

Acid stress response in *Escherichia coli*: mechanism of regulation of *gadA* transcription by RcsB and GadE

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ABSTRACT

Escherichia coli can survive extreme acid stress for several hours. The most efficient acid resistance system is based on glutamate decarboxylation by the GadA and GadB decarboxylases and the import of glutamate via the GadC membrane protein. The expression of the corresponding genes is controlled by GadE, the central activator of glutamate-dependent acid resistance (GDAR). We have previously shown by genetic approaches that as well as GadE, the response regulator of the Rcs system, RcsB is absolutely required for control of *gadA/BC* transcription. In the presence of GadE, basal activity of RcsB stimulates the expression of *gadA/BC*, whereas activation of RcsB leads to general repression of the *gad* genes. We report here the results of various *in vitro* assays that show RcsB to regulate by direct binding to the *gadA* promoter region. Furthermore, activation of *gadA* transcription requires a GAD box and binding of an RcsB/GadE heterodimer. In addition, we have identified an RcsB box, which lies just upstream of the -10 element of *gadA* promoter and is involved in repression of this operon.

INTRODUCTION

Escherichia coli is a neutrophilic bacteria that can survive for a long time at very low pH (<2.5). This property is due to three clearly defined acid resistance (AR)

systems: glutamate-dependent, arginine-dependent and oxidative systems (1). The glutamate-dependent (Gad) system is the most effective. Two glutamate decarboxylase isoforms, GadA and GadB, consume intracellular protons in decarboxylating glutamate, and GadC, an integral membrane protein, exchanges extracellular glutamate for the decarboxylation product, γ -amino butyric acid (GABA) (2,3). The *gadB* and *gadC* genes are cotranscribed, while *gadA* forms an operon with *gadX*, which encodes an activator of the *gad* system (4). *gadA* and *gadX* belong to the acid fitness island (AFI), a 15-kb region unique to *E. coli* and *Shigella* species, which specifies 12 proteins and a small RNA that contribute to *E. coli* acid resistance in different ways (5). One of these genes, *gadE*, codes for a master regulator of glutamate-dependent acid resistance (GDAR) belonging to the LuxR-like family (6,7). GadE is the key activator of *gadA* and *gadBC* genes. It binds to a GAD box, a 20-bp consensus sequence centered 63 bp upstream of the transcriptional start sites of these genes (8). Regulation of *gadE* expression is extremely complex. At least nine regulators appear to converge on the *gadE* promoter region to either activate or repress its transcription in response to different environmental conditions (1).

Previously, we demonstrated by genetic approaches that RcsB, a response regulator, is another essential regulator of *gadA/BC* transcription (9). RcsB is part of the RcsCDB phosphorelay, a signal transduction system conserved in members of the *Enterobacteriaceae*. This system was first identified as a regulator of capsule synthesis and is involved in pathogenesis, development of biofilms, and the regulation of envelope composition or trafficking (10). RcsB can also be activated independently of the

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phosphorelay, by binding of different co-regulators: RcsA, the main one, and RmpA, TviA and PhoP (11–14). Presumably, this allows multiple inputs into RcsB-mediated regulation of specific promoters.

However, the ability of RcsB to stimulate *gadA/B* expression is independent of its Rcs partners, both that involved in the phosphorelay and the RcsA activator. In addition, although the activity of RcsB requires a functional *gadE* allele, its effect is not mediated by activation of *gadE* transcription, as is the case for the majority of other regulators of GDAR. In contrast, activation of RcsB, either through the phosphorylation pathway or by RcsA, leads to general repression of *gad* gene, including *gadA*, *gadB* and *gadE* itself, and a corresponding reduction in acid resistance (9).

The goal of the present study was to investigate the mechanism of regulation of the *gad* genes by GadE and RcsB. We show that RcsB exerts direct control over *gadA/BC* expression. RcsB is a critical partner of GadE and the binding of both regulators as a heterodimer to the GAD box activates *gadA* transcription. In addition, we identified an RcsB box located directly upstream from the –10 element of the *gadA* promoter, which contributes to the repression of *gadA* transcription upon Rcs activation.

MATERIALS AND METHODS

Strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. The *rscA* and *rscCDB* deletions were constructed as described by Datsenko and Wanner (15). The whole ORFs were deleted and replaced by the chloramphenicol resistance (*cat*) gene. To construct MPC398 strain, we first transduced $\Delta rscCDB::cat$ into MC4100 *lon*[–] strain (UGM2). The resistance gene was eliminated by using a FLP helper plasmid (15). Then, we transduced $\Delta rscA::cat$ into the previous strain and finally a $\Delta gadE::kan$ (8).

pHGadE plasmid was constructed by amplifying *gadE* orf from MG1655 strain (gi:948023) using *gadEup* and *gadEdown* primers. The polymerase chain reaction

(PCR) product was digested with NdeI and BamHI and cloned in the corresponding sites of pAPT156 (16).

pMRcsB plasmid was constructed by amplifying *rscB* orf from MG1655 strain (gi:947441) using *rscBupM* and *rscBdownM* primers. The PCR product and the pMALc2E plasmid were digested by BamHI and HindIII enzymes, and ligated.

pRSgadA-lacZ plasmids were constructed as follows. PCR-generated fragments were cloned between the BamHI and EcoRI sites of pRS550 (17). The *gadA* promoter from –101 to +24 relative to the transcription start of the *gadA* gene was amplified with the primers *gadAup* and *gadAdown*. The introduction of the mutations M1, M2 and M3 in the *gadAp* promoter region was done by PCR using primers carrying the corresponding mutations.

Oligonucleotide sequences (5' to 3')

gadAup (GTTTATATTATAAAAAGTCCG), *gadAdown* (GAACCTCCTTAAATTTATTTG), *gadEup* (GGAATTCCATGCATCACCATCACCATCACATGATTTTTCTCATGACG), *gadEdown* (CGCGGATCCCTAAAAT AAGATGTG), *lacZ24* (CGCCAGGGTTTTCCCAGTCACGAC), *rscBupM* (CGCGGA TCCAACAATATGAA CGTAATTATGCC), *rscBdownM* (CGCAAGCTTTT AGTCTTTATCTGCCG GACT).

Purification of MBP-GadE and MBP-RcsB fusion proteins

Escherichia coli MPC398 containing the pMF533 or the pMRcsB plasmid expressing MBP (Maltose Binding Protein product of the *malE* gene) fused to GadE (Uniprot: P63204) or RcsB (Uniprot: P69407) protein respectively (8; our work), was grown at 37°C with aeration in 1 l of LB broth supplemented with 0.2% glucose and 100 µg/ml ampicillin. The purification procedure is described in Ma *et al.* (8).

Purification of (His)₆-RcsB and (His)₆-GadE fusion proteins

Escherichia coli MPC398 containing pHRcsB or pHGadE was grown at 37°C with aeration in 1 l of LB broth

Table 1. Bacterial strains and plasmids in this study

	Genotype	Source or reference
Strains		
UGM2	MC4100 <i>lon</i> [–] (Δ PstI)	Lab. collection
MPC398	MC4100 <i>lon</i> [–] $\Delta rscCDB$ $\Delta rscA::cat$ $\Delta gadE::kn$	This study
MPC295	<i>trpDC::putPA1303-Km-gadA::lacZ</i> (–165 to +788)	(9)
MPC297	<i>trpDC::putPA1303-Km-gadB::lacZ</i> (–203 to +788)	(9)
MPC299	<i>trpDC::putPA1303-Km-gadE::lacZ</i> (–804 to +331)	(9)
CF6343	MG1655 $\Delta lacIZ$ (M1ul)	Lab. Collection
Plasmids		
pHRcsB	pFabI, p15A-derived vector carrying $\phi(lacPUV5-his_6-rscB)$	(20)
pHGadE	p15A-derived vector carrying $\phi(lacPUV5-his_6-gadE)$	This study
pMF533	pMALc2E (New England Biolabs) containing <i>malE-gadE</i> fusion	(8)
pMRcsB	pMALc2E (New England Biolabs) containing <i>malE-rscB</i> fusion	This study
pRS550	ColE1 derived cloning vector, Ap ^R , Kn ^R	(17)
pRSgadA-lacZ	pRS550 containing <i>gadAp</i> fused to <i>lacZ</i>	This study

supplemented with 0.2% glucose and 75 µg/ml of spectinomycin. At $OD_{600} \sim 0.3$, IPTG was added to a final concentration of 0.5 mM. The culture was incubated for an additional 3 h. Cells were then treated with lysozyme (0.75 mg/ml) for 30 min and disrupted by sonication in 10 ml of column buffer (50 mM NaP buffer pH 7, 300 mM NaCl). Lysates were centrifuged at 12 000g for 30 min. The supernatants were loaded onto 1 ml TALON columns (Ozyme). The loaded columns were washed with 20 ml of column buffer and 5 ml of column buffer plus 10 mM imidazole. The proteins were eluted with 5 ml of column buffer containing 150 mM imidazole. Samples with highest protein contents were combined and dialyzed against column buffer containing 40% glycerol. Protein concentrations were determined by Bio-Rad protein assays.

Electrophoretic mobility shift assay

Probes were PCR-generated with ^{32}P 5' end-labeled primers and purified using S300 columns (GE Healthcare). With proteins, 5000 c.p.m. per reaction were incubated at room temperature for 30 min in 10 µl of binding buffer (110 mM NaP pH 7, 10 mM EDTA, 20 mM NaCl, 1 mM DTT, 50 mM potassium glutamate, 1 µg poly-(dI-dC) (Sigma), 7 µM bovine serum albumin). Samples were loaded onto 5% TBE non denaturing gels and electrophoresed at room temperature in 0.5 X TBE. After migration, gels were dried, exposed to Fujifilm sensitive screen. Quantifications were made using Multi Gauge Software.

DNase I footprinting

Experiments were performed using protocol described by Francez-Charlot *et al.* (18). Reactions contained 5–10 nM of purified *gadA* promoter-containing templates which have been generated by PCR using *gadA*up and *gadA*down primers. *gadA*up primer was end-labeled with [γ - ^{32}P] ATP by polynucleotide kinase.

In vitro transcription assays

Reactions (14 µl) were performed in buffer containing 20 mM Tris-HCl pH 8, 100 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 50 µg/ml bovine serum albumin (BSA), 5% glycerol, 0.1 mg/ml heparin, 200 µM ATP, 200 µM CTP, 200 µM GTP, 10 µM UTP and 5 µCi of [α - ^{32}P] UTP (Promega). Linear DNA templates were generated by PCR using *gadA*up and *lacZ*24 primers from pRS*gadA*lacZ plasmid, gel purified using a Qiagen gel extraction kit; 20 nM of templates were used per reaction. When required GadE and RcsB proteins were added, then the reaction was initiated by the addition of 0.3 U of RNAP (Tebu). After 10 min at 30°C, samples were analyzed by denaturing gel electrophoresis. Transcript products were quantified using a Phosphorimager (Fujifilm) and Multi-Gauge software.

Beta-galactosidase assay

Cells were grown in LBG (LB plus 0.4% glucose) at 37°C. Overnight cultures were diluted 7000-fold, grown for

five/10 generations, prior to addition of 500 µM IPTG. The cultures were sampled at different intervals for assay of β -galactosidase activities (19).

RESULTS

Both RcsB and GadE directly regulate *gadA* transcription

To test whether RcsB acts directly at *gadA* promoters, we carried out an *in vitro* transcription assay using linear DNA fragment containing the *gadA* promoter region fused to *lacZ* as template. This assay was based on His-tagged version of RcsB and a MBP-tagged version of GadE (8,20). With RNA polymerase alone, a transcript of the expected size was visible (Figure 1, lane 1). The signal was weak, in agreement with the observation that *in vivo* the activity of the *gadA* promoter is low in the absence of either GadE or RcsB (9). Addition of MBP-GadE to the reaction did not appreciably change the amount of transcript (Figure 1, lane 2). In contrast, in the presence of MBP-GadE, the addition of 3 or 30 nM of (His)₆-RcsB (Figure 1, lanes 7 and 8, respectively) strongly stimulated *gadA* transcription (up to 7-fold). This result therefore indicates that GadE and RcsB act synergistically to stimulate transcription of *gadA*. At higher concentrations of (His)₆-RcsB, stimulation of *gadA* transcription by RcsB in the presence of GadE declined, essentially disappearing at 3 µM RcsB (Figure 1, lane 10). This behavior implies that the ratio of the two regulators is important for the balance activation/repression. Furthermore, (His)₆-RcsB addition alone (Figure 1, lanes 3–6) had a concentration dependent negative effect on *gadA* transcription, indicating that RcsB alone directly represses the basal level of *gadA* transcription.

RcsB and GadE protection of the *gadA* regulatory region

A GAD box, located 16 bp upstream of the -35 element of the *gadA* promoter, is required for the activity of GadE regulator (Figure 2A; Ref. 8). We had previously noticed that the left part of the GAD box was similar to the half site of the consensus RcsB box, and suggested that both GadE and RcsB might bind to it in order to stimulate *gadA* transcription (9). In addition, we identified a putative RcsB box located immediately upstream of the -10 element that could be required for the repression of

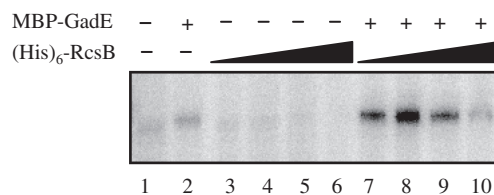


Figure 1. Synergistic regulation of *in vitro* transcription initiation at the *gadA* promoter by GadE and RcsB. The *gadA* promoter-containing linear template was pre-incubated as indicated with RNA polymerase alone (lane 1), plus 760 nM MBP-GadE (lane 2), plus 3, 30, 300 or 3000 nM (His)₆-RcsB (lanes 3–6, respectively), or plus MBP-GadE and (His)₆-RcsB at the same concentrations (lanes 7–10, respectively). The figure shows a representative autoradiogram.

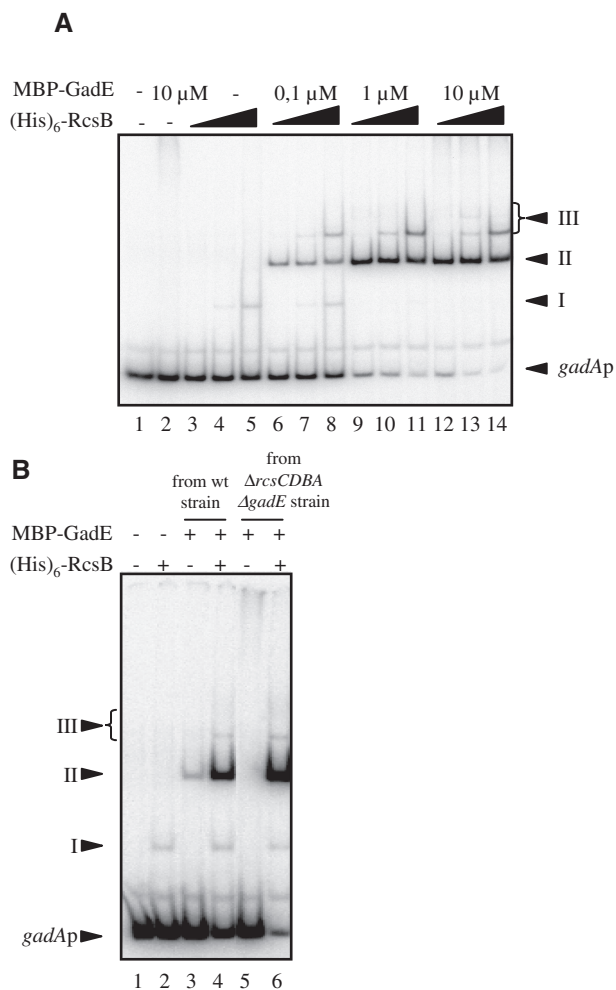


Figure 3. Binding of MalE-GadE and (His)₆-RcsB to the *gadA* promoter region. (A) EMSA with *gadA* promoter region-templates (from -101 to +24 relative to the +1 start of transcription). No protein (lane 1), 10 μ M MBP-GadE (lane 2), 0.1, 1 and 10 μ M (His)₆-RcsB plus 0.1 μ M MBP-GadE (lanes 6–8) or plus 1 μ M MBP-GadE (lanes 9–11), plus 10 μ M MBP-GadE (lanes 12–14). (B) EMSA using protein from wt or *rsc* mutants with *gadA* promoter region templates. Proteins were purified from either a wild-type strain (lanes 3 and 4) or a Δ rscA Δ rscCDB Δ gadE strain (lanes 2, 5 and 6). No protein (lane 1), 1 μ M (His)₆-RcsB (lane 2), 3 μ M MBP-GadE (lanes 3 and 5), 3 μ M MBP-GadE and 1 μ M (His)₆-RcsB (lanes 4 and 6).

These results suggested that GadE and RcsB act synergistically in binding to the *gadA* regulatory region and that both proteins interact directly.

RcsB/GadE heterodimer binds to the GAD box

The formation of complex II could result either to binding of both proteins or to binding of only one after modification by the other. The presence of GadE and RcsB in complexes II and III was tested by using combinations of His-tagged and MBP-tagged versions of GadE and RcsB. Owing to the differences in fusion protein molecular weights [\sim 20 kDa for (His)₆-GadE versus \sim 62 kDa for MBP-GadE, and \sim 23.6 kDa for (His)₆-RcsB versus \sim 66 kDa for MBP-RcsB], migration patterns were expected to depend on the combination of proteins

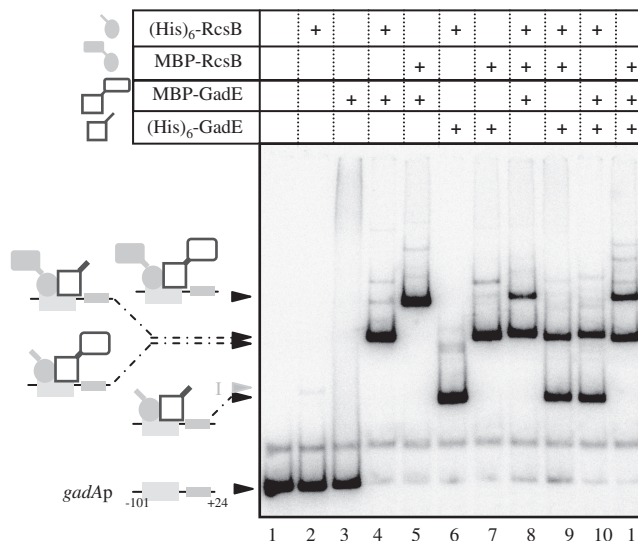


Figure 4. RcsB and GadE bind GAD box as a heterodimer. EMSA was performed as described in Figure 3 using the following proteins at 1 μ M: (His)₆-RcsB, MBP-RcsB, MBP-GadE and (His)₆-GadE. Schematic representations of complexes II obtained are presented on the left-hand side.

present in the different complexes. As shown in Figure 4, this was the case. Complex II exhibited lower mobility when an MBP-tagged version of each protein was used instead of the corresponding His-tagged form (Figure 4, compare complex II migrations in lanes 4 and 5, or 4 and 6, or 5 and 7 or 6 and 7), as expected if complex II consisted of a RcsB/GadE oligomer bound to the *gadA* regulatory region. Combinations of three proteins, both tagged versions of one and one tagged version of the other, gave only two major retarded complexes II in this assay (Figure 4, lanes 8–11). The lack of additional shifted bands indicated that equivalent amounts of RcsB and GadE bound to the *gadA* promoter region. Taking into account that both regulators belong to protein families known to bind DNA as a dimer (23,24) and the size of the protected region at the GAD box, we could reasonably conclude that complex II is the result of an RcsB/GadE heterodimer sitting on the *gadA* regulatory region, at the GAD site.

Binding activities of RcsB and GadE at the *gadA* promoter

The affinity of the RcsB/GadE heterodimer for GAD box was estimated by EMSA with increasing concentrations of RcsB in reactions containing a saturating concentration of GadE (2.5 μ M). With this approach, the apparent binding constant of the RcsB/GadE heterodimer was calculated to be \sim 10 nM. We noticed that a large proportion of MBP-GadE formed homodimers in solution (data not shown). Hence, to obtain enough GadE monomers and to favor the RcsB/GadE heterodimer formation, we used a saturating concentration of GadE. We could not estimate the apparent binding constant of the RcsB homodimer for the RcsB box because even with 10 μ M RcsB, only 6% of the probe was retarded (Figure 3A; lane 5). This indicates that the activation of *gadA*

transcription could take place at low concentrations of both proteins, whereas repression of *gadA* transcription would require higher level of RcsB.

Independent binding on the RcsB and the GAD boxes

In an effort to dissect protein–protein and DNA–protein interactions, various mutations were introduced into the GAD box or in the RcsB box (Figure 5A) and their effects were analyzed by EMSA (Figure 5B). The M1 mutation changes a highly conserved G to a C. This G is also highly conserved in the RcsB box. As shown by the EMSA in Figure 5B (lanes 5–8), this mutation caused a dramatic diminution of complexes II and III, indicating that this G is critical for the binding of the RcsB/GadE heterodimer to the GAD box. In contrast, the formation of complex I was not compromised, in agreement with the idea that this complex was formed by RcsB bound to the RcsB box. The M2 mutation also introduced a modification TT to CC in the right-hand part of the GAD box, but in a less conserved region. This mutation caused a small decrease in the affinity of the RcsB/GadE proteins (Figure 5B, compare lanes 4 and 12), indicating that these nucleotides are not critical for binding RcsB/GadE. Again, no effect on the binding of RcsB to the RcsB box was observed. Finally, the M3 mutation replaced the conserved A in the RcsB box with a G. This mutation completely inhibited complex I formation by RcsB (Figure 5B, lane 15), but had no impact on the formation of complexes II and III by RcsB and GadE (Figure 5B, lane 16). Taken together, these results indicate that binding to one box was largely independent of binding to the other box. It should be pointed out that,

as for the major complex II, the formation of minor complexes III was mainly independent of the RcsB box, indicating that these slow migrating complexes III do not result principally to simultaneous occupation of the Rcs and GAD boxes. This was confirmed by EMSA with a shorter DNA fragment containing only the GAD box. This fragment was still able to form two retarded complexes whose migrations were equivalent to that of complexes II and III (Supplementary Figure S1).

Effects of point mutations on transcriptional activity of *gadA* in vivo

The *in vivo* transcriptional activity of the *gadA* promoter was monitored using plasmid-borne transcriptional fusions between *gadA* regulatory region (–101 to +24) and *lacZ* (Figure 6). As previously reported (9), in a wild-type background, overproduction of (His)₆-GadE activates the transcription of *gadA*, whereas overproduction of (His)₆-RcsB represses. In contrast, the

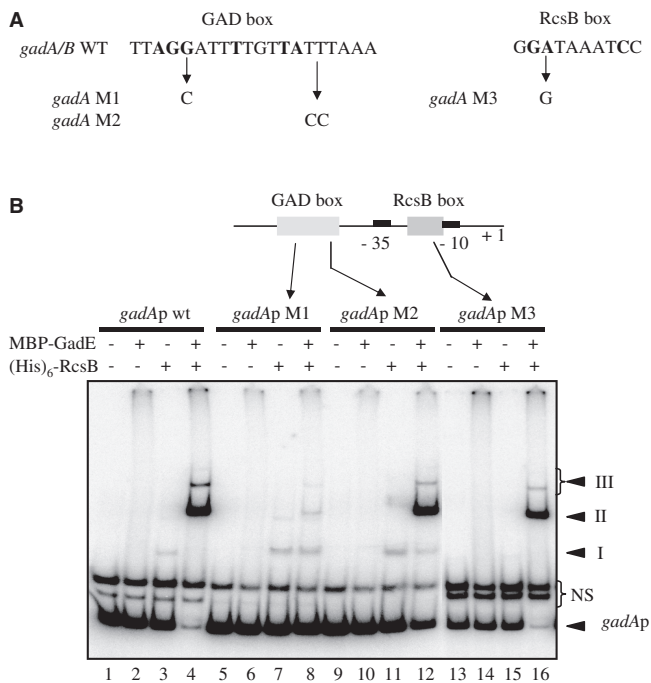


Figure 5. Effects of GAD or RcsB box mutations on RcsB and GadE binding. (A) Point mutations introduced into the GAD and RcsB boxes. Conserved bases are shown in bold. (B) EMSA of *gadA* promoter fragment mutants in the presence of (His)₆-RcsB (5 μM), and/or MBP-GadE (4 μM). NS: non-specific DNA.

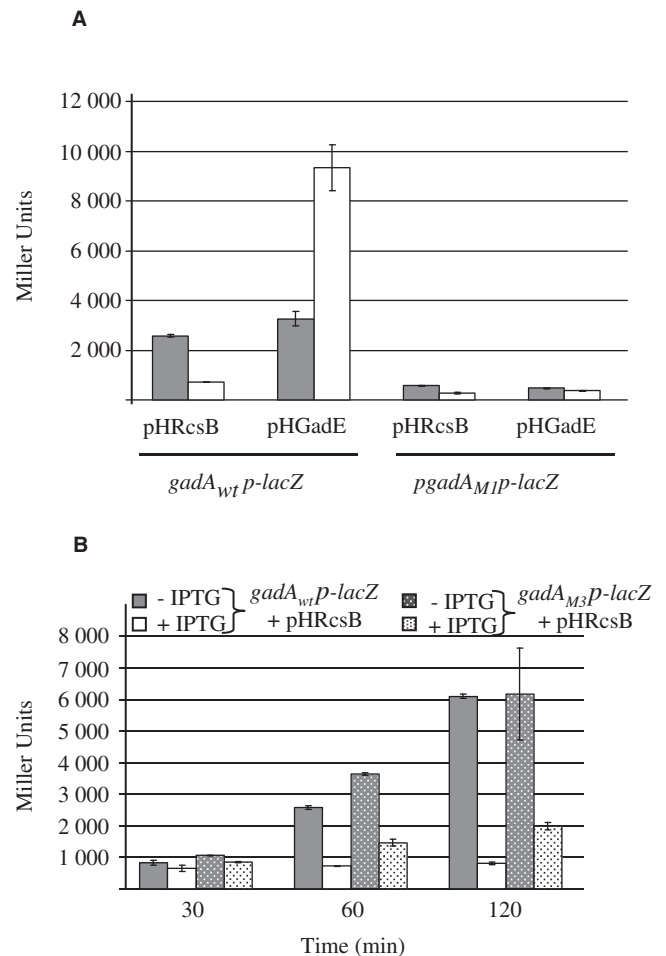


Figure 6. Effects of RcsB or GAD box mutations on the transcriptional regulation of *gadA* in vivo. (A) β-Galactosidase activities of transcriptional *gadA-lacZ* fusions (either wild-type or M1 mutant promoter) with or without overexpressed (His)₆-GadE or (His)₆-RcsB. Strains carrying *gadAp-lacZ* and either pHGadE or pHRcsB plasmids with (white bars) or without (gray bars) 0.5 mM IPTG added for 1 h. (B) Same experiment as in A for *gadA_{wt}p-lacZ* and *gadA_{M3}p-lacZ* fusions with or without induction of RcsB synthesis. Activities were measured 30, 60 and 120 min after IPTG addition.

construction with the inactivated GAD box (mutation M1) displayed a lower intrinsic activity that was no longer stimulated by GadE (Figure 6A). Repression of the β -galactosidase activity was still observed after 1 h of overproduction of RcsB, even if the promoter was no more activated. This repression indicated that the GAD box is not involved in repression of *gadA* expression by RcsB.

With the M3 fusion (mutation in the RcsB box), the basal activity of the *gadA* promoter was slightly higher and RcsB repression little affected (Figure 6B). In the case of the mutant promoter, the transcription level was more than twice that of a wild-type promoter after 120 min of RcsB over expression (2000 versus 800 Miller units). However, a repression was still observed with this mutant promoter indicating that at least part of the RcsB-dependent repression of *gadA* is observed without normal binding of RcsB to the RcsB box. This was, however, consistent with our previous finding that overproduction of RcsB leads to general repression of the *gad* genes, including those coding for the activators of *gadA* expression (9). Therefore, in the case of the M3 fusion, the repression observed after RcsB overproduction was probably due to a lower level of GadE and/or GadX(W).

DISCUSSION

In this study, we report novel findings that shed light on the highly intricate regulation of glutamate-dependent acid resistance in *E. coli* (Figure 7). In particular, we have provided evidence that RcsB is a critical co-activator along with GadE of the *gadA* transcription. Genetic approaches had shown that in addition to the central regulator GadE, the response regulator RcsB is strictly required to activate the expression of the three genes *gadA/BC* and to develop resistance to low pH (9). Here, we demonstrate through *in vitro* assays that RcsB and GadE act directly and synergistically to activate *gadA* transcription (Figure 1). Localization of the binding site responsible for this activation by genetic (Figure 6) and

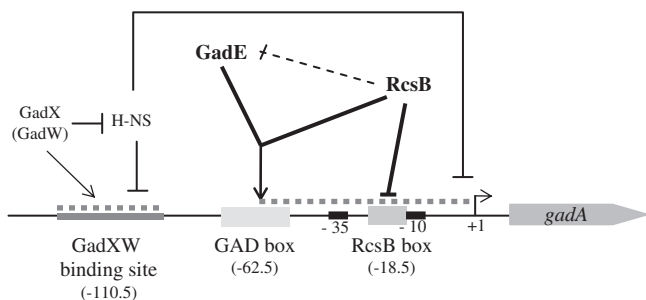


Figure 7. Regulatory model of *gadA* transcription. Schematic representation of *gadA* regulatory region with the GadXW (33), GAD and RcsB boxes (gray boxes). The H-NS binding sites responsible for *gadA* repression are represented as dotted line (37). Direct regulation is shown as a continuous arrow. Repression of *gadA* transcription by RcsB, in which a direct effect has not been confirmed, is shown as a dotted arrow. This putative feed-forward loop could coordinate *gadA* expression.

footprinting (Figure 2) experiments confirmed the identity of the GAD box previously identified (8). However, our data demonstrate that the GadE protein alone is not able to bind this GAD box, and that GadE requires the formation of hetero-oligomers with the co-regulator RcsB to bind this site in the *gadA* regulatory region (Figures 2–5). These data contradict previous reports that GadE alone could bind to the GAD box (8,21,22). As shown in Figure 3B, this discrepancy can be explained by contamination of MBP-GadE overproduced in wild-type strains by RcsB protein.

Importantly, we demonstrate here that RcsB and GadE both bind to the GAD box. Indeed, retarded complexes II and III, formed in EMSA with *gadA* promoter region in presence of both RcsB and GadE contain both proteins (Figure 4) and are abolished by mutation of a highly conserved nucleotide in the GAD box (Figure 5). Although our experiments do not define the stoichiometry of the proteins in the protein/DNA complexes, it is likely by analogy with other response regulators and LuxR-like activators (23,24) that the GAD box can accommodate one RcsB/GadE heterodimer. At present, the difference between complexes II and III is not clear, but the GAD box appeared sufficient to generate these complexes (Figures S1 and 5). We note that formation of complex III depended on RcsB concentration (Figure 3), which could be consistent with secondary binding of RcsB to the already bound RcsB/GadE or alternatively to the RcsB box, this latter corroborates the DNase I protection of the two boxes (Figure 2). The RcsB/GadE heterodimer binds the GAD box with an apparent binding constant of 10 nM, which is 10-fold lower than that measured for the RcsA/RcsB heterodimer (25). The point mutations introduced into the GAD box, on either the right or the left side, did not enable definition of the half-binding site for each partner. Although we note the left half-site is very similar to an RcsB site, further studies will be needed to settle this issue.

It is still not known whether both RcsB and GadE are directly involved in the DNA recognition process or whether one protein stimulates the binding of the other through conformational change triggered by protein-protein interaction. Heterodimerization of transcriptional regulators is prevalent in eukaryotic regulation mechanisms, where it allows the recognition of additional DNA targets (26). However, it is quite uncommon in prokaryotic systems. An *in vitro* study raised the possibility of interaction between hetero-pairs of response regulators from the OmpR/PhoB subfamily (27), and Knusten *et al.* (28) suggested that a ComE/BlpR heterodimer (these proteins belong to the LytR subfamily of response regulator) recognizes a hybrid DNA motif for convergent gene regulation. A RcsB/GadE heterodimer would be the first example of heterodimer formation between a LuxR-like transcription regulator (GadE) and a NarL/FixJ response regulator (RcsB). This noncanonical interaction is sustained by data obtained in the interactome analysis of *E. coli* (29), in which SPA-RcsB was purified with GadE (previously named YhiE). In agreement with this *in vivo* interaction, we found that RcsB co-purified with MBP-GadE (data not shown). All these strongly support

that there is a specific interaction between GadE and RcsB. Both regulators displayed in their C-terminal region a motif related to the LuxR-type DNA-binding HTH (Prosite PS50043 HTH_LUXR_2). It is conceivable that these regulators interact through this motif since structural studies indicated that the fourth helix in this motif facilitates dimerization (30,24). The question arises as to whether GadE ever binds efficiently to DNA on its own or always needs a co-regulator or alternatively does it need a signal to become active. As shown by Sayed and Foster (31), this regulator collaborates with a still unknown co-activator to stimulate its own transcription. However, it remains possible that GadE regulates some target genes independently of co-regulators. For example, we noticed that GadE tends to form dimers during purification. Further studies are needed to understand how GadE controls transcription of ~40 genes, including the ones in the AFI (21).

RcsB can function as an activator of *gadA/B*, although we had previously shown that the overproduction of RcsB or its activation through the Rcs phosphorelay or RcsA pathways represses *gadA/BC* transcription and results in GDAR reduction (9). In the present work, we identified an RcsB box displaying a sequence characteristic of canonical RcsB binding sites, located just upstream of the -10 element of the *gadA* promoter (Figures 2 and 7). This box is probably involved in the repression of *gadA* transcription by obstructing RNA polymerase binding to the promoter. However, the affinity of RcsB for the RcsB box is very low, several orders of magnitude below that of the RcsB/GadE heterodimer for the GAD box, suggesting that the basal level of RcsB is more efficiently oriented to the activation of the *gadA/BC* expression. This is coherent with the results of our *in vitro* transcription assay where in the presence of the two regulators RcsB behaved as an activator at lower concentration and as a repressor at higher concentration (Figure 1). It is however conceivable that either a cofactor or the phosphorylation of RcsB increases the affinity of RcsB for the RcsB box, thus making the direct repression more efficient. A potential co-regulator could be RcsA, since heterodimerization with RcsB was shown to considerably enhance DNA binding efficiency (32,24). On the other hand, our genetic data indicated that the effect of RcsA is minor for *gad* repression: its overproduction decreased GDAR 2-fold compared with a 20-fold decrease upon RcsB overexpression (9). Furthermore, repression of *gadA/BC* transcription seemed to combine a direct effect, probably resulting from binding of RcsB to the RcsB box, and an indirect one, since overexpression of RcsB also repressed transcription of *gadE* itself, as well as of *gadX* and *gadW* (Figure 7; Castanié-Cornet, M.P. and Cam, K. unpublished data) which encode AraC-like regulators involved in *gadE* and *gadA/BC* transcription activation (33,34,35). Sequence analysis revealed a putative RcsB box upstream the -10 element of the *gadE* promoter that could allow RcsB to control a feed-forward loop over *gadA* transcription (36). This putative RcsB-GadE-*gadA* network belongs to the minority of the so-called Coherent-type 2, in which RcsB (X node) will prevent the activation of *gadA* (Z node) directly and indirectly by repressing

gadE expression (Y node). However, we have so far no evidence that this site is important for *gadE* repression.

Finally, what could be the physiological rationale of the dual effect of RcsB on expression of the Gad system? RcsB has been shown to activate gene expression upon the formation of biofilms and it may control a feed-forward loop involved in *gad* transcription, allowing repression of GDAR upon Rcs activation following interactions of cells with surfaces, and more generally after envelope perturbation. The purpose of this negative regulation could be to avoid costly runaway expression of *gad* genes in conditions where cell membrane properties or membrane proteins are modified or damaged, conditions known to activate Rcs system.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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