***Escherichia coli* acid resistance: cAMP receptor protein and a 20 bp *cis*-acting sequence control pH and stationary phase expression of the *gadA* and *gadBC* glutamate decarboxylase genes**

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Acid resistance is an important feature of both pathogenic and non-pathogenic *Escherichia coli*. It enables survival in the acidic regions of mammalian gastrointestinal tracts and is largely responsible for the small number of bacteria required for infection/colonization. Three systems of acid resistance have been identified, the most efficient of which requires glutamic acid during pH 2 acid challenge. Three proteins associated with glutamate-dependent acid resistance have been identified. They are glutamate decarboxylase (encompassing two isozymes encoded by *gadA* and *gadB*) and a putative glutamate:γ-amino butyric acid antiporter (encoded by *gadC*). The results confirm that the GadA and GadB proteins increase in response to stationary phase and low environmental pH. The levels of these proteins correspond to concomitant changes in *gadA* and *gadBC* mRNA levels. Fusions between *lacZ* and the *gadA* and *gadBC* operons indicate that this control occurs at the transcriptional level. Western blot, Northern blot and fusion analyses reveal that regulation of these genes is complex. Expression in rich media is restricted to stationary phase. However, in minimal media, acid pH alone can trigger induction in exponential or stationary phase cells. Despite this differential control, there is only one transcriptional start site for each gene. Expression in rich media is largely dependent on the alternate sigma factor σ^S and is repressed by the cAMP receptor protein (CRP). In contrast, σ^S has only a minor role in *gad* transcription in cells grown in minimal media. Deletions of the regulatory region upstream of *gadA* provided evidence that a 20 bp conserved region located 50 bp from the transcriptional start of both operons is required for expression.

Keywords: acid resistance, glutamate decarboxylase, pH control, *Escherichia coli*, *rpoS*

INTRODUCTION

Escherichia coli, in its natural environment, endures many different stress conditions including frequent periods of acid stress. Even though the organism prefers a neutral pH for growth, *E. coli* can tolerate extremely

acidic conditions (pH 2–3) for several hours (Gorden & Small, 1993; Waterman & Small, 1996). This is particularly important for the pathogenic *E. coli* that generally must survive the strong acid pH of the stomach to cause disease. We have previously shown that three distinct acid-resistance systems are involved in protecting cells from acidic shock (Castanie-Cornet *et al.*, 1999; Lin *et al.*, 1995, 1996). These systems are active only in the stationary phase of growth. Acid-resistance system 1 relies directly on σ^S , the stationary-phase sigma factor (Lange & Hengge-Aronis, 1991; Loewen &

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Abbreviation: CRP, cAMP receptor protein.

Table 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
EK227	<i>E. coli</i> K-12 wild-type	A. C. Matin*
EK298	(TE2680) F ⁻ λ ⁻ IN (<i>rrnD-rrnE</i>) Δ (<i>lac</i>)X74 <i>rpsL galK2 recD1903::Tn10dTc trpDC::putPA1303</i>	T. Elliott†
EK344	GE1050 Δ <i>crp::Cm</i>	G. Weinstock‡
EF362	K-12 <i>rpoS::Tn10</i>	Castanie-Cornet <i>et al.</i> (1999)
EF614	EK298 <i>trpDC::putPA1303-Km-gadB</i> ⁻²⁰³ :: <i>lacZ</i> (o)(-203 to +788 fusion)	EK298 $\times$ pCR384
EF615	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻¹⁶⁵ :: <i>lacZ</i> (o)(-165 to +788 fusion)	EK298 $\times$ pCR385
EF640	Tet ^S derivative of EF615	
EF647	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻¹⁶⁵ :: <i>lacZ</i> (o) <i>rpoS::Tn10</i>	EF640 $\times$ EF362
EF660	Tet ^S derivative of EF647	
EF663	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻⁵¹ :: <i>lacZ</i> (o)(-51 to +788 fusion)	EK298 $\times$ pCF402
EF666	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻⁸⁶ :: <i>lacZ</i> (o)(-86 to +788 fusion)	EK298 $\times$ pCF403
EF676	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻¹⁶⁵ :: <i>lacZ crp::Cm</i>	EF615 $\times$ EK344
EF677	EK298 <i>trpDC::putPA1303-Km-gadA</i> ⁻¹⁶⁵ :: <i>lacZ rpoS::Tn10 crp::Cm</i>	EF647 $\times$ EK344

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Hengge-Aronis, 1994), and will protect cells at pH 2.5 in minimal media. The two other systems involve specific amino-acid decarboxylases and rely on exogenously supplied glutamate (acid-resistance system 2) or arginine (acid-resistance system 3) for protection during acidic shock in minimal media. These systems are thought to provide acid resistance by consuming intracellular protons via the amino acid decarboxylation reaction and also by gradual alkalinization of the medium.

Two operons, *gadA* and *gadBC*, have been identified as participating in the glutamate-dependent acid-resistance system 2 (Castanie-Cornet *et al.*, 1999; Hersh *et al.*, 1996). The *gadA* and *gadB* genes encode highly homologous glutamate decarboxylase isoforms (Smith *et al.*, 1992), whereas *gadC* encodes a membrane-associated glutamate: γ -amino butyric acid (GABA) antiporter that exchanges exogenous glutamate for intracellular GABA. Glutamate decarboxylase production has been shown to increase in response to acid, osmotic and stationary phase signals (Castanie-Cornet *et al.*, 1999; De Biase *et al.*, 1999). De Biase *et al.* (1999) have shown that the histone-like protein HN-S acts as a negative regulator of *gad* expression and reported that both operons are only induced in stationary phase, with enhanced expression occurring at acid pH (De Biase *et al.*, 1999). Expression was reported to be totally dependent on σ^S and was not observed in exponential phase cells. However, glutamate-dependent acid resistance, which requires Gad, does not depend on σ^S (Castanie-Cornet *et al.*,

1999; Lin *et al.*, 1995). Western blot analyses conducted in our laboratory have revealed that acid induces GadA and GadB production in exponential phase cells grown in minimal media even in *rpoS* mutants devoid of σ^S (Castanie-Cornet *et al.*, 1999). A reasonable hypothesis that would account for these apparently conflicting results is that multiple regulatory factors influence *gad* expression and that growth in complex or minimal glucose media determines which regulator dominates. We now provide evidence that *gad* transcription is controlled by several additional factors and that these factors differ depending upon the growth phase and environment. The factors involved include σ^S , the cAMP receptor protein (CRP), σ^{70} and a 20 bp conserved region of the *gad* operon.

METHODS

Bacterial strains and culture media. The bacterial strains used in this study are listed in Table 1. *E. coli* was grown at 37 °C in either minimal E medium (Vogel & Bonner, 1956), E medium containing 0.4% glucose (EG) or in complex medium Luria-Bertani broth (LB) buffered with either 100 mM MES (pH 5.5) or MOPS (pH 8). Antibiotics were used at the following concentrations: ampicillin, 50 μ g ml⁻¹; kanamycin, 25 μ g ml⁻¹; streptomycin, 100 μ g ml⁻¹; tetracycline, 20 μ g ml⁻¹; chloramphenicol, 30 μ g ml⁻¹.

Genetic and molecular techniques. Transductions with P1vir, transformations using CaCl₂ and conjugations were performed according to standard protocols (Miller, 1992). Gen-

Fig. 1. Sequence analysis and alignment of *gadA* and *gadB* promoter regions. The transcriptional start is in bold and indicated by +1. The ATG start codon is underlined. Primers used to generate *gad-lacZ* fusions are indicated by arrows. Not shown is oligonucleotide 109, which is located within the coding region of *gadA* and *B* at nucleotide +788 (codon 253). Boxes around nucleotides indicate the -10 and -35 recognition sequences. The 20 bp regulatory region is indicated by a box between the *gadA* and *gadB* sequences.

eral DNA manipulations were carried out as described by Sambrook *et al.* (1989). β -Galactosidase assays were performed according to Miller (1992). Results presented are representative of triplicate experiments.

Construction of *gad-lacZ* transcriptional fusions. Transcriptional fusions of *gadA* and *gadB* to *lacZ* were constructed by cloning PCR-generated fragments of *gadA* and *gadB* into the *EcoRI* site of the pRS551 vector (Simons *et al.*, 1987). The PCR-generated fragments were made using *gadA* primers 201 (specific to *gadA*) and 109 (5'-CGAACGGTGCCAGGAAG-CC-3') and *gadB* primers 202 (specific to *gadB*) and 109 (Fig. 1). This created fragments extending from bp -164 to +788 (codon 253) relative to the transcription start for *gadA* (pCF384) and from bp -203 to +788 (codon 253) for *gadB* (pCF385). These plasmids were then used to introduce the *lacZ* fusions into a *put* gene engineered in *E. coli* as described by Elliott (1992). After *XhoI* digestion of these plasmids, linearized DNA was transformed into EK298. Kan^R Amp^S Cm^S transformants were obtained following recombination of the plasmid into the chromosome. This resulted in mero-diploid strains containing intact *gad* genes as well as *gadA-lacZ* or *gadB-lacZ* transcriptional fusions located at the *putPA* operon (EF615 and EF614 respectively). Similarly, *lacZ* fusions to truncations of the *gadA* promoter region were made using oligonucleotides 261/109 (bp -85 to +788, containing a putative pH control region) and 260/109 (bp -51 to +788, containing the -10 and -35 promoter recognition regions but lacking the pH control region). These fragments were also cloned into pRS551, forming plasmids pCF403 (region from -85 to +788) and pCF402 (region from -51 to +788). These plasmids were linearized as described above and transformed into EK298 to construct chromosomal fusions.

Purification of nucleic acids, and Northern and Western blotting. Total RNA was isolated from cells grown under different conditions using the RNeasy kit (Qiagen). Aliquots of 5 μ g were subjected to electrophoresis in a MOPS formaldehyde 1% agarose gel (Sambrook *et al.*, 1989). The 1.4 kb *gad* probe, which hybridizes to both *gadA* and *gadB*, was generated by PCR using oligonucleotides 121 (5'-GGAGTT-CGAAATGGACCAGAAG-3') and 122 (5'-AGTTTCGGG-TGATCGCTGAG-3'), and corresponds to the entire ORF of *gadA* or *gadB*. This fragment was uniformly labelled by

including 50 μ Ci [γ -³²P]dCTP in the PCR reaction. Primer extension analysis of the *gad* transcriptional start sites was performed as described by Genosys using oligonucleotide 233 (5'-CTCGTCGACACGTGAATCGAGTAGTTC-3'). Western blot analysis was performed using antibodies and conditions described previously (Castanie-Cornet *et al.*, 1999).

RESULTS

Differential production of Gad in minimal and complex media

Western blot data presented in Fig. 2(a) (lanes 1 and 2) illustrate that glutamate decarboxylase levels were induced by acid in mid-exponential phase cells grown in minimal media, confirming results from our earlier

Fig. 2. Western blot analysis of Gad levels. Wild-type (EK227) and *rpoS* mutant (EF362) strains were grown overnight in LB or EG and diluted 1/200 into fresh medium at specified pH values. Cultures were grown to OD₆₀₀ 0.5–0.7 (EP, exponential phase) or 1.5–2 (SP, early stationary phase), aliquots were removed and subjected to Western blotting using anti-glutamate decarboxylase antibodies. (a) Gad expression in EG minimal medium at pH 7 and 5.5 (final pH values). (b) Gad expression in LB complex media buffered at pH 8 and 5.5. The lower band represents a cross-reacting band that is not the product of either *gad* gene.

Fig. 3. Northern blot analysis of *gad* transcription. Wild-type *E. coli* (EK227) and *rpoS* mutant (EF362) cultures were grown overnight either in EG minimal medium, pH 7, or in LB. Dilutions (1/500) were made in fresh medium at different pH values. At OD₆₀₀ 0.5 (EP, exponential phase), 4 ml each culture was harvested, and at OD₆₀₀ 1.3–1.5 (SP, stationary phase), 2 ml was harvested. Total RNA from these cultures were prepared as described in Methods. Five micrograms total RNA was loaded in each lane, except for lanes 15 and 16, where only 4 µg was loaded. The probe used to detect *gad* mRNA hybridizes to both the *gadA* and *gadBC* mRNAs.

study (Castanie-Cornet *et al.*, 1999). However, as originally reported by De Biase *et al.* (1999), we also confirmed that acid induction was not observed in mid-exponential phase cells grown in complex, rich media (Fig. 2b, lanes 1 and 2). Fig. 2(a) (lanes 3 and 4) demonstrates that the acid induction observed in minimal medium did not require σ^S but that stationary-phase induction at neutral pH in either minimal glucose or complex media was σ^S dependent (Fig. 2a, compare lanes 5 and 7 and Fig. 2b, compare lanes 5 and 7). The focus of this study is to determine why acid pH induces exponential phase cells to produce glutamate decarboxylase in minimal glucose but not in complex LB media and why σ^S is only required for stationary phase expression at neutral pH.

Gad production by acid-stressed, exponential phase cells is transcriptionally controlled

To determine whether the media-dependent regulation exerted over Gad production was due to transcriptional control, we performed Northern-blot experiments on mRNA extracted from wild-type and *rpoS* mutant strains at different stages of growth and in different media. The results presented in Fig. 3 indicate that both *gadA* and *gadBC* behaved in a similar manner, and that transcript levels from each increased more than 10-fold in exponential phase, minimal media grown cells exposed to acidic pH (Fig. 3, compare lanes 5 and 7). The acid-induced increase in *gad* mRNA levels was not dependent on σ^S in exponential, minimal glucose grown cells (Fig. 3, lanes 6 and 8). In contrast, acid induction of *gad* expression in complex media occurred mainly in stationary phase cells (Fig. 3, compare lanes 9 and 11) compared to exponential phase cells (Fig. 3, lanes 1 and 3) and was primarily RpoS-dependent (Fig. 3, compare lanes 10 and 12).

Control of *gad-lacZ* transcriptional fusions

To gain further insight into *gad* regulation, *gadA-lacZ* and *gadB-lacZ* transcriptional fusions were constructed. These fusions were inserted into the chromosome at the *putPA* operon, creating merodiploid strains (EF614, *gadB-lacZ*, and EF615, *gadA-lacZ*). β -Galactosidase activities were then measured at different stages of growth in minimal or complex media. Fig. 4(a) presents the results obtained during exponential phase (OD₆₀₀ 0.5). In minimal (EG) medium, both fusions showed increased transcription at acidic pH (sixfold for *gadB-lacZ* and eightfold for *gadA-lacZ*). However, during mid-exponential growth in complex (LB) medium, no acid induction was observed. The results were different if activities were measured once cells entered stationary phase (OD₆₀₀ 1.2). In this case, acid induction in LB medium was evident as 20- and 40-fold increases for *gadB-lacZ* and *gadA-lacZ* constructs, respectively (Fig. 4b; EF614 and 615). These results agree with the Western and Northern blot analyses noted above indicating that exponential phase cells exhibit acid induction of the *gad* genes in minimal but not in complex media.

Acid and stationary phase controls utilize a single transcriptional start site

De Biase *et al.* (1999) reported the presence of single transcriptional start sites for *gadA* and *gadB* in stationary phase-grown cells. We wondered whether the different *gad* induction profiles observed in minimal and complex media reflected different mRNA transcriptional start sites for stationary phase and acid-induced *gad* expression. Consequently, primer extension analyses to detect transcriptional start sites were performed on mRNA extracted from exponential phase wild-type cells grown in minimal media at pH 5.5. An antisense oligonucleotide (oligonucleotide 233) able to hybridize with both *gadA* and *gadB* was used for this purpose. The start sites, represented as +1 in Fig. 1, were identical to those identified by De Biase *et al.* (1999), indicating that there is only one start site for each *gad* gene regardless of the inducing condition (data not shown).

Effect of *rpoS* on *gad* transcription

We have shown previously that *rpoS* mutations only have a minor effect on glutamate-dependent acid resistance in acid-adapted cells (Castanie-Cornet *et al.*, 1999) and demonstrate here that σ^S controls stationary phase but not acid-induced expression of the *gad* genes. Fig. 4 illustrates that an *rpoS* mutation (strain EF647) had no effect on the acid pH induction of *gadA* transcription in minimal media in either log (Fig. 4a) or stationary phase (Fig. 4b). However, an *rpoS* mutation did prevent mid-exponential phase transcription of *gadA* in neutral pH minimal media. Thus, mid-exponential-phase neutral pH induction of *gad* in minimal media was most likely due to the early stages of σ^S accumulation known to begin in mid-exponential phase and, as will be shown below, to the absence of a

Fig. 4. Effects of media, pH, *rpoS* and *crp* on *gad-lacZ* expression. β -Galactosidase activities were measured during mid-exponential phase (a) and stationary phase (b) growth. Exponential phase cells were prepared by diluting overnight cultures (pH 7) 1:200 into fresh medium including LB pH 7, LB pH 5.5 and EG pH 7. For the LB cultures, when the OD_{600} reached 0.5 ($\sim 3 \times 10^8$ c.f.u. ml⁻¹), aliquots were assayed for β -galactosidase activity according to Methods. Early stationary phase cultures were assayed when OD_{600} reached 1.2–1.5. For expression in minimal media, exponential phase cells were grown to OD_{600} 0.2 at pH 7, resuspended in EG pH 7 and EG pH 5.5 media, and induced at that pH for 2 h before assay. Final OD_{600} did not exceed 0.4. ND, Not determined. Experiments were performed in triplicate and individual mean values are presented over each bar. Error bars represent standard error of the mean.

negative regulation present in mid-exponential phase LB cultures that prevents σ^S -dependent *gad* expression. It is clear from the results that in minimal media σ^S only plays a minor role in directing acid-induced transcription of *gad* in exponential and stationary phase cells (Fig. 4, EF647, EG). However, in complex media, the *rpoS* mutation prevented most of the acid pH induction of *gad* transcription observed in stationary phase cells (Fig. 4, strain EF647, LB). Consequently, σ^S appears to be the major sigma factor used for directing *gad* transcription for cells grown in complex, but not minimal, media. The dependence on σ^S for expression in complex media is consistent with the findings of De Biase *et al.* (1999). Minimal media cultures, however, must be able to utilize a different sigma factor under acid conditions. Since only one promoter for each gene appears to be involved regardless of the inducing condition, that other sigma factor is most likely to be σ^{70} , whose recognition sequence is very similar to that of

σ^S . The fact that mid-exponential phase minimal glucose-grown cells induce *gad* in the absence of σ^S while mid-exponential LB-grown cells do not also suggests that more is involved in regulating *gad* than simply swapping σ^{70} for σ^S . Either growth in LB subjects the *gad* genes to a negative regulatory control system not present in minimal glucose cultures or growth in minimal glucose engages an additional positive regulator of *gad*.

CRP is responsible for the repression in complex media of *gadA* transcription

The difference in the induction pattern observed between minimal and complex media indicated that either there is an inhibiting molecule(s) in LB or the glucose present in EG is helping to induce *gadA* and *gadB* in response to acidic pH. The complex versus minimal media effect on *gad* expression suggested the involve-

Fig. 5. Identification of the *gadA* pH response region. Strains EF615 (*gad* bp -164 to $+788$ fused to *lacZ*), EF666 (-85 to $+788$ fusion) and EF663 (-51 to $+788$ fusion) were grown overnight at neutral pH in LB or EG, and diluted 1/200 in fresh medium, LB pH 7 or pH 5.5 and EG pH 7. For the LB cultures, once the OD_{600} reached 0.2–0.5 (exponential phase) (a) and 1.2–1.5 (stationary phase) (b) aliquots were removed and β -galactosidase activities were assayed. For the minimal EG exponential phase cultures, once the OD_{600} reached 0.2, cultures were spun and resuspended in an equal volume of EG pH 7 and EG pH 5.5 and induced for 2 h. The OD_{600} did not exceed 0.4. Experiments were performed in triplicate and individual mean values are presented over each bar. Error bars represent standard error of the mean.

ment of CRP and/or cAMP as a negative regulator of *gadA* transcription in complex LB media. To test this hypothesis, we introduced a *crp* mutation into the *gadA-lacZ* fusion strains and monitored β -galactosidase activities following growth in minimal and complex media at neutral and acidic pH. The results obtained with strain EF676 (Fig. 4a) confirmed a role for CRP as a negative regulator of *gadA* transcription in that the *crp* mutation derepressed *gadA-lacZ* expression in LB grown cells under both pH conditions and did so independently of growth phase (compare EF676 and EF615 in Fig. 4). These results suggest that in complex media CRP represses *gadA* transcription and inhibits induction by acidic pH. However, it is unlikely that CRP is directly involved in pH control since an *rpoS crp* mutant still exhibits acid induction (EF677, Fig. 4). The high level of expression seen in the *crp* mutant was essentially due to RpoS-directed transcription since an *rpoS* mutation reduced *gadA-lac* expression to near normal levels (strain EF677, Fig. 4).

Identification of a regulatory region in *gadA*

The promoter regions of *gadA* and *gadB* exhibit considerable homology up to bp -72 relative to the $+1$ transcriptional start. However, the sequences quickly diverge upstream of position -72 . Of particular interest was a 20 bp block of identity between bp -53 and -72 that we predicted might be involved in the pH control of *gad* transcription (Fig. 1). To address this question,

transcriptional fusions between *gadA* promoter regions containing or lacking this region were made with *lacZ*. EF663 was constructed to contain a fusion between the *gadA* -51 to $+788$ region (missing the pH regulatory region) whereas the fusion constructed in EF666 contained the region between -85 to $+788$, which includes the putative pH regulatory element. β -Galactosidase activities presented in Fig. 5 indicate that strain EF663 (lacking the control region) did not exhibit acid or stationary phase regulation. However, EF666 (containing the control region) exhibited both acid and stationary phase controls. This result confirms that acid control centres on the 20 bp region upstream of the -35 site. Curiously, the overall expression of the -85 to $+788$ fusion (EF666) was two to three times greater than the -166 to $+788$ fusion (EF615), suggesting that the region between -85 and -166 contains a negative element acting on *gadA* transcription.

DISCUSSION

E. coli has maximized the probability of surviving periods of severe acid stress by developing redundant mechanisms for inducing the *gad* genes. Strategies include σ^S -dependent stationary phase induction even in the absence of an immediate acid stress and σ^S -independent acid induction in exponential phase. CRP appears to control whether or not the σ^S -dependent induction mechanism will operate. Under conditions where cAMP levels are high (e.g. rapid growth in LB),

CRP appears to prevent σ^S -dependent transcription of the *gad* genes. This would be physiologically desirable since under rapid growth conditions it is unlikely that severe levels of acid will be encountered. Upon entering stationary phase or when growing on glucose, cAMP levels are low, thereby allowing σ^S -dependent *gad* expression. However, exponential phase minimal glucose grown cells, which possess low levels of cAMP, do not require σ^S for acid-induced *gad* expression. The reason for σ^S -independent, exponential phase induction of *gad* is not intuitively obvious since exponential phase cells that overexpress Gad remain acid sensitive. It is possible that Gad protein produced in exponential phase cells must undergo some form of stationary phase processing in order to become active.

There is a potential, albeit weak, CRP binding site within the 20 bp conserved control region located between -52 bp and -73 bp from the transcriptional start site. This site possesses 9 of the 16 consensus nucleotides associated with CRP binding. The presence of this site may explain the ability of CRP to repress σ^S -dependent expression. The fact that a fusion lacking this region did not exhibit acid induction suggests that an unknown positive regulator also binds to this area. A possible candidate for this regulator is *yhiX*, which is located downstream of *gadA*. The YhiX protein appears to be a member of the AraC family of regulators and was indirectly implicated as a *gad* regulator in gene array studies (Tao *et al.*, 1999). In contrast, *mppA*, encoding a periplasmic murein peptide-binding protein, was experimentally shown to negatively control Gad synthesis but the level at which this regulation may occur has not been investigated (Li & Park, 1999).

The main conclusions derived from this study are that 1) acid and stationary phase induction of *gadA* and *gadB* expression occurs at the transcriptional level; 2) a single promoter drives the expression of each gene regardless of the inducing condition; 3) the sigma factor σ^S is required for stationary phase induction, but not acid induction, of *gad* expression; 4) CRP is a negative regulator of σ^S -dependent *gad* expression; and 5) a conserved 20 bp sequence located between -52 and -73 bp in the *gadA* and *gadBC* promoters is essential for both acid and stationary phase induction.

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