**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 &

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Abbreviation: CRP, cAMP receptor protein.
glutamate-dependent acid resistance, which requires not observed in exponential phase cells. However, system 2 (Castanie-Cornet et al., 1999) has 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 $\sigma^E$ and was not observed in exponential phase cells. However, glutamate-dependent acid resistance, which requires Gad, does not depend on $\sigma^E$ (Castanie-Cornet et al., 1995; 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 $\sigma^E$ (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 $\sigma^F$, the cAMP receptor protein (CRP), $\sigma^{70}$ 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 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 CaCl$_2$ and conjugations were performed according to standard protocols (Miller, 1992). Gen-

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### Table 1. Bacterial strains used in this study

<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>F $\lambda$ IN (rrnD–rrnE) Δ(lac)X74 rpsL. gak2 recD1903::Tn10dTc trpDC::pV11303</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 rpoS::Tn10</td>
<td>Castanie-Cornet et al. (1999)</td>
</tr>
<tr>
<td>EF614</td>
<td>EK298 trpDC::pV11303–Km–gadB::lacZ (o)(−203 to +788 fusion)</td>
<td>EK298 × pCR384</td>
</tr>
<tr>
<td>EF615</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ (o)(−165 to +788 fusion)</td>
<td>EK298 × pCR385</td>
</tr>
<tr>
<td>EF640</td>
<td>Tet$^R$ derivative of EF615</td>
<td>EF640 × EF362</td>
</tr>
<tr>
<td>EF647</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ (o) rpoS::Tn10</td>
<td>EF647 × EF362</td>
</tr>
<tr>
<td>EF660</td>
<td>Tet$^R$ derivative of EF647</td>
<td>EF647 × EF362</td>
</tr>
<tr>
<td>EF663</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ (o)(−51 to +788 fusion)</td>
<td>EK298 × pCF402</td>
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<tr>
<td>EF664</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ (o)(−86 to +788 fusion)</td>
<td>EK298 × pCF403</td>
</tr>
<tr>
<td>EF676</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ crp::Cm</td>
<td>EF676 × EF362</td>
</tr>
<tr>
<td>EF677</td>
<td>EK298 trpDC::pV11303–Km–gadA::lacZ rpoS::Tn10 crp::Cm</td>
<td>EF677 × EF362</td>
</tr>
</tbody>
</table>

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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′-GGATCCTGGGAGTCTAGAATC-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^r^ transformants were obtained following recombination of the plasmid into the chromosome. This resulted in me- 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). 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 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′-GAGTTCGGAATATGACGGAAG-3′) and 122 (5′-AGTTTCCGGTGATCGTCGAG-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′-CTCGTACCTCGAATCGATGAGTC-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.](image)

![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 OD_600 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.](image)
posed to acidic pH (Fig. 3, compare lanes 5 and 7). The transcript levels from each increased more than 10-fold dependent on media. The results presented in Fig. 3 indicate that both strains at different stages of growth and in different media. (Fig. 3, lanes 6 and 8). In contrast, acid induction of gadBC 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.

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 σS 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 σS 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 $\sigma^S'$, 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 $\sigma^S'$ 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 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–lacZ 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 σS-dependent stationary phase induction even in the absence of an immediate acid stress and σS-independent acid induction in exponential phase. CRP appears to control whether or not the σS-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 since under rapid growth conditions it is unlikely 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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