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# GadE (YhiE) activates glutamate decarboxylase-dependent acid resistance in *Escherichia coli* K-12

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## Summary

**Commensal and pathogenic strains of *Escherichia coli* possess three inducible acid resistance systems that collaboratively protect cells against acid stress to pH 2 or below. The most effective system requires glutamate in the acid challenge media and relies on two glutamate decarboxylases (GadA and B) combined with a putative glutamate:γ-aminobutyric acid antiporter (GadC). A complex network of regulators mediates induction of this system in response to various media, pH and growth phase signals. We report that the LuxR-like regulator GadE (formerly YhiE) is required for expression of *gadA* and *gadBC* regardless of media or growth conditions. This protein binds directly to the 20 bp GAD box sequence found in the control regions of both loci. Two previously identified AraC-like regulators, GadX and GadW, are only needed for *gadA/BC* expression under some circumstances. Overexpression of GadX or GadW will not overcome a need for GadE. However, overexpression of GadE can supplant a requirement for GadX and W. Data provided also indicate that GadX and GadE can simultaneously bind the area around the GAD box region and probably form a complex. The *gadA*, *gadBC* and *gadE* genes are all induced by low pH in exponential phase cells grown in minimal glucose media. The acid induction of *gadA/BC* results primarily from the acid induction of *gadE*. Constitutive expression of GadE removes most pH control over the glutamate decarboxylase and antiporter genes. The small amount of remaining pH control is governed by GadX and W. The finding that *gadE* mutations also diminish the effectiveness of the other two acid resistance systems suggests that GadE influences the expression of additional acid resistance components. The number of regulatory proteins (five), sigma factors (two) and regulatory feedback loops focused on *gadA/BC* expression make this one of the most intensively regulated systems in *E. coli*.**

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## Introduction

Any pathogen that journeys through the stomach to cause disease needs a strategy to survive extreme low pH stress. Some organisms, such as *Vibrio cholerae*, must be ingested in huge numbers (>$10^9$ organisms) in the hope that some will survive the pH 1.5 to pH 2.5 gastric environment. However, pathogenic as well as commensal strains of *Escherichia coli* are exceptionally well adapted to survive extremely acidic pH even at low cell numbers. *E. coli* achieves this through three acid resistance (AR) systems (Lin *et al*., 1995; 1996; Hersh *et al*., 1996; Castanie-Cornet *et al*., 1999). These systems enable an infectious dose as low as 10–100 organisms (Giannella *et al*., 1972; 1973; Peterson *et al*., 1989). AR system 1 is a glucose-repressed, acid-induced system typically observed using Luria–Bertani (LB) broth cultures. Unidentified components of this system are controlled by the alternative sigma factor RpoS and the cyclic AMP receptor protein (CRP) (Castanie-Cornet *et al*., 1999). AR system 1 does not require any external amino acids to function at low pH. In contrast, AR systems 2 and 3 are amino acid decarboxylase/antiporter systems that use extracellular glutamate (AR 2) or arginine (AR 3) to protect cells during extreme acid challenges.

Of the three mechanisms, the glutamate-dependent AR system (AR 2) is the most efficient. Known components of this system include two isozymes of glutamate decarboxylase encoded by *gadA* and *gadB*, and a membrane-associated glutamate::γ-aminobutyric acid (GABA) antiporter (GadC) (Smith *et al*., 1992; Castanie-Cornet *et al*., 1999; De Biase *et al*., 1999). The decarboxylase and antiporter orchestrate a process of internal proton consumption that maintains a pH suitable for cell survival (H. Richard *et al*., in preparation). The *gadB* and *gadC* genes are arranged as an operon (*gadBC*) at 33.8 min (1570.1 kb) on the *E. coli* genome. The *gadA*

gene is located at 78.98 min (3665.2 kb) as the lead gene in an operon with *gadX*. The *gadX* cistron encodes an AraC-like activator of *gadBC and gadA* (Hommais *et al*., 2001; Shin *et al*., 2001; Ma *et al*., 2002; Tramonti *et al*., 2002; Tucker *et al*., 2002).

Based on the amount of regulatory control focused on this system, *E. coli* must rely heavily on glutamate-dependent acid resistance. The *gadA/BC* genes are induced in response to acid, osmotic and stationary phase signals (Castanie-Cornet *et al*., 1999; De Biase *et al*., 1999). Known regulators that affect *gadA/BC* expression include the sigma factor RpoS, two AraC-like regulators (GadX and GadW), cAMP and cAMP receptor protein (CRP). The level of involvement for each regulator varies depending upon the growth phase and medium. Stationary phase induction, especially in rich medium (LB), seems clear. The goal is to increase the production of the GadX regulator, which, in turn, activates *gadA/BC* expression. As cells enter stationary phase, the levels of the RpoS sigma factor increase, an increase that occurs somewhat earlier in moderately acidic cultures (pH 5.5). The increase in RpoS drives expression of *gadX* from a proximal promoter located downstream of *gadA.* The resulting increase in GadX protein then activates the *gadA* and *gadBC* promoters (Ma *et al*., 2002; Tramonti *et al*., 2002). However, the expression of *gadX* is tempered by the product of *gadW*, a gene downstream of *gadX*. The *gadW* gene produces another AraC-like regulator (Ma *et al*., 2002). GadW represses *gadX* but can also directly activate the *gadA/BC* promoters under different situations.

The global regulator cyclic AMP receptor protein (CRP) administers additional control over GadX production, tying it to the carbon energy state of the cell. CRP does this by repressing RpoS production (Castanie-Cornet and Foster, 2001; Ma *et al*., 2002). A final repressor implicated in stationary phase control is the histone-like protein H-NS, although its relative importance may be strain dependent (De Biase *et al*., 1999; Ma *et al*., 2002; Tramonti *et al*., 2002).

Stationary phase induction of *gadA/BC* occurs regardless of whether cells were grown in rich or minimal media. In contrast, exponential phase expression is very media dependent. Log phase, LB-grown cells do not express *gadA/BC* unless the levels of GadX are elevated by mutation or by an overproducing *gadX* plasmid. However, exponential phase cells grown in minimal salts glucose media will express *gadA/BC* to high levels during growth under acidic conditions (pH 5.5). We show here that log phase expression in minimal media is RpoS and GadX independent, a finding that suggested the existence of another regulator. Previous studies demonstrated that a 20 bp *cis*-acting DNA sequence (GAD box) located in the promoter regions of *gadA* and *gadBC* is essential for the expression of these genes under any condition (Castanie-Cornet *et al*., 1999). However, GadX only partially contacts this region (Tramonti *et al*., 2002). This finding also suggested the existence of an unknown, even more critical regulator.

Multiple gene array studies have implicated *yhiE*, a gene encoding a putative regulator, as participating in acid resistance. Although predicted to be a regulator of acid resistance, how it was involved has not been explored (Masuda and Church, 2002; 2003; Tucker *et al*., 2002; Nishino *et al*., 2003). The goals of the current study were to examine whether *yhiE* contributes to acid resistance through direct control over *gadA/BC* expression. The results show that YhiE, renamed here as GadE, is a critical acid-induced regulator of *gadA/BC* expression.

## Results

### *Acid induction of* gadA/BC *expression in minimal salts glucose media is RpoS and GadXW independent*

Previous reports indicated that *gadA/BC* expression in LB-grown cells is RpoS dependent (Ma *et al*., 2002; Tramonti *et al*., 2002). Induction occurs during entry into stationary phase coincident with the stimulation of RpoS synthesis. RpoS subsequently drives the expression of GadX, a required activator for *gadA/BC* transcription in LB. However, the situation in minimal salts glucose media (EG) is very different. Figure 1A and B illustrates that *gadA/BC* expression in LB occurs primarily in stationary phase cells and depends on RpoS. Figure 1C and D, in contrast, shows that exponential phase cells grown in minimal EG medium exhibit RpoS-independent, acid-induced *gadA/BC* expression.

Figure 1E reveals that *gadA/BC* expression in EG medium is also *gadX* and *gadW* independent. This was in contrast to LB, in which *gadA/BC* expression required one or the other AraC-like regulator (Ma *et al*., 2002). GadX and W were not entirely without effect in minimal media, however. Unlike LB, where they were needed to activate *gadA/BC*, GadX and GadW acted as negative regulators in minimal media (in exponential phase) such that removing them allowed additional, acid-induced expression (Fig. 1E, lanes 1 and 2 versus 3 and 4). The results demonstrated that the expression of *gadA/BC* in minimal glucose media depends on a regulator other than RpoS, GadX or GadW.

### *GadE (YhiE) is required for* gadA/BC *expression during exponential growth*

Evidence presented earlier indicated that a putative regulatory protein encoded by *gadE* (*yhiE*) was somehow involved in *E. coli* acid resistance (Masuda and Church, 2002; Tucker *et al*., 2002). The acid resistance system

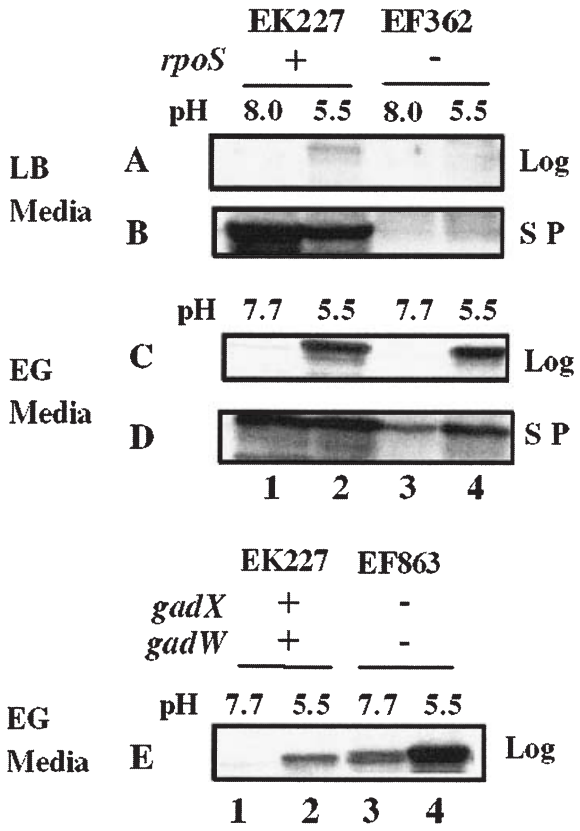


**Fig. 1.** GadA/B production in minimal glucose does not require RpoS, GadX or GadW. Cells were grown in LB (A and B) or minimal glucose (C–E) to exponential (A, C and E) or stationary phase (B and D). Cultures were grown under acid or alkaline conditions as shown. Strains and pertinent genotypes are shown. SDS-PAGE was performed on 5 μg samples and probed with anti-GAD antibody as described in *Experimental procedures*.

affected and the basis of GadE(YhiE) involvement were not determined. A clue was provided by computer analysis, which indicated that GadE (YhiE) belonged to the LuxR family of transcriptional regulators. This led to an investigation of whether this putative regulatory protein affected acid resistance by influencing *gadA/BC* expression. Figure 2A and B (lanes 1 and 2 versus 3 and 4) shows that cells grown to exponential phase in minimal glucose media required GadE (YhiE) for acid-induced expression of *gadA* and *gadBC*. Northern blot analysis (Fig. 2A) indicated that the effect occurred at the transcriptional level for both *gadA* and *gadBC* operons. Because of its direct participation in *gadA/BC* control, we will refer to this gene as *gadE*.

It was possible that the effect of *gadE* on *gadA/BC* expression resulted from a polar effect of the deletion on a downstream gene. To eliminate this possibility, a plasmid that expressed *gadE* under the control of its native promoter was introduced into a *gadE* mutant, and the effect on GadA/B production was monitored by Western blot. As shown in Fig. 2B, lanes 5 and 6, this plasmid restored acid-induced *gadA/BC* expression.

### *GadE is also required for stationary phase expression of* gadA/BC

We demonstrated previously that either GadX or GadW was required by stationary phase cells to express *gadA/BC* (Ma *et al*., 2002). To determine whether GadE was also needed, we tested a *gadE* mutant for stationary phase production of glutamate decarboxylase. As predicted, the *gadE* mutant failed to express any GAD (Fig. 3A, lanes 1 and 2 versus 3 and 4). However, overproducing GadE from a plasmid successfully suppressed the stationary phase requirement for GadX and W (Fig. 3B, lanes 3 and 4 versus 5 and 6).

### *GadX function is epistatic to GadE*

As neither GadX nor GadW was required for *gadA/BC* expression in log phase EG but GadE was essential, it appeared that GadE does not work through GadX or W

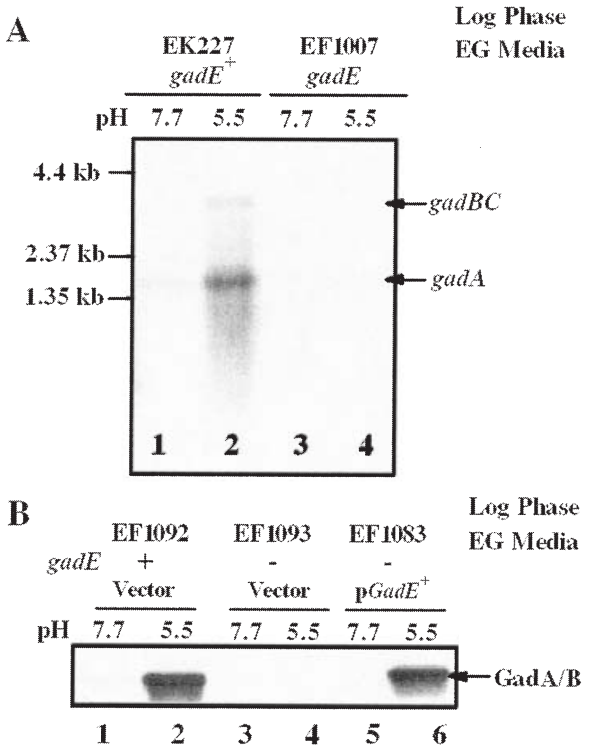


**Fig. 2.** Acid induction of *gadA* and *gadBC* requires GadE.
A. Northern blot analysis. RNA extracted from exponential phase, minimal glucose media-grown cells (*gadE*$^+$ and *gadE*$^-$) was probed for *gadA* and *gadBC* messages as described in *Experimental procedures*. Samples of 5 μg of total RNA were loaded per well.
B. Western blot analysis of GadA and B production in *gadE*$^+$ and *gadE*$^-$ strains containing an empty vector (pPCRScript) or vector in which *gadE*$^+$ was cloned (pPCRScript YhiE$^+$). The cloned *gadE*$^+$ was expressed from its native promoter. Western analysis was performed as in Fig. 1. Growth pH values for different cultures are shown.

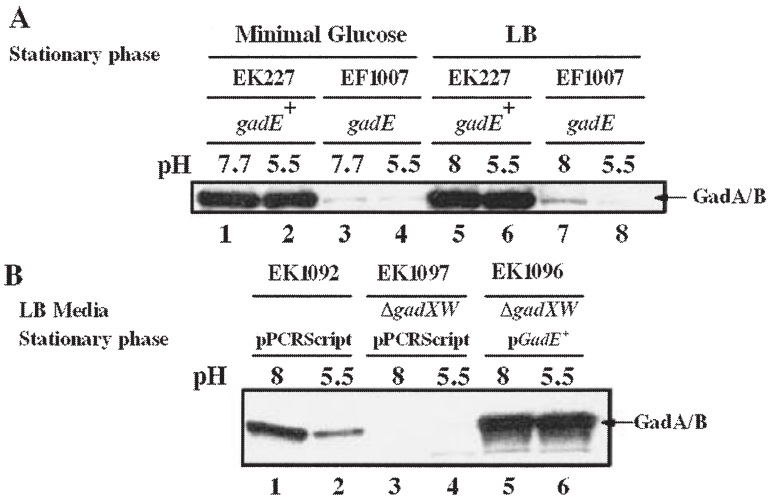


**Fig. 3.** GadE is required for stationary phase expression of *gadA/BC* and, when overexpressed, will suppress the *gadXW* requirement in LB. Western blot analysis of GadA/B production.
A. Stationary phase production of GAD in *gadE*$^+$ and *gadE*$^-$ mutants grown in minimal glucose ($OD_{600}$ 2) or LB ($OD_{600}$ 3) media.
B. Effect of overproducing GadE on the GadXW phenotype. pGadE$^+$ is pPCRScript YhiE$^+$ (GadE). Western analysis was performed as in Fig. 1.

(Fig. 1E). The data above also indicate that overexpressing GadE can supplant the stationary phase requirement for GadX or W (Fig. 3B). This could mean that either GadX or GadE alone can drive expression, or that E is essential and X only helps GadE under some situations. To distinguish between these possibilities, we examined whether or not overexpressing GadX could eliminate a need for GadE. A *gadW crp* double mutant that overexpresses GadX was used for this study (Ma *et al*., 2002). GadW directly represses *gadX* whereas Crp indirectly represses *gadX* by lowering RpoS levels. Figure 4 shows that GadX was highly expressed in a *crp gadW* mutant (Fig. 4B) but that GadE remained essential for *gadA/BC* expression(Fig. 4A). Thus, GadX cannot supplant GadE as an activator. These data suggest that GadX facilitates the use of GadE (YhiE), probably at the 20 bp Gad box, and does not directly activate *gadA/BC* expression.

### *Purified GadE binds the 20 bp GAD box*

A previous study demonstrated that the 20 bp GAD box sequence present in the *gadA* and *gadBC* promoters was essential for the expression of those genes (Castanie-Cornet and Foster, 2001). However, DNA footprint analysis revealed that the positive regulator GadX only partially protected this sequence (Tramonti *et al*., 2002). It was considered possible that GadE contacted the Gad box more directly. To address this question, a MalE–GadE hybrid protein was engineered, purified and used in gel shift experiments. Figure 5 reveals that GadE can bind to both *gadA* and *gadB* promoters with equal affinity. Unlabelled DNA from the *gad* promoter successfully competed with labelled DNA for binding (Fig. 5, lanes 7 and 14). Thus, GadE does bind the *gad* promoters.

To show that GadE binds to the 20 bp GAD box, 32 bp fragments that included the GAD box and 6 bp flanking regions from the *gadA* or *gadB* promoters were synthesized. In addition, equal-sized fragments were made from regions situated to the left and right of the *gadA* GAD box but that did not carry the GAD box. MalE–GadE failed to shift either of the fragments missing the GAD box (Fig. 6B, lanes 2, 3, 5 and 6) but definitely bound to the GAD box fragments (Fig. 6, lanes 8, 9, 13 and 14). Binding was specific as unlabelled GAD box fragments from either *gadA* or *gadB* successfully competed with the radiolabelled GAD box fragments for MalE–GadE. The evidence clearly indicates that GadE binds to the GAD box sequence.

A somewhat different situation was observed for GadX. MalE–GadX binds to the 32 bp Gad box fragments (20 bp Gad box plus 6 bp flanking sequences) but, unlike GadE, it also binds to adjacent 32 bp fragments that do not contain the Gad box (Fig. 6C). This is consistent with the broad GadX footprint reported earlier. However, the highest affinity is still to the GAD box area. Differences in the 6 bp flanking areas between *gadA* and *gadB* account for the higher affinity of GadX for the *gadA* area. The results also indicate that GadX and GadE can bind independently to this region.

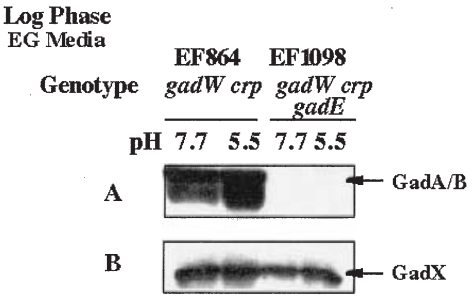


**Fig. 4.** Overexpression of GadX does not suppress a *gadE* mutant phenotype. Cells were grown in minimal media containing 0.1% casamino acids and prepared for Western blot analysis of GadX or GadA/B as described in Fig. 1.

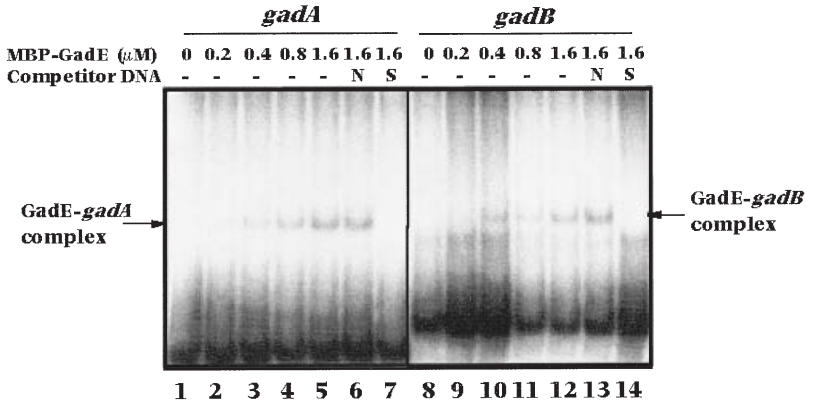


**Fig. 5.** Electrophoretic gel shift analysis of GadE interactions with the *gadA* and *gadB* promoters. Promoter fragments from *gadA* (242 bp; −164 to +78) and *gadBC* (281 bp; −203 to +78) were used in gel shift experiments with purified MalE–GadE protein (see *Experimental procedures*). Lanes are marked with the concentration of hybrid MalE–GadE used and indicate whether the reaction contained unlabelled non-specific (N) or specific (S) inhibitor.

*GadE is acid induced and involved in acid induction of* gadA/BC

The *gadA/BC* operons are acid induced in minimal media during exponential growth (Figs 1C and E and 2B). The cause of this pH control is not known. Although growth pH appears to affect GadW activity (Ma *et al*., 2002), GadW is not required for pH control (Fig. 1E, lanes 3 and 4). We explored the possibility that pH control could lie with the expression of *gadE*. Northern blot results presented in

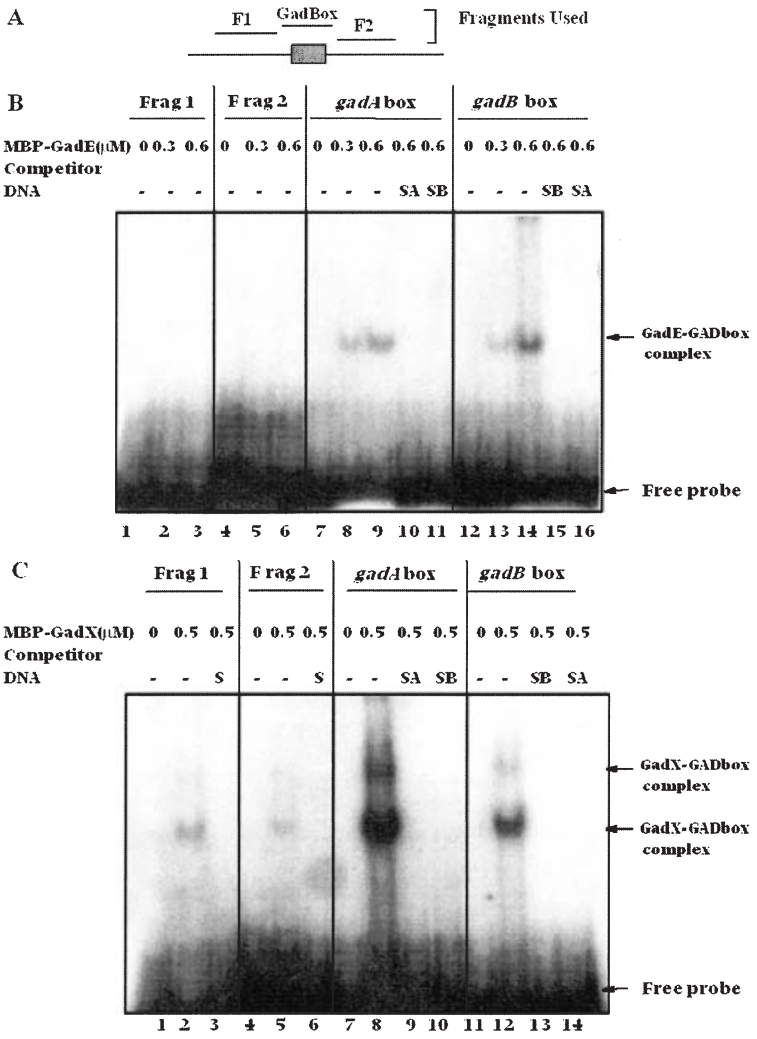


**Fig. 6.** GadE binds specifically to the 20 bp GAD box.
A. Illustration of 32 bp fragments used for EMSA. F1 and F2 flank the GAD box of *gadA* but do not contain the GAD box. The Gad box fragments also contain 6 bp sequences flanking the 20 bp GAD box.
B and C. Gel shift experiment using different 32 bp fragments and different concentrations of MalE–GadE (B) or MalE–GadX (C). GadA box and GadB box represent 32 bp fragments encompassing the 20 bp GAD box from *gadA* and *gadB* respectively. SA and SB are unlabelled, competitive 32 bp GadA box and GadB box fragments respectively.

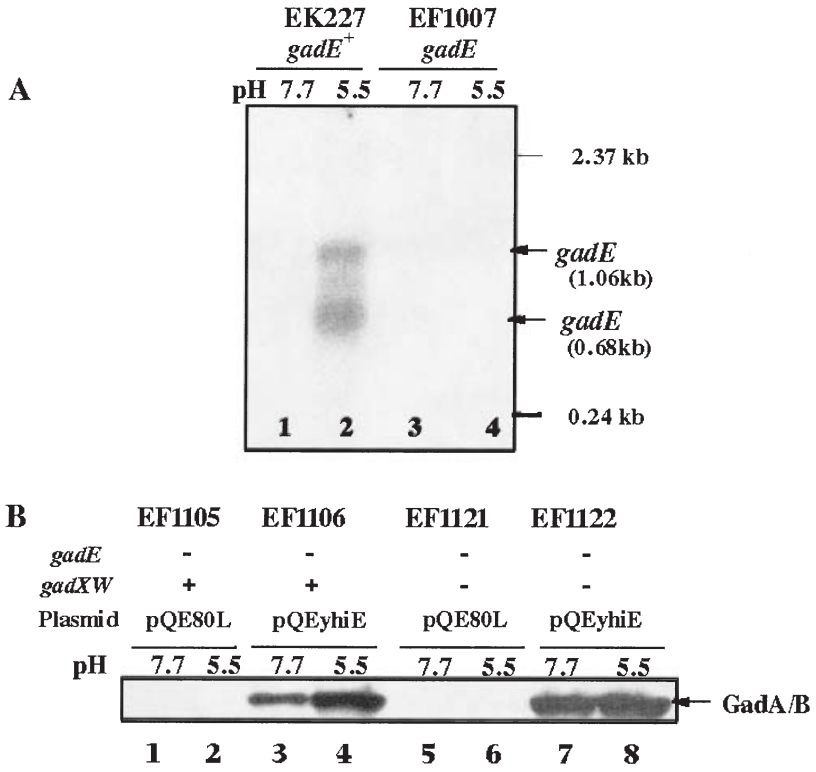


**Fig. 7.** Acid induction of the *gadE* locus is responsible for acid induction of *gadA/BC*.
A. Northern blot analysis of *gadE* expression. Cells were grown to exponential phase ($OD_{600}$ = 0.4) in minimal glucose media at the pH values indicated. Samples of 5 μg of total RNA were loaded per well.
B. Western blot analysis of GadA/B acid induction in cells with a constitutively expressed GadE. Cells were grown to log phase in minimal glucose containing 1.5 mM IPTG to induce *gadE*. Western analysis was performed as in Fig. 1.

Fig. 7A revealed that *gadE* is itself acid induced and produced as two transcripts of 0.68 kb and 1.06 kb. The acid induction of *gadE* may therefore be responsible for the pH-dependent expression of *gadA/BC*.

To confirm this link, *gadE* was removed from pH control and placed under the control of an IPTG-regulated promoter in plasmid pQE80L (the vector and *gadE* construct were kindly supplied by N. Masuda). Normally, the *gadE* (Fig. 7A) and *gadA/BC* genes (Fig. 2A) are not expressed in log phase cells growing at pH 7.7 in minimal glucose. However, Fig. 7B (lane 3) illustrates that artificially inducing GadE at pH 7.7 leads to an increase in GAD production, supporting the hypothesis that pH control of *gadE* accounts in large part for the pH control of *gadA/BC*.

### *GadX and GadW impose a second level of acid control*

The data presented above clearly indicate that the acid induction of *gadE* correlates with acid induction of the *gadA/BC* genes. However, GadX and W have also been implicated in pH control (Ma *et al*., 2002). Closer examination of Fig. 7B (lanes 3 and 4) reveals that, even when GadE was constitutively expressed, some pH control over *gadA/BC* expression remained. To examine whether residual pH control in this experiment resulted from GadXW, we overexpressed GadE in a Δ*gadXW* strain and re-examined GadA/B production by Western blot. The results confirmed that the GadXW system repressed *gadA/BC* to some degree at pH 7.7 even when GadE was overproduced (Fig. 7B, lanes 7 and 8). Thus, GadXW and GadE exert separate forms of pH control over *gadA/BC*.

### *The conditional requirement for GadX or W in minimal glucose-grown stationary phase cultures is related to GadE level*

In contrast to log phase (pH 5.5) ,where neither GadX nor W are needed for *gadA/BC* expression, the Western blot in Fig. 8A (lanes 7 and 8) indicates that, in stationary phase EG, there is a need for either X or W in addition to GadE for maximum GadA/BC expression. However, neither GadX nor GadW is required for *gadE* expression (data not shown). A Northern blot study was performed using minimal media-grown cells to determine whether the GadX/W requirement might be related to the level of *gadE* expression. The results indicate that stationary phase growth conditions dictating a need for X or W also produce low levels of *gadE* (Fig. 8B). However, when *gadE* levels were elevated, as in log phase pH 5.5-grown cells (Fig. 8B), neither GadX nor W had much influence on *gadA/BC* expression.

### *GadE and GadX simultaneously bind the* gad *promoter region*

The results above indicated that, under certain circum-

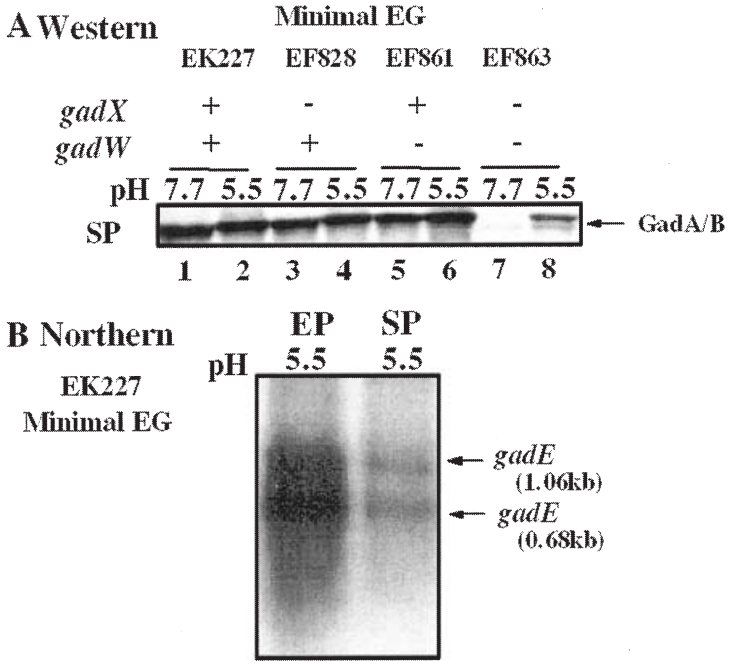


**Fig. 8.** Maximum expression of *gadA/BC* in stationary phase minimal media requires either GadX or GadW.
A. Western blot analysis of GadA/B production in stationary phase, minimal media-grown cells. Western analysis was performed using 5 μg of total protein per lane as in Fig. 1.
B. Northern blot analysis of *gadE* transcripts from cells grown in minimal glucose to exponential phase (lane 1) or stationary phase (lane 2). Samples of 5 μg of total RNA were loaded per well.

stances, GadX and GadE are required for optimal expression of the *gadA/BC* genes. The data also showed that each protein binds strongly to the 20 bp Gad box region, although GadX can weakly bind adjacent areas. We next used a supershift experiment to test whether both proteins can simultaneously bind the 32 bp Gad box fragment described in Fig. 6. The results showed that either one or the other protein could bind, but not both (data not shown). We then used the entire 242 bp *gadA* promoter region and asked the same question. The results, presented in Fig. 9, clearly demonstrated that the presence of both proteins caused a supershift of the *gadA* promoter fragment. Regardless of whether GadX (lanes 2–6) or GadE (lanes 9–13) levels were held constant while levels of the other protein were allowed to increase, supershifts were observed. Thus, although only one protein can occupy the smaller 32 bp fragment, the larger fragment accommodates both.

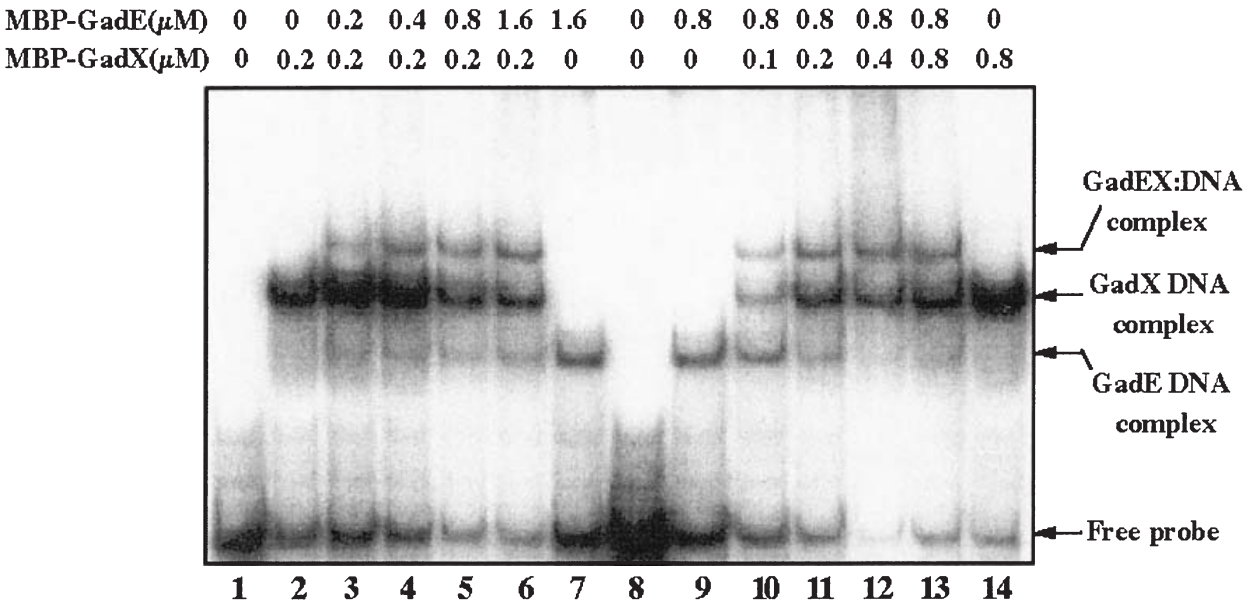


**Fig. 9.** GadX and GadE supershift the *gadA* promoter. Experimental conditions were as described in Fig. 5.

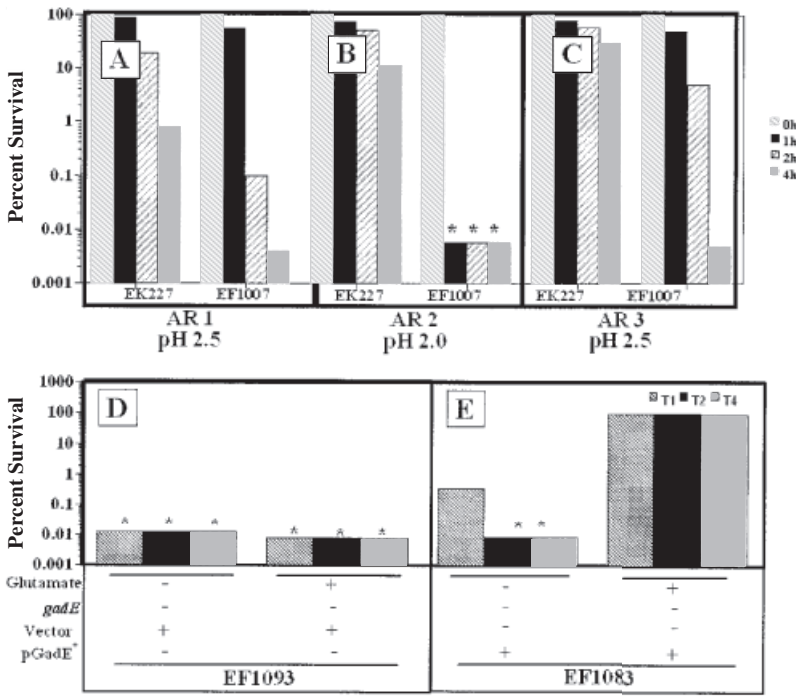


**Fig. 10.** Effect of *gadE* on acid resistance.
A. Cells grown in LB MES pH 5.5 to stationary phase were diluted 1:1000 into minimal media pH 2.5.
B. Cells grown in LB glucose were diluted 1:1000 in pH 2.5 minimal media with 0.7 mM glutamate.
C. Cells grown in BHIG but diluted into pH 2.5 minimal media with1.5 mM arginine.
D. Glutamate-dependent acid resistance of a *gadE* mutant containing empty vector and vector containing *gadE*$^+$ (E). First bar set in panels D and E, cells grown at pH 7.7. Second bar set, cells grown at pH 5.5. *Viable count below the detection limit.

*GadE affects all three acid resistance systems*

Previous reports have shown that a *gadE* (*yhiE*) mutant is defective in acid resistance but never addressed which of the three known acid resistance systems was affected. Consequently, we tested the effect of a *gadE* (*yhiE*) mutation on the function of these systems. The mutation was expected to have a dramatic effect on the glutamate-dependent system as *gadE* (*yhiE*) was required for GadA/BC production. This prediction was confirmed (Fig. 10B). Figure 10A and C shows that *gadE* also had a significant effect on the glucose-repressed (AR 1) and the arginine-dependent (AR 3) systems. By 4 h of acid challenge, AR system 1 was about 1000-fold less effective, whereas the arginine-dependent system was 1000- to 10 000-fold less effective at acid protection.

Western blot analysis of the AdiA (arginine decarboxylase) protein needed for AR system 3 revealed that *gadE* did not affect the production of this protein (data not shown). As knocking out *gadC* or *gadA/B* does not affect the other two systems (Castanie-Cornet *et al*., 1999), the results argue that *gadE*, in addition to affecting GadA/BC synthesis, may affect the expression of other, unknown genes, the products of which are of general importance to acid resistance.

To show that the general effect of a *gadE* mutation on acid resistance was not the result of polar effects on downstream genes, a *gadE* mutant strain containing the pYhiE$^+$ (GadE$^+$) plasmid was tested for acid resistance. Figure 10E demonstrates that the plasmid successfully complemented the acid resistance defect seen at pH 5.5 in Fig. 10D.

## Discussion

Acid resistance is a critical survival strategy for *E. coli* and other select microorganisms (Sanders *et al*., 1998; Small and Waterman, 1998; Cotter *et al*., 2001). It is useful as the organism traverses the gastric acid barrier, but it can also play an important role in surviving organic acid stresses that result from self-generated fermentation products or products made by neighbours in a mixed microbial community. Although the basic physiology of glutamate-dependent acid resistance seems simple, i.e. the decarboxylation of glutamate consumes a proton, the regulatory network guiding its synthesis is extremely complex (Fig. 11). Previous studies have implicated *gadX*, *gadW*, *crp*, *rpoS*, *hns* and *evgA* as some of the regulators involved in modulating *gadA/BC* expression. The results presented here indicate that there is an absolute requirement for GadE as an activator. The data shown indicate that this protein directly binds to the 20 bp GAD box sequence required for activation.

The expression of *gadE* is acid induced in log phase cultures grown in minimal glucose media, presumably through the EvgSA two-component system (Masuda and Church, 2003). Our results indicate that *gadE* induction subsequently activates expression of the *gadA* and *gadBC* transcriptional units. However, there is very little

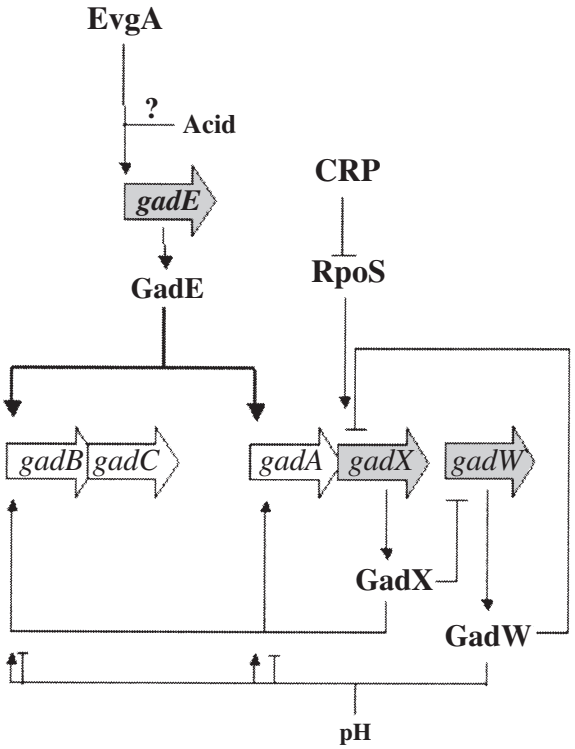


**Fig. 11.** Regulatory network governing *gadA/BC* expression and glutamate-dependent acid resistance. Thin arrows indicate positive control. T lines indicate negative control.

expression of *gadA/BC* in log phase cultures grown in rich media regardless of pH. Transcription in this media not only requires GadE but needs GadX as well (Shin *et al*., 2001; Ma *et al*., 2002; Tramonti *et al*., 2002). Transcription of *gadX* is primarily driven by the alternative sigma factor sigma S (RpoS), levels of which are very low in log phase LB-grown cultures. In addition to low RpoS levels, log phase expression of *gadX* is repressed by GadW (Ma *et al*., 2002). However, as cells enter stationary phase, sigma S levels rise and increase the production of GadX. This increase occurs regardless of external pH conditions.

Why *gadA/BC* transcription requires GadX in some situations but not others may be related to the level of GadE present in the cell. Increasing GadE diminishes the need for GadX or W. It has been shown that both GadX and GadW bind to the *gadA/BC* promoter regions and that the 20 bp GAD box enhances GadX binding. These proteins may interact with GadE in some way at the promoter and influence GadE's ability to activate transcription of the *gadA/BC* genes. Indeed, the evidence presented in Fig. 9 suggests that GadX and GadE may form a complex around the Gad box. It is also possible that another negative regulator prevents the expression of *gadA/BC* in complex media and that GadX or W, two AraC-like regulators, can help GadE to overcome this putative repressor.

The picture emerging of *E. coli* acid resistance is complex. Gene array analyses have found that GadX controls at least eight genes in addition to *gadA/BC* (Tucker *et al*., 2003). Another recent study has indicated an acid resistance regulatory pathway emanating from the two-component regulatory system EvgAS (Masuda and Church, 2002). Data presented in that study suggested that EvgA regulates *ydeO*, the product of which regulates *gadE* (*yhiE*). The authors used overexpression of EvgA artificially to activate acid resistance in pH 2.5 LB. Both *ydeO* and *gadE* (*yhiE*) mutants were found to be defective in EvgA-stimulated acid resistance as tested in LB, although which acid resistance system was affected was not discerned. The data presented here using natural induction conditions clearly show that the primary effect of *gadE* (*yhiE*) is through its direct control over *gadA/BC* expression. However, in contrast to the gene array study, results using targeted Northern blot analysis have revealed that *ydeO* does not significantly affect *gadE* (*yhiE)* or *gadA/BC* expression under these conditions (data not shown). An explanation for this apparent discrepancy is that the array study exposed the YdeO–GadE (YhiE) connection by overexpressing the EvgA response regulator, an artificial induction strategy. However, multiple signals may activate *gadE* expression, and YdeO may sense and deliver one of them. Even if that is the case, that hypothetical signal pathway is not needed to activate *gadE* under the conditions examined here (minimal versus LB media).

It is becoming apparent that acid resistance in general and the *gadA/BC* genes in particular are among the most highly regulated gene systems in *E. coli*. Components involved include a two-component signal transduction system (EvgAS), three activators (GadE, X, W), two sigma factors (sigma S and sigma 70), two global regulators (Crp, H-NS) and at least one regulatory loop (GadX–GadW). In addition, the TorS regulator appears to play some role under anaerobic conditions (Bordi *et al*., 2003). Close monitoring by these multiple regulatory circuits suggests that the glutamate decarboxylase and glutamate:GABA antiporter system is vitally important to *E. coli* survival. Expression and activation of the system must be closely tied to the physiological state of the cell and conditions in the environment. These regulatory networks allow the cell to anticipate possible acid pH disasters even if the crisis never comes. Stationary phase cells, for example, need not encounter any level of low pH in order to be forearmed. Elucidating the signals sensed by the AraC-like regulators GadX and GadW and the manner in which *gadE* is induced by low pH will provide critical insight into how glutamate-dependent acid resistance ties into the physiology of the cell.

**Table 1.** Bacterial strains and plasmids in this study.

| Strains | Genotype | Source or reference |
|---|---|---|
| EK227 | K-12 wild type λ- F- | A. C. Matin (Stanford University) |
| EK551 | K-12 (MG 1655) Δ*yhiE*::Km | Tucker *et al*. (2002) |
| EK584 | (TB1) *ara* Δ (*lac-proAB*)*rpsL* (Δ80*lacZ* ΔM15)*hsdR* | New England Biolabs |
| EK585 | (XL10-Gold Kan) *recA hsdR endA* | Stratagene |
| EF362 | K-12 (EK227) *rpoS*::Tn*10* | Castanie-Cornet *et al*. (1999) |
| EF828 | K-12 (EK227) Δ*gadX* | Ma *et al*. (2002) |
| EF861 | K-12 (EK227) Δ*gadW*::Km | Ma *et al*. (2002) |
| EF863 | K-12 (EK227) Δ*gadXW*::Km | Ma *et al*. (2002) |
| EF864 | K-12 (EK227) Δ*gadW*::Km Δ*crp*::Cm | Ma *et al*. (2002) |
| EF908 | K-12 (EK227) Δ*gadXW* | Ma *et al*. (2002) |
| EF1007 | K-12 (EK227) Δ *gadE*::Km | EK227 × EK551 |
| EF1079 | K-12 (EK227)/pPCR-Script Amp *yhiE* (*gadE*)$^+$ | This study |
| EF1083 | K-12 (EK227) Δ*gadE*::Km/pPCR-Script Amp *yhiE* (*gadE*)$^+$ | This study |
| EF1092 | K-12 (EK227)/pPCR-Script Amp SK(+) | This study |
| EF1093 | K-12 (EK227) Δ*gadE*::Km/pPCR-Script Amp SK(+) | This study |
| EF1095 | K-12 (EK227) Δ*gadW* Δ*crp*::Cm | EF864 × pCP20 |
| EF1096 | K-12 (EK227) Δ*gadXW*::Km/pPCR-Script Amp *yhiE* (*gadE*)$^+$ | This study |
| EF 1097 | K-12 (EK227) Δ*gadXW*::Km/pPCR-Script Amp SK(+) | This study |
| EF1098 | K-12 (EK227) Δ*crp::Cm* Δ*gadW* Δ*gadE*::Km | This study |
| EF1105 | K-12 (EK227) Δ*gadE*::Km/pQE80L | This study |
| EF1106 | K-12 (EK227) Δ*gadE*::Km/pQE*yhiE*(*gadE*) | This study |
| EF1120 | K-12 (EK227) Δ*gadXW* Δ*gadE*::Km | EK551 × EF908 |
| EF1121 | K-12 (EK227) Δ*gadXW* Δ*gadE*::Km/pQE80L | This study |
| EF1122 | K-12 (EK227) Δ*gadXW* Δ*gadE*::Km/pQE*yhiE* (*gadE*)$^+$ | This study |
| Plasmids | | |
| pQE80L | | Masuda and Church (2003) |
| pQE*yhiE*(*gadE*) | | Masuda and Church (2003) |
| pMALc2E | | New England Biolabs |
| pMF533/pMALc2E*gadE* | | This study |
| pPCR-Script Amp SK(+) | | Stratagene |
| pPCR-Script Amp *yhiE*(*gadE*) | | Tucker *et al*. (2002) |
| pCP20 | FLP recombinase | Datsenko and Wanner (2000) |

## Experimental procedures

### *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) (Vogel and Bonner, 1956), complex medium, Luria–Bertani broth (LB) medium buffered with either 100 mM MOPS (pH 8) or 100 mM morpholinethanesulphonic acid (MES, pH 5.5), LB containing 0.4% glucose (LBG) and brain–heart infusion broth containing 0.4% glucose (BHIG). The minimal glucose media was supplemented with 0.1% casamino acids where indicated. Antibiotics were used at the following concentrations: ampicillin, 60 μg ml$^{-1}$; kanamycin, 25 μg ml$^{-1}$; tetracycline, 20 μg ml$^{-1}$; carbenicillin, 100 μg ml$^{-1}$; and chloramphenicol, 30 μg ml$^{-1}$. All strains were grown at 37°C with aeration.

### *Genetic and basic molecular techniques*

Phage P1 transduction, transformation with $CaCl_2$ and electroporation were performed by standard methods (Miller, 1992). General DNA manipulations were carried out as described earlier (Sambrook *et al*., 1989). Deletion mutations were carried out by the method reported by Datsenko and Wanner (2000). The list of oligonucleotide primers used is listed in Table 2.

### *Western blot analysis*

Strains were grown at 37°C in media containing the required antibiotics as indicated. Conditional expression of GadE from pQE*yhiE* (*gadE*) was induced by adding 1.5 mM IPTG to the media containing 100 μg carbenicillin before inoculation. Expression of *gadE* from the pPCR Script Amp *yhiE* (*gadE*) clone with a native promoter was induced in indicated media with 100 μg ml$^{-1}$ ampicillin. At $OD_{600}$ of 0.4 (log phase) or 3.8 (late stationary phase), cells were collected by centrifugation and then resuspended in 0.01% sodium dodecyl sulphate (SDS) solution. Protein concentrations were determined using Bio-Rad Protein Assay reagent. Samples (5 μg of protein) were mixed with 2× SDS-PAGE loading buffer (125 mM Tris, pH 7.0, 20% glycerol, 10% β-mercaptoethanol, 6% SDS, 0.2% bromophenol blue), boiled for 5 min and then separated by PAGE on 10% Tris-HCl Criterion gels (Bio-Rad). After semi-dry electrophoretic transfer of proteins onto polyvinylidene difluoride (PVDF) membranes (Millipore), the membranes were incubated overnight with 5% powdered milk in TBST buffer (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.05% (v/v) Tween 20) to block non-specific protein interactions. The membranes were probed with one of the following primary antibodies: rat anti-GAD (Castanie-Cornet *et al*., 1999) or rabbit anti-GadX (kindly provided by S. Shin and J. Kaper) followed by the appropriate monoclonal secondary antibodies coupled to peroxidase (Sigma): anti-rat (1:10 000) or anti-rabbit (1:4000). Antibody-tagged protein bands on the probed

**Table 2.** Oligonucleotide primers used in this study.

| Name | Sequence |
|---|---|
| oligo 201 | 5′-CAGCAATGTTTGGGCGATTTTTATTAC-3′ |
| oligo 202 | 5′-GATAATTCAGGAGACACAGAATGCG-3′ |
| oligo 233 | 5′-CTCGTCGACACGTGAATCGAGTAGTTC-3′ |
| oligo 377 | 5′-GGAGTTCGAAATGGACCAGAAG-3′ |
| oligo 378 | 5′-AGTTTCGGGTGATCGCTGAG-3′ |
| oligo 379 | 5′-GGTGTCGACTATGAACCTGCTTCCCATCGACTAC-3′ |
| oligo 507 | 5′-CAATACGCAAACCGCCTCTCC-3′ |
| oligo 508 | 5′-AGTGAATCCGTAATCATGGTCATA-3′ |
| oligo 540 | 5′-CAAGTTATGATTTTTCTCATGACGAA-3′ |
| oligo 541 | 5′-CTAAAAATAAGATGTGATACCCAG-3′ |
| oligo 572 | 5′-TTCTGCTTAGGATTTTGTTATTTAAATTAAGC-3′ |
| oligo 573 | 5′-GCTTAATTTAAATAACAAAATCCTAAGCAGAA-3′ |
| oligo 574 | 5′-ATAACATTAGGATTTTGTTATTTAAACACGAG-3′ |
| oligo 575 | 5′-CTCGTGTTTAAATAACAAAATCCTAATGTTAT-3′ |
| oligo 586 | 5′-GTTAAATGTTTATATTATAAAAAGTCGTTT-3′ |
| oligo 587 | 5′-AAACGACTTTTTATAATATAAACATTTAAC-3′ |
| oligo 588 | 5′-CTGTAATGCCTTGCTTCCATTGCGGATAAA-3′ |
| oligo 589 | 5′-TTTATCCGCAATGGAAGCAAGGCATTACAG-3′ |

membranes were detected using an ECL Western blot detection kit (Amersham).

### *Northern blot analyses*

Total RNA was extracted from log phase cultures ($OD_{600} = 0.4$; $2 \times 10^8$ cells $ml^{-1}$) grown under either alkaline or acidic conditions in LB or minimal media. Extractions were performed using the RNeasy kit (Qiagen). RNA concentrations were determined by measuring the OD values at 260 and 280 nm. Aliquots of RNA (5 μg) were denatured at 65°C for 15 min and separated by electrophoresis through a denaturing formaldehyde–agarose (1.2%) gel as described previously (Sambrook *et al*., 1989). The RNA was then transferred onto a positively charged nylon membrane (Amersham Pharmacia) and baked at 80°C for 2 h. The membranes were probed with a 1.4 kb *gadA/B* probe or a 0.534 kb *gadE* probe generated by polymerase chain reaction (PCR) using oligos 377/378 and oligos 540/541 respectively. Probes were labelled with [α-$^{32}$P]-dCTP (Amersham) using a random-primed DNA labelling kit (Boehringer Mannheim). The *gadA/B* probe corresponds to the entire open reading frame (ORF) of *gadA* or *gadB* and hybridizes to both *gadA* and *gadB*. As a control, the membranes were also hybridized with a 23S ribosomal RNA probe (oligo 379) end-labelled with [γ-$^{32}$P]-ATP.

### *Purification of MBP–GadE fusion protein*

The *gadE* ORF with the start codon deleted was excised from pQE*yhiE* (kindly provided by N. Masuda and G. M. Church) using *Bam*HI and *Hin*dIII and religated into *Bam*HI and *Hin*dIII sites located downstream of MalE in plasmid pMALc2E (New England Biolabs). The resulting plasmid, pMF533, was transformed into *E. coli* K-12 strain TB1 (New England Biolabs). Cells containing pMF533 were grown in 500 ml of LB broth with 0.2% glucose and 100 μg $ml^{-1}$ ampicillin at 37°C with aeration. A final concentration of 0.3 mM IPTG was added to the culture when the optical density at $OD_{600}$ reached 0.5. The culture was incubated for an additional 3 h to induce expression. Cells were pelleted by centrifugation at 8000 *g* for 15 min, washed once with 50 ml of iced column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl and 1 mM EDTA) with 1 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) and resuspended in 10 ml of iced column buffer containing 1 mM PMSF. The cell suspension was passed three times through a French pressure cell (20 000 p.s.i.). Cell lysates were centrifuged at 10 000 *g* for 30min to remove the insoluble fraction. The supernatants were diluted 1:5 with the iced column buffer in the presence of PMSF. Diluted cell extracts were loaded onto 10 ml amylose columns (New England Biolabs), which were pre-equilibrated with 80 ml (8 column volumes) of column buffer. The loaded column was washed with 200 ml of iced column buffer. MBP–GadE protein was eluted with 20 ml of column buffer containing 10 mM maltose and collected in fractions of 1.5 ml each. Samples of the three highest protein content fractions were combined and desalted through a PD-10 Sephadex column (Amersham Pharmacia). The protein was eluted with desalting buffer (10 mM $NaH_2PO_4$, pH 6.8, 0.1 mM EDTA and 0.2 M NaCl), and glycerol was added to a final concentration of 50% for storage. The protein was purified to homogeneity based on a Coomassie-stained SDS-PAGE (Bio-Rad).

### *Electrophoretic mobility shift assay (EMSA)*

The ability of MalE–GadE protein to bind the *gadA* and *gadB* promoters was tested using EMSA. The promoter target fragments were amplified with oligos 201/233 (*gadA*) and 202/233 (*gadB*) respectively. The PCR-generated *gadA* promoter fragment extends from bp −164 to +78, whereas the *gadB* promoter extends from bp −203 to +78 relative to the transcriptional start sites. Both fragments include the 20 bp GAD box region implicated in *gad* expression. Two 32 bp GAD box fragments contained the 20 bp GAD box sequence flanked by 6 bp on each end from the *gadA* or *gadB* promoters. In addition, two 32 bp control fragments immediately upstream and downstream of the 32 bp GAD box sequence of *gadA* were also used for EMSA. The 32 bp double-stranded DNA fragments were prepared by annealing two complementary oligonucleotides (oligos 572/573 for GAD box A; 574/575 for GAD box B; 586/587 for control fragment 1 and 588/589 for control fragment 2) for 10 min at 90°C in TEN buffer (1 mM Tris-HCl, pH 8.0, 1 mM EDTA and 100 mM NaCl). All fragments were end-labelled with [γ-$^{32}$P]-ATP by T4 polynucleotide kinase.

Radiolabelled DNA probes (5 ng, 5000 c.p.m.) were incubated with MalE–GadE fusion protein at room temperature for 30 min in 20 μl of binding buffer (20 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 50 mM potassium glutamate, 0.01 mM EDTA, 1 mM $NaH_2PO_4$, 20 mM NaCl, 1 mM dithiothreitol and 30 mg $ml^{-1}$ salmon sperm DNA). Samples were loaded onto 5% TBE non-denaturing 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.

### *Acid resistance assays*

To test for AR system 1, cells were grown in LB MES (pH 5.5) and LB MOPS (pH 8) for 22 h. Cultures grown in LB containing 0.4% glucose were used to test AR system 2, whereas

cells grown in BHIG were used to test AR system 3. The above three stationary phase cultures were diluted 1:1000 into prewarmed EG pH 2.5 medium to test acid resistance (final cell concentration of $2 \times 10^6$ ml$^{-1}$). Dilutions were made in unsupplemented EG pH 2.5 for AR 1, EG pH 2.0 supplemented with 0.7 mM glutamate for AR 2 and EG 2.5 medium containing 1.5 mM arginine for system 3. Viable counts were determined at time 0, 1, 2 and 4 h after acid challenge.

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