**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

Marie-Pierre Castanie-Cornet† and John W. Foster

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 σS and is repressed by the cAMP receptor protein (CRP). In contrast, σS 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*

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**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 σS, the stationary-phase sigma factor (Lange & Hengge-Aronis, 1991; Loewen &
glutamate-dependent acid resistance, which requires not observed in exponential phase cells. However, two operons, gadA and also by gradual alkalinization of the medium. Protons via the amino acid decarboxylation reaction provide acid resistance by consuming intracellular shock in minimal media. These systems are thought to (acid-resistance system 3) for protection during acidic supplied glutamate (acid-resistance system 2) or arginine amino-acid decarboxylases and rely on exogenously (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-5 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 σ5 and was not observed in exponential phase cells. However, glutamate-dependent acid resistance, which requires Gad, does not depend on σ5 (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 σ5 (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 σ5, the cAMP receptor protein (CRP), σ70 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 µg ml⁻¹; kanamycin, 25 µg ml⁻¹; streptomycin, 100 µg ml⁻¹; tetracycline, 20 µg ml⁻¹; chloramphenicol, 30 µg ml⁻¹.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EK227</td>
<td><em>E. coli</em> K-12 wild-type</td>
<td>A. C. Matin*</td>
</tr>
<tr>
<td>EK298</td>
<td>(TE2680) F′ ÷ IN (rntD−rntE) Δ(lac)X74 rpsL. gadK2 recD193::Tn10ΔTc trpDC::putPA1303</td>
<td>T. Elliott†</td>
</tr>
<tr>
<td>EK344</td>
<td>GE1050 Δacr::Cm</td>
<td>G. Weinstock‡</td>
</tr>
<tr>
<td>EF362</td>
<td>K-12 rpsO::Tn10</td>
<td>Castanie-Cornet * et al. (1999)</td>
</tr>
<tr>
<td>EF614</td>
<td>EK298 trpDC::putPA1303–Km–gadB−::lacZ (o)(−203 to +788 fusion)</td>
<td>EK298 × pCR384</td>
</tr>
<tr>
<td>EF615</td>
<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ (o)(−165 to +788 fusion)</td>
<td>EK298 × pCR385</td>
</tr>
<tr>
<td>EF640</td>
<td>Tet derivative of EF615</td>
<td>EF640 × EF362</td>
</tr>
<tr>
<td>EF647</td>
<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ (o) rpoS::Tn10</td>
<td>EF640 × EF362</td>
</tr>
<tr>
<td>EF660</td>
<td>Tet derivative of EF647</td>
<td>EF640 × EF362</td>
</tr>
<tr>
<td>EF663</td>
<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ (o)(−51 to +788 fusion)</td>
<td>EK298 × pCF402</td>
</tr>
<tr>
<td>EF666</td>
<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ (o)(−86 to +788 fusion)</td>
<td>EK298 × pCF403</td>
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<tr>
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<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ crp::Cm</td>
<td>EF615 × EK344</td>
</tr>
<tr>
<td>EF677</td>
<td>EK298 trpDC::putPA1303–Km–gadA−::lacZ rpoS::Tn10 crp::Cm</td>
<td>EF647 × EK344</td>
</tr>
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eral DNA manipulations were carried out 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'-GGAAACCGTGCCAGGAAGCC-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 pput gene engineered in E. coli as described by Elliott (1992). After XhoI digestion of these plasmids, linearized DNA was transformed into EK298. KanR AmpR CmR transformants were obtained following recombination of the plasmid into the chromosome. This resulted in merodiploid 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 RNaseasy kit (Qiagen). Aliquots of 5 µg 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'-GGATTTCCGAAAATGCGAGAAG-3') and 122 (5'-AGTTTCCGGTGATCGCTGAG-3'), and corresponds to the entire ORF of gadA or gadB. This fragment was uniformly labelled by including 50 µCi [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'-CTCGTCGACCTGAGGAGATGTCTCC-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. 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.

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.
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 $\sigma^S$ but that stationary-phase induction at neutral pH in either minimal glucose or complex media was $\sigma^S$ 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 $\sigma^S$ 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 $\sigma^S$ 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). $\beta$-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 (OD$_{600}$ 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 (OD$_{600}$ 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 $\sigma^S$ 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 $\sigma^S$ accumulation known to begin in mid-exponential phase and, as will be shown below, to the absence of a
negative regulation present in mid-exponential phase LB cultures that prevents $\sigma^S$-dependent gad expression. It is clear from the results that in minimal media $\sigma^S$ 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, $\sigma^S$ appears to be the major sigma factor used for directing gad transcription for cells grown in complex, but not minimal, media. The dependence on $\sigma^S$ 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 $\sigma^\alpha$, whose recognition sequence is very similar to that of $\sigma^S$. The fact that mid-exponential phase minimal glucose-grown cells induce gad in the absence of $\sigma^S$ while mid-exponential LB-grown cells do not also suggests that more is involved in regulating gad than simply swapping $\sigma^\alpha$ for $\sigma^S$. 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 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-
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^A-independent acid induction in exponential phase. CRP appears to control whether or not the σ^A-dependent induction mechanism will operate. Under conditions where cAMP levels are high (e.g. rapid growth in LB),
CRP appears to prevent $\sigma^S$-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 stationary phase or when growing on glucose, cAMP severe levels of acid will be encountered. Upon entering since under rapid growth conditions it is unlikely that gad genes 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 $\sigma^S$-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 $\sigma^S$ is required for stationary phase induction, but not acid induction, of gad expression; 4) CRP is a negative regulator of $\sigma^S$-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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