JOURNAL OF BACTERIOLOGY, Apr. 1996, p. 2216–2223 Vol. 178, No. 8
0021-9193/96/$04.00+0

Autoactivation of the *marRAB* Multiple Antibiotic Resistance Operon by the MarA Transcriptional Activator in *Escherichia coli*

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Received 18 October 1995/Accepted 4 February 1996

Transcriptional activation of the promoters of the *mar*/*soxRS* regulons by the sequence-related but independently inducible MarA and SoxS proteins renders *Escherichia coli* resistant to a broad spectrum of antibiotics and superoxide generators. Here, the effects of MarA and SoxS on transcription of the *marRAB* promoter itself were assayed in vitro by using a minimal transcription system and in vivo by assaying β-galactosidase synthesized from *marR*::*lacZ* fusions. Purified MarA and MalE-SoxS proteins stimulated *mar* transcription about 6- and 15-fold, respectively, when the RNA polymerase/DNA ratio was 1. Purified MarA bound as a monomer to a 16-bp "marbox" located 69 to 54 nucleotides upstream of a putative RNA initiation site. Deletion of the marbox reduced MarA-*mar* binding 100-fold, abolished the stimulatory effects of MarA and SoxS on transcription in vitro, and reduced *marR*::*lacZ* synthesis about 4-fold in vivo. Deletion of upstream DNA adjoining the marbox reduced MarA binding efficiency 30-fold and transcriptional activation 2- to 3-fold, providing evidence for an accessory marbox. Although MarA and the *mar* operon repressor, MarR, bound to independent sites, they competed for promoter DNA in band shift experiments. Assays of *marR*::*lacZ* transcriptional fusions in *marRAB* deletion or *soxRS* deletion strains showed that the superoxide generator paraquat stimulates *mar* transcription via *soxRS* and that salicylate stimulates *mar* transcription both by antagonizing MarR and by a MarR-independent mechanism. Thus, transcription of the *marRAB* operon is autorepressed by MarR and autoactivated by MarA at a site that also can be activated by SoxS.

The MarA and SoxS proteins are transcriptional activators of at least a dozen promoters, the *mar* and *soxRS* regulons, that render *Escherichia coli* resistant to a variety of antibiotics and superoxide-generating agents. Genes known to be part of both regulons (denoted as the *mar*/*soxRS* regulon) include *fpr*, *fumC*, *inaA*, *micF*, *nfo*, *sodA*, *soi-17*/*19*, *zwf*, and three others identified by two-dimensional polyacrylamide gel electrophoresis (PAGE), while *acnA*, *acrAB*, and *pqi-5* have so far been identified only for one or the other regulon (2, 5, 8, 13–15, 18, 21, 22, 24, 25, 27, 35, 42).

The remarkable overlap of the regulons is reflected in other properties of the two activators. MarA and SoxS are members of the AraC subfamily of helix-turn-helix transcriptional activators and have more than 45% sequence homology with each other (1, 6, 11, 12, 43) and with a third protein, Rob, which can also activate antibiotic and superoxide resistance via at least some of the same genes (3, 19, 39). Purified MalE-SoxS (9, 18), native SoxS (23), and near-native MarA (MarA with a single histidine residue at its amino terminus [18]) have all been shown to bind specifically to regulon promoter regions. MalE-SoxS and MarA have also been shown to activate transcription of *mar*/*soxRS* regulon genes and to recognize a specific 21-bp *zwf* promoter "soxbox" sequence in vitro (10, 18). Furthermore, both proteins are "ambidextrous" (17, 18) in that their mechanism of activation requires the carboxy-terminal domain of the alpha subunit of RNA polymerase (16) at some promoters but not at others. For six genes of the *mar*/*soxRS* regulon, this requirement for the alpha carboxy-terminal domain correlates strictly with the location of the soxbox relative to the −35 promoter hexamer (17, 18).

The expression of these proteins is, however, controlled by different mechanisms. *marA* is negatively regulated as a part of the *marRAB* operon, which is autorepressed by MarR (2, 6) and derepressed by phenolic compounds such as salicylate (7, 37, 40). The binding of *mar* promoter DNA to purified MarR and to a MalE-MarR fusion protein has been demonstrated (29, 37). The MarR protein was shown to bind to the *mar* promoter region at two sites, between the presumptive −35 and −10 transcription signals and just 5′ to the first codon of marR (29). The affinity of MarR binding to its promoter is greatly reduced when it binds the ligand, salicylate (29). *soxS* is positively regulated by SoxR, which is itself activated by superoxides ($O_2^{\cdot -}$) that are generated by redox-cycling xenobiotics such as paraquat (14). *soxR* and *soxS* are divergently transcribed with the *soxR* promoter embedded in the *soxS* structural gene (1, 43). Interestingly, SoxS binds to the *soxS* promoter in vitro and negatively autoregulates expression of *soxS* (33).

While either *soxRS* or the *mar* operon can be activated in the absence of the other (13, 35), evidence that they interact has recently been reported. Miller et al. (32) found increased levels of *mar* mRNA in a constitutively active *soxR* mutant and suggested that *mar* expression may be stimulated by SoxS. This prompted us to test the *mar* promoter for the presence of a soxbox. We report here the existence of a "mar/soxbox" upstream of the −35 hexamer which binds MarA and enables activation of *mar* transcription by MarA and SoxS both in vitro and in vivo. Thus, the *mar* operon has the remarkable property that its first gene product, MarR, represses its transcription whereas the second gene product, MarA, stimulates its transcription.

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MATERIALS AND METHODS

Materials. Purified near-native MarA and MarR (each with a single histidine residue N terminal to the native protein) and the hybrid MalE-SoxS proteins

were prepared as previously described (9, 18, 29). Each was >95% pure as judged by sodium dodecyl sulfate-PAGE (SDS-PAGE). Restriction enzymes, deoxynucleotides, T4 DNA ligase, polynucleotide kinase, alkaline phosphatase, and Vent exo$^-$ DNA polymerase were purchased from New England Biolabs. Oligonucleotide primers were purchased from Operon Technologies. [γ-^{32}P]ATP (6,000 Ci/mol) was purchased from Dupont. Other chemicals were from Sigma Chemical Corp.

Plasmid and strain construction. Standard bacterial and molecular techniques were used throughout this study (31, 36). Plasmid pRGM258 (18) is a pTA108 (41) derivative containing the *mar* region (see Fig. 4) from nucleotide (nt) 1312 through 2769 (6) with a linker nucleotide (T) at nt 1311 and lacking two of three pTA108 *Bam*HI sites (nt 1 and 246). The pRGM258 nucleotides are numbered to keep *mar* sequence numbering the same as that of Cohen et al. (6). pRGM261 was constructed to convert the *mar* **C**AATTC sequence to a unique *Eco*RI restriction site (**G**AATTC) at nt 1370 to 1375 as follows. (i) Amplification by PCR, using pRGM258 DNA as template and pairs of primers corresponding to nt 1278 to 1300 and 1386 to 1358 in which nt 1370 was changed from G to C and nt 1699 to 1681 and 1358 to 1386 in which nt 1370 was changed from C to G, gave two oligonucleotides that were purified by electrophoresis in low-melting-point agarose. (ii) The oligonucleotides were mixed in a PCR mixture as templates by using primers from nt 1278 to 1300 and 1699 to 1681. (iii) The purified DNA fragment was digested with *Bam*HI (nt 1306) and *Sal*I (nt 1640) and cloned in the corresponding region of pRGM258.

mar promoter deletion mutants were constructed by digestion of pRGM261 with *Bam*HI and *Eco*RI and religation with appropriate synthetic DNA containing nt 1306 to 1311 of the vector and nt 1312 to 1370 of the *mar* sequence (thereby reestablishing the wild-type **C**AATTC sequence at nt 1370 to 1375). The synthetic DNAs were prepared from complementary oligonucleotides (Operon) with the following deletions: nt 1311 to 1328 in pRGM279 (Δ*marO279*), nt 1311 to 1333 in pRGM287 (Δ*marO287*), nt 1311 to 1338 in pRGM288 (Δ*marO288*), nt 1329 to 1346 in pRGM280 (Δ*marO280*), and nt 1347 to 1364 in pRGM281 (Δ*marO281*). These plasmid constructs were transformed into the Δ*mar inaA1*::*lacZ* strain N7962 (35), giving strains N8279, N8287, N8288, N8280, and N8281, respectively. The wild type in the series is N8258, carrying pRGM258. The sequences were verified by using Circumvent DNA sequencing kits (New England Biolabs).

marR::*lacZ* translational fusions were constructed from these pRGM261-derived promoter region deletion plasmids as follows. The plasmids were digested with *Rsa*I, and the nt 1240 to 1453 fragments were purified and ligated to pRGM276, an Amp^s derivative of the translational fusion vector pRS552 (38) that had been partially digested with *Sma*I. (Plasmids pRGM276 and pRGM275 [see below] were derived from pRS552 and pRS551 [38], respectively, by digestion with *Pst*I and *Sca*I and religation.) Recombinants that produced a blue-green colony on Luria-Bertani (LB) broth (35) plates supplemented with 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 30 μg of kanamycin per ml were selected, and their sequences were verified. The β-galactosidase is fused to MarR after the third amino acid of MarR.

marR::*lacZ* transcriptional fusions were constructed by digestion of the transcriptional fusion vector pRGM275 (see above) with *Bam*HI and alkaline phosphatase and religation with the purified *Bam*HI insert from the corresponding translational fusion plasmids. The appropriate recombinant plasmids were identified by the blue, Kan^r transformants of GC4468 obtained on LB–X-Gal–kanamycin. The plasmids are numbered pRGM351, -352, -353, -354, -355, and -356 and have the promoter deletions found in plasmids pRGM258, -279, -287, -288, -280, and -281, respectively.

Single copies of the transcriptional fusions were constructed in phage λ and inserted at *att*λ by the method of Simons et al. (38). Phage λRS45 was grown on the pRS551 derivatives and gave recombinants λRGM351 to -356, corresponding to pRGM351 to -356. Recipient cells were either *marRAB*$^+$ *soxRS*$^+$ Kan^s (GC4468), yielding strains N8581 to -8586; Δ*marRAB soxRS*$^+$ Kan^s (N7840), yielding strains N8591 to -8596; *marRAB*$^+$ Δ*soxRS* Kan^r (DJ901 [14]), yielding strains N8611 to -8616; or Δ*marRAB* Δ*soxRS* Kan^r (JHC1096 [13]), yielding strains N8621 to -8626. In the former two cases, blue, Kan^r colonies were selected; in the latter, lysogens were detected on the basis of their blue color on LB–X-Gal plates.

β-Galactosidase assays. Cells were treated with inducers for 1 h at 32°C and assayed for β-galactosidase activity as previously described (31, 35).

In vitro transcription assays. In vitro transcription analyses were carried out by a modification (17) of the procedure of Kajitani and Ishihama (20). Briefly, commercial RNA polymerase holoenzyme (Epicentre Technologies, Madison, Wis.) was incubated with template DNA for 15 min at 37°C to form open complexes, nucleoside triphosphates and heparin were added, and the mixtures were incubated to allow a single round of transcription. Transcription assays contained 2 nM *mar* DNA, 2 nM *gnd* DNA (a MarA/SoxS nonresponsive promoter serving as an internal control), and, except where noted otherwise, 4 nM RNA polymerase. When present, the concentration of MarA protein was 67 nM and the concentration of MalE-SoxS fusion protein was 200 nM. The *mar* promoter templates were obtained by PCR amplification of *mar* DNA using one primer corresponding to nt 1730 to 1714 (286 bp into *marR*) and the other corresponding to nt 1278 to 1300 for amplifying *mar* DNA from plasmids or nt 1300 to 1320 (over 80 bp upstream of the putative −35 promoter hexamer) for amplifying *mar* chromosomal DNA (*mar*[chr]) (10). The transcription products were separated by electrophoresis and quantitated with a Molecular Dynamics Phosphorimager.

Gel mobility shift and footprinting. Gel mobility shifts in the presence and absence of 5 mM sodium salicylate (29) and footprint analyses (4) were carried out by using a *mar* promoter fragment ^{32}P labelled at nt 1306 and extending to nt 1502. The number of MarA molecules bound to *mar* DNA was determined (34) by using a *mar* promoter fragment ^{32}P labelled at nt 1306 and extending to nt 1463 and polyacrylamide concentrations of 4, 6, 8, and 10%. Footprinting on the complementary strand employed a fragment labelled at nt 1460 starting with a 5′-labelled primer corresponding to nt 1460 to 1430 and an unlabelled primer corresponding to nt 1278 to 1300 and amplified by PCR for 20 cycles: 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. Incubation mixtures for DNase I digestion contained DNA in 100 μl with 920 ng of MarA, 13 μg of MarR, or both and 0.03 U of DNase I. In the absence of protein, 0.005 U of DNase I was added, and incubations stopped at the times indicated.

RESULTS

Transcriptional activation in vitro of *mar* by MarA and MalE-SoxS. The observation of Miller et al. (32) that overexpression of *soxS* increased *mar* mRNA levels in vivo suggested that the *mar* promoter, like promoters of the *soxRS* regulon, may be transcriptionally activated by SoxS. To test this, the effect of MalE-SoxS protein on *mar* transcription was measured in vitro. Initial assays were carried out with a molar ratio of RNA polymerase to total DNA of 10 and MalE-SoxS present at the concentration that yielded maximum stimulation of target gene transcription. Under these conditions of excess RNA polymerase, MalE-SoxS enhanced *mar*[chr] transcription ~1.3-fold (data not shown). However, when RNA polymerase was limiting (ratio of RNA polymerase to total DNA, 1) the extent of *mar* transcription stimulated by MalE-SoxS was much greater, about 5-fold, or 12- to 15-fold after normalization to transcription from the *gnd* DNA internal control (*gnd* is not part of the *mar* or *soxRS* regulon) (Table 1).

Having previously demonstrated that promoters that respond to MalE-SoxS also respond to MarA (18), we tested purified MarA for its ability to stimulate *mar* transcription. As with MalE-SoxS, MarA enhanced *mar* transcription only ~1.3-fold when RNA polymerase was in 10-fold excess (Fig. 1A), but when RNA polymerase was limiting, MarA stimulated *mar* transcription by 4.1- to 4.9-fold, or 5.5- to 6.3-fold when normalized to *gnd* (Fig. 1B; Table 1). Thus, in a minimal in vitro system under conditions of both limiting and excess RNA polymerase, both MalE-SoxS and MarA transcriptionally activate the *mar* promoter.

MarA-*mar* DNA complex. In view of the strong stimulation of *mar* transcription by MarA, the binding of MarA to a 197-bp end-labelled *mar* promoter fragment was examined by gel mobility shift studies. In most studies a single retarded complex was observed in the gels (Fig. 2A, lanes 2 to 5). This binding was sequence specific as shown by the ability of unlabelled *mar* promoter DNA, but not salmon sperm DNA, to compete for MarA (data not shown). Occasionally, a second, more slowly migrating complex was seen at the highest MarA concentrations (Fig. 2A, lane 1). As this was not consistently found, we were unable to determine by gel retardation whether the binding in the second complex was also site specific.

To determine the number of MarA molecules bound to the single complex, electrophoretic mobility shift assays were carried out at different gel concentrations (34). The molecular weight of the bound protein was determined to be 13,500 or close to the expected molecular weight of a MarA monomer (15,500). This is consistent with our observations that MarA exists in solution as a monomer (30) and with our finding that Rob and MalE-SoxS bind as monomers to the *zwf* and *fumC* promoters (19). The dissociation constant (K_D) for the single MarA-*mar* complex is calculated to be $\sim 2 \times 10^{-8}$ M in the absence of nonspecific competitor DNA.

TABLE 1. Activation of *mar* transcription by MalE-SoxS and MarA

DNA template	Ratio[a]															
	MalE-SoxS[b]								MarA[c]							
	Expt 1		Expt 2		Expt 3		Avg		Expt 1		Expt 2		Expt 3		Avg	
	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]	Raw	Normalized[d]
mar[chr][e]	5.7	10	5.0	19			5.3	15	6.0	5	3.8	5.9			4.9	5.5
mar258			7.3	17	2.2	6.9	4.7	12			4.0	6.5	4.3	6.0	4.1	6.3
Δ*mar279*	5.3	6	3.2	16	5.6	8.9	4.7	10	4.2	4.9	16.0	9.7			10.0	7.3
Δ*mar287*			7.4	20			7.4	20			7.5	7.4			7.5	7.4
Δ*mar288*			5.2	20	4.6	16	4.9	18			4.7	9.9	6.2	6.1	5.5	8.0
Δ*mar280*	2.9	2.4	2.0	4.3	2.0	4.3	2.3	3.7	3.4	3.1	3.8	3.2	4.5	3.3	3.9	3.2
Δ*mar281*	0.6	0.9	0.3	1.0	0.28	0.7	0.4	0.84	1.1	1.1	1.9	0.63	0.8	0.84	1.0	0.87

[a] Of counts incorporated into RNA in the presence of protein to those incorporated in the absence of protein.
[b] 6 pmol.
[c] 2 pmol.
[d] To the transcription of *gnd*.
[e] Template obtained by PCR from wild-type chromosomal DNA as described in Results.

Independent binding of MarA and MarR with *mar*. Since MarR binds to the *mar* promoter region at two different sites (29), we compared the binding of MarA with that of MarR. Two complexes of MarR with *mar* were seen (Fig. 2A, lanes 6 to 9). The mobility of the first, faster-migrating MarR complex corresponded roughly to that of the ephemeral second complex of MarA with *mar* (Fig. 2A, see arrow). Since the purified MarA and MarR proteins used here are similar in size (calculated molecular weights, 15,500 and 16,100, respectively), MarR appears to bind as a dimer at each of its two sites, as previously suggested (29).

To determine whether MarA influenced the binding of MarR and vice versa, the effect of incubating both proteins with *mar* DNA was analyzed (Fig. 2A, lanes 10 to 13 and 14 to 17). In addition to the bands that were previously seen with MarA or MarR alone, new bands were observed. The complexes are most readily interpreted as corresponding to *mar* bound with (i) one MarA monomer (A), (ii) one MarR dimer (R2), (iii) mixed trimers (monomeric MarA plus dimeric MarR [AR2]), (iv) two MarR dimers (R4), or (v) mixed pentamers (monomeric MarA and two MarR dimers [AR4]). Thus, MarA and MarR appear to bind to independent sites.

Nevertheless, quantitation of the bands reveals some competition between the proteins for binding. Fewer MarR-bound complexes were found in the presence (Fig. 2A, lanes 10 to 13 and 14 to 17) than in the absence of MarA (lanes 6 to 9). For example, in lane 7, 13% of the DNA was unbound, 28% was complexed to MarR dimers (R2), and 59% was complexed to tetramers (R4). If MarA were noncompetitive with MarR, then the sample to which MarA was added (lane 11) should contain no more than 13% monomer (A) complexes and the

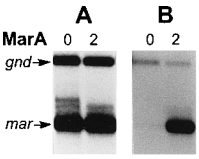

FIG. 1. MarA activation of in vitro *mar* transcription. In vitro transcription was carried out in a mixture containing *mar* and *gnd* DNA fragments in the presence and absence of 2 pmol of purified MarA protein per 30-μl reaction mixture. (A) Transcription carried out at a molar ratio of RNA polymerase to total DNA of 10; (B) transcription carried out at a molar ratio of RNA polymerase to total DNA of 1.

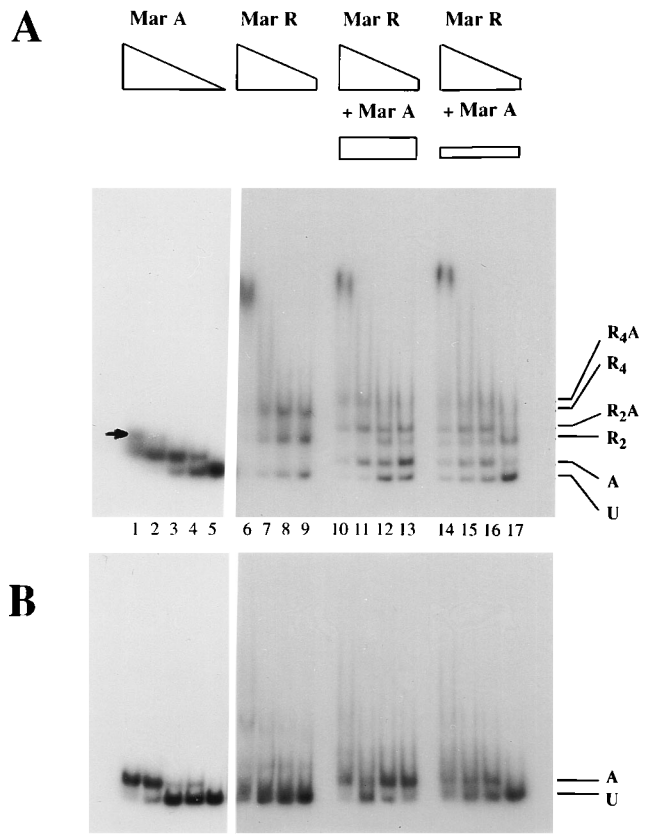

FIG. 2. Electrophoretic mobilities of *mar* promoter complexes with MarA and MarR. (A) A 197-bp ^{32}P-labelled wild-type *mar* promoter fragment (nt 1306 to 1502) was incubated in 10 μl for 30 min alone (lane 5); with 23, 12, 5.5, or 2.76 ng of MarA (lanes 1 to 4, respectively); with 260, 130, 66, or 33 ng of MarR alone (lanes 6 to 9, respectively); with the amounts of MarR in lanes 6 to 9 plus 12 ng of MarA (lanes 10 to 13, respectively); or with the amounts of MarR in lanes 6 to 9 plus 5.5 ng of MarA (lanes 14 to 17, respectively). (B) Samples identical to those in panel A were incubated with 5 mM sodium salicylate in the binding buffer. The samples were then subjected to electrophoresis without (A) or with (B) salicylate in the gels and gel buffer. Following electrophoresis and drying of the gels, the DNA was visualized by radioautography. The hypothesized numbers of MarA or MarR molecules in each complex are indicated by subscripts in the designations.

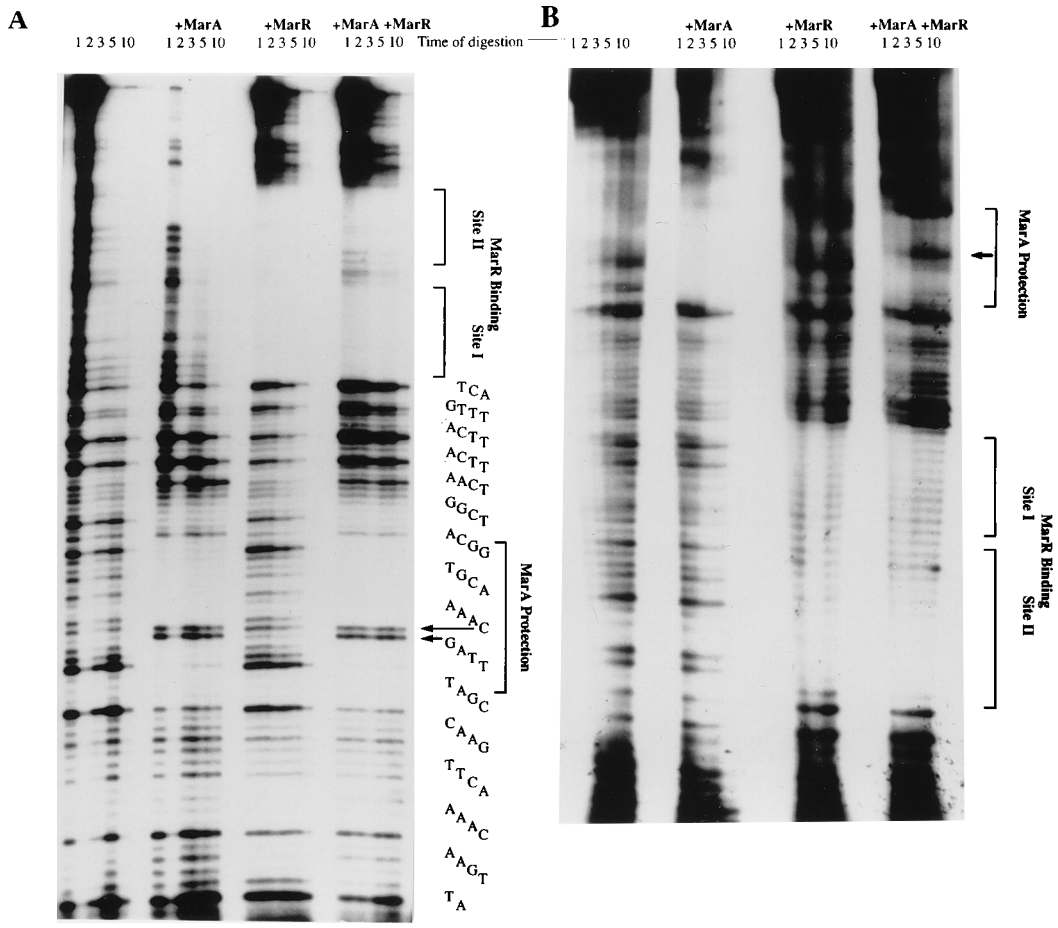

FIG. 3. DNase I footprint analysis of the *mar* promoter in the presence of MarA and MarR. (A) Analysis of the strand proceeding from upstream of the −35 signal towards the *marR* gene. Regions of protection by MarA and MarR and sites of hypersensitivity induced by MarA are indicated. Note the lack of MarA protection at the bottom of the gel, where the sequences deleted in Δ*marO280* are clearly visible. (B) Analysis of the complementary strand.

ratio of trimers (AR2) to pentamers (AR4) should remain 1:2 (28 to 59%). In fact, in lane 11, 34% of the DNA moved as monomers, 27% migrated as trimers, and only 29% migrated as pentamers. This indicates that the binding of MarA competes with the binding of MarR even though MarA and MarR bind to different sites in the promoter.

Effects of salicylate on binding. Since MarR complexes with the promoter region are disrupted when MarR binds to salicylate (29), an inducer of the *mar* operon (7), the effect of salicylate on MarA complexes was examined. When the samples analyzed above were subjected to electrophoresis in the presence of 5 mM salicylate (Fig. 2B), the complexes with MarR were clearly abolished, confirming the previous report. However, salicylate had only a marginal effect on the MarA complexes under the same conditions.

MarA binding site. To determine where MarA binds to *mar*, DNase I footprint analyses were carried out with MarA alone, MarR alone, or both (Fig. 3). MarA was found to protect a 16-bp site from DNase I digestion and rendered two positions within that region hypersensitive to DNase I (Fig. 3 and 4). The hypersensitivity suggests that MarA may bend the DNA. No protection was seen elsewhere. MarR alone bound to the sites

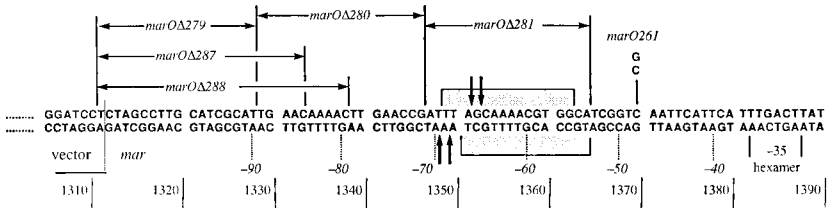

FIG. 4. Sequence and map of the upstream portion of the *mar* promoter showing the *mar* nt 1312 to 1390 sequence and the 7 bp of the vector that precede it in plasmid pRGM258, the extents of the *mar* deletions described in this article, the location of the *marO261* C:G to G:C transversion in pRGM261, and the putative −35 hexamer. The bases on each strand protected by MarA from DNase I activity (shading) and sites rendered DNase I hypersensitive by MarA (vertical arrows) are indicated. The negative numbers indicate the distance from the putative mRNA initiation site at nt 1418.

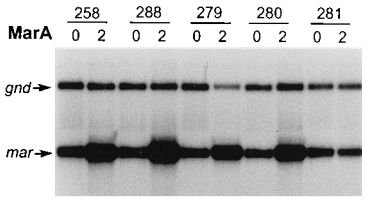

FIG. 5. Effects of upstream deletions on MarA activation of *mar* transcription in vitro. Transcription reactions were carried out on DNA templates with the indicated promoter region deletions under conditions of limiting RNA polymerase in the presence and absence of purified MarA.

I and II previously described (29). The protection by MarA was not altered by the presence of MarR or vice versa, as could be anticipated from the band mobility shift assays described above.

Surprisingly, this "marbox" at nt 1349 to 1364 (−69 to −54 relative to the putative RNA initiation site at nt 1418) is just downstream of a sequence at nt 1322 to 1337 that resembles the degenerate soxbox consensus sequence even better than does the marbox (9). To analyze the role in transcription of the MarA binding site and of these upstream sequences, a series of deletions in the region from nt 1312 to 1364 (Fig. 4) were introduced into pRGM258, a plasmid containing the wild-type *mar* region from nt 1312 to 2769. PCR-amplified DNA fragments were assayed for their abilities to act as templates for in vitro transcription and for the stimulation of transcription by MarA and MalE-SoxS (Fig. 5; Table 1). Transcription of Δ*marO279* DNA, with a deletion of *mar* nt 1312 to 1338 (and nt 1311 of the vector), was stimulated by both MarA and MalE-SoxS to the same extent as that of the wild type (approximately fivefold). Δ*marO281*, with a deletion of the region protected by MarA from DNase I (nt 1347 to 1364), retained template activity for transcription but was not stimulated by MarA or MalE-SoxS. This result confirms the importance of the mar/soxbox at nt 1349 to 1364 for activation of the *mar* promoter.

Curiously, deletion of the 18 bp (nt 1329 to 1346) lying just upstream of the MarA binding site in Δ*marO280* diminished the stimulation of transcription by MarA (to 3.9-fold) and MalE-SoxS (to 2.3-fold). An imperfect (10 of 16 bp) direct repeat of the protected site is present at nt 1329 to 1344 and a less imperfect (14 of 20 bp) direct repeat of nt 1342 to 1361 is present at nt 1322 to 1341. Conceivably, nt 1329 to 1346 includes an accessory MarA binding or loading site which is important for transcriptional activation. Binding of MarA to such a site might be the cause of the second complex occasionally seen by gel mobility shifts (Fig. 2A, lane 1), but binding there may not be strong enough to be detected by footprint analysis. Two other promoter region deletions, Δ*marO287* and Δ*marO288*, with the same 5′ end point as Δ*marO279* but which extend through nt 1333 and 1339, respectively, had no detectable effect on *mar* transcriptional activation by MarA or MalE-SoxS (Table 1).

In view of the diminished activation of transcription by MarA and MalE-SoxS with the Δ*marO280* template, gel retardation assays were performed to compare the binding of MarA to these promoter deletion DNAs. The affinity of MarA for the wild-type promoter fragment was approximately 30-fold greater than for Δ*marO280* and 100-fold greater than for Δ*marO281* (data not shown). This suggests a role in binding for both sequences.

Activities of *marR*::*lacZ* transcriptional fusions. In vivo activation of the *mar* operon by MarA or SoxS was assessed by measuring the β-galactosidase activities of single-copy *marR*::*lacZ* transcriptional fusions integrated at *att*λ in wild-type, *marRAB* deletion, *soxRS* deletion, or doubly deleted strains following exposure to known *mar* or *soxRS* inducers (Table 2). Deletion of the *marRAB* structural genes abolishes both MarR-specified repression and MarA-specified activation. Similarly, the *soxRS* deletion eliminates the possibility of SoxS activation. The effect of deletion of the marbox and adjacent sequences on the expression of the *marR*::*lacZ* transcriptional fusions was investigated to evaluate further the function of this region. The results, summarized as follows, show that *mar* can be activated in vivo via either MarA or SoxS acting on the marbox promoter region.

(i) All of the *marR*::*lacZ* promoter region deletions except for Δ*marO279* adversely affected promoter activity irrespective of the presence or absence of *marRAB* or *soxRS*. The Δ*marO280* and Δ*marO281* deletions were the most severely affected, but even the Δ*marO287* and Δ*marO288* deletions had reduced activities. Thus, both basal and derepressed levels of *mar* promoter activity are dependent on sequences between nt 1329 and 1364 (−89 to −54). Similar effects of these deletions on β-galactosidase synthesis from *marR*::*lacZ* translational fusions carried on multicopy plasmids have been observed (data not shown), suggesting that the action of MarA on the *mar* promoter affects transcription primarily.

(ii) Salicylate derepresses *mar* transcription in vivo (7), pre-

TABLE 2. β-Galactosidase activities of *marR*::*lacZ* transcriptional fusions incubated with the indicated inducers for 1 h at 32°C

Parental strain and *mar* promoter (strain)	β-Galactosidase		Fold stimulation by[a]:		
	Sp act[b]	Activity relative to wt[c]	SAL	PQ	SAL + PQ
GC4468 (*mar*$^+$ *sox*$^+$)					
wt (N8581)	92	1.0	12	1.5	16
Δ*279* (N8582)	94	1.0	13	1.7	13
Δ*287* (N8583)	62	0.68	11	1.9	16
Δ*288* (N8584)	35	0.38	20	2.5	28
Δ*280* (N8585)	22	0.23	10	1.3	14
Δ*281* (N8586)	21	0.22	6.3	0.7	5.9
N7840 (Δ*mar sox*$^+$)					
wt (N8591)	1,130	1.0	2.2	1.7	2.7
Δ*279* (N8592)	1,080	0.95	2.8	2.0	3.4
Δ*287* (N8593)	750	0.66	2.2	2.1	3.7
Δ*288* (N8594)	640	0.57	3.0	2.7	4.9
Δ*280* (N8595)	520	0.46	2.4	1.4	2.8
Δ*281* (N8596)	430	0.38	2.7	0.99	2.4
DJ901 (*mar*$^+$ Δ*sox*)					
wt (N8611)	103	1.0	12	0.92	10
Δ*279* (N8612)	96	0.93	13	1.0	13
Δ*287* (N8613)	74	0.72	13	1.0	12
Δ*288* (N8614)	36	0.35	23	1.1	24
Δ*280* (N8615)	48	0.47	10	1.1	9.6
Δ*281* (N8616)	17	0.17	9.8	1.1	9.2
JHC1098 (Δ*mar* Δ*sox*)					
wt (N8621)	1,150	1.0	3.1	1.2	3.4
Δ*279* (N8622)	1,440	1.3	2.6	1.2	2.7
Δ*287* (N8623)	820	0.71	2.8	0.96	3.1
Δ*288* (N8624)	740	0.64	3.2	1.0	3.4
Δ*280* (N8625)	590	0.51	2.8	1.1	3.3
Δ*281* (N8626)	580	0.50	2.5	1.1	3.0

[a] SAL, 5 mM sodium salicylate; PQ, 500 μM paraquat.
[b] Standard deviation, approximately 15%.
[c] wt, wild type.

sumably by binding to and inactivating MarR, as in vitro (29). In agreement with this, the effect of salicylate on *marR*::*lacZ* transcription was greatest in *marR*$^+$ cells even when segments of the nt 1312 to 1364 promoter region were deleted from the *marR*::*lacZ* reporter fusion. Interestingly, the Δ*marO288* deletion responded exceptionally well to induction by salicylate in *marRAB*$^+$ cells, suggesting that increased production of MarA can overcome the Δ*marO288* defect.

Surprisingly, even in Δ*marRAB* strains, in which *marR*::*lacZ* transcription was derepressed about 12-fold, salicylate further stimulated *marR*::*lacZ* expression two- to threefold whether in a *sox*$^+$ or Δ*soxRS* background and independently of deletions in the nt 1312 to 1364 region. This *marR*-independent effect might be due to the presence of a secondary MarR-like repressor that is also inactivated by salicylate. Several such candidates with homology to MarR, such as MprA (also known as EmrR [26]), have recently been identified (40). Alternatively, salicylate might enhance the activity of RNA polymerase at this promoter either directly or by inducing the expression of an unknown activator that does not require the nt 1312 to 1364 region.

(iii) Genetically manipulated overexpression of *soxS* or treatment of *soxRS*$^+$ cells with redox-cycling compounds such as paraquat has been found to increase *mar* operon transcription (32, 37). Paraquat, which induces *soxS* expression, induced *mar* transcription 1.5- to 2.7-fold but not in Δ*soxRS* strains and not if the *mar* promoter marbox was deleted. Paraquat only minimally stimulated *mar* transcription from the Δ*marO280* promoter, also suggesting a role for nt 1339 to 1346 in recognizing SoxS, as seen above in vitro (Table 1; Fig. 5). This strongly implicates the following chain of events: activation of SoxR by paraquat-generated superoxides activates the synthesis of SoxS, which in turn activates the *mar* promoter, provided that the mar/soxbox sequences between nt 1339 and 1364 are present. In the absence of treatment with *soxRS* inducers, basal *soxRS* expression does not appear to contribute significantly to *mar* expression, as was found previously for *inaA1*::*lacZ* expression (35).

Consistent with these hypotheses, simultaneous treatment of the cells with paraquat and salicylate was more effective in inducing *marR*::*lacZ* transcription than treatment with salicylate or paraquat alone. As above, the effect of paraquat was dependent on *soxRS* and the region between nt 1339 and 1364, while the effects of salicylate could be separated into MarR-dependent and MarR-independent categories.

Kinetics of *marR*::*lacZ* induction by salicylate. The ability of MarA to autoactivate the *mar* promoter was studied as a function of time following induction by salicylate. Wild-type cells carrying wild-type or promoter region deletion *marR*::*lacZ* transcriptional fusions were induced with 5 mM salicylate, and β-galactosidase activity was measured (Fig. 6). In each case, the relative activities of the uninduced cells were those seen previously (Table 2), and in each case elevated transcription was seen in 10 min and reached maximum by 30 min. Interestingly, while the noninduced level of β-galactosidase in strain N8584 which carries the Δ*marO288* promoter deletion was less than 40% of that of the wild type, after 30 min of salicylate treatment it was over 60% of the induced wild-type activity. On the other hand, *marR*::*lacZ* fusions carrying promoter deletions Δ*marO280* and Δ*marO281* were reduced to 20% or less of the wild-type activity both before and following induction by salicylate. Why the induced synthesis appears to peak at 30 min and why the Δ*marO288* promoter responds so well to induction (Table 2) are not understood.

Effect of *mar* promoter region deletions on *inaA1*::*lacZ* expression. Since the *mar* promoter region deletions affect *mar* promoter activity, they should also affect the amount of MarA synthesized and thus the expression of *mar*/*soxRS* regulon genes. To test this, multicopy *marRAB*$^+$ plasmids with promoter region deletions were transformed into the Δ*mar* strain N7962 which contains a *mar*/*soxRS* regulon reporter gene *inaA1*::*lacZ* (35) and β-galactosidase activities were measured. Salicylate induced β-galactosidase synthesis seven- to ninefold in all of the strains except those with Δ*marO280* or Δ*marO281* promoters, for which the induction was only about fivefold (Table 3). Thus, maximum activation of the *mar* regulon promoter *inaA* depends on the autostimulatory effect of MarA on the *mar* operon.

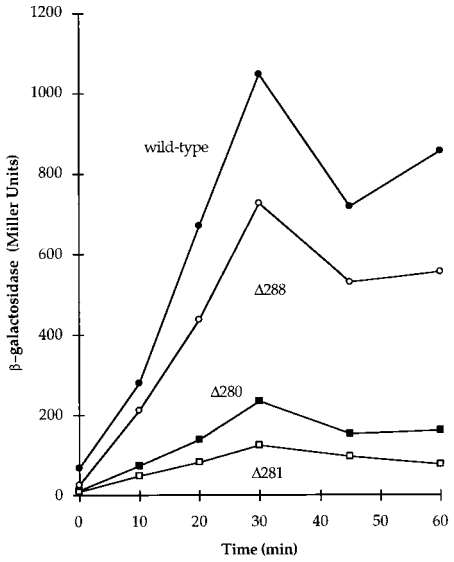

FIG. 6. β-Galactosidase synthesis by a wild-type strain (GC4468) with a wild-type (filled circles), Δ*marO288* (open circles), Δ*marO280* (filled squares), or Δ*marO281* (open squares) *marR*::*lacZ* single-copy transcriptional fusion following the addition of 5 mM sodium salicylate at 32°C. Standard deviation, about 15%.

DISCUSSION

Transcriptional activation in vitro. The report (32) that overexpression of SoxS induced *mar* transcription in vivo led us to test for transcriptional activation of *mar* by purified MarA and MalE-SoxS proteins. Both proteins transcriptionally activated the promoter in vitro, but the extent was greatest when the ratio of RNA polymerase to promoter was 1 (Fig. 1 and Table 1). This is consistent with a role for MarA in stabilizing

TABLE 3. β-Galactosidase activities of the *inaA1*::*lacZ* transcriptional fusion in a *mar* deletion strain carrying *marRAB*$^+$ plasmids with the indicated *mar* promoter deletions

Plasmid (*mar* promoter)	β-Galactosidase (Miller units)[a]		
	Uninduced	With 5 mM salicylate	Fold increase
pRGM258 wt	47	421	8.9
pRGM279 Δ*279*	46	420	9.1
pRGM287 Δ*287*	61	438	7.2
pRGM288 Δ*288*	42	353	8.3
pRGM280 Δ*280*	39	190	4.9
pRGM281 Δ*281*	36	170	4.7

[a] Standard deviation, approximately 15%.

the binding of RNA polymerase to the promoter, an interaction that was seen in vitro for SoxS and RNA polymerase at *nfo* and *micF* promoters (23).

Identification of a marbox in the *mar* promoter. Gel mobility assays indicated that MarA bound to *mar* as a monomer. Footprinting experiments identified a sequence at −69 to −54 (nt 1349 to 1364) which contained sites that MarA either protected from or rendered hypersensitive to DNase I (Fig. 4). The hypersensitive sites might be indicative of the DNA bending that has been found for MarA and MalE-SoxS bound to *zwf* and *fumC* DNA (44). We surmise, from the distance of this marbox (centered at −61.5) to the putative −35 transcriptional signal (17, 18), that activation of *mar* by MarA requires interaction with the carboxy-terminal domain of the alpha subunit of RNA polymerase, i.e., that MarA is a class I activator (16) of *mar* as it is for *zwf*.

The marbox sequence is critical for *mar* transcriptional activation by both MarA and MalE-SoxS in vitro since marbox deletion (Δ*marO281*) DNA was not activatable by either protein. Furthermore, deletion of the marbox severely reduced its ability to bind MarA in vitro and reduced *mar*::*lacZ* expression about fourfold in vivo (Table 2). This suggests that the Δ*marO281* promoter cannot respond to MarA in vivo, and so it may be used to distinguish MarA-dependent activation from other causes. Indeed, paraquat, which induces SoxS expression (14), induced *mar* transcription twofold only when the marbox and *soxRS* were present (Table 2). This indicates that the marbox is also required for activation of *mar* by SoxS. In contrast, salicylate induces *mar* transcription (about 12-fold) by two discernible modes. The principal effect (about sixfold) is due to inactivation of MarR, as previously found (7, 29, 37), but a lesser (twofold) effect is *marRAB* and marbox independent. This could be due to inactivation by salicylate of an ancillary *mar* repressor, such as EmrR (26, 40), or to a novel, direct effect of salicylate on *mar* transcription.

A marbox accessory region. Promoter region deletion analysis shows that the region adjacent to the marbox from nt 1329 to 1346 (−89 to −72) also plays a role in binding and activation. In the absence of this region (Δ*marO280*), binding of MarA decreased about 30-fold and transcriptional activation by MarA and MalE-SoxS was reduced about 3-fold (Table 1). Deletion (Δ*marO279*) of the 17 *mar* base pairs further upstream (nt 1312 to 1328) had no significant effect on transcriptional activation in vitro or in vivo, while deletion of nt 1312 to 1333 in Δ*marO287* or of nt 1312 to 1338 in Δ*marO288* had no significant effect in vitro but had a small effect in vivo. This implies that the sequence between nt 1329 and 1346 plays an "accessory" role in MarA and MalE-SoxS transcriptional activation of, and MarA binding to, the *mar* promoter. Interestingly, although the region from nt 1327 to 1344 (−91 to −74) contains an imperfect direct repeat (12/18 identity) of the marbox region at nt 1347 to 1364, this sequence was not protected from DNase I attack by MarA (Fig. 3). How the accessory region enhances transcriptional activation remains to be determined.

MarA-MarR interference. Purified MarA bound promoter DNA as a monomer (estimated K_D, $\sim 2 \times 10^{-8}$ M; Fig. 2) at a site distinct from those bound by MarR (Fig. 3). Nevertheless, the binding studies suggest that MarA can reduce MarR binding to the promoter (Fig. 2). If this occurs in vivo, it could be a second mechanism by which MarA affects *mar* transcription.

Physiological consequences of the *mar* promoter marbox. These studies revealed two unique features of *marRAB* operon regulation. (i) The first gene of the operon encodes a repressor of its transcription, while the second gene encodes an activator. This would seem to poise *mar* for rapid activation upon loss of repression. However, we did not observe a major effect of the presence of the marbox on *mar* induction by salicylate (Fig. 6). (ii) The presence of the marbox renders the operon subject to control by homologous transcriptional activators such as SoxS. As observed above (Table 2) and previously (35), basal levels of SoxS do not contribute significantly to *mar* expression. However, we have recently found (30) that wild-type levels of Rob account for half of the basal level of *mar* expression, so that in strains with both Δ*mar* and *rob*::*kan* mutations the marbox plays no significant role in *mar*::*lacZ* expression. Furthermore, the transformation of this MarA- and Rob-deficient strain with a plasmid that overexpresses MarA (28) increased *mar*::*lacZ* expression fourfold, and this increase was marbox dependent (30). Thus, the *mar* promoter's marbox attunes *mar* expression to the levels of three transcriptional activators, MarA, Rob, and SoxS.

Whether MarA influences the levels of SoxS or Rob is not known. Since overexpression of *soxS* or *rob* engenders multiple antibiotic and superoxide resistance even in the absence of *mar* (3, 32, 35), MarA is clearly not obligatory. However, the observation that SoxS binds to, and seems to negatively regulate, its own promoter (33) raises the possibility that MarA and Rob could also negatively regulate *soxS*.

ACKNOWLEDGMENTS

This research was supported in part by Public Health Service grant GM27113 from the National Institutes of Health (R.E.W.).

We thank Shilen Lakhani and Ben Bruce for excellent technical assistance.

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