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marA Locus Causes Decreased Expression of OmpF Porin in Multiple-Antibiotic-Resistant (Mar) Mutants of *Escherichia coli*

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Mar (multiple antibiotic resistant) mutants of *Escherichia coli* express chromosomally mediated resistance to a variety of structurally unrelated hydrophilic and hydrophobic antibiotics. Insertion of transposon Tn*5* into the *marA* locus at min 34.05 on the chromosome completely reverses the Mar phenotype (A. M. George and S. B. Levy, J. Bacteriol. 155:531–540, 1983). We found that among changes in the outer membrane of Mar mutants, porin OmpF was greatly reduced, although Mar mutants were more resistant than cells lacking only OmpF. Transduction of the *marA* region from a Mar strain, but not a wild-type strain, led to loss of OmpF. P1 transduction of *marA*::Tn*5* into a Mar mutant partially restored OmpF levels. Therefore, OmpF reduction required a mutation in the *marA* region. Mar mutants of an *ompF-lacZ* operon fusion strain expressed 50 to 75% of the β-galactosidase activity of the isogenic non-Mar parental strain, while Mar mutants of a protein fusion strain expressed less than 10% of the enzyme activity in the non-Mar strain. These changes were completely reversed by insertion of *marA*::Tn*5*. The responsiveness of OmpF-LacZ to osmolarity and temperature changes was similar in Mar and wild-type strains. Although some transcriptional control may have been present, OmpF reduction appeared to occur primarily by a posttranscriptional mechanism. The steady-state levels of *ompF* mRNA were twofold lower and the mRNA was five times less stable in the Mar mutant than in the wild-type strain. Expression of *micF*, which lowers *ompF* mRNA levels, was elevated in Mar strains, as revealed by a *micF-lacZ* fusion. Studies with strains deleted for the *micF* locus showed that the *marA*-dependent reduction of OmpF required an intact *micF* locus. Our findings suggest that the *marA* locus directly or indirectly increases *micF* expression, causing a posttranscriptional decrease in *ompF* mRNA and reduced amounts of OmpF.

The outer membrane of gram-negative bacteria forms a permeability barrier for the entry and exit of various compounds, including nutrients, metabolites, and antibiotics. Most hydrophilic compounds traverse the outer membrane via membrane-spanning protein pores called porins (26). In *Escherichia coli* K-12, the major outer membrane porins are OmpF and OmpC (26). The porins also serve as receptors for certain bacteriophages (4). The expression of OmpF and OmpC in *E. coli* is tightly regulated in a coordinated fashion so that the total amount of porin remains constant (32). The level of each porin fluctuates in response to environmental signals, such as temperature and osmolarity (18, 19, 32), by means of the *ompR* and *envZ* gene products (10, 11). OmpF levels are highest under conditions of low temperature or low osmolarity (18, 19, 32). Many reports have described mutations which reduce or eliminate the OmpF porin from the outer membrane, rendering the cell more resistant to antibiotics, including tetracycline, chloramphenicol, β-lactams, and hydrophilic quinolones (12, 15, 28, 29), and to bacteriophages (4, 6, 28).

While investigating the mechanisms of chromosomal multiple antibiotic resistance (Mar) derived in *E. coli* by stepwise selection on increasing concentrations of tetracycline or chloramphenicol (7), we observed that OmpF was greatly reduced in the outer membrane of all Mar mutants. The reversal of the Mar phenotype by insertion of transposon Tn*5* into the *marA* locus at min 34.05 of the chromosome (8) also led to a restoration of OmpF. In this communication we characterize *marA* control over OmpF expression.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, and medium. The bacterial strains used in this study are listed in Table 1 or below. Bacteriophages TuIa and TuIb, originally from C. Schnaitman, were obtained from A. Wright. Plasmid pmicB21, containing the *micF-lacZ* fusion (24), was kindly provided by M. Inouye, and pBR272, containing the cloned *ompF* gene (23), was obtained through T. J. Silhavy. Unless otherwise stated, cells were grown in L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose per liter).

Mutant isolation, nomenclature, and P1 transduction. Multiple-antibiotic-resistant (Mar) mutants were isolated by plating approximately 1 ml of a stationary-phase culture of *E. coli* on MacConkey or L agar plates containing tetracycline (2 to 5 μg/ml) or chloramphenicol (5 to 10 μg/ml) (7, 8). Following 2 to 4 days of incubation at 30°C, resistant colonies which arose were reisolated on the same medium, and the resultant clones were stored at −70°C in phosphate-buffered saline (pH 7.5) containing 20% glycerol. Strains resistant to higher levels of tetracycline or chloramphenicol were subsequently selected in stepwise fashion on antibiotic-containing L agar plates. Mutants derived for use in this study were designated according to the antibiotic used for selection, Tc or Cm for tetracycline and chloramphenicol, respectively, followed by the level (in micrograms per milliliter) used for selection and the number of steps required. For example, a mutant of strain MH513 derived by selection on tetracycline (3 μg/ml) in a single step was designated MH513-Tc3-1. The mutation frequency of one step was on the order of 10^{-6} to 10^{-8}. Mar mutants inactivated by P1 transduction (22) of *marA*::Tn*5* from AG1025 (8) were des-

TABLE 1. *E. coli* strains[a]

Strain	Genotype or description	Reference
AG100	*argE3 thi-3 rpsL xyl mtl supE44* Δ(*gal-uvrB*)	7
AG102	Mar derivative of AG100 expressing Tc^r	7
AG1025	Same as AG102, but containing *marA*::Tn*5*	8
CT001	*hpcR*	30
MH513	Δ*lacU169 rpsL relA thiA flbB* Φ (*ompF-lacZ*$^+$)*16-13*	10
MH621	Δ*lacU169 rpsL relA thiA flbB* Φ(*ompF-lacZ*)*16-21*	10
JF701	*aroA357 ilv-227 metB65 his-53 purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx-63 ompC264*	6
JF703	Same as JF701 except *ompC*$^+$ *ompF254 aroA*$^+$	6
PLK1253	*trpR trpA9605 his-29 ilv pro arg thyA deoB* (or *deoC*) *tsk rac zdd-230*::Tn*9*, *zde-234*::Tn*10*	2
LM111	Chloramphenicol-sensitive derivative of PLK1253	This study
LM111-Cm80-5	Mar derivative of LM111	This study
SPC101	P1 transductant of *zde-234*::Tn*10* from LM111-Cm80-5 into AG100; chloramphenicol resistant	This study
SPC102	P1 transductant of *zde-234*::Tn*10* from LM111-Cm80-5 into AG100; chloramphenicol sensitive	This study
MC4100	F$^-$ Δ*lacU169 araD rpsL relA thi fibB*	A. Wright
SM3001	MC4100 Δ*micF1*	22
DME555	F$^-$ Δ*araD139* Δ(*argF-lac*)*U169 rpsL150 rclA1 fibB5301 ptsF25 deoC1 thiA1* Δ*lamB106* Δ*ompF80* Tn*10*	T. Silhavy
MH621 Δ*micF*	Same as MH621 but Δ*micF1*	This study

[a] All strains listed are *E. coli* K-12 strains, except CT001, which is an *E. coli* C strain.

ignated with the strain name followed by Kan; for example, MH513-Tc3-1-Kan is MH513-Tc3-1 (*marA*::Tn*5*).

MICs. Susceptibility to the various antibiotics was determined by a gradient plate method (3) on PenAssay agar (Difco) or L agar. Cultures in logarithmic growth phase in L broth were used, and the MIC was defined by the limit of confluent growth following incubation of plates for 24 to 40 h at 30°C. All antibiotics were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Protein analysis of outer membranes. A method similar to that described before (16) was used to isolate outer membranes. Cells grown to the mid- to late logarithmic phase at 30°C were harvested by centrifugation, washed with 10 mM sodium phosphate (pH 7), and sonicated in 1 ml of the same buffer at an A_{530} of 17 with a fine-tip probe in a Branson sonicator. Unlysed cells were removed by centrifugation (5,000 × *g*, 5 min) at 4°C, and total membranes were pelleted subsequently (18,000 × *g*, 1 h). Membranes were suspended in 0.67 ml of 0.5% sodium lauryl sarcosinate (5) in phosphate buffer and incubated at 22°C for 30 min. The insoluble outer membranes were pelleted (18,000 × *g*, 1 h) and solubilized by heating in 0.05 M Tris chloride (pH 6.8)–4% sodium dodecyl sulfate (SDS)–2% β-mercaptoethanol–10% glycerol at 100°C for 4 min. Samples (8 μl) for protein analysis were separated by polyacrylamide gel electrophoresis (PAGE) at 22°C in a 7% polyacrylamide gel containing 8 M urea and 0.1% SDS as described before (20) but with 0.01% ammonium persulfate and 0.21% bisacrylamide. Gels were soaked in 7% acetic acid–5% methanol to fix proteins and to remove urea and were then stained with 0.1% Coomassie brilliant blue R250 (Bio-Rad Laboratories).

Bacteriophage titrations. Bacteriophage TuIa or TuIb was incubated in L broth with 0.2 ml of cells grown to logarithmic phase at 30°C for 20 min, the mixture was plated in a soft overlay of L agar, and plates were incubated at 30°C overnight to allow plaque formation. Strains lacking OmpF or OmpC were included as controls to verify phage specificity.

β-Galactosidase assays. β-Galactosidase was assayed at 30°C with the substrate *O*-nitrophenyl-β-D-galactoside (ONPG), and activity was expressed in arbitrary units as described by Miller (22). One unit represents 0.222 μmol of ONPG converted per min per A_{600} unit of cells. Although strain AG100 (and its derivatives) is *lac*$^+$, under the growth conditions used here endogenous levels of β-galactosidase were negligible.

RNA analysis. RNA was prepared from cells by a modification of a method described previously (31). Cells were grown at 30°C to an A_{530} of 0.8 to 1.0, centrifuged (4,000 × *g*, 5 min), and concentrated 25-fold in L broth. The cultures, with and without rifampicin (200 μg/ml; Sigma), were shaken at 30°C, and 0.25-ml samples were removed directly to 0.6 ml of 65°C lysing buffer (0.06 M sodium acetate and 1.25% SDS in diethyl pyrocarbonate-treated H_2O). The tubes were inverted gently three times, and 0.75 ml of 65°C phenol (saturated with 50 mM sodium acetate [pH 5.2]) was added. The samples were incubated for 15 min at 65°C, with gentle inversion every 3 min, followed by 15 min on ice. They were then centrifuged (15,000 × *g*, 5 min, 4°C), and 0.6 ml of the aqueous phase was precipitated with 0.5 volume of 0.3 M sodium acetate–3 volumes of ethanol and stored at −70°C.

Samples of the ethanol precipitate were centrifuged (15,000 × *g*, 30 min, 4°C), and the pellets were washed with 70% ethanol and dried. The pellets were redissolved with vigorous agitation in 15% deionized glyoxal–50% dimethyl sulfoxide–10 mM sodium phosphate buffer, pH 6.6, made with diethyl pyrocarbonate-treated water, incubated at 50°C for 1 h, and subjected to electrophoresis through 1.0% agarose in 10 mM sodium phosphate buffer, pH 6.6, for 4 h at 100 V with constant buffer recirculation at 4°C. The RNA was transferred from the gel to a nylon membrane (GeneScreen Plus; New England Nuclear) with 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate) for 18 h. Prehybridization of the membrane in 1% SDS–1 M sodium chloride–10% dextran sulfate for 2 h at 60°C was followed by hybridization for 18 h at 60°C with the 5.3-kilobase (kb) *Eco*RI-*Hin*dIII fragment from pPR272 (23), which contains the *ompF* coding sequence. This fragment was labeled with [^{32}P]dCTP by using a random primed DNA-labeling kit (Boehringer Mannheim). The membrane was then washed twice for 5 min with 2× SSC at 20°C, twice for 30 min in 2× SSC–1% SDS at 60°C, and twice for 30 min in 0.1× SSC at 20°C. The membrane was blotted dry and exposed to X-ray film (Kodak XAR-5) at −70°C with two intensifying screens. The density of bands on exposed film was determined with an LKB scanning laser densitometer.

RESULTS

Selection of Mar mutants of AG100 and CT001. Consistent with earlier results (7, 8), Mar mutants of *E. coli* K-12 strain

TABLE 2. Antibiotic susceptibility

Strain	Type[b]	MIC (μg/ml)[a]						
		Tetracycline	Chloramphenicol	Ampicillin	Penicillin G	Rifampicin	Nalidixic acid	Puromycin
AG100	P	1.2	3.6	6.7	17	1.6	2.1	56
AG102	M	10.8	18.0	21.4	77	18	7.6	330
AG1025	I	1.4	4.0	7.5	22	1.8	2.4	67
MH513	P	1.6	4.2	9.7	24	1.4	1.7	56
MH513-Tc3-1	M	12.0	16.2	21.1	58	22	4.9	200
MH513-Tc3-1-Kan	I	1.5	3.8	9.2	24	2.1	1.6	56
MH621	P	1.1	4.2	10.3	32	2.2	1.6	44
MH621-Tc2-1	M	9.6	19.4	23.6	91	26	6.7	340
MH621-Tc2-1-Kan	I	1.2	4.6	10.0	31	2.4	1.8	44

[a] Average of two determinations, which were within 10% of each other.
[b] P, Parental; M, Mar; I, Tn*5*-inactivated Mar.

AG100 and *E. coli* C strain CT001 were obtained at frequencies between 5×10^{-6} and 5×10^{-7} on MacConkey agar supplemented with low amounts of tetracycline or chloramphenicol. They were cross-resistant to tetracycline, chloramphenicol, ampicillin, penicillin, rifampicin, nalidixic acid, and puromycin (data for AG100, Table 2). Except in Tn*5*-containing strains, there was no decreased susceptibility to kanamycin (data not shown).

Outer membrane protein changes associated with Mar mutants. We compared the outer membrane proteins from *E. coli* C and *E. coli* K-12 strain AG100, their Mar mutants, and their Tn*5*-inactivated derivatives by SDS-PAGE. Proteins insoluble in 0.5% sodium lauryl sarcosinate were operationally defined as outer membranes (5). Changes were evident in Mar mutants, including the appearance of prominent new Mar-related proteins (designated Mrp1 and -3 to -6) (Fig. 1).

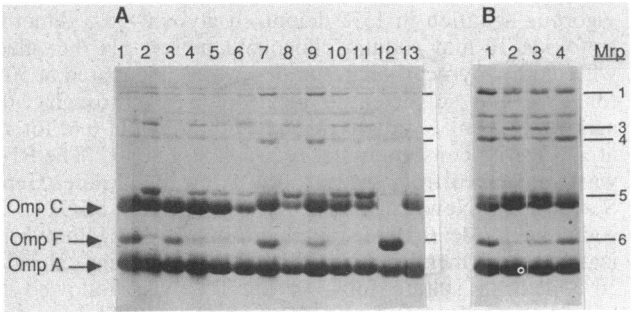

FIG. 1. SDS-urea-PAGE of outer membrane proteins. (A) Proteins prepared from parental strains, Mar mutants, and *marA*::Tn*5*-inactivated derivatives of CT001 (lanes 1–6) and AG100 (lanes 7–11); CT001 (lane 1); CT001-Tc80-7 (lane 2); CT001-Tc80-7-Kan (lane 3); CT001-Tc5-1 (lane 4); CT001-Tc50-5 (lane 5); CT001-Tc200-12 (lane 6); AG100 (lane 7); AG102 (lane 8); AG1025 (lane 9); AG100-Tc5-1 (lane 10); and AG100-Tc50-5 (lane 11). The positions of OmpC, OmpF, and OmpA were determined by using porin mutants JF701 and JF703 (lanes 12 and 13, respectively). Dashes refer to Mar-related protein (see below). (B) Proteins prepared from AG100 (lane 1), AG100-Tc 2.5-1 (lane 2), SPC101, a *zde-234*::Tn*10* transductant of AG100 which was fivefold more resistant to chloramphenicol than was AG100 (lane 3), and SPC102, a *zde-234*::Tn*10* transductant of AG100 which had the same susceptibility to chloramphenicol as did AG100 (lane 4). Mrp (Mar-related protein) bands 1, 3, 4, 5, 6 are marked. The top fourth of the gel, bearing no protein bands, was omitted from the figure.

Of note was the loss of the major outer membrane porin OmpF in Mar mutants of both *E. coli* C (Fig. 1A, lanes 2, 4, 5, and 6) and *E. coli* K-12 (Fig. 1A, lanes 8, 10, and 11). Outer membranes prepared from *E. coli* K-12 strains missing OmpC or OmpF were used as standards (Fig. 1A, lanes 12 and 13, respectively). The outer membrane protein profile of low-, intermediate-, and high-level Mar mutants revealed that maximal OmpF loss occurred in the first-step Mar mutants (Fig. 1A, lanes 4 and 10). Insertion of *marA*::Tn*5* into a Mar strain resulted in partial (40 to 70%) restoration of OmpF (Fig. 1A, lanes 3 and 9). Wild-type antibiotic susceptibility was completely restored by this insertion (AG1025, Table 2). Insertion of *marA*::Tn*5* into a non-Mar strain had no effect on OmpF or antibiotic susceptibility (data not shown). The residual band in the OmpF region in Mar mutants (Mrp6, e.g., Fig. 1A, lane 2) proved not to be OmpF. Its migration rate was slightly slower than that of OmpF, and it was still present in Mar stains (but not in non-Mar strains) following insertion of Tn*5* into *ompF* (data not shown).

Some Mar strains also manifested a partial loss of OmpC which was reversed by the *marA*::Tn*5* insertion (Fig. 1). The efficiency of plating of OmpC-specific bacteriophage TuIb was not detectably altered by these changes, however (data not shown).

The sensitivity of strains to the OmpF-requiring bacteriophage TuIa confirmed the losses and gains of OmpF seen on gels. In L broth at 30°C, this phage had a 10^3 lower titer on Mar strain AG102 than on the wild-type parent AG100. The titer on the *marA*::Tn*5* revertant AG1025 was the same as for the wild type, but the plaques were more turbid.

The insertion mutation *zde-234*::Tn*10* (min 34.2) is located close to the *marA* gene defined by *marA*::Tn*5* at min 34.05 (8). We wished to know whether the region near Tn*10* from a Mar strain was sufficient to lower OmpF. We therefore transduced *zde-234*::Tn*10* from Mar mutant LM111-Cm80-5 into wild-type strain AG100 by using P1 phage. Of 24 Tn*10*-bearing transductants, 9 showed a lowering of OmpF almost as great as that seen in a Mar strain and expressed 3.7 to 5.5 times the resistance of the wild-type strain to chloramphenicol (Fig. 1B, lane 3). The rest of the transductants showed no change in OmpF (Fig. 1B, lane 4). We conclude that a mutation(s) linked to min 34.2 in Mar strains is sufficient to cause an OmpF decrease. The *marA* locus is the likely site for such a mutation.

TABLE 3. β-Galactosidase activity of *ompF-lacZ* fusion strains[a]

Strain	β-Galactosidase activity (U)	% of wild-type activity
Operon fusion strains		
MH513	486	100
MH513-Tc3-1	362	74
MH513-Tc10-2	252	52
MH513-Tc20-3	278	57
MH513-Tc30-4	242	50
MH513-Cm7-1	377	78
MH513-Cm40-3	360	74
MH513-Tc3-1-Kan	460	95
MH513-Cm7-1-Kan	491	101
Protein fusion strains		
MH621	2,843	100
MH621-Tc2-1	199	7.0
MH621-Tc5-2	176	6.2
MH621-Tc10-3	151	5.3
MH621-Cm7-1	208	7.3
MH621-Cm15-2	109	3.8
MH621-Cm30-3	102	3.6
MH621-Tc2-1-Kan	3,473	122
MH621-Tc5-2-Kan	3,298	116
MH621-Cm7-1-Kan	2,650	93
MH621-Cm15-2-Kan	2,899	102

[a] All cultures were grown in L broth at 30°C. Values represent the average of three or more experiments.

Resistance levels and β-galactosidase activity of Mar mutants bearing *ompF-lacZ* fusions. To determine whether *marA*-related reduction of OmpF was regulated transcriptionally or translationally, we used two *ompF-lacZ* fusions. MH513 contains an operon fusion in which the *lacZ* gene is under the transcriptional control of the *ompF* promoter (10). This fusion strain makes an intact β-galactosidase product. MH621 contains a protein fusion in which the *lacZ* coding sequence is fused to the 5′ portion of the *ompF* coding sequence; a small portion of the *ompF* translation signals and a small amino-terminal portion of the OmpF protein are included (10). In both strains, the normal *ompF* gene is deleted. The amount of *ompF-lacZ* hybrid protein product of MH621 is under both the transcriptional and translational control signals known to govern expression of native OmpF (10).

The frequency (10^{-7}) of Mar mutants obtained from fusion strains on low levels of tetracycline (2 to 3 μg/ml) or chloramphenicol (7 μg/ml) and the antibiotic resistance phenotype of the Mar mutants (Table 2) were comparable to those obtained with the nonfusion strains. Transduction of *marA*::Tn*5* into the Mar mutants containing the *ompF-lacZ* fusions led, as in other strains, to a restoration of antibiotic susceptibility (Table 2).

The β-galactosidase activity of MH513, MH621, their respective Mar mutants of various resistance levels, and their *marA*::Tn*5*-inactivated derivatives was assayed during growth at 30°C (Table 3). Mar mutants derived from the operon fusion strain MH513 expressed 50% or more of the β-galactosidase levels of the parent, MH513. This was true whether tetracycline or chloramphenicol had been used as the selective agent for the Mar mutants. The modest reduction in β-galactosidase activity in Mar strains carrying this fusion was reversed by transduction of *marA*::Tn*5* into the strain (Table 3).

The β-galactosidase activity of wild-type MH621, containing the *ompF-lacZ* protein fusion, was about fivefold higher than that of wild-type MH513 bearing the operon fusion (Table 3). It has been suggested that such a result arises from more efficient translation initiation of the mRNA containing the *ompF* translational initiation signals rather than from the *lacZ* signals (10). Mar mutants derived from the protein fusion strain MH621 had a more dramatic reduction in β-galactosidase expression than did those from the operon fusion strain MH513 (Table 3). Mar mutants of MH621 obtained by selection on low-level tetracycline (2 μg/ml) or chloramphenicol (7 μg/ml) showed only 7% of wild-type β-galactosidase levels. Higher-level Mar mutants of MH621 isolated in a stepwise fashion to increased levels of resistance to tetracycline or chloramphenicol showed only slight, additional decreases in β-galactosidase activity (Table 3). Inactivation of MH621 Mar mutants with *marA*::Tn*5* from AG1025 restored β-galactosidase activity to approximately that of the wild type (Table 3). The results with both the *ompF-lacZ* operon fusion and the *ompF-lacZ* protein fusion suggested that the primary level of *marA* control over OmpF expression was posttranscriptional.

Previously, P1 transduction experiments suggested that the mutant *marA* region alone could lower OmpF levels (see above). We performed a similar transduction experiment to determine the influence of this region on the amount of OmpF-LacZ fusion protein. *zde-234*::Tn*10* from wild-type LM111 or from Mar mutant LM111-Cm80-5 was transduced into wild-type fusion strain MH621 by using P1 phage. Among 32 Tn*10*-containing transductants from the donor Mar strain, 17 had 0 to 20% of the wild-type level of β-galactosidase activity, a reduction similar to that seen in first-step Mar fusion strains. Among 10 transductants from a wild-type donor, none showed lowering of β-galactosidase activity. Therefore, a mutant locus or loci near min 34.2 in a Mar mutant, when transduced into MH621, was completely responsible for reducing OmpF-LacZ expression; the locus from a non-Mar mutant was ineffective. Presumably the mutant locus was the same one that lowered intact OmpF protein (see above) and is probably *marA*.

Effect of osmolarity and temperature on β-galactosidase activity. The levels of native OmpF fluctuate in the cell in response to osmolarity and temperature (18, 19, 32). Conditions of low osmolarity or low temperature lead to increased production of the OmpF porin. We assayed β-galactosidase in MH513, MH621, and Mar mutants of these fusion strains grown under various culture conditions (Table 4). When the strains were grown in NBS (nutrient broth [Difco Laboratories] containing 15% sucrose), the β-galactosidase activity decreased compared with the activity of the strain grown at lower osmolarity in nutrient broth alone (Table 4). Although the absolute levels of β-galactosidase activity produced by the Mar mutants were lower than that by the wild type for both the operon and especially the protein fusion, the relative change caused by altered osmolarity was similar for Mar and wild-type strains (Table 4).

When Mar and wild-type fusion strains were grown at 30 or 37°C, a lower β-galactosidase activity was observed at the higher temperature for all strains tested (Table 4). Once again, although the absolute activity of a Mar mutant was lower than that in the corresponding wild-type strain, the relative changes in each due to a change in temperature were similar. These data indicated that although overall OmpF expression was reduced in the Mar mutants, the residual production was still responsive to environmental signals of osmolarity and temperature.

***ompF* mRNA levels in Mar mutants.** Among the possible mechanisms for posttranscriptional *marA* control of OmpF

TABLE 4. Effect of osmolarity and temperature on β-galactosidase activity of *ompF-lacZ* fusion strains[a]

Strain	β-Galactosidase activity (U)		Activity ratio, NB/NBS	β-Galactosidase activity (U)		Activity ratio, 30°C/37°C
	NB	NBS		30°C	37°C	
MH513	419	193	2.2	486	319	1.5
MH513-Tc3-1	337	146	2.4	362	208	1.7
MH513-Cm7-1	320	122	2.6	377	276	1.4
MH621	2,958	293	10.1	2,843	1,026	2.8
MH621-Tc2-1	601	109	5.5	199	66	3.0
MH621-Cm7-1	546	95	5.8	108	55	3.8

[a] For osmolarity measurements, strains were grown at 30°C in either nutrient broth (NB) or nutrient broth containing 15% sucrose (NBS). For temperature measurements, all strains were grown in L broth. Values represent the average of three experiments.

expression would be instability of *ompF* mRNA in Mar mutants. We examined *ompF* mRNA levels in wild-type AG100 and its Mar derivative AG100-Tc2-1. Total RNA prepared from these strains was separated in gels and transferred to nylon membranes for hybridization with a radiolabeled *ompF* probe. The largest hybridizing fragment, corresponding to the intact *ompF* mRNA, was seen in both strains at time zero (before rifampicin addition); however, the amount of hybridizing RNA in the wild type was approximately twice that in the mutant (Fig. 2). After addition of rifampicin to the culture to stop mRNA initiations, the amount of intact *ompF* mRNA decreased with an approximate half-life of 15 min in the wild-type strain and 3 min in the mutant. Since results with Mar mutants of the operon fusion strain had indicated that transcription initiations were nearly normal, we concluded that *ompF* mRNA stability was the primary cause of OmpF reduction in the Mar mutants. Similar results were obtained when we examined the levels of *ompF-lacZ* mRNA in MH621 and its first-step mutant MH621-Tc2-1 by using a *lacZ* probe (data not shown).

***micF-lacZ* levels in Mar mutants.** One proposed mechanism for regulating *ompF* mRNA stability involves *micF*, a gene producing an RNA transcript whose sequence is complementary to the 5′ portion of the *ompF* mRNA (24). It has been suggested that hybridization of *micF* RNA to *ompF* mRNA prevents the translation of *ompF* mRNA and facilitates its degradation (24). The *micF* gene (min 47) has a common operator sequence with *ompC* but is transcribed in the opposite direction (24). We introduced plasmid pmicB21, containing a *micF-lacZ* fusion (24), into AG100, LM246, and LM249. The resulting β-galactosidase activity in the Mar strain LM246 was more than six times that in the wild-type strain AG100 or the *marA*::Tn*5*-inactivated Mar strain LM249 (Table 5).

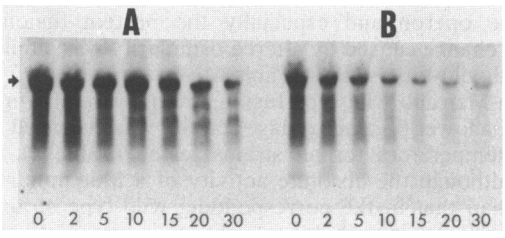

FIG. 2. Autoradiogram of *ompF*-hybridizing RNA prepared from AG100 (A) and AG100 Tc2-1 (B). RNA was subjected to electrophoresis and transferred to a nylon membrane before hybridization with the ^{32}P-labeled *ompF* probe. Numbers below each lane refer to minutes following the addition of rifampicin to the culture. The zero-minute sample was removed before rifampicin addition. Arrow points to intact *ompF* mRNA.

The increase in *micF* expression in a Mar strain might be the direct cause of *ompF* mRNA instability. It was possible, however, that the enhanced transcription of *micF* (as revealed by the *micF-lacZ* fusion in the Mar mutant) represented solely the effect, not the cause, of the *ompF* mRNA reduction. That this was not so was shown by introduction of the *micF-lacZ* fusion plasmid into the OmpF-deficient strain LM218, bearing *ompF*::Tn*5*. β-Galactosidase activity increased only twofold over that in wild-type AG100, suggesting some effect of OmpF loss on *micF*, but the enzyme activity was still threefold lower than in the Mar mutant (Table 5). Insertion of *ompF*::Tn*5* into the Mar mutant did not further increase the level of *micF-lacZ*-dependent β-galactosidase activity (Table 5). It was possible that the residual amino-terminal portion of *ompF* in the *ompF*::Tn*5* strains was activating *micF*. However, by using DME555, bearing an *ompF* deletion, we found a level of *micF-lacZ* activation similar to that seen with the *ompF*::Tn*5* strain (data not shown). This suggested that the residual portion of *ompF* did not activate *micF*.

To ascertain directly the contribution of *micF* to the *marA*-dependent reduction of OmpF, we compared the production of OmpF protein in strains containing and lacking *micF*. SM3001 is deleted for the *micF* locus (21). We introduced low-copy-number plasmids containing a cloned 9-kb *Pst*I fragment with either the wild-type (pmarA$^+$) or mutant (pmarA) *marA* locus (S. P. Cohen, H. Hächler, and S. B. Levy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, abstr. no. A8, p. 2; manuscript in preparation) into SM3001 and its parent strain, MC4100, which contains *micF*. Outer membrane protein preparations from these strains were analyzed by urea-SDS-PAGE, and the amount of OmpF protein relative to OmpA was determined by scanning laser densitometry. The OmpF/OmpA ratios in strains MC4100 (pmarA$^+$), MC4100(pmarA), SM3001(pmarA$^+$), and SM

TABLE 5. β-Galactosidase activity of strains containing a *micF-lacZ* fusion plasmid[a]

Strain[b]	Relevant genotype	β-Galactosidase activity (U)
AG100	*marA*$^+$ (no plasmid)	32
AG100mic	*marA*$^+$ (pmicB21)	604
LM218mic	*marA*$^+$ *ompF*::Tn*5* (pmicB21)	1,211
LM246mic	*marA* (pmicB21)	3,812
LM249mic	*marA*::Tn*5* (pmicB21)	836
LM250mic	*marA ompF*::Tn*5* (pmicB21)	3,828

[a] All cultures were grown in L broth at 30°C. Values represent the average of four experiments.

[b] LM246 is a specific subclone of AG100-Tc2.5-1. LM249 is LM246 inactivated by *marA*::Tn*5*. LM250 is LM246 inactivated by *ompF*::Tn*5*. *ompF*::Tn*5* from MH450 (10) was introduced by P1 transduction.

TABLE 6. β-Galactosidase activity of *ompF-lacZ* fusion strains with wild-type and mutant *marA* plasmids[a]

Strain	β-Galactosidase activity (U)
MH621	2,541
MH621(pmarA$^+$)	2,599
MH621(pmarA)	205
MH621 Δ*micF*	2,810
MH621 Δ*micF*(pmarA$^+$)	2,541
MH621 Δ*micF*(pmarA)	2,811

[a] All cultures were grown in L-broth at 30°C. Values represent the average of two or more determinations.

3001(pmarA) were 0.323, 0.189, 0.355, and 0.396, respectively. Relative to the OmpF levels in the presence of the wild-type *marA* plasmid, the mutant *marA* plasmid caused a greater than 40% decrease in OmpF when present in MC4100 but no reduction when present in SM3001.

We also determined the effect of Δ*micF* on production of the OmpF-LacZ fusion protein in strain MH621. The *micF* deletion was transduced into MH621 by linkage to kanamycin resistance. The wild-type and mutant *marA* plasmids were introduced into MH621 and MH621 Δ*micF*, and β-galactosidase activity was assayed (Table 6). The wild-type *marA*$^+$ plasmid pmarA$^+$ had no effect on β-galactosidase activity in either MH621 or MH621 Δ*micF*. In MH621, the mutant *marA* plasmid pmarA caused a greater than 10-fold decrease in β-galactosidase activity, consistent with the results from a chromosomal mutant *marA* locus (Table 3). However, in the absence of *micF*, no decrease was observed.

The results with the *micF* deletion strains suggest that *micF* is absolutely required for the *marA*-dependent reduction of OmpF. We conclude from these studies that Mar strains manifest both a decrease in *ompF* mRNA stability and an increase in *micF* transcription. It appears likely that the activation of *micF* brings about the reduction in *ompF* mRNA stability.

DISCUSSION

Chromosomal Mar mutants of *E. coli* were found to have greatly reduced amounts of OmpF porin. Laboratory-derived mutants and clinical isolates of *E. coli* with reduced amounts of OmpF have previously been linked to resistance to various antibiotics, including the β-lactams (12), tetracycline (28), chloramphenicol (29), and hydrophilic fluoroquinolones (13, 15), all of which are involved in the Mar phenotype. While OmpF loss may account for some of the Mar-related resistances at low levels, it does not account for all of them. Mar mutants are more resistant than *ompF* mutants. In addition, insertion of *marA*::Tn*5* into Mar strains completely restored wild-type antibiotic susceptibility but only partially restored OmpF, and rifampicin, a lipophilic antibiotic involved in the Mar phenotype, probably does not enter through this pore. Also, high-level Mar resistances appear to involve other changes, such as an efflux system for tetracycline (8).

The reduced steady-state levels of *ompF* mRNA (Fig. 2) suggested that decreased transcription was occurring. However, the rapid degradation of the mRNA and the results with the *ompF-lacZ* fusions (Table 3) suggested that the primary mechanism is posttranscriptional. In line with this suggestion, we found elevated expression of *micF* in Mar mutants, as revealed by a *micF-lacZ* fusion (Table 5). *micF* appears to destabilize *ompF* mRNA by producing an RNA transcript whose 3′ end is complementary to the 5′ portion of the *ompF* mRNA (24). When *micF* is present in multicopy, *ompF* mRNA levels and OmpF porin are greatly reduced (21, 24). It has been proposed that hybridization of *micF* RNA to the *ompF* mRNA prevents mRNA translation, promoting more rapid degradation in vivo than for mRNA which is being actively translated (24). Recently it has been demonstrated that the reduced OmpF levels in an *E. coli tolC* mutant (25) are associated with overexpression of *micF* RNA, increased amounts of OmpC, and decreased amounts of *ompF* mRNA without notable alteration in the rate of *ompF* transcription (23). The increased *micF-lacZ* expression in Mar mutants (Table 5) also suggested that the reduction of *ompF* mRNA and OmpF in Mar mutants was at least partially the result of *micF* activation. Unlike findings with the *tolC* mutant, however, under our growth conditions Mar mutants did not show an increase in OmpC (Fig. 1). Therefore, *micF* activation in Mar mutants may be independent of OmpC expression, although the two loci are reported to be normally under common transcriptional control (24).

The level of OmpF in a cell fluctuates according to the osmolarity and temperature of its environment (18, 19, 32). This fluctuation is mediated through the *ompB* locus, which encodes the *ompR* gene product, a transcriptional activator (17, 27), and the *envZ* gene product, a membrane-spanning protein which may serve as the environmental sensor (10). Although the amount of intact OmpF was greatly reduced in Mar mutants, our studies with the fusion strains revealed that the remaining activity was still subject to regulation by osmolarity and temperature to the same magnitude as in the non-Mar strains. Therefore, the *ompR-envZ* regulation of *ompF* did not appear to be affected by *marA*.

A locus near *zde-234*::Tn*10* (min 34.2) was able to lower OmpF and OmpF-LacZ when transduced from a Mar mutant but not from a wild-type strain. Although not enough transductants were examined to definitively map the mutated locus which lowers OmpF, the frequency of cotransduction of this locus with *zde-234*::Tn*10* was consistent with a location at *marA* (8). Others have described mutant loci, *norB* (14) and *cfxB* (14a), obtained by selection of *E. coli* on norfloxacin and ciprofloxacin, respectively, each of which mapped to a region near min 34 and caused a reduction in OmpF. These loci may be identical to *marA*. We note that the *marA* region did not offer enough resistance to be selected directly on tetracycline or chloramphenicol following transduction, which explains the previous failure (8) to achieve such transduction. Inactivation of *marA*::Tn*5* partially restored wild-type OmpF levels and completely restored levels of the OmpF-LacZ fusion protein and its mRNA. Therefore, the *marA* locus was clearly required for lowering of OmpF in Mar strains. That intact OmpF was only partially restored by *marA*::Tn*5* may reflect the presence in Mar strains of a locus independent of *marA* which acts on the intact *ompF* gene, mRNA, or protein but not on *ompF-lacZ*.

We hypothesize that *marA* lowers OmpF by increasing *micF* expression and thereby destabilizing *ompF* mRNA. This occurs without altering OmpC. *marA* joins a growing number of genetic loci which affect OmpF expression (10, 20, 24, 25). Such diversity of genes points to the critical role of this porin in normal cell function.

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