

Many Chromosomal Genes Modulate MarA-Mediated Multidrug Resistance in *Escherichia coli*[∇]

Cristian Ruiz and Stuart B. Levy*

Center for Adaptation Genetics and Drug Resistance and Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, Massachusetts 02111

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Multidrug resistance (MDR) in clinical isolates of *Escherichia coli* can be associated with overexpression of *marA*, a transcription factor that upregulates multidrug efflux and downregulates membrane permeability. Using random transposome mutagenesis, we found that many chromosomal genes and environmental stimuli affected MarA-mediated antibiotic resistance. Seven genes affected resistance mediated by MarA in an antibiotic-specific way; these were mostly genes encoding unrelated enzymes, transporters, and unknown proteins. Other genes affected MarA-mediated resistance to all antibiotics tested. These genes were *acrA*, *acrB*, and *tolC* (which encode the major MarA-regulated multidrug efflux pump AcrAB-TolC), *crp*, *cyaA*, *hns*, and *pcnB* (four genes involved in global regulation of gene expression), and the unknown gene *damX*. The last five genes affected MarA-mediated MDR by altering *marA* expression or MarA function specifically on *acrA*. These findings demonstrate that MarA-mediated MDR is regulated at multiple levels by different genes and stimuli, which makes it both complex and fine-tuned and interconnects it with global cell regulation and metabolism. Such a regulation could contribute to the adaptation and spread of MDR strains and may be targeted to treat antibiotic-resistant *E. coli* and related pathogens.

Historically, infectious diseases have been the leading cause of mortality and morbidity until the discovery of antibiotics in the 20th century. However, the overuse and misuse of antibiotics have favored the emergence and spread of bacteria that are resistant to multiple antibiotics, threatening the treatment of bacterial infections worldwide (26, 27).

Multidrug resistance (MDR) can occur through the acquisition of extrachromosomal DNA, such as plasmids or transposons, via chromosomal mutations in genes coding for proteins targeted by the drug (e.g., mutations in *gyrA* that confer resistance to fluoroquinolones), or via altered expression of intrinsic mechanisms, such as efflux pumps that expel multiple classes of antibiotics out of the cell (2). Although increased intrinsic resistance usually produces moderate resistance levels, it can generate resistance to a wide array of antibiotics and other toxic chemicals. Moreover, this type of resistance may be a “stepping stone” to higher levels of resistance, such as in MDR clinical isolates having a combination of mutations in target genes and increased drug efflux (1, 16).

The *Escherichia coli marA* gene, which forms part of the chromosomal *marRAB* operon, was the first global regulator of intrinsic MDR discovered (18, 22). It has been extensively studied because of the widespread interest in *E. coli* as a major source of intestinal infections in developing countries and because of outbreaks of food poisoning and extraintestinal infections in industrialized countries (23). Moreover, *marA* orthologs or *marA*-like regulators are widespread among bacteria, including many clinically relevant pathogens, such as *Salmonella enterica*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Yersinia pestis*

and other *Enterobacteriaceae*, *Neisseria gonorrhoeae*, and *Staphylococcus aureus* (1, 2, 16).

The product of *marA*, the transcription factor MarA, directly activates or represses many chromosomal genes. MarA produces intrinsic multiple antibiotic resistance (Mar) mainly by upregulating the expression of *acrAB* and *tolC*, which encode AcrAB-TolC, the major multidrug efflux pump in *E. coli* (28, 39), and by upregulating *micF*, a regulatory antisense RNA which downregulates *ompF* translation and thus the OmpF porin-mediated outer membrane permeability to antibiotics. MarA also upregulates superoxide resistance (*acnA*, *fumC*, *fpr*, *sodA*, and *zwf*) and DNA repair (*nfo*), among other effects (5, 6).

Overexpression of *marA* is often found in MDR clinical isolates and is the result of spontaneous mutations in the autorepressor *marR* or in the DNA-binding sites of MarR (16, 31). Overexpression of *marA* also occurs when MarR is inactivated by chemicals such as salicylate (10, 46) or by cellular proteins such as TktA (13). Moreover, transcription of *marA* is activated by MarA and by the MarA homologs SoxS and Rob, mostly in the presence of the helper protein Fis (32). The intracellular level of MarA depends also on the Lon protease, which constitutively degrades MarA (21).

At a single-gene level, the function of MarA on at least one operon, *hdeAB*, which is involved in acid resistance, depends also on growth phase, pH, and other regulators of *hdeAB* (42). However, little is known about how the function of MarA on MDR is regulated at a global level. Here, we report that many genes and environmental stimuli are involved in a fine-tuned modulation of MarA-mediated MDR by affecting *marA* expression or function.

MATERIALS AND METHODS

Growth conditions. All cultures were grown in LB medium (10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter) at 37°C with agitation. Antibiotics

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance and Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Phone: (617) 636-6764. Fax: (617) 636-0458. E-mail: stuart.levy@tufts.edu.

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TABLE 1. Bacterial strains and plasmids used in this study

Group	Genotype or relevant characteristic(s)	Reference or source
<i>E. coli</i> strains used for mutagenesis		
HdeA100	GC4468 <i>zdd-239::Tn9 delI738</i> (Δ 39 kb, including <i>marRAB</i> locus) (Chl ^r) <i>hdeABp::lacZ</i> (Amp ^r) on λ at <i>latt</i> site/pMB102	42
HdeA100 mutants	HdeA100 mutagenized using EZ-TN5 <R6K γ ori/KAN-2> or EZ-TN5 <Kan-2> transposomes; Amp ^r Chl ^r Kan ^r	This study
<i>E. coli</i> strains used for multidrug resistance and gene expression experiments		
BW25113	Wild type; F ⁻ λ^- Δ (<i>araD-araB</i>)567 Δ <i>lacZ</i> 4787(<i>::rmB-3</i>) <i>rph-1</i> Δ (<i>rhaD-rhaB</i>)568 <i>hsdR514</i>	CGSC; 3
JW5248	BW25113 Δ <i>marR::kan</i> ; Kan ^r	CGSC; 3
CR1000	BW25113 Δ <i>marR</i>	This study
CR2000	BW25113 Δ <i>marRA</i>	This study
CR1000/CR2000 inactivation mutants	Kan ^r	P1 HdeA100 mutant \times CR1000/CR2000
Complementation plasmids		
pNTR-SD (control)	<i>Ptac lacI^q</i> ; low copy number; Amp ^r	Mobile plasmid collection
pNT3- <i>crp</i>		NBRP (<i>E. coli</i>) (NIG, Japan); 43
pNTR-SD- <i>hns</i>		
pNTR-SD- <i>cyaA</i>		
pNT3- <i>pcnB</i>		
pNTR-SD- <i>acrB</i>		
pNTR-SD- <i>tolC</i>		
pNTR-SD- <i>damX</i>		
Other plasmids		
pCP20	Plasmid for excision of <i>kan</i> and <i>cat</i> markers by FLP-mediated site-specific recombination; Amp ^r Chl ^r	12
pKD3	Template for amplifying the <i>cat</i> gene; Chl ^r	12
pKD46	λ Red recombinase expression plasmid; Amp ^r	12
pMB102	<i>ori colE1 lacI lacZp::marA</i> ; Amp ^r	40
pNN608	Single copy; <i>acrAp-lacZ</i> ; Chl ^r	30

were used at 100 μ g ml⁻¹ (ampicillin) and 25 μ g ml⁻¹ (chloramphenicol and kanamycin).

Strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. The Δ *marR* knockout parental strain CR1000 was obtained using plasmid pCP20 as previously described (12) to remove the kanamycin resistance gene of strain JW5248 (*E. coli* BW25113 Δ *marR::kan*; Keio collection [3]). The Δ *marRA* knockout parental strain CR2000 was constructed using the λ Red recombinase method (12) and primers MarRAdeIF2 (5'CCAGCGATCTGTTCAATGAAATATTCCATTGGGTCGCTTAATCCATATGGTGTAGGC TGGAGCTGCCTC) and MarRAdeIR2 (5'TTGCCTCAGTGACGTTGTCCAGC TTTTCAACTAGCTGTTGTAATGATTTAAATGGGAATTAGCCATGGTCC) (the sequences corresponding to plasmid pKD3 are in bold). Briefly, these primers were used to generate a PCR product of the *cat* gene of pKD3 with sequences flanking *marRA* at both ends. This product was used to replace wild-type *marRA* in *E. coli* BW25113, and the *cat* gene was then removed using plasmid pCP20. In-frame deletion of *marR* in CR1000 and *marRA* in CR2000 was confirmed by PCR amplification and sequencing using specific primers flanking the deleted genes.

Genetic procedures. PCR, phage P1 transduction, and plasmid or transposome electroporation were performed according to standard procedures (45). DNA sequencing was performed at Tufts University Core Facility. β -Galactosidase assays for studying the expression of the *acrA-lacZ* transcriptional fusion in single-copy plasmid pNN608 were performed as previously described (42). Bacterial two-hybrid assays for studying interactions between MarA and other proteins were performed by the adenylate cyclase system/ β -galactosidase selection method, as previously described (13).

Mutagenesis and identification of mutants with reduced MarA function. *E. coli* HdeA100 (Table 1) (42) is a Δ *marRAB* strain that has *marA* in a plasmid, cloned under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter, and bears a *hdeABp-lacZ* chromosomal transcriptional fusion. This strain produces blue colonies on LB X-Gal (5-bromo-4-chloro-3-

indolyl- β -D-galactopyranoside) plates and white colonies on LB X-Gal-IPTG plates because MarA represses *hdeAB* expression. To find genes important for MarA function, HdeA100 cells were electroporated with the EZ-TN5 <Kan-2> or EZ-TN5 <R6K γ ori/KAN-2> kanamycin resistance transposome (Epicentre) and plated on LB containing ampicillin, kanamycin, 0.5 mM IPTG, and 40 μ g ml⁻¹ X-Gal. Mutant colonies in which MarA failed to repress *hdeAB* (blue colonies) were selected and re-purified, and their phenotype was reconfirmed.

Transposome insertion points were identified by arbitrarily primed PCR (AP-PCR) or by direct genomic sequencing and confirmed by PCR amplification and sequencing using specific primers flanking the transposome-disrupted genes. AP-PCR was performed essentially as described by Das et al. (11), using the arbitrary primers described by Gonin et al. (19) (Arb1 and Arb6 in round 1 and Arb2 in round 2) and the specific primers TNF3 (5'GGCAAAGCAAAAGTTCAAATCACC) in round 1 and TN5F2 (5'GTCCACCTACAACAAAGCTC TCATCAACCGTGG) in round 2. The final AP-PCR products were purified using a QIAquick PCR purification kit (Qiagen) before sequencing. Direct genomic sequencing was performed at the Tufts University Core Facility, using the internal transposome primers included in the Epicentre transposome kit and genomic DNAs obtained using a Wizard Genomic DNA purification kit from Promega.

MIC determinations. Mutations were transferred from strain HdeA100 (Δ *marRAB hdeABp-lacZ*, with *marA* cloned in a plasmid) to the parental strains CR1000 (Δ *marR*) and CR2000 (Δ *marRA*) by P1 transduction and were reconfirmed by PCR before their effects on antibiotic susceptibility to cefoxitin, norfloxacin, chloramphenicol, and minocycline were studied. The different mutants were grown overnight in liquid cultures and their MICs determined using Mueller-Hinton (M-H) plates and Etest strips (AB Biodisk) according to the manufacturer's specifications. MIC readings were performed after incubation for 20 h at 37°C. M-H plates were supplemented with ampicillin and 0.5 mM IPTG in plasmid complementation experiments, with 1 mM cyclic AMP (cAMP) (44) in complementation experiments of *cyaA* mutants with cAMP, and with 300 mM

NaCl or 0.4% D-glucose in the environmental stimuli experiments. Etest strips were a generous gift from AB Biodisk (Solna, Sweden).

Gene expression experiments. Mutants showing reduced MarA-mediated MDR were studied by reverse transcription followed by real-time quantitative PCR (RT-qPCR) to determine the expression levels of *marA*, *acrA*, *tolC*, and *micF*. These genes comprise both class I (*marA* and *acrA*) and class II (*tolC* and *micF*) MarA-activated promoters (33). *gapA*, which encodes the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) enzyme, was used as an endogenous reference gene according to the method of Viveiros et al. (47). The specific primers for real-time qPCR of these genes are described elsewhere (47). These primers were also used to generate the PCR products used to perform qPCR standard plots for each gene.

Briefly, overnight cultures of the different CR1000 (Δ *marR*)- and CR2000 (Δ *marRA*)-derivative mutants were grown overnight, diluted 1:1,000 in fresh LB, and grown for 3 to 4 h to an optical density at 600 nm (OD_{600}) of about 0.3. Then, total RNA in the cultures was stabilized using RNAlater bacterial reagent (Qiagen) and isolated after lysozyme-proteinase K digestion of bacteria by using an RNeasy minikit (Qiagen) and On-Column DNase I digestion (Qiagen) according to the manufacturer's specifications. RNAs were then treated with RQ1 RNase-free DNase from Promega to remove any remaining DNA and repurified with the RNeasy minikit, and their purity from proteins and concentration was determined using a NanoDrop ND-1000 spectrophotometer. Reverse transcription was performed using the SuperScript III first-strand synthesis system from Invitrogen, the specific primers aforementioned, and 200 ng of RNA. RT-associated reactions without reverse transcriptase (RT⁻ reactions) were used as controls to confirm the lack of contaminating DNA in the RNA samples. The cDNAs obtained from the RT⁺ and RT⁻ reactions were quantified after 45 cycles using a Mx3000P detection system (Stratagene) with 25- μ l reaction mixtures of primers (300 nM each), cDNA (4 μ l of a 1:10 dilution of the RT⁺ or RT⁻ reaction mixture), and 2 \times QuantiTect SYBR green qPCR master mix from Qiagen (12.5 μ l). Absolute transcript numbers were calculated using standard plots obtained by qPCR of serial dilutions of gene-specific, gel-purified PCR products of known concentrations.

***marA* mRNA polyadenylation and half-life experiments in the parental strain and the *pcnB* mutant.** Polyadenylation of *marA* transcripts was determined by RT-PCR performed as described above, using the *marA*-specific forward primer and an oligo(dT)₂₀ primer as the reverse primer, followed by agarose gel analysis of the amplification products. For the *marA* mRNA half-life experiments, overnight cultures of each strain were diluted 1:1,000 in fresh LB and grown for 3 to 4 h to an OD_{600} of about 0.3. Then, RNAs were extracted before (time zero) and after addition of rifampin (500- μ g ml⁻¹ final concentration, which stops RNA synthesis) and culture incubation at 37°C for 0.5, 1, 2, and 4 min. The amount of *marA* transcripts was determined by RT-qPCR as described above. Half-life was defined as elapsed time $\times \log_2$ (beginning amount/final amount).

Statistical analysis. Statistically significant differences in MDR or gene expression were determined by Student's *t* test (two independent samples with equal variance, with two-tailed distribution), using Microsoft Excel 2003 software.

RESULTS AND DISCUSSION

Identification of 24 different genes whose inactivation alters MarA function. We used random transposome mutagenesis to find chromosomal genes affecting MarA-dependent MDR. We could not directly screen for mutants with altered MDR, since we would obtain many mutants that affected MDR by mechanisms unrelated to MarA. Therefore, we chose an alternative primary screening method which detected reduction of MarA function. As detailed in Materials and Methods, we used the HdeA100 strain (Δ *marRAB hdeABp-lacZ*, with *marA* cloned in a plasmid) to select mutants in which MarA had lost its ability to repress the expression of the *hdeABp-lacZ* fusion (blue colonies). After screening 11,000 mutants (about 2.5 hits per gene), we found 24 different chromosomal genes whose inactivation prevented *hdeAB* repression by MarA. They encoded unrelated proteins, including transcriptional regulators (*csfG*, *crp*, *hns*, and *ompR*), enzymes (*appA*, *cyaA*, *degP*, *maoC*, *metL*, *pcnB*, *recD*, *treC*, and *ynfE*), transport proteins (*acrA*, *acrB*,

mhpT, *nikD*, and *tolC*), and others (*alx*, *damX*, *metV*, *yfdG*, *yibL*, and *yniD*) (see Table 3).

Our assumption was that some of these genes would also affect MarA function on genes other than *hdeAB* and, thus, would be important for MarA-mediated MDR. On the other hand, the effects of some of them on MarA may be specific for *hdeAB* or may be the result of an indirect effect on the complex regulatory network of *hdeAB* (see reference 42), or some might affect the expression or copy number of the IPTG-inducible, *marA*-containing plasmid in the strain HdeA100. Such genes, identified just because of the screening method used, were not expected to affect MarA-mediated MDR; however, some may still be relevant for MarA-mediated MDR if, independently, they were also involved in regulation of *marA* expression or MarA function (for example, global regulators and other genes with pleiotropic effects).

Since the goal of this work was to find genes important for MarA-mediated MDR, the identified genes were not studied with regard to how they affected *hdeAB* repression by MarA. Instead, we performed a second screen, involving antibiotic resistance, to find which of them affected MarA-mediated MDR. This second screen was not performed in the HdeA100 background because of the aforementioned limitations and because, in this strain, *marA* is cloned in a multicopy IPTG-inducible plasmid. In the HdeA100 strain, therefore, the promoter and copy number of *marA* are not physiological, and ampicillin is needed in the plates to maintain the plasmid, which may alter the effects of the antibiotics tested in the second screen.

For the second screen, therefore, we transferred the mutations in the identified genes to the parental strains CR1000 (*E. coli* BW25113 Δ *marR*; overexpresses *marA*) and CR2000 (*E. coli* BW25113 Δ *marRA*; MarA free). These two strains were chosen for several reasons. There is almost no phenotypic difference in antibiotic susceptibility between a wild-type strain and a Δ *marRA* strain (Table 2) because the expression of *marA* in a wild-type strain (BW25113) is strongly repressed by MarR. A system in which *marA* expression is not repressed is necessary to study MarA-mediated MDR. In a wild-type strain, such derepression can be achieved by adding to the culture medium chemicals such as salicylate, known to induce *marA* expression by inactivating MarR (see the introduction). However, salicylate affects cell growth, MDR, and gene expression independently from MarA, which would complicate our studies and their interpretation. A more appropriate system for our screen involved the use of a Δ *marR* parental strain, which constitutively overexpresses *marA* at physiological levels from its native location in the chromosome and, thus, is similar to MDR clinical isolates that overexpress *marA* because they have mutations in *marR*. As a comparing strain, we chose the Δ *marRA* parental strain instead of the wild-type strain because both strains are physiologically similar (similar antibiotic susceptibilities) and because the Δ *marRA* strain is completely MarA free so it is the best reference for antibiotic susceptibility and gene expression levels in the absence of MarA. Moreover, using this strain instead of the wild type prevents genes that indirectly affect *marA* expression via MarR, e.g., proteins that interact with MarR, from being found in our screen (13).

After transferring the mutations in the identified genes to the Δ *marR* and Δ *marRA* parental strains, we examined the

TABLE 2. MICs and levels of MarA-mediated MDR in the wild-type and parental strains

Strain	Description or genotype	MIC ($\mu\text{g/ml}$) ^{a,b}			
		FOX	NOR	CHL	MIN
BW25113	Wild type	4.0	0.09	10.7	3.3
CR2000	BW25113 ΔmarRA (MarA-free parental strain)	3.2	0.07	8.1	2.4
CR1000	BW25113 ΔmarR (MarA-overproducing parental strain)	10.6	0.21	23.9	4.9

^a The results are presented as the average MICs (the standard errors of the means were 0 to 15% [$n = 3$ to 9]) for cefoxitin (FOX), norfloxacin (NOR), chloramphenicol (CHL), and minocycline (MIN). MarA mediated low-level resistance to all four antibiotics, as shown by the significant increase in MICs in the ΔmarR parental strain compared to the levels for the ΔmarRA parental and wild-type strains. MarA also mediated resistance to other antibiotics tested (ampicillin, cephalothin, nalidixic acid, rifampin, erythromycin, tetracycline, and doxycycline) but had no effect on susceptibility to aminoglycosides, trimethoprim-sulfamethoxazole, imipenem, or fosfomycin (data not shown).

^b The CR1000 MIC/CR2000 MIC ratios were as follows: for FOX, 3.3; for NOR, 3.1; for CHL, 3.0; and for MIN, 2.0. These ratios represent 100% MarA-mediated resistance to each antibiotic (see Table 3), that is, the increase in the MIC resulting from physiological overexpression of *marA* in the ΔmarR parental strain (CR1000) compared to the MIC for the ΔmarRA parental strain (CR2000).

susceptibilities of the resulting strains to four structurally and functionally unrelated bactericidal (cefepime and norfloxacin) or bacteriostatic (chloramphenicol and minocycline) antibiotics whose increased MICs were MarA dependent (their MICs were increased in the ΔmarR parental strain compared to the level for the ΔmarRA parental strain) (Table 2).

Fifteen identified genes affect MarA-mediated antibiotic resistance. Compared to the levels for the ΔmarR and ΔmarRA parental strains, inactivation of 15 of the 24 identified genes reduced MarA-mediated resistance to at least one antibiotic by 50% or more; in contrast, inactivation of 9 of the 24 genes (*cspG*, *ompR*, *appA*, *metL*, *yfnE*, *mhpT*, *abx*, *metV*, and *yfdG*) did not significantly alter MarA-mediated antibiotic resistance (Table 3).

Inactivation of 7 of the 15 genes affecting resistance reduced MarA-mediated resistance to only one antibiotic: these genes were *nikD*, *degP*, *recD*, and *yibL* (chloramphenicol), *maoC* and *yniD* (minocycline), and *treC* (norfloxacin) (Table 3). This finding suggests that these genes do not affect *marA* expression or MarA function on genes essential for the Mar phenotype, such as *acrAB* or *tolC*. In contrast, these seven genes may have a role in some MarA-mediated cell responses that are important only for certain antibiotics or may be necessary for such responses to be effective. These genes were not further studied.

Inactivation of the other eight genes (*crp*, *hns*, *cyaA*, *pcnB*, *acrA*, *acrB*, *tolC*, and *damX*) decreased MarA-mediated MDR to all the antibiotics tested (Table 3). This was expected for *acrA*, *acrB* and *tolC* because of the major role of AcrAB-TolC in the Mar phenotype but was not known for the other five genes. Because of their general effect in MarA-mediated MDR, these genes were studied in more detail (see Fig. 1 to 4).

***crp* and *cyaA*.** CRP is one of the major global transcriptional regulators in *E. coli*. It is involved in regulation of catabolic operons in response to the energetic status of the cell and in other functions. CRP directly activates or represses the transcription of about 200 genes, although it probably interacts with many other low-affinity binding sites in the chromosome. The activity of CRP is triggered by binding of the second messenger cAMP, whose conversion from ATP is catalyzed by the adenylate cyclase enzyme CyaA in response to glucose starvation and other stresses (references 20 and 35 and references therein).

Inactivation of *crp* or *cyaA* moderately reduced MarA-mediated MDR to all the antibiotics tested (Table 3). Complementation of these mutants by the *crp* or *cyaA* gene on a

plasmid restored MarA-mediated MDR (Fig. 1). Also, addition of 1 mM cAMP to the culture medium complemented the *cyaA* inactivation, whereas it had no effect on the parental or *crp* strains (data not shown). Inactivation of *crp* or *cyaA* had previously been shown to increase MDR to oxacillin, macrolides, and crystal violet in a strain also inactivated for the major pump AcrAB-TolC because CRP-cAMP represses the expression of the accessory multidrug efflux pump MdtEF (38). In contrast, we show here that CRP-cAMP contributes to MDR in *E. coli* in the presence of MarA and the AcrAB-TolC pump.

The reason for such a reduction in MarA-mediated MDR in the *crp* and *cyaA* mutants seems to be that the expression of *marA* (Fig. 2A) and, in consequence, the MarA induction of *acrA*, *tolC*, and *micF* (Fig. 2B) were significantly reduced in both mutants. Earlier studies had demonstrated that CRP-cAMP binds to and activates *marRAB* transcription *in vitro* but not in cells grown in minimal medium (20, 49). Our results agree with those *in vitro* results and suggest that activation of *marA* expression by CRP-cAMP does occur in cells grown in rich medium, which is important for full *acrA*, *tolC*, and *micF* activation by MarA and thus for MarA-mediated MDR. However, indirect effects of CRP-cAMP on regulation of *marA* expression cannot be ruled out, given the many genes regulated by CRP-cAMP.

Therefore, *crp* and *cyaA* play a role in MarA-mediated MDR by being involved in upregulation of *marA* expression and, in consequence, of MarA-regulated genes.

***hns*.** H-NS is a nucleoid-associated DNA-binding protein that plays a major role in the organization of the bacterial chromosome and in regulation of gene expression. It affects the transcription of over 5% of *E. coli* genes, usually acting as a repressor or as a gene silencer. H-NS plays a pleiotropic role in bacterial response to environmental stimuli such as temperature, osmolarity, pH, and starvation (17).

Inactivation of *hns* strongly reduced MarA-mediated MDR to all the antibiotics tested (Table 3). Addition of *hns* on a plasmid restored (cefepime) or significantly increased (norfloxacin, chloramphenicol, and minocycline) MarA-mediated MDR (Fig. 1). H-NS has previously been shown to decrease MDR in a strain inactivated for the major multidrug efflux pump AcrAB-TolC. This occurred because H-NS represses *acrEF*, *mdtEF*, and *emrKY* expression. These three operons are not expressed in a wild-type strain grown in LB and encode three TolC-dependent MDR efflux pumps whose activity is detected only in the absence of AcrAB-TolC (37). In contrast,

TABLE 3. Effect of genes whose inactivation prevented repression of *hdeAB* by MarA on MarA-mediated MDR

Protein/function	Gene	Locus ^b	Level of MarA-mediated MDR (% of parental level) ^a			
			FOX	NOR	CHL	MIN
Transcriptional regulators						
Cold shock protein CspG	<i>cspGp^c</i>	b0990	54	91	93	75
Cyclic AMP receptor protein	<i>crp</i>	b3357	21	33	29	38
DNA-binding protein H-NS	<i>hns</i>	b1237	19	23	20	35
OmpR transcriptional dual regulator	<i>ompR</i>	b3405	67	77	93	100
Enzymes						
Acid phosphatase	<i>appA</i>	b0980	68	84	93	100
Adenylate cyclase	<i>cyaA</i>	b3806	43	41	46	32
Serine protease Do	<i>degP</i>	b0161	94	91	50	100
Putative ring-cleavage enzyme of phenylacetate degradation	<i>maoC</i>	b1387	100	61	75	40
Aspartate kinase/homoserine dehydrogenase	<i>metL</i>	b3940	120	91	100	100
Poly(A) polymerase I; RNA modification	<i>pcnB</i>	b0143	10	23	33	30
RecBCD DNA helicase/exonuclease	<i>recD</i>	b2819	80	77	50	65
Trehalose-6-phosphate hydrolase	<i>treC</i>	b4239	94	46	85	100
Oxidoreductase subunit paralog of DmsA	<i>ynfE</i>	b1587	94	68	93	100
Transport						
AcrAB-TolC multidrug efflux system	<i>acrA</i>	b0463	16	0	8	40
AcrAB-TolC multidrug efflux system	<i>acrB</i>	b0462	6	9	8	40
Putative propionic acid transporter	<i>mhpT</i>	b0353	68	91	100	100
Subunit of nickel ABC transporter	<i>nikD</i>	b3479	68	68	50	125
TolC outer membrane channel	<i>tolC</i>	b3035	0	0	0	40
Others						
Putative membrane protein	<i>alx</i>	b3088	120	68	100	65
Predicted membrane-anchored protein	<i>damX</i>	b3388	40	46	50	30
tRNA-methionine	<i>metV</i>	b2816	74	91	75	65
CPS-53 (KpLE1) prophage; bactoprenol-linked glucose translocase (flippase)	<i>yfdG</i>	b2350	68	91	93	65
Conserved protein	<i>yibLp^c</i>	b3602	68	68	50	100
Predicted unconserved protein	<i>yniDp^c</i>	b4535	94	77	125	50

^a Abbreviations: FOX, cefoxitin; NOR, norfloxacin; CHL, chloramphenicol; MIN, minocycline. MarA-mediated MDR for each mutant was calculated as the fold increase in MIC for the $\Delta marR$ background (CR1000-derivative mutant) compared to the MIC for the $\Delta marRA$ background (CR2000-derivative mutant) by using the $\Delta marR$ MIC/ $\Delta marRA$ MIC ratio. This ratio for the parental strains (the MIC for the $\Delta marR$ parental strain CR1000 divided by the MIC for the $\Delta marRA$ parental strain CR2000) for each antibiotic (shown in Table 2, footnote b) was considered to represent 100% MarA-mediated antibiotic resistance, whereas a ratio of 1 (with the MIC for the $\Delta marR$ background being equal to the MIC for the $\Delta marRA$ background) was considered to represent 0% MarA-mediated antibiotic resistance. These two reference points were used to calculate the percentage of MarA-mediated antibiotic resistance for each mutant according to its $\Delta marR$ MIC/ $\Delta marRA$ MIC ratio. When inactivation of a gene significantly reduced MarA-mediated resistance to an antibiotic compared to the level for the parental strain ($P < 0.01$, associated with reductions of 50% or more), the corresponding value is highlighted in bold. For parental strains, $n = 9$; for mutants, $n = 2$ to 5.

^b The locus tags refer to accession numbers (<http://biocyc.org/ecocyc/index.shtml>).

^c The insertion of the transposome was in the promoter of the gene. All the other insertions were within the open reading frame (ORF) of the genes.

we found that H-NS increases MDR in *E. coli* in the presence of MarA and an intact AcrAB-TolC pump.

When we studied the *hns* mutant at the level of gene expression, we found that *marA* expression and MarA-dependent induction of *tolC* and *micF* expression were unaffected (Fig. 2). However, the level of *acrA* induction by MarA was half that in the parental strain (Fig. 2B). This finding indicates that the reduced MarA-mediated MDR observed in the *hns* mutant is caused by a decrease in the AcrAB component of the AcrAB-TolC multidrug efflux pump. Such a specific contribution by H-NS to MarA induction of *acrA*, but not the other MarA-regulated genes tested, is of interest because H-NS is known to have no effect on regulation of *acrA* *per se* (37). Moreover, such an effect also occurs on *hdeAB*, which is regulated by MarA and H-NS both independently and synergistically (42). Decreased induction of *acrA* expression by MarA in the *hns* mutant (Fig. 2B) did not involve alteration of *marA* expression (Fig. 2A), and no protein-protein interaction between H-NS and MarA was found in two-hybrid experiments (data not

shown). Thus, this effect is either indirectly mediated by other H-NS-regulated genes or attributable to synergic MarA binding or function at the *acrA* promoter caused by H-NS effects on DNA topology. An effect of DNA topology changes on *acrA* expression has not been reported before; however, DNA looping or changes in DNA supercoiling produced by H-NS, even when bound far from a promoter, can alter the DNA affinity of other transcription factors (reference 17 and references therein).

Therefore, *hns* plays an essential role in MarA-mediated MDR by being necessary for full MarA induction of *acrA* expression.

***pcnB*.** The *pcnB* gene encodes the enzyme poly(A) polymerase I (PAP I), which, together with polynucleotide phosphorylase and other proteins, is involved in polyadenylation, processing, and degradation of the vast majority of *E. coli* mRNA transcripts in exponential phase (reference 36 and references therein).

Inactivation of *pcnB* strongly reduced MarA-mediated

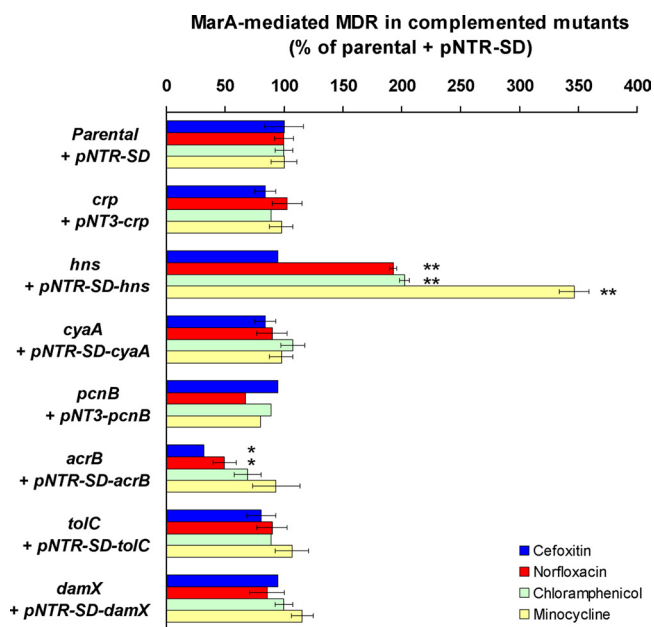


FIG. 1. Complementation of genes whose inactivation reduced MarA-mediated MDR to all antibiotics tested. Those genes whose inactivation reduced MarA-mediated MDR to all antibiotics tested (Table 3) were added back on a plasmid to their respective mutants to study if MarA-mediated MDR was restored. All plasmids were derivatives of the control plasmid pNTR-SD (Table 1). The percentage of MarA-mediated MDR was calculated as explained in Table 2, footnote *b*, and Table 3, footnote *a*, with 100% MarA-mediated antibiotic resistance defined as the ratio resulting from dividing, for each antibiotic, the MIC for the $\Delta marR$ parental strain CR1000 bearing the pNTR-SD plasmid by the MIC for the $\Delta marRA$ parental strain CR2000 bearing pNTR-SD. The results are presented as the average \pm the standard error of the mean ($n = 4$). Statistically significant differences are shown as * ($P < 0.05$) or ** ($P < 0.01$).

MDR to all the antibiotics tested (Table 3); addition of *pcnB* on a plasmid to this mutant restored MarA-mediated MDR (Fig. 1). The reason for such a reduction in MarA-mediated MDR in the *pcnB* mutant seems to be that the levels of *marA* transcripts (Fig. 2A) and, by consequence, MarA induction of *acrA*, *tolC*, and *micF* (Fig. 2B) were strongly reduced in this mutant compared to the levels for the parental strain. Further experiments showed that *marA* transcripts were polyadenylated both in the parental strain and in the *pcnB* mutant (RT-PCR products of the same size were obtained; data not shown). Moreover, we found no significant differences in the half-lives of the *marA* mRNA transcripts between the parental strain and the *pcnB* mutant (Fig. 3). These findings suggest that the reduction in *marA* transcript levels found in the *pcnB* mutant compared to the level for the parental strain is not the result of a decrease in *marA* mRNA stability but rather the result of a decrease in *marA* transcription. Considering that a role for PAP I in transcription has not been reported, the effect of PAP I on *marA* expression may be indirectly mediated by other proteins, such as SoxS and Rob, two activators of *marA* transcription whose mRNA stabilities have been found to be increased after overproduction of PAP I (36).

Therefore, *pcnB*-encoded PAP I plays an important role in MarA-mediated MDR by being necessary, probably indirectly, for full *marA* expression.

acrA, *acrB*, and *tolC*. Upregulation of the expression of the AcrAB-TolC multidrug efflux pump is the main basis for MarA-mediated MDR (28, 39), which explains why inactivation of *acrA*, *acrB*, or *tolC* dramatically reduced MarA-mediated MDR to all the antibiotics tested (Table 3). Such reduction was similar for each of these three genes (Table 3). This fact suggests that other minor multidrug efflux pumps in which AcrA or TolC are involved (28) do not play a role in MarA-mediated MDR under the conditions tested here.

These three genes may have additional effects on the MarA system besides their role in antibiotic efflux, since we found in our first screen that their inactivation also reduced MarA repression of *hdeAB* by an unknown mechanism. Therefore, we studied them in more detail to determine if they have other effects on MarA-mediated MDR.

To study additional effects of the AcrAB component of the AcrAB-TolC pump, we focused on the *acrB* mutant. As expected, complementation of this mutant with the *acrB* gene on a plasmid restored MarA-mediated MDR (Fig. 1). However, such a complementation was only partial for two antibiotics (cefoxitin and norfloxacin), which suggested that the inactivation of *acrB* might be also affecting the expression of the upstream *acrA* gene. In fact, when we studied the *acrB* mutant at the level of gene expression (RT-qPCR), we found that *acrA* transcriptional activation by MarA was indeed significantly reduced (Fig. 2B). However, *marA* expression (Fig. 2A) and *tolC* and *micF* activation by MarA (Fig. 2B) were not affected. To study whether this reduction in *acrA* activation by MarA was produced by the insertion of the transposome in *acrB* or by the absence of the AcrB protein, we added a single-copy plasmid bearing an *acrA-lacZ* transcriptional fusion to the parental and *acrB* mutant strains. In the parental strain, MarA activation of *acrA-lacZ* expression obtained by β -galactosidase assays (not shown) was similar to MarA activation of *acrA* expression found by RT-qPCR, showing the equivalence of both assays; however, in the *acrB* mutant, *acrA-lacZ* activation by MarA was no longer reduced compared to the level for the parental strain (not shown), in contrast to the RT-qPCR results. This finding shows that the reduced *acrA* activation by MarA in the *acrB* mutant compared to the level for the parental strain observed by RT-qPCR was produced artifactually by the *cis* transposome insertion in *acrB* and not by the absence of the AcrB protein.

Complementation of the *tolC* mutant with *tolC* on a plasmid restored MarA-mediated MDR, as expected (Fig. 1). When we studied this mutant at the level of gene expression, we found that *marA* expression was significantly increased (2.7-fold) compared to the level for the parental strain (Fig. 2A). This result agrees with a recent publication by Rosner and Martin (41). Using a different method (*lacZ* fusions) to study gene expression, they found that *marA* and *soxS* expression, Rob activity, and the expression of several genes belonging to the *mar* regulon were increased in a *tolC* mutant (41). They did not find the mechanism by which inactivation of *tolC* affects *marA* expression, but they propose that TolC is involved in one or more pumps responsible for effluxing cellular metabolites; in a *tolC* mutant, these metabolites would not be eliminated and would trigger the activation of the MarA/SoxS/Rob system in order to upregulate TolC-mediated efflux and restore homeostasis (41). The metabolites and pump(s) involved are still

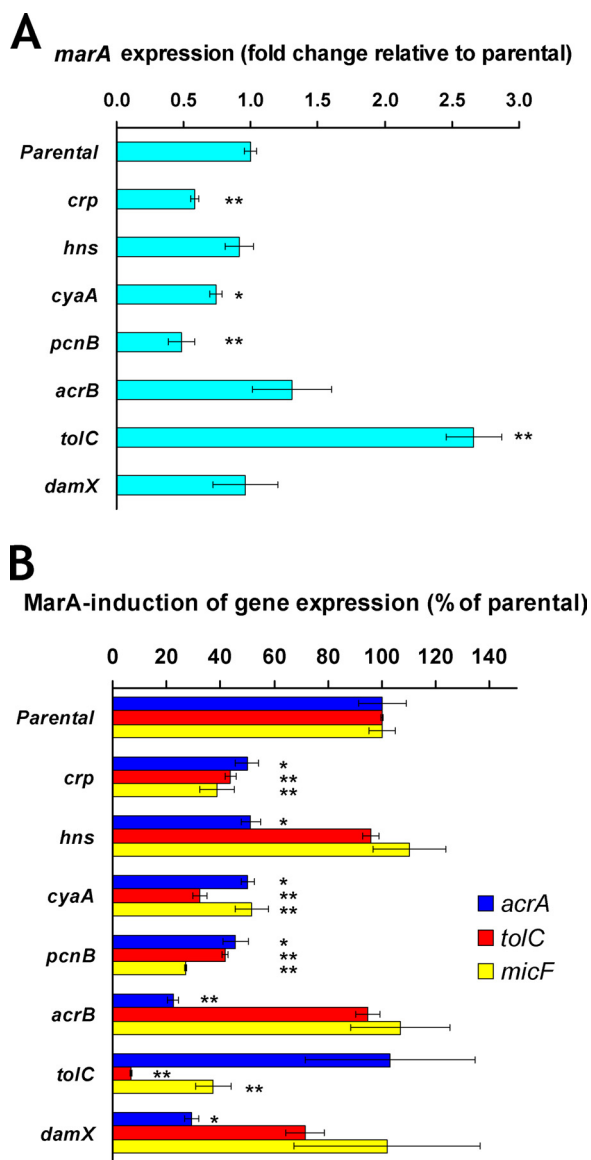
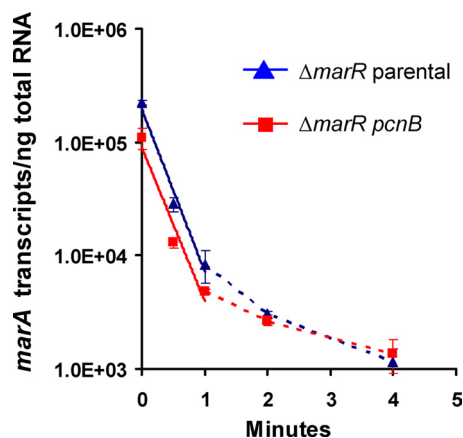


FIG. 2. Gene expression in mutants with reduced MarA-mediated MDR to all antibiotics tested. The effect of each inactivated gene on the expression of *marA*, *acrA*, *tolC*, *micF*, and *gapA* was measured by RT-qPCR (see Materials and Methods). The results are presented as the average \pm the standard error of the mean ($n = 3$ to 5). Statistically significant differences for a mutant compared to the level for the parental strain are shown as * ($P < 0.05$) or ** ($P < 0.01$). (A) Fold change in *marA* expression in different $\Delta marR$ -derivative mutants relative to the level for the $\Delta marR$ parental strain CR1000. Fold 1 means no change in *marA* expression in a mutant compared to the level for the parental strain. *gapA* was used as an endogenous reference gene, and its expression was not affected by *marA* (*gapA* expression was the same in the $\Delta marR$ and $\Delta marRA$ parental strains; data not shown). Except for *pcnB*, none of the inactivated genes tested significantly affected the expression of *gapA*. The *pcnB* mutant showed reductions in both *gapA* and *marA* levels. However, such reductions seem to be an effect specific for these genes and not a general effect produced by *pcnB* inactivation on all RNAs, since inactivation of *pcnB* in the $\Delta marRA$ background produced the same reduction in *gapA* levels but did not decrease the levels of the other genes studied (*acrA*, *tolC*, and *micF*) (data not shown). (B) Effect of inactivation of each gene on MarA induction of *acrA*, *tolC*, and *micF* expression. The percentage of MarA induction of gene expression for each mutant was calculated by comparing the $\Delta marR/\Delta marRA$ ratio (in terms of the number of tran-



scripts per ng of RNA) observed for the mutant to that observed for the parental strain, as explained for the percentage of MarA-mediated MDR in Table 3, footnote a.

unknown (41). However, they found that the mechanism by which *tolC* inactivation alters *marA* expression is independent of the major AcrAB-TolC pump, since inactivation of *acrAB* did not produce the changes in *marA* expression and other changes found when *tolC* is inactivated (41). Our findings agree with their result, since we also found no alteration of *marA* expression in the *acrB* mutant (Fig. 2A).

Interestingly, despite *marA* expression being higher in the *tolC* mutant (Fig. 2A), the degree of activation of *acrA* and *micF* expression by MarA in this mutant was equal to or less than that observed in the parental strain (Fig. 2B). However, in both the $\Delta marR$ and the $\Delta marRA$ backgrounds, the absolute numbers of *acrA* and *micF* transcripts per ng of RNA in the *tolC* mutant were 2- to 4-fold higher than those observed in the parental strain (data not shown). These findings may be explained by the increased *soxS* expression and Rob activity also found in a *tolC* mutant (41). SoxS and Rob are two MarA homologs that are functionally similar to MarA with respect to their binding sites and the genes that they regulate. Thus, their increased amount/activity in the *tolC* mutant (41) would explain the higher levels of *acrA* and *micF* expression in absolute numbers that we found in the *tolC* mutant compared to the level for the parental strain. Moreover, the functional overlapping of these regulators with MarA may explain why the level of *marA* expression was higher in the *tolC* mutant than in the parental strain but the level of MarA induction of *acrA* and *micF* was not higher. Induction of the expression of *acrA* and *micF* by additional amounts/activity of SoxS and/or Rob (41)

scripts per ng of RNA) observed for the mutant to that observed for the parental strain, as explained for the percentage of MarA-mediated MDR in Table 3, footnote a.

would impede additional induction of these genes by MarA, since all three regulators have the same binding site. These findings may be also explained by the different response that some genes have *in vivo* to changes in the concentration of MarA (34).

Therefore, *acrA*, *acrB*, and *tolC* are essential for MarA-mediated MDR because of the role of the AcrAB-TolC pump in antibiotic efflux. Moreover, *tolC* is involved in regulation of *marA* expression by an unknown and probably indirect mechanism.

damX. The *damX* gene encodes a predicted membrane-anchored protein conserved among many *Enterobacteriaceae*. Little is known about its regulation and function. Lyngstadaas et al. (29) found that overproduction of DamX interfered with cell division. However, they suggested that such an effect could be indirect or nonspecifically produced by nonphysiological amounts of DamX, since they found no differences between a wild-type and a *damX*-inactivated strain. Leclerc et al. (25) found that the *damX* homolog in *Salmonella* might be involved in adherence to and invasion of human intestinal cells.

We found that inactivation of *damX* moderately decreased MarA-mediated MDR to all the antibiotics tested (Table 3). This is the first report of a role for *damX* in *E. coli* when expressed at physiological levels. Addition of *damX* on a plasmid restored MarA-induced MDR (Fig. 1). Inactivation of *damX* had no effect on *marA* expression or on MarA induction of *tolC* and *micF* (Fig. 2). However, it dramatically decreased MarA induction of *acrA* expression (Fig. 2B). This finding indicates that the reduction in MarA-mediated MDR observed in this mutant was the result of a decrease in the AcrAB-TolC multidrug efflux pump. Considering that *marA* expression was unaffected (Fig. 2A), that no protein-protein interaction between DamX and MarA was found in two-hybrid experiments (data not shown), and that DamX is a predicted membrane protein, the effect of DamX on *acrA* induction by MarA is likely to be indirect.

Therefore, *damX* contributes to MarA-mediated MDR by specifically enhancing, probably indirectly, MarA induction of *acrA* expression.

The identified genes can mediate the adaptation of MarA-mediated MDR to environmental stimuli. Understanding how the genes found here affect MarA-mediated MDR provides a better knowledge of how *marA* functions in *E. coli*. Since the expression/activity of many of these genes/proteins are known to be regulated by different environmental stimuli, we studied whether these genes played a role in adaptation of MarA-mediated MDR to different environmental conditions. As examples, we focused on *crp*, *cyaA*, and *hns* (Fig. 4).

As mentioned above, CRP-cAMP regulates bacterial response to glucose starvation and other stress conditions. In the presence of glucose, CyaA does not catalyze the conversion of ATP to cAMP and CRP is not activated. As shown in Fig. 4, we found that addition of glucose (0.4%, wt/vol) to the medium indeed reduced MarA-mediated MDR to all the antibiotics tested in the parental strain. Such a reduction was similar to that observed for the *crp* and *cyaA* mutants in the absence of glucose. On the contrary, addition of glucose did not significantly alter MarA-mediated MDR in the *crp* and *cyaA* mutants (Fig. 4). Thus, MarA-mediated MDR is greater under glucose

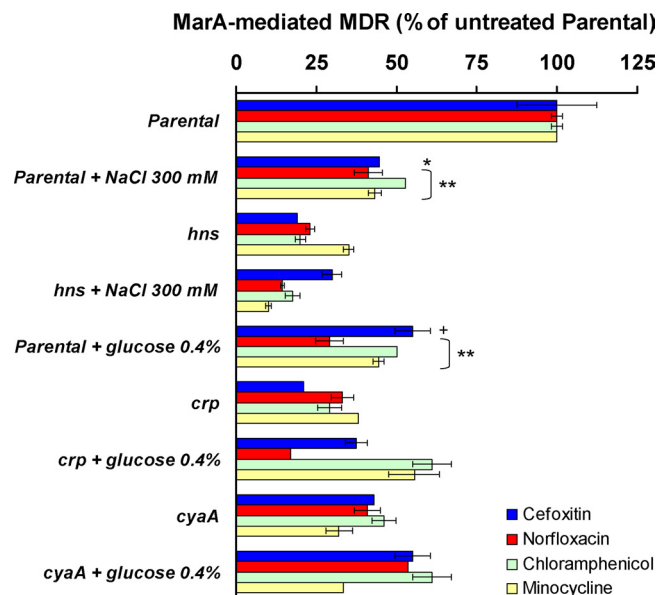


FIG. 4. Effects of *crp*, *cyaA*, and *hns* on adaptation of MarA-mediated MDR to environmental stimuli. The percentage of MarA-mediated MDR was calculated as explained in Table 3, footnote *a*. The results are presented as the average \pm the standard error of the mean ($n = 3$). When different conditions for the same strains were compared, addition of 0.4% glucose or 300 mM NaCl was found to produce statistically significant differences in MarA-mediated MDR in the parental strain (P was <0.01 for all antibiotics except for cefoxitin, in which case P was <0.05 for NaCl and P was <0.1 for glucose; these statistical significances are shown in the figure as “**,” “*,” and “+,” respectively), whereas these additions produced no differences in the mutants studied. Levels of MarA-mediated MDR were significantly different between the parental strain and the mutants in the absence of glucose and NaCl ($P < 0.01$ [see Table 3]; these statistical significances are not indicated in this figure), but no significant differences between them were found in the presence of glucose or NaCl.

starvation conditions than in the presence of glucose, and such an effect is dependent on *crp* and *cyaA*.

H-NS regulates bacterial response to osmolarity and other stresses. H-NS activity strongly decreases at high osmolarity (e.g., 300 mM NaCl) because changes in H-NS protein structure and/or DNA curvature reduce H-NS affinity for its DNA-binding sites (reference 14 and references therein). We found that addition of 300 mM NaCl to the medium reduced MarA-mediated MDR to all the antibiotics tested in the parental strain (Fig. 4). This reduction was similar to that observed in the *hns* mutant without added NaCl. On the contrary, addition of NaCl did not significantly alter MarA-mediated MDR in the *hns* mutant (Fig. 4). Thus, high osmolarity reduces MarA-mediated MDR in a *hns*-dependent way.

Additional comments. The results presented here demonstrate the importance of 15 chromosomal genes in modulating antibiotic resistance mediated by the transcriptional regulator MarA in *E. coli*. These genes encode unrelated transcriptional regulators, enzymes, transporters, and unknown proteins and can affect MarA response in general (by altering *marA* expression or MarA function) or specifically only for some antibiotics. Moreover, some of these genes can mediate the adaptation of MarA-mediated MDR to different environmental stimuli.

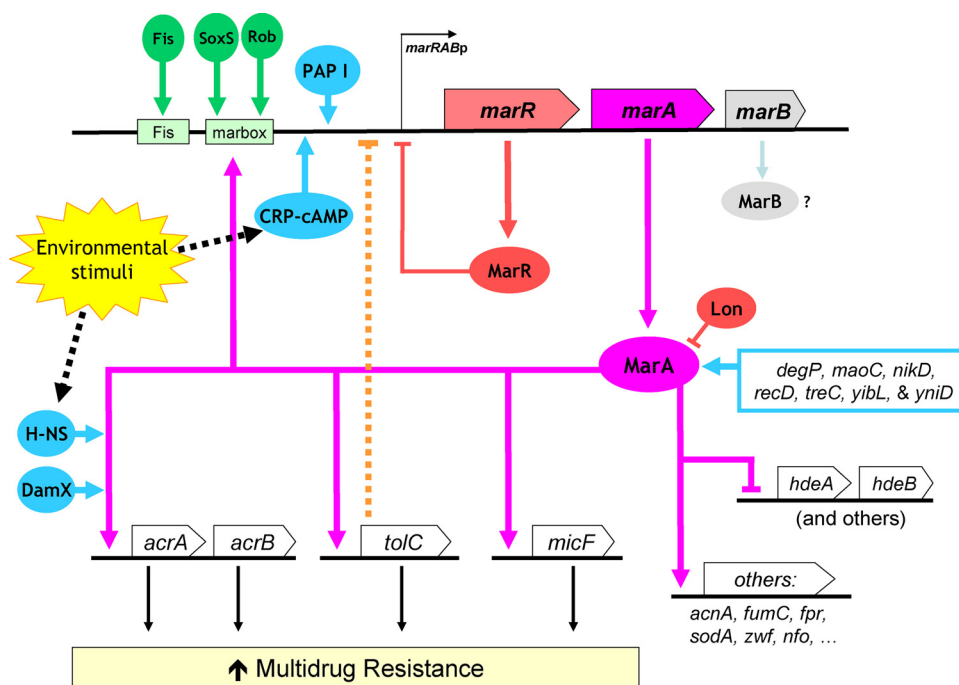


FIG. 5. Regulation of MarA-mediated MDR. The figure was produced using data from the literature (see main text) and the results obtained here. Functional interactions, either direct or indirect, are represented as arrows for activation/induction and as “-” for repression. New genes found here to affect MarA-mediated MDR are in blue.

These and previous findings show that MarA-mediated MDR is regulated at multiple levels, including *marA* transcription, MarA protein stability, and MarA function on specific promoters (Fig. 5). They also show an interconnection of the MarA system with global regulation and cell metabolism. Why does the MarA system have such a multifaceted regulation? It may be a consequence of the complexity of antibiotic action and cell responses to antibiotics, including secondary targets, signaling effects, and indirect effects, such as antibiotic-induced generation of hydroxyl radicals or changes in gene expression and cell metabolism (8, 15, 24). On the other hand, such a regulation would allow integration of different signals as well as transitory and fine-tuned adaptation of the MarA-mediated response to different conditions, which would ensure that such a response is always adjusted to the cell needs. This idea agrees with reports showing that *marA* expression changes when cells are grown in different media or growth phases (4).

This complex regulation has additional implications. Modification of *marA* expression (directly or indirectly, e.g., via *crp*, *cyaA*, *pcnB*, or *tolC*) is a mechanism of generally altering the expression of the MarA regulon, which may thus affect other MarA-regulated functions such as virulence (1, 6, 9). On the other hand, altering MarA activity only on some genes allows a specific modification of only certain MarA functions. Here, we found that *hns* and *damX* specifically alter MarA activation of *acrA* expression and, thus, MarA-mediated MDR. We had previously found that other regulators of *hdeAB* were able to modify MarA activity on *hdeAB* (42). Therefore, gene-specific alteration of MarA activity by other proteins seems not unusual, which may explain why some genes are differently activated *in vivo* by the same concentrations of MarA, especially

when these differences are poorly correlated with the *in vitro* affinity of MarA for their promoters (34).

The complexity and adaptability of the MarA response may favor the spread of MDR strains with mutations in the autorepressor *marR*. Also, the different levels of MDR found among different clinical isolates mutated in *marR* (e.g., reference 31) may be explained by additional mutations in the genes found here to modulate MarA-mediated MDR. Moreover, these findings raise the question of whether the effect of other MDR regulators, such as AcrR, a repressor of *acrA*, or the MarA-homologs SoxS and Rob, as well as *marA* orthologs or *marA*-like regulators in other clinically relevant pathogens, are also subject to this kind of fine-tuned regulation. Such a complex regulation of MDR may exist also in other bacteria, especially among *Enterobacteriaceae*. However, the specific regulatory details may be different since other species have different genes and regulatory networks, including additional MarA homologs and additional MDR regulators, and the genes found here to alter MarA-mediated MDR may have different effects. For example, in contrast with what was found for *E. coli*, inactivation of *tolC* in *S. enterica* does not affect *marA* expression but results in overexpression of the *marA* homolog *ramA* (48).

Finally, inhibitors of multidrug efflux pumps and inhibitors of MDR and virulence regulators, including MarA, have been proposed to enhance the effectiveness of antibiotics (7, 28). A potential alternative would involve the use of inhibitors of the expression or activity of genes that enhance *marA* expression or function. This approach would offer additional advantages, such as the possibility of inhibiting only some MarA functions or the possibility of synergistic effects, since some of these genes also have a role in MDR or virulence.

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