The *Escherichia coli* AraC-family regulators GadX and GadW activate *gadE*, the central activator of glutamate-dependent acid resistance

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Escherichia coli can survive pH 2 acid stress by using several acid resistance systems. The most efficient of these employs glutamate decarboxylase (GadA/GadB) to consume protons, and an antiporter (GadC) to exchange the intracellular decarboxylation product for external glutamic acid. Expression of the essential transcriptional activator of this system, GadE, is controlled by several regulators in a hierarchical fashion. In this study, two additional activators have been identified. The AraC-family regulators GadX and GadW, previously found to activate *gadA/BC* *in vitro*, are now shown *in vivo* to directly activate *gadE* expression, which, in turn, activates the *gadA/BC* genes. *In vivo* results using *E. coli* and *Salmonella enterica* show that these regulators actually have little direct effect on *gadA* and *gadBC* promoters. The numerous *gadE* induction pathways converge on a 798 bp control region situated upstream of the *gadE* promoter region. Deletions of this control region exposed the region between –798 and –360 nt (relative to the translational start) to be required for maximum *gadE–lacZ* expression in Luria–Bertani (LB) medium and to be the primary focus of GadX and GadW control. The GadE protein itself, which binds to three GAD box sequences present between –233 and –42 nt, helped activate GadE expression in LB, but only when the –798 to –360 region was absent. These regulatory regions and proteins appear to integrate a variety of physiological signals that forecast a need for GadE-dependent gene expression and acid resistance.

Received 16 February 2007

Revised 4 May 2007

Accepted 10 May 2007

INTRODUCTION

Stomach acid, pH 2 or less, kills most bacteria (Smith, 2003). However, *Escherichia coli* survives this acidity nearly as well as the stomach pathogen *Helicobacter pylori* (Gorden & Small, 1993; Lin *et al.*, 1996). Acid resistance (AR) appears to contribute to the low infectious dose of pathogenic *E. coli* and aids the gastric passage of commensal strains (Giannella *et al.*, 1973; Price *et al.*, 2004).

Four *E. coli* AR systems have been described, three of which, the glutamate-, arginine- and lysine-dependent systems, are based on amino acid decarboxylation reactions (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995; Richard & Foster, 2003). The decarboxylases consume an intracellular proton while removing CO₂ from their amino acid substrates. Decarboxylation products are then expelled by specific antiporters in exchange for new substrate (Gong *et al.*, 2003; Iyer *et al.*, 2003). This process changes internal pH and electrical charge in ways that enable the cell to survive extreme acid stress (Richard & Foster, 2004).

Glutamate-dependent AR (GDAR) is the most effective system. It is composed of two isoforms of glutamate decarboxylase, GadA and GadB (Smith *et al.*, 1992), and the membrane-bound antiporter GadC that exchanges glutamate for the decarboxylation product γ -aminobutyric acid (GABA) (Richard & Foster, 2004). Induction of GadABC is multifactorial and includes the AraC-like regulator GadX, formerly YhiX (Hommals *et al.*, 2001; Ma *et al.*, 2002; Shin *et al.*, 2001; Tramonti *et al.*, 2003; Tucker *et al.*, 2002, 2003), the AraC-family proteins GadW (Ma *et al.*, 2002; Tramonti *et al.*, 2006) and YdeO (Masuda & Church, 2003), the LuxR-family regulator GadE (Hommals *et al.*, 2004; Ma *et al.*, 2003b), the EvgSA two-component regulatory system (Masuda & Church, 2002), the alternative sigma factor RpoS (Castanie-Cornet *et al.*, 1999), the signal GTPase MnmE (Gong *et al.*, 2004), as well as RcsF, TorR, Crp and H-NS (Bordi *et al.*, 2003; Castanie-Cornet *et al.*, 2006; De Biase *et al.*, 1999; Ma *et al.*, 2002; Tramonti *et al.*, 2002). This large number of regulators probably reflects a need to connect induction of GDAR to many facets of cell physiology, but makes the definition of regulatory circuits difficult.

GadE is required to induce *gadA* and *gadBC* under all circumstances (Ma *et al.*, 2003b). Most of the other

Abbreviations: AR, acid resistance; EMSA, electrophoretic mobility shift assay; GDAR, glutamate-dependent AR.

regulators influence the system by controlling *gadE*. However, which regulators are used differs depending on growth phase and medium. For instance, EvgA, YdeO and GadE itself form a branched regulatory loop that activates *gadE* during exponential growth in pH 5.5 minimal medium (Ma *et al.*, 2004). However, neither EvgA nor YdeO is required for *gadE* expression in stationary phase. A second circuit involving MnmE activates *gadE* during stationary-phase growth in Luria–Bertani (LB) medium containing glucose. However, in LB lacking glucose, another, unknown circuit takes over (Gong *et al.*, 2004). It was suspected that this alternative *gadE* control circuit might involve RpoS, GadX and GadW – three regulators required to induce GadABC during growth in LB (Ma *et al.*, 2002; Tramonti *et al.*, 2002, 2006).

RpoS activates *gadY*, a gene located downstream of *gadX*, whose small-RNA (sRNA) product stabilizes *gadX* mRNA (Opdyke *et al.*, 2004). The resulting increase in GadX somehow activates *gadA* and *gadBC* (Giangrossi *et al.*, 2005; Ma *et al.*, 2002; Shin *et al.*, 2001; Tramonti *et al.*, 2002, 2006). GadX and GadW bind and activate the *gadA* and *gadBC* promoters *in vitro* (Tramonti *et al.*, 2006). However, overexpression of GadX *in vivo* does not induce *gadA* or *gadB* in the absence of a *gadE*⁺ allele, suggesting that the *in vitro* results may not reflect *in vivo* function (Gong *et al.*, 2004). Microarray studies indicate that GadX may actually activate *gadE*, which, in turn, would activate *gadA* and *gadBC* (Hommais *et al.*, 2004; Tucker *et al.*, 2003; Weber *et al.*, 2005). The current study uses *in vivo* approaches to reexamine the roles of GadX, GadW and RpoS in controlling AR.

METHODS

Strains, plasmids, oligonucleotides and growth media. Strains and plasmids used in this study are listed in Table 1. Constructions of merodiploid chromosomal *gadE*–*lacZ* and *gadA*–*lacZ* transcriptional fusions have been described previously (Ma *et al.*, 2002, 2003b, 2004).

LB medium was buffered with either 100 mM MOPS (pH 8.0) or MES (pH 5.5), and supplemented with 0.4% glucose (LBG) as indicated. Cultures were grown at 37 °C with aeration (225 r.p.m.). Antibiotics used were ampicillin (Ap), 60 µg ml^{−1}; kanamycin (Km), 50 µg ml^{−1}; tetracycline (Tc), 30 µg ml^{−1}; and chloramphenicol (Cm), 40 µg ml^{−1}. Exponential-phase cells were prepared via a 1:1000 dilution of 18 h stationary-phase cultures into fresh media followed by growth to OD₆₀₀ 0.3–0.4. Stationary-phase cultures were grown for 18 h.

AR was tested by diluting an overnight culture (LB or LBG, pH 5.5 or pH 8) to 2 × 10⁶ c.f.u. ml^{−1} into pH 2.5 minimal E salts glucose medium (EG medium) containing 1 mM glutamic acid, and percentage survival over time was determined by viable counting (Lin *et al.*, 1995).

Molecular procedures. P1 transduction, β-galactosidase assays, DNA manipulations, CaCl₂ transformation and electroporation were performed by standard methods (Miller, 1992; Sambrook *et al.*, 1989). Results are means of triplicate experiments and fell within 10% of the stated values.

Transfer of *lacZ* fusions from *E. coli* to *Salmonella enterica* serovar Typhimurium was accomplished by conjugally transferring a plasmid

containing P22 phage from TE1335 (EK297) into EF1285 and EF1286 (Elliott, 1992). The resulting phage lysates were used to transduce restrictionless *S. Typhimurium* SF586 (JR501; Bullas & Ryu, 1983). The resulting constructs (JF4971 and JF1502) contained the *gadE*–*lacZ* fusions inserted into the *put* locus. P22HT105-int was subsequently used to transfer the fusions into the wild-type strain UK1 to make JF4972 and JF5003 (Curtiss *et al.*, 1991).

MBP–GadX was purified, and electrophoretic mobility shift assays (EMSAs) were performed as described previously (Ma *et al.*, 2004; Shin *et al.*, 2001). EMSA was conducted with the *gadE* sequence between −682 and −355 generated by PCR with oligo-598 (5′-CTAGTGATTTC AACCTACT-3′) and oligo-601 (5′-ATGTAATCCG-ATTTAAATATCGAG-3′). A *lacP* control sequence [oligo-507 (5′-CCATACGCAAACCGCTCTCC-3′), oligo-508 (5′-AGTGAAT-CCGTAATCATGGTCATA-3′)] was run in parallel.

RESULTS

RpoS activates *gadE*

A chromosomal *gadE*–*lacZ* fusion extending from −804 to +331 relative to the *gadE* start codon was engineered into the *trp* operon, producing a merodiploid strain with intact *gadE*⁺ at its normal location. Expression of this fusion was dependent on RpoS during growth in LB, but was RpoS-independent during growth in LBG (Fig. 1a). A previous study found that MnmE is required for optimal expression of *gadE* during growth in LBG (Gong *et al.*, 2004). The effect of RpoS on AR paralleled its effect on *gadE*–*lacZ* (Fig. 1b). Thus, RpoS may affect AR primarily by influencing GadE production. RpoS does not affect AR through any *gadE*-independent mechanism, since overexpressing GadE in an RpoS mutant still activates AR.

Why glucose reduced *gadE* expression yet produced a level of AR equivalent to that of cells grown without glucose is not clear. Additional control mechanisms that increase GadE mRNA or protein stability may produce enough GadE to fully activate GDAR.

GadX and GadW activate *gadE*

Individual mutations in *gadX* or *gadW* had little effect on *gadE*–*lacZ* expression during growth in LB (no glucose). However, a Δ *gadXW* mutation dramatically reduced expression (Fig. 2a, wild-type vs *gadXW*). Thus, GadX and GadW had redundant effects. However, during growth in glucose (pH 5.5), GadX and GadW activation of *gadE* was additive (Fig. 2b).

Fig. 2 also reveals that *gadE*^{−804 to +331}–*lacZ* is acid-induced during growth in LBG but not in the absence of glucose (Fig. 2a, b, wild-type cells). Part of that acid induction appears to involve GadX and GadW acting as partial negative regulators of *gadE* at pH 8 in glucose. This may be due to the previously demonstrated cross-repression of *gadX* and *gadW* on each other (Ma *et al.*, 2003b). Since *gadE* expression is RpoS-independent during growth on glucose (Fig. 1a), but remains dependent on

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or characteristic	Reference or source
Strains		
EK227	K12 wild-type λ - F-	A.C. Matin, Stanford University
EK592	MG1655	G. Church
EF362	<i>rpoS</i> ::Tn10	Castanie-Cornet <i>et al.</i> (1999)
EF825	K12 $\Delta(lacA-Z)::cat$	Ma <i>et al.</i> (2002)
EF830	K12 $\Delta gadX \Delta lac::cat$	Ma <i>et al.</i> (2002)
EF839	K-12 $\Delta gadX \Delta lac::cat$ <i>trpDC::putPA1303-Km-gadA-lacZ(O)</i>	Ma <i>et al.</i> (2002)
EF911	K-12 $\Delta gadW \Delta lac::cat$	Ma <i>et al.</i> (2002)
EF912	K-12 $\Delta gadXW \Delta lac::cat$	Ma <i>et al.</i> (2002)
EF921	K-12 $\Delta gadW \Delta lac::cat$ <i>trpDC::putPA1303-Km-gadA-lacZ(O)</i>	Ma <i>et al.</i> (2002)
EF933	K-12 $\Delta gadXW \Delta lac::cat$ <i>trpDC::putPA1303-Km-gadA-lacZ(O)</i>	Ma <i>et al.</i> (2002)
EF1007	K12 $\Delta gadE::Km$	Ma <i>et al.</i> (2003b)
EF1032	K12 $\Delta(yhiXW::Km) \Delta crp::Cm$ <i>gadE2::Tn10(dTc)</i>	Gong <i>et al.</i> (2003)
EF1073	K12 <i>trmE::Tn10(dTc)</i>	Gong <i>et al.</i> (2003)
EF1243	MG1655 $\Delta lac::cat$ $\Delta gadE::Km$	Ma <i>et al.</i> (2002)
EF1285	EK298 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	Ma <i>et al.</i> (2004)
EF1288	EK298 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	Ma <i>et al.</i> (2004)
EF1303	MG1655 $\Delta lac::cat$ <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	Ma <i>et al.</i> (2004)
EF1304	MG1655 $\Delta lac::cat$ <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	Ma <i>et al.</i> (2004)
EF1305	MG1655 $\Delta lac::cat$ <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	Ma <i>et al.</i> (2004)
EF1338	EF1243 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	EF1243 X EF1303
EF1339	EF1243 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	EF1243 X EF1305
EF1346	EF1243 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	EF1243 X EF1304
EF1357	EF825 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	EF825 X EF1285
EF1358	EF830 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	EF830 X EF1285
EF1359	EF911 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	EF911 X EF1285
EF1360	EF912 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-804 to +331}	EF912 X EF1285
EF1361	$\Delta lac::cat$ <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	EF825 X EF1286
EF1362	EF830 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	EF830 X EF1286
EF1363	EF911 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	EF911 X EF1286
EF1364	EF912 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-360 to +331}	EF912 X EF1286
EF1365	$\Delta lac::cat$ <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	EF825 X EF1288
EF1366	EF830 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	EF830 X EF1288
EF1367	EF911 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	EF911 X EF1288
EF1368	EF912 <i>trpDC::putPA1303-Km-gadE-lacZ(0)</i> ^{-195 to +331}	EF912 X EF1288
EF1535	<i>rpoS</i> ::Tn10	EK592 X EF362
EF1572	EF1357 <i>rpoS</i> ::Tn10	EF1357 X EF362
Plasmids		
pCR2.1	TA cloning vector	Qiagen
pQE80L	4.8 kb cloning vector	Masuda & Church (2003)
pBAD24	4.5 kb cloning vector	Guzman <i>et al.</i> (1995)
pMF533	pMalc2E containing 528 bp <i>gadE</i> + engineered with <i>Bam</i> HI and <i>Hind</i> III sites	Z. Ma and J. W. Foster, unpublished data
pRS552	12.4 kb cloning vector	Simons <i>et al.</i> (1987)
	pPCR-Script Amp <i>yhiE(gadE)</i>	Tucker <i>et al.</i> (2002)
	pPCR-Script Amp <i>yhiX(gadX)</i>	Tucker <i>et al.</i> (2002)
pSGF540	1022 bp <i>gadA</i> fragment cloned into pRS552	This study

GadX and/or GadW (Fig. 2b), factors other than RpoS must activate GadX during growth in LBG.

RpoS does not appear to activate *gadE* through a GadXW-independent pathway, since *rpoS*, $\Delta gadXW$ and *rpoS* $\Delta gadXW$ mutants are equally acid sensitive, and the overexpression of RpoS does not induce *gadE* in a $\Delta gadXW$ mutant (data not shown)

The -360 to -804 region is required for GadX and GadW activation of *gadE*

The intergenic region controlling *gadE* expression is large (798 bp). Deletions removing sequences from -804 to -360, or to -195 nt upstream of the translation start, were used to determine where GadX and GadW had effects. Sequences between -360 and -804 were required for

Fig. 1. Effect of RpoS on *gadE-lacZ* expression and AR during growth with and without glucose. (a) Wild-type (EF1357) and *rpoS* (EF1572) mutant strains containing *gadE*^{-804 to +331}-*lacZ* operon fusions were assayed for β-galactosidase activity. Cells were grown overnight in pH 5.5 buffered LB with and without 0.4% glucose. Values are means of triplicate experiments with SEM. (b) Wild-type (EK592) and *rpoS* mutant (EF1535) strains were tested for AR by diluting an overnight culture (LB or LBG, pH 5.5) to approximately 2×10^6 c.f.u. ml⁻¹ into pH 2.5 EG containing 1 mM glutamic acid, and percentage survival over time was determined by viable counting. Assays were performed at 1 h (grey bars), 2 h (cross-hatched bars) and 4 h (black bars) of acid challenge.

maximal *gadE* expression in LB with or without glucose (Fig. 3, a vs b, d vs e). GadX or GadW alone activated this expression (Fig. 3a, d, fourth bars).

It also appeared that *gadX* and *gadW* influenced *gadE* expression in the deletion strains (Fig. 3b, e). However, it will be shown below that this dependence is indirect due to GadE autoinduction from the wild-type *gadE*⁺ allele also present in these strains. The results indicate that the major target for GadX and GadW lies between -804 and -360 nt.

The -360 to -804 region inhibits GadE autoactivation

GadE can autoactivate by binding to three GAD box sequences between -233 and -42 nt, shown in Fig. 4(a). Autoinduction was previously seen during exponential growth in minimal glucose media, but not during growth in LB (Ma *et al.*, 2004). Fig. 4 illustrates that in LB at pH 5.5 (black bars) *gadE* did not autoactivate unless the -360 to -804 region was removed, at which point *gadE* caused a 10-fold decrease in expression (Fig. 4b, c, filled bars, set 1 vs 4, set 2 vs 5). At pH 8 (open bars) without glucose, some GadE-dependence was noted even in the full-length fusion (Fig. 4b, open bars, set 1 vs 4). However, GadE-dependence was still enhanced by removing the -360 and -804 bp region (open bars, set 2 vs 5).

These results help explain data above in which *gadX* and *gadW* influenced expression of the shorter *gadE-lacZ* fusions (Fig. 3). In the merodiploid strain, GadX and GadW stimulate the wild-type *gadE*⁺ allele, and the resulting GadE protein activates the -360 and -195 *gadE-lacZ* fusions. The Δ *gadXW* mutant no longer makes GadE, so induction of the shorter *gadE-lacZ* fusions will not occur. This was confirmed when the plasmid pQE-GadE did not activate the full-length *gadE*^{-804 to +331}-*lacZ* fusion, even in a Δ *gadXW* mutant, but did activate the -360 to +331 fusion in the Δ *gadXW* mutant (data not shown). Thus, an unknown regulatory protein may limit *gadE* autoactivation by binding between -360 and -804 nt, creating a need for GadX or GadW.

Fig. 2. Effects of GadX and GadW on *gadE* expression in *E. coli*. Cells were grown for 17 h in LB (a) or LBG (b) buffered to pH 5.5 (filled bars) or pH 8 (open bars). The strains used were wild-type (EF1357), *gadX* (EF1358), *gadW* (EF1359) and Δ *gadXW* (EF1360). Values are means of triplicate experiments with SEM.

Fig. 3. Effects of GadX and GadW on the expression of *gadE* with deletions in the upstream regulatory region. Cells were grown as in Fig. 2 (only pH 5.5 is shown). Relevant *gadX* and *gadW* genotypes are shown below the figure. The parental fusion strains (first bar in each panel) contained *gadE* sequences extending from -804 to +331 (EF1357), -360 to +331 (EF1361) and -195 to +331 (EF1365). -804, -360 and -195 indicate the base-pair position relative to the *gadE* translational start. The *gadX* derivatives of each fusion were EF1358 (-804; a, d), EF1362 (-360; b, e), and EF1366 (-195; c, f). In the same order, the *gadW* derivatives for each fusion were EF1359, EF1363, EF136; and the Δ *gadXW* derivatives were EF1360, EF1364. The -195 *gadXW* fusion was not done. Values shown are the means of triplicate experiments and did not vary by more than 10 %.

GadX binds to the *gadE* control region

Regulators EvgA, YdeO and GadE bind to subfragments of the 798 bp *gadE* upstream control region (Ma *et al.*, 2004). Since GadX control over *gadE* appeared to require the region upstream of -360 bp, we tested whether GadX, purified as a maltose-binding fusion, could bind to this region. The *gadE* fragment between -682 and -355 bp was used along with the unrelated *lacP* fragment as a negative control. The results shown in Fig. 5 reveal that MBP-GadX binds to this region, supporting the hypothesis that GadX can act directly on *gadE*. The binding is specific, since the reaction mix contains poly-dIC as a nonspecific competitor and because GadX did not shift the *lacP* promoter (Fig. 5, left-hand panel). Similar attempts to purify active GadW-MBP were not successful.

GadX and GadW activate *gadE* expression in *S. enterica*

To provide further *in vivo* evidence that GadX and GadW directly activate *gadE*, we moved the *gadE*^{-804 to +331}-*lacZ*

Fig. 4. Effects of a *gadE* mutation on the expression of *gadE* with deletions in the upstream regulatory region. (a) The basic *gadE*-*lacZ* constructs, with known promoter (bent arrow) and GAD boxes (boxes) shown. Upstream regions extend to the base pairs shown relative to the start codon. (b, c) Cells were grown in LB (b) and LBG (c) buffered to pH 5.5 (filled bars) and pH 8 (open bars), as in Fig. 2. The wild-type fusion strains used were EF1303 (-804 to +331), EF1304 (-360 to +331) and EF1305 (-195 to +301). The *gadE* derivatives of these fusions were EF1338 (-804 to +331), EF1346 (-360 to +331) and EF1339 (-195 to +301). Values shown are the means of triplicate experiments and did not vary by more than 10 %.

and *gadE*^{-360 to +331}-*lacZ* fusions from *E. coli* into *S. enterica* serovar Typhimurium. *S. Typhimurium* does not possess *gadA*, *B*, *C* or *E*, and is devoid of many genes whose products are known or suspected to induce *gadE*, including *evgA*, *ydeO*, *gadX* and *gadW*. We transduced the *E. coli* *gadE*-*lacZ* fusions into the chromosomal *put* locus of *S. Typhimurium* UK1, and then introduced plasmids pQE-*gadX* and pMF524, in which *gadX* and *gadW*, respectively, are under *lacP* control, into the *S. Typhimurium* strain and measured *gadE*-*lacZ* expression. Exponential-phase cells

Fig. 5. GadX binds *in vitro* to the *gadE* region. EMSA reactions contained 2–8 μ M purified MBP-GadX. Binding reactions (20 μ l total vol.) contained 20 mM HEPES (pH 8), 5 mM $MgCl_2$, 50 mM potassium glutamate, 0.01 mM EDTA, 1.0 mM sodium dihydrophosphate, 20 mM NaCl, 1 mM DTT, 30 μ g BSA ml^{-1} and 50 μ g poly-dIC ml^{-1} . PCR-amplified, end-labelled fragments of *gadE* were added to 20 000 c.p.m. per reaction. The *gadE* upstream fragment from –682 to –355 was generated using oligo-598 and oligo-601, and fragment 1+2 (oligo-599/oligo-662, –360 to +28). Reactions were performed at room temperature. Glycerol was added to 10% (v/v), and the entire reaction was electrophoresed at room temperature on a 4% acrylamide, non-denaturing gel with 0.5 $\times$ Tris-buffered EDTA containing 1.5% (v/v) glycerol. The two fragment bands seen in the no-protein control were the result of cross-lane leakage of ^{32}P -labelled product.

were used in this experiment to show that other stationary-phase factors, such as RpoS, were not needed. The results shown in Fig. 6(a) reveal that plasmid pQE-*gadX* increased the expression of *gadE-lacZ* almost 10-fold in exponential-phase cultures supplemented with 0.1 mM IPTG. Higher concentrations of IPTG decreased growth of the culture, suggesting that GadX overexpression may have an effect on other *S. Typhimurium* genes (data not shown). GadW, though less effective than GadX, still activated *gadE-lacZ* between four- and fivefold. These results support the hypothesis that GadX and GadW activate *gadE in vivo*. The fact that activation was less than expected (based on the *E. coli* results) suggests that factors in addition to GadX and GadW are important to maximize *gadE* expression. We also found that neither GadX nor GadW activated expression of the –360 to +331 fusion, confirming that sequences upstream of –360 are important for activation by these proteins (data not shown).

GadX and GadW do not activate *gadB* in *S. Typhimurium*

Previously published results suggest that GadX and GadW bind and regulate the *gadA/BC* promoters. GadX and GadW have been reported to activate and repress these promoters under different conditions. In addition, over-expressing MalE-GadX successfully induced *gadA* and *gadB* in *E. coli* (Giangrossi *et al.*, 2005; Gong *et al.*, 2004). These *in vivo* results could be explained as an indirect effect of GadX and GadW activating *gadE*. However, *in vitro* EMSAs and DNA footprint analysis using GadX and GadW indicate that both proteins can bind to the *gadA* and *gadBC* promoters (Ma *et al.*, 2003a, 2002, 2003b; Shin *et al.*,

Fig. 6. Effects of GadX and GadW on the activation of *gadE-lacZ* and *gadB-lacZ* in *S. Typhimurium*. (a) The *gadE-lacZ* fusion was moved into *S. Typhimurium* along with empty vector and the plasmids pQE-*gadX* and pMal-*gadW*. (b) The *gadB-lacZ* fusion was moved into *S. Typhimurium* along with empty vector and the plasmids pQE-*gadX*, pMal-*gadW* and pQE-*gadE*. Cells were grown to exponential phase (OD_{600} 0.3) in LB with (open bars) or without (solid bars) 0.1 mM IPTG. Values shown are the means of triplicate experiments and did not vary by more than 10%.

2001; Tramonti *et al.*, 2002, 2006). More recently, an *in vitro* effect of GadX on the transcription of *gadA* and *gadB* promoters has been demonstrated (Giangrossi *et al.*, 2005). Nevertheless, since the *in vivo* data presented indicate that GadX and GadW activate *gadE*, and that GadE is essential for *gadA* and *gadBC* induction, it seemed possible that the *in vivo* roles of GadX and GadW in inducing *gadA* and *gadBC* may have more to do with their triggering the expression of GadE. Previous *in vivo* support for this hypothesis was reported when overexpression of GadX failed to induce *gadA* or *gadB* in a *gadE* mutant strain (Gong *et al.*, 2004).

This hypothesis was further tested using the *S. Typhimurium* model. A *gadB-lacZ* fusion was introduced into the *Salmonella* chromosome, and then plasmids expressing GadX, GadW or GadE were introduced. The results shown in Fig. 6(b) indicate that GadX and GadW had little or no effect on *gadB* expression, although they did activate *gadE* (Fig. 6a). In contrast, introducing GadE had a tremendous effect on *in vivo gadB* activation.

DISCUSSION

E. coli GDAR operons *gadA* and *gadBC* are subject to a remarkably complex regulation. At least 12 regulatory proteins control this acid survival mechanism, with much of that control levelled at induction of the *gadE* activator (Foster, 2004). Several induction circuits are evident. First, the response regulator EvgA and the AraC-family activator YdeO create a feed-forward *gadE* activation circuit that functions in minimal glucose medium (Ma *et al.*, 2004; Masuda & Church, 2003). The second pathway involves GadE autoinduction, also evident in cultures grown in minimal glucose (Ma *et al.*, 2004). A third pathway requires the MnmE (TrmE) molecular switch GTPase and is most evident when cells are grown in LBG (Gong *et al.*, 2004).

The current report describes two other regulatory pathways that can activate *gadE* expression, primarily in rich media. One pathway is GadX-dependent while the other is GadW-dependent. The GadX and GadW pathways activate *gadE* in stationary-phase LB at any growth pH. Thus, *E. coli* has developed at least five iterative regulatory circuits that assure activation of *gadE* and AR under diverse physiological situations.

GadX and GadW appear to play a role in *gadE* induction regardless of whether glucose is present in the growth medium. During growth in glucose, the two AraC-family regulators collaborate with the MnmE-dependent pathway to effect maximum *gadE* expression. However, GadX and GadW appear to be independent of the MnmE-modulated pathway, since an *mnmE* mutation did not affect the expression of either *gadX-lacZ* or *gadW-lacZ* fusions (data not shown). The results presented also indicate that the alternative sigma factor RpoS indirectly activates *gadE* by first activating *gadX*. However, the fact that RpoS is not

needed for *gadE* expression in glucose, yet GadX and GadW remain important, suggests the presence of an RpoS-independent route for *gadX* induction.

The finding that GadX and GadW do not appear to directly activate *gadA/BC* promoters *in vivo*, but do so indirectly by activating *gadE*, contradicts earlier models, including our own. However, based on microarray reports, this result was not totally unexpected (Tucker *et al.*, 2002, 2003). It is certainly possible, even probable based on the *in vitro* findings, that under some growth condition(s) these regulators will directly bind *gadA/BC in vivo* and modulate GadE-dependent activation, but that growth condition does not seem to be growth in LB.

The discovery that several additional regulatory layers govern the expression of the *E. coli gadE* gene underscores the importance that this organism places on becoming acid resistant. Growth to different cell densities in different media clearly results in different physiological states, some of which may presage encounters with extreme acid stress. The AR regulatory network described here appears to be configured in a way that senses dramatic, and perhaps nuanced, changes in chemical signals that coincide with changing physiology. GadX, GadW and YdeO are all AraC-family regulators with potential sensing modules that likely sense different physiological signal molecules in the cell. Each signal molecule could alter the abilities of these proteins to act as activators of *gadE* or of *gadA/BC*.

It is also important to recognize that the relevance of this system extends beyond GDAR, since GadE is in fact a global regulator that activates numerous genes in addition to *gadA/BC*, and negatively regulates biofilm formation in K12 as well as epithelial attachment in enterohaemorrhagic *E. coli* (EHEC) (Dahan *et al.*, 2004; Hommais *et al.*, 2004; Tatsuno *et al.*, 2003; J. W. Foster, unpublished observation). It is important to learn what signals these regulators sense and then integrate that knowledge into a global view of cell physiology under acid stress.

ACKNOWLEDGEMENTS

The authors would like to thank Jon Audia, David Wood, Herbert Winkler and Michael Spector for support and helpful discussions, and N. Masuda and G. Church, Harvard Medical School, for sharing strains and plasmid constructs.

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Edited by: D. J. Jamieson