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Role of the Multidrug Resistance Regulator MarA in Global Regulation of the *hdeAB* Acid Resistance Operon in *Escherichia coli*▽†

Cristian Ruiz,[1,2] Laura M. McMurry,[1,2] and Stuart B. Levy[1,2,3]*

Center for Adaptation Genetics and Drug Resistance[1] and the Departments of Molecular Biology and Microbiology[2] and of Medicine,[3] Tufts University School of Medicine, Boston, Massachusetts 02111

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MarA, a transcriptional regulator in *Escherichia coli*, affects functions such as multiple-antibiotic resistance (Mar) and virulence. Usually an activator, MarA is a repressor of *hdeAB* and other acid resistance genes. We found that, in wild-type cells grown in LB medium at pH 7.0 or pH 5.5, repression of *hdeAB* by MarA occurred only in stationary phase and was reduced in the absence of H-NS and GadE, the main regulators of *hdeAB*. Moreover, repression of *hdeAB* by MarA was greater in the absence of GadX or Lrp in exponential phase at pH 7.0 and in the absence of GadW or RpoS in stationary phase at pH 5.5. In turn, MarA enhanced repression of *hdeAB* by H-NS and hindered activation by GadE in stationary phase and also reduced the activity of GadX, GadW, RpoS, and Lrp on *hdeAB* under some conditions. As a result of its direct and indirect effects, overexpression of MarA prevented most of the induction of *hdeAB* expression as cells entered stationary phase and made the cells sevenfold more sensitive to acid challenge at pH 2.5. These findings show that repression of *hdeAB* by MarA depends on pH, growth phase, and other regulators of *hdeAB* and is associated with reduced resistance to acid conditions.

MarA, an AraC/XylS transcriptional regulator of *Escherichia coli*, directly activates or represses multiple chromosomal genes. As a result, it affects many functions including multiple-antibiotic resistance (Mar), virulence, and survival (1, 5). MarA is specified by the *marRAB* operon, whose expression is induced by sodium salicylate, menadione, and other chemicals which inactivate the autorepressor MarR (1, 10, 33, 38). Using gene arrays, Pomposiello et al. (33) found that overexpression of MarA from an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid repressed *hdeAB* and other genes involved in acid resistance (*gadA*, *gadB*, *gadC*, *hdeD*, and *slp*), whereas sodium salicylate activated these same genes. Direct repression of *hdeAB* by MarA has been demonstrated previously (37). However, whether MarA affects cell resistance to acid conditions remained unknown.

The *hdeAB* operon of *E. coli* specifies two periplasmic chaperones that are essential for cell survival in rich media at extremely low pH, such as that in the stomach (pH 1.5 to 3). Both chaperones are only active below pH 3 and prevent irreversible aggregation of acid-denatured periplasmic proteins (14, 21, 23, 28). HdeA seems to play a major role at pH 2, whereas HdeB is more active at pH 3 (23). The determined HdeA crystal structure and the model HdeB structure are similar (14, 45), in agreement with their genetic and functional similarities. HdeA is one of the most abundant periplasmic proteins in stationary phase (25).

Expression of *hdeAB* is affected by different environmental conditions. The *hdeAB* operon is induced in stationary phase (39, 44) and at acid pH (19, 43). Transcription of *hdeAB* depends on complex circuits of regulation involving RpoD (σ^{70}), RpoS (σ^{38}), H-NS, cyclic AMP receptor protein, GadE, GadX, GadW, GadY, EvgAS, YdeO, Lrp, MarA, SoxRS, TorRS, and TrmE, among other regulators (see Fig. 1 and references in the legend; see Fig. 2 for the DNA binding sites of some of these regulators in the *hdeAB* promoter).

We have studied how MarA fits into the complex transcriptional control of *hdeAB*. We report the effect of pH, growth phase, and known regulators of *hdeAB* on *hdeAB* expression and repression by MarA, as well as the effect of MarA on the activities of these other regulators. We also describe the effect of MarA on acid resistance.

MATERIALS AND METHODS

Strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Because HdeA and HdeB seem to be mainly important in rich medium (28), we used LB medium (per liter: 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl) at pH 7.0 or buffered to pH 5.5 with 100 mM MES (morpholinoethanesulfonic acid). All cultures were grown at 37°C with agitation. Cells grown overnight (18 h) were subsequently diluted 1:100 in fresh media to an optical density at 600 nm (OD_{600}) of 0.03. According to the growth curves obtained, exponential-phase cells were defined as those obtained from cultures in fresh media grown for 2 to 2.5 h (0.3 to 0.6 OD_{600}) and early-stationary-phase cells as those obtained after 6 h (1.4 to 2.9 OD_{600}). The final pH of the cultures was 7.0 to 8.0 in LB at pH 7.0 and 5.6 to 6.0 in LB at pH 5.5. The antibiotics were used at 100 μg ml^{-1} (ampicillin) and 25 μg ml^{-1} (chloramphenicol, kanamycin, and tetracycline). MarA expression from plasmid pMB102 was induced by inoculation of cells from the overnight cultures into fresh medium containing 0.5 mM IPTG.

Construction of the *hdeAB*p-*lacZ* chromosomal transcriptional fusion and other DNA manipulations. A 372-bp fragment from the *hdeAB* promoter (from −349 to +23 relative to the transcriptional start site) was amplified by PCR using chromosomal DNA from strain AG100 as template, primers HdeALF (5′AGG gaattcAAAATATCGCCAGAGACGAAC, EcoRI site in lowercase) and HdeALR (5′TATggatccAGCCGTCACGAATCAAT, BamHI site in lowercase), and Turbo *Pfu* polymerase (Stratagene, La Jolla, CA), using a T_m of 55°C. The amplified DNA was cloned into the vector pRS415 between the EcoRI and BamHI sites, yielding the plasmid pHdeA415, whose insert sequence was verified

* Corresponding author. Mailing address: Center for Adaptation Genetics and Drug Resistance, 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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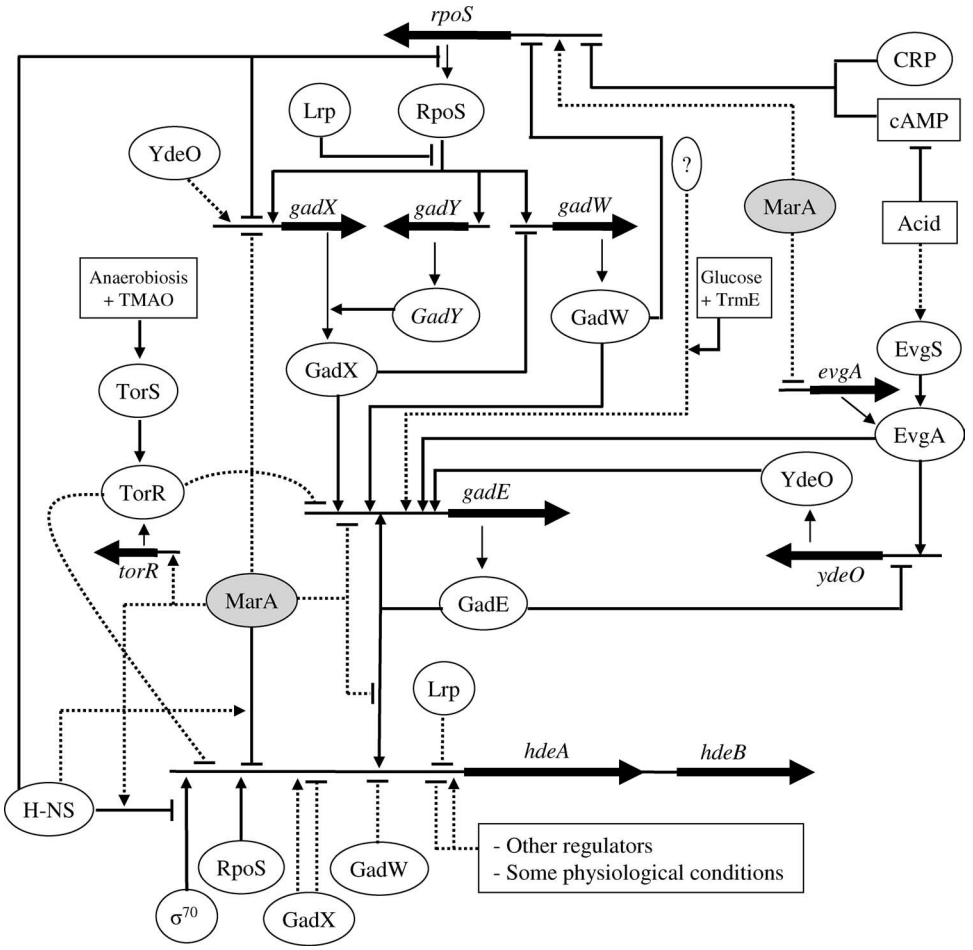

FIG. 1. Regulation of the *hdeAB* operon. The figure has been produced using data from the literature (8, 13, 16, 20, 22, 24, 26, 27, 30, 33, 37, 39, 42, 43, 44, 46) as well as the results obtained in this study. Thick arrows represent genes. Direct regulation is shown as a continuous thin arrow. Indirect regulation, or regulation in which a direct effect has not been confirmed, is shown as a dotted thin arrow. See the supplemental material for a more detailed explanation of this figure. cAMP, cyclic AMP; CRP, cAMP receptor protein.

at the Tufts University Core Facility. Recombination between the *hdeAB*p-*lacZ* region of pHdeA415 and λRZ5 (35) resulted in a lysate bearing λRZ5 (*hdeAB*p-*lacZ*). This was used to infect strain JHC1096, and Amp^r Lac^+ lysogens were selected and purified on LB agar containing ampicillin (50 μg ml^{-1}) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg ml^{-1}). Lysates from these lysogens were then used to infect strain JHC1096, and Amp^r Lac^+ lysogens were again isolated. The resulting strain, HdeA96, was confirmed by PCR, as previously described (34), to have a single copy of the transcriptional fusion located in the λ*att* site on the chromosome. Strain HdeA96 was subsequently transformed with plasmid pMB102 to obtain strain HdeA100. Plasmid $CaCl_2$ transformation and phage P1 transduction to transfer mutations in different *hdeAB* regulatory genes to the strain HdeA100 were performed according to standard procedures (36).

Identification of the putative binding sites for the *hdeAB* regulators. The *hdeAB* promoter was analyzed from positions −168 (transcriptional start site of the divergent gene *hdeD*) to +54 (translation start codon for HdeA) (Fig. 2). Putative binding sites were identified using the "search patterns" utility of Colibri (genolist.pasteur.fr/Colibri/). MarA (37) and H-NS (39) binding sites in the *hdeAB* promoter region have already been identified. The GadE binding site has been located between −158 and +4 but not identified (20). The consensus binding sequences we used were the following: 5′WYAGGMWWWWDYTW YWWW for binding of GadE, GadX, and GadW (gadbox [26, 43]); 5′YAGHA WATTWTDCTR for Lrp (11); and 5′CTGTTCATAT for TorR (2, 40); where D is A, T, or G; H is A, C, or T; M is A or C; R is A or G; W is A or T; and Y is C or T. Only sequences with 70% or higher identity to the consensus sequences were selected.

β-Galactosidase assays. Expression of the *marO-lacZ* and *hdeAB*p-*lacZ* chromosomal reporters was tested using exponential-phase and stationary-phase cells prepared as described above. The assays were performed using cells permeabilized with sodium dodecyl sulfate-chloroform as previously described (29). All assays were performed at least in triplicate; each replicate was the result of an independent assay performed in duplicate. Statistically significant differences were determined by *t* test (two independent samples with equal variance, two-tailed distribution) using Microsoft Excel 2003 software. Unless otherwise stated, differences were considered to be statistically significant only when P was <0.01.

Acid resistance assays at very low pH. The assays were performed using early-stationary-phase cells (OD_{600} of 1.3 to 2.5) prepared as described above and obtained from cultures in fresh media grown for 6 h (cultures in LB broth) or 7 to 8 h (cultures in LB broth containing 5 mM sodium salicylate). Control cells were directly plated on LB pH 7.0 agar after serial dilution in fresh LB broth at pH 7.0. To mimic the conditions that *E. coli* cells would encounter when passing through the stomach, sample cells were diluted to about 10^6 CFU per ml in LB at pH 2.5 (acidified with HCl) and incubated for 2 h at 37°C without shaking. Cells were then serially diluted in fresh LB broth at pH 7.0 and plated on LB pH 7.0 agar plates to determine viability. The assays were performed at least in triplicate.

RESULTS

Effect of stationary phase and pH on *hdeAB* expression and repression by MarA. We chose conditions that are known to

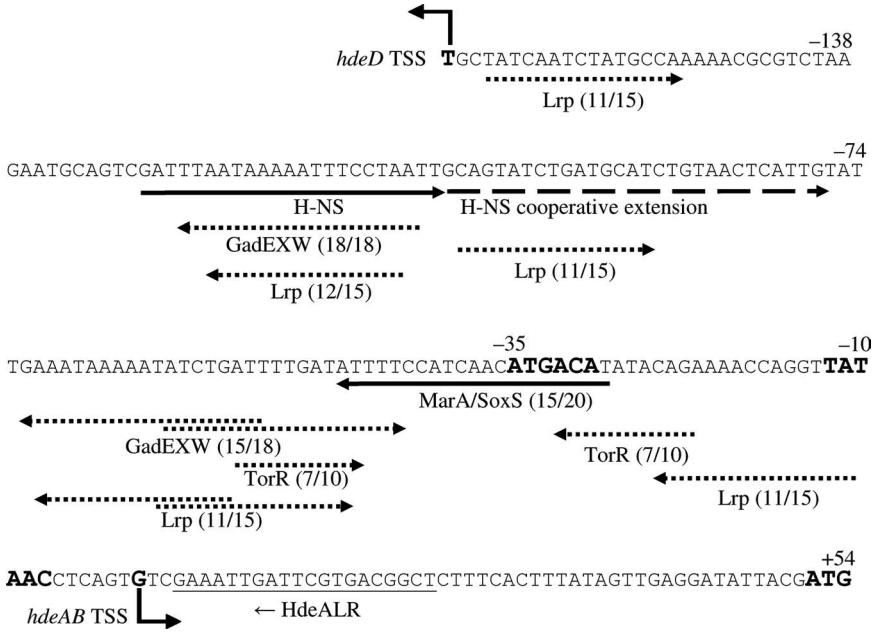

FIG. 2. Binding sites in the *hdeAB* promoter for the selected regulators. The *hdeAB* promoter was analyzed from positions −168 to +54 as described in Materials and Methods. The binding sites of the selected transcriptional regulators are shown below the corresponding sequence as continuous arrows (known) or dotted arrows (putative). In parentheses are the number of nucleotides that match the consensus sequence/number of nucleotides of the consensus sequence. Even though *hdeAB* is transcribed from RNA polymerase containing σ^{70} or σ^{38} (39), its promoter does not have the −10 sequence (CTATACT) found in many promoters regulated by σ^{38} that replaces the −10 sequence (XTATAAT) found in σ^{70} promoters (12). TSS, transcription start site.

have a major effect on the expression of *hdeAB* (exponential versus stationary phase and pH 7.0 versus pH 5.5 [19, 39, 43, 44]) and on the physiology of the HdeA and HdeB proteins (LB rich medium [28]). In a Δ*marRAB* strain bearing an *hdeAB*p-*lacZ* chromosomal reporter, the expression of *hdeAB* at pH 7.0 increased 1.7-fold from exponential phase to early stationary phase (Fig. 3). The β-galactosidase activity obtained and the increase in *hdeAB* expression observed are in agree-

TABLE 1. Bacterial strains and plasmids used in this study

Name	Genotype/relevant characteristics	Reference/source
E. coli strains		
AG100	*argE3 thi-1 rpsL xyl supE44* λ lysogen	15, also see reference 4; this study[a]
CV1008	CV976 *lrp-35*::Tn*10* (Tet^r)	32
DT162	MG1655 Δ*gadX*::*kan*	43
DT203	MG1655 Δ*gadW*::*kan*	43
EF1155	MG1655 Δ*gadE*(*yhiE*)::*kan*	26
GC4468	Δ(*lac*)*U169 rpsL*	9
JHC1096	GC4468 *zdd-239*::Tn*9 del1738* (39 kb deleted, including *marRAB* locus) (Cm^r)	17
LCB621	MC4100 *torR49*::mini-Tn*10* (Tet^r)	31
PS2652	Δ*lacZ169 zch-506*::Tn*10 hns-1001*::Tn*seq1* (Kan^r)	6
SPC105	MC4100 $marO_{11}$-*lacZ* (Amp^r) in λ*att* site	10
ZK1000	ZK126 Δ*rpoS*::*kan*	7
HdeA96	JHC1096 *hdeAB*p::*lacZ* (Amp^r) in λ*att* site	This study
HdeA100	HdeA96 transformed with pMB102	This study
HdeA101	HdeA100 *hns-1001*::Tn*seq1* (Kan^r)	P1 PS2652 × HdeA100
HdeA103	HdeA100 Δ*rpoS*::*kan*	P1 ZK1000 × HdeA100
HdeA105	HdeA100 *lrp-35*::Tn*10* (Tet^r)	P1 CV1008 × HdeA100
HdeA107	HdeA100 *torR49*::mini-Tn*10* (Tet^r)	P1 LCB621 × HdeA100
HdeA109	HdeA100 Δ*gadX*::*kan*	P1 DT162 × HdeA100
HdeA111	HdeA100 Δ*gadW*::*kan*	P1 DT203 × HdeA100
HdeA113	HdeA100 Δ*gadE*::*kan*	P1 EF1155 × HdeA100
Plasmids		
pHdeA415	*ori colE1 hdeAB*p-*lacZ*, AmpR	This study
pJPBH	*ori colE1 lacI*, AmpR	37
pMB102	*ori colE1 lacI lacZp*::*marA*, AmpR	33
pRS415	*ori colE1 lacZ* fusion vector, AmpR	41

[a] Determined in this study to be a single lysogen of λ by PCR as previously described (34).

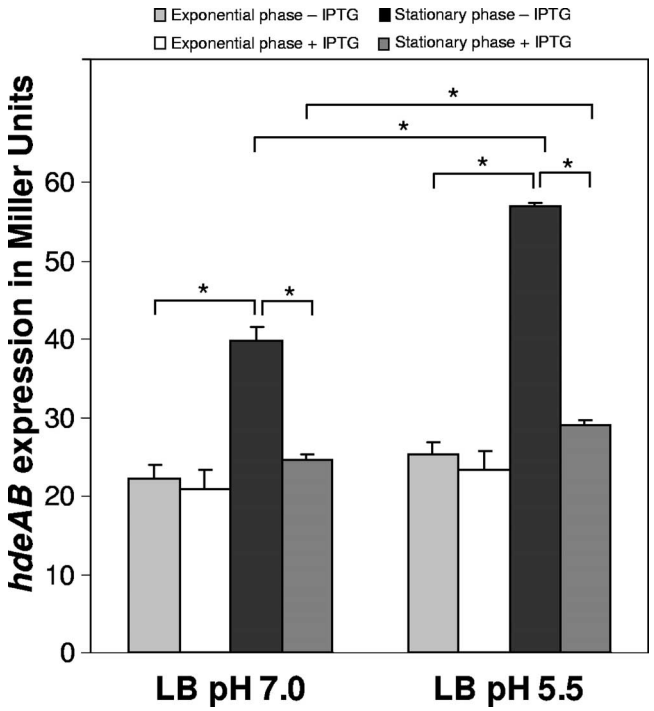

FIG. 3. Effect of MarA on the expression of *hdeAB*. The β-galactosidase activity in Miller units of the *hdeAB*p-*lacZ* chromosomal reporter was determined using HdeA100 cells grown in LB at pH 7.0 (left) or in LB at pH 5.5 (right) to exponential or stationary phase. The assays were performed in the absence of MarA (without IPTG) or in the presence of MarA (with IPTG). The results are presented as the averages ± the standard errors of the means ($n \geq 3$). Statistically significant differences ($P < 0.01$) between related conditions (exponential versus stationary phase at the same pH; pH 7.0 versus pH 5.5 in the same growth phase; and presence versus absence of MarA under the same pH and growth phase) are indicated by asterisks.

ment with a previous report (39). When the assays were performed using late-stationary-phase cells (overnight cultures), the activity found was about 10-fold higher than that in exponential-phase cells (data not shown). Such high expression is consistent with the high amount of HdeA protein reported under similar conditions (25). At pH 5.5, the expression of *hdeAB* in exponential phase showed no significant increase compared to that at pH 7.0, even though other authors have found acid induction in exponential-phase cells grown under different conditions (19, 43). In stationary phase, *hdeAB* expression at pH 5.5 was larger than that at pH 7.0 (a 2.5-fold increase compared to expression in exponential phase at pH 5.5; Fig. 3).

When HdeA100 cells (bearing pMB102) were induced by IPTG to overexpress MarA, we found that MarA prevented nearly all of the 1.7- to 2.5-fold increase in *hdeAB* expression as cells entered stationary phase (Fig. 3). MarA did not repress *hdeAB* in exponential phase but did repress about twofold in stationary phase regardless of the pH (Fig. 3) to nearly the exponential-phase level. With the control plasmid pJPBH, IPTG led to no effect (data not shown).

Effect of known regulators on *hdeAB* expression: H-NS is the main repressor and GadE is the main activator of *hdeAB*. We asked whether the changes in the repression produced by MarA on *hdeAB* occurred via other known regulators of *hdeAB*. Seven regulators were selected for the study: H-NS, RpoS (σ^{38}), and GadE, which are known direct regulators of the *hdeAB* operon (see Fig. 1 and 2); and GadX, GadW, Lrp, and TorR, which are thought to be both direct (not demonstrated, but all of them have several putative binding sites in the *hdeAB* promoter; Fig. 2) and indirect regulators of *hdeAB* (they regulate H-NS, RpoS, or GadE; Fig. 1).

The effects of these regulators on *hdeAB* expression obtained earlier by others cannot be compared since these studies have been performed using dissimilar methods and conditions (in some cases only with microarrays of exponential-phase cells grown in minimal medium). Therefore, before studying their effects on MarA control of *hdeAB* expression, we evaluated the relative importance of each of these regulators individually on *hdeAB* expression under the same conditions. We chose the conditions described above that have a major effect on the expression of *hdeAB* and on the physiology of HdeA and HdeB.

The results obtained and their statistical significance are displayed in Table 2 (data in the absence of MarA). Under all the conditions studied, H-NS was the main repressor of *hdeAB*, although repression by H-NS was about half as great at pH 5.5 as at pH 7.0. GadE was the main activator. GadX was a direct or indirect activator of *hdeAB* only in stationary-phase cells at pH 5.5, but it was a repressor in exponential phase at pH 7.0;

TABLE 2. Activation or repression of *hdeAB* expression by selected regulators in the absence and the presence of MarA[a]

Regulator	Activation or repression in indicated growth phase[b,c]							
	pH 7.0				pH 5.5			
	Exponential		Stationary		Exponential		Stationary	
	–	+	–	+	–	+	–	+
H-NS	**–10.5**	**–9.9**	**–9.9**	**–13.7**	**–6.0**	**–6.1**	**–5.7**	**–8.7**
GadE	**2.9**	**2.9**	**3.5**	**2.2**	**3.7**	**3.7**	**4.0**	**2.3**
GadX	***–1.9***	***–1.5***	1.1	1.2	–1.1	1.0	**1.7**	**1.6**
GadW	**–1.4**	–1.2	–1.3	–1.2	–1.4	–1.2	***–2.3***	***–1.7***
RpoS	–1.2	–1.1	**1.3**	1.1	1.0	**1.2**	**–1.2**	1.0
Lrp	***–1.3***	*1.0*	1.0	1.1	–1.1	–1.1	–1.1	1.0
TorR	–1.1	–1.1	1.0	1.1	1.0	1.1	–1.1	1.0

[a] *hdeAB* expression in LB was determined by measuring the β-galactosidase activity of the *hdeAB*p-*lacZ* chromosomal reporter in the Δ*marRAB* strain HdeA100 (wild type) and in seven HdeA100-derivative strains inactivated for different *hdeAB* regulators (see Table 1). The assays were performed in the absence (−) of MarA (without IPTG) or in the presence (+) of MarA (with IPTG).

[b] Results are the *n*-fold activation (positive values) or repression (negative values) produced by each regulator on *hdeAB* expression and were obtained from a ratio of the *hdeAB* expression in the parental strain and the *hdeAB* expression in the strain inactivated for a particular regulator under the same conditions; e.g., a regulator that increased *hdeAB* expression five times (ratio of the parental strain/inactivated strain of 5) would be represented in the table as "5" and would be said to produce a fivefold activation. On the contrary, a regulator that decreased *hdeAB* expression five times (ratio of the parental strain/inactivated strain of 0.2; thus, ratio of the inactivated strain/parental strain of 5) would be represented in the table as "–5" and would be said to produce a fivefold repression. Note that both regulators would have the same "strength" with respect to their effect on *hdeAB* expression, although in an opposite way. Results in bold indicate that the activation or repression produced by a regulator was statistically significant ($P < 0.01$).

[c] Results in italics ($P < 0.05$) or underlined ($P < 0.01$) indicate that MarA produced a statistically significant change in the activation or repression produced by a regulator under the same conditions (e.g., the repression produced by H-NS on *hdeAB* in stationary phase at pH 5.5 was significantly stronger in the presence of MarA than in the absence of MarA).

TABLE 3. *hdeAB* repression by MarA in strains inactivated for selected regulators of *hdeAB*

Strain	Repression in indicated growth phase[a]			
	pH 7.0		pH 5.5	
	Exponential	Stationary	Exponential	Stationary
WT[b]	–1.1	*–1.7*	–1.1	*–2.0*
hns	–1.1	**–1.2**	–1.1	**–1.3**
gadE	–1.1	**–1.0**	–1.1	**–1.1**
gadX	**–1.3**	–1.7	–1.2	–1.9
gadW	–1.2	–1.7	–1.2	**–2.6**
rpoS	–1.2	–1.5	–1.3	**–2.5**
lrp	**–1.4**	–1.8	–1.1	**–2.3**
torR	–1.1	–1.7	–1.1	–2.0

[a] Results are the *n*-fold repression (since the values are negative) of *hdeAB* expression produced by MarA (the ratio calculated as *hdeAB* expression without MarA divided by *hdeAB* expression with MarA in the same strain and conditions). Results in bold ($P < 0.05$) or in bold and underlined ($P < 0.01$) indicate when the inactivation of a regulator produced a statistically significant change in the repression produced by MarA compared to the repression produced by MarA in the parental strain under the same conditions (same column). *hdeAB* expression was measured as described in the footnotes of Table 2.

[b] Repression of *hdeAB* expression produced by MarA in the parental strain (see Fig. 3) was statistically significant ($P < 0.01$) only in stationary phase (values in italics). WT, wild type.

TABLE 4. Effect of different growth conditions on the expression of *marRAB*[a] with and without the inducer sodium salicylate

Condition	Expression ratio[b]	
	Exponential phase[c]	Stationary phase[c]
No salicylate		
pH 7.0	1.0	1.3
pH 5.5	1.6	1.8
5 mM salicylate for 1 h[d]		
pH 7.0	7.7	1.7
pH 5.5	1.4	1.8
5 mM salicylate, added to diluted stationary-phase cells[e]		
pH 7.0	13.9	27.4
pH 5.5	ND	ND

[a] The expression of the *marRAB* operon in LB medium was studied using the chromosomal *marO-lacZ* reporter of the strain SPC105.

[b] The β-galactosidase activity under each condition was normalized to the activity without salicylate at pH 7.0 in exponential phase (154 ± 7.5 Miller units), defined as expression ratio 1.0. The expression of *marRAB* under all the conditions studied was statistically different ($P < 0.01$) from that found without salicylate at pH 7.0 in exponential phase. The pH of the cultures was unaffected by sodium salicylate.

[c] Exponential- and stationary-phase assays were performed at OD_{600} of 0.3 to 0.6 (achieved 2 h after inoculation from an overnight culture) and at OD_{600} of 1.9 to 2.7 (achieved after 6 h), respectively.

[d] Sodium salicylate was added to the cultures after 2 h of growth (exponential phase) or after 6 h of growth (stationary phase); the reporter expression was measured 1 h later.

[e] Overnight cultures grown without sodium salicylate were used to inoculate fresh media containing sodium salicylate. The cultures needed 4 to 5 h to reach OD_{600} of 0.3 to 0.6 and 17 h to reach OD_{600} of 1.9 to 2.7. The induction in LB at pH 5.5 was not measured because of cessation of cell growth under these conditions (ND).

GadW was a repressor of *hdeAB* mainly in stationary phase at pH 5.5. RpoS had a small effect on *hdeAB* expression and only under certain conditions. Lrp only repressed *hdeAB* in exponential phase at pH 7.0. TorR did not significantly affect *hdeAB* expression.

MarA also controls hdeAB expression by modifying the effect of other regulators of *hdeAB*. We studied whether MarA was able to alter the effect of the other regulators on *hdeAB* expression. Under each condition, we compared the regulator-inactivated strain/parental strain ratio obtained in the presence of MarA with that obtained without MarA (Table 2).

The activity of two major regulators, H-NS and GadE, under these conditions was strongly affected by MarA (Table 2). MarA enhanced *hdeAB* repression by H-NS in stationary phase at both pH 7.0 and pH 5.5, which indicates that MarA assists or collaborates with H-NS to repress *hdeAB* in stationary phase. On the contrary, MarA reduced the ability of GadE to activate *hdeAB* in stationary phase at pH 5.5, which suggests that MarA hinders *hdeAB* activation by GadE.

MarA also reduced the repression produced by GadX and Lrp in exponential phase at pH 7.0 and the repression produced by GadW and RpoS in stationary phase at pH 5.5 (Table 2).

Effect of the other regulators of *hdeAB* on repression by MarA. After finding that MarA was able to alter the effect of the other regulators on *hdeAB*, we studied the converse possibility: whether the absence of the other regulators affected the ability of MarA to repress *hdeAB* (Table 3). A dramatic decrease in the repression produced by MarA in stationary phase was found in the *hns* and *gadE* mutants at both pH 7.0 and pH 5.5 (Table 3). Therefore, both H-NS and GadE were involved in the increased *hdeAB* repression by MarA that occurred as cells enter stationary phase.

On the contrary, repression by MarA increased in the *gadX* and *lrp* mutants in exponential phase at pH 7.0 and in the *gadW*, *rpoS*, and *lrp* mutants in stationary phase at pH 5.5 (Table 3). These results might be due to competition of GadX, GadW, and Lrp with MarA, while the decreased repression by MarA produced by RpoS may be a direct or indirect effect (see Fig. 1).

Effect of growth phase and pH on the expression of *marA* without and with the inducer sodium salicylate. To understand how the native promoter of *marA* responded to growth phase and pH, we used *marO-lacZ*, a chromosomal reporter of the *marRAB* operon (in SPC105). We found that expression of *marA* at pH 7.0 was slightly, but significantly, induced in stationary phase compared to the level in exponential phase; greater induction occurred at pH 5.5 (Table 4). Induction of *marA* expression by sodium salicylate (5 mM) depended also on the growth phase and the pH. At pH 7.0, salicylate produced a strong induction after 1 h when added to cells growing in exponential phase (Table 4), as previously reported (10, 38), whereas it had much less effect when added to stationary-phase cells (as measured relative to expression in cells in stationary phase without salicylate) (Table 4). At pH 5.5, salicylate produced little or no effect on *marA* expression after 1 h when added to cells in exponential or in stationary phase. When cells grown overnight without salicylate were inoculated into medium at pH 7.0 with 5 mM salicylate, cell growth was slower than that without inducer. Under these conditions, the induction of *marA* expression by salicylate was very strong when tested in exponential phase and even stronger in stationary phase (Table 4). Note that this set of cells was exposed longer to salicylate (Table 4). Growth was arrested when overnight

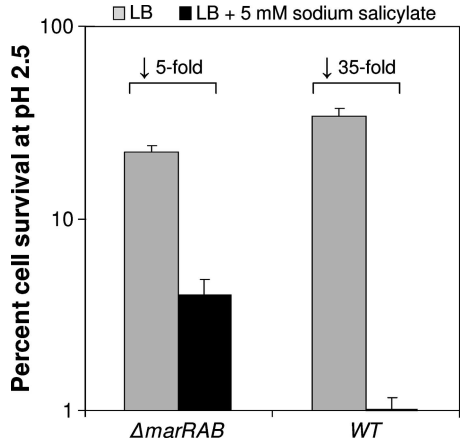

FIG. 4. Effect of MarA on the acid resistance of *E. coli*. The acid challenge at pH 2.5 was performed as described in Materials and Methods, using early-stationary-phase cells grown in LB broth at pH 7.0 without (gray) or with (black) 5 mM sodium salicylate to induce *marRAB*. Two different strains were compared: the Δ*marRAB* strain JHC1096 (Δ*marRAB*) and the wild-type strain GC4468 (WT). The percent survival at pH 2.5 after incubation for 2 h was determined in comparison with untreated samples. The results are presented as the averages ± the standard errors of the means ($n = 3$). The *n*-fold decrease in survival produced by sodium salicylate in each strain (ratio between gray and black bars) is indicated above the bars.

cultures grown without salicylate were diluted into medium at pH 5.5 with 5 mM salicylate.

Overexpression of *marA* decreases acid resistance in *E. coli*. To know whether repression of *hdeAB* by MarA affects acid resistance in *E. coli* under conditions similar to those in the stomach, we compared the survivals at pH 2.5 for 2 h at 37°C of a wild-type strain and a Δ*marRAB* strain (Fig. 4). We used cells grown in LB with or without 5 mM sodium salicylate to early stationary phase (where MarA produces the highest repression of *hdeAB*). In the absence of salicylate, the two strains showed similar survivals (20 to 34%) when grown at both pH 7.0 (Fig. 4) and pH 5.5 (not shown) prior to the acid challenge. However, growing the cells in LB at pH 7.0 in the presence of the *marRAB* inducer salicylate produced a fivefold decrease in the acid resistance of the Δ*marRAB* strain but a 35-fold decrease in the acid resistance of the wild-type strain (ratio between gray and black bars in Fig. 4). These results show that (i) although salicylate induces the expression of *hdeAB* and other acid resistance genes (33), salicylate itself was toxic, decreasing the acid resistance of the cells, and (ii) the strong overexpression of *marA* produced by salicylate in wild-type cells made them sevenfold more sensitive than Δ*marRAB* cells to the acid challenge.

DISCUSSION

We studied the role of MarA in the complex regulation of *hdeAB* and in acid resistance of *E. coli* growing in rich medium at both pH 7.0 and pH 5.5. Overexpression of MarA largely prevented the induction of *hdeAB* expression as cells enter stationary phase, in part because MarA-mediated repression of *hdeAB* increased in stationary phase. Growth phase or pH-dependent changes on transcriptional regulation by MarA have not been reported before for any other member of the *mar* regulon. Thus, we asked if such changes occurred via other regulators of *hdeAB*.

Seven other regulators were selected to study how they affected *hdeAB* repression by MarA. These regulators were chosen because they regulate *hdeAB* directly and/or indirectly (Fig. 1), and because all of them have known or putative DNA binding sites in the *hdeAB* promoter (Fig. 2). MarA is known to bind a "marbox" partially overlapping the −35 hexamer of the promoter (37). Therefore, competition with RNA polymerase for DNA binding and/or direct interactions between MarA and σ^{70}, σ^{38}, or other subunits of RNA polymerase are possible. Moreover, several putative binding sites for the other selected regulators, except H-NS, are adjacent to or partially overlap the marbox (Fig. 2). Thus, interaction and/or competition between MarA and these regulators is also possible.

Before studying how these regulators affected MarA repression on *hdeAB*, we examined their own effect on *hdeAB* under the chosen growth conditions. H-NS was the main repressor of *hdeAB* under all the conditions studied, in agreement with previous findings at pH 7.0 (3, 39). However, *hdeAB* repression by H-NS was significantly lower at pH 5.5, which has not been described before.

GadE was the main activator of *hdeAB* in LB medium under all the conditions studied, whereas GadX repressed or activated *hdeAB* depending on the growth conditions, and GadW repressed *hdeAB*. The different effects that GadX and GadW had under diverse conditions, sometimes opposite to that of GadE, support the idea that they can regulate *hdeAB* independently of GadE. The effect of the Gad regulators on *hdeAB* has not been studied before in stationary phase; thus we can only compare our results with previous data obtained using exponential-phase cells grown in minimal medium (20, 43). In general, our results are in agreement with the previous findings (20, 43), although some differences were found, probably because of the different culture media used. (i) In exponential phase, we found that GadX repressed *hdeAB* in LB medium at pH 7.0 (Table 2), while others observed no significant effect of GadX in minimal medium at pH 7.4 (43). (ii) In exponential phase, we found that GadX produced no effect on *hdeAB* expression in LB medium at pH 5.5 (Table 2), while others reported activation of *hdeAB* in minimal medium at pH 5.5 (20, 43) to be even greater than that produced by GadE (20).

RpoS had little effect on *hdeAB* expression under the conditions studied, probably because of a balance between its direct and indirect effects (Fig. 1). Lrp had little effect on *hdeAB* expression in LB medium, in contrast with the strong repression of *hdeAB* by Lrp that occurs in minimal media (42). TorR had no effect on *hdeAB* expression, which suggests that TorR only regulates *hdeAB* after induction of the TorRS system by trimethylamine *N*-oxide and anaerobiosis (8).

We then found that some of the *hdeAB* regulators examined can affect MarA-mediated repression. Moreover, in turn, MarA can modify the effect of some of these other regulators. This is the first report of functional interactions between MarA and other gene regulators. H-NS and GadE were associated with the increase in *hdeAB* repression by MarA that occurred in stationary phase. Conversely, MarA helped H-NS repression and interfered with GadE activation of *hdeAB* in stationary

phase. Given that the H-NS and MarA binding sites are not close (Fig. 2), and that H-NS can produce complex DNA superstructures in the DNA (39), it is not yet clear how H-NS and MarA interact to repress *hdeAB*.

It seems unlikely that GadE, the main activator of *hdeAB*, assists MarA in repression. More likely, MarA interferes with GadE-mediated activation of *hdeAB* directly (the marbox partially overlaps one "gadbox" [Fig. 2]; thus, MarA and GadE might compete for DNA binding or for interacting with RNA polymerase) and/or indirectly (MarA represses the expression of *gadE* and the *gadE* activators *gadX* and *evgA* [33]).

MarA seems also to compete functionally with GadX, GadW, RpoS, and Lrp under some conditions, which might ensure that *hdeAB* expression is not repressed more than needed. Such interference might be direct, given the partial overlap between the MarA binding site and some binding sites of these regulators (Fig. 2), or indirect by means of an intermediary (Fig. 1).

To know whether repression of *hdeAB* by MarA was correlated with decreased acid resistance, we studied the effect of pH and growth phase on the expression of *marA* and the effect of MarA on acid resistance. We found that, like *hdeAB* expression (Fig. 3), *marA* expression was induced in stationary phase and at acid pH (Table 4). However, this moderate induction was presumably not enough to significantly change *hdeAB* expression, since the acid resistance of the cells remained almost unaffected (Fig. 4); but it might be important in affecting the expression of other genes of the *mar* regulon with a less complex regulation or with marboxes with higher affinity for MarA.

Sodium salicylate induced the expression of *marA* at pH 7.0 but not at pH 5.5 (Table 4), which might relate to a change in its interaction with MarR (5) or to the unknown mechanisms involved in the induction of *marRAB* by acid pH. However, this lack of induction at pH 5.5 could be important to maintain *hdeAB* expression and the resistance of the cells under acid conditions.

At pH 7.0, induction of *marA* expression by salicylate was very strong, mainly when the cells were exposed to salicylate for longer periods of time. This strong induction made stationary-phase cells sevenfold more sensitive to acid challenge at pH 2.5 in the presence of *marA* than in its absence (Fig. 4), consistent with the repression produced by MarA on *hdeAB* (and probably other acid resistance genes) in stationary phase.

Further research is needed to understand the biological meaning of MarA repression of *hdeAB* at pH 7.0 in response to salicylate. Such repression might allow cell resources to be directed to the (unknown, but perhaps more important) MarA-mediated response to salicylate. Once salicylate was no longer present, the fast degradation of MarA (18) would allow a rapid recovery of the expression of *hdeAB* and other acid resistance genes, minimizing the impact of MarA on acid resistance.

In conclusion, MarA plays an active and complex role in the regulation of *hdeAB* and acid resistance in *E. coli* that depends on the growth conditions, on other regulators of *hdeAB*, and on the degree of induction of *marA* expression.

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