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Collaborative Regulation of *Escherichia coli* Glutamate-Dependent Acid Resistance by Two AraC-Like Regulators, GadX and GadW (YhiW)

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An important feature of *Escherichia coli* pathogenesis is an ability to withstand extremely acidic environments of pH 2 or lower. This acid resistance property contributes to the low infectious dose of pathogenic *E. coli* species. One very efficient *E. coli* acid resistance system encompasses two isoforms of glutamate decarboxylase (*gadA* and *gadB*) and a putative glutamate:γ-amino butyric acid (GABA) antiporter (*gadC*). The system is subject to complex controls that vary with growth media, growth phase, and growth pH. Previous work has revealed that the system is controlled by two sigma factors, two negative regulators (cyclic AMP receptor protein [CRP] and H-NS), and an AraC-like regulator called GadX. Earlier evidence suggested that the GadX protein acts both as a positive and negative regulator of the *gadA* and *gadBC* genes depending on environmental conditions. New data clarify this finding, revealing a collaborative regulation between GadX and another AraC-like regulator called GadW (previously YhiW). GadX and GadW are DNA binding proteins that form homodimers in vivo and are 42% homologous to each other. GadX activates expression of *gadA* and *gadBC* at any pH, while GadW inhibits GadX-dependent activation. Regulation of *gadA* and *gadBC* by either regulator requires an upstream, 20-bp GAD box sequence. Northern blot analysis further indicates that GadW represses expression of *gadX*. The results suggest a control circuit whereby GadW interacts with both the *gadA* and *gadX* promoters. GadW clearly represses *gadX* and, in situations where GadX is missing, activates *gadA* and *gadBC*. GadX, however, activates only *gadA* and *gadBC* expression. CRP also represses *gadX* expression. It does this primarily by repressing production of sigma S, the sigma factor responsible for *gadX* expression. In fact, the acid induction of *gadA* and *gadBC* observed when rich-medium cultures enter stationary phase corresponds to the acid induction of sigma S production. These complex control circuits impose tight rein over expression of the *gadA* and *gadBC* system yet provide flexibility for inducing acid resistance under many conditions that presage acid stress.

The acidic pH of the human stomach is a daunting environment for any pathogen. After a meal is ingested, the gastric pH can fall to 2 or less, an acidification sufficient to kill most enteric pathogens. However, commensal and pathogenic strains of *Escherichia coli* have evolved three systems of acid resistance that will, upon induction, protect the organism from pH 2 environments for hours (2, 11, 12). This period is long enough for the organism to breach the gastric acid barrier and gain entrance to the less acidic intestinal environment. The most potent of the three acid resistance systems requires extracellular glutamate to function and is induced either in moderately acid environments (pH 5 to 6) during exponential growth or upon entry into stationary phase at any pH. Extracellular glutamate is not required, however, for induction. The glutamate-dependent acid resistance system includes two isoforms of glutamate decarboxylase encoded by *gadA* and *gadB* that convert intracellular glutamate to γ-amino butyric acid (GABA) and consume a proton in the process. In addition, a dedicated antiporter, GadC, is believed necessary to export the GABA end product out of the cell while simultaneously importing more glutamate as the substrate, although this has not yet been demonstrated. The genes *gadB* and *gadC* form the *gadBC* operon, while *gadA* maps to a different location. How this system actually enables acid resistance is not known, but one proposal is that it affects intracellular pH.

Regulation of the system is extremely complex. Earlier work from this and other laboratories has shown that *gadA* and *gadBC* expression is regulated by separate sigma S (σ^S)-dependent and -independent pathways even though only a single transcriptional start site has been identified for either operon (1, 2, 4). The alternative sigma factor σ^S, encoded by *rpoS*, is used to express a regulon of stress-regulated genes classically associated with entry into stationary phase (6, 7). The σ^S-dependent pathway for *gad* expression is evident in complex-medium-grown cells, whereas σ^S-independent regulation occurs in minimal-glucose-grown cells. The cyclic AMP (cAMP) receptor protein (CRP) and the nucleoid protein H-NS have been implicated as potent repressors of *gad* expression in complex Luria-Bertani (LB) media (1, 4). In addition, a 20-bp conserved DNA sequence, called the GAD box, is located upstream from the *gadBC* and *gadA* promoters and is required for induction (1). Outside this one area, almost no homology is apparent between the two promoters.

The *gadX* (*yhiX*) gene, located downstream of *gadA* and

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TABLE 1. Bacterial strains and plasmids in this study

Strain or plasmid	Genotype	Source or reference
Strains		
EK227	K-12, wild type, λ^- F^-	A. C. Matin
EK344	(GE1050) Δ*crp*::Cm	16
EK395	Δ*cya-1400*::Km λ^- e14$^-$ *relA1 spoT1 thi-1*	J. Kaper
EK432	Δ(*lacA-lacZ*)*515*(::Cat)	3
EK426	K-12 (MG1655) Δ*gadX*::Km	T. Conway
EK437	*hns-206 oppC506*::Tn*10*	C. Higgens
EK441	K-12 (MG1655) Δ*gadW*::Km	T. Conway
EK442	K-12 (MG1655) Δ*gadXW*::Km	T. Conway
EK540	TOPO 10 F^- *mcrA* Δ(*mrr-hsdRMS-merBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 recA1 deoR araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nupG*	Invitrogen
EK541	BL21 Star(DE3): F^- *ompT hsd*$_B$ (r_B^- m_B^-) *dcm rne-131*	Invitrogen
EF362	K-12 (EK227) *rpoS*::Tn*10*	2
EF528	K-12 (EK227) Δ*crp*::Cm	EK227 × EK344
EF615	EK298 *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−165 to +788)	2
EF646	Δ*cya-1400*::Km/pT18-Zip × pT25-Zip	14
EF663	EK298 *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−51 to +788)	2
EF666	EK298 *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−86 to +788)	2
EF678	K-12 (EK227) Δ*crp*::Cm *rpoS*::Tn*10*	EF528 × EF362
EF757	K-12 (EK227) Δ*gadX*::Km	EK227 × EF426
EF825	K-12 (EK227) Δ(*lacA-lacZ*)::Cat	EK227 × EK432
EF828	K-12 (EK227) Δ*gadX*	EF757 × pCP20
EF830	K-12 (EK227) Δ*gadX* Δ(*lacA-lacZ*)::Cat	EF828 × EK432
EF833	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−165 to +788) Δ(*lacA-lacZ*)::Cat	EF825 × EF615
EF839	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−165 to +788) Δ*gadX* Δ(*lacA-lacZ*)::Cat	EF830 × EF615
EF861	K-12 (EK227) Δ*gadW*::Km	EK227 × EK441
EF863	K-12 (EK227) Δ*gadXW*::Km	EK227 × EK442
EF864	K-12 (EK227) Δ*gadW*::Km Δ*crp*::Cm	EF528 × EK441
EF865	K-12 (EK227) Δ*gadXW*::Km Δ*crp*::Cm	EF528 × EK442
EF906	K-12 (EK227) Δ*gadX* Δ*crp*::Cm	EF828 × EF528
EF907	K-12 (EK227) Δ*gadW*	EF861 × pCP20
EF908	K-12 (EK227) Δ*gadXW*	EF863 × pCP20
EF911	K-12 (EK227) Δ*gadW* Δ(*lacA-lacZ*)::Cat	EF907 × EK432
EF912	K-12 (EK227) Δ*gadXW* Δ(*lacA-lacZ*)::Cat	EF908 × EK432
EF919	Δ*cya-1400*::Km/pT18 × pT25	EK395 × pT18 × pT25
EF920	Δ*cya-1400*::Km/pMF497/pMF498	This study
EF921	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−165 to +788) Δ*gadW* Δ(*lacA-lacZ*)::Cat	EF911 × EF615
EF922	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−51 to +788) Δ*gadW* Δ(*lacA-lacZ*)::Cat	EF911 × EF663
EF923	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−86 to +788) Δ*gadW* Δ(*lacA-lacZ*)::Cat	EF911 × EF666
EF928	K-12 (EK227) Δ*cya*::Km	EK227 × EK395
EF929	K-12 (EK227) Δ*gadXW*::Km *hns*::Tn*10* Δ*crp*::Cm	EF865 × EK437
EF931	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−51 to +788) Δ(*lacA-lacZ*)::Cat	EF825 × EF663
EF932	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−86 to +788) Δ(*lacA-lacZ*)::Cat	EF825 × EF666
EF933	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−165 to +788) Δ*gadXW* Δ(*lacA-lacZ*)::Cat	EF912 × EF615
EF934	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−51 to +788) Δ*gadXW* Δ(*lacA-lacZ*)::Cat	EF912 × EF663
EF935	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−86 to +788) Δ*gadXW* Δ(*lacA-lacZ*)::Cat	EF912 × EF666
EF948	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−51 to +788) Δ*gadX* Δ(*lacA-lacZ*)::Cat	EF830 × EF663
EF949	K-12 (EK227) *trpDC*::*putPA1303*-Km-*gadA*::*lacZ*(o) (−86 to +788) Δ*gadX* Δ(*lacA-lacZ*)::Cat	EF830 × EF663
EF957	K-12 (EK227) *hns-206 oppC506*::Tn*10*	EK227 × EK437
Plasmids		
pT18	pBluescript II KS containing T18 C terminus of *B. pertussis cyaA*	9
pT25	pACYC184 containing T25 N terminus of *B. pertussis cyaA*	9
pT18-Zip	pT18 containing leucine zipper domain	9
pT25-Zip	pT25 containing leucine zipper domain	9
pCP20	Flp recombinase	3
pMF497	*gadW-'cyaA* in pT18	This study
pMF498	*cyaA'-gadX* in pT25	This study
pMF499	pET102/D-TOPO containing Trx-GadW-His$_6$	
pMF506	*cyaA'-gadW* in pT25	This study
pMF507	*yhiX-'cyaA* in pT18	This study
pET102/D-TOPO	His-Patch thioredoxin fusion vector	Invitrogen

cotranscribed with it, encodes an AraC- and XylS-like regulator implicated in the control of the *gadAX* and *gadBC* loci (8, 18, 19). Tramonti et al. (20) recently showed that *gadX* was an RpoS-dependent gene and is itself acid induced. GadX was originally ascribed to have both positive and negative regulatory function, depending on the environmental conditions tested (18). However, another gene encoding an AraC-like regulatory protein with possible influence over *gadA* and *gadBC* expression was recently discovered as a result of gene array analysis (D. Tucker and T. Conway, submitted for pub-

lication). The gene, *gadW* (*yhiW*), is located downstream of *gadX* (D. Tucker and T. Conway, unpublished data). GadW is 60 and 30% homologous to GadX in the DNA binding and dimerization domains, respectively. Previous work describing GadX as both a positive and negative regulator did not consider the potential influence of the *gadW* product (18), and the *gadW* array studies did not directly examine effects on protein levels or explore a potential collaboration with GadX. So with the discovery of GadW, it became necessary to reexamine the role of GadX as well as the influence of GadW over *gadA* and *gadBC* expression.

We have taken a direct genetic approach to better define the roles of GadX and GadW in controlling this system. The results indicate that GadW mediates control of the *gadA* and *gadBC* genes by repressing *gadX* and, under some situations, by activating *gadA* and *gadBC*. CRP represses the system largely by inhibiting the production of RpoS, the sigma factor primarily responsible for *gadX* expression. Finally, in contrast to reports using a different background, repression by H-NS was independent of *gadX.* The overlapping control of *gadX* by CRP, RpoS, and GadW makes *E. coli* highly attuned to situations that can lead to lethal acid stress.

TABLE 2. Oligonucleotide primers used in this study

Name	Sequence
Oligo-201	5′-CAGCAATGTTTGGGCGATTTTTATTAC-3′
Oligo-202	5′-GATAATTCAGGAGACACAGAATGCG-3′
Oligo-233	5′-CTCGTCGACACGTGAATCGAGTAGTTC-3′
Oligo-340	5′-CTATGCAATCACTACATGGGAATT-3′
Oligo-341	5′-CTCTATAATCTTATTCCTTCCGCAG-3′
Oligo-377	5′-GGAGTTCGAAATGGACCAGAAG-3′
Oligo-378	5′-AGTTTCGGGTGATCGCTGAG-3′
Oligo-379	5′-GGTGTCGACTATGAACCTGCTTCCCATCGACTAC-3′
Oligo-406	5′-TGCGCCAAAACGTGAATCGAGTAGTTC-3′
Oligo-407	5′-TCGCACCAAAACGTGAATCGAGTAGTT-3′
Oligo-414	5′-ATACATATGACTCATGTCTGCTCGGTGAT-3′
Oligo-415	5′-TAAGAATCCATTCAAATGCGAAATATGTCA-3′
Oligo-444	5′-AAGCTGCAGGGATGCAATCACTACATGGGAATT-3′
Oligo-445	5′-TCAGGATCCCTATAATCTTATTCCTTCCGCAGA-3′
Oligo-446	5′-AAGGGGCCCCATGCAATCACTACAT-3′
Oligo-447	5′-TCAAAGCTTATTAATCTTATTCCTTC-3′
Oligo-448	5′-AAGCTGCAGGGATGACTCATGTCTG-3′
Oligo-449	5′-ATGGGATCCTCAGGAAAAGGTACCT-3′
Oligo-450	5′-AAGGGGCCCCATGACTCATGTCTGCT-3′
Oligo-451	5′-ATGAAGCTTATGGAAAAGGTACCT-3′
Oligo-458	5′-AATAAGATTATAGAGTTTTACT-3′
Oligo-459	5′-CATGAGTCATGATTATCCCTTA-3′
Oligo-465	5′-CACCATGACTCATGTCTGCTCG-3′
Oligo-466	5′-GGAAAAGGTACCTGGCGAATG-3′
Oligo-507	5′-CAATACGCAAACCGCCTCTCC-3′
Oligo-508	5′-AGTGAATCCGTAATCATGGTCATA-3′

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. The media used included minimal E medium containing 0.4% glucose (EG) (21) and complex LB medium buffered with either 100 mM morpholinepropanesulfonic acid (MOPS [pH 8]) or 100 mM morpholineethanesulfonic acid (MES [pH 5.5]). Antibiotics were used at the following concentrations: ampicillin, 60 μg/ml; kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; and chloramphenicol, 30 μg/ml. All strains were grown at 37°C with aeration.

Genetic and molecular procedures. Phage P1 transduction, transformation with $CaCl_2$, and electroporation were performed by standard methods (13). General DNA manipulations were carried out as described earlier (17). Deletion of the kanamycin resistance cassette in the *gadX*, *gadW*, and *gadXW* mutants was carried out by the method reported by Datsenko and Wanner (3). The list of oligonucleotide primers used is given in Table 2.

Western blot analysis. Strains were grown at 37°C in media containing the required antibiotics as indicated. At an optical density of 600 nm (OD_{600}) of 0.4 (log phase) or 3.8 (late stationary phase), cells were collected by centrifugation and then were resuspended in 0.01% sodium dodecyl sulfate (SDS) solution. Protein concentrations were determined using Bio-Rad Protein Assay reagent. Samples (5 μg of protein) were mixed with 2× SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer (125 mM Tris [pH 7.0], 20% glycerol, 10% β-mercaptoethanol, 6% SDS, and 0.2% bromophenol blue), boiled for 5 min, and were then separated by PAGE on 10% Tris-HCl Criterion gels (Bio-Rad). After semidry electrophoretic transfer of proteins onto polyvinylidene difluoride membranes (Millipore Co., Bedford, Mass.), the membranes were incubated overnight with 5% powdered milk in Tris-buffered saline–Tween buffer (150 mM NaCl, 10 mM Tris-HCl [pH 8.0], and 0.05% [vol/vol] Tween 20) to block nonspecific protein interactions. The membranes were probed with one of the following primary antibodies: rat anti-GAD (2), mouse anti-RpoS, and rabbit anti-GadX (kindly provided by S. Shin and J. Kaper), followed by the appropriate monoclonal secondary antibodies coupled to peroxidase (Sigma): anti-rat (1:10,000), anti-mouse (1:3,000), and anti-rabbit (1:4,000). Antibody-tagged protein bands on the probed membranes were detected using an enhanced chemiluminescence Western blot detection kit (Amersham) (2).

Northern blot and primer extension analyses. Total RNA was extracted from log-phase cultures (OD_{600} = 0.4; 2×10^8 cells per ml) grown under both alkaline and acidic conditions in LB medium using the RNeasy kit (Qiagen). RNA concentrations were determined by measuring the optical densities at 260 and 280 nm. Aliquots of RNA (5 μg) were denatured at 65°C for 15 min and were separated by electrophoresis through a denaturing formaldehyde-agarose (1.2%) gel as described previously (17). The RNA was then transferred onto a positively charged nylon membrane (Amersham-Pharmacia) and was baked at 80°C for 2 h. The membranes were probed with a 1.4-kb *gadA* and *gadB* probe or a 0.827-kb *gadX* probe generated by PCR using oligo-377 and -378 or oligo-340 and -341, respectively. Probes were labeled with [α-^{32}P]dCTP (Amersham) by using a random-primed DNA-labeling kit (Boehringer Mannheim). The *gadA* and *gadB* probe corresponds to the entire open reading frame of *gadA* or *gadB* and hybridizes to both *gadA* and *gadB*. As a control, the membranes were also hybridized with a 23S rRNA probe (oligo-379) that was end labeled with [γ-^{32}P]ATP.

For primer extension analysis of the *gad* transcriptional start sites, 1 pmol each of the primers for *gadA* (oligo-406) and *gadB* (oligo-407) was 5′ end labeled with T4 polynucleotide kinase (Promega) and [γ-^{32}P]ATP. Reverse transcription of *gadA* or *gadBC* mRNA was performed using the Qiagen Omniscript reverse transcriptase (RT) kit. Sequencing reactions were carried out using the GIBCO BRL double-stranded DNA cycle-sequencing system and were run in parallel with the cDNA transcripts to map the 5′ ends of *gadA* or *gadBC* mRNA. The promoter and beginning regions of *gadA* and *gadB* genes were used as the templates in the sequencing reactions.

RT-PCR. The RNA used for RT-PCR was extracted using TRIzol Reagent (GIBCO BRL) per the manufacturer's recommendation from cells grown to log phase (OD_{600} = 0.4) in LB medium at pH 8 and 5.5 and in EG medium at pH 7.7 and 5.5 during log phase. RT-PCR was performed with the Superscript One-Step RT-PCR kit (GIBCO BRL). cDNA for *gadX* was prepared using oligo-340 and oligo-341. cDNA for *gadW* was prepared using oligo-414 and oligo-415. To detect potential *gadX gadW* cotranscripts, the pairs oligo-340 (forward primer) and oligo-415 (reverse primer) and oligo-458 and oligo-459 were used to amplify the entire *gadX gadW* region and the shorter intergenic region between *gadX* and *gadW*, respectively. Reaction conditions were designed as follows: cDNA synthesis and predenaturation, 1 cycle of 55°C for 30 min and 94°C for 2 min. PCR amplification involved 40 cycles of denaturation (94°C for 15 s), annealing (55°C for 30 s), extension (72°C for 1 min), and one final extension cycle of 72°C for 10 min. Control PCRs were performed using DNA to confirm primer efficacy. RNA purity was confirmed by performing the reaction in the absence of RT.

Purification of Trx-GadW-His$_6$. The K-12 *gadW*-coding region was amplified with oligo-465 and oligo-466 by using *Pwo* proofreading polymerase (Invitrogen), and the resulting fragment was cloned into the pET102/D-TOPO vector containing the His-Patch thioredoxin leader (pET Directional Expression Kits; Invitrogen). To enable directional cloning, the forward primer, oligo-465, was designed to contain 5′ CACC at the 5′ end, which pairs with the overhang sequence GTGG in the vector. The reverse primer, oligo-466, starts with the codon preceding the *gadW* stop codon (TGA) so that the cloned PCR product will produce an in-frame fusion with the C-terminal His tag. The resulting plasmid, pMF499, was transformed into TOPO 10 cells and, from there, into

BL21 Star(DE3) for expression. The BL21 strain containing pMF499 was grown in 1 liter of LB medium containing 100 μg of ampicillin/ml at 30°C with shaking. When the OD_{600} of the culture reached 0.55, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was incubated for an additional 3 h at 30°C. The cells were harvested by centrifugation and were resuspended overnight in 60 ml of lysis buffer (6 M guanidine, 0.1 M NaH_2PO_4, 0.01 mM Tris-HCl, and 5 mM imidazole) with shaking at 4°C. Insoluble cell debris was removed by centrifugation (8,000 × *g*, 4°C for 15 min), and the supernatant was passed through a 0.45-μm-pore-size filter. The filter-sterilized solution was subsequently incubated with 5 ml of ProBond resin (Invitrogen) at room temperature for 1 h, after which the resuspension was transferred to a Poly-Prep Chromatography column (Bio-Rad). The column was washed with 50 ml of washing buffer (8 M urea, 6 M guanidine, 0.1 M NaH_2PO_4, 0.01 mM Tris-HCl, and 5 mM imidazole), and the protein was eluted with the column buffer (8 M urea, 0.1 M NaH_2PO_4, 0.01 mM Tris-HCl, and 250 mM imidazole [pH 4.9]). Three-milliliter samples of the three highest-protein-content fractions were combined and desalted through a PD-10 Sephadex column (Amersham Pharmacia). The protein was eluted with desalting buffer (10 mM NaH_2PO_4 [pH 6.8], 0.1 mM EDTA, and 0.2 M NaCl), and glycerol was added to a final concentration of 50% for storage. This procedure purified Trx-GadW-His_6 to homogeneity based on Coomassie-stained SDS-PAGE (data not shown). Western blot analysis also failed to detect any GadX in these preparations.

EMSA. The ability of His-tagged GadW protein to bind the *gadA* and *gadB* promoters was tested using the electrophoretic mobility shift assay (EMSA). The promoter target fragments were amplified with oligo-201 and oligo-233 (*gadA*) and oligo-202 and oligo-233 (*gadB*). The PCR-generated *gadA* promoter fragment extends from bp −164 to +78, while the *gadB* promoter extends from bp −203 to +78 relative to the transcriptional start sites. Both fragments include the 20-bp GAD box region implicated in *gad* expression. Both fragments were end labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. A PCR fragment of *lacZ* promoter (oligo-507 and oligo-508) was used as a probe for nonspecific DNA binding. Radiolabeled DNA probes (5,000 cpm) were incubated with GadW fusion protein at room temperature for 30 min in 30 μl of binding buffer (20 mM HEPES [pH 8.0], 5 mM $MgCl_2$, 50 mM potassium glutamate, 0.01 mM EDTA, 1 mM NaH_2PO_4, 20 mM NaCl, 1 mM dithiothreitol, 30 μg of bovine serum albumin/ml, and 30 μg of salmon sperm DNA/ml). Samples were loaded onto 5% Tris-borate-EDTA nondenaturing ready gel (Bio-Rad) and were electrophoresed at room temperature in 0.5% Tris-borate-EDTA buffer with 0.2% glycerol. The gels were dried and exposed to X-Omat Kodak film at −70°C for 3 h.

Bacterial two-hybrid system. The two-hybrid system used to demonstrate in vivo interaction between GadX and GadW is based upon the reconstitution of adenylate cyclase activity of *Bordetella pertussis* CyaA protein (9). Plasmid pT25 is a derivative of pACYC184 that encodes the N-terminal T25 fragment of CyaA (amino acids 1 to 224; CyaA′) with a multicloning site at the C terminus. Expression is controlled by the *lacUV5* promoter. Plasmid pT18 is a derivative of pBluescript II KS (Stratagene), compatible with pT25, that encodes the C-terminal T18 fragment of CyaA (amino acids 225 to 339; ′CyaA) with the multicloning site of pBluescript II KS located at the N terminus. Functional reconstitution of adenylate cyclase activity is achieved if each Cya fragment is fused in frame to one of two interacting proteins. Adenylate cyclase activity was monitored in an *E. coli* strain deficient in endogenous adenylate cyclase (*cya*). The *gadW* gene was PCR amplified (*Pwo* polymerase) from EK227 using oligo-450 and oligo-451, which contain *Apa*I and *Hin*dIII sites, respectively. The *Apa*I and *Hin*dIII sites were used to directionally clone the insert into pT18. This created plasmid pMF497, in which the N terminus of the *gadW* open reading frame was fused in frame with the ′Cya fragment of pT18. The *gadW* stop codon was omitted from oligo-451 to allow read-through into the T18 fragment of Cya to generate a GadW-′Cya hybrid protein. A CyaA′-GadW hybrid was then constructed by amplifying *gadW* with oligo-448 and oligo-449 engineered with *Pst*I and *Bam*HI sites, respectively. Insertion of the PCR fragment into pT25 cut with *Pst*I and *Bam*HI created an in-frame CyaA′-GadW fusion (pMF506).

The *gadX* open reading frame was cloned in frame into the pT25 vector as follows: the *gadX* gene was PCR amplified from *E. coli* K-12 (EK227) with the proofreading *Pwo* polymerase by using oligo-444, which has a *Pst*I site, and oligo-445, which was engineered to contain a *Bam*HI site. The oligonucleotides were designed so that the insert would be in frame with CyaA′ and contain a stop codon after *gadX*. The PCR product was gel purified (Geneclean II kit; Bio 101), digested with *Pst*I and *Bam*HI, and ligated with T4 ligase into similarly digested pT25, resulting in plasmid pMF498. A GadX-′CyaA hybrid was then constructed by amplifying *gadX* with oligo-446 and oligo-447 engineered with *Apa*I and *Hin*dIII sites, respectively. Insertion of the PCR fragment into pT18 cut with *Apa*I and *Hin*dIII created an in-frame GadX-′CyaA fusion (pMF507). Potentially complementing pairs of these plasmids were transformed into the *cya*-deficient strain EK395, and the expression of chromosomal *lacZ* was monitored as an indicator of cAMP production resulting from potential interactions between pairs of Cya fusion protein. All cultures were grown in LB broth in the presence of chloramphenicol, ampicillin, or both as needed, and 0.5 mM IPTG at 30°C with aeration (250 rpm). β-Galactosidase activity was measured as described by Miller (13).

RESULTS

The *gadX* and *gadW* genes constitute separate transcriptional units. Since the *gadW* gene is located downstream of *gadA* and *gadX*, it was necessary to determine if independent promoters drive expression of each gene before analyzing the individual roles of GadX and GadW in controlling *gadA* and *gadBC* expression. If *gadX* and *gadW* are transcribed separately, then individual mutations in these genes could be examined without fear of polar effects. RT-PCR was chosen to identify whether these genes form an operon. As shown in Fig. 1, primers designed to detect the individual messages amplified both *gadX* and *gadW* (lanes 7 and 8). However, primers designed to amplify a potential intercistronic message failed (Fig. 1, lane 9), indicating that *gadX* and *gadW* do not form an operon. Thus, *gadX* and *gadW* mutants can be examined separately for their effects on *gadA* and *gadBC* expression.

GadW inhibits activation of *gadA* and *gadBC* by GadX. Western blot results presented in Fig. 2 illustrate that *gadA* and *gadBC* are normally repressed in cells growing exponentially in complex LB media (Fig. 2A, lanes 1 and 2). However, when GadW was removed through mutation, GadX activated *gadA* and *gadBC* expression under acid or alkaline conditions (Fig. 2A, lanes 5 and 6 versus lanes 7 and 8). Thus, GadW is a negative regulatory protein that inhibits the activation of *gadA* and *gadBC* by GadX. Figure 3 presents evidence reinforcing this conclusion from Northern blot analyses that examined both the *gadA* and *gadBC* transcripts. The results indicate that GadX is not both a positive and negative regulator as previously reported (18). Strain EF861, possessing GadX but lacking GadW, highly expressed the *gadA* and *gadBC* messages under both acid and alkaline conditions. Thus, GadX serves as an activator of *gadA* and *gadBC*, while GadW can inhibit activation.

Interestingly, Fig. 2A and B (lanes 3 and 4 versus lanes 7 and 8) also indicate that the opposite scenario is true: that GadX can prevent GadW induction of *gadA* and *gadBC*. It seems that, while either GadX or GadW can induce *gadA* and *gadBC*, when both AraC-like regulators are present, they inhibit each other's function.

GadW represses expression of *gadX*. The *gadX* gene is expressed from two promoters, P*gadA* and P*gadX* (20). Since GadW inhibits GadX-dependent expression of *gadA* and *gadBC* and since a putative GAD box exists upstream of the *gadX* promoter, we hypothesized that GadW might repress *gadX* expression. Figure 4 presents Northern blot data that confirmed this idea. In mid-log-phase cells, *gadX* is not expressed well at either pH (EK227) but is expressed three- to fourfold higher in a *gadW* mutant (EF861). This increased expression appears to come from the *gadX* promoter, not the *gadA* promoter, since the level of *gadAX* transcript did not increase. It is significant that the same *gadW* mutant also overexpressed the *gadA* transcript under the same conditions (Fig. 3), suggesting that either the *gadAX* cotranscript is processed

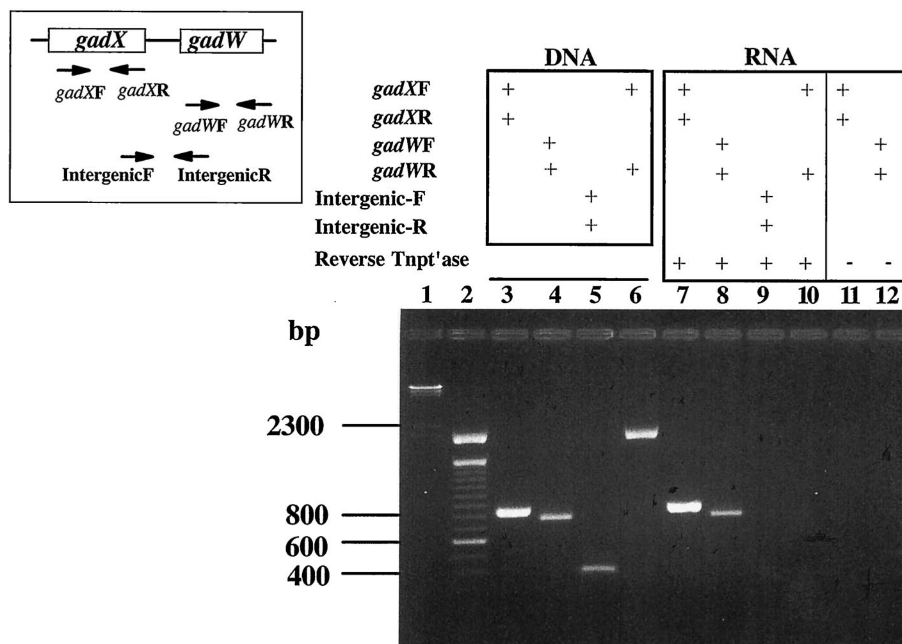

FIG. 1. RT-PCR analysis of *gadX* and *gadW* transcripts. Inset, left, represents the *gadX* and *-W* genes and the location of primers used for RT-PCR. *gadX* forward, oligo-340; *gadX* reverse, oligo-341; *gadW* forward, oligo-414; *gadW* reverse, oligo-415; intergenic forward, oligo-458; and intergenic reverse, oligo-459. PCRs with and without RT (Reverse Tntp'ase) were run, and products were separated on 2% agarose gels. Lane 1, *Hin*dIII-cut lambda DNA; lane 2, 100-bp ladder; conditions for lanes 3 to 12 are shown above the gel. RNA used was from log-phase (pH 5.5), LB-grown EK227 cells. Similar results were obtained using RNA from cells grown at pH 8 and minimal-glucose-grown cells (data not shown).

to separate the two RNA molecules or that there is a terminator between *gadA* and *gadX* that prevents most transcripts originating at P*gadA* from extending into *gadX*.

Purified GadW binds the *gadA* and *gadBC* promoters. The *gadX* and *gadW* products are both implicated as regulators of *gadA* and *gadBC* expression (8, 18–20) (Tucker and Conway, unpublished). EMSAs previously demonstrated that a purified MalE-GadX hybrid protein directly binds a 447-bp promoter fragment of *gadA* encompassing the 20-bp GAD box (18). We have now purified a thioredoxin-His-tagged GadW fusion protein suitable for conducting similar DNA binding assays (see Materials and Methods). The results presented in Fig. 5 illustrate that GadW binds to both the *gadA* (242 bp) and *gadBC* (281 bp) promoters but not to the unrelated *lacZ* promoter. The purified GadW protein was not contaminated with GadX, based on Western blot analysis (data not shown). The results suggest that GadW can act directly on the *gadA* and *gadBC*

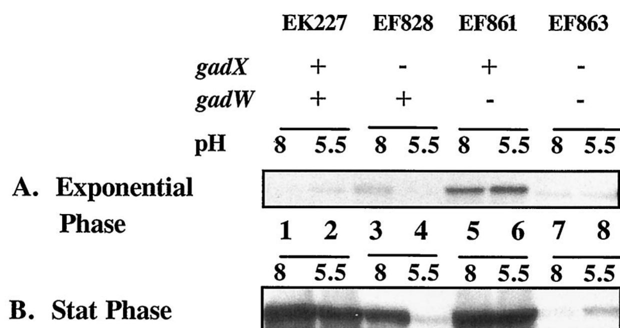

FIG. 2. Effects of GadX and GadW on pH-regulated *gadA* and *gadBC* expression in exponential- and stationary-phase cells grown in LB. Western blot analysis of extracts prepared from cultures grown to mid-log (OD_{600} = 0.4) (A) or stationary (Stat) (OD_{600} = 3.8) (B) phase in LB medium buffered to the pH values indicated. Strains and genotypes are indicated in the figure. Whole-cell proteins were extracted by boiling in SDS, and 5 μg of protein was loaded per sample on SDS–10% PAGE gels. Blots were probed with polyclonal anti-GadA and -B antibody.

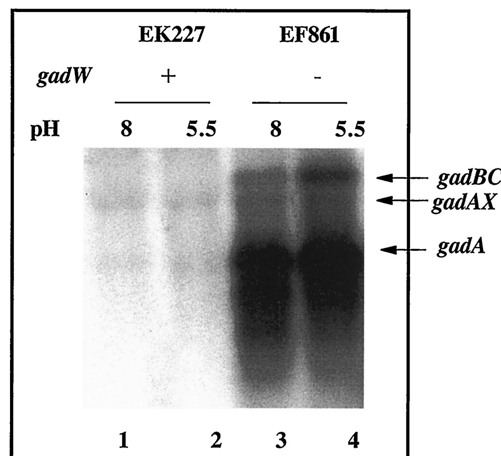

FIG. 3. Northern blot analysis of *gadW* effects on *gadA* and *gadBC* message. Cells were grown to log phase in buffered LB medium. RNA was extracted, and 5 μg was electrophoresed in formaldahyde-agarose gels and probed with a 1.4-kb *gadA* and *-B* probe that can detect *gadAX*, *gadA*, and *gadBC* transcripts. Strains and relevant genotypes are indicated in the figure.

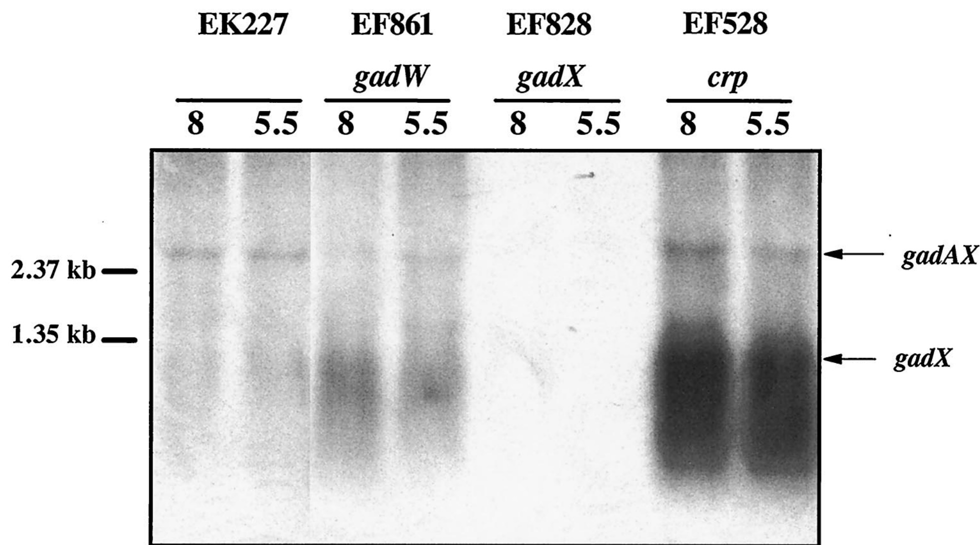

FIG. 4. Northern blot analysis of GadW effects on *gadX* message. Cells were grown to log phase in buffered LB medium. RNA was extracted; 5 μg was electrophoresed in formaldahyde-agarose gels and probed with an 827-bp radiolabled *gadX* probe that can detect *gadAX* and *gadX* transcripts. Strains and relevant genotypes are indicated in the figure. pH is given immediately over the blot.

promoter regions in vivo. The slightly larger band seen in lane 1 of the second panel in Fig. 5 is an artifact of the PCR. It was not present on other GadW EMSA gels. The multiple shifted bands suggest that GadW may bind as a multimer to these promoters.

The conserved 20-bp *cis*-acting regulatory region is required for GadX and GadW control. A 20-bp conserved sequence (TTAGGATTTTGTTATTTAAA) located between −53 and −73 bp from the *gadA* and *gadBC* transcriptional start sites was previously identified as being required for *gadA* and *gadBC* expression (1). To determine if this 20-bp *cis*-acting GAD box region was required for GadX or -W control of *gadA* and *gadBC* expression, *gadX* and *gadW* mutations were tested for effects on the expression of a series of *gadA-lacZ* fusions in which different portions of the *gadA* promoter were deleted. Strains carrying the entire region from −164 to +788 or a truncation that retains the region from −85 to +788, both of which contain the *cis*-acting GAD box sequence, were sub-

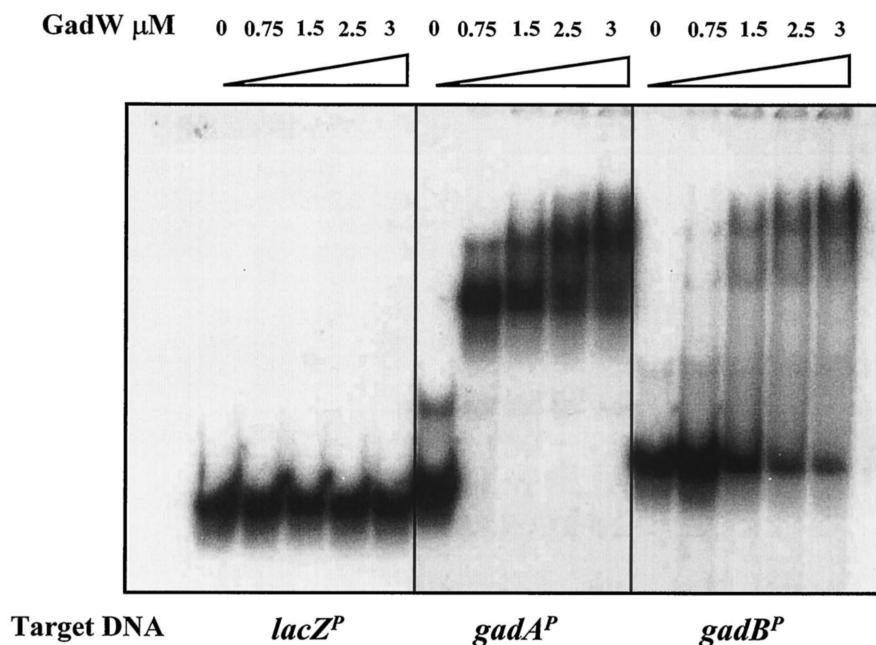

FIG. 5. GadW binds to the *gadA* and *gadB* promoters. EMSA conditions were described in Materials and Methods. Radiolabeled promoter fragments (5,000 cpm) were incubated with purified Trx-GadW-His_6 protein for 30 min at 25°C and were electrophoresed through a 5% nondenaturing polyacrylamide gel.

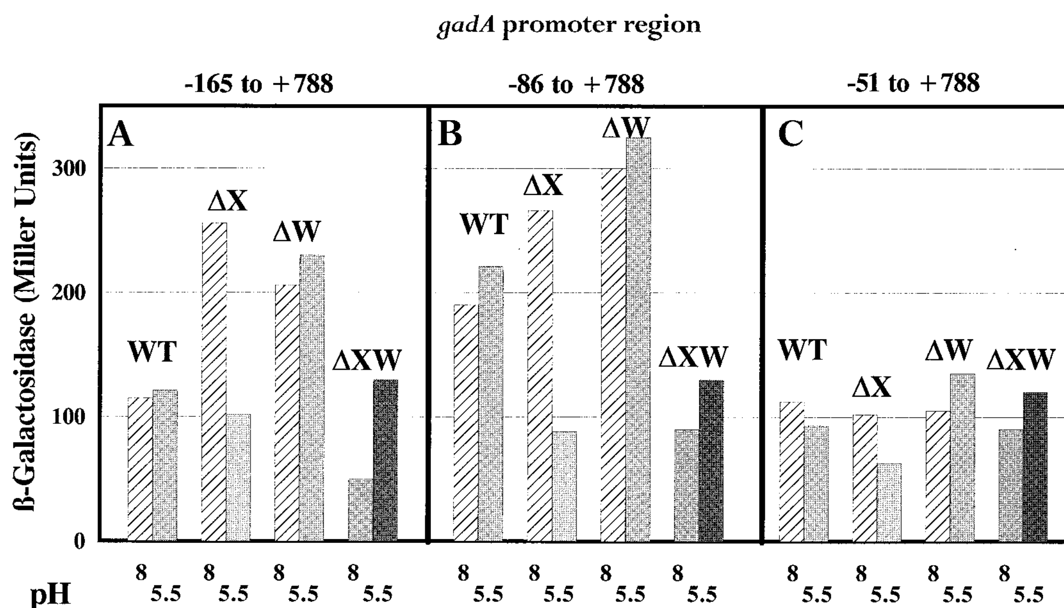

FIG. 6. Effects of promoter deletions on GadX and GadW regulation of *gadA*. Cells were grown to exponential phase in LB medium buffered to pH 8 with 100 mM MOPS or to pH 5.5 with 100 mM MES and were examined for β-galactosidase activity (Miller units). (A) *gadA-lacZ* fusions; (B) *gadA-lacZ* fusions; and (C) *gadA-lacZ* fusions. The length of the *gadA* promoter region (in nucleotides) is given along top of panels. Wild type (WT), EF833, EF932, and EF931; ΔX, EF839, EF949, and EF948; ΔXW, EF933, EF935, and EF934; and ΔW, EF921, EF923, and EF922.

jected to normal control by GadX and GadW (Fig. 6A and B). In contrast to the fusions containing the GAD box, fusions starting at −51 and thus lacking the 20-bp sequence were devoid of X or W control (Fig. 6C).

Another unusual aspect of GadW is seen in Fig. 6A and B. Whereas removing GadW allowed GadX to activate *gadA-lacZ* expression at both growth pH values, removing GadX allowed W to activate expression only at pH 8. This is consistent with what was observed in the Western blots, both in log-phase cultures (Fig. 2A, lanes 3 and 4) and more dramatically in stationary-phase cultures (Fig. 2B, lanes 3 and 4). The data are consistent with a model in which GadW binds the *gadA* and *-B* promoters best at pH 8. This binding could inhibit GadX activity. However, when GadX is missing, GadW could inadvertently activate *gadA* and *gadBC* expression.

GadX and GadW form homodimers in vivo. An important characteristic of many AraC-like regulators is that they form homodimers via an N-terminal dimerization domain (5, 15). Since GadX and -W are predicted to be AraC-like regulators, a two-hybrid approach was taken to demonstrate whether or not either of them dimerizes in vivo. The genes encoding GadX and GadW were fused to the *B. pertussis* CyaA T18 and T25 fragments as described in Materials and Methods. The T18 and T25 fusion pairs were then placed into a Δ*cya* mutant of *E. coli*. If the T25-GadX and GadX-T18 hybrids dimerize in vivo, the two CyaA fragments will be brought together, reconstituting an active adenylate cyclase. Adenylate cyclase activity was then monitored by measuring the expression of the cAMP-dependent *lacZ* gene. The same approach was taken to test GadW dimerization. As shown in Table 3, the two-GadX-hybrid fusions (EF974) increased *lacZ* expression 55-fold over that in control strains (EF919), while the two-GadW-hybrid fusions (EF975) yielded a 45-fold increase. The results indicate that GadX and GadW form homodimers in vivo.

We also noticed that GadX and GadW can form heterodimers in vivo, although this was a much weaker interaction than that of homodimers. Table 3 illustrates that the X-Cya and W-Cya hybrid proteins (EF920 and EF973) generated four- to fivefold-higher levels of β-galactosidase than did controls. The significance of heterodimer formation is not yet apparent.

CRP regulates *gadX*. Previous studies have shown that CRP is a potent negative regulator of *gadA* and *gadBC* expression in log-phase, LB-grown cells (1). Figure 7A illustrates that a *crp* mutation caused overexpression of *gadA* and *gadBC* (EF227 versus EF528) and that overexpression required GadX (EF864 versus EF865). This suggested that CRP might repress *gadA* and *gadBC* indirectly by regulating *gadX* expression. The Northern blot in Fig. 4 (EF528) and the Western blot in Fig. 7B indicate that CRP does repress *gadX* expression at the

TABLE 3. Two-hybrid analysis of GadX and GadW in vivo interactions

Strain	Relevant genotype	β-Galactosidase activity[a]
EK395	Δ*cya*	60
EF919	EK395/pT25/pT18	65
EF646	EK395/pT25-Zip/pT18-Zip	1,570
EF1017	EK395/pT25/pT18-*gadX*	62
EF1018	EK395/pT25/pT18-*gadW*	72
EF1019	EK395/pT25-*gadX*/pT18	68
EF1020	EK395/pT25-*gadW*/pT18	62
EF974	EK395/pT25-*gadX*/pT18-*gadX*	3,600
EF975	EK395/pT25-*gadW*/pT18-*gadW*	2,880
EF920	EK395/pT25-*gadX*/pT18-*gadW*	318
EF973	EK395/pT25-*gadW*/pT18-*gadX*	246

[a] Strains were grown to stationary phase (22 h) at 30°C in LB medium with 0.5 mM IPTG and were assayed for β-galactosidase (Miller units).

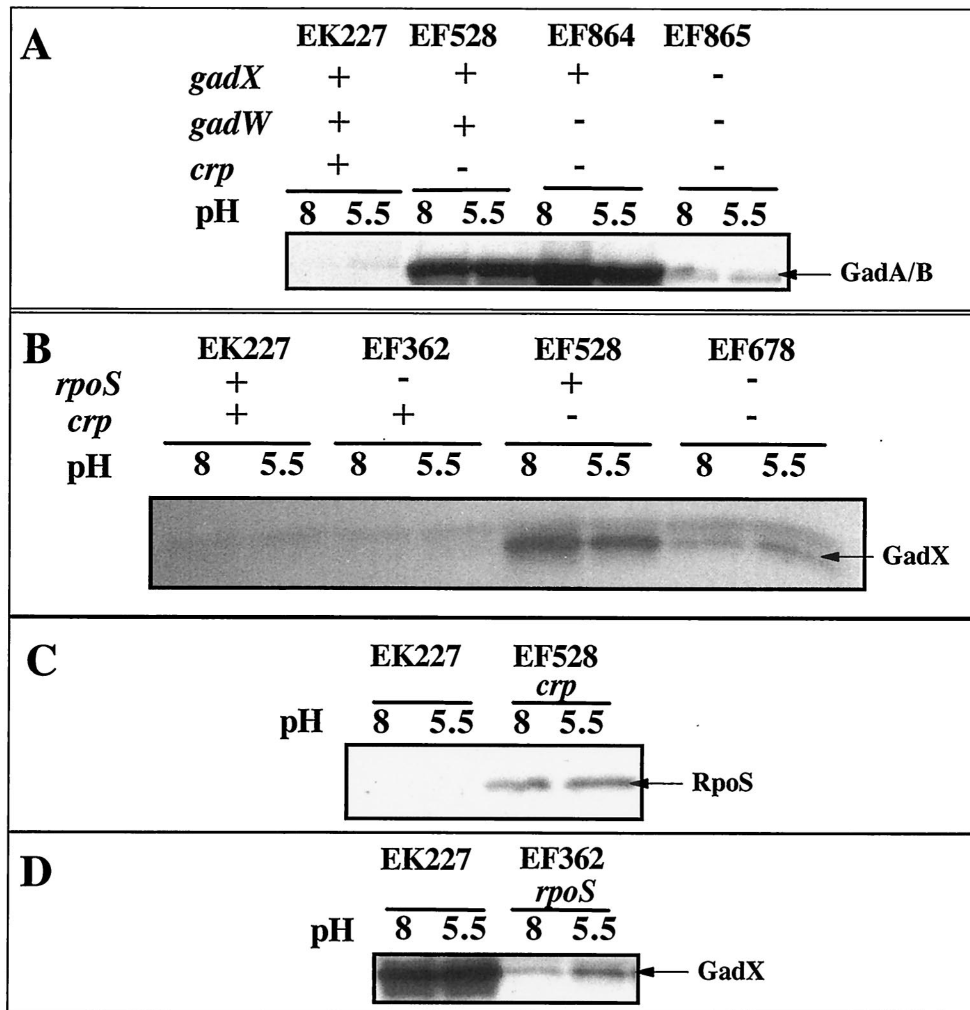

FIG. 7. Western blot analysis of *crp* and *rpoS* effects on GAD, RpoS, and GadX protein levels. Cells were grown in LB medium with MES, pH 5.5, or LB medium with MOPS, pH 8, to log phase (A to C) or to stationary phase (D). Strains used and relevant genotypes are indicated in the figure.

RNA level. The *crp* mutation caused elevated *gadX* message and protein (compare EF528 to EK227). A *crp* mutation did not affect expression of *gadW* (data not shown).

As reported previously, expression of *gadX* is principally driven by the RpoS sigma factor (Fig. 7D, stationary phase), and it is known that CRP represses *rpoS* expression (Fig. 7C, exponential phase) (10, 20). Because of this, the effect of a *crp* mutation on GadX production was tested in an *rpoS* mutant background (Fig. 7B). The results indicate that CRP repression of *gadX* occurs primarily, but not completely, through its control over *rpoS* (Fig. 7B, compare EF528 and EF678). The *rpoS crp* mutant (EF678) still produced elevated levels of GadX compared to the *rpoS* mutant (EF362). The data, in sum, suggest that the majority of CRP control over *gadA* and *gadBC* occurs through its control over RpoS, which, in turn, is needed to maximally express *gadX*.

Acid induction of *gadA* and *gadBC* in complex media is due to the timing of RpoS production. In complex media, GAD production is acid induced as cells enter stationary phase but occurs in equivalent amounts by late stationary phase. We asked whether pH control could be due to altered levels of RpoS rather than through GadX sensing some parameter of pH. Cells were grown to late log phase (OD_{600} = 1.2) in LB medium with MES, pH 5.5, and LB medium with MOPS, pH 8. Under these conditions GAD is observed in the pH 5.5 but not the pH 8 culture. Western blot analysis of RpoS revealed that the levels of this sigma factor were higher at pH 5.5 than at pH 8, suggesting that pH control of GAD production in complex media was indirect, through RpoS effects on GadX production (Fig. 8). The growth rates of the pH 5.5 and pH 8 cultures were equivalent (ca. 30 min). Thus, the accelerated production of RpoS under acidic conditions was not the result of an early entry into stationary phase but was a consequence of low pH.

H-NS conditionally represses *gadA* and *gadBC* expression independent of GadX. Previous reports utilizing *E. coli* strain W3110 convincingly showed that H-NS can also indirectly repress *gadA* and *gadBC* expression by inhibiting RpoS production (4, 20). However, we could not reproduce those results with the Stanford University wild-type K-12 strain in which

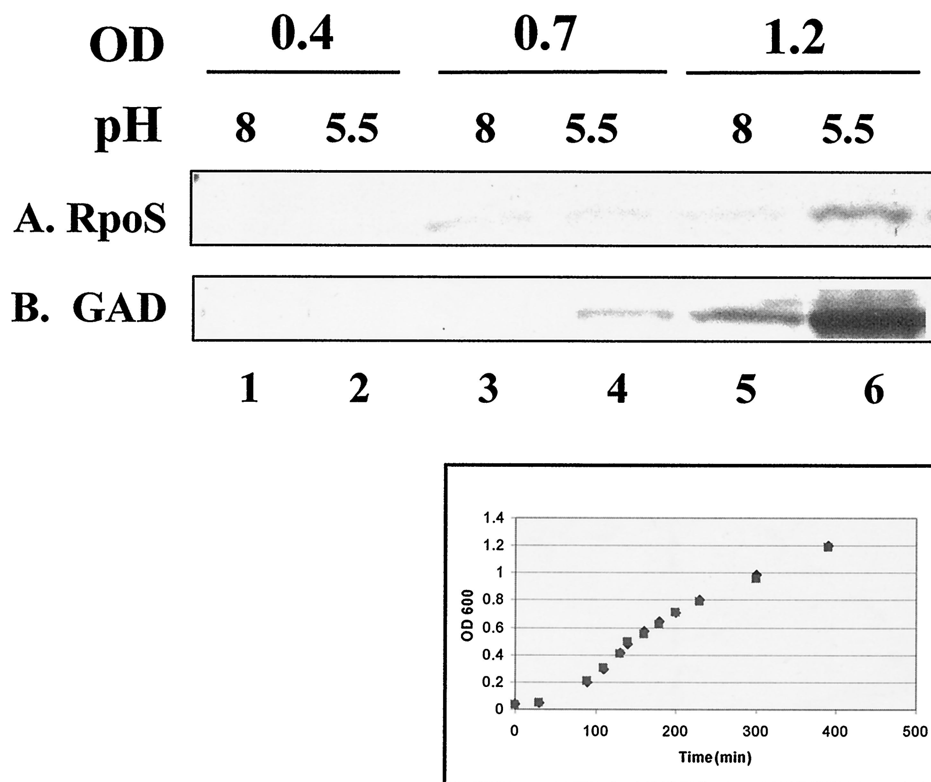

FIG. 8. Low-pH and growth phase induction of glutamate decarboxylase and RpoS in rich media. EK227 was grown to different optical densities in LB media buffered to pH 5.5 or 8. The inset illustrates the growth under both conditions measured as OD_{600}. Extracts were probed by Western blot analysis as described in the Fig. 2 legend. (A) Detection of RpoS; (B) detection of glutamate decarboxylase (GAD).

CRP was the main global regulator (Fig. 9A). We did find that a *crp gadX gadW* mutant (EF865), which produces almost no glutamate decarboxylase when grown to exponential phase in LB medium, would make copious amounts of GAD once an *hns* mutation was introduced (Fig. 9B, compare lane 1 to lane 2 and lane 3 to lane 4). This confirms that H-NS will repress *gadA* and *gadBC*; however, this control did not require *gadX* as reported for the W3110 strain and was not even evident in a *gadX*$^{+}$ culture (data not shown).

DISCUSSION

The glutamic acid decarboxylase and putative GABA:glutamate antiporter system plays a prominent role in *E. coli* acid resistance. While it might seem that regulation of this system should be straightforward with acid pH inducing expression and alkaline pH preventing induction, regulation of the *gadA* and *gadBC* operons is much more complex. Several reports, some seemingly contradictory, have invoked various roles for CRP and H-NS as repressors, σ^{S} and σ^{70} as principal sigma factors, and an AraC-like regulator, GadX, as both inducer and repressor. The data presented in this study concern another AraC-like regulatory protein, GadW, and its collaborative role with GadX in controlling this system prior to and during entry into stationary phase. The study also examines CRP-mediated control of this system. The results add more clarity to the complex model of *gadA* and *gadBC* expression and its regulation. Figure 10 summarizes the basic control circuits mapped for this system.

An earlier report describing GadX as a repressor at pH 8 but an activator at pH 5.5 can now be partly explained (18). The activator-repressor model was based on data obtained from cells grown in minimal glucose media. The repressor effect can also be seen, although less dramatically, in the LB-grown cells represented in Fig. 2A. More GadA and -B protein is seen at pH 8 in the *gadX* mutant (Fig. 2A, lane 3) than is found in the wild type (Fig. 2A, lane 1), originally suggesting that GadX was a repressor at pH 8. The inducer effect at pH 5.5 can be seen in Fig. 2B, where the *gadX* mutant (Fig. 2B, lane 4) produces less GadA and -B protein than does the wild type (Fig. 2B, lane 2), suggesting that GadX was an activator at pH 5.5. However, when GadW is missing, GadX is really only a positive regulator (Fig. 2A, lanes 5 and 6). Repression at pH 8 appears to be due to an effect of pH on GadW function (Fig. 2A and B, lanes 3 and 4).

The fact that GadX and GadW can each independently activate *gadA* and *gadBC* under certain conditions indicates that both proteins are capable of binding to the *gadA* and *gadBC* promoter regions, a conclusion supported in vitro for both GadX (18, 20) and GadW (this report). The data also shows that GadW regulates the *gadX* promoter (Fig. 4). The effects of GadW on *gadA* and *gadBC* expression are not solely through its impact on *gadX* expression, however, since GadW can affect *gadA* and *gadBC* expression even in the absence of

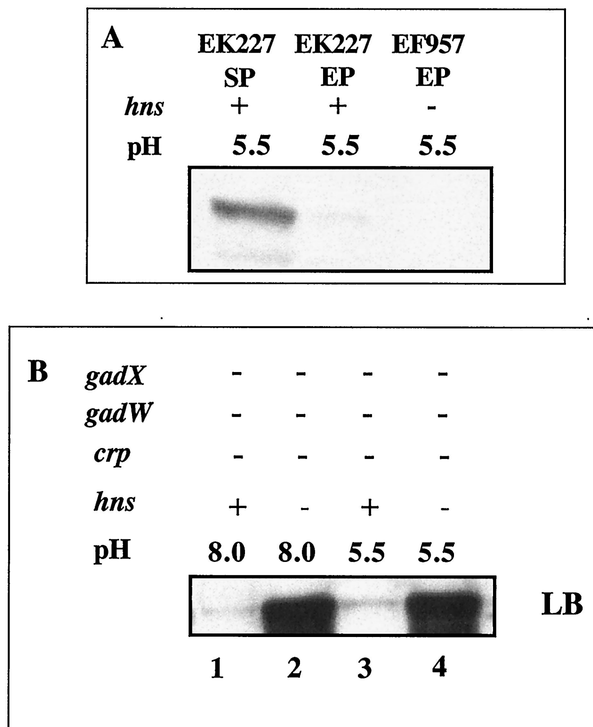

FIG. 9. Effects of H-NS on *gadA* and *gadBC* expression. Cells were grown in complex, buffered LB media to exponential phase. Western blot analysis using anti-GadA and -B antibody was performed as described in the Fig. 2 legend. For panel A, all cells were *gadA* and *gadBC*$^+$ *gadX*$^+$ *gadW*$^+$. For panel B, all cells lacked GadX, GadW, and CRP (*hns*$^+$, lanes 1 and 3, EF865; *hns* mutant, lanes 2 and 4, EF929).

GadX (Fig. 2A and B, compare lanes 3 and 4 with lanes 7 and 8). An interesting question is why does GadW, in the absence of GadX, activate *gadA* and *gadBC* only at pH 8? One model, discussed briefly above, is that GadW might bind *gadA* and *gadBC* promoters best when cells are grown at pH 8, which either prevents GadX from binding or attenuates GadX activation. Alternatively, GadW may not strongly bind the *gadA* and *gadBC* promoter regions if GadX is already bound to them. In this model, GadW, under ordinary circumstances, might primarily temper GadX activity by repressing *gadX*.

The transcriptional organization of the *gadA* locus involves three promoters, one upstream of *gadA*, one upstream of *gadX*, and one upstream of *gadW*. Transcription from P*gadA* can produce a cotranscript with *gadX*, but transcription from P*gadX* does not yield a cotranscript with *gadW* (20) (this report). However, mutations that affect expression from P*gadA* have a large effect on *gadA* message levels but almost no effect on the *gadAX* cotranscript (Fig. 3 and 4 for *gadW* effects and Fig. 4 for *crp* effects). This suggests either that *gadA* and *gadX* are usually transcribed separately and only occasionally form cotranscripts or that the *gadAX* cotranscript is subject to vigorous processing that separates the two messages.

Consistent with their annotation as AraC-like regulators, GadX and GadW will readily form homodimers in vivo (5). The finding that these proteins also appear capable of forming heterodimers may provide insight into the GadX and -W control circuit. Heterodimers could be incorporated into a model that explains GadW inhibition of GadX activity, although equally plausible alternative hypotheses involving competitive DNA binding are possible. Many AraC-like regulators bind ligands that alter their function (5), but whether or not GadX or GadW binds specific cytoplasmic ligands remains unknown.

Expression of *gadA* and *gadBC* in LB media is principally dependent on RpoS. A recent study, confirmed here, demonstrated that expression of *gadX* is RpoS dependent (20). In that same study, *gadX* was uncoupled from RpoS control by placing *gadX* under the control of a Tn*5*-*lacO* promoter-operator element. When *gadX* was expressed from this promoter, the *gadA* and *gadBC* genes were transcribed even in log-phase (pH 7.4), LB-grown cells, a condition ordinarily devoid of *gadA* and *gadBC* expression. This finding suggested that the RpoS effects on *gadA* and *gadBC* expression are indirect, occurring due to an RpoS requirement for *gadX* expression. The result also suggests that the *gadA* and *gadBC* promoters are themselves RpoS independent. The fact that expression of *gadA* and *gadBC* in minimal glucose media is mostly RpoS independent and GadX independent supports that idea (data not shown). However, the result also suggests that another *gadA* and *gadBC* induction pathway exists, one independent of GadX.

We have also found that CRP represses *gadX* expression, which explains the derepressive effect that *crp* mutations have on *gadA* and *gadBC* expression. CRP appears to negatively regulate *gadX* expression by virtue of its effects on RpoS production. The developing model for the control circuit is that, when *E. coli* is actively growing under conditions where cAMP levels are high (e.g., exponential growth in media like LB medium), CRP will repress *gadX* by inhibiting RpoS production. GadW also represses *gadX* under this condition. As cells approach stationary phase, RpoS levels rise, causing increased expression of *gadX.* GadX levels increase and overcome repression of *gadA* and *gadBC* by GadW. Cells grown fermentatively on glucose would have lower cAMP levels and less CRP-dependent repression of the system.

The pH control of this system, at least in rich, undefined media, also appears tied to the pH control of RpoS production. Evidence supporting this model includes the fact that *gadA* and *gadBC* expression in this media requires RpoS and that the levels of glutamate decarboxylase and of RpoS are induced earlier in pH 5.5 cultures entering stationary phase than in pH 8 cultures. It has previously been shown that pH influences translation of *rpoS* message and degradation of RpoS protein in *Salmonella enterica* (14, 22).

The nucleoid protein H-NS has also been identified as a repressor for this system (4, 8, 23). A recent report has demonstrated that H-NS may work to repress the *gadA* and *gadBC* genes by repressing *gadX* (20). However, our results, using a different genetic background, indicate that H-NS still affects *gadA* and *gadBC* expression in a *gadXW* mutant. Thus, H-NS must also act on *gadA* and *gadBC* independently of *gadX.* Another inconsistency between the two studies is that an *hns* mutation in our K-12 strain did not relieve CRP-dependent repression of *gadA* and *gadBC.* Thus, our results argue that CRP, not H-NS, is a master regulator of glutamate-dependent acid resistance. The reason(s) for these discrepancies is not apparent and may be strain dependent.

The question remains: why does the cell expend so much energy to regulate the *gad* genes? At present count there are

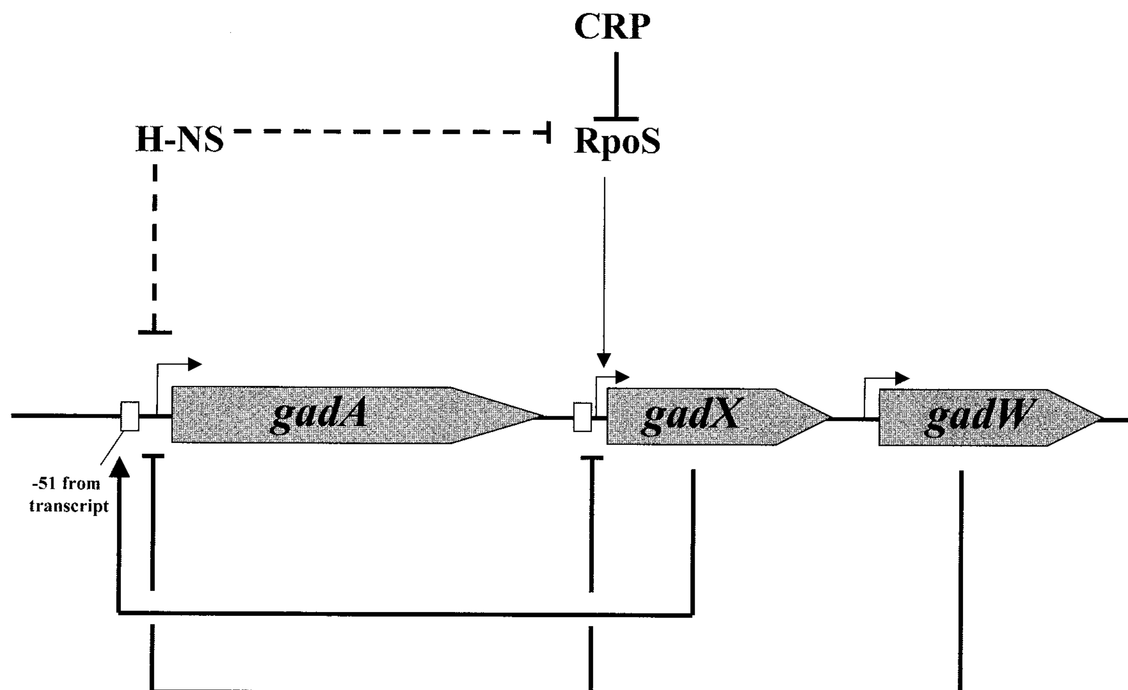

FIG. 10. Model of the GadX and -W and CRP-RpoS control circuit. GadX activates expression from P*gadA*, while GadW inhibits expression (in the presence of GadX) from P*gadA* and P*gadX*. Transcription of the activator gene *gadX* is largely dependent on the RpoS sigma factor, tying expression of the system to various stress conditions that increase RpoS levels. Expression is also influenced by growth on carbohydrates through CRP, which dampens expression of RpoS, an effect more evident in LB-grown cells, where cAMP levels are high, than in cells grown on glucose, where cAMP levels are low. H-NS is also reported to inhibit *gadX* expression but appears capable of inhibiting *gadA* and *gadBC* expression independently of *gadX* control.

two repressors (H-NS and CRP), one activator (GadX), one repressor-activator (GadW), and two sigma factors focused on controlling this system. This complex regulatory network must reflect the importance that *E. coli* places on surviving transient exposures to extreme acid stress, ensuring that the system is in place under any environmental condition that could lead to acid stress. Numerous questions remain regarding the regulatory interactions that take place between these many factors.

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