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Functional Characterization and Regulation of *gadX*, a Gene Encoding an AraC/XylS-Like Transcriptional Activator of the *Escherichia coli* Glutamic Acid Decarboxylase System†

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The *Escherichia coli* chromosome contains two distantly located genes, *gadA* and *gadB*, which encode biochemically undistinguishable isoforms of glutamic acid decarboxylase (Gad). The Gad reaction contributes to pH homeostasis by consuming intracellular H^+ and producing γ-aminobutyric acid. This compound is exported via the protein product of the *gadC* gene, which is cotranscribed with *gadB*. Here we demonstrate that transcription of both *gadA* and *gadBC* is positively controlled by *gadX*, a gene downstream of *gadA*, encoding a transcriptional regulator belonging to the AraC/XylS family. The *gadX* promoter encompasses the 67-bp region preceding the *gadX* transcription start site and contains both RpoD and RpoS putative recognition sites. Transcription of *gadX* occurs in neutral rich medium upon entry into the stationary phase and is increased at acidic pH, paralleling the expression profile of the *gad* structural genes. However, P_{T5}*lacO*-controlled *gadX* expression in neutral rich medium results in upregulation of target genes even in exponential phase, i.e., when the *gad* system is normally repressed. Autoregulation of the whole *gad* system is inferred by the positive effect of GadX on the *gadA* promoter and *gadAX* cotranscription. Transcription of *gadX* is derepressed in an *hns* mutant and strongly reduced in both *rpoS* and *hns rpoS* mutants, consistent with the expression profile of *gad* structural genes in these genetic backgrounds. Gel shift and DNase I footprinting analyses with a MalE-GadX fusion protein demonstrate that GadX binds *gadA* and *gadBC* promoters at different sites and with different binding affinities.

The ability to survive in an acidic environment is essential for successful colonization of the mammalian host by both commensal and pathogenic enteric bacteria. These microorganisms are faced with an extremely acidic shock (pH <2.5) during their passage through the stomach and must counteract the deleterious effect of volatile fatty acids while living in the gut. Enteric bacteria have evolved a number of strategies enabling them to overcome the acidic stress, including, among others, the amino acid decarboxylase-based systems (14).

The glutamate decarboxylase (Gad) system has recently gained interest because of its major role in the acid resistance of enteric pathogens such as *Escherichia coli*, *Shigella flexneri*, and *Listeria monocytogenes* (6, 8, 9, 35). The *E. coli* chromosome contains two genes located 2,100 kb apart, *gadA* and *gadB*, encoding two biochemically undistinguishable forms of Gad, differing in five amino acid residues (4, 10).

The chemistry of the Gad-dependent pH neutralizing system is simplified by the folllowing intracellular reaction: glutamate^{-1} + H^+ → γ-aminobutyrate + CO_2. Following decarboxylation, one intracellular proton is consumed, and both products are released by the cell. Gas-liquid partition of CO_2 is pH dependent, the gaseous state being favored at low pH. Moreover, γ-aminobutyrate (GABA; pI ≈ 7.0) is less acidic than glutamate (pI ≈ 3.1), thereby providing local buffering of the extracellular environment.

The logical sequence of these chemical events (decarboxylation followed by GABA export) reflects the physical association of the genes coding for the system components. In *E. coli*, the *gadB* gene constitutes a single transcriptional unit with the downstream *gadC* gene, encoding the putative glutamate/GABA antiporter (9). In a *gadB*-deficient background, the system activity is guaranteed by the second decarboxylase isoform, the *gadA* gene product (6).

Transcript analysis and enzyme activity measurements indicate that expression of *gad* genes is switched on at the stationary phase under oxidative growth conditions and responds positively to acidic and hyper- and hypo-osmotic shocks (9). The extent of *gadA* and *gadBC* expression can be differentiated depending on the culture conditions, and stationary phase and acid pH activate distinct regulatory circuits. The contribution of GadA and GadB to the total amount of cellular glutamate decarboxylase activity, as determined by using *gadA* and *gadB* mutants, reveals that cells grown in exponential phase under acidic conditions express more GadA than GadB, the latter isoform being more abundant in stationary phase at pH 7 (6).

The nucleoid protein H-NS is involved in the negative control of *gadA* and *gadBC* transcription during the exponential growth phase under oxidative conditions, while the alternative sigma factor RpoS is responsible for *gad* expression at the

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† This manuscript is dedicated to the memory of Franco Tatò, prematurely deceased 7 July 2001.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
ATCC 11246	Wild type	American Type Culture Collection (27)
DDBGX	*gadX*::pJP*gadX′* derivative of ATCC 11246 *tet*	This work
DH5αF′	F′ *recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lacZYA-argF*)*U169* (ϕ80d *lacZ*Δ*M15*)	2
JM109	(F′ *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15*) *recA1 endAI gyrA96 thi hsdR17 supE44 relA1* Δ(*lac-proAB*)	2
S17.1	*pro thi hsdR recA* RP4.2 (*tet*::Mu) (*kan*::Tn*7*)	31
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC ptsF25 rbsR*	5
RH90	MC4100 *rpoS*359::Tn*10*	18
YK4122	*trp*$^+$ derivative of YK1100	37
YK4124	*trp*$^+$ *hns-2* derivative of YK1100	37
YK4124*rpoS*	YK4124 *rpoS*359::Tn*10*	9
Plasmids		
pRS415	Operon fusion vector; ColE1 replicon, *lacZYA bla*	32
pJP5608	Mobilizable cloning vector for construction of single-crossover insertional mutants; R6K replicon, *mob tet* Tra$^-$	28
pBluescript SK (pBs)	Multicopy phagemid vector; ColE1 replicon, *lacZ*α *bla*	Stratagene
pBs*gadX*	832-bp amplicon generated from pBsAX, encompassing the entire *gadX* coding sequence, ligated to *Bam*HI and *Hin*dIII sites of pBs	This work
pT	pBs-derived T/A vector for cloning PCR products	20
pT*gadX*	838-bp amplicon generated from pBsAX, encompassing entire *gadX* coding sequence, ligated to pT	This work
pMAL-c2	Phagemid vector for protein fusion and expression; ColE1 replicon, P_{tac} *malE*::*lacZ*α *lacI*^{q} *bla*	New England Biolabs
p*malE*::*gadX*	852-bp fragment encompassing entire *gadX* coding sequence and part of the multicloning site of pBs ligated to the *Eco*RI-*Hin*dIII sites of pMAL-c2 under P_{tac} control	This work
pQE60	Expression vector; ColE1 replicon, P_{T5}-*lacO* RBSII	Qiagen
pQE*gadX*	832-bp fragment encompassing the entire *gadX* coding sequence ligated to the *BamH*I-*Hin*dIII sites of pQE60 under P_{T5}-*lacO* RBSII control	This work
pBsAX	4.1-kb *Cla*I genomic fragment from *E. coli* ATCC 11246, encompassing *gadA* and *gadX* genes, cloned in pBs	10
pBsA	2.8-kb *Cla*I-*Nco*I genomic fragment from *E. coli* ATCC 11246, encompassing *gadA* gene, cloned in pBs	This work
pBsX	2.1-kb *Eco*RI-*Nco*I genomic fragment from pBsAX, encompassing *gadX* gene, cloned in pBs	This work
pBsB	4.5-kb *Hin*dIII genomic fragment from *E. coli* ATCC 11246, encompassing *gadB* gene, cloned in pBs	10
pBsBC	5.5-kb *Eco*RI-*Hin*dIII genomic fragment from *E. coli* ATCC 11246, encompassing *gadBC* operon, cloned in pBs	9
pJP*gadX′*	255-bp *Nco*I-*Hin*fI fragment from pQE*gadX* cloned in pJP5608	This work

stationary phase but not during fermentative growth (6, 7, 9). It has previously been suggested that H-NS could directly bind *gad* promoters, thereby silencing transcription of *gad* genes during the exponential phase (9). Alternatively, H-NS could indirectly repress expression of *gad* genes by negatively acting on RpoS stability (3, 36). Accordingly, acid pH, which is the main stimulus for *gad* induction, also increases the cellular levels of RpoS during the exponential phase (16).

Evidence has recently emerged that the activation of the *gad* system is mediated by the GadX protein (17, 30, 34), a member of the AraC/XylS family of transcriptional regulators (15), encoded by the *gadX* gene (formerly designated *yhiX*), located downstream of *gadA*. GadX expression was positively correlated with both upregulation of *gadA* and *gadBC* genes and resistance to acidic pH (17). Shortly after, a role of GadX in the transcriptional control of the plasmid-encoded regulator (*per*) of enteropathogenic *E. coli* virulence genes was demonstrated (30), linking pH sensing with differential expression of genes involved in acid resistance and synthesis of virulence factors.

In this report, we focus on *gadX* regulation and on the mechanism by which GadX positively controls the expression of the *gadA* and *gadBC* gene system. We provide evidence that GadX is a DNA-binding protein which recognizes the promoters of *gadA* and *gadBC* genes to a different extent and that GadX expression results in upregulation of target genes during exponential growth in rich neutral medium, i.e., when the *gad* system is normally repressed. The expression profile of *gadX* shares features with that of the *gad* structural genes, suggesting that GadX is a terminal component of the H-NS- and RpoS-dependent regulatory cascade responsible for *gadA* and *gadBC* transcriptional control.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. Growth was monitored by optical density measurements at 600 nm (OD_{600}). The media included Luria-Bertani (LB) (2), LBG (LB supplemented with 0.4% glucose and acidified to pH 5.0 with HCl), and LB-MES (LB buffered at pH 5.5 with 100 mM morpholinoethanesulfonic acid) (19). Ampicillin, kanamycin, and tetracycline were added at concentrations of 100, 25, and 12.5 μg/ml, respectively.

Construction of GadX expression systems. The 838-bp DNA fragment encompassing the entire *gadX* gene was generated by PCR using *Vent* polymerase (New England Biolabs) from pBsAX with primers 5′-GG*GGATCC*ATGCAATCACT ACACGGGAATT-3′ and 5′-GG*AAGCTT*CTATAATCTTATTCCTTCCGCA GA-3′ (restriction sites are italicized; the *gadX* start and stop codons are underlined). After digestion with *Bam*HI and *Hin*dIII, the amplicon was ligated to the corresponding restriction sites of pBlueScript (pBs), yielding pBs*gadX*. This construct was used to transform *E. coli* DH5αF′ competent cells and sequenced on both strands. The 832-bp *Bam*HI-*Hin*dIII fragment from pBs*gadX* was then ligated to the corresponding sites of the expression vector pQE60, yielding pQE*gadX*. This construct was used to transform *E. coli* JM109 competent cells.

To generate the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible p*malE*::*gadX* construct, the 838-bp DNA fragment encompassing the entire *gadX* gene was amplified by PCR using *Taq* polymerase (Perkin Elmer) from pBsAX with the primers 5′-GGCATATGCAATCACTACATGGGA-3′ and 5′-CCGG ATCCCTATAATCTTATTCCTTCCG-3′ (the *gadX* start and stop codons are underlined). The amplicon was initially T/A ligated to the pBs-derived T-vector

(20), yielding pT*gadX*, and sequenced on both strands to ensure that mispriming did not occur during amplification. Subsequently, the 852-bp *Eco*RI-*Hin*dIII fragment from pT*gadX*, containing the whole *gadX* open reading frame and part of the multicloning site of pBs, was ligated in frame with the *malE* gene into the corresponding sites of the expression vector pMAL-c2 (New England Biolabs), yielding p*malE*::*gadX*. As a consequence of the cloning strategy, the p*malE*::*gadX* fusion construct contained 18 additional nucleotides in the *malE-gadX* linker region, coding for an extra amino acid sequence (SEFDWH). *E. coli* laboratory strains DH5αF′ and JM109 were used for p*malE*::*gadX* propagation and MalE-GadX expression, respectively.

Construction of *gadX*::*lacZ* transcriptional fusions and β-galactosidase assay. Three transcriptional fusions were generated for the *gadX* gene by cloning into the vector pRS415 (32) PCR-generated DNA fragments obtained using pBsAX as the template and spanning from positions −222, −67, and +18 to position +121, relative to the *gadX* transcription start site. Primers used for PCR amplification were 21 to 23 bases in length and designed to allow directional cloning at the *Eco*RI and *Bam*HI sites of pRS415. PCR amplification (25 cycles) was carried out using *Vent* polymerase as follows: 95°C for 1 min, 52°C for 1 min, and 74°C for 1 min. The amplicons were sequenced on both strands. β-Galactosidase activity was measured according to Miller (24) and expressed as follows: $1{,}000 \times [(OD_{420} - 1.75 \times OD_{550})/(OD_{600}$ culture × reaction time × volume)].

Purification of nucleic acids, blotting analysis, and primer extension. RNA was isolated from cells grown under different conditions at specified optical densities, using a modified hot phenol extraction method (9). The RNA concentration was estimated by measuring the optical density at 260 and 280 nm in 0.1 N NaOH. Electrophoretic conditions, blotting, and labeling of probes were carried out essentially as previously described (9). The *gadA/B* probe was a 1.4-kb PCR-generated DNA fragment corresponding to the entire coding sequence of *gadB*. Membrane hybridization and washing were carried out as previously described (9). The *gadX* probe was the 832-bp fragment obtained from *Bam*HI-*Hin*dIII digestion of plasmid pBs*gadX*. When using the *gadX* probe, membrane prehybridization was as follows: 42°C in 50% formamide–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–0.5% sodium dodecyl sulfate (SDS)–100 μg of denatured herring sperm DNA ml^{-1}. After 20 h of hybridization, the membranes were washed at room temperature twice in 2× SSC–0.05% SDS for a total of 30 min and twice at higher stringency in 1× SSC–0.1% SDS for a total of 20 min. Northern blot filters were exposed to Kodak X-Omat UV autoradiographic films for a period varying from a few hours to 10 days, depending on the strain under investigation.

Primer extension analysis was performed with a 21-mer complementary to the region spanning from nucleotides +101 to +121 relative to the *gadX* transcription start point, and using RNA extracted from *E. coli* ATCC 11246 grown to the stationary phase in LB-MES, pH 5.5. The entire procedure was essentially as previously described (9).

Genetic procedures. For construction of the *gadX* knockout, a 255-bp *Nco*I-*Hin*fI fragment, obtained from pT*gadX* and encompassing codons 21 to 105 of *gadX*, was treated with Klenow polymerase to fill recessed ends and blunt-end ligated in the multicloning site of the 6.2-kb mobilizable suicide vector pJP5608 (28). This construct, designated pJP*gadX*′, was conjugated from *E. coli* S17.1 (λ*pir*) to wild-type *E. coli* ATCC 11246. Exconjugants were selected for the tetracycline resistance marker carried by the suicide plasmid. Nine exconjugants were obtained, all carrying site-specific insertion of the suicide construct into *gadX*, as confirmed by Southern blot hybridization analysis of the *Cla*I-digested chromosomal DNA (9).

Purification of MalE-GadX fusion protein. Overnight cultures of *E. coli* JM109(p*malE*::*gadX*) were diluted 100-fold in 125 ml of fresh LB medium containing 0.2% glucose and 100 μg of ampicillin per ml and incubated at 37°C with aeration. When the culture density reached an OD_{600} of ≈0.5, the expression of MalE-GadX was induced by addition of 0.3 mM IPTG, and incubation was continued for an additional 4 h at 37°C. Cells were harvested by centrifugation at $4{,}100 \times g$ in a Sorvall GSA rotor at 4°C for 30 min and resuspended in 10 ml of T200 buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, and 200 mM NaCl). Resuspended cells were disrupted by sonication on ice and centrifuged at $8{,}000 \times g$ in a Sorvall SS-34 rotor at 4°C for 20 min to remove cell debris. The supernatant was loaded at a flow rate of 15 ml/h onto a 5-ml column of amylose resin (New England Biolabs) preequilibrated in T200 buffer at 4°C. After loading, the column was washed with T200 buffer at the same flow rate until the protein content of the flowthrough returned to baseline (60 ml of T200 buffer).

Bound proteins were eluted with 15 ml of T200 buffer containing 10 mM maltose. Of the 12 fractions (1 ml each) collected, four (3 to 6) contained the highest concentration of MalE-GadX, as judged by SDS-polyacrylamide gel electrophoresis (PAGE) analysis. These fractions were pooled, dialyzed against T100 buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, and 100 mM NaCl) and loaded at a flow rate of 15 ml/h onto a 5-ml heparin-agarose column preequilibrated at 4°C in T100 buffer. After loading, the column was washed with T100 buffer at the same flow rate until the protein content of the flowthrough returned to baseline (45 ml of T100 buffer). The bound MalE-GadX protein was eluted with a 40-ml linear gradient from 100 to 600 mM NaCl in T buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA) and collected in 20 fractions of 2 ml each. MalE-GadX-containing fractions were pooled, concentrated by passage through a Centricon-30 ultrafiltration unit (Millipore), and stored at 4°C.

Electrophoretic mobility shift assay and DNase I footprinting. DNA mobility shift assays were conducted using the *gadA*, *gadBC*, and *gadC* promoter (P) regions encompassing positions −176 to +77 (P*gadA*), −173 to +77 (P*gadBC*), and −120 to +142 (P*gadC*), relative to the transcription start sites of the individual genes (9). P*gadA* and P*gadBC* were transferred from the corresponding promoter-probe constructs in pRS415 (9) into the *Eco*RI and *Bam*HI sites of pBs to yield pBsP*gadA* and pBsP*gadBC*, respectively. P*gadC* was excised by *Pst*I-*Cla*I digestion of pBsB (10; this work) and cloned into the corresponding sites of pBs, yielding pBsP*gadC*. P*gadA* and P*gadBC* fragments were generated by *Eco*RI-*Bam*HI digestion from pBsP*gadA* and pBsP*gadBC*, respectively, while P*gadC* was obtained by *Pst*I-*Sal*I digestion from pBsP*gadC*. This latter digestion was necessary to make possible terminal labeling of the P*gadC* fragment. The 5′ protruding ends of P*gadA*, P*gadBC*, and P*gadC* fragments were filled in with Klenow DNA polymerase (Roche) and [α-^{32}P]dATP, according to standard protocols (2).

Radiolabeled DNA fragments (10 fmol) were incubated with increasing concentrations of MalE-GadX (from 0 to 10 pmol) at 22°C for 30 min in 20 μl of binding buffer (10 mM Tris-HCl [pH 8.0], 50 mM KCl, 0.5 mM EDTA, 6% glycerol, 200 μg of bovine serum albumin [BSA] per ml, and 100 ng of poly[dI-dC] per μl). Samples were loaded onto 5% nondenaturing acrylamide gels in 0.5× TAE (Tris-acetate-EDTA) buffer. Gels were run at room temperature in 0.5× TAE buffer for a total of 4 h at 10 V/cm and immediately exposed to X-ray films.

For DNase I footprinting experiments, 5 ng of PCR-generated P*gadA* and P*gadB* (9) were incubated for 20 min at 25°C with the indicated amounts of purified MalE-GadX in 30 μl of a mixture containing 40 mM HEPES (pH 8.0), 100 mM KCl, 10 mM magnesium acetate, and 0.5 mM dithiothreitol (DTT). After DNase I treatment, the partial digestion products were ethanol precipitated and subjected to 30 cycles of asymmetric PCR using the 5′-^{32}P-end labeled primers, essentially as previously described (12). Primers were gadAfrw (5′-GGGAATTCATCGCCCGAACAGGAATG-3′) and gadBfrw (5′-GGGAATTCAATAACAACACAACACAC-3′ for the *gadA* and *gadB* promoter regions, respectively, and gadABrev (5′-GGGGATCCCGTGAATCGAGTAGTTC-3′) for both. The PCR-generated fragments were separated on a 7% sequencing gel.

Immunoblot analysis and Gad activity assay. Aliquots of 2.5 μg of protein from the soluble fraction of cell lysates were used for Western blot analysis. Samples were run on SDS–10% PAGE and directly electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). Both GadA and GadB were detected with affinity-purified anti-GadA rabbit polyclonal antibodies (9) and horseradish peroxidase-labeled secondary antibody provided with the BM chemiluminescence Western blotting kit (Roche).

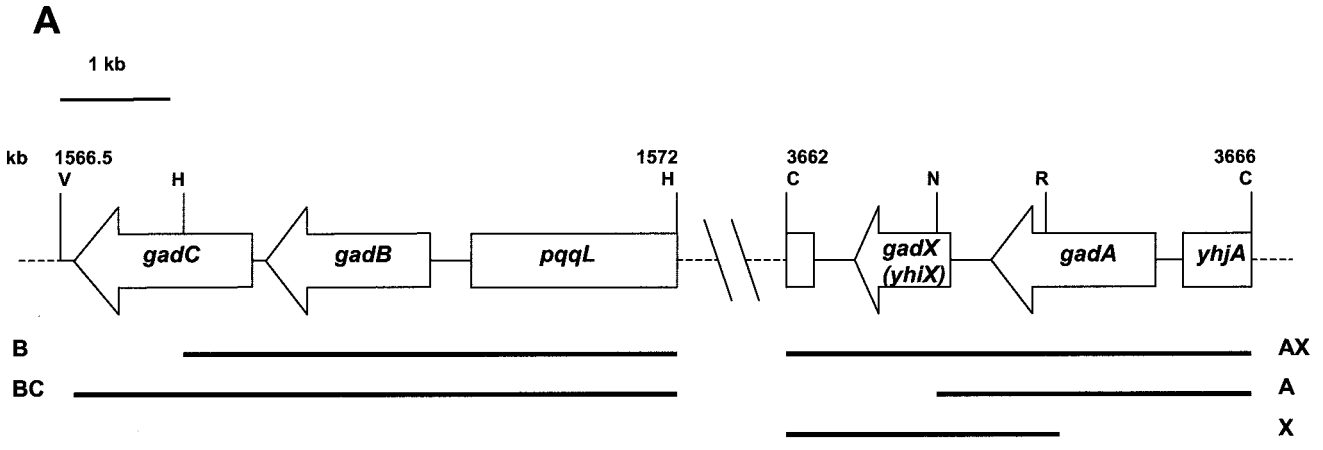
Glutamate decarboxylase activity assays and GABA measurements were performed as previously described (9).

RESULTS

Identification of a regulatory gene of the *gad* system downstream of *gadA*. The physical organization of the *E. coli* ATCC 11246 genomic fragments previously used to clone the entire *gadBC* operon and the *gadA* gene (10) is shown in Fig. 1A. By homology with the *E. coli* K-12 genome sequence (4), the 4.1-kb *Cla*I genomic fragment encompassing the *gadA* gene was later recognized to include the entire *gadX* (*yhiX*) gene as well. The *gadX* gene of *E. coli* ATCC 11246 is predicted to encode a protein of 274 amino acids with an estimated molecular mass of 31,565 Da and a pI of 8.94.

BLASTP analysis (1) disclosed a substantial similarity between the GadX protein and a number of transcriptional regulators belonging to the AraC/XylS family. The GadX variants from *E. coli* K-12 (4) and *E. coli* serotypes O157:H7 (29) and O126:H6 (30) were aligned with GadX from ATCC 11246. A total of 24 amino acid substitutions, mostly conservative, were

A



B

Plasmid	Gad (U/mg)	GABA (mM)	Extracellular pH
pBs	0.4 ± 0.1	0.46 ± 0.24	4.62 ± 0.04
pBsAX	27.6 ± 7.8	5.54 ± 0.19	4.95 ± 0.10
pBsA	12.4 ± 2.9	1.20 ± 0.32	4.65 ± 0.05
pBsX	7.6 ± 1.9	5.51 ± 0.75	4.83 ± 0.05
pBsB	4.7 ± 1.0	2.16 ± 0.30	4.67 ± 0.03
pBsBC	5.0 ± 0.6	5.71 ± 0.70	4.82 ± 0.06

FIG. 1. (A) Chromosomal organization of the *gad* gene system in *E. coli*. Black bars identify the chromosomal regions of *E. coli* ATCC 11246 cloned in the pBluscript (pBs) vector and containing the specified genes: A (*gadA*), B (*gadB*), BC (*gadB* and *gadC*), AX (*gadA* and *gadX*), and X (*gadX*). Numbering (kilobases) is relative to the *E. coli* K-12 map (4). Restriction sites used for cloning the individual chromosomal fragments are indicated: V, *Eco*RV; H, *Hin*dIII; C, *Cla*I; N, *Nco*I; R, *Eco*RI. (B) Intracellular Gad activity, GABA levels, and pHs of the spent medium are given for *E. coli* JM109 carrying the individual constructs. The reported values are relative to those in 24-h cultures in LBG medium (pH 5.0) and represent the means (± standard deviations) of at least five determinations.

detected, none of which occurred within the two helix-turn-helix motifs that represent the DNA-binding signature for AraC/XylS family proteins (data not shown).

Preliminary evidence for the role of *gadX* in the positive regulation of the *gadA* and *gadBC* system was provided by transactivation experiments conducted in *E. coli* JM109. This K-12 strain expresses very low glutamate decarboxylase levels (10) even under inducing conditions, i.e., after 24 h of growth in LBG medium, pH 5.0. Figure 1B shows the glutamate decarboxylase (Gad) activity, the concentration of released GABA, and the pH of the spent medium in *E. coli* JM109 carrying the cloned genomic fragments (Fig. 1A). The multicopy pBsAX plasmid (*gadA-gadX*) directed extremely high Gad and GABA release activities compared with the control vector (pBs). Excision from plasmid pBsAX of the *Nco*I-*Cla*I region downstream of *gadA* yielded plasmid pBsA. *E. coli* JM109(pBsA) expressed 2.2-fold less decarboxylase activity than pBsAX transformants and showed an even more pronounced reduction of the amount of released GABA (4.6-fold decrease). On the other hand, deletion of the whole *gadA* gene, as in plasmid pBsX, caused a substantial decrease in the decarboxylase activity (3.6-fold) without affecting the amount of exported GABA, which equaled that observed for *E. coli* transformants carrying pBsAX. More interestingly, the GABA export activity in the presence of the multicopy *gadX* gene (pBsAX and pBsX) equaled that observed for the multicopy decarboxylase/antiporter *gadBC* system (pBsBC). Gad activity and extracellular pH were similar in pBsX and pBsBC transformants, indicating that the *gadX* gene is involved in transactivation of the *gad* system.

***gadX* gene product is a transcriptional activator of *gad* structural genes.** To correlate GadX expression with the upregulation of *gad* structural genes, we expressed the *gadX* gene under the control of the heterologous T5-*lacO* promoter-operator element and monitored transactivation of *gad* structural genes under conditions in which the *gad* genes are not expressed, i.e., during exponential growth in LB medium at pH 7.4. Expression of the *gad* system was undetectable in exponential cultures of *E. coli* JM109 carrying the control vector pQE60, while substantial *gadA* and *gadBC* transcription and increased glutamate decarboxylase and GABA export activities were observed when the *gadX* gene was provided in *trans* under the control of the exogenous promoter, as in pQE*gadX* (Fig. 2A and B). Due to leakage of the T5-*lacO* hybrid promoter, expression of the *gad* system was detectable even without induction, while addition of 0.1 mM IPTG was detrimental, probably due to a toxic effect resulting from excessive expression of the GadX activator protein.

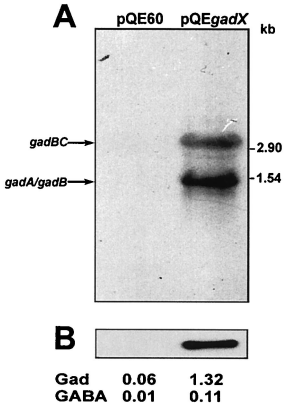


FIG. 2. GadX-dependent activation of *gad* genes. (A) Northern hybridization analysis of *gad* mRNAs extracted from *E. coli* JM109 carrying the pQE60 vector and the expression construct pQE*gadX*. Cells were grown at 37°C in neutral LB medium to the mid-exponential phase ($OD_{600} \approx 0.8$). Aliquots of 10 μg of total RNA were electrophoresed, transferred onto nylon filters, and hybridized with the *gadA/B* probe. Sizes of RNA standards are given on the right. (B) Immunoblot analysis of GadA and GadB expression in whole bacterial lysates (2.5 μg of protein) probed with anti-GadA/B polyclonal antibodies. The decarboxylase activity (Gad, in units per milligram) in the cell lysates and the GABA levels (millimolar) in the growth medium are given for each condition.

Evidence for the involvement of *gadX* in the positive control of *gad* genes in *E. coli* ATCC 11246 was provided by the analysis of *gadA* and *gadBC* expression in a *gadX*-defective background. For this purpose, a *gadX* site-specific mutant was generated by inserting the suicide plasmid pJP*gadX′* within the *gadX* coding sequence of *E. coli* ATCC 11246. The *gadX* mutant, designated DDBGX, was compared with the parental strain for expression of *gad* structural genes under different growth conditions. During the stationary phase at neutral pH, *gadA* and *gadBC* transcripts were approximately fivefold less abundant in DDBGX (*gadX*) compared with the wild-type strain (Fig. 3A, left panel). A more pronounced reduction of transcript levels (eightfold) was observed under inducing conditions, i.e., during the stationary phase in acidic (pH 5.5) buffered LB-MES medium (Fig. 3A, right panel). This effect was also detectable, though to a lesser extent, at the level of protein expression and activity, as shown by immunoblot analysis of GadA/B expression in crude cell lysates and by direct measurements of glutamate decarboxylase activity in *E. coli* ATCC 11246 and in the isogenic *gadX* mutant (Fig. 3B). Thus, while the *gadX* gene product is dispensable for basal *gadA* and *gadBC* transcription, its presence is required for maximum expression of the *gad* gene system in *E. coli* ATCC 11246.

Mapping and characterization of *gadX* promoter region. As a premise to the investigation of *gadX* transcriptional control, primer extension analysis was conducted using total RNA extracted from *E. coli* ATCC 11246 grown to the stationary phase in LB-MES at pH 5.5. Results demonstrate that transcription of *gadX* originates from the T residue located 29 nucleotides upstream of the ATG start codon (Fig. 4). The −35 and −10 hexamers (TTGACT-N_{19}-ATTAAT) match 5 of 6 and 4 of 6 nucleotides, respectively, of the recognition site for RpoD-dependent RNA polymerase. The −10 hexamer overlaps the sequence 5′-CTACATT-3′, which matches 5 of 7 nucleotides of the RpoS consensus and is preceded by a potentially bent DNA region, as expected for RpoS-dependent promoters (11).

Evidence for the existence of an indigenous *gadX* promoter was also inferred by the analysis of reporter gene activity directed by *gadX*::*lacZ* transcriptional fusions in the promoter probe vector pRS415 (32). Strong promoter activity was observed for the DNA fragment encompassing nucleotides −67 to +121 relative to the *gadX* transcription start point. This region is likely to encompass the minimal *gadX* promoter element, since inclusion of the additional 155-nucleotide upstream sequence (−222 to +121 construct) did not significantly alter the reporter gene expression, while an 85-nucleotide deletion (+18 to +121 construct) totally abrogated promoter activity (Fig. 4B).

Transcription of *gadX* gene is activated during stationary phase and responds to acidic shock. As a specific activator of the *gad* gene system, *gadX* can be predicted to respond positively to stimuli which are known to induce expression of the *gadA* and *gadBC* genes. However, a *gadX*::*lacZ* promoter fusion from enteropathogenic *E. coli* was found not to be controlled by growth phase variation in LB medium, though it responded positively to acid induction (30).

To address the issue of *gadX* transcriptional control, total RNA was extracted from *E. coli* ATCC 11246 and MC4100 cells grown to exponential or stationary phase either in standard LB medium (pH 7.4) or in acid-buffered LB-MES medium (pH 5.5). Figure 5 shows that the *gadX* probe detected two mRNA species with apparent sizes of 1.0 and 2.7 kb. The size of the shorter transcript fits well with that predicted for a monocistronic *gadX* mRNA ending at the level of the stem-

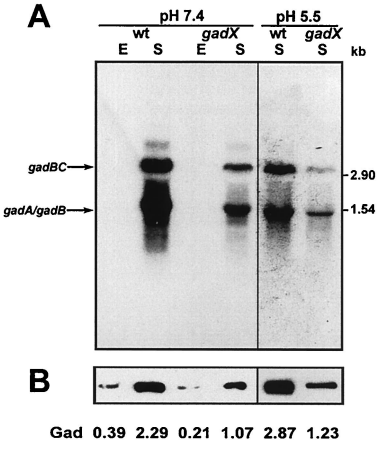


FIG. 3. Effect of *gadX* mutation on the expression of *gad* structural genes and on glutamic acid decarboxylase and GABA export activities. (A) Northern blot analysis of total RNA extracted from *E. coli* strains ATCC 11246 (wild type, wt) and DDBGX (*gadX*) grown at 37°C in neutral LB medium (pH 7.4) or in mildly acidic LB-MES medium (pH 5.5). E, exponential-phase cultures ($OD_{600} \approx 0.5$); S, stationary-phase cultures ($OD_{600} \approx 2.0$). Aliquots of 10 μg of total RNA were electrophoresed, transferred onto nylon filters, and hybridized with the *gadA/B* probe (left panel, 1-day exposure; right panel, 6-h exposure). Sizes of RNA standards are given on the right. (B) Immunoblot analysis of GadA and GadB expression in whole bacterial lysates (2.5 μg of protein) probed with anti-GadA/B polyclonal antibodies. The decarboxylase activity (Gad, in units per milligram) in the cell lysate is given for each condition.

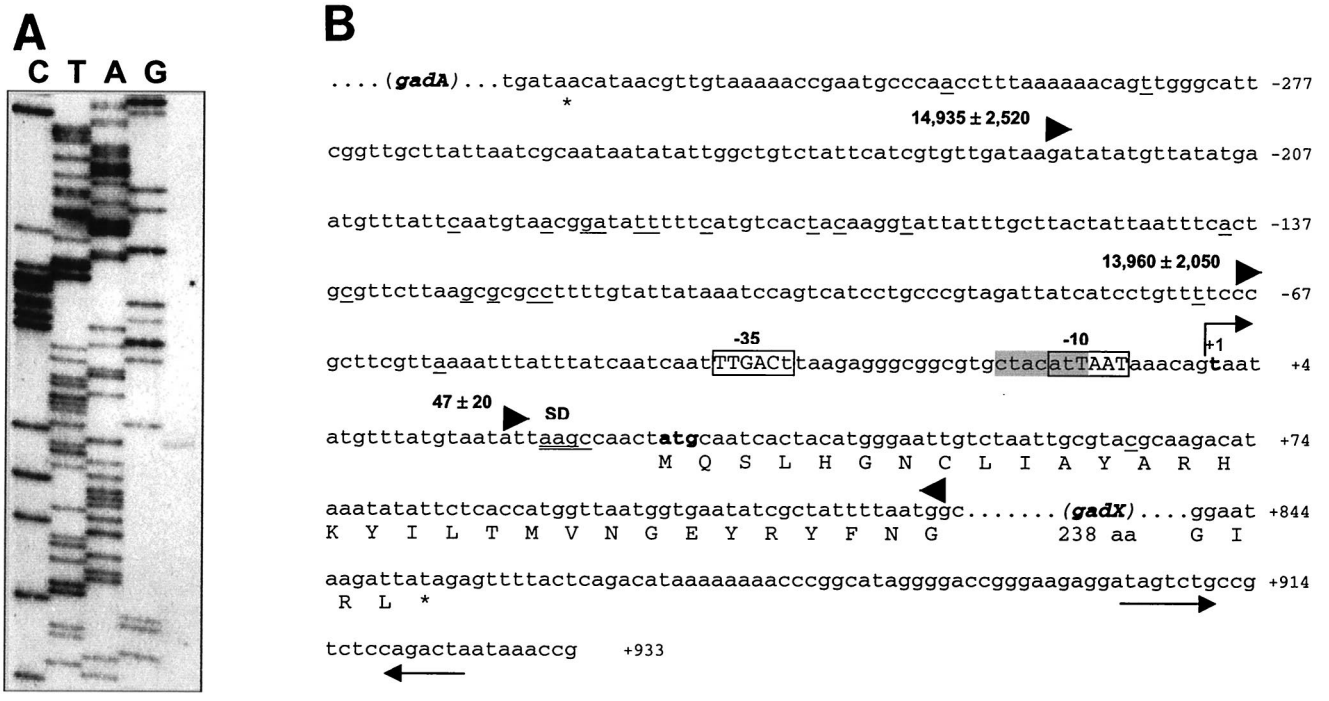


FIG. 4. Promoter mapping and location of the transcription start point of *gadX*. (A) Mapping of the 5′ end of the *gadX* transcript by primer extension. RNA was extracted from stationary-phase cells grown at pH 5.5 and retrotranscribed after priming with a 5′-end-labeled oligonucleotide. Lanes C, T, A, and G are sequencing ladders of pBsAX with the same oligonucleotide used for the primer extension reaction. Sequencing reactions were run in parallel with the cDNA transcript (right lane) to determine exactly the 5′ end of the *gadX* message. (B) Sequence analysis of the *gadX* promoter region in *E. coli* ATCC 11246. The bent arrow indicates the transcriptional start site at the residue in bold, defined as +1. Differences from the *E. coli* RpoD consensus sequences for the −10 and −35 promoter elements are shown as lowercase letters within the boxed regions. The RpoS consensus is shaded in gray. Nucleotide sequence variations between ATCC 11246 and the K-12 strain MG1655 (4) are underlined. The potential Shine-Dalgarno (SD) sequence is double underlined. The triangles define the 5′-to-3′ boundaries of the DNA fragments tested for the ability to direct *lacZ* expression in pRS415 (32). The number preceding each forward-pointing triangle is the β-galactosidase activity value (in Miller units) expressed by exponential-phase cultures of *E. coli* MC4100 carrying the cloned promoter fragment. Sequence numbering is relative to the *gadX* transcription start point.

loop structure centered at position + 915 relative to the *gadX* transcription start point (Fig. 4B). The 2.7-kb mRNA species, accounting for nearly 10% of the total *gadX*-specific messages, is likely to be a dicistronic *gadAX* transcript originating from the *gadA* promoter, approximately 1.8 kb upstream of *gadX*. In fact, a similar-sized mRNA species was previously detected by autoradiography following hybridization with the *gadA/B* probe (9).

Both *gadX* messages were detectable only after entry into the stationary phase, but their relative amounts varied depending on the acidity of the medium. In fact, *gadX* mRNAs were clearly detectable in stationary-phase cultures at pH 5.5, while only the shorter transcript was detected in stationary-phase cultures at pH 7.4 and appeared to undergo rapid degradation (Fig. 5). Thus, the transcription profile of *gadX* appears to be growth phase dependent and coordinated with that of the *gadA* and *gadBC* genes (9), though transcript levels differ quantitatively between the low Gad producer *E. coli* MC4100 and the overproducing wild-type strain ATCC 11246.

To address the issue of whether *gadX* is responsible for transcriptional activation at its own promoter (P*gadX*), the activity of P*gadX*::*lacZ* transcriptional fusions (nucleotides −222 to +121) was tested in *E. coli* carrying in *trans* the multicopy *gadX* gene under the control of its indigenous pro-

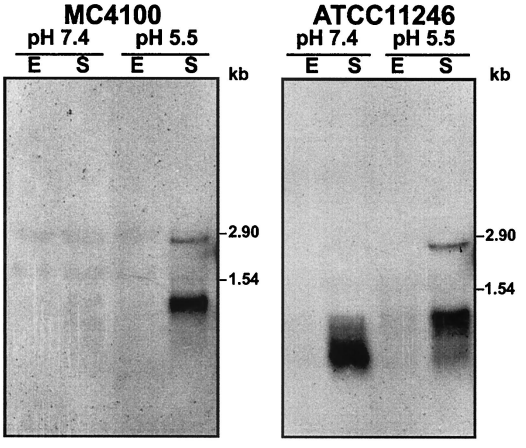


FIG. 5. Analysis of *gadX* transcripts during the growth cycle and under acidic conditions. Total RNA was extracted from *E. coli* strains MC4100 (left) and ATCC 11246 (right) grown at 37°C in neutral LB medium (pH 7.4) or in mildly acidic LB-MES medium (pH 5.5). E, exponential-phase cultures ($OD_{600} \approx 0.5$); S, stationary-phase cultures ($OD_{600} \approx 2.0$). Aliquots of 10 μg of total RNA were electrophoresed, transferred onto nylon filters, and hybridized with the *gadX* probe. Sizes of RNA standards are given on the right of each panel.

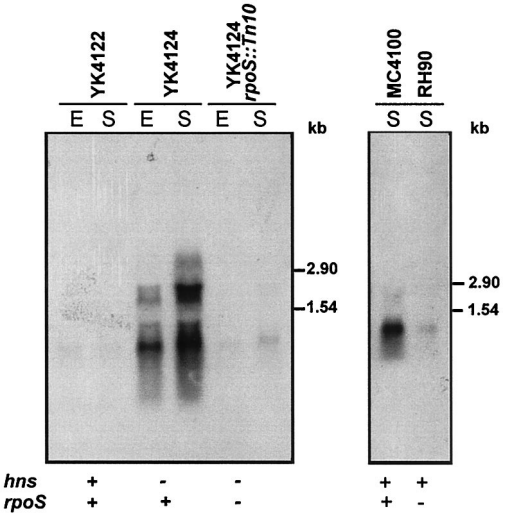


FIG. 6. Effect of *hns* and *rpoS* mutations on transcription of the *gadX* gene. Northern hybridization analysis of total RNA (10 μg in each lane) extracted from the wild-type *E. coli* strain YK4122 and from its *hns* (YK4124) and *hns rpoS* (YK4124 *rpoS*::Tn *10*) mutants (left panel) and from wild-type *E. coli* strain MC4100 and its *rpoS* mutant (RH90) (right panel). Bacteria were grown at 37°C in neutral LB medium to the exponential (E) or stationary (S) phase. Nylon filters were hybridized with the *gadX* probe. Sizes of RNA standards are given on the right of each panel.

moter. It was observed that P*gadX* was equally active both in the presence and in the absence of multicopy *gadX*, irrespective of the growth stage of the culture (data not shown). It can therefore be argued that GadX is not involved in activation of transcription arising from P*gadX*.

***gadX* gene is under transcriptional control of H-NS and RpoS.** The *gadX* gene has recently been identified as an H-NS-repressible gene (17), consistent with the previous observation that H-NS acts as a negative regulator of the *gad* structural genes during the exponential phase (9, 38). Moreover, we and others have also linked stationary-phase transcription of *gadA* and *gadBC* to the activity of the RpoS sigma factor (6, 9). Given that *gadX* expression is also stationary-phase dependent, we investigated the involvement of both H-NS and RpoS in the transcriptional control of *gadX*. The amount of *gadX* transcripts was therefore compared in wild-type *E. coli* K-12 and in *hns*, *rpoS*, and *hns rpoS* mutants.

Figure 6 shows that repression of *gadX* is relieved in the *hns*-defective background, resulting in increased transcription of both *gadX* and *gadAX* mRNAs even during the exponential phase. This effect correlates with the upregulation of both the *gadA* and *gadBC* genes, whose transcription was found to occur during the exponential phase in the *hns* mutant (9). Moreover, RpoS primarily affects *gadX* expression, since the *rpoS* mutation strongly reduced *gadX* transcription irrespective of the *hns* background. Also in this case, the pattern of *gadX* regulation parallels that of the *gadA* and *gadBC* genes (9), indicating that H-NS and RpoS act as master regulators of the whole *gad* gene system.

GadX binding to *gadA* and *gadBC* promoters produces multiple protein-DNA complexes. As a member of the AraC protein family, GadX is expected to bind specific sequence motifs located within or in the proximity of the target promoters. On this assumption, we generated a recombinant derivative of GadX and tested the in vitro binding of this protein to the promoter region of the *gadA*, *gadBC*, and *gadC* genes. To simplify the purification protocol of GadX and to overcome problems deriving from the poor solubility of AraC/XylS family proteins (15), a soluble MalE-GadX fusion was used for in vitro studies. Chimeric MalE-GadX was found to activate the *gad* system in vivo, although to a much lesser extent than the wild-type GadX protein, as expressed from pQE*gadX* and pBsX.

The p*malE*::*gadX* construct was generated by cloning the *gadX* coding sequence in frame with the 3′ terminus of the *malE* gene in the pMAL-c2 expression vector. In doing so, we expected the C-terminal portion of GadX not to be affected by physical hindrance of fused MalE, thereby retaining the helix-turn-helix domains competent for interaction with target DNA. The p*malE*::*gadX* construct was introduced in *E. coli* JM109 and used to overexpress the fusion protein following IPTG induction. SDS-PAGE analysis of crude cell extracts revealed that a 73-kDa protein was expressed upon induction, its mass being consistent with the expected size for the MalE-GadX fusion protein (42 kDa for MalE and 31 kDa for GadX; data not shown).

Given that more than 90% of the fusion protein was recovered from the soluble fraction of the induced cell lysate, this was used as the starting material for affinity purification on an amylose column, followed by chromatography onto a heparin-agarose column to remove residual protein contaminants. The purification protocol yielded approximately 95% pure MalE-GadX, as judged by SDS-PAGE. An attempt was made to remove the MalE moiety from the fusion protein by factor Xa proteolysis, but the results so far achieved indicate that GadX tends to precipitate in aqueous solution after MalE cleavage (data not shown). This feature, which has previously been reported for a number of proteins of the AraC/XylS family (13, 26, 33), led us to use the soluble MalE-GadX fusion protein for preliminary in vitro DNA-binding studies.

DNA probes encompassing the *gadA*, *gadBC*, and *gadC* promoter regions (see Materials and Methods) were used in protein-binding assays with increasing amounts of the MalE-GadX protein in the presence of a 500-fold molar excess of poly(dI-dC) as the competitor (Fig. 7A). Addition of up to 2 pmol of protein to the P*gadA* probe (10 fmol) produced two equally represented DNA-protein complexes with reduced mobility (forms I and II; Fig. 7A, left panel). At higher MalE-GadX concentrations, form II gradually decreased, giving way to a maximally shifted species (form III). A minor species of intermediate mobility between forms I and II also appeared. Then, at more than 8 pmol of MalE-GadX, the whole probe was shifted, prevalently generating forms I and III.

A less complex pattern of MalE-GadX binding was observed with the P*gadB* probe (Fig. 7A, right panel), with the generation of only two forms (II and III). In this case, form II appeared to be prevalent, but the binding affinity of the promoter was apparently lower than that of P*gadA*, as indicated by the fact that a substantial amount of the P*gadB* remained unbound in the presence of 10 pmol of MalE-GadX. Under the same experimental conditions, the mobility of the P*gadC* promoter was not affected by the addition of up to 10 pmol of MalE-GadX, and a shift of P*gadA* and P*gadB* was not observed after

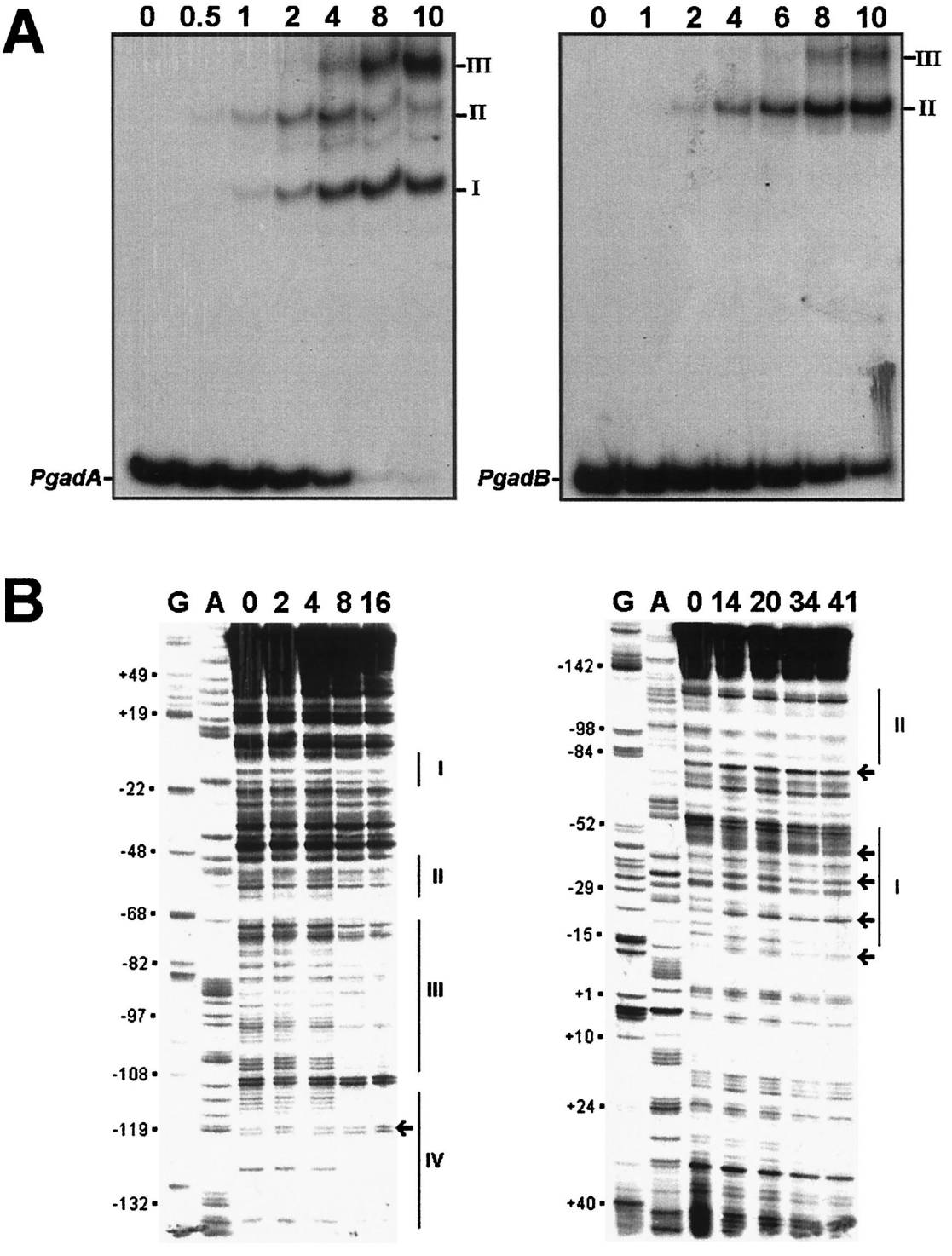


FIG. 7. Identification of MalE-GadX binding sites in *gadA* and *gadBC* promoters. (A) Gel retardation assays of in vitro binding of the purified MalE-GadX protein to the promoter regions of *gadA* (P*gadA*, left) and *gadBC* (P*gadB*, right) genes. The DNA fragments were labeled with [α-^{32}P]dATP by fill-in of 5′ protruding ends. In each binding reaction, 10 fmol of the DNA probe was incubated in a 10-μl volume with increasing amounts (0.5 to 10 pmol) of the MalE-GadX protein, under the conditions described in Materials and Methods. MalE-GadX-bound DNA fragments (forms I, II, and III) were separated from the unbound probe on a 5% polyacrylamide gel run in 0.5× TAE buffer. (B) DNase I footprinting assays. The 265-bp DNA fragments carrying the promoter regions of *gadA* (left) and *gadBC* (right) were incubated with the indicated amounts (picomoles) of MalE-GadX. Samples were processed as described in Materials and Methods using gadAfrw (left panel) and gadABrev (right panel) as the primers. Lanes G and A represent *Taq*I polymerase sequencing reactions using the same primers. The MalE-GadX-protected sites are indicated with vertical lines and labeled with roman numbers from I to IV. Arrows indicate DNase I-hypersensitive sites. (C) Sequence alignment of *gadA* and *gadBC* promoter regions showing the DNase I-protected sites on the coding (full line) and noncoding (dotted line) DNA strands. Sites are indicated above the corresponding sequence. The −35 and −10 hexamers for both *gadA* and *gadBC* are shown in bold type.

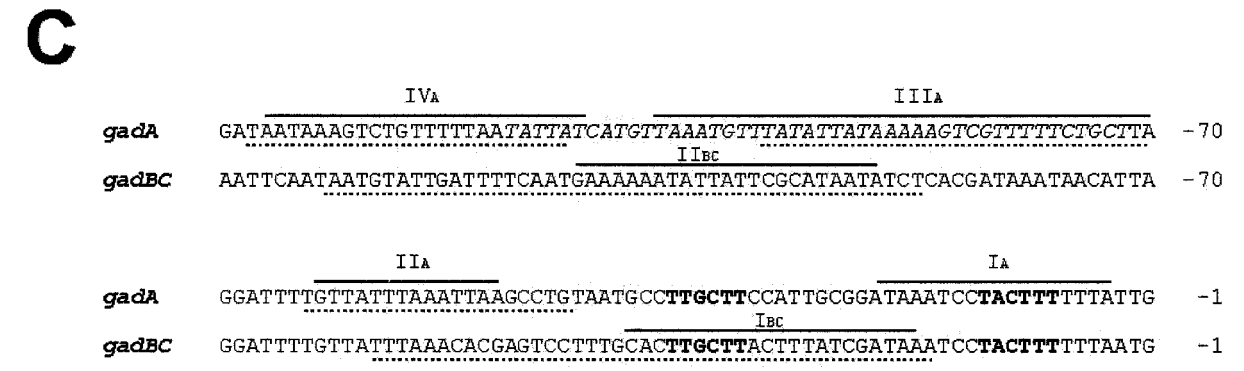


FIG. 7—*Continued*.

replacement of the fusion protein with an equimolar amount of the MalE protein (data not shown).

The observation that MalE-GadX specifically interacts with the promoter sequences of the *gadA* and *gadBC* genes, but not with the intergenic region between *gadB* and *gadC*, prompted us to map the binding sites of GadX on *gadA* and *gadB* promoters by DNase I footprinting assays. Four MalE-GadX binding sites were identified on P*gadA* and two on P*gadB* (Fig. 7B). MalE-GadX extensively protected the *gadA* promoter region (left panel) at positions −4 to −20 (site I), −49 to −62 (site II), −70 to −107 (site III), and −112 to −135 (site IV), relative to the transcription start site. The protected sites have an average length of approximately 22 nucleotides and show, within the limit of accuracy of our analysis, different affinities for MalE-GadX. Almost complete DNase I protection occurred at sites III and IV upon the addition of 8 pmol of MalE-GadX, while a twofold increase in the amount of the protein (16 pmol) was required for binding at sites I and II.

As expected from the gel retardation assay, MalE-GadX also bound the *gadB* promoter region, but only at two sites and with a lower affinity compared with the *gadA* promoter region (Fig. 7B, right panel). MalE-GadX preferentially binds to the distal upstream promoter region of *gadB*, as inferred by the protection exerted by 14 pmol of the protein on an extended region spanning from positions −87 to −131 (site II). Upon addition of more than 30 pmol of MalE-GadX, another site extending from positions −17 to −58 (site I) became protected on P*gadB*.

DISCUSSION

In this work we present evidence for the direct involvement of the GadX protein in transcriptional activation of the *gad* gene system. GadX belongs to the AraC/XylS family of bacterial transcriptional regulators, known to be activators of functions as diverse as sugar catabolism, responses to stress, and virulence (15, 23). The positive regulatory role of GadX was inferred both by transactivation assays of *gad* structural genes under physiological and nonphysiological conditions and by reduced transcription of *gadA* and *gadBC* in a *gadX* mutant. Differences in *gadA* and *gadBC* expression levels under the above conditions were confirmed by the direct measurement of glutamic acid decarboxylase (GadA/B) and GABA export (GadC) activities. Expression analysis of the *gad* gene system in the *gadX* mutant indicates that GadX is required for maximal expression during the stationary phase and acid induction, though it is not essential for basal expression of the system.

Given that expression of *gad* structural genes is silenced during the exponential phase in neutral rich (LB) medium, it was possible to monitor GadX activity only when the culture had entered the stationary phase. At this growth stage in neutral LB medium, *gadA* and *gadBC* are transcribed exclusively by RpoS (9, 7), and their reduced expression in the *gadX* mutant points to an interplay between GadX and RpoS. This is not surprising, inasmuch as other proteins of the AraC/XylS family have been shown to activate transcription directed by alternative sigma factors (21). Our data also suggest that RpoS is not the only sigma factor involved in GadX-mediated activation of the *gad* gene system. In fact, GadX-mediated recruitment of vegetative (RpoD-dependent) RNA polymerase at the *gad*A and *gadBC* promoters could explain the observation that expression of *gadX* under an exogenous promoter suffices for transactivation of the *gad* gene system during the exponential phase in neutral rich (LB) medium. Accordingly, *gadX*-dependent activation of *gad* structural genes was also observed in an *rpoS* background (A. Tramonti and D. De Biase, unpublished data). Moreover, an involvement of GadX in the RpoS-independent, acid-responsive pathway of induction of the *gad* genes (7) is inferred by the evidence that stationary-phase expression of *gadA* and *gadBC* genes in the *gadX* mutant is more drastically reduced at acidic than at neutral pH. However, the observation that transcription of *gadA* and *gadBC* is only partially abrogated in the *gadX* mutant raises the possibility that additional positive regulators are also involved in the transcriptional tuning of the *gad* system, as found for other genes controlled by transcriptional regulators of the AraC/XylS family (22, 25).

As an activator of the *gad* system, *gadX* was expected to respond to one or more of the stimuli known to trigger expression of the *gad* structural genes. As far as stationary phase and acid induction are concerned, *gadX* expression paralleled that of the *gad* structural genes, being stationary phase dependent and acid inducible. Conversely, expression of *gadX* during the stationary phase was not affected by exposure of cells to hyper- and hypo-osmotic environments. The expression of *gadX* was found to be primarily dependent on RpoS, and under our experimental conditions, *gadX* expression was not appreciable in wild-type *E. coli* during the exponential phase, even upon acidic induction. RpoS dependence is also supported by the

observation that the *gadX* promoter is endowed with both sequence and topological features predicting recognition by RpoS (11).

We and others (17) have observed that H-NS acts as a repressor of *gadX* expression, and this could explain silencing of *gadX* during the exponential phase. Given the hierarchy of regulation of the whole *gad* system, a direct activity of H-NS on the *gadX* promoter can be envisaged, resulting in repression of the *gad* structural genes during the exponential phase. However, an antagonistic effect of GadX on H-NS repression, likely resulting from competition at the *gadA* and *gadBC* promoters, should also be taken into account. An additional level of repression of *gad* genes is provided by the cyclic AMP receptor protein (CRP), which was demonstrated to repress RpoS-dependent *gad* transcription during the exponential phase in rich (LB) medium (7). Since the region predicted to interact with CRP overlaps GadX binding sites II and III on P*gadA* (Fig. 7), displacement of the CRP repressor by the GadX activator could account, at least in part, for the upregulation of the system during the exponential phase.

Based on primer extension results and sequence analysis, the *gadX*-specific mRNA is predicted to be approximately 930 nucleotides in length. Accordingly, RNA blot experiments showed that an mRNA species with an apparent size of 1 kb was specifically recognized by the *gadX* probe. In addition to the 1-kb *gadX* transcript, a less abundant *gadAX* bicistronic transcript of approximately 2.7 kb was also detected. Thus, autoregulation of *gadX* expression can be expected to occur through the direct activity of GadX at the *gadA* promoter, leading to *gadAX* cotranscription. Such an autoregulatory loop would imply that expression of both GadA and GadX is coordinated and responsible for superinduction of the whole *gad* system; primary stimuli (stationary phase and acid) acting on P*gadX* would lead to increased expression of GadX and, in turn, to an amplification of the response through GadX-dependent activation of the *gadAX* and *gadBC* promoters.

Conclusive evidence for direct recognition of the *gad* promoters by GadX derives from electrophoretic mobility shift assays, which indicate that MalE-GadX specifically binds P*gadA* and P*gadB* and that binding occurs through the formation of several DNA-protein complexes. These forms were not detected in a preliminary analysis of MalE-GadX binding to the *gadA* and *gadBC* promoters (30). However, the complex profile of promoter recognition by MalE-GadX was confirmed herein by DNase I footprinting analysis. Binding was observed to occur with different affinity at four sites on P*gadA* and two on P*gadB*. The highest-affinity binding was detected at sites III and IV of P*gadA*, the former site encompassing at least 60% of the DNA element which was predicted to be pH responsive (9). Binding of MalE-GadX at sites III and/or IV of P*gadA* could account for the formation of the two predominant protein-DNA complexes (forms II and/or I) observed at the same protein concentration in the electrophoretic mobility shift assay (Fig. 7, left panels).

By doubling the amount of MalE-GadX, two additional protected sites (I and II) were detected on P*gadA*. Interestingly, this is also the concentration threshold at which transition from complex II to complex III occurs. Thus, while complex I is stably retained up to saturating concentrations of MalE-GadX, complexes II and III appear to be mutually exclusive. A plausible explanation for these observations is that, at low protein-DNA ratios, high-affinity binding of MalE-GadX either to site III or IV generates complex I (1:1 protein-DNA ratio), while binding to both sites results in complex II (2:1 protein-DNA ratio). At higher protein-DNA ratios, the coexistence of both forms I and III (Fig. 7, left panels) indicates that MalE-GadX can occupy either one or three of four sites at the same time.

The protection pattern of MalE-GadX on P*gadB* is different from and less puzzling than that observed for P*gadA*. Protection of site II from DNase I digestion occurred within the concentration range used to observe the formation of the DNA-protein complex II in the electrophoretic mobility shift assay (Fig. 7, right panels). The lower electrophoretic mobility of complex II compared with that of complex I in P*gadA* suggests that it originates from binding of two MalE-GadX molecules, and this would be in line with the stoichiometry predicted for complex II of P*gadA*. The amount of MalE-GadX required to protect site I on P*gadB*, however, can also reasonably explain the appearance of complex III in the gel mobility shift assay. In fact, site I is fully protected and complex III is formed when 1 μM MalE-GadX is used, a concentration which is far too high to be significant.

Thus, while both the *gadA* and *gadB* promoters are characterized by the presence of multiple sites of interaction with GadX, they differ significantly in the overall organization (number, position, size, and sequence) of binding sites, which might reflect relevant differences in the regulation of these genes by GadX. Preferential targeting of GadX to *gadA* was inferred from gel mobility shift and DNase I footprinting assays and by the observation that the multicopy *gadA* can titrate out the intracellular pool of the chromosomally encoded GadX activator. Careful analysis of the data shown in Fig. 1B indicates that introduction of multicopy *gadA* (pBsA) in *E. coli* results in exuberant glutamate decarboxylase activity (12.4 U/mg) but low GABA export (1.2 mM). This can be interpreted as the GadX-dependent expression of chromosomal *gadBC* being limited due to withholding of the available GadX activator by the multicopy *gadA* promoter sequence. Conversely, when *gadB* is expressed as a multicopy gene (pBsB), the glutamate decarboxylase activity is noticeably lower (4.7 U/mg), consistent with the lower affinity of GadX for the *gadBC* promoter, but the amount of exported GABA is increased (2.2 mM). This would mean that the intracellular GadX level is sufficient to activate transcription of the chromosomal copies of the *gad* structural genes, including *gadC*, resulting in increased expression of the GadC antiporter. The results obtained with the multicopy *gadAX* contruct (pBsAX) further corroborate this conclusion; in fact, they demonstrate that the titrating effect of the multicopy *gadA* promoter is relieved by virtue of GadX overexpression, and this results in high GadC activity (as inferred from GABA export), similar to what was observed with the multicopy *gadX* (pBsX) and *gadBC* (pBsBC) constructs.

Identification of the effectors involved in GadX activation and further investigation of the interactions between regulatory components of the *gad* system (H-NS, RpoS, CRP, and GadX) may provide further insight into the molecular circuitry underlying the glutamic acid-based acid stress response of *E. coli.*

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