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Purification and Regulatory Properties of MarA Protein, a Transcriptional Activator of *Escherichia coli* Multiple Antibiotic and Superoxide Resistance Promoters

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Expression of the *marA* or *soxS* genes is induced by exposure of *Escherichia coli* to salicylate or superoxides, respectively. This, in turn, enhances the expression of a common set of promoters (the *mar*/*soxRS* regulons), resulting in both multiple antibiotic and superoxide resistance. Since MarA protein is highly homologous to SoxS, and since a MalE-SoxS fusion protein has recently been shown to activate *soxRS* regulon transcription, the ability of MarA to activate transcription of these genes was tested. MarA was overexpressed as a histidine-tagged fusion protein, purified, cleaved with thrombin (leaving one N-terminal histidine residue), and renatured. Like MalE-SoxS, MarA (i) activated the transcription of *zwf*, *fpr*, *fumC*, *micF*, *nfo*, and *sodA*; (ii) required a 21-bp "soxbox" sequence to activate *zwf* transcription; and (iii) was "ambidextrous," i.e., required the C-terminal domain of the α subunit of RNA polymerase for activation of *zwf* but not *fumC* or *micF*. Thus, the *mar* and *soxRS* systems use activators with very similar specificities and mechanisms of action to respond to different environmental signals.

Resistance to a variety of antibiotics and to superoxides can be induced in *Escherichia coli* by treatments which either derepress the *marRAB* operon or activate the *soxRS* system. The *marRAB* operon is negatively autoregulated by MarR, which acts as a sensor and regulator for the operon (2, 4) and which has been shown to bind to two sites in the *marRAB* promoter region in vitro (25). Treatments with aromatic weak acids such as salicylate (5), syringaldehydes (27a), or antibiotics (13) derepress the operon. Salicylate has been shown to bind to MarR and enhance the dissociation of MarR from *mar* promoter DNA in vitro (25). The *soxRS* system is activated in *E. coli* by treatment with superoxide-generating agents or nitric oxide (11, 29, 30, 34). Oxidation of the sensor [2Fe-2S]SoxR protein converts it into a specific activator of *soxS* transcription (14, 14a, 35).

In both cases, the crucial event is the elevated expression of the master regulator, MarA (9) or SoxS (1, 30, 36), which then controls expression of very similar sets of genes called the *mar* or *soxRS* regulons (11, 31, 34, 37). Native SoxS protein (21a) and SoxS in the form of a MalE-SoxS fusion protein have been purified and their binding sites in the promoters of six genes (*zwf*, *fpr*, *fumC*, *micF*, *nfo*, and *sodA*) of the *soxRS* regulon have been identified. Furthermore, a 21-bp "soxbox" lying within the MalE-SoxS binding site just upstream of the *zwf* −35 hexamer has been shown to be required for SoxS activation of *zwf* transcription in vivo and for MalE-SoxS activation in vitro (7, 16). Moreover, when placed upstream of the normally SoxS-unresponsive *gnd* promoter, this soxbox brought *gnd* expression under MalE-SoxS control both in vivo and in vitro (7, 16). With RNA polymerases reconstituted with wild-type or mutant α subunits, SoxS was found to be "ambidextrous," requiring the α C-terminal domain (CTD) for transcriptional activation of the *zwf* and *fpr* promoters but not the *fumC*, *micF*, and *nfo* promoters (16).

Since MarA and SoxS are members of the AraC class of DNA binding proteins and are highly homologous to each other in the putative helix-turn-helix domains (1, 4, 8, 9), it was of interest to determine whether MarA is also a transcriptional activator and whether its mode of action is similar to that of SoxS. We report here the purification of MarA and its ability to stimulate transcription of six *mar*/*soxRS* promoters in vitro. In all respects examined, MarA and SoxS were highly similar.

MATERIALS AND METHODS

Plasmid constructions. Standard procedures were used throughout (27, 32). The nucleotide numbers for the *mar* operon are those of Cohen et al. (4). Plasmid pRGM258 contains the wild-type *marORAB* sequences from nucleotides 1311 to 2769 but is missing a *Bam*HI site within the plasmid. It was generated by digestion of the pTA108-based plasmid pRGM174 (24) with *Sma*I and *Bst*EII (both of which cut within the plasmid sequences) and mung bean nuclease, followed by religation and cloning in strain N7962 (31). To prepare plasmid pRGM223, the entire *marA* gene and a portion of *marB* (nucleotides 1893 to 2495) were amplified by PCR with pRGM258 DNA. The 5′ end of the upstream primer contained the sequence CCCAATTCCAT linked to the *marA* sequence from 1893 to 1915, (generating an *Nde*I restriction site, CATATG, with the ATG corresponding to the first methionine codon of *marA*); the 5′ end of the downstream primer contained the sequence GGCGG linked to the *marB* sequence from 2494 to 2470 (generating a *Bam*HI site). The amplified DNA fragment was digested with *Nde*I and *Bam*HI, ligated to the similarly digested expression vector pET15b (Novagen), and cloned in strain BL21(λDE3), forming strain N8224. DNA sequencing of the entire *marA* insert demonstrated that PCR amplification had not introduced any mutations.

Purification of MarA. Strain N8224 was grown in 2 liters of superbroth at 37°C to an A_{600} of 0.8. IPTG (isopropyl-β-D-thiogalactopyranoside) was then added to 0.4 mM, and vigorous aeration was continued for 3 h. The remaining steps were carried out in the cold. The cells were harvested by centrifugation (~5 g [wet weight]), washed with 25 ml of 50 mM Tris-HCl (pH 7.5)–1 mM EDTA–1 M

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NaCl, frozen overnight, and resuspended in 25 ml of the buffer. The cells were sonically disrupted with eight 15-s bursts and centrifuged at 120,000 × *g* for 30 min. The supernatant fluid was discarded, the pellet was rinsed with 30 ml of 4 M urea–50 mM Tris-HCl (pH 8.5), and centrifuged again at 120,000 × *g* for 30 min. Finally, the pellet was resuspended in 25 ml of 50 mM Tris-HCl (pH 8.5)–6 M guanidinium-HCl and centrifuged for 30 min at 120,000 × *g*. The resulting solution was diluted to 75 ml with 50 mM Tris-HCl (pH 8.5) and passed through a 100-ml bed volume of chelating Sepharose equilibrated with 0.1 M $NiCl_2$ that had been washed three times with water. The column was eluted with a linear gradient of 0 to 1.0 M imidazole in 1 M NaCl–50 mM Tris-HCl (pH 8.5). A sharp peak of MarA protein eluting at ~0.2 M imidazole was collected and extensively dialyzed against buffer A (1 M NaCl–50 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 8.0]–1 mM dithiothreitol–5 mM EDTA–0.1 mM Triton X-100). The protein was concentrated with Amicon membranes to ~45 ml (~0.6 mg/ml) and digested with 500 U of thrombin for 1 h at 0°C. The thrombin was removed by benzamidine Sepharose chromatography, and the histidine tag was removed by Sephadex gel filtration, leaving the MarA protein as wild type except for one N-terminal histidine residue from the expression vector. After extensive dialysis against buffer A in dialysis tubing with a molecular weight exclusion of 8,000, the protein was again concentrated with Amicon membranes and finally was dialyzed against buffer A or buffer A supplemented with 20% glycerol.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 15% polyacrylamide gels, and staining was performed with Coomassie brilliant blue (32).

In vitro transcription. Single-round in vitro transcription reactions and the quantitation of the amounts of test and reference transcripts were carried out with RNA polymerase, the test DNA template, and control *gnd* DNA at a molar ratio of 20:1:1 as described previously (16). DNA fragments containing the promoter regions of *zwf*, *fpr*, *fumC*, *micF*, *sodA*, *nfo*, and *gnd* were prepared by PCR as described previously (16). In preliminary experiments, transcriptional activation of *zwf* was found to be maximal when open complexes were formed in the presence of 1 pmol of MarA. Hence, to ensure that MarA was not limiting, activation reaction mixtures contained 2 pmol of MarA (67 nM); other parameters of in vitro transcription, e.g., salt concentration, were not optimized for each test promoter. Except as noted otherwise, the final concentrations in the 30-μl transcription mixture were RNA polymerase (40 nM), test template, and *gnd* DNA (2 nM [each]), sodium chloride (20 mM), and purified MarA (67 nM). The amount of the test transcript was normalized to the amount of the *gnd* reference transcript.

Commercial *E. coli* RNA polymerase was purchased from Epicentre Technologies (Madison, Wis.). The RNA polymerase holoenzymes reconstituted from purified wild-type and mutant subunits were prepared by the method of Igarashi and Ishihama (15). As reported previously (15), RNA polymerase holoenzyme containing the mutant α subunit was about 25% as active as holoenzyme reconstituted with wild-type subunits. Because of the scarcity of the RNA polymerase reconstituted with mutant α subunits, we did not compensate for the lower level of activity by proportionately increasing the amount of mutant holoenzyme; however, control experiments showed that a fourfold increase in the amount of mutant holoenzyme produced the same amount of transcript as enzyme reconstituted with wild-type subunits (reference 15 and data not shown).

RESULTS

Purification of MarA. MarA was overexpressed as a histidine-tagged fusion protein by IPTG induction of strain N8224. A major band of the mobility expected for the MarA fusion protein was observed on SDS-PAGE of crude cell extracts (Fig. 1). The histidine-tagged MarA fusion protein was purified and renatured, and the histidine tag was removed. From 2 liters of culture, 35 mg of MarA was obtained which appeared as a single homogeneous band of approximately 15,000 Da, the molecular mass of MarA deduced from the *marA* DNA sequence. The preparation of MarA was estimated to be 95 to 97% pure, containing a contaminant with a molecular weight of ~8,000.

Sufficiency of MarA for in vitro transcriptional activation of *zwf*, *fpr*, *fumC*, *micF*, *nfo*, and *sodA*. Previous work has demonstrated that overexpression of MarA in a Δ*mar* strain confers antibiotic resistance similar to that of a *mar*-constitutive strain (9, 24). To determine whether the purified MarA protein affected gene expression, we tested its ability to activate the in vitro transcription of *zwf*, *sodA*, *micF*, and *fumC*, genes known to be under *mar* control in vivo, and of *fpr* and *nfo*, genes not known to be regulated by *mar* in vivo but activated by SoxS in vivo and by MalE-SoxS in vitro. MarA activated transcription

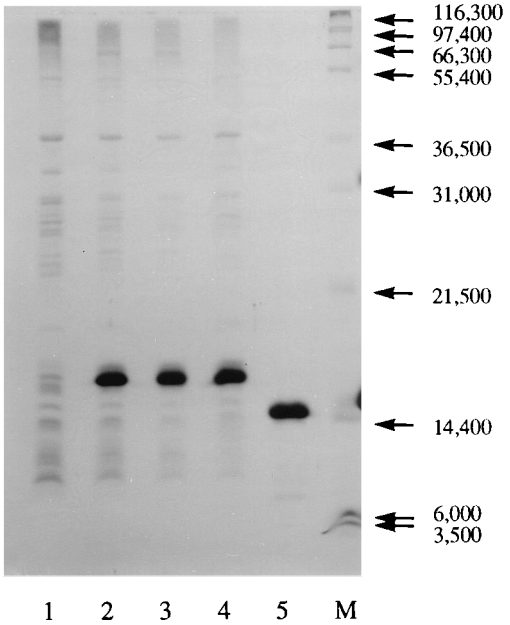

FIG. 1. SDS-PAGE of MarA during purification. The indicated samples were resuspended in loading buffer and subjected to SDS-PAGE. Lanes: 1, 50 μl of a culture of strain N8224 pelleted just prior to induction; 2, 25 μl of the culture pelleted 3 h after induction with IPTG; 3, 25 μl of the crude sonic extract; 4, 25 μl of the pellet after resuspension in 6 M guanidine; 5, 10 μg of the final preparation of MarA after thrombin digestion and removal of the thrombin and the histidine tag. The molecular weights of marker proteins (lane M) are indicated.

of all six test promoters but did not stimulate *gnd* transcription (Fig. 2). After normalization to the *gnd* internal control, the extent of activation of the indicated promoters was as follows: *zwf*, 3.3; *fpr*, 2.7; *fumC*, 4.0; *micF*, 7.3; *nfo*, 3.1; and *sodA*, 1.4.

The finding that purified MarA activates transcription of these promoters in the absence of other factors confirms the in vivo observations that MarA alone, like SoxS, can transcriptionally activate the *mar* regulon (9, 24). Furthermore, as found here for *fpr* and *nfo*, genes previously known to be regulated by only one system (10, 22), such as the *mar*-regulated *acrAB* gene (23) and the *soxRS*-regulated genes *acnA* (12) and *pqi-5* (21), may turn out to be regulated by the other. However, subtle differences between the promoters or other aspects of their regulation could influence the degree of their responsiveness to either activator.

Coincidence of the sites for SoxS- and MarA-mediated activation of *zwf* transcription in vitro. To determine whether transcriptional activation by MarA requires the same sequences as SoxS, we conducted in vitro transcription assays with the same DNA templates used in the previous SoxS studies (7, 16). Like MalE-SoxS, MarA stimulated in vitro transcription approximately threefold from the *zwf* promoter of templates Z1, Z2, Z3, Z4, and Z5, whose 5′ deletion end points reside at positions −140, −86, −76, −67, and −62, respectively (Fig. 3A). Like MalE-SoxS, purified MarA failed to enhance transcription from construct Z6, whose 5′ deletion endpoint lies at position −57. Thus, the 5′ boundaries of the "marbox" and the soxbox for *zwf* transcription are located between positions −58 and −61.

To define further the site required for MarA activation of the *zwf* promoter, in vitro transcription was assayed with DNA templates prepared from constructs Z4G4 and Z5G4, in which *zwf* soxbox-containing sequences −67 to −42 and −62 to −42,

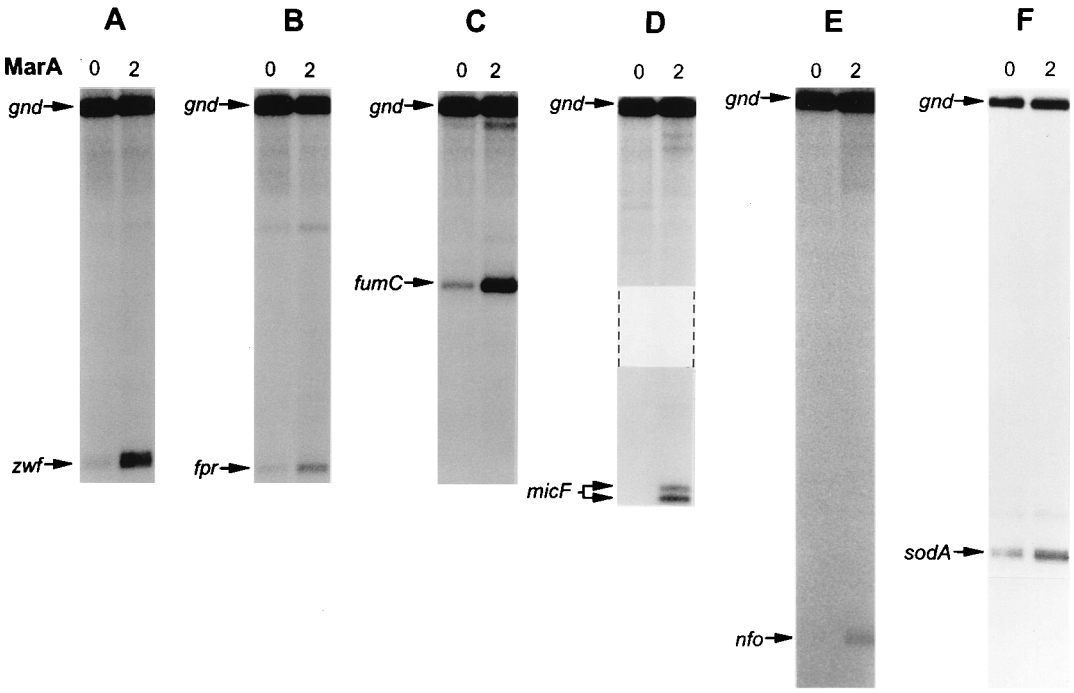

FIG. 2. MarA-dependent activation of in vitro transcription of six promoters also regulated by SoxS. Mixtures of the test and *gnd* reference promoters were transcribed under standard conditions in the absence and the presence of native MarA protein (2 pmol). (A) *zwf*. (B) *fpr*. (C) *fumC*. (D) *micF*. (E) *nfo*. (F) *sodA*.

respectively, have been placed upstream of the *gnd* promoter's −35 hexamer (7). Previously, we found that the extent of transcriptional activation of these hybrid *gnd* promoters by MalE-SoxS was only about 1.5-fold under the standard conditions in which the molar ratio of RNA polymerase to total DNA was 10, but it was about 10-fold at a molar ratio of 1.0 (16). Similar results were obtained with MarA. Under the standard conditions, MarA enhanced transcription from the two hybrid promoters by about 1.5-fold (data not shown), whereas under conditions of limiting RNA polymerase, the extent of activation by MarA was four- to fivefold (Fig. 3B). Thus, SoxS and MarA can utilize the same 21-bp sequence to activate *zwf* transcription.

Ambidextrous transcriptional activation by MarA. We previously showed that MalE-SoxS, like catabolite gene activator protein, is an ambidextrous transcriptional activator in that the CTD of the RNA polymerase α subunit is required for activation of the *zwf* and *fpr* promoters but not for activation of the *fumC*, *micF*, and *nfo* promoters (16). The effect of the α CTD on the ability of purified MarA to activate transcription from the *zwf*, *fumC*, and *micF* templates was tested (Fig. 4). With RNA polymerase reconstituted with wild-type subunits, MarA activated transcription from the three templates described above by factors of 2.5, 2.5, and 10.0, respectively. In contrast, with RNA polymerase reconstituted with the α-235 mutant, whose α subunits lack the 94 amino acid residues from the C terminus, MarA was completely unable to activate *zwf* (0.7-fold activation), although transcription from the *fumC* and *micF* promoters was still activated by 1.4- and 2.4-fold, respectively. Thus, like MalE-SoxS (16), MarA requires the α CTD for activation of *zwf* transcription but not for activation of *fumC* and *micF*. With MalE-SoxS, we have demonstrated that activation does not require the CTD of the RNA polymerase σ^{70} subunit at any of the six promoters (20) and that MalE-SoxS binds DNA as a monomer (38). Recent results (26, 38) suggest that MarA also binds DNA as a monomer.

DISCUSSION

The similarity of MarA and SoxS, predicted from their amino acid sequence homologies and functional similarities in vivo, is extended further by the present studies. For both activators, a 21-bp soxbox sequence is necessary for transcriptional activation of the *zwf* promoter and is sufficient to convert the *gnd* promoter to an activator-responsive form. Both proteins are ambidextrous, i.e., they activate the *zwf* promoter by interacting with the CTD of the α subunit of RNA polymerase but do not require the α CTD to activate the *fumC* and *micF* promoters. Moreover, both MarA and MalE-SoxS, respec-

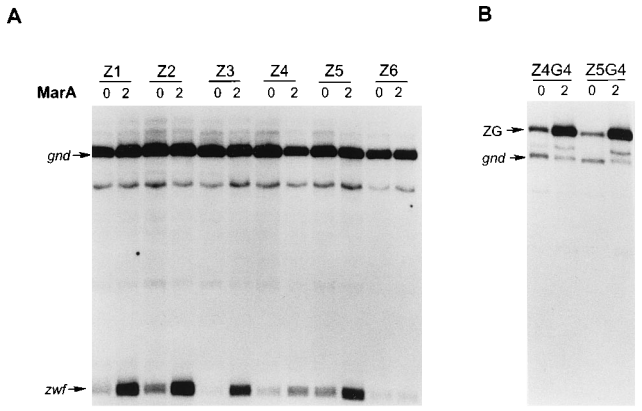

FIG. 3. Identification of the *zwf* sequences required for transcriptional activation by MarA in vitro. (A) Effect of 5′ deletions Z1 to Z6 on MarA activation of *zwf* transcription. Test templates were transcribed in mixtures containing the *gnd* reference promoter in the presence and absence of purified MarA protein (2 pmol). (B) Ability of the 21-bp *zwf* soxbox to confer MarA-mediated transcriptional activation on the heterologous *gnd* promoter in constructs Z4G4 and Z5G4. In these latter assays, the molar ratio of RNA polymerase to total DNA was 1.0.

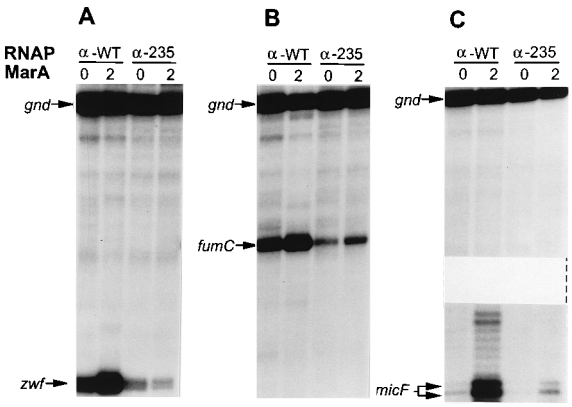

FIG. 4. Effect of deletion of the CTD of the RNA polymerase (RNAP) α subunit on transcriptional activation by MarA. The test promoters *zwf* (A), *fumC* (B), and *micF* (C) were transcribed by RNA polymerase reconstituted with wild-type (α-WT) or α-235 mutant subunits in the absence or the presence of purified MarA protein (2 pmol).

tively, have been shown recently to bend *zwf* DNA by 50° and 45° and *fumC* DNA by 34° and 48° (38). Thus, only subtle differences separate the behavior of MarA from that of MalE-SoxS.

Rob, another protein with sequence and functional homology to MarA and SoxS (3, 9, 33), is able to bind *oriC*, *micF*, *nfo*, *sodA*, and *zwf* in vitro (3). Indeed, in in vitro transcription experiments similar to those described here, purified Rob protein behaved in all respects like MarA and MalE-SoxS (17, 19). However, neither Rob's in vivo function nor its regulation has been elucidated.

The similarity of MarA and SoxS with respect to amino acid sequence homology, DNA recognition, and transcriptional activation properties suggests that they arose initially by gene duplication and then diverged to accommodate the constraints mandated by their specific stimulus-responsive regulatory systems: the salicylate-sensitive negative control mediated by MarR and the superoxide-sensitive positive control mediated by SoxR. Surprisingly, the systems are even more interconnected than this. Miller et al. (28) have found that SoxS activates *mar* transcription in vivo, and we have recently found a marbox sequence in the promoter region upstream of *marRAB* which binds MarA and enables either purified MalE-SoxS or MarA to activate transcription of *marRAB* in vitro (18, 26). Thus, while either system can clearly function when the other is absent (10, 31), they are closely intermeshed, and in a sense, *marRAB* could be considered a component of the *soxRS* regulon. What is not clear is why stimuli as diverse as phenolic compounds, antibiotics, and superoxides trigger essentially the same antibiotic and superoxide resistance response.

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