Sodium regulates *Escherichia coli* acid resistance, and influences GadX- and GadW-dependent activation of *gadE*

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Enteric bacteria must survive the extreme acid of the stomach (pH 2 or less) before entering the intestine where they can colonize and cause disease. *Escherichia coli* is superior to most other *Enterobacteriaceae* in surviving pH 2 acid stress because it has four known acid-resistance systems, the most studied of which depends on glutamic acid. Glutamate-dependent acid resistance requires glutamate decarboxylase isozymes GadA and GadB, as well as a glutamate/ γ -aminobutyric acid antiporter encoded by *gadC*. The regulatory protein GadE is the essential activator of the *gadA* and *gadBC* genes. The transcription of *gadE*, however, is controlled by numerous proteins. Two of these proteins, GadX and GadW, are AraC-family regulators whose sensory input signals are not known. Since Na^+ and K^+ play important roles in pH homeostasis, the contribution of these ions toward the regulation of this acid-resistance system was examined. The results indicated that a decrease in Na^+ , but not K^+ , concentration coincided with diminished acid resistance, and decreased expression of the *gadE*, *gadA* and *gadBC* genes. However, Na^+ -dependent regulation of these genes dissipated in the absence of GadX and GadW. Since Na^+ levels did not regulate *gadX* or *gadW* transcription, it is proposed that GadX and GadW sense intracellular Na^+ concentration or some consequence of altered Na^+ levels.

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INTRODUCTION

The extreme acid environment of the human stomach, which can reach pH 2 or less, is harsh for any enteric organism. Enteric pathogens must survive this level of pH for at least 2 h, the average emptying time for the stomach, before reaching the intestine (Texter, 1968). To overcome this challenge, *E. coli* possesses four known acid-resistance systems (Foster & Moreno, 1999; Gorden & Small, 1993; Hersh *et al.*, 1996; Iyer *et al.*, 2003; Richard & Foster, 2003). Acid-resistance (AR) system 1 (AR1), also referred to as the oxidative system, is a stationary-phase-induced glucose-repressed system that does not require the presence of an amino acid in the challenge medium to function (Castanie-Cornet *et al.*, 1999). The other three systems (AR2, AR3 and AR4) are all amino-acid-dependent systems that require glutamate, arginine and lysine, respectively (Castanie-Cornet *et al.*, 1999; Gong *et al.*, 2003; Iyer *et al.*, 2003; Lin *et al.*, 1995).

All four AR systems provide different levels of protection. AR1 and AR4 provide a minimal level of protection, while AR3 provides moderate protection. The glutamate-dependent (AR2) system is the most robust, and thus the most extensively studied. This system requires glutamate decarboxylase, encoded by *gadA* and *gadB*, and a

glutamate/ γ -aminobutyric acid (GABA) antiporter (GadC) (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1996, 1999; Hersh *et al.*, 1996; Malashkevich *et al.*, 1998; Richard & Foster, 2003). GadA and GadB are isozymes of glutamate decarboxylase, and they are encoded by genes located at different sites on the chromosome (78.98 and 33.8 min, respectively). The *gadB* and *gadC* genes are transcribed as an operon (De Biase *et al.*, 1999). As internal pH becomes acidic, GadA/B converts intracellular glutamate to GABA, with the consumption of a proton and the release of CO_2 . The GABA produced in the decarboxylation reaction is then transported out of the cell through GadC, with the concomitant uptake of glutamate. A continuous cycle of proton consumption through amino-acid decarboxylation raises the internal pH of acid-stressed cells (Castanie-Cornet *et al.*, 1999; Foster & Moreno, 1999; Richard & Foster, 2004). This increase in internal pH is thought to be required for survival in extreme acid stress.

Genetic regulation of this system is complex, and involves numerous regulators. Environmental signals such as acid and osmotic stress, as well as stationary phase signals, are important in inducing *gadA/BC* expression (Castanie-Cornet & Foster, 2001; Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999). Known transcriptional regulators include the alternative sigma factor RpoS, three AraC-family regulators (GadX, GadW and YdeO), cAMP and cAMP

Abbreviations: AR, acid-resistance; GABA, γ -aminobutyric acid; HTH, helix–turn–helix.

receptor protein, GadE (a LuxR-like regulator), TrmE (an Era-like GTPase), PhoP, EvgA/S (a two-component regulatory system) and the RcsCDB phosphorelay (Castanie-Cornet & Foster, 2001; Castanie-Cornet *et al.*, 2006, 1999; De Biase *et al.*, 1999; Gong *et al.*, 2004; Hommais *et al.*, 2001; Ma *et al.*, 2004, 2002, 2003b; Masuda & Church, 2002, 2003; Tramonti *et al.*, 2002; Zwir *et al.*, 2005). The activity of each of these transcriptional regulators leads to the activation or repression of genes involved in glutamate-dependent acid resistance (Foster, 2004).

GadE is the essential activator of the *gadA/BC* genes. Most of the other regulators influence *gadA/BC* expression by controlling the expression of *gadE*. GadX and GadW are two AraC-like regulators that function in this regulatory circuit by controlling the expression of *gadE* and *gadA/BC* (Ma *et al.*, 2003a; Tramonti *et al.*, 2006; Sayed *et al.*, 2007). These proteins are known to activate and repress *gadA/BC* and *gadE* under different growth conditions; however, the signal that influences their activity has yet to be identified (Ma *et al.*, 2002; Tramonti *et al.*, 2002).

Of interest to this study is the role of Na⁺ and K⁺ in regulating *gadA/BC* expression. We hypothesized that one or both of these ions might regulate acid resistance because they play such important roles in housekeeping pH homeostasis systems (Booth, 1985, 1999; Kroll & Booth, 1983; Lewinson *et al.*, 2004; Padan *et al.*, 1981, 2005; Zilberstein *et al.*, 1982). This investigation has revealed that Na⁺ levels affect *gadA/BC* expression during growth into stationary phase. The effects of Na⁺ on *gadA/BC* expression appear to be through the modulation of GadX and GadW activity, which, in turn, controls *gadE* expression.

METHODS

Strains, and culture conditions. The strains used in this study are listed in Table 1. Media included minimal E medium containing 0.4 % glucose (EG) (Vogel & Bonner, 1956), and Na⁺- and K⁺-deficient

media (modified M63 medium; Miller, 1992) containing 15 mM (NH₄)SO₄, 18 µM FeSO₄·7H₂O, 1 mM MgSO₄ and 0.2 % glucose. In addition, the Na⁺-deficient medium (M63K) contained 100 mM KH₂PO₄, while the K⁺-deficient medium (M63N) contained 100 mM NaH₂PO₄. A control medium (M63N/K) containing both KH₂PO₄ and NaH₂PO₄ in equal concentrations (50 mM of each) was also used. M63N⁰/K⁰, which was used for acid challenge only, contained (NH₄)SO₄, FeSO₄·7H₂O, MgSO₄ and 0.2 % glucose, with no added NaH₂PO₄ or KH₂PO₄. All Na⁺- and K⁺-deficient media were made with ultrapure chemicals and double-distilled water. Na⁺ and K⁺ measurements were made using Na⁺- and K⁺-specific combination electrodes (Orion ROSS Na⁺ electrode; ThermoOrion ionplus K⁺ electrode). For acid-resistance assays, the acid challenge medium or H₂O was prepared at pH 2.5 (adjusted with HCl). Cultures were grown at 37 °C, with shaking at 220 r.p.m., overnight (18 h) to stationary phase.

Acid-resistance assays. Acid-resistance assays were performed as described previously (Castanie-Cornet *et al.*, 1999). Briefly, cells were grown overnight in EG, M63K, M63N or M63N/K at pH 5.5. Stationary-phase cultures were diluted 1:1000 into prewarmed pH 2.5 M63K, M63N, M63N/K or H₂O, with or without 1.6 mM glutamate. To determine if Na⁺ or K⁺ was required during acid challenge, cells were grown in minimal EG medium at pH 5.5 to stationary phase. Cells were then harvested, and washed three times in an equal volume of MC buffer (10 mM MgCl₂ and 5 mM CaCl₂) to remove excess Na⁺ and K⁺ from the cells. After the final wash, cells were resuspended in an equal volume of MC buffer, and subsequently diluted 1:1000 into the pH 2.5 challenge media indicated (Fig. 1). At various time points, 10 µl aliquots were removed and serially diluted, and 10 µl of each dilution was plated on Luria-Bertani (LB) agar. The numbers of c.f.u. were determined, and percentage survival was calculated relative to time zero.

Western blot analysis. Cells were cultured overnight in 3 ml of the indicated media (Fig. 2) at pH 5.5. Cells were then harvested by centrifugation at 4500 g for 5 min (4 °C), and resuspended in 50 µl 0.1 % SDS. Protein concentrations were measured using the Bio-Rad protein assay reagent. Protein (5 µg) was separated on 10 % polyacrylamide-SDS gels (Bio-Rad), and then transferred to Immobilon-P (PVDF) membranes with a Semipore transfer cell (Hoefer Scientific) at 100 mA for 2 h. Membranes were blocked with 5 % nonfat milk in Tris-buffered saline (10 mM Tris, pH 8, 150 mM NaCl) containing Tween 20, and incubated with rat (GadA/B) or rabbit (GadC) primary antibodies for 1 h, followed by incubation with anti-rat or -rabbit

Table 1. Strains used in this study

Strain	Genotype	Source or reference*
EK 227	K-12 WT λ ⁻ F ⁻	A. Matin
EK 612 (SS721)	(MC4100) <i>gadX::lacZ</i> (Km ^r)	Shin <i>et al.</i> (2001)
EK 687 (EG16160)	(MG1655) <i>gadW::lacZ</i> (Km ^r)	E. Groisman
EF 833	(EK 227) <i>trpDC::putPA1303-Km-gadA::lacZ</i> (-165 to +788) Δ(<i>lacA-lacZ</i>)::Cat	Ma <i>et al.</i> (2002)
EF 933	(EK 227) <i>trpDC::putPA1303-Km-gadA::lacZ</i> (-165 to +788) Δ <i>gadXW</i> Δ(<i>lacA-lacZ</i>)::Cat	Ma <i>et al.</i> (2002)
EF 952	(EK 227) Δ(<i>lacA-lacZ</i>)	Sayed <i>et al.</i> (2007)
EF 1236	(EK 227) <i>trpDC::putPA1303-Km-gadA::lacZ</i> Δ(<i>lacA-lacZ</i>)::Cat	Gong <i>et al.</i> (2004)
EF 1357	(EK 227) <i>trpDC::putPA1303-Km-gadE::lacZ</i> (-804 to +331) Δ(<i>lacA-lacZ</i>)::Cat	Sayed <i>et al.</i> (2007)
EF 1358	(EK 227) <i>trpDC::putPA1303-Km-gadE::lacZ</i> (-804 to +331) Δ <i>gadX</i> Δ(<i>lacA-lacZ</i>)::Cat	Sayed <i>et al.</i> (2007)
EF 1359	(EK 227) <i>trpDC::putPA1303-Km-gadE::lacZ</i> (-804 to +331) Δ <i>gadW</i> Δ(<i>lacA-lacZ</i>)::Cat	Sayed <i>et al.</i> (2007)
EF 1360	(EK 227) <i>trpDC::putPA1303-Km-gadE::lacZ</i> (-804 to +331) Δ <i>gadXW</i> Δ(<i>lacA-lacZ</i>)::Cat	Sayed <i>et al.</i> (2007)
EF 1387	(EK 227) <i>gadX::lacZ</i> (Km ^R) Δ(<i>lacA-lacZ</i>)	EK 612 × EF 925
EF 1389	(EK 227) <i>gadW::lacZ</i> (Km ^R) Δ(<i>lacA-lacZ</i>)	EK 687 × EF 925

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Fig. 1. Na⁺ and K⁺ effects on AR2 function. (a) Cells were grown (pH 5.5) and challenged (pH 2.5) in the media indicated. Acid challenge was conducted in the presence and absence of glutamic acid HCl. Dilutions of 1:1000 were made from an overnight culture into the challenge media. Cells were not washed for this experiment because they were grown and challenged in the same medium, and therefore there would be no ion carry over. Aliquots (10 µl) were removed at 1 (black bars), 2 (grey bars) and 4 h (white bars), serially diluted, and plated on LB. The numbers of c.f.u. were determined, and percentage survival was calculated relative to time 0. (b) Cells were grown in EG pH 5.5, washed three times in MC buffer, and then diluted 1:1000 into the media indicated, in the absence and presence of glutamic acid. Aliquots were removed at 2 (black bars) and 3 h (grey bars), serially diluted, and plated on LB to determine c.f.u. (a, b) Survival results in the presence of glutamic acid are shown; survival of all cultures challenged in the absence of glutamate was below the level of detection. The bars represent mean values of triplicate assays. Replicate values did not vary by more than 10% from the mean.

secondary antibodies for 1 h at room temperature. The blot was developed with ECL detection reagents (Amersham Pharmacia Biotech).

β-Galactosidase assays. β-Galactosidase assays were performed according to Miller (1992). Cells were cultured in the indicated medium to stationary phase, and 100 µl of the stationary phase culture was used in the assay.

RESULTS

Na⁺ contributes to AR2-mediated survival

Na⁺ and K⁺ have central roles in pH homeostasis during growth at different pH values. It is thought that Na⁺/H⁺

Fig. 2. Western blot analysis of GadA/B and GadC under different growth conditions. Cells grown to stationary phase in the media indicated, at pH 5.5, were harvested, resuspended in 0.1% SDS, and the proteins were separated on a 10% Tris/HCl polyacrylamide gel. Membranes were then probed with either anti-GadA/B- or anti-GadC-specific antibodies.

and K⁺/H⁺ antiporter systems help maintain cytoplasmic pH near 7.8 when cells are growing under alkaline and acidic conditions, respectively (Booth, 1985). Consequently, potential roles for these ions in surviving extreme acid stress were investigated. Cells were grown to stationary phase in M63K, M63N or M63N/K at pH 5.5, and subsequently challenged in the same medium at pH 2.5. When cells were grown and acid challenged in M63N (a high-Na⁺, low-K⁺ condition), survival was 84% after 2 h (Fig. 1a). A similar result was observed when cells were both grown and acid challenged in M63N/K (containing an intermediate level of both Na⁺ and K⁺) (Fig. 1a). However, when cells were grown and challenged in M63K (a low-Na⁺, high-K⁺ condition), survival was only 3% after 2 h at pH 2.5 (Fig. 1a). The data suggest that Na⁺ is important to acid resistance. However, it was unclear whether Na⁺ was more important during cell growth (i.e. during the induction of acid resistance), or during acid challenge (i.e. via the actual mechanism of acid resistance).

As noted above, Na⁺ can function as a counter-ion in antiport mechanisms that directly remove H⁺ from the cytoplasm (Karpel *et al.*, 1991; Kroll & Booth, 1983; Zilberstein *et al.*, 1982). Alternatively, Na⁺ could act at the gene level by influencing the induction of key system components (Rahav-Manor *et al.*, 1992). We first determined whether Na⁺ or K⁺ played a physiological role in the mechanism of acid survival. The *gadA/BC* genes were induced by growth to stationary phase in pH 5.5 minimal EG medium, which is a high-Na⁺, high-K⁺ medium. The induced cells were then extensively washed to remove excess Na⁺ and K⁺, and subsequently challenged in M63N, M63N⁰/K⁰ (containing no added Na⁺ or K⁺) or H₂O, at pH 2.5, in the presence or absence of glutamic acid (Fig. 1b). Remarkably, cells survived equally well under all challenge conditions when glutamic acid was present. This result indicates that neither Na⁺ nor K⁺ plays a major physiological role during pH 2.5 acid stress.

Na⁺ levels affect *gadA/BC* expression

Glutamate decarboxylase and the GadC antiporter are critical components of AR2. To determine whether Na⁺ or K⁺ regulates *gadA/BC* expression, Western blot analyses were performed to examine the levels of GadA/BC proteins. Cells grown to stationary phase in M63K, M63N and M63N/K were harvested, and solubilized in 0.1 % SDS. Cells cultured in media containing high levels of Na⁺ (either M63N or M63N/K) expressed GadA/BC proteins at high levels (Fig. 2, lanes 2 and 3). In contrast, Fig. 2 (lane 1) shows that cells grown in M63K (low Na⁺, high K⁺) poorly expressed the GadA/B decarboxylases and GadC antiporter. This corresponds to the lower level of acid resistance seen under these conditions in Fig. 1(a). These findings suggest that high Na⁺ levels are involved in inducing *gadA/BC* expression.

The expressions of *gadA* and *gadBC* are controlled at the transcriptional, as well as the translational, level (Castanie-Cornet & Foster, 2001; De Biase *et al.*, 1999; Gong *et al.*, 2004). We next examined which of these levels was influenced by high Na⁺ by using *gadA::lacZ* transcriptional and translational fusions. The results shown in Fig. 3(a, b) indicate that cells grown under high-Na⁺ (low-K⁺) conditions (M63N) exhibit a 5.8-fold higher β -galactosidase activity compared with cells cultured in a

high-K⁺ (low-Na⁺) condition (M63K). Cells grown at intermediate Na⁺/K⁺ concentrations (M63N/K) induced *gadA* at a level that was 3.4-fold above the expression seen with cells grown in low-Na⁺ M63K medium (Fig. 3a, b). Because similar fold differences were obtained when a *gadA::lacZ* translational fusion was examined, it appears that Na⁺ levels affected *gadA/BC* transcription, not translation, during growth to stationary phase (Fig. 3b).

One might argue that the ion-dependent expression of this system is due to low K⁺ rather than high Na⁺ levels. That is, high K⁺ levels repress the system, rather than high Na⁺ levels induce it. However, the effects are probably not due to low K⁺, based on results we will show later using *gadX* and *gadW* mutants.

The expression of GadE, the essential activator of *gadA/BC*, is also regulated by Na⁺ levels

Of the known activators of *gadA* and *gadBC*, GadE appears to be the most important. Thus, we wondered whether the effect of Na⁺ levels on *gadA/BC* expression might really be due to an influence of these ions on *gadE* expression. This question was addressed by examining the influence of Na⁺ and K⁺ on the expression of a *gadE::lacZ* transcriptional fusion strain. When cells containing a *gadE::lacZ* fusion were cultured in M63N (high Na⁺), *gadE* expression was threefold higher than that produced when the cells were grown in M63K (low Na⁺), and 1.75-fold higher than levels made during growth in M63N/K (Fig. 3c). The results indicate that *gadE* transcription is regulated by Na⁺ levels. Since GadE is a required activator of *gadA* and *gadBC*, the Na⁺ dependence of *gadA/BC* is likely to be due to Na⁺-dependent regulation of *gadE*.

GadX and GadW affect the Na⁺-dependent regulation of *gadE* and *gadA*

GadX and GadW are two AraC-family regulators that activate the expression of *gadA*, *gadB/C* and *gadE* (Ma *et al.*, 2002; Tramonti *et al.*, 2002; Sayed *et al.*, 2007). Therefore, we questioned whether GadX and/or GadW play a role in the Na⁺-dependent regulation of *gadE*, as well as that of *gadA*. Wild-type (WT) and Δ *gadX/W* strains carrying the *gadA::lacZ* reporter were cultured in M63K, M63N and M63N/K. The results presented in Fig. 4(a) indicate that the Na⁺-dependent induction of *gadA* exhibited by the WT is lost in the Δ XW mutant. A similar pattern was observed when we examined the *gadE::lacZ* reporter strain (Fig. 4b). The Δ *gadX/W* strain again failed to induce *gadE* in the high-Na⁺/low-K⁺ medium (Fig. 4b).

A report by others has shown that GadE alone can activate *gadA/BC* expression *in vivo*, but that GadE is required for GadX or GadW to activate these genes (Sayed *et al.*, 2007). Thus, these data suggest that GadX and GadW affect Na⁺-dependent *gadA* expression indirectly through the Na⁺-dependent regulation of *gadE*.

Fig. 3. *gadA* and *gadE::lacZ* expression is Na⁺/K⁺ dependent. (a–c) Strains containing a *gadA::lacZ* transcriptional fusion (EF 833; *gadA::lacZ_O*) (a), a translational fusion (EF 1236; *gadA::lacZ_P*) (b), and a *gadE::lacZ_O* transcriptional fusion (EF 1357) (c), were grown in the media indicated at pH 5.5 to stationary phase (media supplemented with 0.3 mM tryptophan). These cultures were then assayed for β -galactosidase activity. The data shown are representative of at least three separate experiments; bars represent SEM.

Fig. 4. GadX and GadW affect the Na^+/K^+ -dependent regulation of *gadA* and *gadE*. (a, b) A *gadXW* deletion was introduced into strains containing *gadA::lacZ* (a), and *gadE::lacZ* (b) fusions. The derivative strains were then grown to stationary phase in the media indicated supplemented with tryptophan, at pH 5.5, and assayed for β -galactosidase activity, along with WT strains carrying the same fusions. Black bars, WT; grey bars, *gadXW* deletion mutant. Values are means of triplicate assays and the bars represent SEM.

GadX and GadW differentially affect *gadE::lacZ* expression

The individual effects of GadX and GadW were then analysed to determine whether distinct roles exist for each of these two proteins in *gadE* expression. In WT cells possessing both *gadX*⁺ and *gadW*⁺, *gadE* was induced in high Na^+ /low K^+ (Fig. 5, bar 1 versus bar 5). Either GadX or GadW alone could carry out this activation (Fig. 5, bars 2 and 3 versus bar 4).

The situation was different in low- Na^+ conditions. Here, deleting GadX, while retaining GadW ($\Delta gadX$ *gadW*⁺), actually increased low Na^+ expression of *gadE* compared with WT cells (Fig. 5, bar 5 versus bar 6). This suggests that GadW can activate *gadE* in low Na^+ , and that GadX normally prevents or inhibits this. In contrast, when GadX was retained, and GadW was removed (*gadX*⁺ $\Delta gadW$), there was little effect on low Na^+ expression of *gadE* (Fig. 5, bars 5 and 7). However, GadX was still needed to activate *gadE* in low Na^+ , as seen when comparing the *gadW* and *gadXW* results (Fig. 5, compare bars 7 and 8). These data can be explained as GadX repressing the activity of GadW, while also directly activating *gadE*, under low- Na^+ conditions. Under high- Na^+ conditions, however, GadX and W had an additive effect on the expression of *gadE* (Fig. 5, bars 1 versus bars 2 and 3). As for the effect of these mutations on acid resistance, mutants individually defective in *gadX* or *gadW* remained acid resistant following

Fig. 5. GadX and GadW exhibit a differential effect on the Na^+/K^+ regulation of *gadE* expression. Strains carrying a *gadE::lacZ* transcriptional fusion were introduced with a *gadX*, *gadW* or *gadXW* (same strain as Fig. 4) mutation. These strains were then grown to stationary phase in the media indicated, at pH 5.5, and assayed for β -galactosidase activity, along with the WT. Values are means of triplicate assays and the bars represent SEM.

growth in high Na^+ because of the redundant effects of GadX and GadW on *gadE* induction (50–60 % survival after 4 h at pH 2.5). In low Na^+ , however, *gadX* mutants were actually more acid resistant than the WT, exhibiting 10 % survival versus 1 % survival, respectively, after 4 h at pH 2.5. This occurs because, as shown in Fig. 5, GadW is better at activating *gadE* when GadX is absent.

As noted above, one might argue that the ion-dependent expression of this system is due to low K^+ rather than high Na^+ levels. That is, high K^+ levels repress the system rather than high Na^+ levels induce it. However, the effects are probably not due to K^+ . When both K^+ and Na^+ were high (as in NK medium), the *gadX* and *gadW* phenotypes were similar to those seen using the high- Na^+ /low- K^+ medium, i.e. both GadX and GadW were needed for maximal expression of *gadA* and *gadE* (data not shown). If high K^+ was the sensed condition, one would have expected that the *gadX/gadW* phenotypes observed in M63N/K medium would be similar to those seen in M63K medium. They were not.

Na^+/K^+ levels do not regulate expression of *gadX* and *gadW*

We next examined whether the Na^+ effect on *gadE* and *gadA* expression was due to Na^+/K^+ effects on *gadX* or *gadW* expression. Strains containing *gadX::lacZ* or *gadW::lacZ* fusions were grown in high- Na^+ (M63N), low- Na^+ (M63K) and intermediate- Na^+ (M63N/K) media, and the effects on expression were determined. The results shown in Fig. 6 reveal that Na^+ levels had no significant effect on the transcription of either *gadX::lacZ* or *gadW::lacZ*, suggesting that Na^+ -dependent regulation of *gadA* and *gadE* is due to the post-transcriptional

Fig. 6. *gadX* and *gadW* expression is not Na^+/K^+ dependent. Strains carrying a *gadX::lacZ* fusion (a), and a *gadW::lacZ* fusion (b), were grown to stationary phase in the media indicated, at pH 5.5, and then assayed for β -galactosidase activity.

modulation of GadX and GadW activity, and not to their levels of expression.

DISCUSSION

E. coli is an acid-resistant enteric micro-organism capable of withstanding extreme acid exposure for several hours (Small *et al.*, 1994; Small, 1998). AR2, the glutamate-dependent system, is the most robust of the four systems that protect *E. coli* from acid stresses encountered in the environment and in the stomach of the host (Castanie-Cornet *et al.*, 1999; Iyer *et al.*, 2003; Price *et al.*, 2004). A complex web of regulation controls AR2 expression, and this allows cells to anticipate future exposures to acid stress when growing under a variety of conditions (Castanie-Cornet & Foster, 2001; Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999; Gong *et al.*, 2004; Hommais *et al.*, 2001; Ma *et al.*, 2003a, 2004, 2002; Masuda & Church, 2002, 2003; Tramonti *et al.*, 2002). Production of GadE is the focus of much of this regulatory network. Numerous regulatory proteins are known to control *gadE* expression, including GadE itself, GadX, GadW, CRP, RpoS, YdeO, EvgAS, PhoP and RcsCDB. Despite knowledge of these many regulators, little is known regarding what small molecules influence their activities.

Previous work has shown that cAMP levels affect induction of acid resistance by controlling RpoS production, which, in turn, activates expression of a small RNA (GadY) needed to stabilize *gadX* mRNA (Jung & Kim, 2003; Ma *et al.*, 2003a; Opdyke *et al.*, 2004). In addition to cAMP, microarray studies have implicated PhoP, which senses Mg^{2+} , as a regulator of *gadE* (Zwir *et al.*, 2005). The current work indicates that Na^+ is also involved in this extensive regulatory network. However, it is not clear whether the

system senses internal or external Na^+ levels, since altering external Na^+ concentration will also influence internal Na^+ concentration. For instance, it has been shown that changing external Na^+ levels from 1 mM to 85 mM will change internal Na^+ levels from 5 mM to 14 mM (Lo *et al.*, 2006).

Since GadE is the central activator of *gadA* and *gadBC*, it would seem reasonable that the effect of Na^+ on *gadA/BC* expression would be indirect via an effect on GadE activity or expression. GadE belongs to the LuxR family of transcriptional regulators, with homology in the helix-turn-helix (HTH) DNA-binding domain (Fuqua *et al.*, 1994). The LuxR family of regulators is separated into two classes, both of which depend on a chemical or environmental signal to activate or inactivate them (Nasser & Reverchon, 2007; Withers *et al.*, 2001). However, Na^+ does not appear to be a signal sensed directly by GadE; instead, Na^+ appears to affect the expression of *gadE* via influences over GadX and GadW.

GadX and GadW are two AraC-like regulators that have been shown to regulate *gadA/BC* expression (Ma *et al.*, 2002; Tramonti *et al.*, 2002), and have more recently been established as regulators of *gadE* expression (Sayed *et al.*, 2007). Members of the AraC/XylS family of regulators possess a DNA-binding domain (HTH motif), as well as a dimerization domain (Gallegos *et al.*, 1997). Many of these proteins are also capable of binding ligands, such as arabinose in the case of AraC, that modulate their activity (Gallegos *et al.*, 1997). The data presented indicate that the Na^+ level during growth can modulate the activities of GadX and GadW. However, it is not certain whether GadX and/or GadW actually bind Na^+ , or if external Na^+ somehow alters their activities.

The working model fitting the current data suggests that GadW can activate *gadE* regardless of Na^+ levels. GadX, on the other hand, will activate transcription of *gadE* when Na^+ levels are high. However, when Na^+ levels are low, GadX appears to inhibit GadW activity. Because Na^+ levels did not affect the expression of *gadX* and *gadW* operon fusions (Fig. 6), it seems that Na^+ post-transcriptionally modulates the activity of these two regulators.

The mechanism by which Na^+ modulates GadX and GadW activities is not known. There is some evidence that GadX and GadW can form both homodimers and heterodimers (Ma *et al.*, 2002). Therefore, assuming that the Na^+ effect is post-translational and intracellular, the level of Na^+ inside the cell may change the effectiveness of these interactions, thereby changing the activity of the proteins. It is also possible that this ion does not affect protein-protein interaction, but affects the activity of the protein directly via ion binding. Alternatively, Na^+ may cause some other unknown physiological effect inside the cell that then modulates the activity of GadX and GadW.

It is known that changing external Na^+ levels from 1 mM to 85 mM changes internal Na^+ levels from 5 mM to

14 mM (Lo *et al.*, 2006). Other published measurements indicate that growth pH also alters intracellular Na⁺ levels, changing them from approximately 0.1 $\mu\text{mol (mg protein)}^{-1}$ to 0.4 $\mu\text{mol (mg protein)}^{-1}$ during growth at pH 8.3 and 6, respectively (Shijuku *et al.*, 2001). Thus, even though we cannot rule out the possibility that external Na⁺ levels are responsible for the effects seen, the evidence that external Na⁺ levels and pH affect internal Na⁺ levels is consistent with the idea that an acid-associated increase in internal Na⁺ levels could contribute to the acid induction of the GAD system.

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