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Identification of New Genes Regulated by the *marRAB* Operon in *Escherichia coli*

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Random Tn*phoA* and Tn*lacZ* translational fusions were introduced into an *Escherichia coli* strain with a deletion of the multiple antibiotic resistance (*mar*) locus, complemented in *trans* by a temperature-sensitive plasmid bearing the *mar* locus with a constitutively expressed *mar* operon. Five gene fusions (two with *lacZ* and three with *phoA*) regulated by the *mar* operon were identified by increased or decreased marker enzyme activity following loss of the complementary plasmid at the restrictive temperature. Expression of LacZ from both *lacZ* fusions increased in the presence of the *mar* operon; expression from the three *phoA* fusions was repressed by the *mar* operon. The *lacZ* fusions were mapped at 31.5 and 14 min on the *Escherichia coli* chromosome. One of the *phoA* fusions was located at 51.6 min while the two others mapped at 77 min. Cloning and sequencing of a portion of the fused genes showed all of them to be different. The *phoA* fusions at 77 min were located in a recently identified gene, *slp*, a lipoprotein of unknown function (D. M. Alexander and A. C. St. John, Mol. Microb. 11:1059–1071, 1994). The others showed no homology with any known genes of *E. coli*. The insertions caused small but reproducible changes in the antibiotic susceptibility profile. This approach has enabled the identification of new genes in *E. coli* which are regulated by the *marRAB* operon and involved in the Mar phenotype.

Chromosomally mediated multiple antibiotic resistance (Mar) mutants of *Escherichia coli* constitutively express the regulated *marRAB* operon, which is located at 34 min on the *E. coli* chromosomal map (5, 13). Expression of the operon, consisting of *marR*, *marA*, and *marB*, is normally repressed by MarR but can be induced by diverse compounds such as tetracycline, chloramphenicol, menadione, and salicylates (6, 16, 28). Mar mutants show increased resistance to multiple antibiotics and structurally unrelated compounds (2, 12–14). Selected by low concentrations of a single drug, Mar mutants can attain very high levels of resistance if maintained in contact with the selective agent (12). Insertion of Tn*5* into *marA*, the gene for a protein which causes increased antibiotic resistance (11, 16, 33), completely reversed the resistance phenotype (13).

The *marRAB* operon affects distant chromosomal genes, as revealed by changes seen in proteins separated in two-dimensional gels (14). One effect is to increase expression of *micF* (8) and thereby indirectly decrease expression of *ompF* (8). However, the reduced level of OmpF in Mar mutants is only a small component of antibiotic resistance (6, 7), indicating that other genes are involved in the Mar phenotype. To identify the other genes regulated by the *marRAB* operon and involved in the Mar phenotype, we used a marker fusion approach, with Tn*phoA* (22) and Tn*lacZ* (32). We isolated fusions in which β-galactosidase or alkaline phosphatase expression changed when the *mar* operon was removed. As a result of these studies, we have identified four *mar* locus-regulated (*mlr*) genes, three of which have not previously been described.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Bacterial strains and plasmids used in this study are listed in Table 1. The 1.24-kbp *Bsp*HI deletion in the *mar* locus in strain ASS110 was made by homologous recombination following methods described previously (17), with pWY4, a derivative of the temperature-sensitive plasmid pMAK705, bearing the deletion. The presence of the deletion in the recipient strain was verified by Southern blot analysis using the deleted fragment as a specific probe. The strain was made *recA1* by P1 transduction of the mutation in association with *srl*::Tn*10* from LM303 as previously described (23) and selected on plates containing tetracycline. The presence of the *recA1* mutation in the recipient strain was verified by its sensitivity to killing by irradiation with 300 ergs of 254-nm UV light per cm^2. Cells in which Tn*10* had been spontaneously excised (making them tetracycline susceptible) were identified after enrichment for Tc^s derivatives among cells growing logarithmically in 7.5 μg of tetracycline per ml after the addition of 20 μg of ampicillin per ml to kill the Tc^r growing cells. The *recA1* tetracycline-sensitive strain was called ASS111.

P1*vir* came from the laboratory collection. λTn*5phoA* and λTn*5lacZ* (pr) were obtained from B. Wanner (Purdue University, West Lafayette, Ind.). These phages are defective in both replication and lysogenization (λ c1857 b221 Pam3 *rex*:Tn*phoA*) (32). The λTn*5lacZ* (pr) used was λTn5A′ −4 (32), which contains 254 bp in common with λTn*5phoA* in the left end and has the kanamycin resistance (Kan^r) gene from Tn*5*. Strain AW1045 came from A. Wright of our department. In the text, the insertions will be referred to as Tn*phoA* and Tn*lacZ*.

Plasmid pAS10 was constructed by cloning a 2.5-kbp PCR-generated fragment from the mutant (*marR5*) *mar* locus on pHHM193 into the temperature-sensitive plasmid pMAK705. The resulting plasmid constitutively expressed the *marRAB* operon as well as transcriptional unit 1, consisting of *orf64* and *orf157* (see reference 5).

Media and chemicals. Cells were grown in LB broth (10 g of tryptone per liter, 5 g of yeast extract per liter, 5 g of NaCl per liter). Selective media were prepared by using the following antimicrobial agents at the indicated concentrations: chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml; and tetracycline, 12 μg/ml. The blue dyes 5-bromo-4-chloro-3-indolyl phosphate (X-P) and 5-bromo-4-chloro-3-indole-β-D-galactopyranoside (X-Gal), for detecting alkaline phosphatase and β-galactosidase activities, respectively, were obtained from Sigma Chemical Co. (St. Louis, Mo.) and used at a concentration of 40 μg/ml.

Antibiotic susceptibility assays. Antibiotic susceptibility was measured by a gradient plate method (9) on LB agar with cultures grown to logarithmic growth phase in LB broth. The MIC was estimated by the limit of confluent growth after incubation of plates for 36 to 48 h at 30°C.

Genetic techniques. Chromosomal and plasmid DNA isolation, DNA transformation, DNA transfer and hybridization, analysis with restriction endonucleases, T4 DNA ligase, and DNA labeling were performed as described previously (4, 16, 26).

DNA sequencing was performed (27) with a Sequenase kit (U.S. Biochemical

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Reference or source
E. coli		
AW1045	Derivative of MC4100, F$^-$ *araD139* Δ*lacU169 rpsL relA thi* Δ*phoA E15* Δ(*ara-leu*)*7679*	Andrew Wright
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ* Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	18
LM303	*met pro srl*::Tn*10 recA1*	Laboratory collection
ASS110	AW1045 with 1.24-kbp *Bsp*HI *mar* locus-specific deletion (Δ(*orf64/157 marORAB*)	This study
ASS111	ASS110 *recA1*	This study
ASS112	AS111 bearing a *mar*-regulated *lacZ* gene fusion in *mlr1*	This study
ASS113	AS111 bearing a *mar*-regulated *lacZ* gene fusion in *mlr2*	This study
ASS114	AS111 bearing a *mar*-regulated *phoA* fusion in *mlr3*	This study
ASS115	AS111 bearing a *mar*-regulated *phoA* fusion in *slp*	This study
ASS116	AS111 bearing a *mar*-regulated *phoA* fusion in *slp*	This study
Plasmids		
pUC18	Multicopy vector, Ap^r	34
pMAK705	Temperature-sensitive cloning vector, Cm1^r	17
pWY4	pMAK705 bearing a 9-kb *Pst*I fragment from the chromosomal *mar* region in which a 1.24-kbp *Bsp*HI *mar* locus deletion was made	33
pHSG415	Temperature-sensitive low-copy-number vector, Ap^r Cm^r Km^r	16
pHHM193	pHSG415 carrying the *mar* region on a 9-kbp chromosomal fragment with a point mutation in start codon of MarR (GTG)(*marR5*)	5
pAS1	pUC18::(prc::Tn*5*)	29
pAS10	pMAK705 bearing *orf64 orf157 marO marRAB* on a 2.5-kbp PCR product from pHHM193	This study
pAS42	pUC18 carrying a 10-kb *Sal*I fragment and a 6-kbp *Sal*I fragment from ASS112, Km^r	This study
pAS43	pUC18 carrying an 8-kbp *Sal*I fragment from ASS113, Km^r	This study
pAS44	pUC18 carrying a 5.2-kbp *Bam*HI fragment from ASS114, Km^r	This study
pAS45	pUC18 carrying a 9-kbp *Sal*I fragment from ASS115, Km^r	This study
pAS46	pUC18 carrying a 9-kbp *Sal*I fragment from ASS116, Km^r	This study

Corp.). The oligonucleotide 5′-CCAGAACAGGGCAAAAC-3′ was used to sequence the upstream chromosomal DNA junctions of *phoA* and *lacZ* insertions cloned into plasmids.

For hybridization to the gene mapping membranes (Takara Biochemical Inc., Berkeley, Calif.), 100 μl of chloroform-isoamyl alcohol (24:1) and 100 μl of diethylpyrocarbonate (to inactivate possible nucleases) were added to each 100 μl of solution containing the ^{32}P-labeled DNA probes. After hybridization, membranes were washed twice in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) for 5 min at room temperature, followed by two 30-min washes in 2× SSC plus 1% sodium dodecyl sulfate (SDS) at 65°C and two 15-min washes in 0.1× SSC at room temperature. The membranes were exposed to X-ray film overnight. Membranes were not allowed to dry during exposure in order to permit removal of the radiolabeled probes by boiling in 0.5% SDS for 5 to 10 min.

Isolation of Tn*phoA* and Tn*lacZ* insertions. To isolate mutants with chromosomal Tn*phoA* or Tn*lacZ* insertions, we infected 5×10^8 ASS111 cells bearing plasmid pAS10, grown to logarithmic phase at 30°C in the presence of chloramphenicol, with 5 μl of phage lysate:λTn*5phoA* at a titer of 2×10^{10} PFU/ml (multiplicity of infection, around 0.2) or 5×10^{10} PFU of λTn*5lacZ* (pr). Cells infected by the phages were plated at 30°C on kanamycin to identify chromosomal insertions. The transfection frequency was 0.5×10^{-4} for λTn*5phoA* and 0.8×10^{-4} for λTn*5lacZ* (pr). Of the Kan^r colonies from the mutagenesis, 2% with λTn*5phoA* were blue on X-P agar and 11% from the mutagenesis with λTn*5lacZ* (pr) were blue on X-Gal agar.

β-Galactosidase and alkaline phosphatase assays. Whole cells were grown to logarithmic phase at 30°C in LB medium. β-Galactosidase activity was assayed by using ONPG (*o*-nitrophenyl-β-D-galactopyranoside) as the substrate after permeabilization with SDS-chloroform and incubation for 60 min, as previously described (13). The alkaline phosphatase activity of cultures was measured in SDS-chloroform-treated cells by using *p*-nitrophenol phosphate as the substrate (15). In both assays, one unit represents 0.222 μmol of substrate converted per min per A_{600} unit of cells.

For *phoA* fusions, the levels of activity in the membrane and supernatant fractions were also determined. Pellets from cells grown to logarithmic phase were resuspended in 50 mM Tris-HCl (pH 8)–10 mM EDTA (pH 8)–100 μg of lysozyme per ml and disrupted by gentle sonication on ice. After ultracentrifugation at 40,000 × *g* for 1 h, membrane pellets were resuspended by sonication in 0.2 M Tris (pH 8)–2% Triton X-100. The alkaline phosphatase activities of the supernatant and membrane fractions were assayed without SDS-chloroform.

Cloning of *phoA* and *lacZ* fusions. Total chromosomal DNA of the ASS112, ASS113, ASS115, and ASS116 strains was digested with *Sal*I, ligated with similarly digested pUC18, and transformed into strain DH5α with selection for simultaneous ampicillin and kanamycin resistance, forming the plasmids pAS42, pAS43, pAS45, and pAS46, respectively (Fig. 1). The chromosomal DNA from ASS114 was digested with *Bam*HI and ligated with *Bam*HI-digested pUC18, by using the same selection procedure described above; plasmid pAS44 was obtained (Fig. 1). Chromosomal DNA and the DNA of the corresponding plasmids digested with the same enzymes were separated by electrophoresis in agarose, blotted to a nylon membrane, and probed with a ^{32}P-labeled 0.92-kbp *Pst*I fragment of Tn*5* from plasmid pAS1 (29). The same pattern of hybridization was seen in each of the original insertion mutants and its derivative plasmid (data not shown), confirming that the correct fragments had been cloned.

Nucleotide sequence accession numbers. Three partial gene sequences of *mlr1*, *mlr2*, and *mlr3* identified in ASS112 (*mlr1*::Tn*lacZ*), ASS113 (*mrl2*::Tn*lacZ*), and ASS114 (*mlr3*::Tn*phoA*) were assigned GenBank accession no. ECU09712, ECU09713, and ECU09714, respectively.

RESULTS

Identification of Tn*phoA* and Tn*lacZ* insertions into genes controlled by the *mar* locus. A total of 2,100 fusions (820 *phoA* fusions and 1,280 *lacZ* fusions) in ASS111 were randomly selected at 30°C and screened by replica plating at 42°C for a change of color accompanying the loss of temperature-sensitive pAS10 containing the *mar* locus. Five fusions were identified and studied further. Two of these fusions, ASS112 and ASS113, containing a *lacZ* fusion, showed a loss of color upon loss of the *mar* locus on plasmid pAS10 at 42°C. The other three mutants, ASS114, ASS115, and ASS116, containing a *phoA* fusion, showed an increase in color upon loss of the *mar*-containing plasmid. The *mar* dependence of each strain was verified by a return to the original color phenotype upon reintroduction of pAS10 into the cured strain (Tables 2 and 3). In the negative control, introduction of the vector alone was done.

Mapping of fusions regulated by the *mar* operon on chromosome of *E. coli*. We used endonuclease digestion with *Sal*I or *Bam*HI, which cleaves within Tn*5*, leaving an intact Kan^r gene (3) to clone the junctional chromosomal fragments into pUC18. A nylon membrane containing the ordered miniset

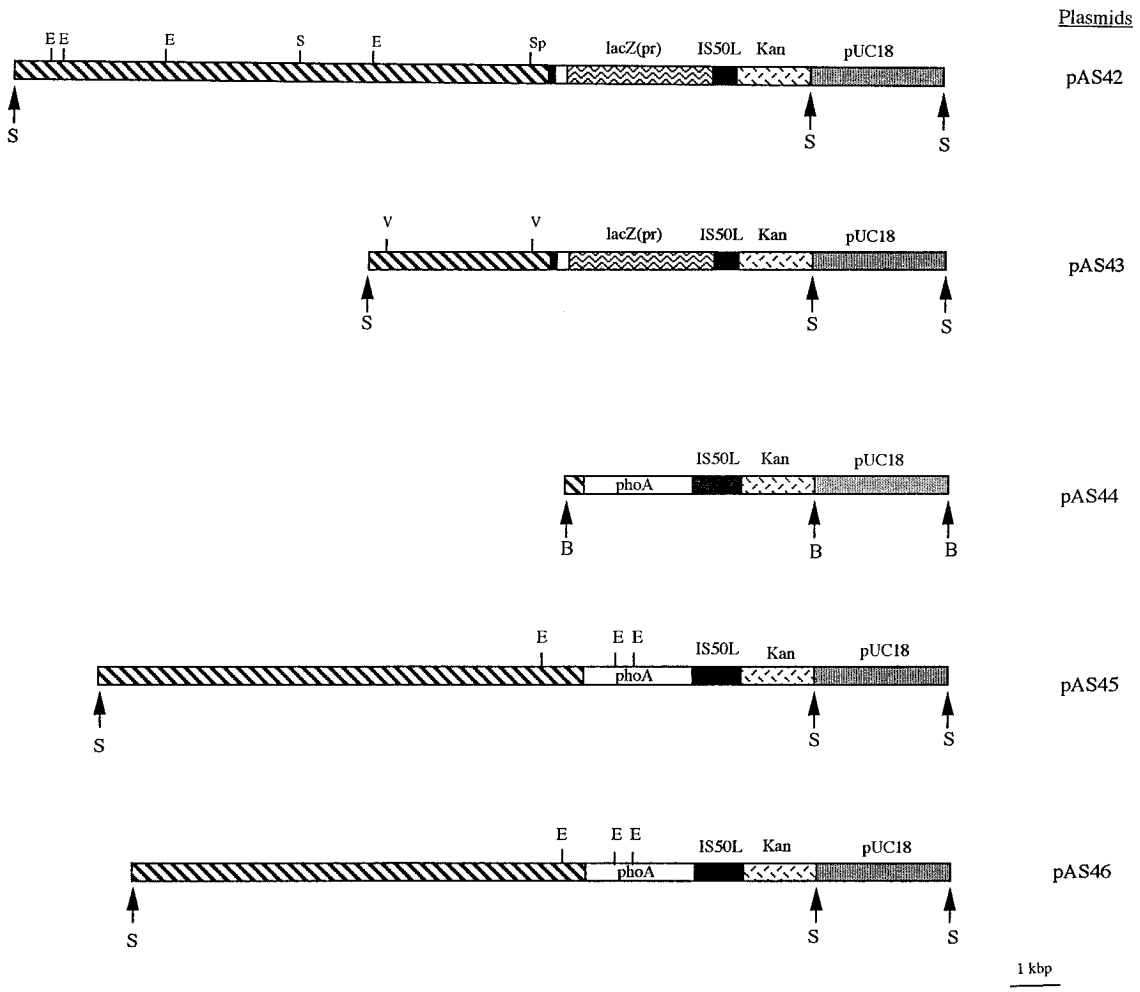

FIG. 1. Restriction enzyme map of plasmids carrying *mar*-regulated insertions. pAS42 and pAS43 contain a *lacZ* gene fusion. The hatched bar indicates chromosomal DNA; the open bar upstream of the *lacZ* gene corresponds to 254 bp of the *phoA* gene. pAS44, pAS45, and pAS46 contain *phoA* fusions. E, *Eco*RI; B, *Bam*HI; S, *Sal*I; Sp, *Sph*I; V, *Eco*RV.

collection of clones of a Kohara collection of phages was hybridized with the radiolabeled chromosomal DNA Tn*5* junctional fragments cloned from the five fusions (Fig. 1). The 3.2-kbp *Eco*RI-*Sph*I chromosomal fragment from the plasmid pAS42 derived from the *lacZ* fusion contained in strain ASS112 hybridized with the overlapping phages 273 and 274, which contained sequences from 31.5 min of the *E. coli* chromosomal map (20). The 3.6-kbp *Eco*RV chromosomal fragment in pAS43, cloned from the *lacZ* fusion in strain ASS113, was mapped to the overlapping phages 166 and 167 containing sequences from 14 min of the *E. coli* map.

TABLE 2. β-Galactosidase activity of Tn*lacZ* gene fusions in the presence or absence of constitutively expressed *mar* operon

Strain	Insertion site	Plasmid[a]	β-Galactosidase activity (U)[b]
ASS112	*mlr1*	None	0.2 ± 0.03
		pAS10	18.8 ± 3.5
		pMAK705	0.3 ± 0.05
ASS113	*mlr2*	None	2.6 ± 0.4
		pAS10	102.5 ± 18
		pMAK705	1.4 ± 0.2

[a] pMAK705, vector alone; pAS10, vector with *mar* locus.
[b] Values are the means ± standard deviations for three independent determinations.

The *phoA* fusion contained in strain ASS114 was mapped at 51.6 min by using the 5.2-kbp *Bam*HI fragment from plasmid pAS44, which hybridized with phages 413 and 414. The fragment used for the hybridization also contained the *phoA* gene and, therefore, also hybridized with phages 142 and 143 corresponding to 1A10 and 6A12 from the Kohara collection.

Finally, the *phoA* fusions present in ASS115 and ASS116 were mapped by using the 9-kbp *Eco*RI-*Sal*I fragment from the plasmids pAS45 and pAS46, respectively (Fig. 1). This band is the same in both plasmids and hybridized in both cases with the overlapping phages 607, 608, and 609, indicating that both fusions are at 77 min of the *E. coli* map. The genes identified were given the designation *mlr1* to *mlr5* (mar locus regulated).

Sequencing and gene identification of cloned junctional chromosomal fragments. About 200 bp of Tn*phoA* or Tn*lacZ* junctional chromosomal fragments from each plasmid were sequenced. The sequences of all five were different. The DNA and all possible putative protein sequences obtained were compared with other DNA and protein sequences in the GenBank

TABLE 3. Alkaline phosphatase activity of Tn*phoA* fusions in the presence or absence of constitutively expressed *mar* operon

Strain	Insertion site	Plasmid[a]	Alkaline phosphatase activity (U)[b]
ASS114	*mlr3*	None	30.2 ± 4.5
		pAS10	7.2 ± 0.5
		pMAK705	26.5 ± 2.5
ASS115	*slp*	None	37.2 ± 6.3
		pAS10	0.3 ± 0.04
		pMAK705	39.5 ± 8.5
ASS116	*slp*	None	29.3 ± 4
		pAS10	0.3 ± 0.04
		pMAK705	26.6 ± 3.5

[a] pMAK705, vector alone; pAS10, vector with *mar* locus.
[b] Values are the means ± standard deviations based on three independent determinations.

TABLE 4. Changes in MICs for Tn*5* insertion mutants

Strain	Fold difference in MIC of[a]:				
	AMP	TET	NAL	NOR	CML
ASS112					
Alone	+1.3	+2.1	+1.9		
+ pAS10		−0.7	+1.4	−0.5	
ASS113					
Alone		−0.8			
+ pAS10		−0.7			
ASS114					
Alone	−0.8	−0.8			
+ pAS10	+1.4	+1.2	+1.3		
ASS115					
Alone	−0.7	−0.8	−0.7	−0.8	
+ pAS10		−0.8	−0.8		
ASS116					
Alone	−0.8	−0.8	−0.7	−0.8	+1.2
+ pAS10		−0.8	−0.7	−0.7	

[a] Fold difference in the MICs for the strains indicated as compared to ASS111 in the presence and absence of pAS10; only differences of ≥20% are noted. Results are the means for determinations for three gradient plates. The calculated MICs (in micrograms per milliliter) of the following antibiotics for ASS111 and ASS111(pAS10), respectively, were 1.8 and 8.1, ampicillin (AMP); 0.6 and 4.1, tetracycline (TET); 0.005 and 0.08, norfloxacin (NOR); 0.4 and 4.1, nalidixic acid (NAL); and 1.0 and not done, chloramphenicol (CML) (chloramphenicol could not be tested in pAS10-containing strains because of Cml[r] on pAS10). +, increase in MIC; −, decrease in MIC.

by using the FASTA method in the Genetics Computer Group package of sequence analysis programs (24). Homology was noted between the fragments on pAS45 and pAS46 and the newly described *slp* gene for a lipoprotein in *E. coli* (1). No homology was found for the other cloned fragments with genes in the data bank. Three partial gene sequences (identified in ASS112 [*mlr1*::Tn*lacZ*], ASS113 [*mlr2*::Tn*lacZ*], and ASS114 [*mlr3*::Tn*phoA*]) were assigned the accession numbers given above.

Expression of *mar*-regulated gene fusions. The strains containing the *lacZ* and *phoA* fusions were examined for β-galactosidase and alkaline phosphatase expression in the presence or absence of the pAS10 plasmid. The β-galactosidase activity in strain ASS112 was increased 94-fold by the pAS10 plasmid (Table 2), but no change was observed with the plasmid vector alone (pMAK705). Enzyme activity in ASS113 was increased 40-fold by the plasmid containing the *mar* operon. These results suggest that the *mar* locus is needed for expression of the genes fused with *lacZ* in these strains.

In contrast, a repressive effect of the *mar* locus was observed with the *phoA* fusions present in the ASS114, ASS115, and ASS116 strains (Table 3). A large effect was observed in ASS115 and ASS116, with 126- and 98-fold-less alkaline phosphatase activity, respectively, in the presence of plasmid pAS10 than that in the strain alone or in the presence of vector plasmid pMK705. A much smaller effect (a 4.3-fold decrease) was noted with the ASS114(pAS10) strain.

A change in expression of LacZ and PhoA was also observed on X-P and X-Gal plates when only MarA, the putative activator protein encoded by the *marA* gene of the *mar* operon (11, 31, 33), was introduced on a high-copy-number plasmid into the five different strains. No change was observed when the repressor of the *mar* operon, MarR, was introduced on a high-copy-number plasmid (data not shown). The latter findings indicate that the genes can be regulated by MarA alone, although an additive role for other genes in the *mar* locus cannot be ruled out.

The physical location of the *mar*-responsive *phoA* fusion proteins was determined by cell fractionation. In ASS115, approximately 80% of the alkaline phosphatase activity was membrane associated. In ASS114 and ASS116, most of the activity was released into the supernatant. Since PhoA must be periplasmic to be active, the fusions are presumably in a membrane or periplasmic protein. For ASS115 and AS116, the gene product, Slp, is known to be periplasmic and probably is also located in the outer membrane (1).

Antibiotic susceptibility phenotype. Since the transposon insertions were responsive to the *mar* operon, we examined the effect of each insertion on antibiotic susceptibility. All fusions showed an altered antibiotic susceptibility profile, whether in the absence or presence of the *mar* operon on pAS10 (Table 4). In the absence of the plasmid, effects independent of the *mar* locus could be seen. Although most differences were small (10 to 40%), some were as high as twofold. Compared with the parental ASS111 strain, ASS112 consistently showed decreased susceptibility of 2-fold to tetracycline and nalidixic acid and of about 1.3-fold to ampicillin, with little, if any, change in susceptibility to chloramphenicol and norfloxacin. ASS113 showed increased susceptibility to tetracycline, but little change in susceptibility to the other antibiotics. The three PhoA fusions, ASS114 to ASS116, showed increased susceptibility to ampicillin, tetracycline, nalidixic acid, and norfloxacin (Table 4).

When plasmid pAS10, bearing the complementing *mar* locus, was added to ASS111, resistance levels to all antibiotics increased. The plasmid, however, was unable to restore the wild-type drug susceptibility profile in the insertion strains. Compared with ASS111(pAS10), mutant ASS112(pAS10) showed increased susceptibility to ampicillin, tetracycline, and norfloxacin but retained a decreased susceptibility to nalidixic acid. For ASS113 with pAS10, the MIC of tetracycline was less than that for the wild type, but the MICs of the other drugs were approximately the same as those for the wild type (~10% less). The plasmid in ASS114 produced MICs of all antibiotics that were the same as or higher than the MICs for the wild type (Table 4). In ASS115 and ASS116, the plasmid produced similar results, with MICs of all drug tested being generally the same as or slightly less than those for the wild type.

DISCUSSION

To clarify the involvement of chromosomal loci other than *marRAB* in the chromosomal multiple antibiotic resistance (Mar) phenotype, we used Tn*phoA* and Tn*lacZ* elements to isolate and characterize *mar*-responsive fusions in a specially constructed *E. coli* recipient. The recipient strain, ASS111 (pAS10), lacked the two chromosomal transcriptional units of the *mar* locus (*orf64*/*orf157* and the *marRAB* operon) but contained a curable (temperature-sensitive) plasmid bearing the *mar* locus with a constitutively expressed *marRAB* operon. Five gene fusions regulated by the *mar* locus were thereby identified. The *mar* locus activated the expression of β-galactosidase in two of these fusions and repressed the expression of alkaline phosphatase in the other three fusions. These changes were also caused by *marA* alone, strongly suggesting that MarA is the regulatory protein, although a role for other genes in the *mar* locus has not been fully excluded.

MarA is believed to be a transcriptional activator of genes involved in the Mar phenotype, since alone it causes increased resistance to multiple antibiotics (5, 11, 31, 33). It has a helix-turn-helix motif and a high degree of similarity to SoxS (64.5%), a known transcriptional activator of the *soxRS* regulon (2). Like SoxS, constitutive expression of the *marRAB* operon gives enhanced expression of glucose-6-phosphate dehydrogenase and Mn-containing superoxide dismutase, enzymes that are induced in response to superoxide radical-generating conditions, and also enhanced antibiotic resistance (2, 14).

Our present results show that the *marRAB* operon regulates expression positively and negatively. It is not yet clear whether both activities are an effect (direct or indirect) of MarA or whether MarB might also be involved. Recent studies have shown that the drug resistance mediated by MarA can be increased 20 to 40% when MarB is also present (31). The effect of MarA with or without MarB on response to oxidative stress or weak acids has not yet been studied.

MarA (with or without MarB) might act directly both as a repressor and an activator for the same locus, as do some other regulatory proteins (19). A well-characterized activator, AraC protein (10), acts positively and negatively on its promoter region (19). As a repressor, the AraC protein appears to block the entry of RNA polymerase to its promoter. As an activator, AraC in the presence of arabinose enhances expression. OmpR can activate or repress the *ompF* gene depending on osmolarity conditions and the strength of the promoter (30). Another protein, Lrp (the leucine-responsive regulatory protein), can be either an activator or repressor at Lrp target genes (21). One of the best examples is the repressor of phage lambda, which both represses lambda lytic gene expression, maintaining the lysogenic state, and positively regulates its own synthesis (25). Alternatively, the repression or activation function of *marRAB* (through MarA) might be indirect. For example, it might activate one gene which in turn downregulates another. Such is the path by which OmpF expression is decreased by *mar* operon-directed increased expression of *micF* (8). Whether the upregulation of *micF* itself by *marRAB* is direct is not yet known.

Cloning and sequencing of *mar*-regulated fusions revealed that three are newly identified genes located at min 14.1, 31.5, and 51.3 on the *E. coli* genetic map. Sequence analysis showed that the fusions in the ASS115 and ASS116 strains, both of which mapped to min 77, have occurred in overlapping locations in the same gene, the recently described *slp* gene (1). Expression of this gene is decreased by a constitutively expressed *marRAB* operon but is increased by catabolite repression and starvation (1). Neither its role in antibiotic susceptibility nor why loss of its expression leads to increased susceptibility to multiple, structurally unrelated antibiotics has been described (Table 4). That one of the fusions leads to an apparent membrane location and the other does not presumably relates to the different sites of insertion in the *slp* gene (1).

The function of the other genes is not known. However, some of the insertion strains showed increased susceptibility to tetracycline, ampicillin, nalidixic acid, and chloramphenicol (Table 4) while another (*mlr1*::Tn*lacZ*) appeared to cause increased resistance. The clarification of their function and possible contribution to other phenotypes associated with the *mar* locus, under study in this laboratory, will improve the understanding of their role in the pleiotropic phenotype mediated by the *mar* operon in *E. coli* (2, 5, 6, 28, 31).

The results obtained verify the approach used as a means of defining new and previously described genes under control by the *mar* operon and thus in the Mar regulon. Their identification will improve understanding of antibiotic interactions with bacterial cells and the basis for intrinsic cell resistance to environmental stresses.

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REFERENCES

1. **Alexander, D. M., and A. C. St. John.** 1994. Characterization of the carbon starvation-inducible and stationary phase-inducible gene *slp* encoding an outer membrane lipoprotein in *Escherichia coli*. Mol. Microbiol. **11:**1059–1071.
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. **Auerswald, E.-A., G. Ludwig, and H. Schaller.** 1981. Structural analysis of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. **45:**107–113.
4. **Beji, A., D. Izard, F. Gavini, H. Leclerc, M. Leseini-Delstanche, and J. Krembel.** 1987. A rapid chemical procedure for isolation and purification of chromosomal DNA from gram-negative bacilli. Anal. Biochem. **162:**18–23.
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. **Cohen, S. P., L. M. McMurry, D. C. Hooper, J. S. Wolfson, and S. B. Levy.** 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) *Escherichia coli* selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob. Agents Chemother. **33:**1318–1325.
8. **Cohen, S. P., L. M. McMurry, and S. B. Levy.** 1988. *marA* locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of *Escherichia coli*. J. Bacteriol. **170:**5416–5422.
9. **Curiale, M. S., and S. B. Levy.** 1982. Two complementation groups mediate tetracycline resistance determined by Tn*10*. J. Bacteriol. **151:**209–215.
10. **Dunn, T., S. Hahn, S. Ogden, and R. Schleif.** 1984. An operator at −280 base pairs that is required for repression of *araBAD* operon promoter: addition of DNA helical turns between the operator and promoter cyclically hinders repression. Proc. Natl. Acad. Sci. USA **8:**5017–5020.
11. **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.
12. **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.
13. **George, A. M., and S. B. Levy.** 1983. Gene in the major cotransduction gap of *Escherichia coli* linkage map required for the expression of chromosomal resistance to tetracycline and other antibiotics. J. Bacteriol. **155:**541–548.
14. **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 *Esche-*

richia coli. J. Bacteriol. **173:**4433–4439.
15. **Gutierrez, C., J. Barondess, C. Manoil, and J. Beckwith.** 1986. The use of transposon TnphoA to detect genes for cell envelope proteins subject to a common regulatory stimulus. Analysis of osmotically regulated genes in *Escherichia coli*. J. Mol. Biol. **195:**289–297.
16. **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.
17. **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.
18. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
19. **Hoopes, B. C., and W. R. McClure.** 1987. Strategies in regulation of transcription initiation, p. 1231–1240. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
20. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *Escherichia coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
21. **Lin, R., R. D'Ari, and E. B. Newman.** 1992. λ p*lac*Mu insertions in genes of the leucine regulon: extension of the regulon to genes not regulated by leucine. J. Bacteriol. **174:**1948–1955.
22. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
23. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Pearson, W., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
25. **Ptashne, M.** 1992. A genetic switch. Phage λ and higher organisms. Blackwell Scientific Publications, Cambridge, Mass.
26. **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.
27. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
28. **Seoane, A., and S. B. Levy.** 1993. Reversal of MarR binding to the regulatory region of the *marRAB* operon by structurally unrelated inducers, abstr. H-26, p. 204. *In* Abstracts of the 93rd General Meeting of the American Society for Microbiology. American Society for Microbiology, Washington, D.C.
29. **Seoane, A., A. Sabbaj, L. M. McMurry, and S. B. Levy.** 1992. Multiple antibiotic susceptibility associated with inactivation of the *prc* gene. J. Bacteriol. **174:**7844–7847.
30. **Tsung, K., R. E. Brissette, and M. Inouye.** 1990. Enhancement of RNA polymerase binding to promoters by a transcriptional activator, OmpR, in *Escherichia coli*: its positive and negative effects on transcription. Proc. Natl. Acad. Sci. USA **87:**5940–5944.
31. **White, D. G., W. Yan, and S. B. Levy.** 1994. Functional characterization of the chromosomal multiple antibiotic resistance (*mar*) locus in *Escherichia coli*, abstr. A104, p. 20. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
32. **Wilmes-Riesenberg, M. R., and B. L. Wanner.** 1992. Tn*phoA* and Tn*phoA′* elements for making and switching fusions for study of transcription, translation, and cell surface localization. J. Bacteriol. **174:**4558–4575.
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. A26, p. 5. *In* Abstracts of the 92nd General Meeting of the American Society for Microbiology 1992. American Society for Microbiology, Washington, D.C.
34. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.