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Transcriptional Expression of *Escherichia coli* Glutamate-Dependent Acid Resistance Genes *gadA* and *gadBC* in an *hns rpoS* Mutant†

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Resistance to being killed by acidic environments with pH values lower than 3 is an important feature of both pathogenic and nonpathogenic *Escherichia coli*. The most potent *E. coli* acid resistance system utilizes two isoforms of glutamate decarboxylase encoded by *gadA* and *gadB* and a putative glutamate:γ-aminobutyric acid antiporter encoded by *gadC*. The *gad* system is controlled by two repressors (H-NS and CRP), one activator (GadX), one repressor-activator (GadW), and two sigma factors (σ^S and σ^{70}). In contrast to results of previous reports, we demonstrate that *gad* transcription can be detected in an *hns rpoS* mutant strain of *E. coli* K-12, indicating that *gad* promoters can be initiated by σ^{70} in the absence of H-NS.

Commensal and pathogenic *Escherichia coli* must survive transit through the acidic conditions of the stomach, where the pH is normally between 2 and 3, before they can colonize a mammalian host.

The most effective *E. coli* acid resistance system is dependent upon glutamate and involves two isoforms of glutamate decarboxylase (GAD) encoded by *gadA* and *gadB* that convert intracellular glutamate to γ-aminobutyrate, consuming one intracellular proton in the reaction. *gadB* is transcribed in an operon with *gadC*, which encodes an antiporter that is proposed to import glutamate inside the cell while simultaneously exporting γ-aminobutyrate (9, 15, 20).

The regulation of the *gad* system is extremely complex. The expression of all of the *gad* genes is regulated by separate σ^S-dependent and -independent pathways, despite only one transcriptional start site being identified for each locus (4, 5, 6). The alternate sigma factor, σ^S, encoded by *rpoS*, is associated with the stationary-phase expression of *gad* in cells grown in rich medium, whereas σ^S-independent regulation occurs in cells grown in minimal-glucose medium.

The levels of expression of the two GAD isoforms and *gadC* are greatly enhanced in an *hns* deletion background compared to levels found in wild-type cells of *E. coli* (6, 17, 24, 25). The histone-like protein H-NS is a major component of the bacterial nucleoid which influences a variety of cellular processes, such as transcription, recombination, and replication (19, 26). The mechanism underlying gene regulation by H-NS is due to either transcriptional silencing through preferential binding to AT-rich curved DNA sequences often found upstream of *E. coli* promoters or to changes in DNA supercoiling (23, 24). H-NS silencing of gene expression is relieved by environmental signals, such as changes in osmolarity, growth phase, low temperature, anaerobiosis, and pH (2). H-NS has also been implicated in the posttranscriptional regulation of σ^S expression, as

σ^S accumulation is observed in exponential phase in an *hns* mutant (3, 23). This suggested that H-NS has an indirect effect on *gad* expression. However, it has now been shown that H-NS represses *gad* expression directly (11).

It has been established that the expression of the *gad* system is mediated by the GadX protein, a member of the AraC/XylS family of transcriptional regulators, encoded by *gadX* (located downstream of *gadA*) (10, 13, 17). Expression of *gadX* is primarily driven by σ^S (11, 17). GadX was originally shown to play a central role in the H-NS control of genes required in glutamate-dependent acid resistance and to bind to the *gadA* and *gadBC* promoters (13, 17). However, repression of the *gad* genes by H-NS has now been demonstrated to be independent of GadX (11). GadW, another AraC-like regulator, mediates control of the *gadA* and *gadBC* genes by repressing *gadX* and can also activate the *gad* genes directly in rich medium at pH 8 in stationary phase (11). The cyclic AMP receptor protein (CRP) represses the system by inhibiting the production of σ^S. This complex regulatory network maintains tight control over expression of the *gad* system but also provides flexibility for inducing acid resistance under a variety of conditions that precede exposure to acid.

Transcription of *gadA* and *gadBC* in an *hns rpoS* background. De Biase et al. (6) have reported that *gad* transcription is abrogated in an *hns rpoS* double mutant. They suggest, however, that *gad* promoters can be recognized by H-NS and transcribed by σ^{70}, as GAD expression driven by multicopy plasmids was not completely shut off in an *rpoS* mutant. Consistent with this observation, the *gad* genes are expressed in *Salmonella enterica* serovar Typhimurium LT2 (6), which carries a weak *rpoS* allele with a rare UUG start codon (21). More recently it has been implied that *gad* expression can be driven by σ^{70} in cells grown to mid-exponential phase in minimal medium (4).

Because of this inconsistency we investigated the expression of the *gad* genes in isogenic *hns* (22), *rpoS* (15), and *hns rpoS* (23) mutants of *E. coli* K-12 MC4100 (14) by performing Northern hybridization with the internal fragments of both *gadB* and *gadC* (Fig. 1). Due to the high DNA sequence identity (98%) between *gadB* and its isoform *gadA* (16), the *gadB*

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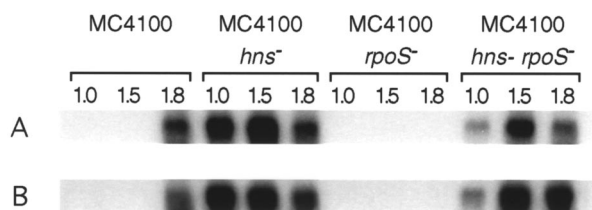
FIG. 1. Northern analysis of *gadB* and *gadC* transcription in *E. coli* MC4100 and isogenic *hns*, *rpoS*, and *hns rpoS* mutants. Total RNA was extracted from cultures of each strain at the growth curve points of A_{600} of 1.0, 1.5, and 1.8. The membrane was probed with the ^{32}P-labeled internal fragments of *gadB* (A) and *gadC* (B).

probe was used to detect the transcripts of both genes. MC4100 is a commonly studied laboratory strain and can be observed to be still undergoing rapid division at an A_{600} of 1.0 (see Fig. 2).

Total RNA was prepared from cultures grown in Luria-Bertani (LB) medium at various stages of the growth curve (A_{600} of 1.0, 1.5, and 1.8) by using TRIzol reagent as described by the manufacturers (Gibco) and by 1 h of DNase treatment (Boehringer Mannheim). RNA samples (30 μg) were heated for 5 min at 65°C, fractionated on 1.2% formaldehyde-agarose gels, and blotted onto nylon membranes (Amersham). Membranes were hybridized with the internal fragments of *gadB* and *gadC* and were nick translated with [α-^{32}P]deoxynucleoside triphosphates (Amersham).

Primers used for generating the 1.0-kb internal fragment of *gadB* were 5′ TCC GCT GCA CGA AAT GCG CGA CGA TGT CGC A 3′ and 5′ AAC CTG GTA AGA GGC GTT CTG TAC TTT GGT 3′. Primers used for generating the 1.2-kb internal fragment of *gadC* were 5′ TCT GGG TCC GAG ATG GGG ATT TGC AGC GAT 3′ and 5′ TGG TGA ACG TCG
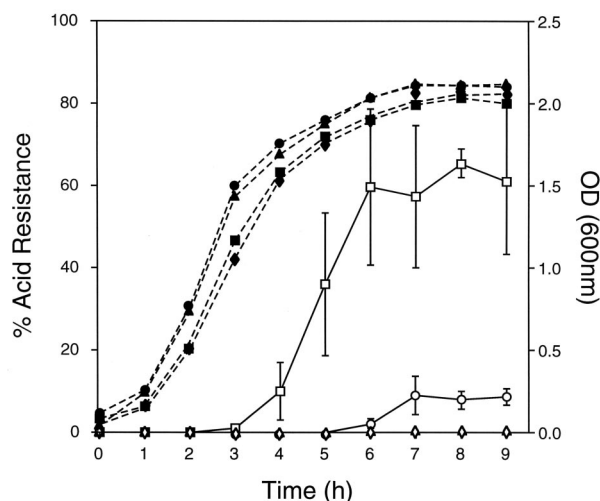

FIG. 2. Effect of *hns* mutation on the expression of acid resistance in *E. coli* MC4100. Cultures were diluted 1:1,000 and were grown in LB medium. Results were taken from a single experiment performed in triplicate. The percentage of acid resistance was determined along the growth curve for strains MC4100 (circles), MC4100 *hns* (squares), MC4100 *rpoS* (diamonds), and MC4100 *hns rpoS* (triangles). Specific acid resistance and optical densities (OD) at 600 nm are indicated by open and closed symbols, respectively.

ACG CGG GTG CAG GAA GAA 3′. PCR amplification was carried out as described previously (20).

Transcripts of all *gad* genes were absent in the *rpoS* mutant as well as in the exponentially grown parental strain, whereas transcripts of *gad* genes were detected in stationary-phase cultures ($A_{600} = 1.8$) of the parental strain (Fig. 1). The sizes of the mRNA obtained corresponded to those predicted from the open reading frames of both *gadA/B* and *gadC* (data not shown). We also observed a higher-molecular-weight transcript that corresponded with the deduced length of a polycistronic *gadBC* message, but this transcript was weaker than either the *gadA/B* or *gadC* transcript (data not shown). As previously reported by De Biase et al. (6), transcription of the *gadA* and *gadBC* genes was strongly enhanced in the *hns* mutant and occurred during exponential growth.

In contrast to De Biase et al. (6), however, we were able to detect transcripts of both *gadA* and *gadBC* in an *hns rpoS* mutant. The presence of transcripts in an *hns rpoS* double mutant suggests that the promoters of all *gad* genes can be recognized by σ^{70} in vivo. This has been demonstrated for other σ^{S}-dependent genes (1, 12, 23). The stronger signal present at an A_{600} of 1.0 in the *hns* mutant compared to that of the *hns rpoS* strain may be explained by the accumulation of σ^{S} in mid-exponential phase in this strain (3, 23).

The *hns rpoS* mutant used in this study was provided by T. Mizuno (Nagoya University), and we have confirmed that it is defective at the *rpoS* locus as acid resistance can be restored by complementation with the wild-type *rpoS* gene (data not shown). The detection of *gad* transcripts in this strain is compatible with the observation of a previous study which showed the expression of transcripts and proteins from other σ^{S}-dependent genes (*csgBA* and *hdeAB*) in similar *E. coli* K-12 *hns rpoS* mutant strains (1).

Possible explanations for the differences between the observations of De Biase et al. (6) and those reported here are the different growth conditions and the strains used in the two studies. De Biase et al. (6) examined a different *E. coli* K-12 *hns rpoS* mutant, YK4122, and grew it to early exponential phase for 2 h ($A_{600} = \sim0.4$) and to stationary phase for 5 h ($A_{600} = \sim2.1$). For stationary-phase induction we grew our cultures overnight for 16 h ($A_{600} = 1.8$). It is possible that their incubation times were too short to detect significant levels of *gad* transcription in this strain. Under these same growth conditions they were only able to detect extremely weak signals of *gad* transcription in the MC4100 K-12 strain.

Expression of acid resistance in an *hns* mutant of *E. coli*. To determine whether the increased transcriptional expression of the *gad* genes in *hns* and *hns rpoS* mutants correlated with relief of growth phase dependence of acid resistance, the expression of acid resistance was assayed along the growth curve for the isogenic *hns*, *rpoS*, and *hns rpoS* mutants and the wild-type parent of *E. coli* K-12 grown at 37°C in LB medium (Fig. 2). LB medium for extreme acid exposure (pH 2.5) was adjusted for pH by using HCl. Cultures were adjusted to the appropriate concentrations (1×10^5 to 5×10^5 CFU/ml) by serial dilutions in phosphate-buffered saline, and an aliquot was added to the acidified LB medium and was incubated for 2 h at 37°C with aeration. Control dilutions from the original culture were plated on LB agar and were grown overnight at 37°C. Colony counts were then compared to those obtained

from the same culture exposed to the acidified medium to determine the percentage of survival. Acid resistance was defined as the percentage of the number of bacteria surviving the acid treatment compared to the initial number of inoculum exposed. Values shown for percentage of survival represent the mean of a single experiment performed in triplicate.

The *hns* mutant exhibited induction of acid resistance in mid-exponential phase (A_{600} = 1.2) some 3 h earlier than the parent strain. This acid resistance was not constitutive and implies that σ^S is still required for the induction of expression. This finding is supported by the observation that CRP-dependent repression of *gadA* and *gadBC* is not relieved in an *hns* mutant (11), indicating that CRP is a master regulator of glutamate-dependent acid resistance. The *hns rpoS* double mutant did not exhibit the acid resistance phenotype, reflecting previous observations by De Biase et al. (6). This was despite the fact that we have shown that this strain produces transcripts of *gadA* and *gadBC* as well as other acid resistance genes (*hdeAB*) (20), which are also expressed much earlier in the growth phase by this strain (24). The *hns rpoS* mutant still expressed GAD enzyme activity when cells were permeabilized with Triton X-100 (data not shown).

A repressing action of H-NS on *gadA* and *gadBC* promoters, preventing a successful transcription-inducing complex with σ^{70} during the exponential growth, can be predicted. In stationary phase the σ^S levels increase, and this can overcome H-NS repression, probably by more efficient recognition of curved promoter regions required for H-NS binding. The region upstream of many σ^S-dependent promoters is often AT rich and shows intrinsic DNA curvature (8). Thus, σ^S may relieve H-NS repression at these promoters. A -10 consensus sequence (CTATACT) was determined for σ^S-dependent promoters based on the comparison of characteristic promoters known to be under the control of σ^S and those that can be recognized in vitro by both σ^S and σ^{70} (7). Sequences similar to this consensus were identified by primer extension mapping of the transcriptional start points of *gadA* and *gadBC* (4, 6).

σ^S appears to be the major sigma factor used for directing *gad* transcription for cells grown in complex, but not minimal, medium. Minimal-medium cultures, however, must be able to utilize a different sigma factor under acidic conditions (4). Since only one promoter for each gene appears to be involved regardless of the inducing conditions, the other sigma factor is suggested to be σ^{70} (4). In accordance with this, a *crp gadX gadW* mutant which produced no GAD when grown to exponential phase in LB medium made copious amounts of GAD once an *hns* mutation was introduced (11).

Together these results suggest that additional genes that are likely to be dependent upon σ^S for their expression contribute to glutamate-dependent acid resistance, a conclusion endorsed in previous reports (5, 6, 18). Some of these reports have also indicated that the *gad* decarboxylase/antiporter system would create a futile proton cycle, since transport of glutamic acid brings into the cell a proton that is consumed by its decarboxylase (5, 18). To compensate, a mechanism for generating endogenous glutamate would be required. Glutaminases have been previously hypothesized to play a role in glutamate-dependent acid resistance (5). Identification of additional genes and further investigation of the interactions between regulatory components of the *gad* system will be important for un-

derstanding the complex mechanism of glutamate-dependent acid resistance.

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