The *Escherichia coli* Transcriptional Regulator MarA Directly Represses Transcription of *purA* and *hdeA*

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SUMMARY

The *Escherichia coli* MarA protein mediates a response to multiple environmental stresses through the activation or repression *in vivo* of a large number of chromosomal genes. Transcriptional activation for a number of these genes has been shown to occur via direct interaction of MarA with a 20 bp degenerate asymmetric “marbox” sequence. It was not known whether repression by MarA was also direct. We found that purified MarA was sufficient *in vitro* to repress transcription of both *purA* and *hdeA*. Transcription and electrophoretic mobility shift experiments *in vitro* using mutant promoters suggested that the marbox involved in the repression overlapped the –35 promoter motif and was in the “backward” orientation. This organization contrasts with that of the class II promoters activated by MarA, in which the marbox also overlaps the –35 motif but is in the “forward” orientation. We conclude that MarA, a member of the AraC/XylS family, can act directly as a repressor or an activator, depending on the position and orientation of the marbox within a promoter.
The *Escherichia coli* MarA protein, a member of the AraC/XylS family of transcriptional regulators, mediates cellular responses to stress through the differential control of a large number of chromosomal genes comprising the mar regulon (1-5). MarA causes decreased susceptibility to structurally unrelated antibiotics, organic solvents, household disinfectants and oxidative stress agents (1). The MarA homologs SoxS and Rob in *E. coli* are also stress-response proteins (2).

Transcriptional activation of those mar regulon members that have been studied occurs when MarA binds as a monomer to the promoter region at a degenerate asymmetric 20 bp DNA sequence known as the “marbox” (6-7). The mechanisms by which MarA activates members of the mar regulon and the details of MarA, SoxS and Rob interactions with the marbox DNA have been investigated (for example, (6, 8-11)). MarA was originally described as an activator. Its expression directly induced transcription of all previously studied mar regulon members except *ompF*, whose down-regulation resulted indirectly from MarA activation of the antisense RNA *micF*, which then inhibited the translation of *ompF* (12, 13). Repression by MarA has recently been shown *in vivo* for several dozen genes by two independent macroarray studies, although the gene overlap between the two studies was not large (4, 5). To determine whether the repression by MarA was direct or occurred by an indirect action, we focused on two genes, *purA* and *hdeA*.

In the intact cell, transcription of *purA* was decreased in cells constitutively producing MarA (4). Expression of *hdeA* was repressed by induced expression of MarA (supplementary material in (5)). *purA* codes for adenylosuccinate synthase, which plays an important role in the *de novo* pathway of purine nucleotide biosynthesis and is required for adenosine monophosphate...
synthesis from inosine -5’-monophosphate (14). An association between purA and virulence has also been proposed (15, 16). hdeA encodes a periplasmic protein involved in acid resistance (17, 18). In Shigella flexneri and E. coli, both acid resistant organisms, hdeA is present, but it is absent in Salmonella, which is merely acid tolerant (17).

We show here that MarA directly represses both purA and hdeA. Thus MarA is a dual regulator capable of both activation and repression, depending upon the promoter.
EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions — *Escherichia coli* K12 strains and plasmids used in this study are shown in Table I. Strains were routinely grown at 37°C in LB broth (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl).

General Molecular Biology Manipulations — Preparation of genomic and plasmid DNA and purification of PCR products and of DNA fragments from agarose gels were carried out with Qiagen products: QIAamp Tissue kit, QIAprep spin Miniprep kit, QIAquick PCR purification kit and Qiaquick gel extraction kit, respectively, following the manufacturer's instructions. Restriction enzyme digestions followed standard procedures (19). PCR amplification utilized either *Taq* polymerase (Gibco BRL – Life Technologies) or TripleMaster Polymerase Mix (proofreading) (Eppendorf) in a GeneAmp® PCR System 9700 thermocycler (PE Applied Biosystems, Perkin Elmer). Oligonucleotide synthesis and DNA sequencing were performed by the Tufts University Core Facility.

RNA Preparation and Northern Analysis — Total RNA was extracted from cells using the Qiagen RNAeasy kit. Two micrograms of RNA were fractionated by electrophoresis in a denaturing formaldehyde agarose gel, which was then blotted onto a Hybond-N (Amersham) membrane. RNA was visualized by both ethidium bromide staining of the gel and by methylene blue staining of the membrane. *purA* or *hdeA* probes were amplified by PCR using AG100 chromosomal DNA as template. For *purA*, a ~1270 bp region was amplified using the primer pair F2/P-Rev (GAAAACGATTGGCTGAAC/AAGGTGGATTCAGACCAG). For *hdeA*, a ~396 bp region was amplified using the primer pair HdeA1/HdeA2
Hybridization of membrane-bound RNA to the \textit{purA} or \textit{hdeA} probes was performed as described previously (4).

\textbf{PCR Amplification of Promoter Regions Used in Transcription in Vitro} — The promoter region of the \textit{nfnB}-luciferase fusion in pSP-\textit{nfnB1} was amplified with the primer pair NFN-F1//lucR (CCCGGTACCCTTCGCGATCTGTCAACG//CTTCCACGGATAGAATGG), producing a 261 bp product with a 105 nt transcript (20). All other PCR products were generated using AG100 chromosomal DNA. The wild type 225 bp \textit{purA} promoter-containing template was amplified using primer pair \textit{purApF}//\textit{purApR} (GGAAAACGGTTTGCTGAAC//CGTTCAAGTCAGAAGATCG). PCR products containing mutated \textit{purA} marboxes were made using reverse primer \textit{purApR} together with a mutated forward primer. The 5' end of a mutated forward primer corresponded to the 5' end of the PCR product shown in Fig. 5; its arbitrary 3' terminus was either …AACTCTG-3' (for D2F and D3F) or …GAAAAGC-3' (for all others). All \textit{purA} templates yielded a transcript of 105 nt. For \textit{hdeA}, a 193 bp PCR product of the promoter region was amplified using the primer pair HdeA1a//HdeA2a (TCTGATGCATCTGTAACTCA//GAAGCAGACCACCAAGAATA). Mutant PCR products were made with the reverse primer HdeA2a together with primers beginning at the 5' end shown in Fig. 6. All \textit{hdeA} transcripts were 94 nt long. The 167 bp \textit{gnd} promoter-containing PCR product used the primers GNDF//GNDR (TCGCAACTTTGATCGAAT//TACATACTCCTGTCAGGT); the transcript was 55 nt long. All PCR products were gel-purified before being used in the transcription reactions \textit{in vitro}. 
Transcription in Vitro — N-terminal polyhistidine-tagged MarA (His$_6$-MarA) protein was purified essentially as described by Jair et al. (21) and was a generous gift of Victoria Bartlett and Michael Alekshun (Paratek Pharmaceuticals, Boston, MA). Single round (5 min) transcription in vitro was carried out essentially as described (22) in 30 µl using PCR products as DNA templates. The Reaction Buffer at pH 7.8 contained 50 mM Tris.HCl, 0.1 mM EDTA, 3 mM magnesium acetate, 0.1 mM dithiothreitol, 20 mM sodium chloride, and 250 µg/ml bovine serum albumin. Other components of the pre-incubation mixture were E. coli RNA polymerase holoenzyme (sigma 70; Epicentre), 40 nM; the RNAsé inhibitor SUPERase In™ (Ambion), 500 U/ml; test DNA (purA, hdeA, or nfnB promoter-containing DNAs) and control DNA (gnd promoter-containing DNA) each at 2 nM. In most experiments involving mutant templates with lower transcription levels, the concentration of each test DNA was raised to 4 nM or 6 nM. Purified His$_6$-MarA, diluted as needed in Reaction Buffer, was added to the pre-incubation mixture on ice to give 200 nM; the buffer in which His$_6$-MarA was prepared was similarly diluted and added to the control. Open complexes were allowed to form at 37°C for 15 min, when a heparin/nucleotide mixture was added to initiate transcription: the final concentrations were heparin, 1.2 mg/ml and nucleotides, 0.3 mM UTP, 0.96 mM ATP, CTP, GTP with ($\alpha$-32P)-UTP at 0.5 µCi/ml (Perkin Elmer Life Sciences). After 5 min, an equal volume of Ambion gel loading buffer II was added to stop the reaction, the samples were boiled for 3 min, chilled, and fractionated by electrophoresis on a 7% polyacrylamide/ 8 M urea gel in Tris-borate-EDTA pH 8.3.

Quantification of Transcript Levels — Radioactivity was detected by exposure of membranes/dried gels to Kodak BioMax MS X-ray film; alternatively, exposure to a storage
phosphor screen was followed by scanning with a Molecular Dynamics Storm PhosphorImager. Film images for methylene blue-stained membranes showing ribosomal RNA were scanned into Adobe Photoshop and the bands were quantified using NIH Image 1.62. Transcripts from both northern blots and from transcription reactions in vitro were quantified after PhosphorImager scanning by ImageQuant (Molecular Dynamics). The amounts of the two major transcripts produced, in vivo by hdeA or in vitro by purA, were combined. The amount of each test transcript was normalized by the level of the ribosomal RNA (for northerns) or gnd transcript (for in vitro transcriptions).

Identification of Putative Marboxes — Putative marboxes were identified using the “search patterns” utility of Colibri (http://genolist.pasteur.fr/Colibri/) with the 20 bp degenerate consensus sequence A[CAT]RGCA[RG]N, where R = A or G; W = A or T; Y = T or C; and N = A, T, G, or C (7). Only marboxes between 150 bp upstream and 100 bp downstream of the transcriptional start that had 15 or more matches and the 5th C invariant were considered.

Electrophoretic Mobility Shift Assays (EMSA) for purA — Binding of purified His6-MarA to DNA from the purA promoter region was measured by EMSA. The DNA used for fragments of 107-185 bp was amplified by PCR using a chromosomal AG100 template, while the 20 - 29mers encompassing marboxes were made by annealing equimolar concentrations of complementary DNA oligomers. The forward oligomers for the 20 bp marboxes were marbox 1 (AGTGCAAAAAAGTGCTGTAAC), marbox 2 (TTGAGTGCAAAAAAGTGCTGT), and “marbox 3” (AGGTCATTTTTGAGTGCAA). The forward oligomer for the wild type 29 bp
fragment comprising overlapping marboxes 1 and 2 was TTTTGAGTGCAAAAAAGTGCTGTAACTCTG. Two mutant oligomers were designed such that all 4 bp of RE1 of either marbox 1 or marbox 2 were mutated; the mutations are shown in Fig. 5, MB1.1F and MB2.1F respectively. In all cases, DNA was labelled at the 3’ end with digoxigenin-11-ddUTP (DIG gel shift kit, Roche Molecular Biochemicals) and the EMSA reactions were performed as described previously (20).
RESULTS

Transient Induction of MarA Caused a Decrease of purA and hdeA Expression in Cells — The earlier E. coli DNA macroarray experiments showing that constitutively-produced MarA reduced expression of purA (4) were confirmed subsequently using northern assays (data not shown). To determine whether this repression was a result of MarA function, as opposed to a stress-related artifact associated with the constitutive overproduction of MarA, we controlled expression of marA with the IPTG-inducible lac promoter in plasmid pMB102 (5) in the mar-deleted strain JHC1096 (Table 1). In the same experiment we also examined the expression of hdeA, which had been observed in another macroarray study to be repressed upon MarA induction in cells (5). After 1 hr induction of MarA by IPTG, a decline was seen in the levels of transcripts of purA (Fig.1A) and hdeA (Fig. 1B). Typically the decrease at 1 mM IPTG was ~ 3 fold for purA and 6 – 25 fold for hdeA, with less IPTG required for hdeA. Each of the two bands in Fig. 1B probably includes both genes of the hdeAB operon (23, 37); therefore hdeB was probably also repressed. No decrease was seen in cells bearing the vector control (pJPBH) (Fig. 1A & 1B). These results show that the decrease in purA and hdeA expression was caused, directly or indirectly, by transiently synthesized MarA rather than by a stress reaction due to constitutively overabundant MarA.

Purified MarA Repressed Transcription of purA and hdeA in Vitro — To determine whether repression was direct or indirect, we tested the effect of purified His\textsubscript{6}-MarA protein on transcription of purA and hdeA in vitro. This protein, engineered to facilitate purification and hereafter referred to as MarA, had been shown previously to activate nfnB transcription in vitro (20). The DNA templates for the single round transcription reactions were PCR products bearing
a promoter, its transcriptional start, and 55 to 105 bp downstream. The transcriptional start sites of the purA gene (14) and the hdeAB operon (23) have been defined. We mixed the promoter-containing DNA of purA or hdeA with that of the control gnd (a promoter that is unresponsive to MarA) (21,24,25,26). The promoter for the MarA-activated gene nfnB (20), also mixed with gnd DNA, was tested in parallel as a positive control for MarA activity. In the presence of 200 nM MarA, there was a 5 - 6 fold activation in nfnB transcription, as expected (Fig. 2). As anticipated, MarA did not affect the expression from the control gnd promoter. The same concentration and batch of MarA protein, on the other hand, repressed both purA and hdeA (Fig. 2). The average repression ratio was 0.44 for purA (see Fig. 5, WT) and 0.60 for hdeA (see Fig. 6, WT) relative to controls without MarA. These results showed that MarA by itself could directly repress purA and hdeA transcription.

DNA Sequences Involved in Binding by MarA: EMSA — Activation of transcription by MarA involves binding of this protein as a monomer to a degenerate 20 bp “marbox” located upstream from or overlapping the –35 motif within the promoter (6, 10). It seemed likely that for repression, a marbox in the promoter region would also be involved, although perhaps with a different location or orientation.

The purA promoter region contained five consensus marboxes (see Experimental procedures for criteria); all were upstream of the translational start site. By EMSA using PCR products covering various regions of the purA promoter, we showed that MarA bound with similar affinity to fragments spanning bp –140 to +45; –121 to –14; and –96 to +45, relative to the transcriptional start (data not shown). Therefore binding occurred between bp –96 and –14, eliminating three of
the marboxes from consideration. Remaining were marbox 1 (forward; bp –49 to –30, 15/20 consensus bp) and the overlapping marbox 2 (backward; bp –33 to –52, 15/20 consensus bp) (see Fig. 5). Further mobility shift assays with 20 bp DNA fragments comprising the individual marboxes showed that MarA did not bind to a “control” poor consensus backward “marbox 3” (bp –42 to –61; only 12/20 consensus bp), but did bind to marboxes 1 and 2. The affinity for marbox 2 ($K_d < 100$ nM) was greater than that for marbox 1 ($K_d > 400$ nM) (Fig. 3A), suggesting that marbox 2 was preferred.

It was not straightforward to further distinguish the relative importance of marboxes 1 and 2 since they overlapped at 17 of their 20 bp (see Fig. 5). We therefore took advantage of the two highly conserved marbox “recognition elements”, RE1 and RE2, of 4 bp each which are particularly critical for activation by SoxS (27) and presumably by MarA. The crystal structure of MarA bound to marbox DNA shows the two recognition helices of the protein in contact with the RE elements of the marbox (10). We mutated all four bp of RE1 of either marbox 1 or marbox 2 (see Experimental procedures and Fig. 5) in 29 bp duplexes containing both purA marboxes 1 and 2. The mutations in the RE1 of one marbox did not affect the RE1 of the other. We found that MarA still bound to mutated purA marbox 1, but did not bind to mutated marbox 2 (Fig. 3B). This result confirmed the importance of marbox 2.

In the case of hdeA, there were six consensus marboxes (Fig. 4; see Experimental procedures for criteria). However, EMSA experiments were not conclusive since a shift in mobility was seen for only a very small fraction of hdeA promoter DNA, even with MarA at 750 nM.
DNA Sequences Involved in Repression by MarA: Transcription Assays in Vitro — For purA, the EMSA analyses had identified marbox 2 as more important for MarA binding but did not prove its involvement in MarA-mediated transcriptional repression. Therefore we repeated the in vitro transcription reactions using promoters with mutations in marboxes 1 and 2 to look for loss of repression. Both deletions and point mutations were used. Some of these mutations caused a reduction in transcription itself (see Discussion), so a doubling or tripling in the amount of template DNA (including with wild type controls) was used to enhance the amount of transcript (see Experimental procedures).

For purA, changing 3 bp in RE2 of marbox 2 did not prevent repression (Fig. 5, MB2.2F), nor did deletions of 7 or 14 bp in marboxes 1 and 2 (Fig. 5, purD2F, purD3F, DelF). Truncation of the final 6 bp of marbox 2 decreased but did not eliminate repression (Fig. 5, purApF2). The unexpected repression still seen in spite of marbox deletions and truncation is discussed below. Nevertheless, repression was clearly prevented by the alteration of all 4 bp of RE1 in marbox 2 (Fig. 5, MB2.1F), while no effect was seen for mutations in RE1 of marbox 1 (Fig. 5, MB1.1F, mut1F). These latter results again pointed to marbox 2, and not marbox 1, as critical for repression of purA.

For hdeA, a template missing marbox 1, the last 13 bp of marbox 6, and the last 6 bp of marbox 5 still showed repression (Fig. 6, MB5.5), as did a template missing all of marbox 5 (and consequently the last 2 bp of marbox 3) (Fig. 6, ΔMB5). It appeared therefore that marboxes 1, 5 and 6 were not needed for repression. Not surprisingly, mutations of the last two bp of marbox 3 did not block repression (Fig. 6, MB3a). However, alteration of all 4 bp of RE2 of marbox 3 did
block repression (Fig. 6, MB3b). Therefore marbox 3 was necessary for repression of hdeA. To investigate the role of RE1 of marbox 3, two of its four bp (within the −35 hexamer) were changed, but this reduced basal levels of transcription such that quantification was not possible. When the −35 hexamer (atgaca) was mutated to the consensus hexamer (ttgaca), transcription increased notably; repression by MarA still occurred (Fig. 6, MB3e), even though the mutation also affected one bp of RE1 of marbox 3. When this single mutation in the −35 hexamer was then combined with the RE2 mutations of MB3b, transcription remained high and repression was again lost (Fig. 6, MB3k). These results together indicated that marbox 3, and particularly its RE2, was critical for repression of hdeA by MarA. No further mutations were attempted in RE1 as it completely overlapped the −35 hexamer.
DISCUSSION

Using transcription analysis in vitro we have demonstrated that purified MarA protein was sufficient to downregulate expression of purA and hdeA via the promoter region of these genes. How the same protein is able both to activate and to repress transcription probably relates to the identity and location of DNA sequence motifs that are recognized by MarA. For our studies here, we presumed that marboxes within the promoter region would be involved. By a combination of EMSA and transcription experiments in vitro using wild type and mutant marboxes, we found that the marbox most likely to contribute to repression of both purA and hdeA overlapped the –35 promoter motif and was oriented in the “backward” direction. “Class II” promoters activated by MarA also have marboxes overlapping the –35 motif, but these marboxes are in the forward direction (6). Presumably the two different marbox orientations result in two types of interactions between MarA, RNA polymerase, and/or DNA, one leading to activation and the other to repression.

In the case of purA, marbox 2 was defined as critical by its affinity for MarA in EMSA and by the fact that point mutations in RE1 of marbox 2 prevented both the mobility shift and the repression by MarA in vitro. Although deletions within this marbox did not prevent repression, this result could readily be explained (for the 7 bp deletions) by the formation of new backward marboxes from the resultant joined sequences, with consensus bp of 15/20 (D2F) or 16/20 (D3F) (see Fig. 5). In the case of the 14 bp deletion (DelF), the newly created marbox was 1 bp too short, but it had 14 consensus bp. However, mutant template purApF2, lacking the terminal 6 bp of the marbox, was still somewhat repressed (Fig. 5). That finding suggested that only the first part of this marbox was needed for repression. Transcription using the templates with deletions...
was considerably reduced, possibly due to destruction of a potential “UP element” (28) comprising bp –41 to –57 in the purA promoter.

For hdeA, even though repression of transcription was seen in vitro, EMSA experiments showed minimal binding of MarA to the hdeA promoter region. Discrepancies between DNA binding as measured by EMSA and by other means have also been observed for other promoters, for example ybjC (29) and inaA (6). We therefore depended upon the transcription assays in vitro to define the particular marbox critical for repression of hdeA. Specifically, upstream marboxes 1, 5, and 6 were not required. Marbox 3, overlapping the –35 promoter motif, was critical for repression, since there was no repression if mutations were placed in all four bp of its RE2 (Fig. 6). The role of RE1 of marbox 3 remained unclear since RE1 completely overlapped the –35 hexamer and could not be extensively mutagenized. The last two bp of marbox 3 could be deleted without eliminating repression in vitro (ΔMB5), suggesting that, as with purA, an entire 20 bp marbox may not be needed for repression (Fig. 6). Of note, RE2 was important for repression of hdeA but not purA.

Two relatives of MarA were reported to directly repress transcription of other promoters, but the marbox involved was not determined. IscR, a newly recognized member of the AraC family of transcriptional regulators and a “distant” MarA relative (20 % identity, 40 % amino acid similarity), was able to function in vitro as a transcriptional repressor of the iscRSUA operon (30). Intriguingly, we had observed downregulation of the same promoter by constitutive expression of MarA (4) (iscS = b2530). Also, the MarA homolog SoxS repressed its own
expression in whole cells and bound its own promoter \textit{in vitro} (31); no further studies of this observation have been reported.

The physiological significance of the repression of \textit{purA} and \textit{hdeA} by MarA is not known. Other proteins also repress \textit{purA} and \textit{hdeA}. The \textit{purA} promoter is repressed by PurR in the presence of purines (32) and the promoter of \textit{hdeA} is repressed by HN-S (33). The \textit{hdeA} promoter can be recognized by both sigma 70 and RpoS (23), and MarA can activate both sigma 70- and RpoS-dependent promoters (34). Interestingly, GadX binds to a region in the \textit{hdeA} promoter (35) which overlaps the MarA binding site, marbox 3. The repression of \textit{purA} by MarA was quantitatively similar \textit{in vivo} and \textit{in vitro} while that of \textit{hdeA} was greater \textit{in vivo} (~ 6 - 25 fold) than \textit{in vitro} (~ 1.7 fold). Possibly repression \textit{in vivo} of \textit{hdeA} by MarA is aided by a cellular product that is constitutive or is induced by MarA.

From our results we postulate that MarA represses by binding to a region of the promoter which overlaps the RNA polymerase –35 binding motif. Certain other repressors also have binding sites that overlap or lie between the RNA polymerase binding motifs; they may provide models for MarA action (36, 37). LexA sterically blocks the binding of RNA polymerase to the \textit{uvrA} promoter (38). MerR bends the –10 motif of the \textit{merT} promoter away from RNA polymerase, causing a reduction in the rate of open complex formation (39, 40). Arc protein at the \textit{P}_{ant} promoter of bacteriophage P22 slows the rate of open complex formation (41).

Although our sample of genes directly repressed by MarA is small, the location and orientation of the marboxes in the two repressed genes studied here are unlike that in either of the two
classes of the MarA-activated genes. We have postulated that it is the backward marbox overlapping the −35 motif which uniquely gives rise to repression. Other cases in which proteins activate some promoters and repress others have been described (37). However, we have found no previous example in which the change between activation and repression relies on the orientation of the binding site.
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REFERENCES


# TABLE I. Bacterial strains and plasmids used in this study

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<td>GC4468</td>
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FIG. 1. Northern blots of purA and hdeA transcripts following induction of MarA.

Total RNA was prepared from cultures of strain JHC1096 (ΔmarRAB) bearing either pJPBH (vector) or pMB102 (marA) which had been induced at A_600=0.5 with increasing concentrations of IPTG (0, 0.25, 0.5, 1 mM) for 1 hr. Loading of rRNA bands was visualized by methylene blue staining (shown below the northern blots).

A, Hybridized with a 32P-labeled internal fragment of the purA gene. The size of the band is 1.2 kb. B, Hybridized similarly with hdeA, using the same RNA on a different blot. The sizes of the bands are 0.6 kb and 0.9 kb.

FIG. 2. Effect of MarA on transcription in vitro of purA, hdeA, and nfnB.

Mixtures of test DNA templates (either purA, hdeA or nfnB) with control template (gnd) were incubated for transcription in vitro using 32P –UTP (see Experimental procedures) without (−) or with (+) 200 nM of purified MarA. Samples were fractionated by polyacrylamide/urea gel electrophoresis. The gel was dried and exposed to film. The larger of the two major bands for nfnB and purA represents the predicted full length runoff transcript.

FIG. 3. Electrophoretic mobility shift of purA promoter regions by MarA.

The concentrations of MarA used were 0, 100, 200, or 400 nM (lanes 0, 1, 2, 4 respectively). A, Wild type DNA. The DNA fragments were 20 bp duplexes, each comprising marbox 1, marbox 2, or the less conserved control “marbox 3”. B, Mutations in marbox 1 or marbox 2. The DNA fragments were 29 bp duplexes comprising both marboxes 1 and 2. An unmutated fragment (wild type) was the control, while the other two fragments were mutated at all 4 bp of the RE1 of
either marbox 1 or marbox 2 (these mutations are shown in Fig. 5 in MB1.1F and MB2.1F, respectively). C, complex of MarA/DNA; F, free DNA.

FIG. 4. **Putative marboxes within the hdeA promoter.**

Six putative marboxes were found using the criteria described in Experimental procedures. The direction of the arrows depicts the orientation of the marboxes. The sequences in bold indicate the forward and reverse primers used in the amplification of the wild-type PCR fragment. RNA polymerase recognition sequences are boxed. The G in bold with the kinked arrow indicates the transcriptional start site (23). The translational start site is boxed and in bold.

FIG. 5. **Effect of mutations in the purA promoter upon repression in vitro by MarA.**

Residues are numbered with respect to the transcriptional start site, which is +1. The wild type sequence (WT) is shown at the top, with overlapping, oppositely-oriented marboxes 1 (forward arrow) and 2 (reverse arrow); the −35 promoter hexamer is beneath a bracket. Each succeeding line represents a mutant template. All templates began at the left-most residue shown except for the wild type template, which began at bp −120. All templates ended at bp +105. Mutations are shown by bold upper case. Deletions are located by a vertical tick/arrow, with the number of bp deleted shown beside the template name. Residues conforming to the marbox consensus (29) for marbox 2 are underlined. The effect of MarA is expressed as the ratio of purA transcription in the presence of MarA divided by purA transcription in its absence, normalized for any differences in the transcription of gnd. The lower the ratio, the more the repression. Values represent an average of 2 to 12 experiments. Stdev, standard deviation. RE, marbox recognition element. (+), repressed. (−), not repressed.
FIG. 6. Effect of mutations in the *hdeA* promoter upon repression in vitro by MarA.

See the legend of Fig. 5 for format. The arrow indicates marbox 3. The wild type template began at bp –99. All the other templates had 5’ start sites as shown. All templates ended at bp +94. Values represent an average of 4 to 12 experiments.
Fig. 1

A.

B.
Fig. 3

A.

marbox 1  marbox 2  "marbox 3"

0  1  2  4  0  1  2  4  0  4

C  F

B.

Wild type  Mutated marbox 1  Mutated marbox 2

0  1  0  1  2  4  0  1  2  4

C  F
Fig. 4

```
-99
tctgatgcatactgtaactcatgtattgaata
   6...

  5
aaaatatctgttggatatattttcatcaacatgacata
   ...6

  4...
...4

tacagaaaccaggttatataaactcagtgtcgaatttgt
   -10

  3

tcgtaacggtctttcactttatgtaggagattaccg

  2

[atg] aaa aat gta tta ggc gtt att ctt ggt
   +94

   ggt ctg ctt ctt c
```
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Thamarai Schneiders, Teresa M. Barbosa, Laura M. McMurry and Stuart B. Levy

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