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Genes of the GadX-GadW Regulon in *Escherichia coli*

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Acid in the stomach is thought to be a barrier to bacterial colonization of the intestine. *Escherichia coli*, however, has three systems for acid resistance, which overcome this barrier. The most effective of these systems is dependent on transport and decarboxylation of glutamate. GadX regulates two genes that encode isoforms of glutamate decarboxylase critical to this system, but additional genes associated with the glutamate-dependent acid resistance system remained to be identified. The *gadX* gene and a second downstream *araC*-like transcription factor gene, *gadW*, were mutated separately and in combination, and the gene expression profiles of the mutants were compared to those of the wild-type strain grown in neutral and acidified media under conditions favoring induction of glutamate-dependent acid resistance. Cluster and principal-component analyses identified 15 GadX-regulated, acid-inducible genes. Reverse transcriptase mapping demonstrated that these genes are organized in 10 operons. Analysis of the strain lacking GadX but possessing GadW confirmed that GadX is a transcriptional activator under acidic growth conditions. Analysis of the strain lacking GadW but possessing GadX indicated that GadW exerts negative control over three GadX target genes. The strain lacking both GadX and GadW was defective in acid induction of most but not all GadX target genes, consistent with the roles of GadW as an inhibitor of GadX-dependent activation of some genes and an activator of other genes. Resistance to acid was decreased under certain conditions in a *gadX* mutant and even more so by combined mutation of *gadX* and *gadW*. However, there was no defect in colonization of the streptomycin-treated mouse model by the *gadX* mutant in competition with the wild type, and the *gadX gadW* mutant was a better colonizer than the wild type. Thus, *E. coli* colonization of the mouse does not appear to require glutamate-dependent acid resistance.

To colonize the mammalian gastrointestinal tract, *Escherichia coli* must survive passage through the acidic environment of the stomach. This barrier to colonization apparently is overcome by three distinct systems for acid resistance (AR), which overlap in ways that protect both growing and nongrowing cells from acid over a broad range of environmental conditions (4). The mechanism of glucose-repressed AR (system 1) is not known. The two amino acid decarboxylase-dependent AR systems are thought to consume protons that leak into the cell during acid stress by decarboxylating glutamate (system 2) or arginine (system 3), which are transported into the cell in exchange for their respective decarboxylation products (23). The most effective *E. coli* AR system is dependent on glutamate and comprises at least three genes, *gadA* and *gadB*, encoding isoforms of glutamate decarboxylase, and *gadC*, encoding a γ-aminobutyrate antiporter. It is clear that additional genes must participate in the glutamate-dependent AR, but these genes remain to be identified (4).

Growth in acidified medium, (27), treatment with acetate (1), and entry into stationary phase (21) all cause induction of *gadBC*, *gadA*, and other genes in the *gadA* region. The regulation of these genes is complex and involves the transcription factors RpoS (3, 14), cyclic AMP (cAMP)-cAMP receptor protein (CRP) (3), HN-S (11), and EvgA (15). Additionally, the *gadX* gene, located immediately downstream of *gadA*, encodes an AraC-type transcription factor that is thought to be an activator of the glutamate-dependent AR genes. This hypothesis is based primarily on evidence that overproduction of GadX induces *gadA*, *gadB*, *hdeAB*, and *hdeD* (11, 22, 26). We previously identified a highly conserved 20-bp sequence element upstream of *gadA* and *gadB* that is required for pH-dependent control of their transcription (3, 14). This 20-bp sequence might be the target of binding by GadX. However, DNA footprinting indicates that the purified MalE-GadX fusion protein binds nonconserved sequences in the *gadA* and *gadB* promoter regions that only partially overlap the 20-bp sequence element (26). Thus, the mechanism by which GadX activates *gadA* and *gadB* is unclear.

We recently identified three acid-inducible transcription factor genes besides *gadX* in the *gadA* region, *gadW* (*yhiW*), *yhiE*, and *yhiF* (27). Mutational analysis indicates that *yhiE*, but not *yhiF*, has a significant effect on the AR phenotype (15, 27). Analysis of the gene targets of EvgA identified a fifth transcription factor gene, *ydeO*, which, when mutated, also confers loss of AR (15). YdeO is an AraC-like transcription factor, as

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are GadX and the product of the *gadW* gene, which lies immediately downstream of *gadX*. Results we report elsewhere indicate that GadW inhibits the GadX-dependent activation of *gadA* and *gadBC* transcription, yet GadW activates their transcription in the absence of GadX (14). GadW binds the *gadA* and *gadB* regulatory regions in vitro, and GadX and GadW form heterodimers in vivo (14).

Thus, it is becoming clear that regulation of the genes involved in glutamate-dependent AR is complex, perhaps involving a regulatory cascade that integrates the various environmental signals that impinge on these genes (15). The available evidence points to GadX, working together with GadW, as being directly involved in the pH-dependent activation of the glutamate-dependent AR genes (14). Therefore, GadX and GadW may function at the bottom of this regulatory cascade. However, there has been no attempt to systematically identify the gene targets of the GadX-GadW regulon. In this study, we deleted the *gadX* and *gadW* genes by allelic replacement with antibiotic resistance cassettes, both individually and in combination, and demonstrated that the *gadX* and *gadX gadW* mutants are reduced in their survival in acid. To identify other genes under GadX-GadW control, we compared the whole-genome expression profiles of the wild-type parent strain with those of the mutants growing in logarithmic phase at pH 7.4 and in acidified minimal growth medium. A regulon of 15 genes in 10 GadX-GadW-dependent transcription units was characterized by reverse transcriptase mapping of the operons and sequence comparison of their respective regulatory regions.

MATERIALS AND METHODS

Bacterial strains and growth. Mutants were constructed by allelic replacement with a kanamycin resistance cassette, as previously described (7). Phage P1 transduction, transformation with $CaCl_2$, and electroporation were performed by standard methods (20). All cultures used for genomic expression profiling were grown in the minimal medium developed for *E. coli* proteome studies (19). Glucose (0.2%) was the sole carbon and energy source. Morpholinepropanesulfonic acid (MOPS) was used as the buffer for pH 7.4 media, and morpholineethanesulfonic acid (MES) was used to buffer the pH 4.5 and 5.5 media. Cultures were grown aerobically with 300-rpm agitation at 37°C in 50 ml of medium in 250-ml Fleakers (Corning, Acton, Mass.). Growth was monitored by measuring the optical density at 600 nm (DU-530 Life UV/visual spectrophotometer; Beckman Instruments Inc., Fullerton, Calif.). Antibiotics were used at the following concentrations: ampicillin, 60 μg/ml; kanamycin, 25 μg/ml. All strains were grown at 37°C with aeration.

Gene expression profiling and treatment of data. Since both the rate of growth and total yield of biomass were affected by alterations in the medium pH, the shape of the growth curve was used to determine the midpoint in logarithmic growth for cell harvest. The methods used for handling whole-genome *E. coli* arrays and data analysis have been described in detail previously and are available on our website (http://www.ou.edu/microarray) (6). Total RNA was extracted from cell culture (4 to 5 ml) diluted (1:1) into ice-cold RNA-Later (Ambion) immediately upon culture sampling, and purified with RN-easy columns (Qiagen). RNA samples were treated with DNase (RNase-free DNase I; Ambion) for 1 h at 37°C to remove any residual DNA, and the DNase-treated samples were repurified with RN-easy columns (Qiagen). Total RNA and the C-terminal primer set (Sigma-GenoSys) were used to synthesize radiolabeled [α^{33}-P]dCTP cDNA (first-strand synthesis) with Superscript II reverse transcriptase (Invitrogen). Matched pairs of *E. coli* Panorama membrane arrays from consecutive printing runs were used throughout this study (Sigma-GenoSys). Hybridization and stripping of membranes were described previously (5, 25, 27). Phosphorimages of hybridized membranes were analyzed in ArrayVision to obtain raw spot intensity data (Imaging Research). The raw data were normalized by expressing individual spot intensities as a fraction of the sum of all gene-specific spot intensities in each image, and the data were analyzed by using semiautomated Visual Basic programs in Microsoft Excel as has been described (6). Clustering algorithms were executed in DecisionSite for Functional Genomics software (Spotfire, Inc.).

RT-PCR. DNase-treated RNA (500 pg) from MES (pH 4.5)-grown *E. coli* cultures, isolated for macroarray analysis (described above), served as the template for reverse transcriptase PCR (RT-PCR) analysis. RT-PCR primers (18- to 20-mers) were designed to produce PCR fragments of predetermined size if the mRNA sample contained a template which encompassed the intervening regions between the genes of interest. Cotranscription of *hdeA* and *hdeB* (see Fig. 4, lane C) was evaluated with primers *hdeA>hdeB* (5′-TCAACTCCTGGACCTGTGAAG-3′) and *hdeB>hdeA* (5′-AATTCGGCAAGTCATTAGATGC-3′). Cotranscription of *yhiD* and *hdeB* (Fig. 4, lane B) was evaluated with primers *hdeB>yhiD* (5′-ATGACCTGCCAGGAATTTATTG-3′) and *yhiD>hdeB* (5′-ACGTCAGCAAAACCATATTTCG-3′). Cotranscription of *yhiD* and *hdeA* (Fig. 4, lane A) was evaluated with primers *yhiD>hdeB* and *hdeA>hdeB*. Cotranscription of *hdeD* and *yhiE* (see Fig. 4, lane D) was evaluated with primers *hdeD>yhiE* (5′-GTTATTGGTGTGCTGGATATCG-3′) and *yhiE>hdeD* (5′-TGATACTTTCTTTGCGGCTAAC-3′). Cotranscription of *gadW* and *gadX* (see Fig. 4, lane F) was evaluated with primers *gadX>gadW* (5′-GTGTAGAATGCAACGTGCTTTG-3′) and *gadW>gadX* (5′-AATGGCAAACTGTCAGCTCATC-3′). Cotranscription of *gadX* and *gadA* (see Fig. 4, lane G) was evaluated with primers *gadA>gadX* (5′-GTACGACCTCTCTGAACGTCTG-3′) and *gadX>gadA* (5′-GATTTAATGCCTCCTCCTTGAG-3′). Cotranscription of *gadW* and *gadA* (see Fig. 4, lane E) was evaluated with primers *gadW>gadX* and *gadA>gadX*. Cotranscription of *yhiS* and *slp* (see Fig. 4, lane I) was evaluated with primers *yhiS>slp* (5′-AAGCCTTTACGACACTCTCCCG-3′) and *slp>yhiS* (5′-AATGAAAGGCTGAGGATGAGTG-3′). Cotranscription of *slp* and *yhiF* (see Fig. 4, lane J) was evaluated with primers *slp>yhiF* (5′-ATCAAAGGCAATAACCAACCTG-3′) and *yhiF>slp* (5′-GATGAGTGCGACAAAATCAATG-3′). Cotranscription of *yhiS* and *yhiF* (see Fig. 4, lane H) was evaluated with primers *yhiS>slp* and *yhiF>slp*. RT-PCR was performed with Qiagen's OneStep RT-PCR kit by following manufacturer's instructions. RT-PCR products were visualized on 0.8% Tris-borate-EDTA–ethidium bromide-stained agarose gels. The RT-PCR product fragment sizes were determined by comparison to the Kb DNA ladder (Stratagene), and these fragment sizes were subsequently compared to the predicted fragment sizes. Negative control RT-PCRs were performed to verify that no contaminating DNA was present to serve as a template for artifactual RT-PCR products. Control primers were designed to amplify regions containing the divergently transcribed genes *hdeD* (see Fig. 4, lane K) and *hdeAB* (see Fig. 4, lane L). These primers did not amplify a fragment during RT-PCR due to the lack of corresponding template mRNA. The control primers were *hdeA>hdeD* (5′-CCCTGAACATCTAAAACCGCATCTTC-3′) and *hdeD>hdeA* (5′-TTTAACCGCCCATAGCATCAACGC-3′) and *yhiD>hdeB* (5′-GCCTTTCATTGAACGCTGACGATACC-3′) and *hdeD>hdeB* (5′-CAGGCAATTGCCGCGAGTATAAGACG-3′).

Acid resistance assay. The glutamate-dependent acid resistance system was tested by growing cells overnight (22 h) in Luria broth (LB)-MOPS (pH 8.0) and LB-MES (pH 5.5) or minimal medium E containing 0.4% glucose (EG) (28) at pH 7.7 and 5.5. The overnight cultures were diluted 1:1,000 into prewarmed EG (pH 2.5) containing 1.5 mM sodium glutamate, resulting in a final culture pH of approximately 2.5. Viable-cell counts were determined at 0, 1, 2, and 4 h after the acid challenge (12). Acid challenge was carried out for 1, 2, and 4 h at 37°C.

Mouse colonization assay. The method used to compare the large intestine-colonizing abilities of *E. coli* strains in mice has been described previously (24, 29). Briefly, three male CD-1 mice (5 to 8 weeks old) were given drinking water containing streptomycin sulfate (5 g/liter) for 24 h to eliminate resident facultative bacteria (17). Following 18 h of starvation, the mice were fed 1.0 ml of 20% (wt/vol) sucrose containing 10^{10} CFU each of two LB-grown *E. coli* MG1655 strains, depending on the experiment. After the bacterial suspension was ingested, both food (Charles River Valley rat, mouse, and hamster formula) and streptomycin-water were returned to the mice, and 1 g of feces was collected after 5 and 24 h and on odd-numbered days at various times. Mice were housed individually in cages without bedding and were placed in clean cages daily. Fecal samples (no older than 24 h) were homogenized in 1% Bacto Tryptone, diluted in the same medium, and plated on MacConkey agar plates (Difco) containing streptomycin sulfate (100 μg per ml) and nalidixic acid (100 μg per ml) when determining MG1655 Str^r Nal^r CFU or on MacConkey agar plates containing streptomycin sulfate (100 μg per ml) and kanamycin sulfate (40 μg per ml) when determining MG1655 Str^r Δ*gadX*::Km^r or MG1655 Str^r Δ*gadXW*::Km^r CFU. All plates were incubated for 18 to 24 h at 37°C prior to counting. The $\log_{10}$ mean number of CFU per gram of feces ± the standard error of the $\log_{10}$ mean of the CFU per gram of feces was calculated for each set of three mice at each time point.

TABLE 1. Bacterial strains and plasmids employed in this study

Strain or plasmid	Genotype	Source
E. coli MG1655 CGSC6300	K-12 F$^-$ λ$^-$ *rph fnr*	CGSC[a]
DT203	MG1655 Δ*gadW*::Kan^r	Allelic replacement
DT162	MG1655 Δ*gadX*::Kan^r	Allelic replacement
DT169	MG1655 Δ*gadX* (Kan$^-$)	Allelic replacement
DT232	MG1655 Δ*gadXW*::Kan^r	Allelic replacement
MG1655 Str^r	MG1655 Str^r	This study
MG1655 Str^r Nal^r	MG1655 Str^r Nal^r	This study
DT162 Str^r	MG1655 Str^r Nal^r Δ*gadX*::Kan^r	Allelic replacement
DT232 Str^r	MG1655 Str^r Nal^r Δ*gadXW*::Kan^r	Allelic replacement
EK227	K-12 wild type	A. C. Matin
EF757	K-12 Δ*gadX*::Kan^r	EK227 × DT162[b]
EF828	K-12 Δ*gadX*	EF757 × DT169
EF861	K-12 Δ*gadW*::Kan^r	EK227 × DT203
EF863	K-12 Δ*gadXW*::Kan^r	EK227 × DT232

[a] CGSC, *E. coli* Genome Stock Center.
[b] Constructed by P1 transduction.

RESULTS

Experimental design. The goal of the gene expression profiling experiments was to identify target genes under GadX control and to investigate the role of the associated transcription factor, GadW. A growing body of evidence indicates that GadX regulates genes known to be involved in glutamate-dependent AR (11, 22, 26) and other genes that are induced by acid (27). Previously, we identified conditions that favor induction of the glutamate-dependent AR system 2, while repressing or failing to induce the other systems. Under these conditions, nine genes in the 14-kb *gadA* region (*slp* to *gadA*) of the genome were induced by acid in the wild-type strain, including known genes of glutamate-dependent AR system 2, but not genes of the other AR systems (systems 1 and 3) (27). The experimental design is based on the mode of regulation of the three AR systems. AR system 1 is catabolite repressed by glucose and induced in complex medium; arginine-dependent AR system 3 is regulated by CysB and induced only in stationary phase; glutamate-dependent AR system 2 is independently regulated by extracellular pH, HN-S, RpoS, and EvgA (3, 4, 8, 15). Cultures were grown to log phase on acidified glucose minimal medium, conditions that favor acid induction of the glutamate-dependent AR system components, while repressing or failing to induce the other systems.

In addition to induction by acid and GadX control, the adjacent AraC-like transcription factor GadW plays a role in regulating the glutamate-dependent AR system (14). Therefore, we generated isogenic *gadX*, *gadW*, and *gadX gadW* mutants by allelic replacement with a kanamycin resistance cassette. As listed in Table 1, these are the Δ*gadX*::Kan^r, Δ*gadW*::Kan^r, and Δ*gadXW*::Kan^r strains, respectively. To minimize potential polarity effects of the *gadX* lesion on *gadW* expression, a Kan$^-$ *gadX* Kan$^-$ mutant was constructed by removing the Kan^r cassette with FLP recombinase (7); this is the Δ*gadX* strain (Table 1). Mutant and wild-type cells were grown at pH 7.4, 5.5, and 4.5, for a total of 16 experiments. Growth curves for the wild-type strain under these conditions have been published elsewhere (27); growth of the mutant strains was not significantly different from that of the wild type (data not shown). In the first of two sets of experiments (Table 2, experiments 1, 3, 5, 6, 8, and 10), the expression profile of the *gadX* mutant was compared to that of the wild type. The cultures in experiments 1, 3, 6, and 8 were duplicated (biological replicates), and one of the RNA samples was labeled and hybridized twice, while the other was hybridized three times, for a total of five technical replicates, which were averaged for subsequent analysis. The cultures in experiments 5 and 10 were grown once at pH 4.5, and the RNA was labeled and hybridized twice. The second set of experiments (Table 2, experiments 2, 4, 7, 9, and 11 to 16) compared the four mutants to the wild type; the cultures were grown once at pH 5.5 or 7.4, and the RNAs were labeled and hybridized twice to matched membrane pairs.

A total of 20 different two-condition, pairwise comparisons of the data were made. Comparisons of the wild-type strain grown in acid versus neutral conditions were published previously and are shown here for comparison to the mutants (27). Three general types of two-condition comparisons yielded useful information: pairwise comparison of acid versus neutral conditions within each strain, of the mutants versus the wild type in acid conditions, and of the mutants versus the wild type in neutral conditions. Side-by-side presentation of the data in computer-generated visualizations (heat maps generated with Spotfire software) made it possible to compare the various two-condition and replicate experiments (Fig. 1).

Cluster analysis of array data. The overriding goal of this work was to identify genes associated and coregulated with the glutamate-dependent AR phenotype. Based on evidence that GadX is involved in controlling this AR regulon and that the genes of interest are acid inducible, the following criteria for membership in the regulon were established: (i) expression is affected by mutation of *gadX* and (ii) induction occurs in acid conditions.

We began analysis of the data by clustering all 20 of the two-condition comparisons by using standard clustering algorithms (9). Application of *k* mean clustering requires the user to select the number of clusters. The simplest consideration of any two-condition comparison is that gene expression can remain unchanged, increase in the experimental condition, or increase in the control condition. Thus a *k* cluster value of 3

TABLE 2. Experimental replication

Expt	Genotype of strain	pH	No. of replicates	
			Biological	Technical
1	Wt[a]	7.4	2	5
2	Wt	7.4	1	2
3	Wt	5.5	2	5
4	Wt	5.5	1	2
5	Wt	4.5	1	2
6	*gadX*	7.4	2	5
7	*gadX*	7.4	1	2
8	*gadX*	5.5	2	5
9	*gadX*	5.5	1	2
10	*gadX*	4.5	1	2
11	*gadX* (Kan$^-$)	7.4	1	2
12	*gadX* (Kan$^-$)	5.5	1	2
13	*gadW*	7.4	1	2
14	*gadW*	5.5	1	2
15	*gadX gadW*	7.4	1	2
16	*gadX gadW*	5.5	1	2

[a] Wt, wild type.

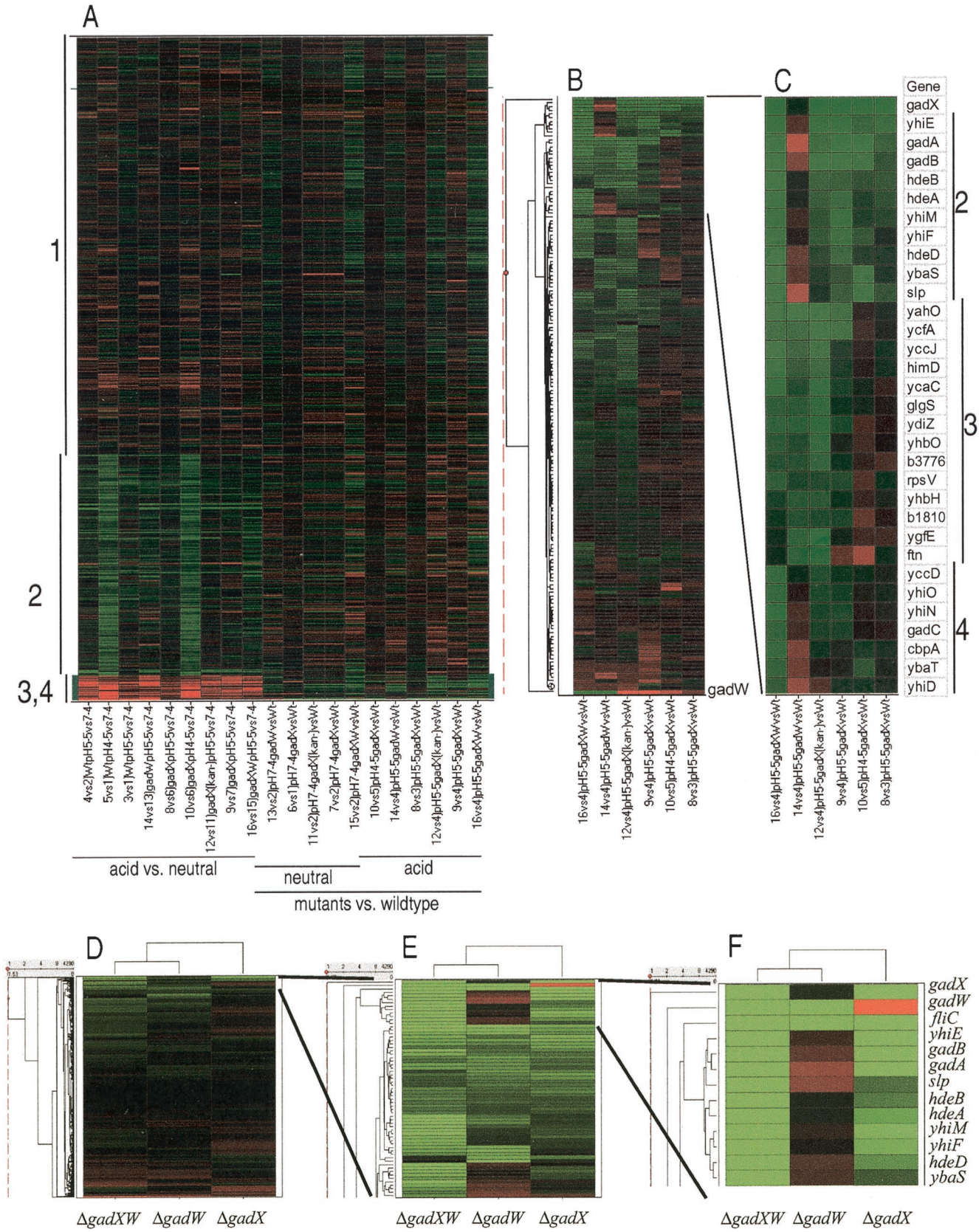

FIG. 1. Cluster analysis of array data. (A) *k* mean cluster analysis ($k = 4$) of all 4,290 genes in 20 experimental comparisons (see text for details); cluster numbers are shown to the left. The horizontal lines each represent one gene. (B) Hierarchical cluster analysis of genes in *k* mean clusters 3 and 4 for comparisons of mutants versus the wild type under acid conditions. (C) Expanded view of the four most significant clusters from the hierarchical cluster analysis shown in panel B; genes and cluster numbers are at the right. (D) Hierarchical cluster analysis of mutants versus the wild type under acid conditions (see text for details). (E and F) Expanded views of hierarchical cluster analysis shown in panel D. Ratios are displayed colorimetrically: bright red, genes with ≥2.2-fold-higher expression (~2 standard deviations) at the experimental time point than the control; bright green, ≤2.2-fold-lower expression at the experimental time point than the control; black, no change in expression.

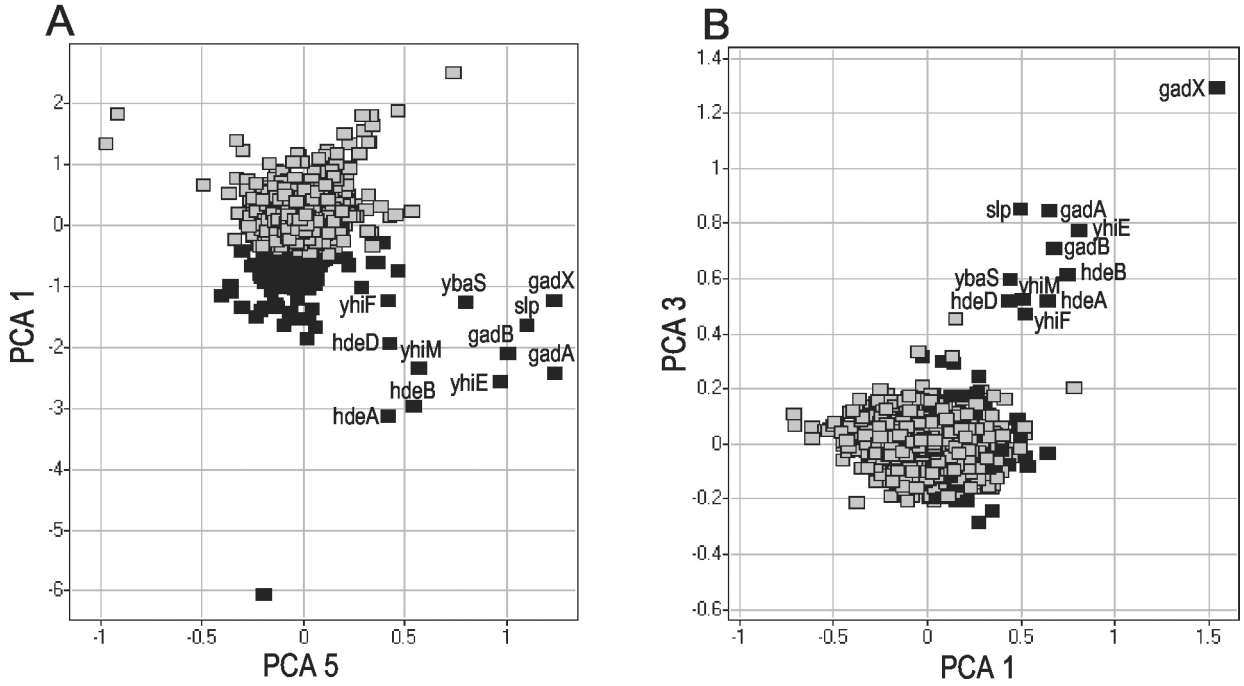

FIG. 2. PCA of array data. (A) Plot of PC-1 versus PC-5 from PCA of all 4,290 genes and 20 comparisons. PC-1 sorts the data primarily by response to the medium pH, and PC-5 sorts the data primarily by response to the *gadX* mutation (11 principal components [PCs] preserved 98.0% of the variability in the data). (B) Plot of PC-1 versus PC-3 from PCA of the mutants versus the wild type in acid conditions. PC-1 sorts the data primarily by the *gadXW* effect, and PC-3 sorts the data primarily by the *gadX* effect (three PCs preserved 85.1% of the variability). Black squares, acid-inducible genes (162); grey squares, all other genes. Putative GadX regulon members are labeled on the plots.

would seem to be the most appropriate, but the results of this analysis placed the *asr* gene alone in one cluster because of its extreme induction by acid. When the *k* cluster value was adjusted to 4, *asr* again was alone in a cluster; the remaining genes were sorted into three clusters on the basis of their pH-dependent behavior, the dominant variable in these experiments (Fig. 1A). There were 2,698 genes whose expression was essentially unchanged in the data sets (cluster 1), 1,431 that were more highly expressed in the neutral pH control conditions (cluster 2), and 161, in clusters 3 and 4, that were more highly expressed in acid (without consideration of statistical significance). We note that all of the 28 acid-inducible genes identified by rigorous statistical analysis (27) were contained in these clusters.

The 161 genes identified by *k* cluster analysis as being acid inducible, together with *yhiD*, which was added to this set because it is cotranscribed with *hdeA* and *hdeB* (see below), were further analyzed by hierarchical clustering of related data columns (experimental categories indicated at the bottom of Fig. 1A). Clustering of the data sets for the mutants versus the wild type at pH 7.4 did little to shed further light on their regulation (data not shown). Hierarchical clustering of the mutants versus wild type in acid pH conditions was more revealing. The regulatory genes themselves, *gadX* and *gadW*, clustered at opposite ends of the data set, as their expression was substantially different from that of all other genes. This result is not surprising, given that one or the other was deleted in the strains being compared (Fig. 1B). The second hierarchical cluster contained 10 genes that appeared to be regulated by GadX; all but 2 of these genes (*ybaS* and *gadB*) are adjacent to *gadA* on the genome, and all are significantly induced in acid conditions (Fig. 1C). The third cluster contained 14 acid-inducible genes that were not significantly regulated by GadX or GadW. The fourth cluster contained seven additional acid-inducible genes that appeared to be regulated by GadX and GadW. Hierarchical clustering of all 4,290 genes (rather than just the 162 acid-inducible genes) in the comparisons of the mutants versus wild type under acid conditions also resulted in a single cluster containing the 10 target genes, together with *gadX* and *gadW* (Fig. 1D to F).

Principal-component analysis (PCA) was used to validate the results of hierarchical clustering. PCA employs an algorithm that reduces a complex data set to its principal components (reduced dimensional space) by replacing the original data columns with a smaller number of new columns containing their eigenvectors (18). The individual principal components tend to reflect the experimental variation introduced by just one variable in a complex data set. Application of this algorithm revealed that the entire data set (all 4,290 genes and 20 pairwise comparisons) could be reduced to 11 principal components that preserved 98.0% of the variability in the data. Nearly one-half (49.4%) of the variability was retained in PC-1, which sorted the data primarily by acid inducibility. Maximum segregation of the data by the response to mutation of *gadX* was observed in PC-5. A two-dimensional plot of PC-1 versus PC-5 segregated *gadX* and the 10 target genes, confirming the results obtained by hierarchical clustering (Fig. 2A). Further, hierarchical clustering of the 11 principal components (eigenvectors generated by PCA of the entire data set) resulted in a primary cluster containing the same 10 genes, together with

TABLE 3. Gene expression ratios for GadX-GadW-regulated genes

Gene	Log gene expression ratio[a]									
	9 vs 4, pH 5.5, *gadX* vs Wt	14 vs 4, pH 5.5, *gadW* vs Wt	16 vs 4, pH 5.5, *gadXW* vs Wt	7 vs 2, pH 7.4, *gadX* vs Wt	13 vs 2, pH 7.4, *gadW* vs Wt	15 vs 2, pH 7.4, *gadXW* vs Wt	4 vs 2, Wt, pH 5.5 vs 7.4	9 vs 7, *gadX*, pH 5.5 vs 7.4	14 vs 13, *gadW*, pH 5.5 vs 7.4	16 vs 15, *gadXW*, pH 5.5 vs 7.4
ybaS	−0.171	0.112	**−0.662**	0.114	0.010	−0.172	**0.520**	**0.235**	**0.622**	0.030
ybaT	−0.026	0.132	−0.243	0.034	−0.033	−0.056	0.186	**0.114**	**0.350**	0.000
gadC	−0.145	0.069	−0.260	−0.008	0.009	−0.031	0.241	**0.103**	**0.301**	0.011
gadB	**−0.425**	0.152	**−0.792**	0.004	0.022	−0.112	**0.884**	**0.455**	**1.015**	0.205
yhiM	**−0.330**	0.060	**−0.653**	0.005	0.102	−0.200	**0.956**	**0.621**	**0.915**	**0.503**
yhiN	−0.172	0.077	**−0.356**	0.055	0.044	−0.028	**0.432**	**0.205**	**0.465**	0.104
slp	−0.200	**0.210**	**−0.828**	0.123	0.031	−0.120	**0.710**	**0.388**	**0.888**	0.050
yhiF	**−0.300**	0.023	**−0.627**	−0.056	0.063	−0.073	**0.746**	**0.501**	**0.706**	0.192
yhiD	−0.083	0.176	−0.210	0.053	0.100	0.099	0.247	0.111	**0.323**	−0.063
hdeB	**−0.217**	−0.029	**−0.926**	−0.101	0.180	**−0.637**	**1.287**	**1.171**	**1.077**	**1.036**
hdeA	**−0.273**	−0.031	**−0.771**	−0.105	**0.189**	**−0.677**	**1.309**	**1.141**	**1.089**	**1.215**
hdeD	**−0.234**	0.126	**−0.641**	0.002	0.084	−0.174	**0.942**	**0.706**	**0.984**	**0.523**
yhiE	**−0.362**	0.090	**−0.975**	0.096	0.110	−0.127	**1.114**	**0.656**	**1.094**	**0.314**
gadW	**0.434**	**−0.734**	**−0.800**	**0.784**	−0.106	−0.143	**0.610**	**0.260**	−0.017	−0.046
gadX	**−1.051**	−0.067	**−1.122**	−0.251	0.145	−0.317	**0.804**	0.000	**0.592**	0.000
gadA	**−0.421**	**0.227**	**−0.866**	−0.034	−0.040	−0.282	**0.908**	**0.521**	**1.175**	**0.325**

[a] Boxhead arrangement is as follows: experiments involved, condition or gene(s), genes or conditions compared. Values in boldface are statistically significant ($P < 0.05$) and are ≥3 standard deviations from the mean log ratio for 4,290 genes.

gadX (data not shown). PCA of the *gadX*, *gadW*, and *gadXW* mutants versus the wild type grown in acid conditions revealed that three principal components preserved 85.1% of the variability. From this analysis a two-dimensional plot of PC-1, which sorts the data primarily by the *gadXW* effect, versus PC-3, which sorts the data primarily by the *gadX* effect, again segregated the same 10 target genes (Fig. 2B). Taken together, the cluster analysis results indicate that at least eight genes in the *gadA* region, plus *ybaS* and *gadB*, fulfill the criteria established for inclusion in the GadX regulon: acid induction and regulation by GadX (Table 3).

Mode of regulation by GadX and GadW. Of the 10 target genes identified by cluster and PCA, 8 were expressed at significantly higher levels in the wild type than the *gadX* mutant strains during growth in acid conditions, confirming that GadX functions as a transcriptional activator (loss of GadX in the mutants leads to decreased target gene activation; Table 3, column 1 [i.e., first column after the left column]). This result was confirmed for the *gadA* gene product by Western analysis (data not shown) and further confirms previous reports that acid induction of GadA is diminished in *gadX* mutants (14, 22). When strains were grown under neutral (noninducing) conditions, the *gadX* mutation did not have a statistically significant effect on expression of the target genes (with the exception of *gadW*) compared to expression in the wild type (Table 3, column 4). Thus, GadX appears to function as a transcriptional activator under acidic conditions. GadW, on the other hand, had little effect on expression of the genes in question, with the exception of statistically significant up-regulation of *slp* and *gadA* in the *gadW* mutant compared to the wild type under acid conditions (Table 3, column 2) and of *hdeA* under neutral conditions (Table 3, column 5). Where mutation of *gadW* was observed to have a significant effect, expression of the target genes was higher in the *gadW* mutant than in the wild type.

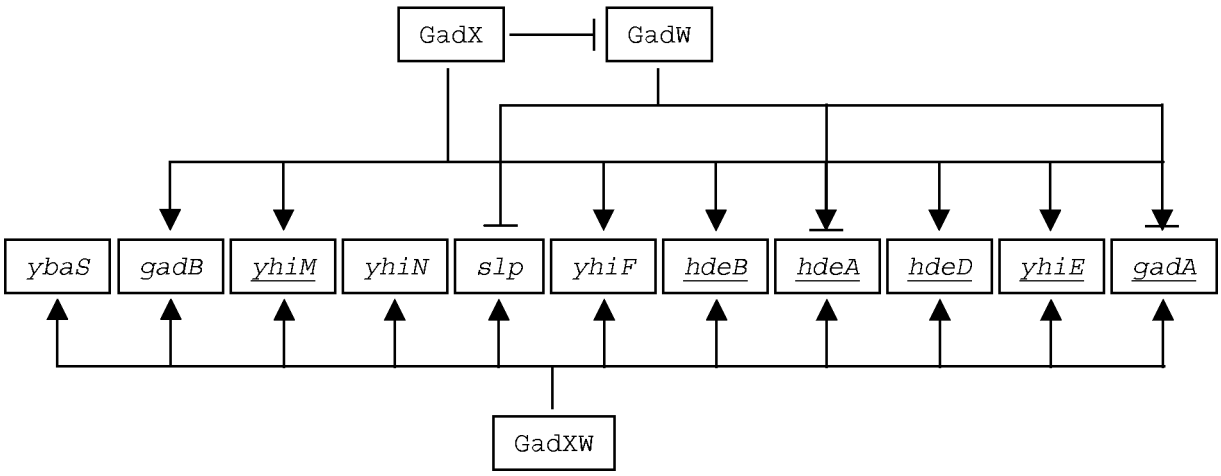

FIG. 3. Model of genetic regulation by GadX and GadW. Target genes are all acid inducible; underlined genes are acid inducible in the *gadX gadW* mutant. Cross lines, negative control; arrows, positive control.

This result for the strain lacking GadW but possessing GadX suggests that GadW functions as a repressor of at least some GadX-activated genes.

Compared to the *gadX* mutant, the *gadX gadW* double mutant showed diminished pH-dependent regulation of the target genes and some genes were no longer significantly induced by acid (Table 3, compare columns 8 and 10). In addition, the *gadX gadW* mutant showed an exaggerated decrease in expression compared to the wild type: the expression ratios for the *gadX gadW* mutant of all target genes were substantially higher than the expression ratios for the *gadX* mutant ($\log_{10}$ expression ratios of −0.6 to −1.0 and −0.2 to −0.4, respectively; Table 3, compare columns 3 and 1). We conclude from these results that GadX is a transcriptional activator and that GadW is either a coactivator with GadX or an inhibitor of GadX-dependent activation. The diminished response to pH in the *gadX gadW* mutant suggests that GadW may be involved in signal transduction of the acid environment to the target operon promoters, although the pH response of some of the genes is not lost entirely in the *gadX gadW* mutant. The regulation of the target genes by GadX and GadW is summarized in Fig. 3. Note that this model (Fig. 3) is based solely on the array data and must be constrained by independent analysis of target gene expression.

Transcript mapping of GadX-dependent operons. It became evident, during the preceding analysis, that a number of the target genes are arranged in operons. Cotranscription of the *gadBC* operon from a single pH-inducible promoter has been described (3). Since the *ybaS* and *ybaT* genes are separated by only 3 bp, their cotranscription was assumed and not tested. We used RT-PCR mapping of transcripts from cells grown at pH 4.5 to confirm whether or not the remaining target genes are arranged in operons. In this way, cotranscription of the *hdeAB-yhiD* operon was established (Fig. 4, lanes A to C). Likewise, cotranscription of *slp-yhiF* was established (Fig. 4, lane J). We found no evidence for a read-through product from *gadA* to *gadW*, indicating that *gadA*, *gadX*, and *gadW* do not constitute an operon (Fig. 4, lane E). However, the analysis does indicate cotranscription of *gadA* and *gadX* (Fig. 4, lane G), as well as of *gadX* and *gadW* (Fig. 4, lane F). The latter result is in conflict with the report by Ma et al. (14) that *gadX* and *gadW* are not cotranscribed. There was no evidence for cotranscription of *hdeD* and *yhiE* or *yhiS* and *slp* (Fig. 4, lanes 4 and 9). In summary, the data indicate that there are 10 operons containing 15 GadX-regulated genes (Fig. 5).

Analysis of putative binding sites. Preceding the *gadA* and *gadB* genes is a perfectly conserved 20-bp element. Deletion of this sequence results in loss of pH-dependent induction of *gadA* (3, 14). Therefore, we used this 20-bp element as a query in a pattern search of the *E. coli* MG1655 genome (Colibri website: http://genolist.pasteur.fr/Colibri). By using this strategy, it was possible to identify a reasonable match upstream of each of the suspected GadX-dependent transcription units (Fig. 6). Alignment of these elements revealed a conserved 18-bp consensus sequence that is A-T rich and fairly degenerate. For example, two putative 18-bp elements were identified in the regulatory region between the divergent *hdeD* and *hdeAB-yhiD* operons; the element upstream of *hdeAB-yhiD* was a relatively poor match. In summary, all of the target genes identified by array analysis are located in transcription units

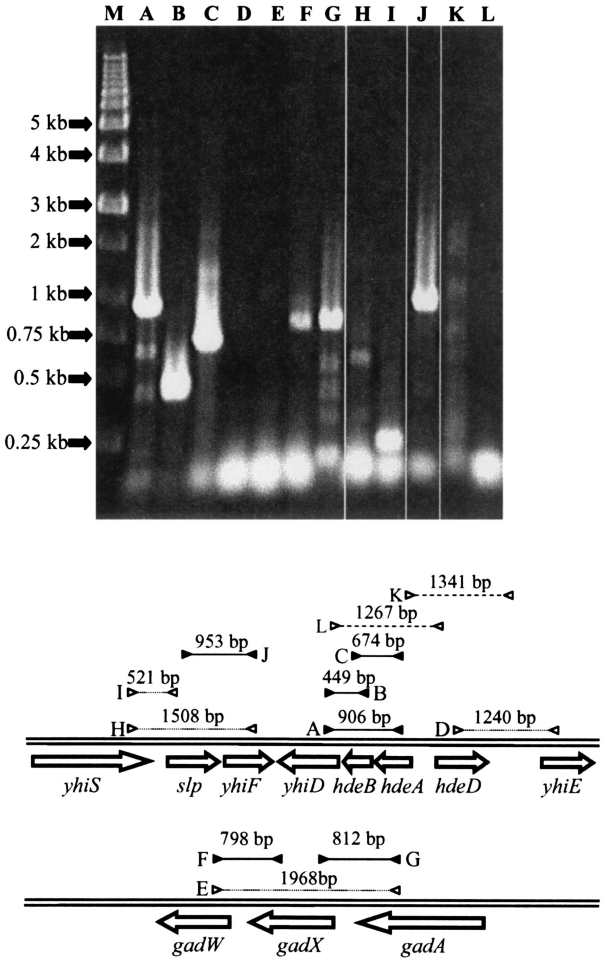

FIG. 4. RT-PCR analysis of *E. coli* MG1655. Template RNA was isolated from mid-logarithmic-phase cells grown in minimal glucose medium at pH 4.5. Lanes: M, Stratagene Kb DNA ladder with sizes indicated on the left; A, *hdeA* to *yhiD*; B, *hdeB* to *yhiD*; C, *hdeA* to *hdeB*; D, *hdeD* to *yhiE*; E, *gadA* to *gadW*; F, *gadX* to *gadW*; G, *gadA* to *gadX*; H, *yhiS* to *yhiF*; I, *yhiS* to *slp*; J, *slp* to *yhiF*; K and L, negative controls. See Materials and Methods for details. Predicted RT-PCR fragments are shown at the bottom; letters match the corresponding lanes in the gel, and lines connect arrowheads that indicate primer locations. Solid lines between primers indicate that a cotranscription product was detected, dotted lines indicate reactions in which no product was made, and dashed lines indicate lack of a product in negative controls.

preceded by the degenerate 18-bp element. No other conserved sequences were identified within the putative regulatory regions of the target genes.

Physiology of the GadX-GadW regulon. Since GadX and GadW appear to be required for regulating glutamate-dependent AR, *gadX*, *gadW*, and *gadX gadW* mutants were subjected to acid challenge to test the prediction that they would have diminished AR (Fig. 7). Log-phase cells grown at pH 5.5, while showing moderate induction of the *gadA* and *gadB* genes, still remain acid sensitive (4). Stationary-phase cells demonstrate the highest glutamate-dependent AR due to higher production of glutamate decarboxylase and/or other required proteins. Since the goal of the acid challenge experiment was to test the prediction that *gadX* and *gadW* affect glutamate-dependent

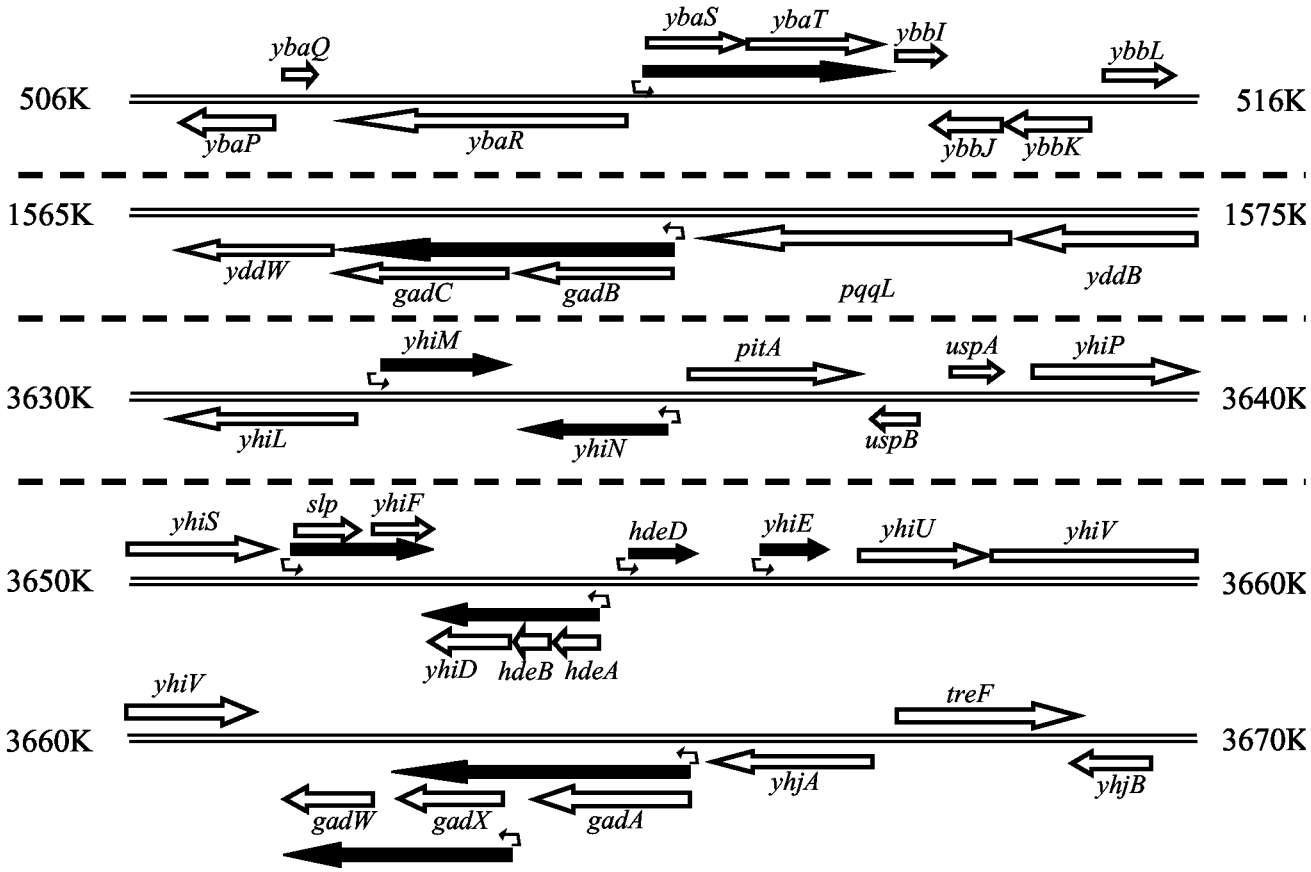

FIG. 5. Gene map of GadX-regulated genes in *E. coli* MG1655. The approximate map locations of the genes in the *E. coli* genome are shown by the numbers at the ends of each line. Dashed line, presence of intervening sequence between sections of the map; solid arrows, transcription of single genes or operons, as determined by RT-PCR analysis shown in Fig. 4; bent arrows, approximate location of the conserved sequence element required for pH-dependent regulation of *gadA*. Open arrows indicate sizes and locations of genes.

AR, we used conditions and cells that exhibit this resistance (i.e., stationary-phase cells).

When cells were pregrown in minimal medium to stationary phase at either pH 5.5 or 7.7, there was no effect of the *gadX* mutation on their ability to survive acid challenge for up to 4 h at pH 2.5 in medium containing glutamate (Fig. 7A). The increased resistance of the *gadW* mutant following 2 and 4 h of acid challenge suggests that GadW represses an AR function that is important in minimal medium. The data indicate that in minimal glucose medium either GadX or GadW is sufficient for AR. The loss of both GadX and GadW had a substantial effect on AR: the *gadX gadW* mutant was acid sensitive when pregrown at pH 7.7 and less acid tolerant than the wild type when pregrown at pH 5.5. When *gadW* mutant cells were

Gene/Operon	Location	Misses	Conserved Sequence
gadAX	-99	0	gtcgtttttctgc**TTAGGATTTTGTTATTTA**aattaagcctgta
gadBC	-99	0	acgataaataaca**TTAGGATTTTGTTATTTA**aacacgagtcctt
hdeAB-yhiD	-112	3	ttgaaataaaaat**AT**ct**GATTTTG**a**TATTTT**ccatcaacatgac
hdeD	-96	0	tcagatactgcaa**TTAGGAAATTTTTATTAA**atcgactgcattc
slp-yhiF	-100	2	atataaacatcag**ACAGG**t**TTA**c**GTTACTAT**caggcatatcacc
ybaST	-131	2	ggctcgatttgca**TCAGGATTA**g**AC**a**TTTAT**ctctttgttttcc
yhiE	-94	1	gggtaaagttctt**ATAGGC**g**TTTACTATATT**gaacaacgattcg
yhiM	-139	0	ataagaattaatc**TTAGGATAAATTTTTATT**tatcatggccttt
yhiN	-94	1	tatcttatataat**TCAGGCAAATACTTC**c**TT**ttagtaatattga
gadXW	-93	3	cctgttctcccgc**TT**c**G**tt**TAAATTTATTTA**tcaatcaatttga
Consensus:			**WYAGGMWWWWDYTWYWWW**

FIG. 6. Alignment of sequences upstream of operons regulated by GadX-GadW. Numbering of nucleotides is with respect to the start codons. Boldface uppercase letters indicate conserved nucleotides. The consensus sequence is shown below. Standard International Union of Biochemistry/International Union of Pure and Applied Chemistry nucleotide base abbreviations are used: W, A or T; Y, T or C; M, A or C; D, G, A, or T.

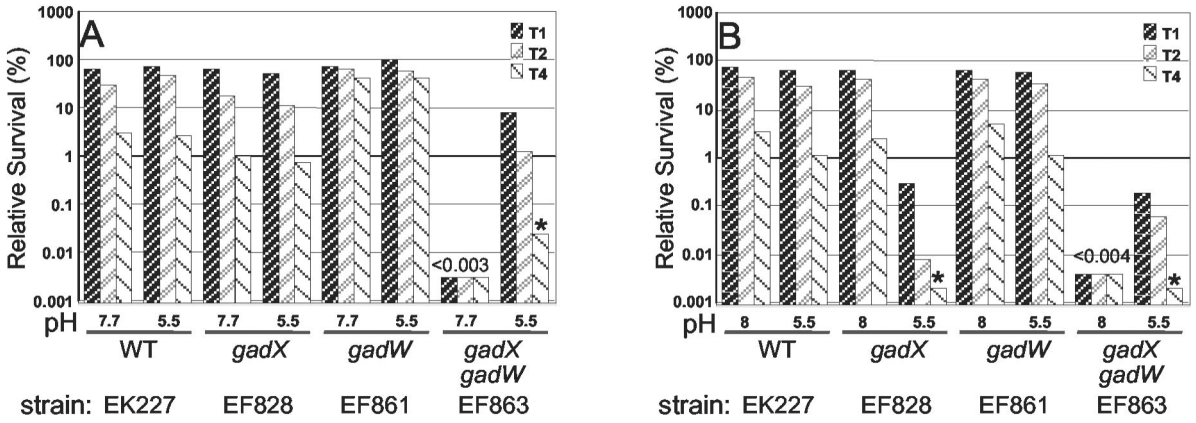

FIG. 7. The glutamate-dependent acid resistance system was tested by growing cells overnight (22 h; optical density at 600 nm = 3.8) in EG at pH 7.7 and 5.5 (A) and LB-MOPS (pH 8) and LB-MES (pH 5.5) (B). The overnight cultures were diluted 1:1,000 into prewarmed EG (pH 2.5) with 1.5 mM sodium glutamate. Viable-cell counts were determined at 0, 1, 2, and 4 h after the acid challenge (12). Asterisks, no surviving bacteria at the lowest dilution tested. WT, wild type.

pregrown to stationary phase in LB at either pH 5.5 or 8, there was no effect on their ability to survive acid challenge (Fig. 7B). The *gadX* mutant was significantly less acid tolerant than the wild type when pregrown at pH 5.5, and the *gadX gadW* mutant was even more acid sensitive when pregrown at pH 8 or 5.5. These results clearly indicate that *gadX* is required, at least under some conditions, to induce glutamate-dependent AR.

The ability of *E. coli* to tolerate acid in the stomach is predicted to play an important role in colonization of the large intestine. Therefore, we tested the ability of *gadX* and *gadX gadW* mutants to compete with their parent strains in the streptomycin-treated mouse model (Fig. 8). In this assay, mice were coinoculated with 10^{10} CFU each of *E. coli* MG1655 Str^r Nal^r and of the mutants in an *E. coli* MG1655 Str^r background. The Nal^r and Kan^r phenotypes used to distinguish the competing strains have no effect on colonization (23). The *gadX* mutant colonized at a level very similar to that for the parent strain early in the experiment and then appeared to have a modest advantage after 9 days. The *gadX gadW* mutant had a very obvious advantage over the parent strain, which continued to decline in numbers after the first day of the experiment. These surprising results suggest that glutamate-dependent AR is not necessary for colonization of the mouse large intestine.

DISCUSSION

There are 28 *E. coli* genes induced during log phase in acidified minimal glucose medium (27). In the present study we provide evidence that GadX regulates 15 pH-inducible genes that comprise 10 separate transcription units. Immediately downstream of *gadX* is the gene *gadW*, which also encodes an AraC-like transcription factor. We show here that GadW influences target gene activation by GadX, perhaps through physical interaction with GadX.

It has long been recognized that cells exposed to acid during logarithmic phase or allowed to enter stationary phase in complex growth medium are far more AR than are cells that have not been so adapted (4, 10). Given the importance of AR in the ecology of *E. coli*, it is not surprising that several regulatory inputs, involving at least four global regulators, HN-S, CRP, RpoS, and EvgA, regulate the AR response. It has been suggested that these regulatory inputs are integrated by GadX, which serves as an activator of the glutamate-dependent AR genes (14, 15, 26). Individual studies of GadX-regulated genes have involved primarily *gadA* and *gadBC*. To focus on the direct involvement of GadX or GadW in their regulation and limit the involvement of RpoS, CRP, or HN-S, the strategy most often employed is to use genetic backgrounds with mutations of the corresponding global regulatory genes. The pleiotropic, indirect effects of these mutations on gene expression across the genome are not considered during studies of individual genes. However, we deemed this strategy to be unsuitable for genome-wide expression studies of GadX-GadW-regulated genes. Rather, we used strains with wild-type copies of the global regulators and sought to employ growth conditions that minimized the involvement of their respective global regulatory networks. In this study, the cells used for expression profiling were harvested in logarithmic phase during growth on neutral or acidified minimal glucose medium. These growth conditions minimized regulation of the AR genes by RpoS and CRP (4, 14). Thus, for inclusion in the GadX-regulated gene set, genes had to meet two criteria: (i) acid induction and (ii) GadX-dependent regulation.

Application of complementary clustering algorithms led to a single conclusion regarding the identity of genes regulated by GadX. Ten of these genes significantly and consistently clustered together, and 5 genes were consistently placed in closely related clusters. Transcript mapping and sequence analysis led to the conclusion that the 15 genes comprise 10 transcription units (Fig. 5). All of the genes and operons identified in this study fulfill the criteria established for inclusion in the GadX regulon: they were all induced by acid and regulated by GadX. Although it was always placed in significant clusters, *gadW* failed to cluster with the other 15 genes, perhaps because the *gadX* mutations affected expression of *gadW* (Table 3, columns 1 and 4). Thus, cluster analysis demonstrated that the GadX-dependent genes, in addition to *gadA*, *gadBC*, and *gadX*, are regulated in a similar fashion, yet none of the other genes are known to be associated with glutamate-dependent AR. Additional genes of the GadX regulon may remain to be discovered

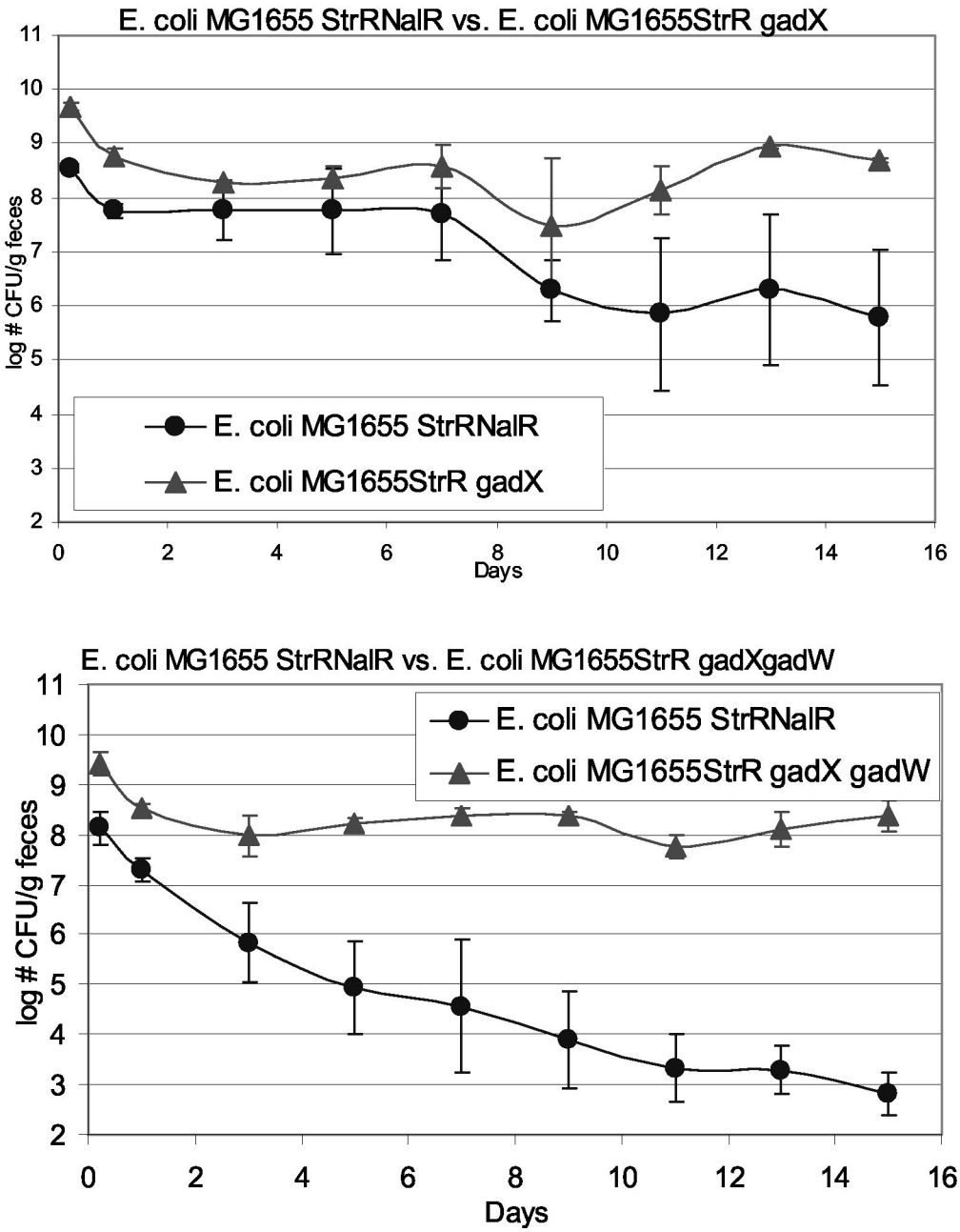

FIG. 8. Colonization of the mouse large intestine. (Top) Three mice were fed 10^{10} CFU of MG1655 Str^r Nal^r and 10^{10} CFU of MG1655 Str^r Km^r Δ*gadX*::Km^r; (bottom) three mice were fed 10^{10} CFU of MG1655 Str^r Nal^r and 10^{10} CFU of MG1655 Str^r Km^r Δ*gadX-gadW*::Km^r. At the indicated times, fecal samples were homogenized, diluted, and plated as described in Materials and Methods. Error bars, standard errors of the $\log_{10}$ means of fecal samples from each set of three mice.

as alternative growth conditions and genetic backgrounds are investigated.

In addition to assigning target genes to the GadX regulon, the data confirm that GadX functions as a transcriptional activator and implicate GadW as being involved. These data are summarized in Fig. 3. In four biologically replicated experiments, 11 genes in the 14-kb *gadA* region (*slp* to *gadA*) were expressed at statistically higher levels in the wild type than in the *gadX* mutants under acid conditions. Mutation of *gadW* had a far less significant effect on expression of the target genes, although three genes were expressed at higher levels in the mutant than in the wild type, a response that is characteristic of a negative regulator. In addition, loss of both GadX and GadW had a dramatic effect on target gene expression. The *gadW*::Kan^r array experiments were biologically repeated only once, and the results must be verified. We recently characterized strains possessing GadX but lacking GadW or possessing GadW but lacking GadX and showed that GadW can inhibit GadX activation of *gadA* and *gadBC* transcription and translation and vice-versa (14). These results suggest that GadX and

GadW act together to integrate the signal(s) that leads to activation of the genes of the GadX regulon. Our recent finding that GadX and GadW physically interact in a two-hybrid experiment supports this suggestion (14). Gene expression profiling does not indicate the mechanism(s) by which GadX and GadW carry out their regulatory roles or the pH-dependent signal that modulates their activities, but it does identify the repertoire of genes regulated by GadX-GadW. Characterization of these genes should provide additional insights into the regulation of the GadX regulon.

The *gadA* and *gadBC* genes are preceded by a perfectly conserved 20-bp element that is required for their pH-dependent regulation (3). The seven other GadX-dependent operons are preceded by similar 18-bp sequences, some of which are rather poor matches to the perfectly conserved 20-bp sequence element (Fig. 6). A recent report showed that a MalE-GadX fusion protein footprints the *gadA* and *gadB* promoters in regions that have no sequence similarity to each other and only partially overlap the 20-bp element, suggesting that the 20-bp sequence is not the target of binding by GadX (26). Certainly, the location of the 20-bp sequence element upstream of the mapped *gadA* and *gadB* promoters is consistent with binding by an activator, but it may serve as a binding site for yet another regulator, and GadX-GadW may influence that binding. We have thoroughly analyzed the putative regulatory regions of the 10 operons regulated by GadX-GadW and identified no other conserved sequences. Thus, the mechanism by which GadX and GadW regulate their pH-inducible target genes, including those identified in this study, is still very much an open question.

The involvement of at least three additional transcription factors in the regulation of the GadX-GadW target genes is implicated. Based on the finding that overproduction of the two-component regulator EvgA identified target genes involved in AR, including putative transcription factor genes, it was recently suggested that induction of the AR genes is controlled by a complex regulatory cascade (15). EvgA overproduction activates the expression of 37 genes, including 11 of the 15 genes identified in this study as being controlled by GadX and GadW. Among the genes regulated by EvgA are *gadX* and *ydeO*, which encode AraC-like transcription factors similar to GadW, and *yhiF* and *yhiE*, which encode LuxR-like transcription factors (27). All of these regulatory genes, with the exception of *ydeO*, are acid inducible (Table 3, column 7). Strains lacking YhiF, which apparently regulates dicarboxylate metabolism (2), exhibit normal AR (15, 27). Strains lacking YhiE (15, 27) or YdeO (15) lose AR. We show in this study that *gadX gadW* mutants display reduced AR. Thus, there appear to be several transcription factors that are required for inducing AR and that presumably serve to activate transcription of AR genes (this remains to be tested).

We favor a model of AR gene control in which GadX and GadW are intermediates in a regulatory cascade and serve to integrate signals received by the cells indicating an acid environment, entry into stationary phase (RpoS), and medium composition (CRP), as well as additional unknown signals (HN-S and EvgA). This would leave the role of direct activation of target genes to one of the other transcription factors. In support of this hypothesis, we tested whether overproduction of YhiE could rescue AR in the *gadX gadW* mutant and found that it did (data not shown). Thus, we propose a complex regulatory cascade in which global regulators (RpoS, CRP, HN-S, EvgA, etc.) influence the expression levels and/or activities of GadX and GadW, which in turn activate the expression and/or activities of transcription activators (e.g., YdeO and YhiE) that directly activate subsets of target genes involved in AR. One prediction of this model is that YhiE directly activates the glutamate-dependent AR genes. This cascade would allow the cell to integrate various physiological processes that are collectively important in AR. We are currently testing this hypothesis.

Given the role of GadX in activating genes involved in glutamate-dependent AR, more specifically, the diminished induction in *gadX* mutants, the decrease in acid tolerance shown by *gadX* and *gadX-gadW* mutants was expected. Numerous researchers have predicted that the ability to tolerate the acid environment of the stomach is an important virulence factor for *E. coli* O157:H7, contributing to the low infectious dose of this enteric pathogen (12, 13). By extension, it can be hypothesized that AR is an important factor for colonization of the mammalian large intestine by commensal *E. coli* strains. To our knowledge this hypothesis has not been previously tested. Surprisingly, our results proved otherwise: *E. coli gadX* and *gadX gadW* mutants are, in fact, more fit for competing in colonization than the wild-type parent strain. It is worth noting that *Vibrio cholerae* induces lysine carboxylase-dependent AR in the mouse intestine and that acid-adapted cells exhibited a major competitive advantage over unadapted cells; however, the *V. cholerae cadA* mutant was also more fit for colonization than its parent strain (16). Perhaps induction of the numerous genes of the GadX regulon imposes an energy burden on the wild-type cell that decreases its fitness relative to that of a mutant that is unable to induce the regulon. Although we were unable to provide evidence for involvement of the glutamate-dependent AR system in colonization, the redundancy of the three *E. coli* AR systems may provide for passage through the acid stomach, even in the absence of one of them. The diversity of *E. coli*'s response to acid conditions, though not necessarily the effectiveness of any one of them, could be important for its survival in the environment, but this remains to be proven.

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