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Characterization of MarR, the Repressor of the Multiple Antibiotic Resistance (*mar*) Operon in *Escherichia coli*

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The *marRAB* operon is one of two operons in the *mar* locus of *Escherichia coli* that are divergently transcribed from a central regulatory region, *marO*. The *marRAB* operon, transcribed from $marO_{II}$, controls intrinsic resistance or susceptibility to multiple antibiotics and is inducible by structurally unrelated compounds such as tetracycline and chloramphenicol (S. P. Cohen, H. Hachler, and S. B. Levy, J. Bacteriol. 175:1484–1492, 1993). To clarify the role of the operon in response to environmental signals, its transcription was studied under different conditions, using a $marO_{II}$-*lacZ* transcriptional fusion introduced into the chromosome of wild-type or *mar*-deleted cells. In wild-type cells, uncoupling agents (such as carbonyl cyanide *m*-chlorophenylhydrazone) and different redox-cycling compounds (e.g., menadione and plumbagin) induced expression from the $marO_{II}$-*lacZ* fusion two- to sevenfold. In the *mar*-deleted strain, LacZ expression from the fusion was 10-fold higher than in wild-type cells. This activity was temperature sensitive (3-fold lower at 42 than at 30°C) and decreased 20-fold with the introduction of the gene for MarR. Structurally different compounds which induce the *mar* operon in wild-type cells reversed the MarR repression of $marO_{II}$-*lacZ* expression. To determine the size of MarR, it was fused to MalE as a MarR fusion protein of 144 amino acids [MarR(144)] or of 125 amino acids (deleted of 19 amino acids at the N terminus) [MarR(125)]. Only the MarR(144) fusion showed repressor ability. The purified MarR(144) fusion, but not the MarR(125) fusion, bound specifically to *marO* in vitro, as revealed by gel retardation, with an apparent dissociation constant of 5×10^{-9} M. MarR, therefore, controls expression of the *marRAB* operon presumably by binding to *marO*. MarR repression in cells can be reversed by different compounds, facilitating the response of bacteria to multiple environmental stress conditions.

The *mar* locus, at 34 min on the *Escherichia coli* chromosome, consists of two divergently expressed transcriptional units (*marCD* [*orf64*, *orf157*] and *marRAB*) from a central operator/promoter site, *marO* (5). The regulated *marRAB* operon controls intrinsic multiple antibiotic resistance and susceptibility in *E. coli* (5, 31, 33). Multiple antibiotic resistance (Mar) mutants, selected by low concentrations of a single drug, such as tetracycline or chloramphenicol, constitutively express the operon as a result of a mutation in *marO* (the putative upstream regulatory region) or *marR* (the gene for the repressor) (2, 5). Mar mutants show decreased susceptibility to the selective agent as well as to other structurally and functionally unrelated antibiotics and inhibitory agents (2, 5, 10–12). Increased contact of Mar mutants with the selective agents leads to mutants with very high resistance to many drugs (10).

Transcription of the normally repressed *marRAB* operon can be induced by structurally different compounds such as tetracycline, chloramphenicol, or salicylates (6, 14), leading to increased expression of a 1.0- and a 1.4-kbp specific mRNA (5, 6, 14). Insertion of transposon Tn*5* into the first codon of *marA* prevents transcription of *marA* and *marB*, whose expression is required for the Mar resistance phenotype (9, 31, 33). The Tn*5* insertion causes a complete reversal of the Mar phenotype, even in those Mar mutants originally having high levels of resistance (11). In strains deleted of the *mar* locus, both transcriptional units are needed in *trans* to produce the full Mar phenotype (31).

In this study, we used a $marO_{II}$-*lacZ* transcriptional fusion to determine what other external conditions affected the expression of the *mar* operon. To define the true start codon (of two possible codons) of MarR, we tested different *marR* mutants and purified, as fusions to MalE, a MarR of 144 amino acids [Mar(144)] and a MarR of 125 amino acids lacking 19 putative N-terminal amino acids [Mar(125)]. Only MarR(144) repressed $marO_{II}$-*lacZ* and showed specific binding to *marO* in vitro.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study as well as their relevant properties are listed in Table 1. The transducing phage P1*vir* (22) was used for strain constructions. Deletion of a 1.24-kbp *Bsp*HI fragment in the *mar* locus of SPC105 was constructed via homologous recombination. Plasmid pWY4, a derivative of the temperature-sensitive cloning vector pMAK705 (15) bearing a 9-kb fragment of the *mar* region of *E. coli* with a 1.24-kbp *Bsp*HI deletion of the *mar* locus (Δ*mar*1.24) (30, 31), was used. The resulting strain ASS121 was made *recA* as described previously (30). In ASS121, therefore, there is no active *marRAB* operon. MO100 was constructed by P1 transduction of a 39-kbp deletion including all of the *mar* locus (from 33.6 to 34.3 min; Δ*mar*39) from strain PLK1738 into SPC105, using chloramphenicol selection for a linked Tn*9* as described previously (14). The presence of the deletions in recipient strains was verified by Southern blot analysis using the *mar* operon-specific probes: the 1.24-kbp *Bsp*HI probe for ASS121 and the 9-kbp *Pst*I probe for MO100.

Media and chemicals. Bacterial cells were routinely grown in L broth containing (per liter) 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of glucose. Tetracycline hydrochloride, chloramphenicol, ampicillin, nalidixic acid, dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), menadione, phenazine methosulfate, paraquat (methyl viologen), plumbagin (5-hydroxyl-2-methyl-1,4-naphthoquinone), sodium benzoate, and acetaminophen were purchased

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant properties	Reference or source
E. coli strain		
SPC105	MC4100 (Δ*lacU169 araD rpsL relA thi flbB*) containing an inserted chromosomal $marO_{II}$-*lacZ* fusion on λ as well as the wild-type *mar* locus	6
MO100	SPC105 with 39-kbp deletion including the *mar* locus; *zdd-230*::Tn*9* (Cm^r)	This study
ASS121	SPC105 with 1.24-kbp *Bsp*HI *marRAB* operon-specific deletion	This study
TB1	F^- *ara* Δ(*lac-proAB*) *rpsL* (Str^r)[φ80dΔ(*lacZ*)*M15*]*hsdR*(r_K^- m_K^+)	New England Biolabs
PLK1738	*trpR trpA his-29 ilv pro-2 arg-427 thyA deo tsx gyrA rac zdd-230*::Tn*9* (Cm^r); deletion from 33.6 to 34.3min (Δ*mar* locus)	17
Plasmids		
pHSG415	Temperature-sensitive, low-copy-number vector, Ap^r Cm^r Km^r	16
pHHM183	pHSG415 carrying wild-type *mar* region on a 9-kbp chromosomal fragment	14
pHHM191	pHHM183 with a point mutation in *marR* (*marR2*)	5
pHHM193	pHHM183 with a point mutation in the start codon of *marR* (GTG) (*marR5*)	5
pMAK705	Temperature-sensitive cloning vector for homologous recombination studies	15
pMarR	pUC18 carrying wild-type *marO* and *marR* on a 850-bp *Dra*I fragment from pHHM183	5
pMarR2	pUC18 carrying wild-type *marO* and mutant *marR2* on a 850-bp *Dra*I fragment from pHHM191	This study
pMarR5	pUC18 carrying mutant wild-type *marO* and mutant *marR5* on a 850-bp *Dra*I fragment from pHHM193	This study
pMAL-C2	Expression vector for creating fusion proteins, containing *malE* gene of *E. coli* (minus its signal sequences) under control of *tac* promoter and $lacI^q$ repressor	New England Biolabs
pAS30	pMAL-C2 fused to a PCR product that encodes a MarR(144) (bp 1444 to 1878 in reference 5)	This study
pAS31	pMAL-C2 fused to a PCR product that encodes a MarR(125)	This study

from Sigma Chemical Co. (St. Louis, Mo.). Sodium salicylate came from Fisher Scientific (Fair Lawn, N.J.). Where needed, the pH was adjusted with NaOH.

DNA manipulations. Plasmid DNA isolations, transformations, restriction endonuclease digestions, DNA probe preparation, transfer, and hybridization were performed as previously described (14, 28). Chromosomal DNA was isolated as described by Beji et al. (3). PCR was performed by using *Taq* polymerase in a Perkin-Elmer Cetus DNA thermal cycler. DNA sequence was determined by the method of Sanger et al. (29), using a Sequenase sequencing kit (U.S. Biochemical, Cleveland, Ohio).

β-Galactosidase assays. The effects of different compounds on β-galactosidase expression from a transcriptional fusion of *lacZ* to the $marO_{II}$ promoter, which controls transcription of the *marRAB* operon ($marO_{II}$-*lacZ* fusion) (6), were tested in cells grown fresh at 30°C in LB medium (L medium with 10 g of NaCl and no glucose) to an A_{600} of 0.2. Following growth in each compound for 60 min, cells were assayed for β-galactosidase activity after permeabilization with sodium dodecyl sulfate (SDS)-chloroform as previously described (28, 30). To determine the effect of temperature, cells were grown for 60 min at 30, 37, or 42°C, centrifuged out of the growth medium, resuspended, and tested in assay buffer at pH 7.0, 30°C. Results (see Tables 2 to 5) are presented as the means of at least three separate experiments ± standard deviation.

Analysis of the MarR protein. PCR was used to synthesize the coding sequence for two different putative MarR proteins with different possible start sites (giving a protein of 144 amino acids or a protein of 125 amino acids) (5). Each PCR product contained *Eco*RI and *Hin*dIII linkers by which the *marR* gene was cloned into pMAL-C2 (New England Biolabs) and fused in frame with the C terminus of the *malE* gene of *E. coli*, resulting in the expression of a maltose-binding protein (MalE) fusion protein. The MalE signal sequence is not present on pMAL-C2. The two resulting pMAL-C2 derivatives, plasmids pAS30 (ORF144) and pAS31 (ORF125), were introduced into strain TB1 (New England BioLabs) to give TB1(pAS30) and TB1(pAS31). To produce the fusion proteins, the strains were grown at 37°C in 1 liter of LB medium containing ampicillin (100 μg/ml) to an A_{600} of ~0.5, and isopropyl thiogalactopyranoside (IPTG) was added to a final concentration of 0.3 mM. After incubation for 2 h, the cells were harvested by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in 15 ml of PBS, and sonicated in short pulses of 15 s. The disrupted cells were centrifuged at 10,000 × *g* for 20 min, and the supernatants (crude extract) were used to purify the fusion proteins by substrate affinity chromatography using immobilized amylose as instructed by the manufacturer. The protein concentration was estimated by UV absorbance at 280 nm, assuming that an A_{280} of 1 represented 1 mg of protein per ml. The fusion proteins were examined by SDS-polyacrylamide gel electrophoresis (PAGE) (6).

In vitro DNA binding assay. The 405-bp *Tha*I fragment in the *marO* region (5) of the *mar* operon had been cloned into the *Sma*I site of pMLB1109, creating pMLB1109-II, in which the fragment has flanking *Eco*RI and *Bam*HI sites (6). Following digestion with *Eco*RI and *Bam*HI, the 415-bp *Eco*RI-*Bam*HI DNA fragment containing the sequence for the *marO* region (5) was 3′ end labeled by filling in the recessed terminus by using Klenow DNA polymerase with [α-^{32}P]dATP. This DNA was purified by a Nick column (Pharmacia) and precipitated with isopropanol, and the amount was estimated by A_{260} and by comparison with the intensities of DNA from a standard DNA ladder after agarose gel separation and ethidium bromide staining. The [α-^{32}P]DNA (10^{-11} M) was incubated with different concentrations of purified MalE-MarR fusion protein (in Tris-EDTA buffer containing 1 mg of bovine serum albumin per ml) in 25 μl of 4 mM Tris-HCl–*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-NaOH (pH 7.9)–75 mM KCl–1 mM EDTA–5 mM dithiothreitol–15% (vol/vol) glycerol for 20 min at 37°C. Lower concentrations of glycerol (9%) or no glycerol did not change the binding results (13). The reaction mixtures were subjected to electrophoresis at 4°C and 35 mA in 5% native polyacrylamide gels (prepared and run in 20 mM Tris-HCl [pH 8]–3 mM sodium acetate [pH 7.9]–1 mM EDTA) which were prerun at 35 mA for at least 90 min. The gels were dried, and autoradiography was performed. No screens were used. The apparent dissociation constant (K_d) was obtained by plotting the fraction of radioactivity found retarded in specific protein-DNA complexes (data obtained through densitometry) versus the log of the concentration of MalE-MarR(144) fusion and calculating K_d from the concentration of protein at which half of the DNA was bound to MarR.

RESULTS

Effect of temperature on LacZ expression from the $marO_{II}$-*lacZ* fusion. The Mar phenotype is temperature sensitive (10). To determine if this sensitivity is at the level of operon expression, we examined the effect of temperature changes (at pH 7.0) on LacZ expression from a transcriptional fusion of *lacZ* to $marO_{II}$ (6). This fusion was present on a λ lysogen in the chromosome of wild-type and *mar*-deleted strains. On the basis of LacZ activity, 30% less transcription of the uninduced *mar* operon in wild-type SPC105 was observed at 42°C than at 30°C (Table 2). When the *mar* operon was induced in SPC105 by sodium salicylate (5 mM), the temperature effect was more

TABLE 2. Effects of temperature on LacZ activity in *E. coli* strains (with and without the *mar* operon) bearing a $marO_{II}$-*lacZ* fusion

Temp (°C)	Mean β-Galactosidase activity (Miller units) ± SD			
	SPC105	SPC105 + salicylate (5 mM)	ASS121 (Δ*mar*1.24)	MO100 (Δ*mar*39)
30	1,118 ± 72	10,178 ± 644	11,572 ± 208	12,200 ± 232
37	915 ± 59	5,123 ± 570	8,654 ± 387	9,933 ± 111
42	771 ± 26	2,950 ± 148	4,035 ± 95	4,267 ± 70

TABLE 3. Effects of different compounds (tested at optimal concentrations) on β-galactosidase of the $marO_{II}$-*lacZ* fusion

Compound	Concn (mM)	Relative β-galactosidase activity[a]			
		SPC105 (wild type)	MO100 (Δ*mar*39)	ASS121 (Δ*mar*1.24)	ASS121 (pMarR)
Uncouplers					
Carbonyl cyanide *m*-chlorophenylhydrazone	0.5	2.0	1.02	1.05	
Dinitrophenol	0.5	7.2	1.03	0.77	5.5
Chelators					
EDTA	1	1.1	1	1.02	
EGTA	1	1.3	0.96	1.04	
Redox-cycling agents					
Menadione	1.45	3.2	1.1	0.9	2.5
Phenazine methosulfate	0.15	2.2	0.95	1.0	
Paraquat	1.3	2.7	0.98	1.2	
Plumbagin	0.03	5.4	1.1	1.2	
Others					
Salicylate	5	8.8	1.1	1.1	4.5
Benzoate	15	7.2	0.94	1.1	2.5
Acetaminophen	10	3.5	0.97	1.2	2.0

[a] Ratio of activity with inducer to activity without inducer tested at 30°C and pH 7.

TABLE 4. Effect of wild-type and mutant MarR on LacZ expression from the $marO_{II}$-*lacZ* fusion in strains with or without the *marRAB* operon

Strain	Plasmid	Mean β-Galactosidase activity (Miller units) ± SD	
		LB	% of control[a] value
SPC105(wt[b])	None	1,116 ± 55	100
	pHHM183 (wt MarR)[c]	418 ± 23	37
	pHHM191 (mutant MarR2)[c]	2,785 ± 106	249
	pHHM193 (mutant MarR5)[c]	1,417 ± 93	126
	pMarR (wt MarR)[d]	114 ± 3	10
	pMarR2 (mutant MarR2)[d]	4,051 ± 124	362
	pMarR5 (mutant MarR5)[d]	2,915 ± 133	261
ASS121 (Δ*mar*1.24)	None	11,612 ± 361	100
	pHHM183	1,544 ± 97	13
	pHHM191	10,203 ± 352	88
	pHHM193	11,587 ± 390	100
	pMarR	556 ± 38	5
	pMarR2	9,519 ± 236	82
	pMarR5	12,029 ± 250	103

[a] SPC105 or ASS121 without any plasmid.
[b] wt, wild type.
[c] Low-copy-number plasmid; cloned 9-kb fragment containing the entire *mar* region.
[d] High-copy-number plasmid; cloned *marO* and *marR* only.

marked: 70% reduction at 42°C. Temperature sensitivity was similarly examined in strains ASS121 and MO100, which contain the $marO_{II}$-*lacZ* fusion but no *mar* locus (Table 2). The constitutive expression of β-galactosidase in ASS121 and MO100 was about 10 times greater than in the wild-type strain SPC105. The LacZ expression observed at 30°C in LB decreased by 65% at 42°C (Table 2). These results suggested that increased temperature reduced transcription dependent on *marO* or affected the stability of the RNA transcript. This effect appears greater in the absence of the wild-type *mar* operon presumably because the repression effect of MarR (4, 5, 10) is absent in these strains. No effect of temperature on LacZ activity in a Lac^+ strain was noted (data not shown), and so the effect was not due to changes in thermostability of LacZ.

Induction of the $marO_{II}$-*lacZ* fusion by multiple compounds. We next investigated the inducibility of $marO_{II}$-*lacZ* by different compounds (Table 3). Each compound was initially tested at different concentrations to determine the amount which produced optimal induction without inhibiting growth. In this group, plumbagin optimally induced at the lowest concentration (0.03 mM). The largest increase in expression was seen with salicylate, dinitrophenol, and benzoate at their optimal non-growth-inhibitory concentrations, as has been reported previously (6). The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone showed much less induction at the same concentration as the uncoupler dinitrophenol. Chelators were not inducers. The inducing effect of acetaminophen has been reported elsewhere (6). Other redox-cycling compounds, menadione, phenazine methosulfate, and paraquat, were also inducers of $marO_{II}$-*lacZ* expression (Table 3).

In MO100 and ASS121 (deleted of the *mar* operon), $marO_{II}$-*lacZ* was derepressed, with no detectable induction by these compounds (Table 3). This finding indicated that control of *marRAB* expression was located exclusively in the *mar* locus, presumably *marR* (2, 5).

MarR controls inducible expression of the *mar* operon by multiple compounds. To verify that MarR controlled inducible expression of *marRAB*, we introduced wild-type MarR, specified by pMarR, into ASS121. LacZ activity in ASS121 was repressed 20-fold (Table 4). The effects of different inducing compounds on this repression, in the absence of other genes in the *mar* locus, were examined. In the presence of the multicopy pMarR, the known inducers tested (at optimal noninhibitory concentrations) caused increased expression of the $marO_{II}$-*lacZ* fusion (Table 3). The fold induction was less than in the wild-type strain SPC105 presumably because of the multiple copies of MarR. The results showed that other genes in the *mar* locus (*marC* [*orf64*], *marD* [*orf157*], *marA*, and *marB*) were not required for the induction of $marO_{II}$-*lacZ* expression by multiple compounds.

Effects of two different *marR* mutants on β-galactosidase expression from the $marO_{II}$-*lacZ* fusion. For *marR*, there are two potential translation start sites, producing ORF125 or ORF144 (5). The actual start site was not known. The *marR* mutation in pHHM193 (designated *marR5*) lies in what would be the initiation codon of ORF144; therefore, the first codon of ORF144 would change from an initiator codon GTG (Met) to a noninitiator codon GTA (Val). If MarR corresponded to ORF144, this change should prevent initiation of *marR* translation at this site and produce no repressor. If MarR comprised ORF125, however, the mutation would be upstream in the adjacent *marO* and repressor activity should not be affected. The *marR* mutation on pHHM191 (designated *marR2*) would be internal for both ORF144 and ORF125 (5) and yield a mutant repressor whatever the start site. To assay for repressor activity, pHHM193 and pHHM191 as well as wild-type *marR* on pHHM183 were introduced individually into SPC105.

Wild-type *marR* on the low-copy-number plasmid control pHHM183 decreased expression of $marO_{II}$-*lacZ* in SPC105 by 63% (Table 4) as expected. Neither mutant plasmid caused decreased LacZ expression; rather, an increase in *mar* operon expression as measured by LacZ was observed (Table 4). The increase probably reflects partial titration of chromosome-specified MarR by *marO* sequences on the plasmids, as previously described (5). In ASS121, which has the 1.24-kb deletion in the *mar* locus, pHHM183 decreased expression by 87%.

TABLE 5. Effects of two putative sizes of MarR on LacZ expression in strains bearing a $marO_{II}$-*lacZ* fusion with or without the *marRAB* operon

Strain[a]	Mean β-Galactosidase activity (Miller units) ± SD
SPC105 (wild type)	1,063 ± 17
SPC105(pAS30)	140 ± 30
SPC105(pAS31)	1,175 ± 78
ASS121 (Δ*mar*1.24)	11,470 ± 128
ASS121(pAS30)	613 ± 54
ASS121(pAS31)	14,181 ± 440

[a] pAS30 bears *marR* of ORF144 and pAS31 bears *marR* of ORF125, each fused to MalE.

pHHM191 in ASS121 caused some (~12%) decrease in LacZ activity; pHHM193 caused no change in LacZ expression (Table 4). These results are consistent with the absence of repressor in pHHM193-containing cells and with partial repressor activity specified by pHHM191.

To determine further the correct size for MarR, we isolated a 0.8-kbp *Dra*I fragment from pHHM183, pHHM191, and pHHM193. This fragment, containing only the sequence for *marO* and *marR*, was cloned into the *Hin*dII site of the high-copy-number vector pUC18, producing plasmids pMarR (wild type), pMarR2, and pMarR5.

In multicopy on pMarR, the wild-type *marR* lowered expression of LacZ activity from the operon fusion by 90% in SPC105 and 95% in ASS121 (Table 4). The mutant *marOR* constructs on the high-copy-number plasmids led to an increase in LacZ activity in SPC105 (Table 4). In ASS121, however, only pMarR2, not pMarR5, caused a decrease (18%) in LacZ activity. The absence of repressor activity from pMarR5 in ASS121 further suggests that the increase in $marO_{II}$-*lacZ* expression caused by this plasmid (and its low-copy-number version pHHM193) in SPC105 comes from titration of chromosomal wild-type MarR by multiple wild-type *marO* on the plasmids (5). These results were consistent with the premise that the "*marR5*" mutation resided in the first codon of *marR*. The greater increase in LacZ expression in SPC105 by *marR2* (on pHHM191 or pMarR2) than by *marR5* suggests that in addition to *marO*, there was an interference by mutant MarR2 protein with wild-type MarR activity, perhaps due to mixed multimers (see below). The findings indicate that *marR* would initiate at bp 1444 (5) to give a protein of 144 amino acids.

Confirmation that MarR is a repressor protein containing 144 amino acids. We verified that the size of the Mar repressor is 144 amino acids by using an independent second method. PCR products corresponding to MarR(144) or MarR(125) were fused genetically to the carboxy terminus of MalE. The resulting clones, pAS30 [containing MalE fused to MarR(144)] and pAS31 [containing MalE fused to (MarR(125)] were transformed into SPC105 or ASS121. In ASS121, plasmid pAS30 repressed transcription from *marO* by 95% (Table 5). No repression was seen with pAS31. In SPC105, the results were similar (Table 5). This finding showed that pAS30, but not pAS31, expressed an active repressor. DNA sequence analysis of the PCR product used to construct pAS31 confirmed that the only difference between *marR* on pAS30 and that on pAS31 was the N-terminal sequence deleted in pAS31. No other mutations in *marR* on pAS31 were seen.

That cells bearing pAS30 or pAS31 indeed each made a fusion protein was verified by SDS-PAGE analysis of total cell protein. Expression of each fusion protein (20% of the total protein) was observed under IPTG induction (Fig. 1). After

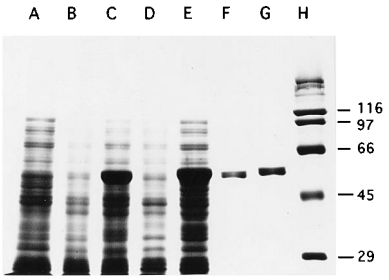

FIG. 1. SDS-PAGE (SDS-8% polyacrylamide gel) of proteins from *E. coli* TB1 without or with plasmids specifying IPTG-inducible MalE-MarR(125) and MalE-MarR(144) before and after purification by affinity chromatography. The gel was stained with Coomassie brilliant blue. Lanes A to E (total cells): A, TB1; B, TB1(pAS30); C, TB1(pAS30) induced by IPTG (0.3 mM) and expressing MalE-MarR(144); D, TB1(pAS31); E, TB1(pAS31) induced by IPTG and expressing MalE-MarR(125). Lane F, 6 μg of purified MalE-MarR(125); lane G, 7 μg of purified MalE-MarR144; lane H, molecular weight standards (positions are indicated in kilodaltons).

the two fusion proteins were purified by affinity chromatography (Fig. 1, lanes F and G), the estimated molecular masses from SDS-PAGE were 56,000 and 58,000 Da for products specified by pAS31 and pAS30, respectively. This result agrees well with calculated values of 56,500 and 58,500 Da.

Binding of purified MarR to *marO* in vitro. The ability of MarR to bind to *marO* DNA in vitro was examined by a gel retardation assay (19) using the two different fusion proteins, MalE-MarR(125) and MalE-MarR(144). The fusion proteins were purified from crude cytoplasmic extracts by elution from immobilized amylose (Fig. 1). The MalE-MarR(125) fusion did not show binding to DNA (10 pM) in this assay at the highest concentration used (40 nM) (Fig. 2). However, MalE-MarR(144) at this concentration and lower caused retardation of the DNA fragment (Fig. 2). Excess unlabeled *marO* DNA completed with the labeled *marO* fragment (Fig. 2), whereas similar and higher amounts of pUC18 DNA did not (data not shown) (13). These findings confirmed the specificity of the binding assay. At higher concentrations of protein, the retarded DNA was found in three locations (Fig. 2, a, b, and c). The apparent dissociation constant for MalE-MarR(144), around 5×10^{-9} M, was estimated from the concentration of protein needed to retard 50% of the radiolabeled DNA.

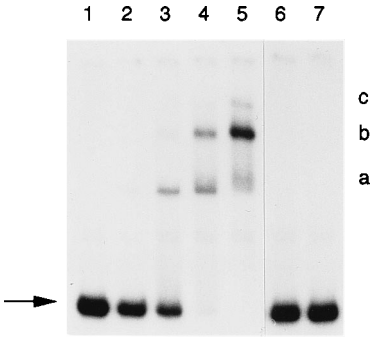

FIG. 2. Specific binding of MarR(144) protein to *marO* DNA. The 415-bp *Eco*RI-*Bam*HI fragment was used as a source of *marO* DNA. *marO* DNA (10 pM) was incubated without (lane 1) or with the following concentrations of purified fusion protein MalE-MarR(144): lane 2, 0.8 nM; lane 3, 4 nM; lane 4, 20 nM; lane 5, 40 nM; lane 6, 20 nM plus 800 pM unlabeled *marO* DNA; lane 7, 40 nM MalE-MarR(125). The arrow designates free *marO* fragment. a, b, and c designate different complexes of *marR*(144) with the *marO* fragment.

TABLE 6. Proteins showing identity or similarity to MarR[a]

Protein	Size (amino acids)	% Identity to MarR	% Similarity to MarR	Reference
MprA (EmrR)	176	27.97	41.95	7, 20a
17Kdaprs or 17Kdapap	166	26.95	49.64	21
PecS	166	26.95	48.93	25
SlyA	146	24.26	52.94	20
HpcR	148	18.93	50.00	26

[a] Initial protein homology searches were carried out by using BLAST (1). Amino acid sequence alignments were then performed by using the GAP program (University of Wisconsin Genetics Computer Group).

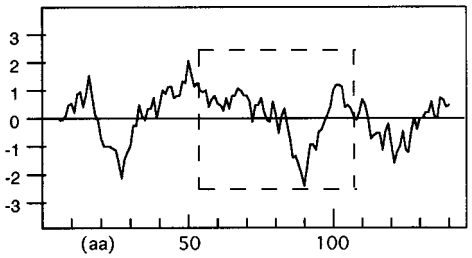

FIG. 3. Hydropathy plot for MarR(144). The Kyte-Doolittle option of DNA Strider, version 1.2, was used to generate a hydropathy analysis based on the *marR*(144) sequence. The hatched box designates the area of greatest similarity to other proteins in the database (see Table 6).

DISCUSSION

This study demonstrates that MarR, the first protein specified by the *marRAB* operon (5), is a 144-amino-acid repressor protein which inhibits transcription of the operon (Table 5) and responds to a variety of structurally different inducers (Table 3). Repression of $marO_{II}$-*lacZ* by cloned MarR in *mar* operon-deleted strains was reversed by multiple inducers used at the same concentration at which they were active in derepression of the chromosomal *mar* operon (Table 3). Therefore, MarR, in the absence of other components of the *mar* locus, can control inducible expression in wild-type cells caused by structurally unrelated compounds. This induction was abolished when MarR was not present.

MarR had been proposed to act on a putative operator/promoter region called *marO* upstream from the start site of *marR* (5). The present work shows that indeed MarR (as MalE-MarR) binds to *marO* (Fig. 2). Its apparent dissociation constant, as judged by a gel retardation assay, was around 5×10^{-9} M. This dissociation constant is comparable to that of other known repressors to their cognate operator regions, e.g., 5.7×10^{-10} M for *lac* repressor (8), 5×10^{-9} M for P22 Arc repressor, which is involved in the switch between lysis and lysogeny of *Salmonella* bacteriophage P22 (4), and 5×10^{-11} M for MerR, the regulatory protein of the mercury resistance operon (24).

The DNA sequence had shown two possible start sites for *marR* (5). We determined the correct size for MarR as 144 amino acids by protein fusion and repressor activities, using the two possible ORFs of 125 and 144 amino acids. The shorter MarR product of 125 amino acids did not show repressor activity and as a fusion to MalE did not bind *marO* in the gel retardation experiments, showing that the additional N-terminal 19 amino acid residues unique to ORF144 are required for the repressor to bind to *marO* DNA.

MarR does not have any known DNA binding motif (23). However, it shows homology to a group of similarly small proteins whose functions are diverse (Table 6). Of these proteins, three (MprA [EmrR], PecS, and HpcR) act as negative regulators and one (SlyA) is a cytolysin. Two others, 17Kdaprs and 17Kdapap, found downstream of the *prs* and *pap* pili operons, respectively, have 85% nucleotide sequence identity with each other and have unknown functions. Of particular interest, all of the proteins show strongest homologies and similarities in hydropathy plots to a particular hydrophilic 50-amino-acid domain within MarR (amino acids 56 to 107) (Fig. 3), suggesting that this region serves similar purposes in all of these proteins.

The gel retardation assay also showed that as MarR concentrations increased, complexes of three different mobilities were observed (Fig. 2). These results are consistent either with up to three binding sites occupied by MarR on a single *marO* fragment or with concentration-dependent oligomeric structure for MarR bound to *marO*. Other repressors, e.g., Arc repressor of bacteriophage P22 (4) and *tet* repressor of the tetracycline resistance operon (18), have been shown to function as oligomers. The greater interference with wild-type repressor in SPC105 cells by mutant pMarR2 than by pMarR5 (Table 4) (both contain *marO*) suggests that MarR2 forms heterologous, less functional multimers with wild-type MarR, again suggesting an oligomeric structure for MarR with *marO*.

Besides affecting antibiotic resistance, the *mar* locus affects cellular responses to oxidative stress and weak acids (2, 6, 12). In this regard, recent data show that the gene called *inaR* (32), which regulates a weak acid-inducible *inaA* locus (at 48.6), is an allele of the *marR* gene (27).

MarR-independent transcription of *marRAB* (as assayed by using $marO_{II}$-*lacZ*) is greater at 30°C than at 37°C. This finding suggests that the *mar* locus may be more adapted to responding to environmental signals likely to occur at lower temperatures rather than to responding to those in the human intestinal tract at 37°C, even though it was antibiotic resistance at 37°C, which led to the original discovery of the locus (11). In view of the data presented here, the designation *mar* could stand for "multiple adaptational response" as well as "multiple antibiotic resistance."

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ariza, R. R., S. P. Cohen, N. Bachhawat, S. B. Levy, and B. Demple.** 1994. Repressor mutations in the *marRAB* operon that activate oxidative stress genes and multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **176:**143–148.
3. **Beji, A., D. Izard, F. Gavini, H. Leclerk, M. Lescini-Detstancche, and J. Krembel.** 1987. A rapid chemical procedure for isolation and purification of chromosomal DNA from gram-negative bacilli. Anal. Biochem. **162:**18–23.
4. **Brown, B. M., J. U. Bowie, and R. T. Sauer.** 1990. Arc repressor is tetrameric when bound to operator DNA. Biochemistry **19:**11189–11195.
5. **Cohen, S. P., H. Hächler, and S. B. Levy.** 1993. Genetic and functional analysis of the multiple antibiotic resistance (*mar*) locus in *Escherichia coli*. J. Bacteriol. **175:**1484–1492.
6. **Cohen, S. P., S. B. Levy, J. Foulds, and J. L. Rosner.** 1993. Salicylate induction of antibiotic resistance in *Escherichia coli*: activation of the *mar* operon and a *mar*-independent pathway. J. Bacteriol. **175:**7856–7862.
7. **del Castillo, I., J. E. Gonzalez-Pastor, J. L. San Millan, and F. Moreno.** 1991.

Nucleotide sequence of the *Escherichia coli* regulatory gene *mprA* and construction and characterization of *mprA*-deficient mutants. J. Bacteriol. **173:** 3924–3929.

8. **Fried, M. G., and D. M. Crothers.** 1981. Equilibria and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6524.
9. **Gambino, L. S. J. Gracheck, and P. F. Miller.** 1993. Overexpression of the MarA positive regulator is sufficient to confer multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **175:**2888–2894.
10. **George, A. M., and S. B. Levy.** 1983. Amplifiable resistance to tetracycline, chloramphenicol, and other antibiotics in *Escherichia coli*: involvement of a non-plasmid-determined efflux of tetracycline. J. Bacteriol. **155:**531–540.
11. **George, A. M., and S. B. Levy.** 1983. Gene in the major cotransduction gap of *Escherichia coli* K-12 linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J. Bacteriol. **155:**541–548.
12. **Greenberg, J. T., J. H. Chou, P. Monach, and B. Demple.** 1991. Activation of oxidative stress genes by mutations at the *soxQ*/*cfxB*/*marA* locus of *Escherichia coli*. J. Bacteriol. **173:**4433–4439.
13. **Gustafson, J., and S. B. Levy.** Unpublished data.
14. **Hächler, H., S. P. Cohen, and S. B. Levy.** 1991. *marA*, a regulated locus which controls expression of chromosomal multiple antibiotic resistance in *Escherichia coli*. J. Bacteriol. **173:**5532–5538.
15. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. **171:**4617–4622.
16. **Hashimoto-Gotoh, T., F. C. H. Franklin, A. Nordheim, and K. N. Timmis.** 1981. Specific purpose plasmid cloning vectors. I. Low copy number, temperature-sensitive, mobilization-deficient pSC101-derived containment vectors. Gene **16:**227–235.
17. **Hill, T. M., J. M. Henson, and P. L. Kumpel** 1987. The terminus region of the *Escherichia coli* chromosome contains two separate loci that exhibit polar inhibition of replication. Proc. Natl. Acad. Sci. USA **84:**1754–1758.
18. **Hillen, W., G. Klock, I. Kaffenberger, L. V. Wray, and W. S. Reznikoff.** 1982. Purification of the *tet* repressor and *tet* operator from the transposon Tn *10* and characterization of their interaction. J. Biol. Chem. **257:**6605–6613.
19. **Lane, D., P. Prentki, and M. Chandler.** 1992. Use of gel retardation to analyze protein-nucleic acid interactions. Microbiol. Rev. **56:**509–528.
20. **Libbey, S. J., W. Goebel, A. Ludwig, N. Buchmeier, F. Bowe, F. C. Fang, D. G. Guiney, J. G. Songer, and F. Heffron.** 1994. A cytolysin encoded by *Salmonella* is required for survival within macrophages. Proc. Natl. Acad. Sci. USA **91:**489–493.

20a. **Lomovskaya, O., K. Lewis, and A. Matin.** 1995. EmrR is a negative regulator of the *Escherichia coli* multidrug resistance pump EmrAB. J. Bacteriol. **177:**2328–2334.

21. **Marklund B. I., J. M. Tennent, E. Garcia, A. Hamers, M. Boga, F. Linberg, W. Gaastra, and S. Normark.** 1992. Horizontal gene transfer of the *Escherichia coli pap* and *prs* pili operons as a mechanism for the development of tissue-specific adhesive properties. Mol. Microbiol. **6:**2225–2242.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Pabo, C. O., and R. T. Sauer.** 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. **61:**1053–1095.
24. **Parkhill, J., A. Z. Ansari, J. G. Wright, N. L. Brown, and V. O. O'Halloran.** 1993. Construction and characterization of a mercury-independent MerR activator ($MerR^{AC}$): transcriptional activation in the absence of Hg(II) is accompanied by DNA distortion. EMBO J. **12:**413–421.
25. **Reverchon, S., W. Nasser, and J. Robert-Baudouy.** 1994. *pecS*: a locus controlling pectinase, cellulase and blue pigment production in *Erwinia chrysanthemi*. Mol. Microbiol. **11:**1127–1139.
26. **Roper, D. I., T. Fawcett, and R. A. Cooper.** 1993. The *Escherichia coli* C homoprotocatechuate degradation operon: *hpc* gene order, direction of transcription and control of expression. Mol. Gen. Genet. **237:**241–250.
27. **Rosner, J. L., and J. L. Slonczewski.** 1994. Dual regulation of *inaA* by the multiple antibiotic resistance (Mar) and superoxide (SoxRS) stress response systems of *Escherichia coli*. J. Bacteriol. **176:**6262–6269.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Seoane, A. S., and S. B. Levy.** 1995. Identification of new genes regulated by the *marRAB* operon in *Escherichia coli*. J. Bacteriol. **177:**530–535.
31. **White, D. W., W. Yan, and S. B. Levy.** Functional characterization of the chromosomal multiple antibiotic resistance (Mar) locus in *Escherichia coli*, abstr. A-104, p. 20. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
32. **White, S., F. E. Tuttle, D. Blankenhorn, D. C. Dosch, and J. L. Slonczewski.** 1992. pH dependence and gene structure of *inaA* in *Escherichia coli*. J. Bacteriol. **174:**1537–1543.
33. **Yan, W., S. P. Cohen, and S. B. Levy.** 1992. Three putative proteins in the *mar* operon mediate intrinsic multidrug resistance in *Escherichia coli*, abstr. 25, p. 5. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.