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MINIREVIEW

Regulation of Chromosomally Mediated Multiple Antibiotic Resistance: the *mar* Regulon

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Multidrug resistance in bacteria is generally attributed to the acquisition of multiple transposons and plasmids bearing genetic determinants for different mechanisms of resistance (48, 62). However, descriptions of intrinsic mechanisms that confer multidrug resistance have begun to emerge. The first of these was a chromosomally encoded multiple antibiotic resistance (*mar*) locus (Fig. 1) in *Escherichia coli* (45, 46). Mar mutants of *E. coli* arose at a frequency of 10^{-6} to 10^{-7} and were selected by growth on subinhibitory levels of tetracycline or chloramphenicol (45, 46). These mutants exhibited resistance to tetracyclines, chloramphenicol, penicillins, cephalosporins, puromycin, nalidixic acid, and rifampin (45). Later, the resistance phenotype was extended to include fluoroquinolones (25, 105), oxidative stress agents (7, 51), and, more recently, organic solvents (8, 49, 144).

The expression of the Mar phenotype is greater at 30°C than 37°C (45, 127). Continued growth in the same or higher antibiotic concentrations led to increased levels of resistance, thus demonstrating an amplifiable multiple antibiotic resistance phenotype (45). Both high- and low-level resistances were decreased or completely reversed by a Tn*5* insertion into a single locus at 34 min (1,636.7 kb) on the *E. coli* chromosome, called the *mar* locus (46). The genetic basis for high-level resistance is only partially attributed to the *mar* locus, since transduction of the locus from high- or low-level *mar* mutants produced only a low level of multidrug resistance (94).

The *mar* locus consists of two divergently positioned transcriptional units that flank the operator *marO* (Fig. 1) in *E. coli* (22, 24, 112) and *Salmonella typhimurium* (133). One operon encodes MarC, a putative integral inner membrane protein (Fig. 1) without any yet apparent function, but which appears to contribute to the Mar phenotype in some strains (see below) (49, 143, 144). The other operon comprises *marRAB*, encoding the Mar repressor (MarR), which binds *marO* and negatively regulates expression of *marRAB* (22, 90, 127), an activator (MarA), which controls expression of other genes on the chromosome, e.g., the *mar* regulon (22, 41, 126), and a putative small protein (MarB) of unknown function (Fig. 1).

The *marRAB* operon responds to a variety of compounds (7, 24, 52, 54, 99, 119, 127) including tetracycline and chloramphenicol (54). Deletion or inactivation of the *marRAB* operon results in increased susceptibility to multiple antibiotics, a variety of oxidative stress agents, and organic solvents (22, 24, 46, 51, 54, 144).

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Since salicylate and acetylsalicylate induced a reversible "phenotypic antibiotic resistance" to multiple antibiotics (118), a connection was made between phenotypic antibiotic resistance and *mar* (24); salicylate induced expression of *marRAB* (24). These studies also extended the spectrum of inducers to include acetaminophen, sodium benzoate, 2,4-dinitrophenol (an uncoupling agent), and cinnamate (a salicylate precursor in plants) (24). The uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone and redox-cycling compounds, menadione and plumbagin, are also inducers of *marRAB* transcription (127).

Initially it was thought that 7.8 kb of chromosomal DNA was needed to generate constitutive *mar* mutants (*mar*^{c}) in a strain bearing a large (39 kb) chromosomal deletion (54). However, this finding was shown to be strain specific, and ~1.1 kb of *mar* sequence, containing *marO*, *marR*, and *marA* sequences, was sufficient to select a *mar*^{c} mutant (91, 134).

mar mediates tetracycline resistance through an energy-dependent efflux system (45). Mar mutants are resistant to fluoroquinolones through a combined decrease in cell influx, e.g., a decrease in the porin OmpF, and an intrinsic efflux system (25). Chloramphenicol resistance in Mar mutants is also attributed to active efflux, which is enhanced over an intrinsic efflux system (96). The Mar phenotype is linked to overexpression of the *acrAB* locus; deletion of *acrAB* confers increased susceptibility to multiple drugs (85, 106) and organic solvents (144) in wild-type or Mar strains (85, 106, 144). These findings suggest that the *acrAB* efflux system is a major mechanism of Mar-mediated resistance (106). However, since the tetracycline (45) and fluoroquinolone (26) efflux systems were only saturated by the respective drug and not by others, it is probable that other drug-specific efflux systems are involved, particularly in high-level multidrug-resistant mutants.

TRANSCRIPTION OF THE *mar* LOCUS

Mutations in *marO* (Fig. 2) or *marR* (see below) (7, 8, 22, 51, 54, 87, 91) led to increased expression of *mar*-specific transcripts (7, 22, 24, 54, 87). Two transcripts, with sizes of ~1.1 and ~0.9 kb, arose from the *marRAB* operon (22, 24, 54, 112) and were also inducible by a number of different compounds including salicylate and tetracycline (see above).

Initial attempts to identify the transcriptional start site of *marRAB* were hindered (112), presumably because of extensive secondary structure (unpublished data using references 152 and 153). The transcriptional start site of the *marRAB* transcript was mapped by primer extension to nucleotide 1418 within *marO* (133) (numbering based on the original description of the *mar* locus [22]), a finding that is consistent with the "+1 rule" of transcriptional initiation (107). Other studies

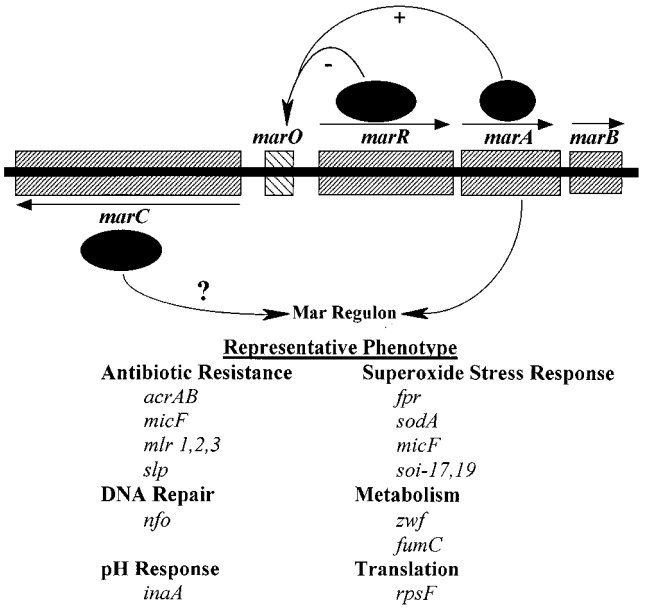

FIG. 1. Genetic organization of the *mar* locus in *E. coli*. *marC* and *marRAB* are transcribed divergently from *marO*. The repressor MarR (144 amino acids) negatively controls *marRAB* expression by binding to *marO*. MarA (127 residues) activates transcription of *marRAB* by binding to *marO* and other operons of the Mar regulon. MarB (72 amino acids) has an unknown function, but it may affect MarA. MarC (221 residues) encodes a putative inner membrane protein with multiple transmembrane-spanning helices and has an unknown function. Genes with known functions that respond to MarA and that are thus within the *mar* regulon include *acrAB* (a stress-induced efflux system) (83–85), *zwf* (glucose-6-phosphate dehydrogenase) (51, 63, 121), *micF* (antisense RNA controlling OmpF expression) (5, 20, 21, 57, 63, 100), *fpr* (ferredoxin reductase) (63, 79), *sodA* (Mn-containing superoxide dismutase) (51, 63, 136), *rpsF* (small ribosomal protein S6) (51, 124), and *fumC* (fumarase C) (7, 63, 78). Those of unknown function that respond to MarA include *inaA* (a weak acid-inducible gene) (119, 146), *slp* (a carbon starvation- and stationary phase-inducible lipoprotein) (2, 126), *mlr1*, *-2*, and *-3* (*mar* locus regulated genes) (126), and *soi-17* and *soi-19* (superoxide-inducible genes) (51, 69).

identified nucleotide 1417 as the *marRAB* transcriptional start point (112). These very similar findings provide experimental support for the original positioning of the −10 and −35 hexamers for *marRAB* (22) (Fig. 2).

The transcriptional control of the *marRAB* operon may be affected by the "marbox" sequence, a *cis*-acting element within *marO* identified as a MarA binding site (see below) (92). The marbox may be recognized by multiple proteins with known

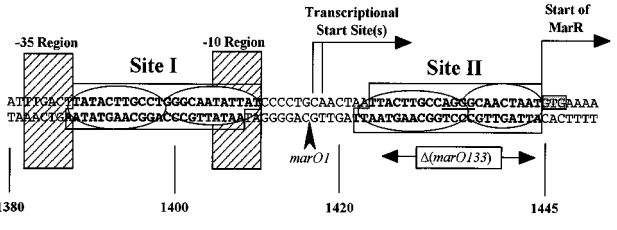

FIG. 2. MarR binding sites and other regulatory elements within *marO*. Sites I and II are the experimentally determined MarR binding regions (92). Numbering is according to Cohen et al. (22). The *marO133* contains a 20-bp deletion including site II (91); *marO1* contains a 20-bp tandem duplication between nucleotides 1416 and 1417 (54). The probable −35 and −10 hexamers of the *marRAB* operon are within the hatched boxes (22). The transcriptional start site(s) of the *marRAB* operon is at bp 1417 (112) or bp 1418 (133). The translational start of MarR, at nucleotide 1445, is shaded, and the putative MarR ribosome binding site is underlined within site II.

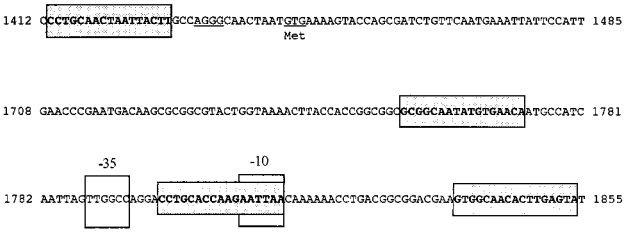

FIG. 3. Locations of a putative secondary promoter and putative marbox/soxbox site(s) distal to or within *marR*. Numbering is according to Cohen et al. (22). Only those regions of *marR* containing a putative marbox/soxbox site(s) are shown. The *marR* ribosome binding site and GTG translational start codon are underlined. The putative activator binding sites with sequence homology to the marbox (TNNGCAANNNNNNNCW) (92) and soxbox (ANNGCAYNNNNNNNCWA) (34, 76) sequences (where N is any base, W is either A or T, Y is a pyrimidine, and R is a purine) are within the shaded boxes. Potential −10 and −35 hexamers are indicated.

activator functions (see below) (63, 92), which may act differentially to control expression of the *mar* locus. An increase in *soxS* expression resulted in elevated levels of *mar*-specific transcripts (99). While this may be attributable to SoxS activation at a site within *marO* (see below), it may have resulted from a generalized stress response.

The molecular basis for the second smaller transcript (see above), for example, an internal promoter in *marR* (see below), degradation, or a processing event, is yet unclear (112). Findings have suggested that sequences within *marR* may enhance *marAB* expression (91). There are four putative "marbox/soxbox" consensus sequences in or near *marR*: one located proximal to *marR* and three within the *marR* coding sequence, at nucleotides 1413 to 1429, 1757 to 1773, 1798 to 1814, and 1838 to 1854 (Fig. 3). All of these sequences exhibit 76% or greater identity to the consensus marbox/soxbox sequences (Fig. 3 legend, and see below). If any sequence plays a role in *marAB* expression, the most likely would be that at nucleotides 1757 to 1773 within *marR*, just proximal to sequences that resemble potential −35 and −10 hexamers (Fig. 3). The spacing between this site and the potential −35 and −10 hexamers is similar to that found between the marbox and the −35 and −10 hexamers of *marRAB* (see below). However, the spacing between the putative −35 and −10 hexamers is shorter than is usually found in *E. coli* promoters.

An internal promoter has been described for the *emr* locus of *E. coli* (80), which encodes a multidrug efflux pump (82). This internal promoter occurs within the first structural gene of the operon (80), is growth-phase regulated (81), and is not responsive to the operon's repressor (80). A "secondary" promoter not under the control of MarR could ensure the constitutive presence of small amounts of MarA, which may be needed for low constitutive expression of the *marRAB* locus and other components of the *mar* regulon (see below). Phenotypic evidence for the basal expression of the *mar* locus in wild-type cells exists, since a deletion of *mar* leads to an increased susceptibility to multiple antibiotics and oxidative stress agents (22, 24, 51, 54).

The transcriptional termination signals of *marRAB* may involve a putative RNA stem-loop structure at nucleotides 2542 to 2559 located immediately downstream of the *marB* coding sequence. These sequences, which resemble a rho-independent transcriptional terminator (117), may facilitate pausing of the transcription complex and subsequently promote the transcriptional termination of highly expressed *marRAB* transcripts (91).

In *E. coli*, *marC* is inducible by tetracycline (22, 112) and

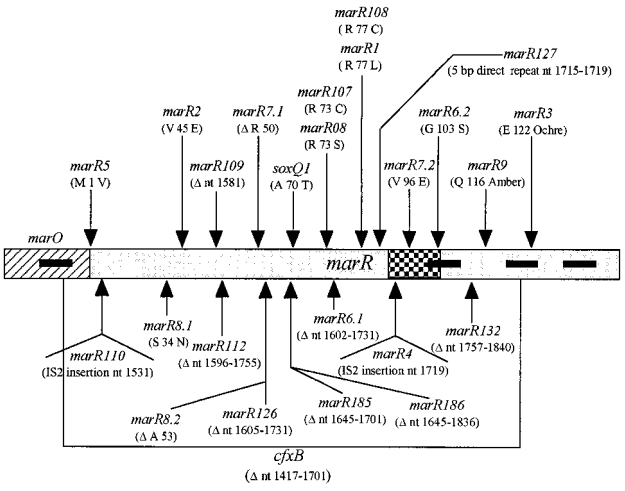

FIG. 4. Mutations within *marR*, the repressor of the *marRAB* locus. The *cfxB* (7, 51, 60), *soxQ1* (7, 51), *marR1* (22, 45), and *marR2*, *marR3*, *marR4*, and *marR5* (54) mutants have been described (22, 127). The quinolone-resistant *E. coli* clinical *marR* mutants (strains KM-D [*marR 6.1*, *6.2*], KM-F [*marR 7.1*, *7.2*], and J28 [*marR 8.1*, *8.2*]) all possessed both point mutations and deletions (87). While both *marR* mutations within these clinical isolates may function together to produce a Mar phenotype, the possibility that a single mutation may suffice cannot be ruled out (87). *marR9* is a Mar mutant of ML308-225 (145). The *marR107*, *marR108*, *marR109*, *marR110*, *marR112*, *marR126*, *marR127*, *marR132*, *marR185*, and *marR186* (91) and *marR08* (8) mutations have been characterized. The locations of the putative marbox/soxbox sites (darkened boxes) (see Fig. 3) and the conserved motif at amino acids 92 to 104 (checkered box) (see text) (99, 127, 135) are indicated. nt, nucleotide.

chloramphenicol (112). Induction of *marC* by salicylate was not detected in *E. coli* but was observed in *S. typhimurium* (133). Transcription from *marC* results in a single, ~0.9-kb transcript initiated from a region proximal to the putative promoter of *marC* at nucleotide 1266 (112).

MarR REPRESSION OF THE *marRAB* OPERON

That *marR* encoded the repressor of the *marRAB* operon was demonstrated when the locus was first identified (21, 22). In the absence of an inducer, MarR negatively regulates transcription of *marRAB* (22) by binding to *marO* (Fig. 2) (90, 127). The MarR-*marO* interaction is highly specific with an apparent K_d of $\approx 10^{-9}$ M (90, 127).

MarR is a member of a family of regulatory proteins, many of which are involved in sensing phenolic compounds (133). Currently, there are a number of MarR homologs (98). To date, a clear DNA binding motif for this family is not known. Computer analysis has revealed a conserved $AspXArgX_5$(Leu/Ile)$ThrX_2Gly$ segment (the amino acids are given the standard three-letter abbreviation, and X represents any residue), which lies at amino acids 92 to 104 in MarR, that may be important (98, 127, 133). Proteins within the MarR family may have similar functions, in that repression of the *marRAB* operon (analyzed by *marO-lacZ* fusions) can be achieved by other members of the family (133), for example, by Ec17kd (88) and MprA (EmrR) (29).

DNA footprinting experiments have shown that MarR protects both strands of *marO* from DNase I cleavage at two locations, sites I and II; MarR presumably binds as a dimer at each of these two sites (Fig. 2) (90). This proposal is in accord with the positioning of the transcriptional (22, 112, 133) and putative translational (22) start sites of the *marRAB* operon and *marR*, respectively (Fig. 2). Specifically, site I is located close to the −35 and −10 hexamers and site II spans the putative MarR ribosome binding site (Fig. 2) (90). Thus, MarR would negatively regulate expression of the *marRAB* locus as well as repress synthesis of itself (7, 22, 90).

In an *E. coli* strain bearing the *cfxB* mutation (Fig. 4) (7, 51, 59), in which site II but not site I of *marO* (Fig. 2) and most of *marR* are deleted (Fig. 4) (7), wild-type MarR synthesized in *trans* restored repression and thus antibiotic susceptibility (7). Another *E. coli* mutant, *marO133*, lacking only site II (Fig. 2), and presumably MarR (since the ribosome binding site proximal to *marR* was deleted [Fig. 2] [90]), also displayed a constitutive Mar phenotype (91). In this mutant as well, MarR synthesized in *trans* repressed expression of the Mar phenotype and purified repressor bound to site I of *marO* in vitro (90). These findings strongly suggest that site I may suffice to repress *marRAB* expression.

marR mutations (7, 8, 22, 45, 51, 54, 87, 91, 127) (Fig. 4), which render the repressor inactive, are scattered throughout the protein and have not particularly helped define the minimal sequences necessary for MarR function and specificity (Fig. 2). None of these mutations occur at any of the strictly conserved amino acids within the previously described motif in MarR (see above). This conserved region may represent the DNA binding domain, and residues outside of this region may confer promoter specificity and/or effector molecule binding. The N terminus is required for negative control; deletion of the first 19 amino acids of MarR eliminated repressor activity (127). Although it is not clear which mutations affect protein stability, many mutants (e.g., *marR1* and *soxQ1*) still exhibit inducibility (7, 24).

Upon exposure to a variety of structurally dissimilar compounds, MarR repression is alleviated (7, 24, 90, 127, 134). While this may result from direct inactivation of MarR by the inducers, such a phenomenon has only been demonstrated with salicylate at high concentrations (5 mM) (90). MarR binds to salicylate with a K_d of 0.5 to 1.0 mM (90). The binding of tetracycline to MarR was also observed, but at a concentration of greater than 10 mM (90). The repressor activities of MarR homologs, MprA (EmrR) (29, 80) and Ec17kd (88), are also antagonized by the presence of sodium salicylate (80, 135). However, another MarR homolog, MexR (115), did not appear to be responsive to salicylate (138).

These studies in vitro do not explain how other inducers, such as tetracycline or chloramphenicol, may inactivate MarR function. Each may interact directly with MarR, in a manner analogous to the interactions of multiple structurally dissimilar compounds with BmrR in *Bacillus subtilis* (1). While salicylate may affect MarR, the true (natural) inducer of the *mar* operon in *E. coli* remains to be determined. Some unidentified cellular product generated upon exposure to any one of these compounds may be involved. However, given the disparity in chemical structure [although all contain an aromatic ring(s)] and the variety of intracellular targets of these inducing compounds, the identity of a possible single metabolic intermediate is not obvious.

MarA, THE MASTER ACTIVATOR

From early studies, the *mar* locus (Fig. 1) was shown to control expression of other chromosomal genes (21, 22, 46). Expression of MarA in a wild-type or a *mar* locus-deleted strain conferred a Mar phenotype (22, 41, 91) (Fig. 1). Initially, the length of MarA was proposed to be 129 amino acids (22); however, it is more likely to be 127 amino acids. The spacing between the ribosome binding site and the initiation codon suggests that translation of the protein should begin at the second methionine within the open reading frame encoding

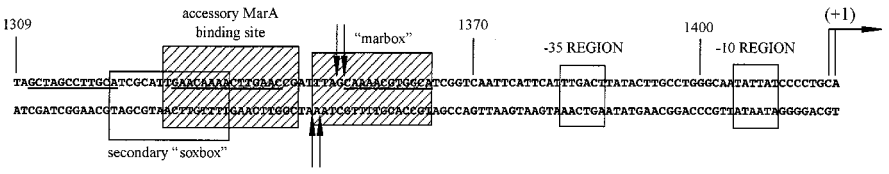

FIG. 5. MarA binding sites and putative transcription factor responsive regions within *marO*. Numbering is according to Cohen et al. (22). MarA, binding to the marbox at nucleotides 1349 to 1364, autoactivates *marRAB* expression and induces DNase I hypersensitivity at the sites indicated with arrows (92). A secondary putative soxbox, originally identified as the *cis*-acting element responsible for SoxS binding (34, 76) (nucleotides 1322 to 1337), and an accessory MarA binding site (nucleotides 1329 to 1346) are indicated (92). A Fis binding site is underlined at nucleotides 1330 to 1344 in the accessory MarA binding site (89). The other underlined regions at nucleotides 1311 to 1322 and 1353 to 1364 are similar to the XylS regulatory motifs, conferring XylS (a MarA homolog) responsiveness, in *P. putida* (39). The probable −35 and −10 hexamers of *marRAB* are boxed (22). The transcriptional start point(s) of *marRAB* is indicated (112, 133).

MarA. This observation, reported previously (6, 41), awaits experimental confirmation.

MarA is a member of the XylS/AraC family of transcriptional activators (38). Proteins within this family activate many different genes, some of which produce antibiotic and oxidative stress resistance or control microbial metabolism and pathogenesis (38). Such proteins are represented in a number of different genera (Table 1), as evident from a recent BLAST (3) search of the National Center for Biotechnology Information (NCBI) databases.

All proteins within the XylS/AraC family possess a 21-residue putative helix-turn-helix DNA binding domain (38). Since the second helix of the MarA helix-turn-helix motif contains a number of positively charged amino acids, an unusual feature of proteins with a helix-turn-helix DNA binding domain, it is speculated that this helix represents the "recognition" portion of the helix-turn-helix motif and that the primary mode of MarA-DNA recognition is nonspecific. The number of intracellular MarA targets and the variety of induced phenotypes support this proposal. The XylS/AraC family also possess a conserved C-terminal region, containing a consensus sequence of unknown function (38).

Most members of the XylS/AraC family possess a second domain, which may be located in either the N or C terminus, that participates in the binding of an effector molecule (38). However, MarA, like SoxS and OrfR (125) (also designated TetD from the Tn*10* transposon [15]), is considerably smaller than most other proteins in this family and apparently lacks an effector binding domain (22). Recent studies have revealed that a "cryptic" effector domain, similar to that found in AraC, resides in an alternate reading frame within *marR* (55). Thus, evolution may have altered this domain from the N terminus of MarA in order to form MarR or, conversely, incorporated the domain to produce the single larger AraC/XylS family members.

Since overexpression of MarA caused multiple changes in the cell (7, 21, 41, 51, 119), its ability to bind a number of promoters was expected (63). A 16-bp MarA binding site (Fig. 5), referred to as the "marbox," was found within *marO* (see Fig. 3 legend) (92). It is very similar to the "soxbox" identified as the *cis*-acting element required for SoxS binding (34, 76), with only two differences, at the first and seventh nucleotides (see Fig. 3 legend) relative to the consensus soxbox sequence (Fig. 5). One of these differences occurs within the conserved GCAY motif of the soxbox (see Fig. 3 legend), which represents the core SoxS binding sequence (76). The marbox maintains an adenine residue at a position occupied by a pyrimidine (Y) of this conserved motif (see Fig. 3 legend) (92). These subtle differences between the marbox and soxbox sequences may allow the marbox to be recognized by other transcriptional activators (see below) (92).

Purified MarA activates the transcription of a number of genes in vitro (Fig. 1) (63). It presumably binds (K_d, $\sim 2 \times 10^{-8}$ M) as a monomer to the marbox in *marO* (Fig. 5) (92) and activates transcription of *marRAB* (63, 92). MarA is "ambidextrous" for *marRAB* and *zwf* expression, since activation requires the C-terminal domain of the α subunit of RNA polymerase (63). For other genes, e.g., *fumC* and *micF*, this requirement is not seen (63). The transcriptional activation of *marRAB* by MarA in vitro is maximal when the concentration of RNA polymerase is limiting, suggesting that MarA may also recruit and/or stabilize the binding of RNA polymerase to responsive promoters (92). MarA renders two sites within *marO* hypersensitive to DNase I digestion, presumably as a result of DNA bending (92). MarA is also thought to induce conformational changes within the promoters of other responsive genes (151).

Deletion of the marbox in *marO* severely reduced the *marO*-MarA interaction and eliminated transcriptional activation by either MarA or MalE-SoxS in vitro (92). The presence of the soxbox (see Fig. 3 legend) is essential for either MarA or SoxS transcriptional activation of *zwf* (63). Other in vitro experiments demonstrated that hybrid promoters containing the soxbox confer a MarA responsiveness to genes not normally under the control of this protein (63).

The binding of MarA to *marO* occurs independently of MarR binding: MarA to the marbox (Fig. 5) and MarR to sites I and II (Fig. 2). These interactions are moderately competitive (92); the presence of MarA reduced the amount of MarR bound to *marO* (92). In addition, the presence of salicylate abolished the MarR-*marO* complex and had only a slight effect on the MarA-*marO* interaction (92).

In addition to the marbox, other regulatory elements may exist within *marO* (Fig. 5) (87). Sequences including an accessory MarA binding site and a putative secondary soxbox (Fig. 5) that is identical to the consensus SoxS binding site (34, 76) have been described (92). As seen with the marbox deletion (see above), elimination of the accessory MarA binding site impaired the *marO*-MarA interaction and reduced the MarA and MalE-SoxS-dependent transcriptional activation of the *mar* operon in vitro (92). Moreover, in cells, the absence of the accessory MarA binding site decreased expression of the *marRAB* operon and *mar* regulon as revealed by *marR*::*lacZ* and *inaA1*::*lacZ* fusions, respectively (92).

The minimal DNA sequences necessary for the transcriptional activation of XylS-regulated promoters in *Pseudomonas putida* have been determined (39); *marO* has similar motifs (Fig. 5). Two sequences located at positions −107 to −96 and −65 to −54, relative to the transcriptional start site of *marRAB* (Fig. 5), resemble the regulatory elements found in *P. putida* (39).

Given the multiple *cis*-acting elements within *marO* (Fig. 5), it has been suggested that the *marRAB* promoter can accommodate a number of MarA homologs (92). In particular, another MarA homolog, Rob, which binds *oriC* in *E. coli* (129), may substantially contribute to the basal levels of *mar* expres-

sion (89, 92), and this property is attributable to the presence of the soxbox (64). Overexpression of Rob in *E. coli* confers multiple antibiotic resistance (6, 103), and purified Rob is capable of binding stress-inducible promoters in vitro (6). An increase in SoxS expression confers multiple antibiotic resistance, and this phenotype is at least partially dependent on an intact *mar* locus (99). These results demonstrate the importance of MarA and Rob in controlling expression of the *marRAB* locus and suggest a minor role, if any, for SoxS.

In addition to the MarA homologs, Fis, a protein involved in recombination, DNA replication, and transcriptional activation (35), also binds *marO* (89), and this binding is dependent on the sequence at nucleotides 1330 to 1344 (Fig. 5).

Given the similarity in binding specificities and induced phenotypes of MarA, SoxS, and Rob (6, 33, 34, 63, 75, 98, 99, 144), the roles of these three proteins (and perhaps others yet to be found) may overlap, depending on the stress being confronted by the microbe. Little is known about how these proteins may function together in order to activate single chromosomal genes in the cell. Their discovery suggests that global chromosomal regulation of resistance to antibiotics and other toxic agents is a common survival function in *E. coli* and other bacteria.

A ROLE FOR MarB AND MarC IN MULTIPLE ANTIBIOTIC RESISTANCE?

The *E. coli marB* would encode a small, 72-amino-acid protein of unknown function (22). It possesses a putative ribosome binding site (22) and is transcribed (22, 24, 54, 112). Excluding the *S. typhimurium* homolog, which itself is only 42% identical (133), there are no other MarB-related proteins deposited within the NCBI databases. This finding is intriguing given the apparent conservation of multiple MarA (Table 1) and MarR homologs (98, 135).

In a *mar* locus-deleted strain complemented with partially deleted *marR* and full-length *marA* and *marB* sequences, multiple antibiotic resistance and constitutive expression of a MarA-responsive reporter construct (*inaA1*::*lacZ*) were observed (91). In the absence of *marB*, multiple antibiotic susceptibility increased and the expression of the reporter construct decreased (91). These latter phenotypes, however, were not produced by *marB* in *trans* (91). Moreover, a frameshift mutation in the 5′ region of *marB* did not reduce constitutive expression of the reporter construct described above (91). These results suggest that *marB* exerts a *cis*-acting effect on MarA activity (91). We found a 10-fold increase in the amount of a MalE-MarA fusion when produced from a *malE-marAB* construct (encoding MarA as a MalE fusion and MarB expressed alone) compared to that from a *malE-marA* construct without *marB* (unpublished data). Overexpression of *marB* alone had no effect on intrinsic drug resistance (143). When added to *marA* in a tandem construction, there was a small increase in the Mar phenotype (143). While the presence of *marB* may affect MarA activity or transcript stability, it may have a yet undefined function. Since it is conserved in *S. typhimurium*, and yet is much more divergent compared to the other components of the *mar* locus, some host-specific function other than transcript and/or protein stability may be involved.

In the initial descriptions of the *mar* locus, the DNA sequence proximal to *marRAB* was proposed to encode two individual peptides designated ORF64 and ORF157 (22). Subsequent resequencing, suggested by findings in *S. typhimurium* (133), revealed a single open reading frame that is now termed *marC*, a putative integral inner membrane protein consisting of 221 residues with multiple transmembrane spanning helices (50, 144). Recent analysis of the NCBI databases identified other MarC homologs. In addition to its counterpart in *S. typhimurium* (133), MarC is homologous to a hypothetical protein in the *E. coli* AdhE-OppA (67) and Asd-GntU (109) intergenic regions. Moreover, two hypothetically translated gene products in the *Methanococcus jannaschii* genome (16) are homologous to MarC.

Although the precise function of MarC is not known, *marC* cloned along with *marRAB* was needed to confer protection against the rapid bactericidal effects of the fluoroquinolones (50) and to produce organic solvent tolerance in a large chromosomal deletion strain (144). This finding, however, may be attributed to a greater production of MarA in constructs bearing *marC* and *marRAB* compared to those bearing *marRAB* alone, as detected with anti-MarA polyclonal antibodies (unpublished data) (144). Given the apparent resemblance of MarC to a membrane-bound transport protein, MarC may play a role alone or in association with other cellular proteins in drug influx and/or efflux.

TABLE 1. Bacterial MarA homologs[a]

Gram-negative bacteria		Gram-positive bacteria
Escherichia coli		*Lactobacillus helveticus*
MarA (22)	*Klebsiella pneumoniae*	**U34257** (31)
OrfR (15, 125)	**RamA** (46)	
SoxS (4, 149)		*Azorhizobium caulinodans*
AfrR (147)	*Haemophilus influenzae*	S52856 (43)
AraC (132)	Ya52 (36)	
CelD (108)		*Streptomyces* spp.
D90812 (101)	*Yersinia* spp.	**U21191** (71)
FapR (68)1)	CafR (40)	AraL (19)
MelR (142)	LcrF (58) or VirF (27)	
ORF_f375 (110)		*Streptococcus mutans*
RhaR (42, 111, 137, 141)	*Providencia stuartii*	MsmR (122)
	AarP (86)	
RhaS (104)		*Pediococcus pentosaceus*
Rob (129)	*Pseudomonas* spp.	RafR (74)
U73857 (32)	MmsR (131)	
XylR (130)	TmbS (28)	*Photobacterium leiognathi*
YijO (13)	XylS (97)	LumQ (77)
	Xys1,2,3,4 (9, 10)	
Proteus vulgaris		*Bacillus subtilis*
PqrA (60)		AdaA (102)
		YbbB (117)
Salmonella typhimurium		YfiF (150)
	Cyanobacteria	YisR (18)
MarA (133)	*Synechocystis* spp.	YzbC (116)
InF (66)	LumQ (65)	
PocR (120)	PchR (65)	

[a] The smaller MarA homologs, ranging in size from 87 (U34257) to 138 (OrfR) amino acid residues, are represented in boldface. References are given in parentheses.

THE MAR REGULON

The Mar regulon encompasses a diverse group of chromosomal genes that are responsive to MarA (Fig. 1). Among known genes are a number of newly discovered *mar* locus regulated (*mlr*) genes identified by using Tn*phoA* and Tn*lacZ* gene fusions (Fig. 1) (126). One of these, *slp*, is a stationary-phase-induced lipoprotein of yet unknown function (2). Increased antibiotic susceptibility was noted in *mlr*-inactivated strains suggesting some role in the intrinsic level of multiple

antibiotic resistance, but their precise function is yet to be determined.

The expression of the AcrAB efflux system is increased in Mar mutants (85). Deletion of the *acrAB* locus in a Mar mutant abolished the Mar phenotype (106). Moreover, overexpression of *acrAB* via a null mutation in *acrR*, the negative regulator of the *acrAB* locus (83), enhanced multiple antibiotic resistance (106). Expression of the AcrAB phenotype is contingent upon the presence of TolC (37), a protein that is postulated to act as an outer membrane efflux channel (12). A *tolC* insertional inactivation reversed the Acr phenotype (37). Since an *acrAB* mutation reversed the Mar phenotype (106), drug resistance is presumably linked to antibiotic export through AcrAB and TolC (37). It is, however, curious that AcrAB appeared inactive in the absence of its putative outer membrane channel. Other export systems, like the Tet protein, function without an outer membrane component (95, 139).

There exists phenotypic similarity between the *mar* and *sox* regulons (4, 7, 51, 98, 99, 144). The *soxRS* locus functions to protect *E. coli* from a variety of reactive oxygen species including nitric oxide, superoxide, and hydrogen peroxide (75) (for an extensive review of this topic, see reference 30). SoxS activates a number of superoxide stress as well as antibiotic resistance genes (51, 98). A menadione-selected mutant, expressing resistance to oxidizing agents, showed multidrug resistance (51) and was later determined to have a deletion in *marR* (Fig. 4) (7). Moreover, *mar* mutants display resistance to oxidative stress agents (7, 51).

inaA, a weak acid-inducible gene located at 48 min on the *E. coli* chromosome (146), is under the control of the Mar and SoxRS stress response systems (119). Plasmid-encoded MarA activates expression of an *inaA1*::*lacZ* fusion in strains deleted of *mar* (92, 119). The proposed *inaA* promoter (146) contains multiple marbox/soxbox sequences (data not shown).

CLINICAL IMPLICATIONS OF THE *mar* LOCUS

Mutations within the *mar* locus leading to constitutive expression of the *mar* operon were identified among clinical isolates of *E. coli* (87). The three mutants studied contained both deletions and point mutations in MarR (Fig. 4) and were resistant to multiple drugs including the fluoroquinolones (87). Other *marR* mutants, none of which were deletions, have been found among European fluoroquinolone-resistant *E. coli* blood isolates (105). These data demonstrate that a mutated *mar* locus can be involved in clinical *E. coli* specimens, although the frequency of its appearance is low (10 to 15% of all quinolone-resistant *E. coli* isolates analyzed).

In the clinical setting, first-step *mar* mutants would be termed susceptible, by the standards of the National Committee for Clinical Laboratory Standards, to most currently used antibiotics (Table 2). However, for some drugs, e.g., tetracycline, nalidixic acid, and rifampin, the early *mar* mutants can achieve levels of clinical resistance (Table 2). We propose that the *mar* locus may be important as a "stepping stone" to higher levels of resistance that in turn result from subsequent mutations elsewhere on the chromosome. First-step *mar* mutants, those selected upon exposure to low concentrations of antibiotics, resist the bactericidal effects of the fluoroquinolones (50) and are more easily selected to a higher level of fluoroquinolone resistance (25).

TABLE 2. *mar*-mediated changes in antibiotic susceptibility

Strain	MIC (μg/ml)[a]						
	Tet	Cm	Amp	Cef	Nal	Nor	Rif
AG100[b]	3	4	2	0.032	6	0.023	6
AG102[b]	16	16	6	0.19	32	1.25	16
Clinical[c]	≥16	≥32	≥32	≥64	≥32	≥16	≥4

[a] Antibiotics are abbreviated as follows: Tet, tetracycline; Cm, chloramphenicol; Amp, ampicillin; Cef, cefotaxime; Nal, nalidixic acid; Nor, norfloxacin; Rif, rifampin.

[b] Antibiotic susceptibilities for the wild-type (AG100 [42]) and *mar* mutant (AG102 [42]) reference strains were determined by E-test, for tetracycline, ampicillin, cefotaxime, and rifampin and aerobic dilution for chloramphenicol, nalidixic acid, and norfloxacin, in this laboratory (113).

[c] Clinical antibiotic resistance levels, determined by aerobic dilution according to the standards of the National Committee for Clinical Laboratory Standards.

OTHER MAR-RELATED AND REGULATORY MULTIDRUG RESISTANCE SYSTEMS

Since the initial description of *mar* (45, 46), other chromosomally located determinants specifying intrinsic multiple antibiotic resistance in gram-negative organisms have been described (6, 23, 44, 47, 52, 53, 56, 70, 82, 84, 86, 98, 103, 114, 123, 140). Some of these are multidrug efflux pumps (56, 82, 84, 114). Others, like *mar*, appear to be regulatory, not structural proteins for resistance (6, 47, 73, 86, 103). By using stringent DNA-DNA hybridization conditions, the presence of *mar* homologs among other members of the family *Enterobacteriaceae* was demonstrated (23). A MarA homolog, PqrA, has been identified in *Proteus vulgaris* (60), where active fluoroquinolone efflux was described (61). MarA-like elements termed RamA and AarP were identified in *Klebsiella pneumoniae* (47) and *Providencia stuartii* (86). The latter engenders a *mar* phenotype in both *P. stuartii* and *E. coli* (86). Another multidrug resistance gene, *romA*, described in *Enterobacter cloacae* (70) may be controlled by *ramA* in *Enterobacter* (47) and, by inference, *marA* in *E. coli.*

Other organisms display multiple antibiotic resistance phenotypes whose mechanisms of resistance suggest a regulatory component. Salicylate induces multiple antibiotic resistance in *Burkholderia* (*Pseudomonas*) *cepacia* (17). Multiple-antibiotic-resistant mutants of *Aeromonas salmonicida* have been isolated following exposure to low levels of antimicrobial compounds (148). In addition, tetracycline, chloramphenicol, and other substances induce a reversible antibiotic resistance in *Flexibacter* sp. (11). The fungus *Mucor racemosus* showed an inducible phenotypic cross-resistance to cycloheximide, trichodermin, and amphotericin B following pretreatment with cycloheximide or trichodermin (72). Further experiments showed that cycloheximide resistance was linked to an induced efflux (128). One may speculate that "*mar*-like" homologs or analogs may exist in these other genera.

In *Mycobacterium tuberculosis*, multidrug resistance is attributed to the sequential selection of resistance to individual drugs following incomplete chemotherapy (14). However, an alternative explanation, based on *mar* in *E. coli*, could envision a first-step *mar* mutation followed by mutations at other loci. We searched unsuccessfully for a MarA homolog in *Mycobacterium smegmatis* mc^2155 using Southern hybridization and degenerate PCR. However, when *marA* from *E. coli* was expressed in *M. smegmatis* mc^2155 under the control of the mycobacterial heat shock *hsp60* promoter, the transformed cells displayed multiple antibiotic resistance at high (37°C), but not low (30°C), temperatures (93). These data suggest that *M. smeg-*

matis mc^{2}155 contains a *mar* regulon and a MarA homolog or analog (93).

As recognition of regulation as a key determinant in multiple antibiotic resistance increases, the likely discovery of Mar-like homologs and analogs in other bacteria and microorganisms is expected. Critical to their discovery is the understanding that various external substances may activate a cell's resistance to multiple toxic agents.

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