positions of OmpC, OmpF, and OmpA, respectively. (c) Amounts of OmpF determined from densitometer tracings of each sample in panels a and b. The amount of OmpF was determined as the ratio of OmpF to OmpA in each sample. This ratio was taken as 100% for each strain grown without sucrose. ○, MC4100; ●, SM3001.

Bacteria were grown at 37°C with shaking in Luria broth (27) without NaCl but with various concentrations of sucrose or procaine as noted in the text.

Preparation of outer membrane proteins. Cells in mid-exponential growth were centrifuged at 10,000 × *g* and resuspended in 4 ml of sodium phosphate (20 mM, pH 7.0). The outer membranes were isolated as described previously (36) and analyzed by 8 M urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (30). The results are presented as a representative experiment of at least three independent experiments.

RNA isolation. Cells were grown overnight at 37°C with shaking in Luria broth without NaCl. Cells from the overnight cultures were diluted into fresh medium with supplements of sucrose or procaine as described in the text. The cultures were grown for approximately 3 h until they had reached an optical density at 600 nm of 0.7 to 0.9. The cells were chilled in ice and centrifuged at 10,000 × *g* for 10 min. Total cellular RNA was isolated by lysing the cells in a guanidine isothiocyanate solution and extracting the lysate with phenol-chloroform as described in the Promega protocol (34). The final RNA solution was treated with RNase-free DNase, and it concentration was determined spectrophotometrically.

Synthesis of radiolabeled RNA probes and ribonuclease protection assay. DNA fragments of *micF* (−32 to +64) and *ompF* (+1 to +135) were cloned into the *Sma*I and *Bam*HI-*Xba*I sites, respectively, of the Bluescript II KS(+) plasmid (Stratagene). The plasmids were linearized with *Pvu*II and used as templates for the in vitro synthesis of radiolabeled RNA probes as described previously (34). Cellular levels of *micF* or *ompF* RNA were determined by the ribonuclease protection assay specified by Ambion (4).

RESULTS

***micF* RNA is essential for osmoregulation of OmpF at low and medium levels of osmolarity.** *micF* antisense RNA is not believed to be a major factor in osmoregulation because a *micF* deletion had little effect on altering the reduced steady-state levels of OmpF protein caused by high-osmolarity conditions (25). We confirmed this by growing the *micF* deletion strain SM3001 and its parent in Luria broth containing 10 or 20% sucrose (Fig. 1). However, when these strains were grown with lower sucrose concentrations, the results were markedly different. The levels of OmpF in the parent were strongly and steadily reduced when these cells were grown in Luria broth with 2 to 6% sucrose, but there was no effect on OmpF synthesis in the *micF* deletion strain (Fig. 2). Similar results were obtained when these experiments were done with rich media other than Luria broth (37).

Effect of osmolarity on *micF* RNA levels. The results presented above strongly indicate that *micF* RNA is required for the negative osmoregulation of OmpF at low-to-medium levels of osmolarity. If this is correct, *micF* RNA levels should sharply increase under these conditions concomitant with the decrease in OmpF. We examined this by measuring the steady-state levels of *micF* RNA in the parent strain grown in the same conditions as those used in determining the amount of OmpF protein. The data in Fig. 3 show a marked increase in *micF* RNA in cells grown in Luria broth when the concentration of sucrose was increased from 0 to 6%. Interestingly, at high sucrose concentrations, when *micF* RNA does not appear to be a major factor in osmoregulation (1, 25) (Fig. 2), *micF* RNA levels were reduced considerably compared with that produced in lower osmolarity (Fig. 3b). Quantification by densitometry of the protein and RNA gels indicates a strong correlation between the increased levels of *micF* RNA and the decrease in OmpF during growth in low to moderate levels of sucrose (Fig. 3c).

Effect of osmolarity on *ompF* mRNA levels. OmpF expression is known to be negatively regulated by osmolarity through the inhibition of transcription by the phosphorylated form of OmpR (13, 46, 48). Our results suggest that this occurs primarily at high levels of osmolarity and that *micF* RNA is responsible for the partial reduction in the amount of OmpF protein found at intermediate (2 to 6% sucrose) osmolarity levels. Data consistent with this were obtained by measuring the steady-state levels of *ompF* mRNA in the parent and in the *micF* deletion strain grown in Luria broth with various amounts of sucrose (Fig. 4). Little or no effect on *ompF* mRNA synthesis was found when MC4100 or SM3001 was grown in the presence of 2 to 6% sucrose. This is in contrast to the strong reduction in *ompF* mRNA found when either strain was grown with high levels of sucrose (Fig. 4). Although the levels of *ompF* mRNA in these experiments were very similar in the *micF*$^+$ and *micF* mutant strains, previous work suggested that

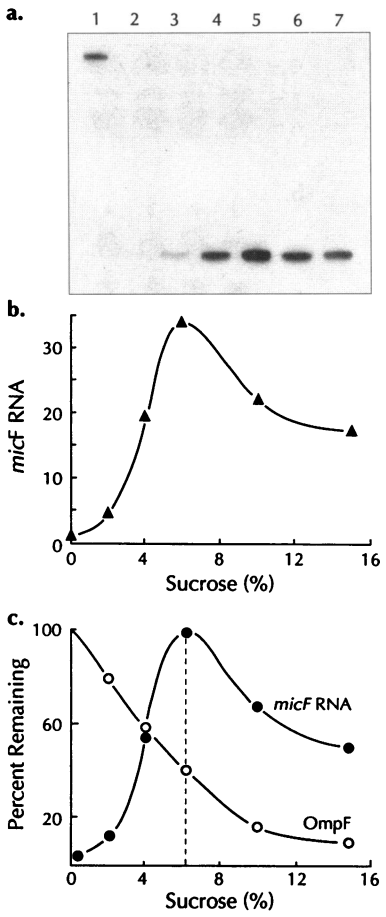

FIG. 3. Effect of increasing concentrations of sucrose on *micF* RNA levels. (a) Ribonuclease protection analysis of *micF* RNA in MC4100 grown in Luria broth supplemented with the following amounts of sucrose: 0%, lane 2; 2%, lane 3; 4%, lane 4; 6%, lane 5; 10%, lane 6; and 15%, lane 7. The probe is shown in lane 1. (b) Amounts of *micF* RNA determined from densitometer tracings of the samples in panel a. (c) Correlation between the levels of *micF* RNA and OmpF expression during growth with increasing amounts of sucrose. ○, densitometer tracings of levels of OmpF shown in Fig. 2a; ●, densitometer tracings of levels of *micF* RNA shown in Fig. 3a.

micF RNA, in addition to inhibiting OmpF translation, also participates in the destablization of *ompF* mRNA (7, 29). Differences in growth and other conditions may account for our failure to detect any additional reduction in *ompF* mRNA in the *micF*$^+$ strain.

Role of *micF* in the reduction of OmpF levels by procaine. Growth of *E. coli* in the presence of procaine and other local anesthetics strongly reduces the level of OmpF in the outer membrane (15, 35). This effect has been reported to be due to transcriptional regulation mediated by EnvZ and OmpR (47, 52) by a mechanism analogous to that involved in osmoregulation (38, 47). We used the *micF* deletion strain to examine whether *micF* RNA, as we have found for osmoregulation, is involved in the negative effect of procaine on OmpF. The data in Fig. 5 show that OmpF levels were strongly reduced in the *micF* mutant and in the parent strain when they were grown in Luria broth and a high concentration (20 mM) of procaine. A reduction in the amount of procaine still resulted in a substantial lowering (20 to 50%) of OmpF in the parent strain (Fig. 5a). However, these levels of procaine had little or no effect on

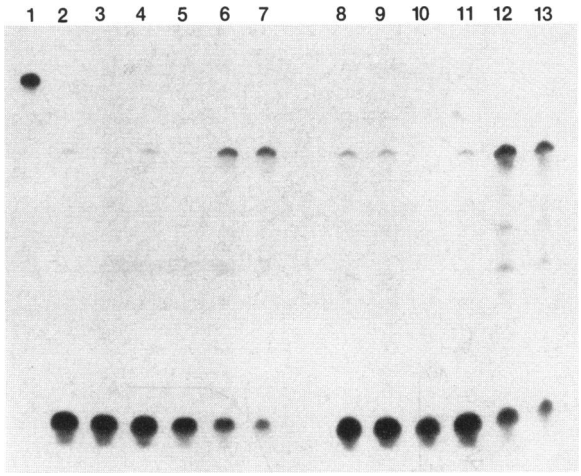

FIG. 4. Effect of increasing concentrations of sucrose on *ompF* mRNA levels. Ribonuclease protection analysis of *ompF* mRNA levels in MC4100 (lanes 2 to 7) and SM3001 (lanes 8 to 13) grown in Luria broth supplemented with the following amounts of sucrose: 0%, lanes 2 and 8; 2%, lanes 3 and 9; 4%, lanes 4 and 10; 6%, lanes 5 and 11; 10%, lane 6 and 12; and 20%, lanes 7 and 13. The probe is shown in lane 1. The data were quantified by using a Bio-Rad imaging densitometer. Little or no reduction in *ompF* RNA levels was found in either strain grown with 2, 4, or 6% sucrose. Growth with 10 and 20% sucrose reduced *ompF* mRNA by 60 and 78% in MC4100 and by 47 and 73% in SM3001, respectively. The nature of the upper band seen in most of the lanes is not known.

the amount of OmpF made in the *micF* deletion strain (Fig. 5b), suggesting that *micF* RNA is essential for the reduction in OmpF found at low procaine concentrations. Consistent with this idea were results which showed a very strong increase in *micF* RNA in the parent strain when low levels of procaine were added to the growth medium (Fig. 6). The extent of this increase was similar to the increase in *micF* RNA when cells were grown in the presence of low concentrations of sucrose (Fig. 3).

DISCUSSION

A number of observations, including the strong reduction in the level of OmpF by a multicopy plasmid that contained *micF*, suggested that *micF* RNA played a major role in osmoregula-

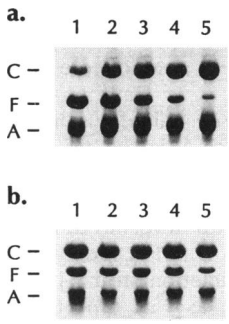

FIG. 5. Effect of increasing amounts of procaine on the expression of OmpF in *micF* deletion mutant. MC4100 (a) and SM3001 (b) were grown in Luria broth and the following amounts of procaine: 0 mM, lane 1; 2 mM, lane 2; 5 mM, lane 3; 10 mM, lane 4; and 20 mM, lane 5. C, F, and A indicate the positions of OmpC, OmpF, and OmpA, respectively.

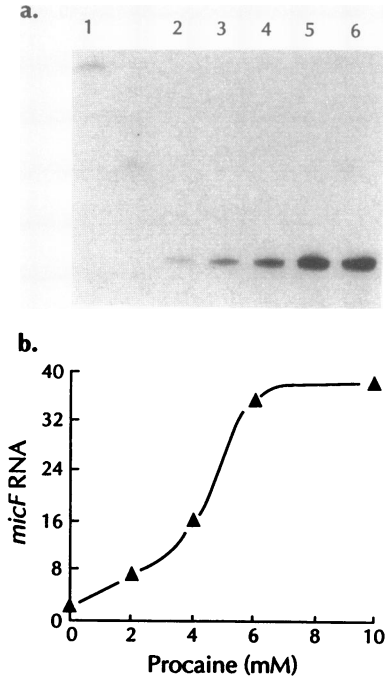

FIG. 6. Effect of increasing concentrations of procaine on *micF* RNA levels. (a) Ribonuclease protection analysis of *micF* RNA in MC4100 grown in Luria broth supplemented with the following amounts of procaine: 0%, lane 2; 2 mM, lane 3; 4 mM, lane 4; 6 mM, lane 5; and 10 mM, lane 6. The probe is shown in lane 1. (b) Amounts of *micF* RNA determined from densitometer tracings of the samples in panel (a).

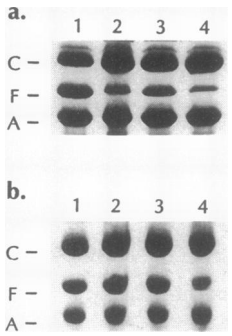

FIG. 7. Osmolarity and procaine act synergistically to inhibit OmpF expression. MC4100 (a) and SM3001 (b) were grown in Luria broth with the following additions: none, lane 1; 4% sucrose, lane 2; 4 mM procaine, lane 3; and 4% sucrose and 4 mM procaine, lane 4. C, F, and A indicate the positions of OmpC, OmpF, and OmpA, respectively.

tion of the OmpF proin protein (29), *micF* RNA, which is highly complementary to the 5′ end of *ompF* mRNA (6, 29), was the first regulatory antisense RNA found to control gene expression in *E. coli* (29). This discovery was a major impetus to work in many laboratories, which showed that numerous genes in prokaryotes are regulated by antisense RNA (45). It was also instrumental in the development of antisense technology for artifically manipulating gene expression in prokaryotic and eukaryotic systems (10, 23, 26). However, shortly after the discovery of *micF* RNA, Matsuyama and Mizushima (25) found that a complete deletion of the *micF* gene had little effect on the steady-state levels of OmpF in cells grown in high-osmolarity medium. Although additional work showed that *micF* RNA contributed to the rapid and complete response of OmpF to osmoregulation, it was concluded that *micF* RNA played only a minor role in this regulatory process (1, 33).

The experiments described in the present work strongly suggest that *micF* RNA is the principal, and possibly sole, mediator of osmoregulation of OmpF when cells are grown in conditions of low-to-moderate levels of osmolarity. Under these conditions, OmpF levels were reduced up to 60% in the parent strain, while OmpF expression was essentially unaffected in the *micF* deletion strain. In medium of high osmolarity, OmpF levels, as was reported earlier (1, 25), were reduced substantially in both strains. This strong negative regulation of OmpF by high osmolarity has been shown to be due primarily to transcriptional control resulting from the binding of phosphorylated OmpR protein to the *ompF* promoter region (13, 46, 48). Taken together, these data suggest that osmoregulation of OmpF is accomplished by two mechanisms. At low-to-intermediate levels of osmolarity, OmpF levels are reduced substantially through translational control by *micF* RNA. As osmolarity is further increased, transcriptional control by OmpR is initiated, and this becomes the primary mechanism for the strong negative regulation of OmpF found at high levels of osmolarity. Consistent with this view are the results of *micF* RNA and *ompF* mRNA measurements in cells grown with increasing amounts of sucrose. Initially, *micF* RNA levels increased markedly concomitant with a strong reduction in OmpF protein (Fig. 3). However, the level of *ompF* mRNA decreased only at higher sucrose concentrations (Fig. 4).

Our results with procaine provide additional evidence for the importance of chromosomal *micF* RNA in the negative osmoregulation of OmpF. It is known that growth of *E. coli* in the presence of procaine strongly reduces OmpF and increases OmpC levels in the outer membrane (15, 35). These effects were shown to be mediated by EnvZ and OmpR (47, 52) and were reported to be due to changes in transcription (38, 47). However, as we have found for osmoregulation, *micF* RNA is required for the strong reduction in OmpF levels caused by low, but not by high, concentrations of procaine. This indicates, as has been postulated previously (38, 47), that osmolarity and procaine inhibit OmpF expression by very similar mechanisms. We suggest that this involves translational control by *micF* RNA at low levels of osmolarity or procaine and transcriptional regulation by OmpR at high levels. This model predicts that these negative effects on OmpF expression by nonsaturating levels of procaine and osmolarity should be additive. The data in Fig. 7 show that this is correct. Small amounts of sucrose or procaine reduced OmpF levels in the parent strain by 33 and 20%, respectively, when added separately and 52% when added together. Significantly, while these levels of sucrose or procaine had no effect on OmpF expression in the *micF* deletion strain, in combination they reduced the amount of OmpF by 36% (Fig. 7).

An early report by Schnaitman and McDonald (44) is consistent with our results. They found that a *micF-ompC* deletion strain was partially constitutive for OmpF synthesis when it was grown in Luria broth supplemented with 2% NaCl. They suggested that chromosomal *micF* RNA was a major factor in the negative osmoregulation of OmpF (44). However, this conclusion was questioned (1, 25) when Mizushima and coworkers (1, 25) reported that a strain with a *micF* deletion retained strong inhibition of OmpF expression when grown in

media of high osmolarity. These seemingly contradictory results can now be explained by our data showing that *micF* RNA is essential for osmoregulation of OmpF at low but not at high levels of osmolarity. Because the experiments of Mizushima and coworkers (1, 25) were done only in high-osmolarity conditions, they failed to identify *micF* RNA as a major component of osmoregulation. On the other hand, Schnaitman and McDonald (44) used intermediate-osmolarity conditions in which both *micF* RNA and OmpR most probably participate in the negative osmoregulation of OmpF. Therefore, a *micF* deletion should partially reduce the negative effects of osmolarity on OmpF expression, and as these workers found (44), this would result in partially constitutive synthesis of OmpF.

There are many other conditions in addition to osmolarity and procaine that lead to increased levels of *micF* RNA and reduced synthesis of OmpF (7–9, 28, 43). At high temperatures, this has been shown to occur through a mechanism distinct from that involved in osmoregulation (11). However, for other conditions, such as a *tolC* mutation (28) or growth in salicylate (40, 43), OmpR and EnvZ appear to be necessary for the strong reduction in OmpF levels. It may be that these conditions also work through the osmoregulation system and use *micF* RNA and high levels of phosphorylated OmpR to block both the translation and transcription of *ompF*.

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