

Characterization of EvgAS-YdeO-GadE Branched Regulatory Circuit Governing Glutamate-Dependent Acid Resistance in *Escherichia coli*

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Received 3 July 2004/Accepted 6 August 2004

Escherichia coli prefers growth in neutral pH environments but can withstand extremely acidic conditions (pH 2) for long periods. Of the four *E. coli* systems that contribute to acid resistance, one, the glutamate-dependent system, is remarkable in its efficacy and regulatory complexity. The resistance mechanism involves the intracellular consumption of protons by the glutamate decarboxylase isozymes GadA and GadB. The antiporter GadC then exports the product, γ -aminobutyric acid, in exchange for fresh glutamate. A microarray study using overexpressed regulators uncovered *evgAS* and *ydeO* as potential regulators of *gadE*, now known to encode the essential activator of the *gadA* and *gadBC* genes. Examination of *evgA* and *ydeO* under normal expression conditions revealed that their products do activate *gadE* expression but only under specific conditions. They were important during exponential growth in acidified minimal medium containing glucose but were unnecessary for *gadE* expression in stationary-phase cells grown in complex medium. The response regulator EvgA activates *gadE* directly and indirectly via induction of the AraC-like regulator *ydeO*. Evidence obtained using *gadE-lacZ* operon fusions also revealed that GadE was autoinduced. Electrophoretic mobility shift assays indicated that EvgA, YdeO, and GadE bind to different regions upstream of *gadE*, indicating they all act directly at the *gadE* promoter. Since GadE controls the expression of numerous genes besides *gadA* and *gadBC*, the relevance of these regulatory circuits extends beyond acid resistance.

Gastric acid is a formidable barrier for gastrointestinal pathogens. Penetrating this barrier requires either a massive assault by large numbers of organisms or a powerful acid resistance (AR) mechanism that allows small numbers of bacteria to survive until the stomach empties its contents into the more alkaline intestine. One remarkable microbe, the stomach pathogen *Helicobacter pylori*, is well regarded for its ability to survive gastric acidity. What is not widely recognized is that *Escherichia coli* is nearly equal to *H. pylori* in this respect, able to survive pH 2 for hours (12, 27).

E. coli possesses four phenotypically distinct systems of AR. AR system 1 is repressed by glucose, is evident in stationary-phase cells, and protects cells in minimal medium (pH 2.5). The other systems are not repressed by glucose and require the addition of glutamic acid (AR system 2), arginine (AR system 3), or lysine (AR system 4) in the pH 2.5 acid challenge medium (4, 11, 12). These amino acid-dependent systems utilize matched decarboxylases and antiporters to protect the cell. The decarboxylases involved are the glutamic acid decarboxylase isozymes GadA and GadB for system 2, the arginine decarboxylase AdiA for system 3, and presumably, the inducible lysine decarboxylase CadA for system 4 (4, 6, 9, 11). These enzymes all contain pyridoxyl phosphate and work by replacing the α -carboxyl groups of their amino acid substrates with a proton recruited from the cytoplasm. The end products are

CO₂ and γ -amino butyric acid, agmatine, and cadaverine, the end products of glutamate decarboxylase, arginine decarboxylase, and lysine decarboxylase, respectively. The cognate antiporters, GadC for glutamate, AdiC for arginine, and CadC for lysine, expel the decarboxylation product in exchange for new amino acid substrate. AR systems 2 and 3 increase intracellular pH and create a positive electrical potential inside the cell (22). AR system 4, a weaker system, has not been examined.

The most effective of these systems is the glutamate-dependent system. For a seemingly simple mechanism, glutamate-dependent AR is subject to extraordinary control. There are at least 10 regulatory proteins known to control the core *gadA* and *gadBC* loci. The *gadA* and *gadBC* genes are induced by growth under acidic conditions or by entry into stationary phase. GadE (formerly *yhiE*), a LuxR-family activator, is the central activator of *gadA* and *gadBC* expression (10, 13). GadE binds to a 20-bp sequence called the GAD box centered –63 bp from the transcriptional start sites of *gadA* and *gadBC* (3, 13). The other regulators form iterative control circuits designed to activate *gadE* expression under different growth conditions. The requirements and roles of these other regulators change with growth phase, aeration, and medium.

One circuit involves cyclic AMP receptor protein (CRP), RpoS, and two AraC-like regulators, GadX and GadW. This circuit plays a prominent role in cells grown in complex medium but also influences expression during growth in minimal medium. The two AraC-like regulators, GadX and GadW, reside downstream of *gadA* but are transcribed, for the most part, by independent promoters (15). GadX and GadW initially appeared to activate and repress the core *gad* genes, depending on the situation (13, 25). However, we now have

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evidence that these regulators activate *gadE*, and thus *gadA* and *gadBC*, during growth in complex medium but can also directly repress *gadA* and *gadBC* (S. Gong, Z. Ma, A. Sayed, and J. Foster, submitted for publication). GadX, GadW, and RpoS, the stress response alternative sigma factor, also form a regulatory loop that is influenced by cyclic AMP and CRP (14, 15, 27, 28).

Members of the second circuit were initially discovered when Masuda and Church found that overexpressing the EvgA response regulator at pH 7 resulted in highly acid-resistant cells (16). A series of gene array studies using strains that overexpressed these regulators suggested a regulatory circuit in which the EvgSA two-component regulatory system activates expression of the AraC-like regulator *ydeO*. YdeO then activates AR. We predicted that this activation occurs by inducing expression of the essential activator *gadE* and tested the proposed linear EvgA-YdeO-GadE regulatory scheme under physiological conditions. The data revealed that an EvgA regulatory circuit does activate *gadE*, but the circuit is used only in exponential-phase cultures growing at low pH (pH 5.5) in a minimal medium containing salts and glucose. It is not needed to activate *gadE* in stationary phase or during growth in a rich medium, such as Luria-Bertani medium (LB). Chromosomal knockout mutations indicate the system is a branched control circuit (diagrammatically summarized in the Discussion) where EvgA activates *gadE* expression directly and indirectly through a *ydeO* feed-forward loop and where *gadE* autoregulates its own expression. Consequently, the EvgA regulatory pathway is more complex than originally thought. The significance of this control extends beyond AR, since GadE, as well as EvgA and YdeO, affect multiple aspects of cell physiology (10, 17).

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) (32), complex LB buffered with either 100 mM morpholinopropanesulfonic acid (MOPS) (pH 8) or 100 mM morpholinethanesulfonic acid (MES) (pH 5.5), LB containing 0.4% glucose (LBG), and brain heart infusion broth (BHI) containing 0.4% glucose (BHIG). When required, the following antibiotics at the following concentrations were used: carbenicillin, 100 μ g/ml; ampicillin, 100 μ g/ml; kanamycin, 25 μ g/ml; tetracycline, 20 μ g/ml; and chloramphenicol, 30 μ g/ml. Where indicated, tryptophan was added at 0.3 mM, and isopropyl- β -D-thiogalactopyranoside (IPTG) was used at 0.3 or 1.5 mM, as indicated. All strains were grown at 37°C with aeration. The growth rates of all strains used were approximately equal when the strains were tested in similar growth media. The average growth rates or doubling times for these isogenic strains were 60 min (\pm 3 min) in minimal EG at pH 7.7, 67 min (\pm 4 min) in EG at pH 5.5, 30 min (\pm 3 min) in BHIG, and 30 min (\pm 3 min) in LBG. Inducing plasmid-containing strains with IPTG did not significantly affect their growth rate relative to those of non-IPTG controls.

Molecular biology techniques. Phage P1 transduction, transformation with CaCl_2 , and electroporation were performed by standard methods (18). General DNA manipulations, kanamycin cassette insertions, and deletions were all performed as described earlier (5, 23). Oligonucleotide primers are listed in Table 2.

Western blot analysis. Strains were grown at 37°C in media containing the required antibiotics as indicated. Genes encoding EvgA, YdeO, or GadE were cloned into the pQE80L vector and conditionally expressed by adding 1.5 mM IPTG to EG containing 100 μ g of carbenicillin. At an optical density at 600 nm (OD_{600}) of 0.4 (log phase) or 3.8 (late stationary phase), cells were collected by centrifugation and then resuspended in 0.01% sodium dodecyl sulfate (SDS) solution. Protein concentrations were determined using Bio-Rad protein assay reagent. Samples (5 μ g of protein) were prepared and subjected to Western blot analysis as described earlier (13). Membranes were probed with rat anti-GAD, followed by the anti-rat secondary monoclonal antibodies coupled to peroxidase (Sigma) (diluted 1:10,000) (4). Antibody-tagged protein bands on the probed

membranes were detected using an ECL Western blot detection kit (Amersham). Results were analyzed by densitometry using Scion Image software.

Northern blot analysis. Total RNA was extracted by using the RNeasy Mini kit (Qiagen) from log-phase cell cultures (OD_{600} of 0.4) grown under both alkaline and acidic conditions in minimal medium containing glucose. RNA (5 μ g) was separated through a denaturing formaldehyde-agarose gel (1.2% formaldehyde-agarose) and subjected to Northern blot analysis as described previously (13, 23). Membranes were probed with a 0.534-kb *gadE* or 0.762-kb *ydeO* probe generated by PCR using oligonucleotide 540 or 541 and oligonucleotide 544 or 545, respectively. Probes were labeled with [α - 32 P]dCTP (ICN) using DECA prime II random-priming DNA labeling kit (Ambion). Both *ydeO* and *gadE* probes correspond to the entire open reading frames (ORFs) of *ydeO* and *gadE*, respectively. For a control, the membranes were also hybridized with a 23S rRNA probe (oligonucleotide 379) end labeled with [γ - 32 P]ATP. Northern blot quantifications were determined by densitometry using Scion Image software.

Primer extension. To determine the *gadE* transcriptional start site, six primers (oligonucleotides 599, 624, 625, 662, 626, and 663) that span the *hdeD-gadE* intergenic region were used for primer extension analysis. One picomole each of the primers was 5' end labeled with [γ - 32 P]ATP T4 using polynucleotide kinase (Promega). Reverse transcription of *gadE* mRNA, isolated from pH 5.5 log-phase cells (EG medium), was performed using the Promega primer extension system. RNA (5 μ g) and end-labeled primers were annealed for 20 min at 58°C, and then 10 U of avian myeloblastosis virus reverse transcriptase was added to the 20- μ l reaction mixture and incubated at 42°C for 1 h. The reaction was halted by adding 20 μ l of kit loading dye. Sequencing reactions were performed using the thermal Sequenase cycle sequencing kit (U.S. Biochemicals) and run in parallel with the cDNA primer extension transcripts to map the 5' end of *gadE* mRNA. A PCR product produced by oligonucleotide 700 or 704, which contains the entire *hdeD-gadE* intergenic region and part of the *gadE* ORF were used as templates in the sequencing reactions.

Construction of *gadE-lacZ* and *evgA-lacZ* transcriptional fusions. Chromosomal *gadE-lacZ* operon fusions were constructed by the method of Elliott (8). An 804-bp fragment containing the *hdeD-gadE* intergenic region plus part of the *gadE* ORF (positions -804 to +28 relative to the *gadE* start codon) was amplified using oligonucleotides 609 and 610 engineered to include EcoRI and BamHI restriction sites, respectively. The fragments were then digested and cloned into EcoRI/BamHI-digested pRS551 (Km^r Amp r), which is a *lacZ* operon fusion vector (26). The resulting plasmid, pMF537, was linearized by XhoI digestion and transformed into the *E. coli* *recD* strain EK298 (Cm^r Km^s) containing a Km^s -*lacZ* cassette inserted into a *putPA* operon that was itself inserted into the *trp* locus of *E. coli*. Recombination between the plasmid and chromosome produced an Amp r Cm^s Km^r merodiploid strain containing an intact *gadE* gene and a *gadE-lacZ* transcriptional fusion located at the *putPA* operon. Correct insertion of the *gadE-lacZ* fusion was confirmed by PCR with oligonucleotide 609 to the *gadE* promoter and oligonucleotide 194 for *lacZ*. The *gadE-lacZ* fusion was transduced to wild-type *E. coli* strains by P1 phage transduction. Additional fusions between *lacZ* and various fragments of the *hdeD-gadE* intergenic region were prepared using oligonucleotide 700 or 704 (positions -804 to +331 relative to start codon ATG), oligonucleotide 702 or 704 (positions -360 to +331), and oligonucleotide 701 or 704 (positions -195 to +331). The PCR fragments from these oligonucleotides were cloned into pRS551, resulting in plasmids pMF555, pMF556, and pMF567, respectively, which were linearized and transformed into strain EK298 as described above.

An *evgA-lacZ* operon fusion was also constructed. A 570-bp DNA fragment containing the *evgA* promoter region was generated by PCR using oligonucleotide 633 or 634 containing engineered EcoRI and BamHI restriction sites, respectively. This fragment was cloned into pRS551, creating plasmid pMF546. A chromosomal merodiploid *evgA-lacZ* transcriptional fusion strain was constructed as described above. β -Galactosidase activities were measured as described previously (18).

Expression and purification of MBP-GadE, MBP-YdeO, and His $_6$ -EvgA fusion proteins. Maltose binding protein (MBP)-GadE fusion protein was purified previously (13). An MBP-YdeO fusion protein was constructed by excising *ydeO* from pQEYdeO (17) using BamHI and HindIII and religating the fragment downstream of MalE in plasmid pMALc2E (New England Biolabs). The resulting plasmid, pMF539, was transformed into strain TB1 (New England Biolabs). MBP-YdeO fusion protein was expressed and purified as described previously for MBP-GadE (13). The protein was purified to homogeneity as determined by Coomassie blue-stained SDS-polyacrylamide gel electrophoresis (PAGE) (data not shown).

To purify EvgA, a His $_6$ -EvgA fusion vector, pQEEvgA, was constructed and transformed into an *evgA5* mutant background as described previously (17). A culture grown overnight was diluted (1:10) into 500 ml of fresh LB medium with

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source, reference, or construction
Strains		
EK298	(TE2680) λ^- F ⁻ IN(<i>rrnD-rrnE</i>) Δ (<i>lac</i>)X74 <i>rpsL galK2 recD1903::Tn10dTc trpDC::putPA1303</i>	8
EK432	Δ (<i>araD-araB</i>)567 Δ (<i>lacA-lacZ</i>)515(<i>::cat</i>) <i>lacIp-4000(lacI^q)</i> Δ (<i>rhaD-araB</i>)568 <i>hsdR514</i>	5
EK551	Δ (<i>gadE yhiE::Km</i>)	29
EK584	TB1ara Δ (<i>lac-proAB</i>) <i>rpsL</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hndR</i>	New England Biolabs
EK592	MG1655 (wild-type K-12)	17
EK593	Δ <i>evgA</i>	17
EK594	Δ <i>evgAS</i>	17
EK595	Δ <i>ydeO</i>	17
EK596	Δ <i>evgAS</i> Δ <i>ydeO</i>	17
EK616	Δ <i>gadE</i> (<i>yhiE</i>)	17
EK619	Δ <i>yhiUV</i>	17
EF1108	K-12/pQE <i>yhiE</i> (<i>gadE</i>)	This study
EF1148	K-12/pQE80L	This study
EF1149	K-12/pQE <i>evgA</i>	This study
EF1151	Δ <i>evgA</i> /pQE <i>evgA</i>	This study
EF1155	Δ <i>gadE</i> (<i>yhiE</i>) <i>::Km</i>	EK592 \times EK551
EF1156	Δ <i>ydeO</i> /pQE <i>evgA</i>	EK595 \times pQE <i>evgA</i>
EF1157	Δ <i>gadE</i> /pQE <i>evgA</i>	EK1155 \times pQE <i>evgA</i>
EF1164	K-12/pQE <i>ydeO</i>	EK592 \times pQE <i>ydeO</i>
EF1165	Δ <i>evgA</i> /pQE <i>ydeO</i>	EK593 \times pQE <i>ydeO</i>
EF1166	Δ <i>gadE::Km</i> /pQE <i>ydeO</i>	EF1155 \times pQE <i>ydeO</i>
EF1206	EK298 <i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) ^a	EK298 \times pMF537
EF1207	TB1ara Δ (<i>lac-proAB</i>) <i>rpsL</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hndR</i>	EK584 \times pMF539
EF1223	EK298 <i>trpDC::putPA1303-Km-<i>evgA-lacZ</i></i> (Oc)	EK298 \times pMF546
EF1238	Δ <i>evgAS</i> Δ <i>ydeO</i> Δ <i>gadE::Km</i>	EK596 \times EK551
EF1239	Δ <i>evgAS</i> Δ <i>ydeO</i> Δ <i>gadE</i>	EF1238 \times pCP20
EF1240	K-12 Δ <i>lac::cat</i>	EK592 \times EK432
EF1241	Δ <i>lac::cat</i> Δ <i>evgAS</i>	EK594 \times EK432
EF1242	Δ <i>lac::cat</i> Δ <i>ydeO</i>	EK595 \times EK432
EF1243	Δ <i>lac::cat</i> Δ <i>gadE</i>	EK616 \times EK432
EF1244	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i>	EF1240 \times EF1206
EF1245	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i> Δ <i>evgAS</i>	EF1241 \times EF1206
EF1246	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i> Δ <i>ydeO</i>	EF1242 \times EF1206
EF1247	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i> Δ <i>gadE</i>	EF1243 \times EF1206
EF1248	Δ <i>lac::cat</i> Δ <i>evgAS</i> Δ <i>ydeO</i> Δ <i>gadE</i>	EF1239 \times EK432
EF1256	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i> Δ <i>evgAS</i> Δ <i>ydeO</i> Δ <i>gadE</i>	EF1248 \times EF1206
EF1257	K-12 Δ <i>evgAS</i> Δ <i>ydeO</i>	EK596 \times EK432
EF1258	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +28ATG} - <i>lacZ</i> (Oc) Δ <i>lac::cat</i> Δ <i>evgAS</i> Δ <i>ydeO</i>	EF1257 \times EF1206
EF1285	EK298 <i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +331ATG} - <i>lacZ</i> (Oc)	EK298 \times pMF555
EF1286	EK298 <i>trpDC::putPA1303-Km-gadE</i> ^{-360 to +331ATG} - <i>lacZ</i> (Oc)	EK298 \times pMF556
EF1288	K298 <i>trpDC::putPA1303-Km-gadE</i> ^{-195 to +331ATG} - <i>lacZ</i> (Oc)	EK298 \times pMF557
EF1294	<i>trpDC::putPA1303-Km-<i>evgA-lacZ</i></i> (Oc) Δ <i>lac::cat</i>	EF1240 \times EF1223
EF1295	<i>trpDC::putPA1303-Km-<i>evgA-lacZ</i></i> (Oc) Δ <i>lac::cat</i> Δ <i>evgAS</i>	EF1241 \times EF1223
EF1296	<i>trpDC::putPA1303-Km-<i>evgA-lacZ</i></i> (Oc) Δ <i>lac::cat</i> Δ <i>ydeO</i>	EF1242 \times EF1223
EF1297	<i>trpDC::putPA1303-Km-<i>evgA-lacZ</i></i> (Oc) Δ <i>lac::cat</i> Δ <i>gadE</i>	EF1243 \times EF1223
EF1303	<i>trpDC::putPA1303-Km-gadE</i> ^{-804 to +331ATG} - <i>lacZ</i> (Oc)	EF1240 \times EF1285
EF1304	<i>trpDC::putPA1303-Km-gadE</i> ^{-360 to +331ATG} - <i>lacZ</i> (Oc)	EF1240 \times EF1286
EF1305	<i>trpDC::putPA1303-Km-gadE</i> ^{-195 to +331ATG} - <i>lacZ</i> (Oc)	EF1240 \times EF1288
EF1348	Δ <i>evgAS</i> Δ <i>ydeO</i> /pQE <i>yhiE</i> (<i>gadE</i>)	EK595 \times pQE <i>yhiE</i> (<i>gadE</i>)
Plasmids		
pCP20	FLP recombinase	5
pQE80L	His tag cloning vector	Qiagen
pRS551	Transcriptional <i>lacZ</i> fusion vector	26
pQE <i>yhiE</i>	<i>gadE</i> (<i>yhiE</i>) ORF cloned into pQE80L, <i>lac</i> promoter	17
pQE <i>evgA</i>	<i>evgA</i> ORF cloned into pQE80L, <i>lac</i> promoter	17
pQE <i>ydeO</i>	<i>ydeO</i> ORF cloned into pQE80L, <i>lac</i> promoter	17
pMALc2E	MBP fusion vector	New England Biolabs
pMF533	pMAL2c <i>EgadE</i>	13
pMF539	pMAL2c <i>EydeO</i>	This study
pMF537	<i>gadE-lacZ</i> fusion ^{-804 to +28ATG} in pRS551	This study
pMF546	<i>evgA-lacZ</i> fusion ^{-420 to +150ATG} in pRS551	This study
pMF555	<i>gadE-lacZ</i> fusion ^{-804 to +331ATG} in pRS551	This study
pMF556	<i>gadE-lacZ</i> fusion ^{-360 to +331ATG} in pRS551	This study
pMF557	<i>gadE-lacZ</i> fusion ^{-195 to +331ATG} in pRS551	This study
pUC <i>evgA</i>	<i>evgA</i> with native promoter in pUC19	17

^a Numbers reflect the extent of upstream sequence fused to *lacZ*.

TABLE 2. Oligonucleotide primers used in this study

Oligo-nucleotide	Sequence
3775'-GGAGTTCGAAATGGACCAGAAG-3'
3785'-AGTTTCGGGTGATCGCTGAG-3'
5405'-CAAGTTATGATTTTCTCATGACGAA-3'
5415'-CTAAAAATAAGATGTGATACCCAG-3'
5445'-CGGGATCCATGTCGCTCGTTTGTCTGTT-3'
5455'-CGCAAGCTTAAATAATCAAATAGCTAAAGC-3'
5865'-GTAAATGTTTATATTATAAAAAAGTCGTTT-3'
5875'-AAACGACTTTTATAATATAAACATTTAAC-3'
5985'-CTAGTGATTTCAACCTACT-3'
5995'-AAGAATCTTTTCGTCATGA-3'
6005'-TTCATTATTTACATCCTTGTGC-3'
6015'-TGGCAATTGGATTGCCAGCTT-3'
6095'-GCCGAAATTCGAATAATTACCCCGTTGTCAC-3'
6105'-GCGGATCCAAGAATCTTCGTCATGAGAA-3'
6245'-GCCAAAAGCCTGTAAAAGAAAAGAATC-3'
6255'-CCTTGTCGGAATCGTTGTCAATATAG-3'
6265'-GTCTGGAGACACGGATATTTATGCAATG-3'
6275'-CGCGAATTCAGGAATCTTACTTAGGATCAATAT-3'
6335'-CGCGAATTCATTAATCTGTTCCACTATTATC-3'
6345'-CCGGATCCGATGACGATATCAGGCTTAAGT-3'
6615'-TTGCCAGCTTAAGTCGAAACAAGG-3'
6625'-ATTCTGGTTGTTATCAGCTTGTA-3'
6635'-TGACTACGGAAAATATCAGCCAT-3'
7005'-TTCCTTGCCGAATTCGAATAATACCCCGTTGTCAC-3'
7015'-CGCACGATGAATTCAGGAATCTTACTTAGGATCAATAT-3'
7025'-TTCCTTGCCGAATTCCTTGCCAGCTTAAGTCGAAACA-3'
7045'-CGCCGGATTGGATCTCTTATGGGGCAAGTGTTCAC-3'
7245'-TCGGACAAGGATGTAATAATGAAAAGGATGAC-3'
7255'-GTCATCTTTCATTATTTACATCCTTGTCGA-3'
7265'-TTCTTATAGCGCTTACTATATTGAAACAACGA-3'
7275'-TCGTTGTTCAATATGTAACGCCTATAAGAA-3'
7285'-CAAACGTTAACTTTTGTGTTGCTATTACAAAGCTGA-3'
7295'-TCAGCTTGTAATAGCAACAAAAGTTAACGTTTG-3'

100 µg of carbenicillin per ml and incubated at 37°C with shaking until reaching an OD₆₀₀ of 0.5. The fusion was induced with 1.5 mM IPTG and incubated for an additional 3 h. The culture was centrifuged for 15 min at 10,000 × g, resuspended in 30 ml of lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole [pH 8.0]), and passed three times through a French pressure cell at 20,000 lb/in². Cell lysates were cleared by centrifugation as described above and passed through a 0.45-µm-pore-size filter. The cleared extract was mixed with 4.5 ml of Ni-nitrilotriacetic acid agarose (Invitrogen) and gently shaken at 4°C for 1 h. The supernatant-agarose mixture was loaded into a column, washed with 65 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole [pH 8.0]). His₆-EvgA protein was eluted with 15 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole [pH 8.0]) and collected in 1.0-ml fractions. Samples (about 3 ml) of the three highest protein content fractions were combined and desalted through a PD-10 Sephadex column (Amersham Pharmacia). The purity of the purified His₆-EvgA was checked by 10% HCl SDS-PAGE (data not shown).

EMSA. Electrophoretic mobility shift assays (EMSA) were used to test whether His-tagged EvgA, MBP-YdeO, and MBP-GadE proteins bound to different regions within the *hdeD-gadE* intergenic sequence. The entire intergenic region and three subfragments were amplified with oligonucleotide 597 or 599 (spanning nucleotides [nt] -804 to +28 relative to the translational start codon), oligonucleotide 627 or 599 (designated F1, spanning nt -195 to +28), oligonucleotide 661 or 662 (designated fragment F2, spanning nt -360 to -190), and oligonucleotide 598 or 601 (designated fragment F3, spanning nt -682 to -355). These fragments were used in binding reactions with purified regulator fusion proteins. All DNA fragments were end labeled with [γ -³²P]ATP by T4 polynucleotide kinase. Radiolabeled DNA probes (5 ng) were incubated with different concentrations of His₆-EvgA, MBP-YdeO, and MBP-GadE fusion proteins at room temperature for 30 min in 20 µl of binding buffer [20 mM HEPES (pH 8.0), 5 mM MgCl₂, 50 mM potassium glutamate, 0.01 mM EDTA, 1 mM NaH₂PO₄, 20 mM NaCl, 1 mM dithiothreitol, 30 µg of bovine serum albumin per ml, 50 µg of poly(dI-dC) per ml]. Where indicated, an excess (100 ng) of specific, unlabeled DNA was added for competitive binding. Samples were loaded onto a 5% Tris-borate-EDTA (TBE) nondenaturing ready gel (Bio-Rad) and electrophoresed at room temperature in 0.5× TBE buffer with 1.2% glycerol. The gels were

dried and exposed to X-Omat Kodak film at -70°C for 3 h. Each EMSA experiment was repeated in triplicate.

AR assays. AR was tested using stationary-phase cultures (OD₆₀₀ of >2.0) and exponential-phase cultures (OD₆₀₀ of 0.4). Stationary-phase cells were prepared for AR system 1 by growth in LB with MES (pH 5.5) and LB with MOPS (pH 8.0) for 22 h. EG minimal medium was used to prepare cells to test AR system 2 (glutamate dependent), while cells grown in BHIG were used to test AR system 3 (arginine dependent). Extracellular glutamate is not needed to induce the glutamate-dependent system. Stationary-phase cultures were diluted 1:1,000 into prewarmed EG medium at pH 2.5 to test AR (final concentration of 2 × 10⁶ cells/ml). Dilutions were made in unsupplemented EG medium (pH 2.5) for AR system 1, EG medium (pH 2.0) supplemented with 1.5 mM glutamate for AR system 2, and EG medium (pH 2.5) containing 1.0 mM arginine for AR system 3.

To test AR in log-phase cells, cultures (10⁸ CFU/ml) were diluted 1:10 into prewarmed EG medium (pH 2.0), yielding a final pH of 2.5 and a final cell density of 10⁷ CFU/ml. Viable counts were determined at time zero, 1, 2, and 4 h after acid challenge. A previous report suggesting that log-phase cells grown in minimal medium were sensitive to acid did not use a sufficiently high cell density to detect resistance (4). A more recent report has shown that log-phase cells grown at pH 5.5 can survive pH 2.5 acid challenge (2). Results are presented as the averages ± standard errors of the means for three experiments.

RESULTS

Overexpression of EvgA, YdeO, and GadE affects only glutamate-dependent AR. A previous study found that overexpressing EvgA or YdeO in log-phase cells grown in LB will induce AR to a level equal to that of LB (pH 2.5) (17). However, *E. coli* contains four distinct systems of AR. The microarray data suggested that two of those systems, the glutamate decarboxylase-dependent (AR system 2) and arginine decarboxylase-dependent (AR system 3) systems, might be affected by EvgA but did not directly test this prediction (17). Consequently, we asked which of the AR systems (AR system 1, 2, or 3) was activated by overexpressing these regulators. The lysine-dependent system (AR system 4) was not examined.

Strains containing the overexpressing plasmids pQE_{evgA}, pQE_{ydeO}, or pQE_{gadE} (*yhiE*) were grown to log phase in minimal medium (pH 7.7) containing glucose, rich LB, or BHIG medium and then tested for the three AR systems. Table 3 presents the results from cells grown on minimal medium containing glucose. In each case, overexpression of the regulatory gene induced only the glutamate-dependent AR system. Similar results were found using BHIG, a medium containing cofactors needed to optimally induce arginine-dependent AR (data not shown). Thus, EvgA, YdeO, and GadE appear to affect only induction of glutamate-dependent AR.

TABLE 3. Effects of EvgA, YdeO, and GadE overexpression on AR mechanisms

Strain	Plasmid	% Survival ^a		
		No addition	Glutamate	Arginine
EF1148	pQE80L	<0.06	<0.06	<0.06
EF1149	pQE _{evgA}	<0.03	100 ± 10	<0.03
EF1164	pQE _{ydeO}	<0.07	85 ± 12	<0.07
EF1108	pQE _{gadE}	<0.06	90 ± 10	<0.06

^a Wild-type cells containing control plasmid pQE80L or plasmids with inserts were grown overnight in EG medium at pH 7.7 with 1.5 mM IPTG and tested for AR. Cells were diluted into fresh EG medium and grown to an OD₆₀₀ of 0.4. At that point the culture was diluted 1/10 into medium at pH 2.5 (final pH) with no supplement (no addition) or containing 1.5 mM glutamic acid or 1.0 mM arginine. The percent survival in EG medium at pH 2.5 4 h after the addition of glutamate or arginine is shown.

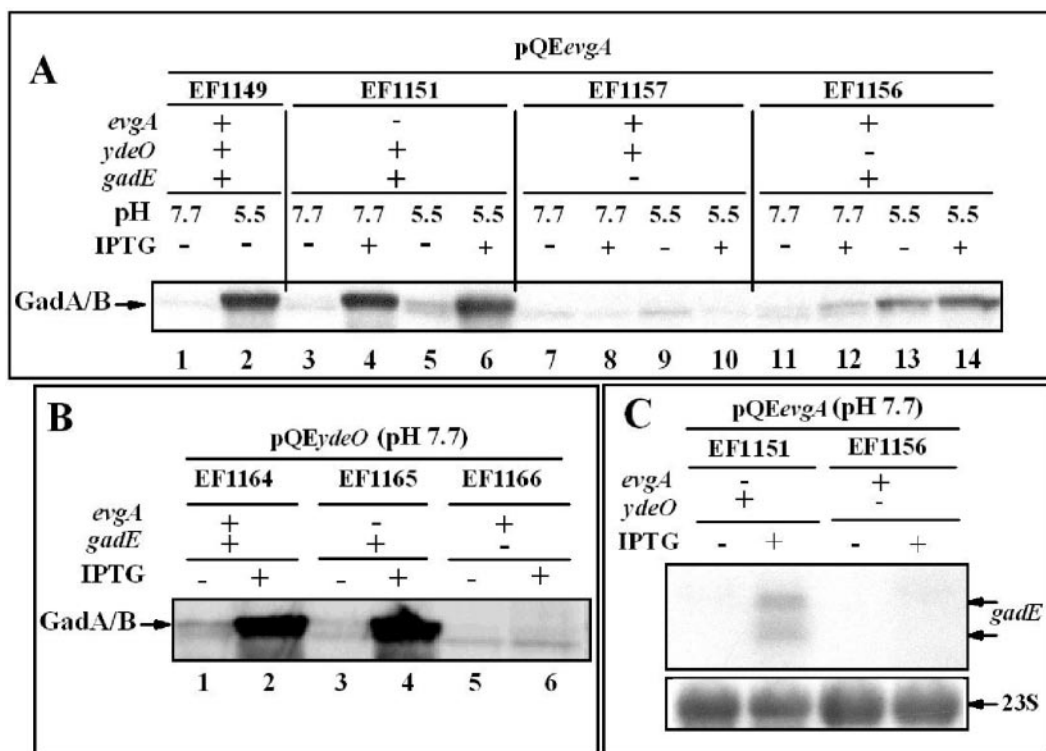


FIG. 1. Induction of GadA or GadB by overexpressing EvgA and YdeO is dependent on GadE. Cells were grown to log phase in minimal EG medium with 1.5 mM IPTG at the pH values indicated. IPTG was added (+) to induce expression of cloned genes *evgA* (A) and *ydeO* (B). Five micrograms of protein from each extract was separated on an SDS–10% polyacrylamide gel and probed with anti-Gad antibody. (C) RNA was extracted from log-phase cells grown in minimal medium at pH 7.7. RNA (5 μ g) was loaded onto 1.2% agarose-formaldehyde denaturing gels and probed for *gadE* mRNA. The smaller, cross-reacting band seen in these figures is not related to either GadA or GadB. The band is still observed in a *gadA gadB* double mutant. 23S rRNA was used as the loading standard in the lower blot.

Overexpression of EvgA or YdeO increases glutamate decarboxylase production at pH 7.7 in a GadE-dependent fashion. The published overexpression study found that the increase in AR caused by overexpressing EvgA was dependent on YdeO and GadE (17). Whether increased AR was the result of concomitant overexpression of glutamate decarboxylase was not tested. Glutamate decarboxylase is essential for high-level AR (4). The results of Western blot analysis presented in Fig. 1 indicate that elevating EvgA levels will increase GAD production in log-phase cells grown in minimal medium (Fig. 1A, compare strains EF1149 [lane 1] and EF1151 [lanes 3 and 4]). The results in Fig. 1A, lanes 4 and 6 versus lanes 8 and 10, illustrate that when EvgA was overexpressed, GAD production increased in a GadE-dependent fashion but was only partially YdeO-dependent (Fig. 1A, lanes 12 and 14). The latter result is the first indication that EvgA may also affect GAD induction through a YdeO-independent route (see below).

The linear EvgA-dependent pathway, as originally proposed, proceeded from EvgA to YdeO to GadE (17). Consistent with this idea, overexpressing YdeO also increased GAD production and relied on GadE to do so (Fig. 1B, compare lanes 2 and 6). The effect was not dependent on EvgA (Fig. 1B, compare lanes 2 and 4). The Northern blot shown in Fig. 1C indicates that EvgA overexpression does induce GadE and that this induction is heavily dependent on YdeO. These results are consistent with the AR phenotypes reported earlier and argue

that overexpression-dependent AR is due, at least in part, to increases in GadE, and thus, GadA, GadB, and GadC (Table 3) (16).

Under natural inducing conditions, *evgA* and *ydeO* contribute to glutamate-dependent AR additively. The overexpression results suggested a linear control circuit proceeding from EvgA to YdeO to GadE, which would then activate the *gadA* or *gadBC* genes (13, 17). However, as powerful as overexpression strategies are at revealing regulatory circuits, abnormally high levels of a regulatory protein could lead to inadvertent, and perhaps physiologically irrelevant, cross talk between regulatory systems (31, 33). Consequently, we tested the relevance of the proposed EvgA-YdeO-GadE circuit under conditions that do not involve overexpression. Mutants defective in these regulators were grown under log-phase and stationary-phase growth conditions that naturally lead to AR. Neither single nor double mutations in *evgA* and *ydeO* affected stationary-phase-induced glutamate-dependent AR (data not shown), but when cells were grown to log phase in minimal medium, *evgA* and *ydeO* mutants exhibited reduced AR (Fig. 2). However, in contrast to what one would predict from a linear regulatory pathway, the *evgA* mutation had a greater effect on AR than the *ydeO* mutation, suggesting that EvgA and YdeO have separable effects on AR (Fig. 2, compare EK592 to EK593 to EK595).

EvgA and YdeO activate GAD expression in exponential-phase cells grown in minimal medium. The previous result established that EvgA and YdeO activate glutamate-depend-

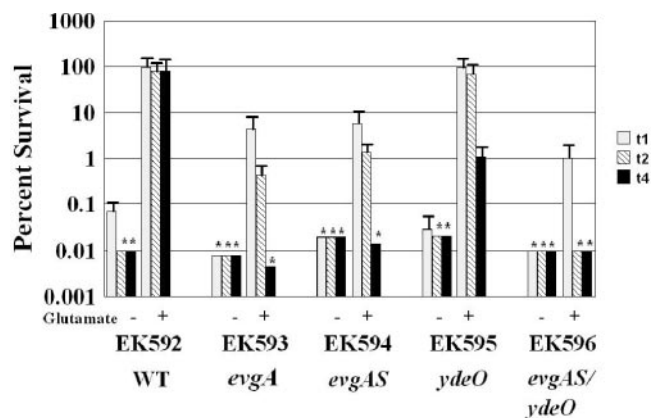


FIG. 2. Effects of *evgA*, *ydeO*, and *gadE* mutations on glutamate-dependent AR. The effects of the individual mutations on AR are shown. Cells were grown to exponential phase in minimal EG medium at pH 5.5 and tested for AR at pH 2.5 as described in Table 3, footnote a. All cultures grown at pH 7.7 were acid sensitive (data not shown). Cells were diluted 1:10 as described in Materials and Methods. The final pH was pH 2.5. The medium either contained no additives (-) or contained glutamate as indicated (+). Asterisks indicate that the result was below the limit of detection indicated by the bar. Viable counts were determined 1, 2, and 4 h after acid challenge (t1, t2, and t4, respectively). WT, wild type.

dent AR under physiological conditions. We next examined whether the AR effects of *evgA* and *ydeO* mutations correlated with decreases in glutamate decarboxylase expression. The *gadA* and *gadBC* genes are normally induced at pH 5.5 in exponential-phase cultures growing in minimal medium containing glucose. Densitometric analysis of GAD Western blots revealed

that *GadA* and *GadB* expression in an *evgA* mutant grown at pH 5.5 was four- to fivefold lower than that in the wild-type control (Fig. 3A, EK594). The *ydeO* mutation lowered expression only approximately twofold (Fig. 3A, EK595). Thus, *EvgA* has a greater effect than *YdeO* on *gadA* or *gadBC* expression.

The effects of *EvgA* and *YdeO* on GAD production also appear additive (Fig. 3A, EK596). If regulation occurred only via the linear *EvgA*-*YdeO*-*GadE* circuit, one would predict that *GadA* or *GadB* expression in an *evgA ydeO* double mutant would be no different than in an *evgA* single mutant. These results paralleled the AR data shown above and suggest that under physiological conditions much of the *EvgA* effect on GAD production occurs by a route other than through *EvgA* control over *ydeO*.

***EvgA* and *YdeO* affect transcription of *gadE*.** Next we tested whether *EvgA* and *YdeO* affected *gadE* expression under physiological conditions. The results of Northern blot analysis shown in Fig. 3B revealed that *EvgA* (compare lanes 1 and 2) had a greater effect on *gadE* transcription than *YdeO* (compare lanes 1 and 3). This finding was consistent with the decarboxylase Western blot (Fig. 3A) and AR (Fig. 2) results. These results also confirm that *EvgA* affects *gadE* expression in *YdeO*-dependent and -independent pathways.

We have, to this point, been assuming that the acid-sensitive phenotypes of *evgA* and *ydeO* mutants were due to the loss of glutamate decarboxylase. However, *evgA* and/or *ydeO* mutants might be acid sensitive because of decreased expression of other genes under their control. Since *GadE* is the essential activator of *gadA* and *gadBC*, we tested whether overexpression of *GadE* could suppress the acid-sensitive phenotype of an *evgA ydeO* mutant. Figure 3C illustrates that the acid-sensitive

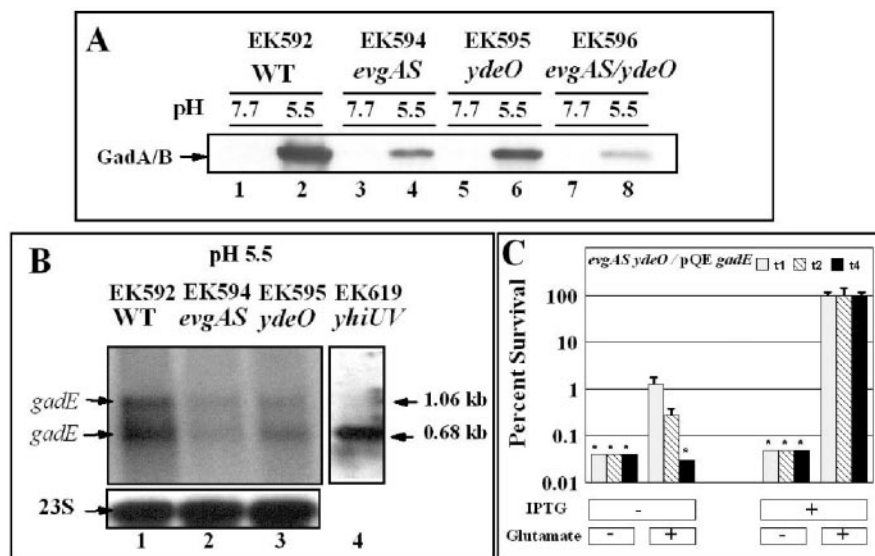


FIG. 3. Effects of *evgA* and *ydeO* on *gadA* and *gadB* expression (A), *gadE* expression (B), and complementation of the *evgA ydeO* AR phenotype by *GadE* (C). Cells were grown to exponential phase (OD_{600} of 0.4) in minimal EG medium and analyzed for *GadA* and *GadB* protein levels by Western blotting (A) as noted in the legend to Fig. 2 and for *gadE* mRNA by Northern blotting (B). Northern blots were performed on 5 μ g of total RNA that was electrophoresed through 1.2% agarose-formaldehyde denaturing gels and probed with a 0.534-kb *gadE* probe. C. Complementation of the *evgA ydeO* acid-sensitive phenotype by *GadE* expression. Cells (EF1348) were grown as in panel A but with (+) or without (-) IPTG to induce the *pQEgadE* plasmid. The slight increase in resistance seen without glutamate is thought to be due to the effects of *GadE* overexpression on target genes other than *gadABC* that aid AR. Viable counts were determined 1, 2, and 4 h after acid challenge (t1, t2, and t4, respectively). WT, wild type.

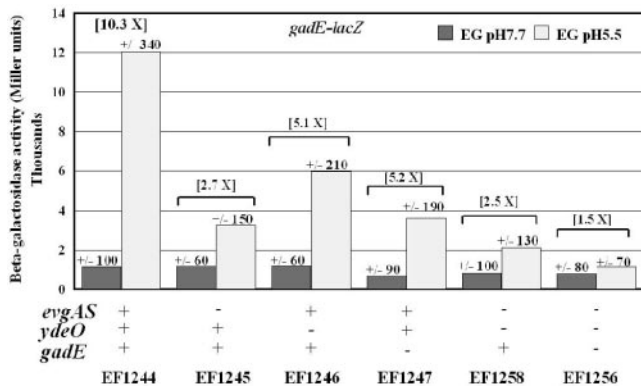


FIG. 4. Effects of *evgAS*, *ydeO*, and *gadE* on *gadE-lacZ* expression. Strains containing a *gadE-lacZ* operon fusion (positions -804 to +28 relative to the *gadE* translational start) inserted at the *trp* operon were grown to exponential phase (OD₆₀₀ of 0.4) in minimal EG medium (containing 0.3 mM tryptophan) at different pH values and assayed for β-galactosidase activity. Values are means ± standard errors of the means (error bars) for four experiments.

phenotype of an *evgA ydeO* mutant was completely reversed by overexpressing GadE. This would not happen if YdeO or EvgA activated other genes necessary for AR. Thus, the acid-sensitive phenotype of *evgA* and *ydeO* mutants appears to be directly and solely due to the loss of *gadE* expression and the subsequent loss of glutamate decarboxylase.

GadE is autoregulated. A variety of *gadE-lacZ* operon fusion strains were constructed to confirm the Northern blot results and begin detailed studies of the *gadE* control region (Fig. 4). The control strain, EF1244, clearly showed that *gadE* expression is induced by acid (10-fold). When *gadE-lacZ* expression was tested in the presence of various mutations, *evgA* had a greater effect on expression than did *ydeO* (Fig. 4, compare EF1245 and EF1246). These results correlate with those of the *gadE* Northern and GAD Western blots shown above and indicate the fusion acts appropriately.

Data from Fig. 3 suggested that EvgA and YdeO might not be the only activators of *gadE*, since an *evgA ydeO* double mutant still exhibited acid-induced expression of *gadA* or *gadB* (Fig. 3A, lanes 7 and 8). Since the *gadE* promoter region contained three potential GAD boxes (see below) and because GadE binds to the known GAD box, we questioned whether *gadE* itself might be that other regulator. To answer this question, we used the chromosomal *gadE-lacZ* operon fusion inserted at the *trp* locus to create a *gadE*⁺/*gadE-lacZ* merodiploid situation. The expression of *gadE-lacZ* in *gadE*⁺ and *gadE* mutant backgrounds was then compared. The data presented in Fig. 4 indicate that GadE does help activate its own expression (Fig. 4, compare EF1244 and EF1247). LacZ production in the *gadE* mutant exhibited a decrease of approximately threefold from that of the wild type under acid conditions but could still be induced by acid (showing a fivefold increase). A strain in which all three regulators were mutated failed to induce *gadE-lacZ* at all (Fig. 4, EF1256). The accumulated data suggest that EvgA, YdeO, and GadE all contribute to *gadE* induction in response to acid. The results also reveal that the *gadE* and *evgA ydeO* mutations lowered pH 7.7 expression, further indication that these genes have a general role in *gadE* expression.

The data in Fig. 4 also indicate that the three regulators have independent effects on *gadE-lacZ* expression. For example, the *ydeO*, *ydeO evgA*, and *ydeO evgA gadE* mutants demonstrate a stepwise decrease in *gadE-lacZ* expression relative to the wild type. The decreases were 2-, 5-, and 12-fold, respectively (12,000 ± 300 Miller units for the wild type, 5,960 ± 150 Miller units for the *ydeO* mutant, 2,100 ± 190 Miller units for the *ydeO evgA* mutant, and 1,100 ± 70 Miller units for the *ydeO evgA gadE* mutant). The accumulated results indicate that each regulator affects *gadE* expression individually and in an additive manner.

Location of the *gadE* promoter. Primer extension analysis was then used to identify the *gadE* transcriptional start site. The results indicate that the start site is an A positioned 21 bp upstream of the translational start codon (Fig. 5). Figure 1C and 3B revealed two *gadE* transcripts, one approximately 0.680 kb and the other around 1.06 kb in size. Attempts to find a second, upstream promoter failed. Six other oligonucleotides spanning the 777-bp region upstream of the identified start site failed to hybridize to either transcript. We have also demonstrated that the second transcript does not represent cross hybridization, since both transcripts disappeared in a *gadE* deletion mutant (13). The second, smaller transcript seen in Fig. 1C and 3B is likely the result of alternative termination or processing sites downstream of *gadE* (see below).

Hommais et al. reported two promoters for *gadE* located 91

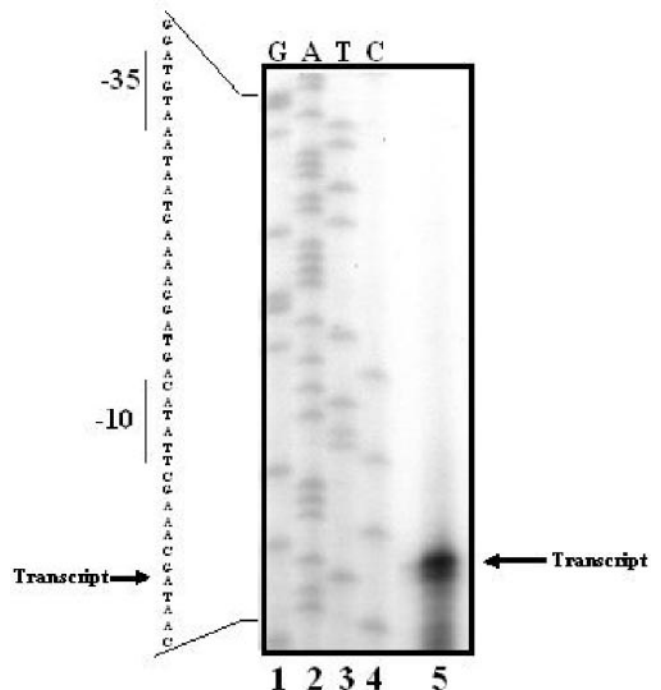


FIG. 5. Primer extension analysis of *gadE* transcription. RNA was extracted from log-phase cells grown in minimal medium at pH 5.5 (OD₆₀₀ of 0.4). The RNA was subjected to primer extension analysis as described in Materials and Methods. Dideoxy sequencing ladder of the fragment used for primer extension is shown in lanes 1 to 4. Primer extensions were performed on RNA extracts using labeled oligonucleotide 624. The DNA sequence using the same oligonucleotide is shown to the left. However, the sequence autoradiograph was reversed to show the sequence of the coding strand.

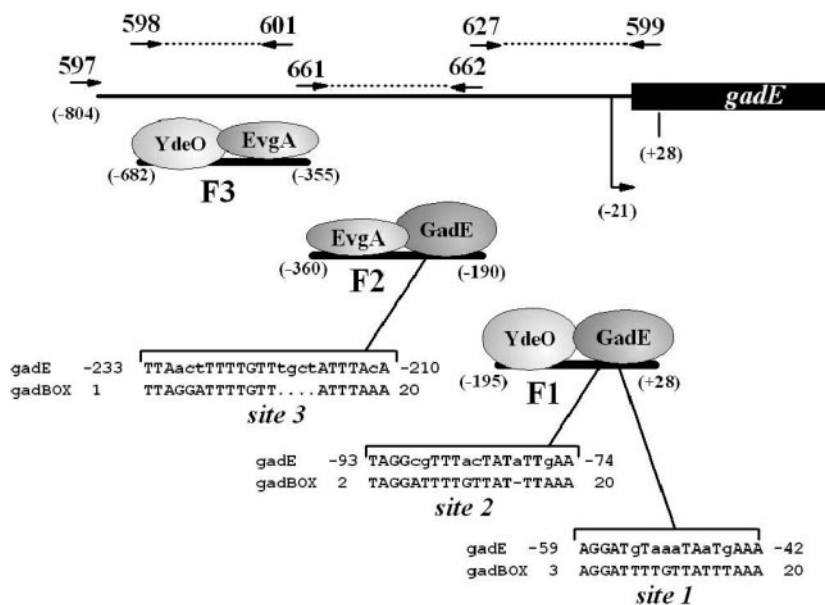


FIG. 6. *hdeD-gadE* intergenic region. The primers used to generate fragments F1, F2, and F3 for EMSA are numbered. Nucleotide distances relative to the *gadE* translational start are noted in parentheses. The location of the *gadE* transcriptional start site is indicated by the bent arrow. Sequences in the *gadE* upstream region that exhibit homology to GAD boxes are identified in fragments 1 and 2 and are shown aligned with the 20-bp GAD box sequence; nonaligned bases are indicated by lowercase letters. EMSA results presented in Fig. 7 are summarized using ovals to represent the different purified regulators.

and 125 bp upstream of the start codon (10). We performed primer extensions using oligonucleotides 624 and 599, which bind to *gadE* mRNA +18 and +5 bp from the ATG start codon, respectively. These oligonucleotides should have easily revealed any significant transcripts within at least 200 bp. Only the transcript starting at -21 bp from the presumed ATG was identified. We cannot offer an explanation for the absence of the other transcripts other than that the earlier study used an *hns* mutant and a plasmid to express *gadE*.

If the two *gadE* transcripts shown in Fig. 3B initiate from the same promoter but reflect different termination sites downstream of *gadE*, then mutations in *evgA* or *ydeO* should reduce both transcripts similarly. Figure 3B illustrates that mutations in either regulator caused parallel decreases of both *gadE* transcripts, although *evgA* had a greater effect on these transcripts than *ydeO* did. We also demonstrated that the larger *gadE* transcript disappears when the *yhiUV* genes, located 338 bp downstream of *gadE*, were deleted (EK619 [Fig. 3B]). The *gadE* ORF itself is 528 bp long. This suggests that termination of the larger transcript occurs within the *yhiU* ORF region.

EvgA, GadE, and YdeO directly bind the *gadE* promoter. Initial EMSA experiments revealed that all three regulators bound to a 748-bp fragment representing most of the 798-bp intergenic region between *hdeD* and *gadE* (data not shown). We then divided this large fragment into three smaller fragments of 223 bp (F1), 171 bp (F2), and 373 bp (F3) as illustrated in Fig. 6. Figure 7 indicates that GadE bound to fragments F1 and F2 (Fig. 7A), EvgA bound to F2 and F3 (Fig. 7B), while YdeO bound to F1 and F3 but not to F2 (Fig. 7C). The results are consistent with all three regulators binding directly at the *gadE* promoter region.

A 20-bp, totally conserved DNA sequence called the GAD box was previously identified upstream of the *gadA* and *gadBC*

genes (3). Each is centered at -63 bp from the transcriptional start sites. The GAD boxes in both of those control regions are essential for expression of the downstream gene and bind purified GadE protein in EMSA experiments (3, 13). Until now, *gadA* and *gadB* were the only two functional GAD boxes known, although others have been predicted (30). Figure 6 illustrates that three potential GAD box sequences were found in fragments 1 and 2, but none were noted in fragment 3. This is consistent with the GadE binding results shown in Fig. 7. Fragment 1 contains two potential GAD box sequences, one site contains 14 of 20 bases (70% identity), while the other site contains 12 of the 20 GAD box bases (60% identity). Fragment 2 possesses one GAD box sequence exhibiting 80% identity to the published GAD box, but the sequence in fragment 2 is interrupted by an imperfect 4-bp repeat TGCT. Site 2 in fragment 1 is centered -63 bp from the *gadE* transcriptional start site, a distance equivalent to the distance of the GAD box sequences of *gadA* and *gadB* from their transcriptional start sites. Of the two other potential GAD boxes, site 1 is only 20 bp from the transcriptional start (overlapping the putative -35 site), while site 3 lies 189 bp from the transcriptional start site.

We then examined whether purified GadE would bind to any of these putative GAD box sequences in vitro. Sequences of 32 to 36 bp containing the different GAD box sites were synthesized and used in EMSA experiments with purified GadE. The results, shown in Fig. 8, reveal that GadE will bind each of the GAD box sites, but not to a fragment lacking a GAD box (fragment F).

To confirm a role for fragments 2 and 3 in regulating *gadE*, we constructed *gadE-lacZ* fusion strains in which one or both of these fragments was missing through deletion. The results presented in Table 4 illustrate that loss of fragment 3 (EF1304) reduced pH 5.5 expression approximately 2.5-fold (from 3,520

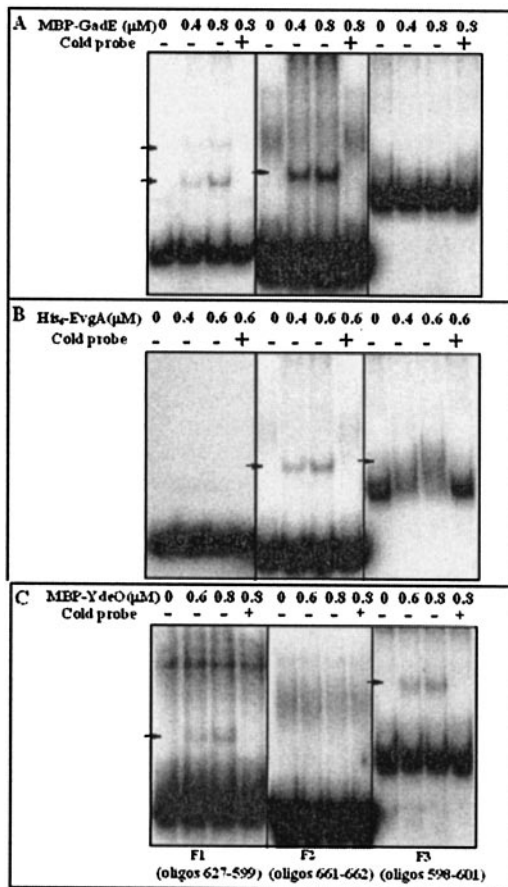


FIG. 7. Electrophoretic mobility shifts of *gadE* promoter region fragments by EvgA, YdeO, and GadE. Fragments corresponding to those described in the legend to Fig. 6 were radiolabeled as described in Materials and Methods, and various concentrations of purified protein were added prior to electrophoresis through a 5% polyacrylamide gel. Some lanes also contained (+) unlabeled, specific competitor DNA (cold probe) as indicated. A. MBP-GadE. B. His₆-EvgA. C. MBP-YdeO. Bands in the middle of lanes 5 to 8 in panels A and C were artifacts of PCR. oligos, oligonucleotides.

Miller units in strain EF1303 to 1,350 Miller units in strain EF1304). Loss of fragments 3 and 2 (strain EF1305) reduced *gadE* expression almost sixfold, indicating that both fragments contribute to overall GadE production. The deletions also lowered pH 7.7 expression. Interestingly, removing these upstream sequences did not eliminate acid induction of *gadE*, suggesting that either all three regulators contribute to pH control (Fig. 4) or another, undetermined factor regulates acid induction of *gadE*. Full-length fusions were expressed differently in the strains in Table 4 (EF1303) than in the strains in Fig. 4 (EF1244) because of the larger amount of *gadE* ORF included in the Table 4 strains.

EvgA-YdeO-GadE form a bifurcated regulatory circuit with partial feedback control. We presented evidence above that EvgA and YdeO directly activate *gadE*. Figure 9A illustrates, using a Northern blot, that *ydeO* is also acid induced and that EvgA is needed to activate *ydeO* expression. When the effect of GadE on *ydeO* expression was examined, we found that GadE exhibited partial negative control over *ydeO* expression (Fig. 9B, compare lanes 2 and 4). An *evgA-lacZ* fusion gene was then

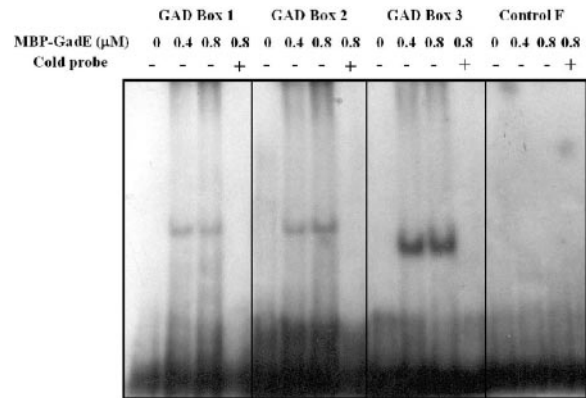


FIG. 8. Binding of MBP-GadE to potential GAD box sequences in the *gadE* control region. Sequences (32 bp) containing GAD box site 1 (oligonucleotide 724 annealed to oligonucleotide 725) and GAD box site 2 (oligonucleotide 726 annealed to oligonucleotide 727) and a 36-bp sequence containing GAD box site 3 (oligonucleotide 728 annealed to oligonucleotide 729) were made and used in EMSA experiments as described in Materials and Methods and in the legend to Fig. 7. The negative-control fragment was a sequence from the *gadA* promoter region made using oligonucleotides 586 and 587 and does not contain a GAD box.

constructed to examine whether this operon was also under acid control and whether YdeO or GadE affected expression. The results indicated that *evgAS* is not acid induced and is not subject to autoinduction and that neither YdeO nor GadE had any major effect on *evgA* expression (data not shown). The fact that EvgA did not autoregulate its expression seems to contradict previous in vitro gel shift results, indicating that purified EvgA caused a shift in an upstream *evgA* sequence that contained a putative EvgA box (17).

DISCUSSION

E. coli is a remarkably acid-resistant neutralophilic microorganism that prefers growth near neutral pH, but is able to withstand transient exposures to pH 2 environments for hours. This property appears designed to protect the organism as it moves through the acidic stomach toward the more hospitable pH environment of the intestine. Glutamate-dependent AR is the most efficient of the four systems that provide this protection (4, 11). The results of this and prior publications indicate that the system is regulated by multiple complex regulatory loops that allow the cell to anticipate future encounters with extreme acid stress. The Crp-RpoS-GadX-GadW loop (called

TABLE 4. Contribution of upstream DNA sequences to *gadE-lacZ* operon fusions

Operon fusion	β-Galactosidase activity ^a		Fold induction
	EG at pH 7.7 ^b	EG at pH 5.5	
EF1303 (-804 to +331) ^c	729 ± 60	3,520 ± 90	5×
EF1304 (-360 to +331)	180 ± 30	1,350 ± 25	7.5×
EF1305 (-195 to +331)	210 ± 50	720 ± 70	3.5×

^a β-Galactosidase activity is expressed in Miller units. Values are the means ± standard errors of the means for three experiments. For ease of construction, the fusions were made to nt +331 rather than +28, as shown in Fig. 5.

^b Cells were grown to exponential phase (approximately 2 × 10⁸ cells/ml).

^c Numbers reflect the extent of upstream sequence fused to *lacZ*.

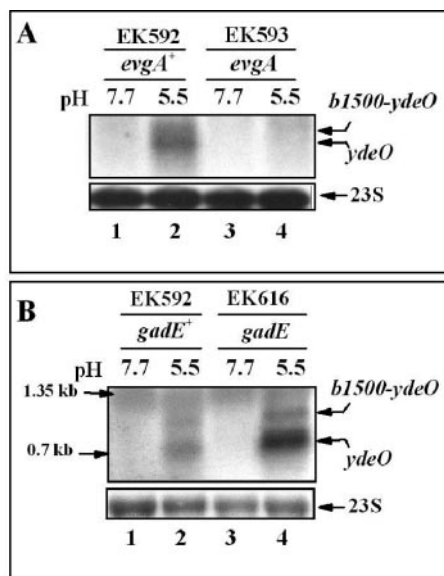


FIG. 9. Northern blot analysis of the effects of EvgA (A) and GadE (B) on *ydeO* expression. Cells were grown to exponential phase, and total RNA was extracted and processed for Northern blot analysis as described in the legend to Fig. 3B. Five milligrams of RNA was run per lane. 23S rRNA was used as the loading control.

the GadXW circuit) is most important in complex medium, while the EvgA-YdeO-GadE activation circuit, described here, is important in exponential-phase cells growing in minimal medium (Fig. 10).

The two-component regulators EvgS (sensor-kinase) and EvgA (response regulator) are highly homologous to the virulence-related BvgAS system of *Bordetella pertussis*. EvgAS is a two-component regulatory system with unknown function but has been the subject of several studies designed to determine its physiological role. The first hint of function was found in a spontaneous mutation that led to constitutively active EvgS and overexpression of the EmrKY multicomponent drug efflux pump (7). Subsequent microarray studies have exposed numerous other genes affected by this constitutively active mutant or by the overexpression of EvgA. Many of the genes uncovered were associated with drug resistance (7, 16, 17, 19, 21). Work by Masuda and Church first implicated EvgAS in control over AR (16, 17).

The EvgA-dependent GadE activation circuit functions in exponential-phase cells growing in minimal medium containing glucose. EvgA directly activates *gadE* without assistance from YdeO, and YdeO can activate *gadE* without EvgA. However, EvgA can also activate *ydeO*. The data further indicates that GadE activates itself and represses *ydeO* in a partial feedback loop. Thus, as GadE is produced, it will begin to shut down the YdeO activation pathway but stimulate its own synthesis. Our results verify an earlier gene array study suggesting that GadE might autoregulate expression (10).

EvgA, YdeO, and GadE proteins all demonstrated an ability to bind different fragments within the 798-bp intergenic region between *hdeD* and *gadE*. GadE binding sites were discovered in the upstream intergenic sequences shown to bind GadE (Fig. 6). However, no sites were found corresponding to a predicted EvgA binding consensus sequence, and nothing is

known of potential YdeO binding sites. The EvgA consensus sequence was recently proposed on the basis of sequence comparisons between six EvgA-regulated genes (17). It is possible that the EvgA binding site is broader than expected. For example, another report found that EvgA could bind upstream of *yhiU* in a region that also lacks the proposed EvgA consensus site (20).

An interesting question posed by these studies is how the three regulators might collaborate to mediate control of *gadE*. The EMSA results indicate that each regulator is capable of binding to different pairs of three regions within the *gadE* promoter region. This pattern could indicate complex DNA looping arrangements, although protein-protein interactions between regulators have not yet been demonstrated. The promoter region for *gadE* (Fig. 6) looks like a classical positively regulated promoter with negligible -35 motif conservation and a weak -10 motif strengthened by TG immediately 5' to the putative -10 motif (1). We predict that these three binding proteins function to facilitate RNA polymerase recruitment and/or binding to this promoter region.

It is also not apparent how the GadE activation circuit shuts off. Efficient activation likely requires interactions between the various regulators and small signal molecules that accumulate under different environmental conditions. There is evidence that, in the absence of signal, GadE protein might naturally degrade and not be replaced. For instance, in spite of what appears to be ample *gadE* mRNA, it has been impossible to observe native intracellular GadE levels by Western blotting. The anti-GadE antibody used easily reveals MBP-GadE in whole-cell extracts, but not native nor His₆-tagged GadE, suggesting that the smaller proteins may be subject to rapid turnover (data not shown). Rapid turnover of GadE coupled with changes in coeffector concentrations as cells leave inducing environments could shut down expression.

The data also do not fully explain the acid induction of *gadE*. Induction is not due to pH effects on the production of EvgA on the basis of *evgA-lacZ* results (data not shown). It is tempting to propose that acid pH alters the phosphorylation status of EvgA, which in turn would influence the acid induction of *ydeO* and *gadE*. However, induction of *gadE* cannot be due solely to a potential change in EvgA activity, since *gadE* was induced by

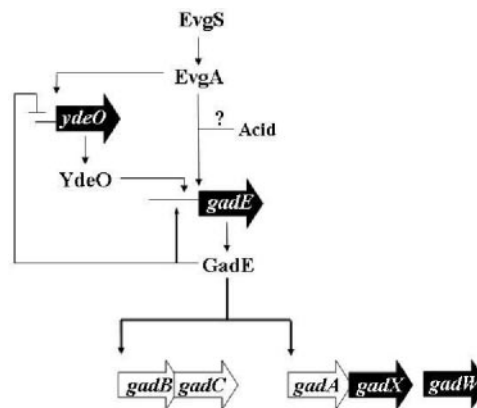


FIG. 10. EvgA-YdeO-GadE branched pathway regulating glutamate-dependent AR. See text for details.

acid in an *evgA* mutant, although to a reduced level (Fig. 4). In fact, no one regulator could be linked to acid induction, although *ydeO* was itself induced by acid (Fig. 9A). The results suggest that these regulators either all contribute to acid induction or there is some other regulatory feature that dictates pH control. Hommais et al. suggest that the promoter regions of *gadE* and other genes within the proposed acid fitness island encompassing *gadE* have a high propensity for helix disruption that might make them easily transcribed (10). They proposed the presence of an efficient locking mechanism involving H-NS that prevents inadvertent expression during normal growth. If this is correct, the decrease in internal pH from 7.8 to around 7.4 during growth at pH 5.5 might be enough to destabilize the locking mechanism. The regulatory proteins noted here could either assist in destabilization to unlock the promoter or perform tasks subsequent to unlocking, such as communicating with RNA polymerase.

The results of the microarray study also suggested that AR caused by YdeO overexpression depended on the *slp-yhiF*, *hdeA*, and *hdeD* genes (17). However, under conditions commonly used to naturally induce AR, none of these genes were required (data not shown). This suggests that another pathway of AR might exist, but the actual inducing conditions remain obscure. A recent report has suggested the existence of one such pathway employing the *asr* product (24).

In addition to demonstrating an EvgA-dependent pathway, the results here suggest the existence of an EvgA-independent pathway that activates *gadE* in stationary-phase cells grown in complex medium. Clearly, the regulatory web governing glutamate-dependent AR is vast, now encompassing 10 regulators.

Why does AR require all this regulation? It is important to note that the induction of AR probably does not occur in the stomach, at least not to a great extent. We predict that induction occurs some time before ingestion (e.g., during stationary-phase growth in the intestine). Thus, one possible reason for this extensive control is that the system may be rigged to induce under many different environmental conditions that could presage an encounter with extreme acid stress, such as gastric acidity. Each growth condition might trigger a different metabolic signal recognized by different regulators within the system. This complexity must reflect the importance of surviving extreme acid stress.

The EvgA-YdeO-GadE circuit also appears to affect cell physiology beyond AR. Gene array studies examining the regulatory reach of the GadX, GadE, and EvgA regulators indicate that each regulator controls numerous genes (7, 10, 17, 19, 21, 30). Some are clearly connected to AR, others display characteristics hinting at a possible connection, while several lack any obvious relationship to acid stress survival. The broad impact these regulators have on gene expression suggests a metabolic importance that remains unexplored.

ACKNOWLEDGMENTS

We thank Patricia Couling for help in preparing the manuscript.

This work was supported by National Institutes of Health award R01-GM61147.

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