JOURNAL OF BACTERIOLOGY, May 1993, p. 2888–2894
0021-9193/93/102888-07$02.00/0

Vol. 175, No. 10

Overexpression of the MarA Positive Regulator Is Sufficient To Confer Multiple Antibiotic Resistance in *Escherichia coli*

LAURA GAMBINO, STEPHEN J. GRACHECK, AND PAUL F. MILLER*

Infectious Diseases, Experimental Therapy Department, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48106-1047

Received 14 December 1992/Accepted 8 March 1993

A genetic approach was undertaken to identify normal bacterial genes whose products function to limit the effective concentration of antibiotics. In this approach, a multicopy plasmid library containing cloned *Escherichia coli* chromosomal sequences was screened for transformants that showed increased resistance to a number of unrelated antibiotics. Three such plasmids were identified, and all contained sequences originating from the *mar* locus. DNA sequence analysis of the minimal complementation unit revealed that the resistance phenotype was associated with the presence of the *marA* gene on the plasmids. The putative *marA* gene product is predicted to contain a helix-turn-helix DNA binding domain that is very similar to analogous domains found in three other *E. coli* proteins. One such similarity was to the SoxS gene product, the elevated expression of which has previously been associated with the multiple antibiotic resistance (Mar) phenotype. Constitutive expression of *marA* conferred antibiotic resistance even in cells carrying a deletion of the chromosomal *mar* locus. We have also found that transformants bearing *marA* plasmids show a significant reduction in *ompF* translation but not transcription, similar to previously described *mar* mutants. However, this reduction in *ompF* expression plays only a minor role in the resistance mechanism, suggesting that functions encoded by genes unlinked to *mar* must be affected by *marA*. These results suggest that activation of *marA* is the ultimate event that occurs at the *mar* locus during the process that results in multiple antibiotic resistance.

The intrinsic level of bacterial susceptibility to antimicrobial agents depends on the affinity of a specific agent for its cellular target as well as the ability of the agent to accumulate within the cellular compartment that contains the target. While the first part of this equation is expected to vary with each specific antibiotic-target combination, the matter of accumulation may depend on global factors that include essentially the same set of cellular processes regardless of the bacterial system. These would be expected to include barriers to permeability as well as active export systems.

A number of reports regarding chromosomal mutations that confer low-level resistance to a variety of antibiotics (10, 14, 19, 29) have appeared. In most cases, however, little is known about either the identity of the gene designated by the resistance mutation or the cellular mechanisms affected by these mutations. Probably the best characterized of these is the *mar* locus in *Escherichia coli* (10, 11). Mutations at *mar* confer resistance to several unrelated antibiotics and have been associated with an increase in the activity of an active efflux mechanism for tetracycline (10). In addition, *mar* mutants have significantly reduced levels of the outer membrane porin OmpF, presumably as a consequence of MicF-mediated translational repression of *ompF* expression (7). Thus, the *mar* locus represents one candidate for an intrinsic genetic system that may play a central role in establishing barriers to the accumulation of foreign compounds.

Nonetheless, the *mar* locus itself is poorly understood at the genetic level. Hachler et al. have determined that approximately 8 kbp of DNA from the *mar* locus is required to restore the ability to select spontaneous *mar* mutants in a deletion strain, but it is not clear which genes included at this site are necessary for drug resistance (15). For this reason, we chose to examine multiple antibiotic resistance by using a different approach: we attempted to identify genes which, when amplified on a multicopy plasmid, would give rise to a Mar phenotype. Such genes might encode factors whose expression is limiting with respect to antibiotic resistance. We describe here one such gene, derived from the *mar* region, which encodes a putative transcriptional activator. Overexpression of this gene product is sufficient to confer an intermediate level of resistance to a number of structurally unrelated agents by at least two mechanisms, the downregulation of *ompF* expression and modulation of other activities encoded outside of the *mar* locus.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and genetic procedures. The bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, cultures were grown in L broth (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl [each per liter]) at 37°C. Antibiotics were added to the following concentrations: ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; chloramphenicol, 20 μg/ml. All antibiotics were from Sigma Chemical Co. (St. Louis, Mo.). Enoxacin (26), a fluoroquinolone, was from an in-house synthesis.

The isolation of mini-Tn*10kan* insertions into plasmids was performed as described previously (20), by using the λNK1316 phage delivery vehicle (20). Construction of specific strains was by P1 transduction (31). Special note should be made regarding the strategy used to construct and verify strains deleted for the *mar* locus. The loss of this locus does not confer an obvious phenotype. Therefore, the *marA* allele containing a mini-Tn*10kan* insertion (plasmid p16 in Fig. 2) was recombined into the chromosome of MC4100 by first subcloning the *SalI-PvuII* fragment from p16 into the temperature-sensitive plasmid pMAK705 and then recovering the desired recombinant strain after resolution of plasmid

* Corresponding author.

TABLE 1. Bacterial strains, plasmids, and phage

Plasmid, strain, or phage	Properties or phenotype	Source or reference
E. coli strains		
MC4100	F⁻ *araD139 ΔlacU169 rpsL relA thi*	T. Silhavy
SK3001	MC4100 Δ*micF1*::*kan*	H. Aiba; 23
PK1738	*trpr trpA9605*(Am) *his-29*(Am) *ilv pro-2 arg-427 thyA deoB* or *deoC tsx gyrA* (Nal^r) *rac zdd-230*::Tn*9* Cm^r del*1738* (Δ*mar*) *sad*	P. Kuempel; 18
B159	MC4100 *marA*::mini-Tn*10kan*	This study
B177	MC4100 *zdd-230*::Tn*9* Cm^r Δ*mar*	This study
MH225	MC4100 ϕ(*ompC'-lacZ*) *10-25*	T. Silhavy; 16
MH513	MC4100 ϕ(*ompF'-lacZ*) *16-13*	T. Silhavy; 16
MH621	MH20 ϕ(*ompF'-'lacZ*)*hyb 16-21*	T. Silhavy; 16
B197	MH621 Δ*micF1*	This study
B202	MH621 *marA*::mini-Tn*10kan*	This study
B194	B180 *zdd-230*::Tn*9* Cm^r Δ*mar* Km^s	This study
MH480	MC4100 Δ(*ompF*)*480*	T. Silhavy; 16
Plasmids		
pBR322	Ap^r Tc^r	3
pBS+	Ap^r	Stratagene
pMAK705	Cm^r; temperature-sensitive replication	E. Olson; 17
p7	Ap^r; 6.2-kbp insert in pBR322; Mar	This study
p9	Ap^r; 2.2-kbp insert in pBR322; Mar	This study
p11	Ap^r; 7.5-kbp insert in pBR322; Mar	This study
p14	p9 *marA*::mini-Tn*10kan*; Ap^r Km^r Mar⁻	This study
p15	p9 with mini-Tn*10kan* in *tet* promoter; Ap^r Km^r Mar⁻	This study
p16	2.3-kbp *SalI-SalI* fragment from p9 into *SalI* site of pBS+	This study
p17	Same as that for p16, but insert is in opposite orientation	This study
p34	*SalI-PvuII* fragment from p14 containing *marA*::mini-Tn*10kan* cloned into *SalI* site of pMAK705; Cm^r Km^r	This study
p37	p9 with deletion of *PvuII* fragment between insert and vector sites	This study
Phage		
λNK1316	Delivery vehicle for mini-Tn*10kan*	N. Kleckner; 20

integrants, as described previously (17). The structure of the *marA*::Tn*10kan* allele in the resulting strain, designated B159, was confirmed by Southern hybridization. The *mar* deletion was then transferred from strain PK1738 (Table 1) into B159 by P1 transduction, with selection for the chloramphenicol resistance conferred by a Tn*9* element that is tightly linked with the *mar* deletion (18). Transductants were then screened for loss of the Tn*10kan* insertion, and these presumptive Δ*mar* strains were further screened for the loss of the *sad* gene (18) on M9 media containing 2.5 mM succinic semialdehyde (22).

Transformation with plasmid DNA was routinely performed by the TSS method (5). Comparison of resistance levels was performed by a gradient plate method (8), except that 30 ml of media was used per layer and the antifoam agent was omitted. Quantitative assessment of resistance was performed by an agar dilution method by using L agar plates supplemented with antibiotics, the concentrations of which varied in twofold increments. These plates were inoculated by spotting 2 μl of a culture suspension containing approximately 10^7 CFU of the test organism per ml. The plates were then incubated overnight at 35°C. The lowest concentration of antibiotic that completely inhibited growth was designated the MIC.

Construction of a plasmid library and identification of multiply antibiotic-resistant clones. Two hundred micrograms of chromosomal DNA from the *E. coli* strain MC4100 was partially digested with the restriction enzyme *Sau*3A and then separated by size on a 10 to 40% sucrose gradient as described previously (2). Seven-hundred-fifty-microliter fractions were removed from the top of the gradient tube, and aliquots were analyzed on an 0.8% Tris-borate-EDTA-agarose gel. A fraction containing DNA fragments in the 5- to 10-kbp range was used as a source of material for cloning. These purified fragments were then ligated with *Bam*HI-digested pBR322 vector, and the ligation products were subsequently transformed into competent library-efficiency DH5α cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Ampicillin-resistant transformants were then screened on supplemented L agar plates for resistance to 10 μg of chloramphenicol per ml. (Vector-transformed cells are sensitive to this level of antibiotic.) Plasmid DNA was purified from prospective positive isolates and then rescreened after transformation into competent MC4100 cells. Confirmed positive clones were then analyzed further.

DNA biochemistry and analysis. Chromosomal DNA was prepared as described by Silhavy et al. (31). Plasmid DNA was prepared by the alkaline lysis method (28). Restriction endonucleases and T4 DNA ligase were from Boehringer Mannheim, and agarose was from FMC Corp. Restriction enzyme analyses, ligations, and agarose gel electrophoresis were performed as described in standard protocols (28). Radiolabeled DNA probes were prepared by the random priming method by using a kit from Boehringer Mannheim and [^{32}P]dATP (Amersham). Transfer of DNA from agarose gels to Nytran membranes (Schleicher & Schuell), and procedures for hybridization and washing of blots were performed as described in the manufacturer's instructions. For mapping experiments in which the Kohara collection of phages (21) was surveyed for homologous sequences, a

nylon membrane containing the ordered miniset collection of recombinant phages was purchased (TaKaRa) and hybridized with radiolabeled probes as described above. DNA sequence analysis was performed by the dideoxy chain termination method of Sanger et al. (30) by using the Sequenase kit (U.S. Biochemicals) and denatured plasmid templates.

Other methods. β-Galactosidase assays were performed as described by Miller (24) by using the chloroform-sodium dodecyl sulfate method. Cultures were grown in L broth to the mid-log phase prior to being assayed for enzyme activity. Computer analyses of nucleic acid and protein sequences were performed on a VAX computer (Digital Equipment Co.) by using the GCG package of sequence analysis programs (9) and on a Macintosh computer by using the MacVector software programs (IBI).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned the accession number L06966.

RESULTS

Cloning of sequences conferring increased antibiotic resistance. To identify genes whose products might be important in establishing the intrinsic level of resistance to antibiotics, we screened a recombinant library of *E. coli* chromosomal DNA sequences cloned into the plasmid pBR322 for transformants with increased levels of resistance to chloramphenicol. Three independent recombinant plasmids that satisfied this criterion were obtained (Fig. 1A). The MIC for chloramphenicol was increased in these strains from 2 μg/ml for pBR322 transformants to 16 μg/ml for cells transformed with the recombinant plasmids shown. Similar increases in resistance were also observed for enoxacin (a fluoroquinolone) and tetracycline. The cloned inserts contained overlapping DNA fragments, indicating that they were probably derived from the same chromosomal locus, a conclusion that was confirmed by hybridization experiments (not shown). Interestingly, the cloned fragments were all oriented in the same direction with respect to the vector *tet* promoter and contained similar but not identical *Sau*3A cloning junctions at their left-hand ends.

Location of the cloned sequences on the physical map of the *E. coli* chromosome. The *Pvu*II-*Cla*I fragment from plasmid p9 (Fig. 1A) was used as a probe to localize the cloned sequences on the *E. coli* chromosome. Hybridization against the miniset collection of Kohara phages resulted in a positive signal against the overlapping phages 305 and 306 (not shown). These phages contain sequences from the 34-min region of the *E. coli* genetic map. This is the same general location as that of the *mar* locus, previously identified by George and Levy as being involved in multiple antibiotic resistance (11).

Localization of the minimal complementation unit. To more precisely define the minimal segment capable of conferring antibiotic resistance, fragments derived from plasmid p9, the smallest of the original resistance plasmids, were subcloned and analyzed for chloramphenicol susceptibility (Fig. 1B). Since a deletion of the sequences to the right of the *Pvu*II site had no effect on the level of resistance (p37), the region involved in the resistance phenotype was clearly localized towards the left half of the insert.

We also sought to isolate mini-Tn*10kan* insertions in p9 that would eliminate the resistance phenotype. Two such insertions were identified and characterized. The first, p14, contained a transposon insertion located to the left of the

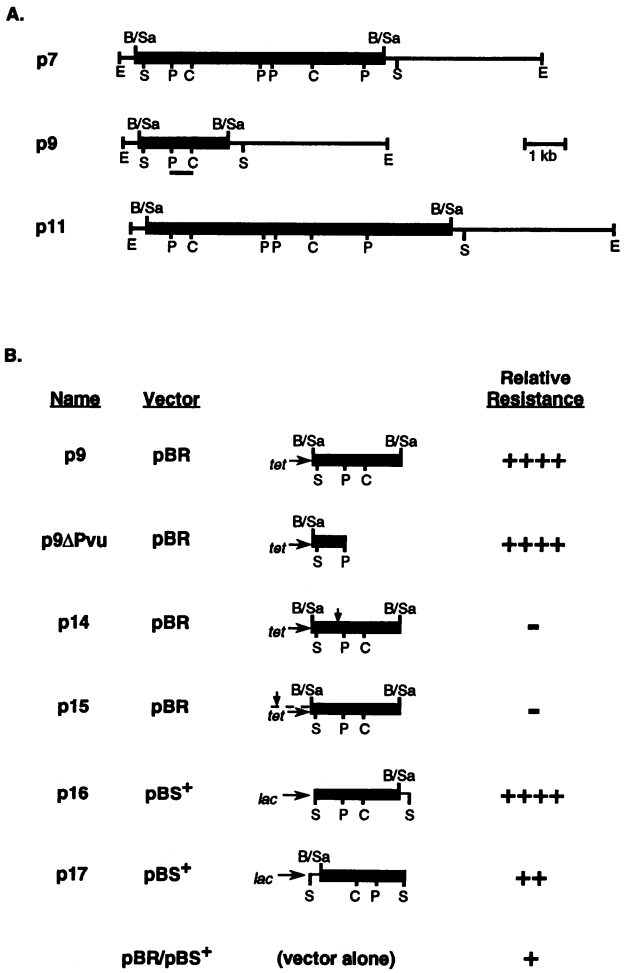

FIG. 1. Analysis of cloned *mar* sequences. (A) Restriction maps of recombinant plasmids conferring multiple antibiotic resistance to wild-type *E. coli* cells. The physical maps of three plasmids capable of conferring chloramphenicol resistance are shown. These plasmids also provide resistance to tetracycline and enoxacin (a quinolone) but not to kanamycin. Abbreviations: B/Sa, *Bam*HI-*Sau*3A junction; E, *Eco*RI; C, *Cla*I; P, *Pvu*II; S, *Sal*I. The heavy bar indicates the cloned *E. coli* chromosomal sequences. Thin lines indicate the pBR322 vector sequences. The bar beneath the plasmid p9 represents the probe used in hybridization experiments against the Kohara collection of overlapping phages (see text). (B) Analysis of antibiotic resistance mediated by derivatives of plasmid p9. Derivatives of this plasmid were constructed to further localize the complementation unit. Only the insert sequences, drawn to the same scale as that in panel A are shown. All constructs were introduced into strain MC4100 and analyzed on gradient plates containing 10 mg of chloramphenicol per ml in the bottom layer. Relative resistance was scored as fractional growth across the gradient, with ++++ indicating growth across the entire plate. ↓, site of mini-Tn*10kan* transposon insertion. Restriction site designations are the same as those described for panel A.

*Pvu*II site, consistent with this portion of the molecule being important for resistance. The second insertion (p15) mapped outside of the cloned DNA to a region of the vector that includes the *tet* promoter. The isolation of this latter insertion offered an explanation for the similar orientation and left-hand cloning junctions observed for the three original resistance plasmids (Fig. 1A): the resistance phenotype

FIG. 2. DNA sequence of the *Sal*I-*Pvu*II fragment from plasmid p9. The deduced protein sequence for the single prominent open reading frame identified is also shown. A potential ribosome binding site (SD), the precise location of the mini-Tn*10kan* insertion in p14 (↓), and a candidate helix-turn-helix motif (4) are indicated.

depends on the expression of cloned sequences from an active, external promoter. This idea also helped to explain another orientation-dependent resistance effect. Subcloning of the *Sal*I fragment from p9 into the plasmid pBS+ in both orientations produced recombinant plasmids that conferred different levels of chloramphenicol resistance (p16 and p17). Greater resistance was observed for the construct in which the insert was oriented in the same direction as the vector-encoded *lac* promoter.

DNA sequence analysis of the resistance determinant. The DNA sequence of both strands of the 704-bp *Sal*I-*Pvu*II fragment was determined (Fig. 2). A single open reading frame encoding a putative 127-amino-acid polypeptide was identified. A computer search of available sequence data bases revealed that the cloned gene is identical to the *marA* gene that was cloned and sequenced by Cohen and coworkers (5a) (GenBank accession no. M96235). The *marA* coding region was analyzed by using the GCG package of sequence analysis programs (9). The predicted polypeptide is very basic, with a computer-predicted pI of 9.3. A potential helix-turn-helix region characteristic of certain transcriptional regulatory proteins (4) was identified in the amino-terminal portion of the molecule.

The sequence data base search also identified a distinct pattern of amino acid sequence similarity between the MarA protein and the products of the *soxS*, *rob*, and *tetD* genes. An alignment of the amino acid sequences of these proteins is shown in Fig. 3. The similarity is particularly striking for the regions that include the predicted helix-turn-helix domains of these proteins, with the second helices being almost completely identical. This observation suggests the possibility of a functional relationship among these proteins.

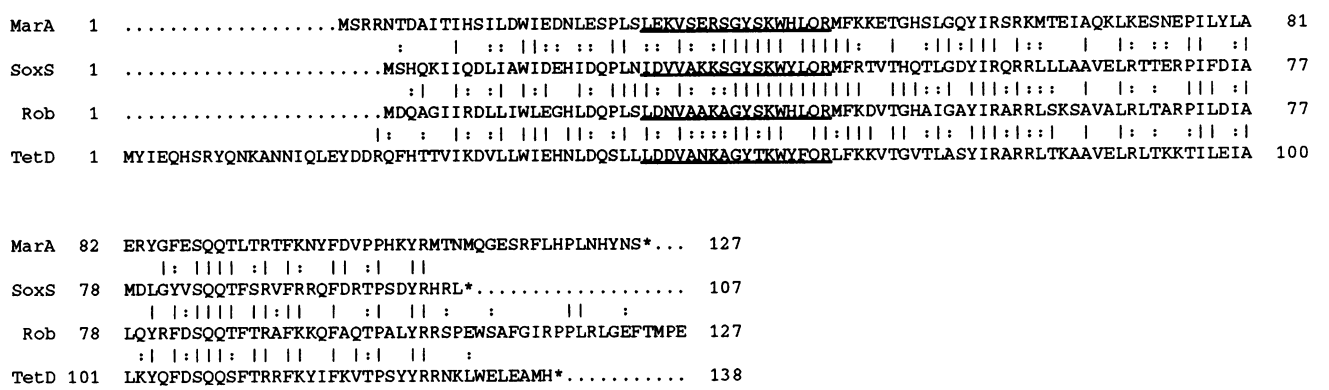

FIG. 3. Alignment of MarA amino acid sequence with SoxS, Rob, and TetD. Alignments are shown over the entire length of each protein for MarA, SoxS, and Tet and for the amino-terminal 127 amino acids of Rob. A vertical line (|) above a given protein sequence indicates an amino acid identity between that protein and MarA, while a colon (:) designates a functionally similar amino acid. The groupings used for functional substitution determination are YWF, QNED, MILV, PAGST, and HKR. Percent identities between MarA and the other proteins are: MarA and SoxS, 39.3% over a 107-amino-acid overlap; MarA and Rob, 49.0% over 104 amino acids; MarA and TetD, 43.1% over 109 amino acids. Candidate helix-turn-helix motifs are underlined.

TABLE 2. p9-induced antibiotic resistance does not require an intact *mar* locus

Strain	Plasmid	Relevant features	Growth (% of gradient)[a]
MC4100	pBR322	Wild type	23
	p9	Wild type; multicopy *marA*	80
B177	pBR322	Δ*mar*	20
	p9	Δ*mar*; multicopy *marA*	80

[a] Values indicate the extent of growth across a linear gradient of 0.0 to 0.25 μg of enoxacin per ml.

Requirement for other *mar* sequences for p9-induced resistance. Since the genetic organization of the *mar* locus has been reported to be complex (15), it seemed possible that other genes whose products are required for *marA*-induced antibiotic resistance would be found there. To address this, we examined the effect of deleting the *mar* locus on the resistance patterns of p9-transformed strains. Strains were compared on enoxacin gradient plates only, since chloramphenicol (Tn*9* Cm^r linked to Δ*mar* in B177 strains) and tetracycline (Tet^r encoded by pBR322) were not usable in these experiments. The *mar* deletion in an otherwise wild-type strain conferred a slightly hypersensitive phenotype compared with that of *mar*$^+$ transformants, suggesting that there is a basal level of activity normally associated with the *mar* locus. However, the p9 plasmid was able to induce an increase in enoxacin resistance in the deletion strain that was indistinguishable from that observed in the wild-type strain (Table 2). Thus, the antibiotic resistance phenotype associated with amplification of the *marA* gene does not require other functions encoded at the *mar* locus. In these and all other experiments performed, transformants containing the p9ΔPvu plasmid (Fig. 1B) behaved indistinguishably from p9-transformed strains, confirming that it is the *marA* sequence on p9 that is responsible for the effects observed (data not shown).

Effects of plasmid p9 on the expression of outer membrane porins. One consequence of a classic *mar* mutation is a decrease in the level of the OmpF porin in the outer membrane (7). To determine if plasmid p9 affects the expression of *ompF*, strains containing various *omp-lacZ* fusions were transformed with this plasmid and analyzed for β-galactosidase activity (Table 3). The most significant effect of p9 compared with that of control strains was its inhibitory effect on the expression of the *ompF-lacZ* translational fusion, consistent with previously described effects of *marA* chromosomal mutations (7). In addition, this inhibition could be completely suppressed by a deletion of *micF*, a negative regulator of *ompF* translation (25). Thus, overexpression of *marA* results in a reduction in *ompF* expression, presumably through the activation of *micF*, consistent with previous reports (7). This downregulation of *ompF* expression did not require other functions encoded at the *mar* locus, since the *mar* deletion did not affect the repression of the *ompF-lacZ* translational fusion mediated by p9 (Table 3).

To determine the effect of this decrease in *ompF* expression on the antibiotic resistance phenotype, the *marA* plasmid p9 or the pBR322 vector were introduced into a strain containing a null mutation in *ompF*, and antibiotic susceptibilities were examined on gradient plates (Table 4). The loss of a functional *ompF* gene had little or no effect on either chloramphenicol or enoxacin susceptibility. In addition, any effect observed was minor compared with that obtained with the original p9 transformants. Moreover, introduction of p9 into the *ompF* mutant further increased resistance to that of the original p9 transformants.

By contrast, a deletion of *micF* had no effect on the resistance of the host strain to either chloramphenicol or enoxacin. The introduction of p9 into the Δ*micF* strain increased resistance to a level that was equivalent to that observed for wild-type (*micF*$^+$) transformants (Table 4). Taken together, the above results suggest that the resistance associated with the p9 plasmid could be explained by the modulation of functions other than a reduction in *ompF* expression, functions encoded outside of the *mar* locus. Apparently, the inhibitory effect of p9 on *ompF* plays only a minor role in this resistance scheme.

DISCUSSION

We have described a new condition by which multiple antibiotic resistance associated with the *mar* locus can be induced in *E. coli*, i.e., overexpression of a transcriptional regulator encoded by this locus. The observations described in this study offer important insights regarding the role of this locus in affecting antibiotic resistance.

First, the transcriptional activator that is described here corresponds to the *marA* gene that has been identified by George and Levy on the basis of a Tn*5* insertion (11). This insertion results in an inability to generate spontaneous *mar* mutants. By comparing the published restriction map of the cloned *mar* locus (15) with the recombinant plasmids described here, it seems certain that the Tn*5* insertion previously described is within the *marA* gene.

The most significant finding is that constitutive overexpression of the MarA activator results in a Mar phenotype even in the absence of an intact *mar* locus. This observation, along with previous molecular cloning work on this region (15), allows us to begin to predict roles for the genes located at this locus. It has been reported that a fairly extensive amount of genetic material, ~8 kbp, is required to generate a classic *mar* mutant (15). Importantly, the Tn*5* insertion in *marA* eliminates all Mar phenotypes associated with such mutations. One possible explanation for these findings is that *marA* is epistatic to other genes encoded at this locus, and the induction of *marA* expression is the ultimate event in a signaling cascade. In this scenario, most typical *mar* mutants would occur either in a gene that affects *marA* expression or in *marA* itself. Our finding that overexpression of *marA* from a multicopy plasmid bypasses the requirement for any other sequences at the *mar* locus is consistent with this model. In addition, the observation that the accumulation of *marA*-specific mRNA species is inducible by certain chemical agents including chloramphenicol, tetracycline, and sodium salicylate (15, 24a) provides direct evidence for a stress-response-like activity associated with this locus. This

TABLE 3. Effect of p9 on the expression of *omp-lacZ* fusions

Strain	Relevant genotype; fusion	β-galactosidase activity[a]	
		pBR322	p9
MH225	Wild type; *ompC* (operon)	1,432	1,080
MH513	Wild type; *ompF* (operon)	480	471
MH621	Wild type; *ompF* (protein)	7,013	225
B197	Δ*micF*; *ompF* (protein)	7,361	6,044
B194	Δ*mar*; *ompF* (protein)	6,776	277

[a] Activity expressed in Miller units as described previously (24).

TABLE 4. Effect of Δ*ompF* and Δ*micF* on antibiotic resistance

Strain	Plasmid	Relevant features	Growth (% of gradient)[a]	
			Chloramphenicol	Enoxacin
MC4100	pBR322	Wild type	19	29
MC4100	p9	Wild type; multicopy *marA*	91	86
MH480	pBR322	Δ*ompF*	27	19
MH480	p9	Δ*ompF*; multicopy *marA*	100	86
SK3001	pBR322	Δ*micF*	24	33
SK3001	p9	Δ*micF*; multicopy *marA*	94	95

[a] Values indicate the extent of growth across a linear gradient containing either 0 to 20 μg of chloramphenicol per ml or 0.0 to 0.25 μg of enoxacin per ml.

scheme would be analogous genetically to other stress-inducible systems in which a regulatory locus encodes both a sensor-transducer and a transcriptional activator (27). The sequences to the right of *marA*, which are required for the establishment of classic *mar* mutants (15), might encode the sensor. Constitutive overexpression of the MarA activator, as in the case described here, would bypass the need for the sensor in the activation of the *mar* system.

It is likely that the effects of classic *mar* mutations on outer membrane permeability are the direct result of increased MarA activity in these cells. The results of the experiments reported here parallel those reported earlier for classic *mar* mutants (6, 7). Specifically, overexpression of MarA from p9 results in decreased *ompF* expression that is dependent on *micF* but which does not require other sequences encoded by the *mar* locus. In addition, gradient plate tests comparing the Δ*micF* and *ompF*::Tn*5* strains with an isogenic wild-type parent demonstrated that modulation of *ompF* expression plays only a minor role in the resistance mechanism. Moreover, high-level resistance imparted by plasmid p9 was observed in all of the backgrounds described above. Thus, another cellular function(s) in addition to the downregulation of *ompF* is affected by MarA overexpression, and this plays a very significant role in the resistance scheme.

What might these other resistance determinants be? One possible candidate is an efflux pump that has been associated with tetracyline resistance in *mar* mutants (10). However, this efflux mechanism appears to be specific for tetracycline, and no increase in the active export of chloramphenicol (10) or quinolones (6) has been detected in *mar* strains. Since *mar* mutants do exhibit a reduced accumulation of all of these compounds, it is likely that barriers to permeability other than reduced levels of OmpF are affected by MarA. In addition, multiple target genes may be regulated by MarA, with different gene products playing distinct roles in mediating resistance towards different antibiotics. For example, while activation of an efflux system may be the most important component of tetracycline resistance, reduced levels of specific porins may promote chloramphenicol or quinolone resistance. All of these effects would be coordinately controlled by *marA*.

MarA shows significant amino acid sequence similarity to other *E. coli* proteins (Fig. 3). Among these proteins, SoxS and TetD are similarly sized, low-molecular-weight molecules whose putative DNA-binding domains are similarly positioned in their primary sequences. The Rob protein is significantly larger, at approximately 35 kDa. The most striking feature from the alignment of these proteins is the strong conservation in the predicted helix-turn-helix motifs, particularly in the second α-helix (Fig. 3) (4). Since this helix has been shown to play a major role in the recognition of the target binding site for certain DNA-binding proteins (4), it is tempting to speculate that these proteins might recognize an overlapping set of operator sequences. In fact, the best characterized of these, SoxS, is particularly intriguing since strains that constitutively express this protein have antibiotic resistance phenotypes similar to those of *mar* mutants (1, 13). In addition, a mutant that is constitutively activated for the Sox system has been mapped to the *mar* region of the chromosome (12). This allele, designated *soxQ1*, confers similar phenotypes to *mar* mutants, in addition to increased resistance to superoxide-generating compounds. It has been proposed that the *soxQ* gene may be part of the *mar* locus and that regulators encoded by the *soxQ-mar* and *soxRS* loci might control the expression of an overlapping set of target genes (12). We have recently found that the antibiotic resistance phenotype associated with constitutive alleles of *soxR* is partially dependent on an intact *mar* locus (24a). We are currently investigating the relationship between *marA* and *soxS* in greater detail. The functions of the putative Rob and TetD proteins are not known. Since the potential exists for the conservation of a DNA binding specificity among the several different gene products mentioned here, it is probable that the target genes controlled by these proteins are important in several stress response or homeostatic systems. Antibiotic-resistant strains carrying the p9 plasmid should provide a useful starting point for the identification of these target genes by classical genetic methodologies.

ACKNOWLEDGMENTS

We thank Eric Olson, Tom Silhavy, Nancy Kleckner, Hirofumi Aiba, and Peter Kuempel for gifts of strains, plasmids, and phages. We are also indebted to E. Olson and T. Silhavy for advice and counsel during the course of this work and to E. Olson for carefully reading the manuscript. We thank our colleagues in the Infectious Diseases Department and the Olson lab for their support.

ADDENDUM IN PROOF

After submission of the manuscript for this article, a DNA sequence analysis of the *mar* region was published by Cohen et al. (5a).

REFERENCES

1. **Amabile-Cuevas, C. F., and B. Demple.** 1991. Molecular characterization of the *soxRS* genes of *Escherichia coli*: two genes control a superoxide stress regulon. Nucleic Acids Res. **19:** 4479–4484.
2. **Ausubel, F. M.** 1989. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. B. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.

4. **Brennan, R. G., and B. W. Matthews.** 1989. The helix-turn-helix DNA binding motif. J. Biol. Chem. **264:**1903–1906.
5. **Chung, C. T., S. L. Niemela, and R. H. Miller.** 1989. One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. USA **86:**2172–2175.
5a. **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., 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.
7. **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.
8. **Cunningham, R. P., S. M. Saporito, S. G. Spitzer, and B. Weiss.** 1986. Endonuclease IV (*nfo*) mutant of *Escherichia coli*. J. Bacteriol. **168:**1120–1127.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
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 the *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. A. 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. **Greenberg, J. T., P. Monach, J. H. Chou, P. D. Josephy, and B. Demple.** 1990. Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **87:**6181–6185.
14. **Gutmann, L., R. Williamson, N. Moreau, M. Kitzis, E. Collatz, J. F. Acar, and F. W. Goldstein.** 1985. Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alterations in outer membrane proteins of *Klebsiella*, *Enterobacter*, and *Serratia*. J. Infect. Dis. **151:**501–507.
15. **Hachler, 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.
16. **Hall, M. N., and T. J. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K-12. J. Mol. Biol. **146:**23–43.
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. **Hill, T. M., J. M. Henson, and P. L. Kuempel.** 1987. The terminus region of the *Escherichia coli* chromosome contains separate loci that exhibit polar inhibition of replication. Proc. Natl. Acad. Sci. USA **84:**1754–1758.
19. **Hooper, D. C., J. S. Wolfson, K. S. Souza, E. Y. Ng, G. L. McHugh, and M. N. Swartz.** 1989. Mechanisms of quinolone resistance in *Escherichia coli*: characterization of *nfxB* and *cfxB*, two mutant resistance loci decreasing norfloxacin accumulation. Antimicrob. Agents Chemother. **33:**283–290.
20. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*, p. 139–180. *In* J. H. Miller (ed.), Bacterial genetic systems. Academic Press, Inc., San Diego.
21. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
22. **Marek, L. E., and J. M. Henson.** 1988. Cloning and expression of the *Escherichia coli* K-12 *sad* gene. J. Bacteriol. **170:**991–994.
23. **Matsuyama, S., and S. Mizushima.** 1985. Construction and characterization of a deletion mutant lacking *micF*, a proposed regulatory gene for OmpF synthesis in *Escherichia coli*. J. Bacteriol. **162:**1196–1202.
24. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24a. **Miller, P. F.** Unpublished observations.
25. **Mizuno, T., M. Chou, and M. Inouye.** 1984. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc. Natl. Acad. Sci. USA **81:**1966–1970.
26. **Nakamura, S., A. Minami, H. Katae, S. Inoue, J. Yamagishi, T. Yoshiyuki, and M. Shimizu.** 1983. In vitro antibacterial properties of AT2266, a new pyridonecarboxylic acid. Antimicrob. Agents Chemother. **23:**641–648.
27. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1989. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49:**479–481.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sanders, C. C., W. E. Sanders, R. V. Goering, and V. Werner.** 1984. Selection of multiple antibiotic resistance by quinolones, β-lactams, and aminoglycosides with special references to cross-resistance between unrelated drug classes. Antimicrob. Agents Chemother. **26:**797–801.
30. **Sanger, F., F. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.