Research Article

The *Escherichia coli* GcvB sRNA Uses Genetic Redundancy to Control cycA Expression

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The *Escherichia coli* sRNA GcvB regulates several genes involved in transport of amino acids and peptides (sstT, oppA, dppA, and cycA). Two regions of GcvB from nt +124 to +161 and from nt +73 to +82 are complementary with essentially the same region of the cycA mRNA. Transcriptional fusions of cycA to lacZ showed the region of cycA mRNA that can pair with either region of GcvB is necessary for regulation by GcvB. However, mutations in either region of gcvB predicted to disrupt pairing between cycA mRNA and GcvB did not alter expression of a cycA-lacZ translational fusion. A genetic analysis identified nts in GcvB necessary for regulation of the cycA-lacZ fusion. The results show that either region of GcvB complementary to cycA mRNA can basepair with and independently repress cycA-lacZ and both regions need to be changed to cause a significant loss of repression.

1. Introduction

The *E. coli* gcvB gene encodes a sRNA of 206 nts [1]. Transcription of gcvB is activated by GcvA when cellular glycine is high and repressed by GcvA when glycine is limiting; repression by GcvA requires the accessory GcvR protein [1]. GcvB regulates cycA, encoding the glycine transport protein [2]. Thus, GcvB regulates its own synthesis by controlling the level of glycine transported into the cell. A ΔgcvB strain shows constitutive synthesis of OppA and DppA, the periplasmic binding protein components of the two major peptide transport systems, SstT, a serine transport system, and CycA, a glycine transport system [1–4]. The *Salmonella enterica* serovar Typhimurium GcvB also regulates OppA and DppA levels and several other genes involved in transport of polar and branched amino acids and general amino acid metabolism [5, 6].

Evidence suggests GcvB regulates its target mRNAs by an antisense mechanism, basepairing with the mRNAs to prevent translation initiation [3–6]. Although it is unclear how extensive pairing between a sRNA and a mRNA must be, research indicates one or two regions of 8-9 basepairs is sufficient for regulation [7]. In cases where basepairing interactions occur, the RNA chaperone Hfq is required, likely to alter RNA secondary structures or to bring together sRNAs and target mRNAs, increasing local RNA concentrations [8–11]. Hfq binds GcvB [11, 12], stabilizing the RNA [5, 13], and loss of Hfq results in the loss of repression of GcvB target mRNAs [2, 4, 5, 13]. For sRNAs studied in detail that regulate by an antisense mechanism, often a single basepair change in the sRNA or its target mRNA results in a loss of regulation by the sRNA (e.g., the sRNA SgrS and its target ptsG mRNA [14]). For GcvB, however, it is surprising that most changes predicted to disrupt pairing with regions of the target mRNAs have little or no effect on GcvB's ability to regulate [2–4].

GcvB homologs contain two conserved sequences of 13 nts (Con-I) and 10 nts (Con-II) (Figure 1(a)) [1, 3, 5]. In addition, a G/T-rich domain that includes the Con-I sequence was shown to be essential for interaction with most GcvB target mRNAs in *E. coli* and *S. enterica* [4, 5, 13]. In *S. enterica*, the Con-II region also pairs with cycA mRNA, possibly inhibiting translation initiation [6]. Analysis of *E. coli* GcvB identified two regions from nt +73 to +82 and from nt +124 to +161 complementary to cycA mRNA (Figures 1(b) and 1(c)). The region from +73 to +82 overlaps Con-I and...
the G/T-rich domain, and the region from +124 to +161 overlaps Con-II (Figure 1(a)). In addition, transcriptional fusions of cycA to lacZ verified the region from −8 to −26 upstream of the AUG start codon, and complementarily with both the +73 to +82 and +124 to +161 regions of GcvB is required for regulation of cycA (Figures 1(b) and 1(c)) [2]. However, changes in either region of GcvB independently did not alter regulation of cycA-lacZ [2]. We devised a genetic selection to identify any nts in GcvB required to regulate cycA-lacZ. In this study, we show the region of GcvB from +73 to +82 as well from nt +124 to +161 is important for regulation of a cycA-lacZ fusion. In addition, both regions can independently repress, suggesting GcvB regulates cycA-lacZ by a mechanism that uses redundancy within GcvB.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Phage. The *E. coli* strains, plasmids, and phage used are listed in Table 1 or are described in the text.

2.2. Media. The complex medium used was Luria-Bertani broth (LB) [19]. Agar was added at 1.5% (w/v) to make solid medium. The defined medium used was the salts of Vogel and Bonner [20] supplemented with 0.4% (w/v) glucose (GM). Ampicillin (Amp) was added at 50 μg mL⁻¹. X-gal was added at 40 μg mL⁻¹.

2.3. DNA Manipulation. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen, Santa Clara, CA). Vent DNA polymerase, Taq DNA polymerase, and restriction enzymes were from New England Biolabs, Inc. (Beverly, MA). T4 DNA ligase was from Roche Diagnostics (Indianapolis, IN). Reactions were as described by the manufacturers.

2.4. Enzyme Assay. β-Galactosidase assays were performed on mid-log phase cells (OD₆₀₀ ~ 0.5) using the chloroform/SDS lysis procedure [19]. Results are the averages of two or more assays with each sample done in triplicate. Results were analyzed using the Student’s *t*-test.

2.5. Random and Site-Directed Mutagenesis of gcvB. Plasmid pGS634 carries the gcvB*142CA+159CC allele on an EcoRI-HindIII fragment [2]. Using pGS634 as template, error-prone PCR was used [21] to amplify DNA containing gcvB. The upstream primer (GcvB-For) was 5′-CTAGGGCGAAAATTCGGGTTGGAATCATGATGGC with an EcoRI site (underlined) and that hybridizes 50 bps upstream of the gcvB transcription start site. The downstream primer (GcvB-Rev) was 5′-GGGGAAGGCTTAAAAAAGGTAGCGAATTTAGGCTACATTTGGTCAAT- the GcvB strain GS1144 lysogenized with λcycA-lacZ. After 1 round of Amp counterselection [19], cells were plated on LB plates + Amp + X-gal. Killing nontransformed lysogens made identification of darker blue colonies efficient. Plasmid DNA was prepared from potential mutants (dark blue transformants) and the DNA sequenced at the Core Facility at the University of Iowa to verify mutations.

Site-directed mutagenesis of gcvB was performed using the PCR “megaprimer” procedure [22] with pGS594 (pGcvB*) as template. Changes were verified by DNA sequence analysis and are predicted by the mfold program [23, 24] to leave the GcvB secondary structure intact.

2.6. Construction of the gcvBΔ74:82 Allele and gcvBΔ74:82 Allele + Additional Mutations. The gcvBΔ74 allele with bp changes that make a strong transcription terminator at t1 and removes sequence distal to t1 was constructed using pGcvB* as template and upstream primer GcvB-For and downstream primer GcvB-t1-5′-GGGGAGGCTTAAAAAAGGTAGCGAATTTAGGCTACATTTGGTCAAT- with a HindIII site (underlined) and that hybridizes beginning at bp +135 in gcvB. The amplified DNA was digested with EcoRI + HindIII, cloned into EcoRI-HindIII digested and gel-purified vector pGS341, replacing the WT gcvA gene. Base changes were verified by DNA sequence analysis and the plasmid-designated pGS642 (pGcvBΔ74) (Figure 2(a)). Mutations in gcvB were then combined with the gcvBΔ74 allele by PCR. Plasmids pGS596 (pGcvBΔ71GGC), pGS602 (pGcvBΔ74+82), pGS629 (pGcvBΔ79GGC), pGS644 (pGcvBΔ142CA+159CC+79GGC), and pGS645 (pGcvBΔ142CA+159CC+80A) were used as templates with upstream primer GcvB-For and downstream primer GcvB-t1+. The amplified DNA fragments were cloned as described for the pGcvBΔ74 allele. Changes were verified by DNA sequence analysis. The plasmids were designated pGS647 (pGcvBΔ71+71GGC), pGS649 (pGcvBΔ74+76AA), pGS653 (pGcvBΔ79GGC+79GGC), pGS654 (pGcvBΔ142CA+159CC+80A), and pGS656 (pGcvBΔ118+80A), respectively (Figure 1).

2.7. Construction of the gcvBΔ74:82 Allele + Additional Mutations. The gcvBΔ74:82 allele with a deletion from bp +74 to +82 was constructed using the PCR “megaprimer” procedure [22]. The new plasmid was designated pGS680 (pGcvBΔ74+82) (Figures 1(a) and 1(c)). Base changes were verified by DNA sequence analysis. Mutations in gcvB in the +124 to +161 region were then combined with the gcvBΔ74:82 allele by the PCR “megaprimer” procedure [22]. The new plasmids were designated pGS682 (pGcvBΔ74:82+142CA), pGS683 (pGcvBΔ74:82+159CC), pGS684 (pGcvBΔ74:82+131CC), pGS697 (pGcvBΔ74:82+142CA+159CC), pGS698 (pGcvBΔ74:82+131CC+142CA), and pGS699 (pGcvBΔ74:82+131CC+159CC) (Figure 1).

2.8. Construction of λcycA-Δ24GG-lacZ, λcycA-Δ29G-lacZ, and λcycA-Δ30T-lacZ Mutations. Plasmid pycA-lacZ carries an *E. coli* cycA-lacZ translational fusion [2]. Using pycA-lacZ as template, PCR “megaprimer” mutagenesis [22] was used to create changes in cycA-lacZ (Figures 1(b) and 1(c)). Base
Figure 1: (a) The gcvA/gcvB promoter region and gcvB gene. Promoter −35 and −10 elements are underlined for gcvA and overlined for gcvB [1, 15]. The GcvA-binding site is indicated by a line [16]. Inverted arrows show stem-loop sequences of putative transcription terminator t1 and terminator t2. A 13 base and a 10 base conserved sequence in gcvB homologs are designated Con-I and Con-II (green bars) [1, 3, 5]. Con-I is part of a larger G/T-rich domain (dashed line) essential for interaction of GcvB with most characterized target mRNAs [4, 5, 13]. Bases in GcvB complementary with cycA mRNA in the +73 to +82 region are in red and in the +124 to +161 region in blue. Changes in gcvB shown not to alter cycA-lacZ expression are below the sequence and are color coded [2]. Two independent changes isolated using ppgvBΔ+142CA+159CC as template and that result in loss of GcvB repression of cycA-lacZ are boxed. (b) Comparison of GcvB from nt +124 to +166 with cycA mRNA. (c) Comparison of GcvB from nt +60 to +96 with cycA mRNA. For (b) and (c), complementarity is indicated with lines and GU bps with dots. Changes in gcvB are shown below the sequences, and changes in cycA are shown above the sequences and are color coded (see text for details).

Figure 2: (a) WT terminator t1 and changes in t1 predicted to increase (↑) transcription termination. Primers used to construct the t1↑ allele delete the sequence distal to the t1↑ changes. (b) Northern analysis of GcvB. RNA was isolated from WT, ΔgcvB, or ΔgcvB transformed with a single-copy plasmid carrying the gcvBΔ↑ allele and probed with either a DIG-labeled GcvB or 5S rRNA-specific DNA probe. Numbers above each lane indicate levels of GcvB relative to WT.
Table 1: Strains, plasmids, and phage.

| Strains*, plasmids, and phage | Relevant genotype | Source or reference |
|-------------------------------|-------------------|---------------------|
| **Strains**                   |                   |                     |
| GS162            | WT               | This lab            |
| GS1144           | ΔgcvB            | [3]                 |
| GS1148           | Δhfq             | [13]                |
| **Plasmids**      |                   |                     |
| pGS341           | Single-copy vector + WT gcvA | [17] |
| pGS594           | Single-copy vector + WT gcvB | This lab|
| pGS596           | pGS594 with a -TGT- to -CCC- change of bps +71 to +73 in gcvB (pgcvB*71CCC) ** | [3] |
| pGS602           | pGS594 with a -TGT- to -AAA- change of bps +76 to +78 in gcvB (pgcvB*76AAA) | [3] |
| pGS629           | pGS594 with a -TGGTT- to -CCCCA- change of bps +79 to +82 and a -TG- to -CA- change of bps +142 and +143 in gcvB (pgcvB*79CCCA) | [4] |
| pGS634           | and a -TG- to -CC- change of bps +159 and +160 in gcvB (pgcvB*142CA*159CC) | [2] |
| pGS634           | Single-copy vector + gcvB<sup>111</sup> allele (see Figure 2 for bp changes) (pgcvB<sup>111</sup>) | This study |
| pGS642           | pGS634 with a -T- to -C- change of bp +79 in gcvB (pgcvB<sup>142CA*159CC*79C</sup>) | This study |
| pGS644           | pGS634 with a -G- to -A- change of bp +80 in gcvB (pgcvB<sup>142CA*159CC*80A</sup>) | This study |
| pGS645           | pGS642 with -TGT- to -CCC- change of bps +71 to +73 in gcvB (pgcvB<sup>71CCC</sup>) | This study |
| pGS647           | pGS642 with -TGT- to -AAA- change of bps +76 to +78 in gcvB (pgcvB<sup>76AAA</sup>) | This study |
| pGS649           | pGS642 with -TGGTT- to -CCCCA- change of bps +79 to +82 in gcvB (pgcvB<sup>79CCCA</sup>) | This study |
| pGS653           | pGS642 with -T- to -C- change of bp +79 in gcvB (pgcvB<sup>111*79C</sup>) | This study |
| pGS655           | pGS642 with -G- to -A- change of bp +80 in gcvB (pgcvB<sup>111*80A</sup>) | This study |
| pGS656           | pGS594 with a deletion from bp +74 to +82 in gcvB (pgcvB<sup>574*82</sup>) | This study |
| pGS680           | pGS680 with -T- to -CA- change of bps +142 and +143 in gcvB (pgcvB<sup>574*82+142CA</sup>) | This study |
| pGS682           | pGS680 with -T- to -CC- change of bps +159 and +160 in gcvB (pgcvB<sup>574*82+159CC</sup>) | This study |
| pGS683           | pGS680 with -TT- to -CC- change of bps +131 and +132 in gcvB (pgcvB<sup>574*82+131CC</sup>) | This study |
| pGS684           | pGS680 with the gcvB<sup>111</sup> change (pgcvB<sup>111*82</sup>) | This study |
| pGS688           | pGS680 with -T- to -CA- change of bps +142 and +143 and a -TG- to -CC- change of bps +159 and +160 in gcvB (pgcvB<sup>574*82+142CA*159CC</sup>) | This study |
| pGS697           | pGS680 with -TT- to -CC- change of bps +131 and +132 and a -TG- to -CC- change of bps +159 and +160 in gcvB (pgcvB<sup>574*82+131CC*159CC</sup>) | This study |
| **Phage**         |                   |                     |
| λgt2             | λ cloning vector; cI857 repressor | [18] |
| λ<sup>cycA</sup>-lacZ | λ vector carrying WT cycA-lacZ translational fusion | [2] |
| λ<sup>cycA<sup>-24GG</sup></sup>-lacZ | λ vector carrying a cycA<sup>-24GG-lacZ</sup> translational fusion with an -AC- to -GG- change at nts −24 and −25 | This study |
2.9. RNA Isolation and Northern Analysis. E. coli strains (Clara, CA) and quantified using a NanoDrop ND-1000 was isolated using an RNeasy Mini Kit (Qiagen, Santa changes were verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa. The intermediate plasmids were designated pcycA-24GG-lacZ, pcycA-29G-lacZ, and pcycA-30T-lacZ. A 5,788 bp EcoRI-MfeI fragment from each plasmid carrying the mutant cycA-lacZ fusions and lacYA genes was then ligated into the EcoRI site of phase lambda [18]. The new phage was designated λycycA-24GG-lacZ, λycycA-29G-lacZ, and λycycA-30T-lacZ. The phage were used to lysogenize appropriate E. coli host strains as described previously [25]. Each lysogen was tested to ensure that it carried a single copy of the λ chromosome by infection with λcl90c17 [26]. All lysogens were grown at 30°C since all fusion phages carry the λI857 mutation, resulting in a temperature sensitive λCl repressor [18].  

2.9. RNA Isolation and Northern Analysis. E. coli strains were grown in 5 mL of LB to mid-log phase. Total RNA was isolated using an RNeasy Mini Kit (Qiagen, Santa Clara, CA) and quantified using a NanoDrop ND-1000 Spectrophotometer. Northern analysis and quantification of RNA were performed as described [13].

3. Results

3.1. Nucleotides in GcvB Important for cycA-lacZ Repression. It was suggested that in S. enterica several regions in GcvB can independently block translation initiation of cycA mRNA [6]. To identify any sequence in E. coli GcvB required to regulate cycA-lacZ, we devised a genetic selection. Since two regions of GcvB from nt +73 to +82 and from +124 to +161 are complementary to cycA mRNA (Figures 1(b) and 1(c)), we biased the selection by disrupting the primary pairing interactions between GcvB and cycA mRNA. If both regions are able to pair with cycA mRNA, disrupting the primary region of interaction would increase the chances of identifying additional nts important for repression. Starting with pGS634 (pgcvB-142CA+159CC) as template error-prone PCR was used to mutagenize gcVb [21]. Transformation of a ΔgcVb strain with the mutagenized DNA allowed us to identify two mutants with increased cycA-lacZ expression (darker blue colonies on X-gal plates). Plasmid DNA prepared from the mutants was sequenced, and two changes in gcVb were identified, a -T- to -C- change at nt +79 and a -G- to -A- change at nt +80 (Figure 1(a), boxed nts). The new plasmids were designated pGS644 (pgcvB-142CA+159CC+79C) and pGS645 (pgcvB-142CA+159CC+80A).

To determine the effects of the mutations on cycA-lacZ expression, the ΔgcvBλycycA-lacZ lysogen was transformed with the new plasmids and control plasmid pgcvB+ and assayed for β-galactosidase. β-galactosidase levels were 2-fold higher in the ΔgcVb lysogen compared to WT and repression was restored in the ΔgcVb[pgcVb+] transformant (Figure 3(a), lanes 1, 2, and 3). In addition, as reported [2], the pgcvB-142CA+159CC allele repressed cycA-lacZ as well as WT gcVb+ (Figure 3(a), lane 4). In the presence of the pgcvB-142CA+159CC-79C and pgcvB-142CA+159CC+80A alleles, β-galactosidase levels were about 2-fold higher than in the control strains (Figure 3(a), compare lanes 3 and 4 with lanes 5 and 6). Of interest, changes at +79 and +80 (although different nts than the -79C and +80A changes) had no effect on cycA-lacZ expression in the absence of the gcVb-142CA+159CC mutation [2], suggesting both regions must be altered to see a loss of GcvB repression.

To determine if each gcVb allele produced comparable levels of GcvB, a Northern analysis was performed. The results showed about the same levels of GcvB for each RNA sample tested except the gcVb-142CA+159CC reli allele, which had about 60% of the WT level (Figure 3(b)). However, the gcVb-142CA+159CC allele showed normal repression of cycA-lacZ (Figure 3(a), lane 4). Thus, loss of repression for the gcVb-142CA+159CC+79C and gcVb-142CA+159CC+80A alleles is not due to reduced levels of the mutant RNAs.

3.2. Sequence Preceding Terminator t1 Is Able to Repress cycA-lacZ. One possibility that could explain the above results is either region of GcvB complementary to the cycA mRNA is sufficient to cause repression and both regions must be changed to see an effect. Two experiments provide results that support this hypothesis. Two Rho-independent terminator sequences can be found in gcVb centered at bp +121 and +189/190, designated t1 and t2, respectively (Figure 1(a)) [1]. Although in vivo and in vitro evidence suggests some termination occurs at t1 [1], no short transcript was detected in either E. coli or S. enterica by Northern analysis [5, 13]. We constructed a gcVb allele where t1 is a better Rho-independent terminator (gcVb t1) (Figure 2(a)). If either region of GcvB complementary to cycA mRNA can pair with the mRNA to cause repression, elimination of sequence distal to t1 should still result in repression of cycA-lacZ. To ensure any regulation observed is not due to read-through of the gcVb t1 allele, all sequence following t1 was deleted (see Materials and Methods). A

Table 1: Continued.

| Strains*, plasmids, and phage | Relevant genotype | Source or reference |
|-------------------------------|------------------|---------------------|
| λycycA-24GG-lacZ             | λ vector carrying a cycA-24GG-lacZ translational fusion with an -A- to -G- change at nt -29 | This study |
| λycycA-29G-lacZ              | λ vector carrying a cycA-29G-lacZ translational fusion with a +142CA+159CC+79C allele showed normal repression of cycA-lacZ as well as WT gcVb+. | This study |
| λycycA-30T-lacZ              | λ vector carrying a cycA-30T-lacZ translational fusion with a +142CA+159CC+80A allele, β-galactosidase levels are about 2-fold higher than in the control strains (Figure 3(a), compare lanes 3 and 4 with lanes 5 and 6). Of interest, changes at +79 and +80 (although different nts than the -79C and +80A changes) had no effect on cycA-lacZ expression in the absence of the gcVb-142CA+159CC mutation [2], suggesting both regions must be altered to see a loss of GcvB repression. | This study |

* All numbering also carry the pheA905 thi araD129 rpsL150 relA1 deoC1 fliB351 ptsF25 rbsR mutations.

**Numbering for gcVb mutations is based on the transcription initiation site as +1. Numbering for the cycA fusions and mutations is based on the A residue in the AUG translation initiation codon as +1 with bases upstream assigned negative values.

### Strain Table 1

| Phage Relevant genotype Source or reference |
|---------------------------------------------|------------------|
| λcycA-29G-lacZ                            | λ vector carrying a cycA-29G-lacZ translational fusion with an -A- to -G- change at nt -29 | This study |
| λcycA-30T-lacZ                            | λ vector carrying a cycA-30T-lacZ translational fusion with a +142CA+159CC+79C allele showed normal repression of cycA-lacZ as well as WT gcVb+. | This study |
| λcycA-30T-lacZ                            | λ vector carrying a cycA-30T-lacZ translational fusion with a +142CA+159CC+80A allele, β-galactosidase levels are about 2-fold higher than in the control strains (Figure 3(a), compare lanes 3 and 4 with lanes 5 and 6). Of interest, changes at +79 and +80 (although different nts than the -79C and +80A changes) had no effect on cycA-lacZ expression in the absence of the gcVb-142CA+159CC mutation [2], suggesting both regions must be altered to see a loss of GcvB repression. | This study |
alleles were grown in LB (+Amp for transformants) to mid-log phase and assayed for β-galactosidase. Numbers above each lane indicate levels of GcvB relative to WT. β-galactosidase was isolated from WT, ΔgcvB, or ΔgcvB transformed with the indicated gcvB alleles and probed with either a DIG-labeled GcvB or 5S rRNA specific DNA probe. Northern Blot showed the gcvB11 allele produced only a short RNA of ~134 nts and at levels about 80% of the WT level (Figure 2(b)). Thus, any change in regulation of cycA-lacZ is likely due to the short RNA rather than a change in the synthesis or stability of the RNA. β-Galactosidase levels were 2.4-fold higher in the ΔgcvB lysogen compared to WT, and repression was restored in the ΔgcvB[pgcvB+] complemented strain (Figure 4, compare lanes 1, 2, and 3). The gcvB11 allele showed ~1.5-fold better repression of cycA-lacZ than the WT gcvB allele (Figure 4, lanes 3 and 4). Although the change was small, it is statistically significant (P value = 0.02 relative the pgcvB+ transformant). The results suggest the region distal to terminator t1 is not necessary for GcvB repression of cycA-lacZ.

Next, we introduced the +79C and +80A changes, as well as several other changes that do not alter cycA-lacZ expression in the full length GcvB, into the gcvB11 allele. The +79C (brown), +80A (purple), +76AAA (green), and +79CCCA (blue) changes reduce complementarity of GcvB with cycA mRNA (Figure 1(c)) and resulted in reduced repression of cycA-lacZ when combined with the gcvB11 allele (Figure 4(a), compare lane 4 with lanes 5–8). The +71CCC change (black) increases complementarity between GcvB and cycA mRNA (Figure 1(c)) and resulted in 1.3-fold increased repression (Figure 4(a), compare lanes 4 and 9). Although the change is small, it is statistically significant (P value = 0.005 relative to the pgcvB11 transformant). A Northern analysis showed about the same amounts of GcvB for each of the RNA samples tested (Figure 4(b)), suggesting altered regulation is not due to altered levels of the mutant RNAs. The results show the region from +70 to +90 is sufficient for GcvB regulation of cycA-lacZ, but changes in this region only result in altered regulation if the region distal to t1 is changed or deleted.

3.3. The gcvB11 Allele Is Dependent on Hfq. It is possible that the truncated GcvB is able to regulate independently of Hfq. To test this possibility, we transformed the Δhfq strain with phfq3+, pgcvB+, and pgcvB11 alleles and assayed for β-galactosidase activity. As shown previously [2], the ΔhfqλcycA-lacZ lysogen showed high levels of β-galactosidase activity and repression was restored in the Δhfqλ[pghfq3+] complemented strain (Figure 4(a), lanes 10 and 11). Both the ΔhfqlpgcvB+ and Δhfqλ[pgcvB11] transformants showed high levels of β-galactosidase activity, suggesting the truncated GcvB still requires Hfq for repression of cycA-lacZ (Figure 4(a), lanes 12 and 13). The results indicate that the Hfq-binding site for GcvB occurs in the region preceding terminator t1.

3.4. Sequence Distal to Terminator t1 Is Able to Repress cycA-lacZ. To determine if the region distal to terminator t1 is able to repress cycA-lacZ, we constructed the gcvBΔ+74:82 allele. This mutation removes the region of GcvB that precedes terminator t1 (Figure 1(c)) and shown above to play a role in regulation of cycA-lacZ in the presence of the gcvB11 allele (Figure 4(a)). Despite the size of the deletion, the mfold program [23, 24] predicts the remaining secondary structure of GcvB to remain intact. The gcvBΔ+74:82 allele showed
1.8-fold better repression of cycA-lacZ than WT gcvB* (Figure 5(a), compare lanes 1 and 4). Next, we introduced the +131CC, +142CA and +159CC changes that do not alter cycA-lacZ expression in the full length GcvB [2], as well as combinations of these changes, into the gcvBΔ+74:82 allele. The gcvBΔ+74:82+131CC and gcvBΔ+74:82+142CA+159CC mutations resulted in >2-fold higher levels of expression than the gcvBΔ+74:82 mutation (Figure 5(a), compare lane 4 with 5 and 10). The remaining mutations showed smaller but statistically significant increases in expression compared to the gcvBΔ+74:82 allele (Figure 5(a), compare lane 4 with lanes 6–9; P values of 0.036, 0.027, 0.004, and 0.014, resp.). A Northern analysis showed about the same levels of expression for transformants grown in LB (+Amp for transformants) to mid-log phase and assayed for β-galactosidase. Transformants were grown in LB (+Amp for transformants) to mid-log phase and assayed for β-galactosidase.

3.5. Regulation Requires GcvB/CycA mRNA Interactions. To confirm altered regulation is due to altered GcvB/cycA mRNA interactions, we constructed a λcyCΔ−24GG-lacZ fusion (an -AC- to -GG- change at nt−24, −25 relative to the cycA AUG start site); the changes reduce pairing of cycA mRNA with the +73 to +82 region of GcvB (Figures 1(b) and 1(c)). A WTλcyCΔ−24GG-lacZ lysogen had −5.5-fold lower levels of expression than the WTλcyCΔ-lacZ lysogen, suggesting the -GG- change affects translation efficiency (Figure 6, compare lines 1 and 14). The WTλcyCΔ−24GG-lacZ and ΔgcvBλcyCΔ−24GG-lacZ lysogens, as well as pgcvB* and pgcvBΔ+74:82 complemented lysogens, showed essentially the same levels of expression, suggesting a complete loss of GcvB regulation (Figure 6, lines 14, 15, 16, and 18). However, the pgcvBΔ−199CC and pgcvBΔ+74:82+159CC alleles, that restore pairing with the cycAΔ−24GG allele, repressed cyCΔ−24GG-lacZ expression about 1.5-fold (Figure 6, compare line 14 with lines 17 and 19). Although the changes are less than the normal 2-fold repression observed for cycA by GcvB, the results are statistically significant (P values of 0.0001 and 0.0028 relative to the WTλcyCΔ−24GG lysogen, resp.) and suggest pairing of GcvB in the +124 to +161 region with cycA mRNA is required for repression.

The WT λcyCΔ−296-lacZ and λcyCΔ−30T-lacZ lysogens showed levels of expression similar to the WTλcyCΔ-lacZ lysogen, suggesting the changes do not dramatically affect translation efficiency (Figure 6, compare lane 1 with lanes 4 and 9). In addition, β-galactosidase levels were about 2-fold higher in each ΔgcvB lysogen compared to its WT control and repression was restored in the ΔgcvB[pgcvB*] and ΔgcvB[pgcvBΔ11] transformants (Figure 6, compare lanes 4–7 and lanes 9–12). This is not unexpected since the cyCΔ−296 and cycAΔ−30T changes disrupt pairing with GcvB in the +73 to +82 region but do not disrupt pairing in the +124 to +161 region (Figures 1(b) and 1(c)). However, the pgcvBΔ+79C and pgcvBΔ+80A alleles, that restore
pairing with the \( \text{cycA}^{-29G} \) and \( \text{cycA}^{-30T} \) alleles, respectively, increased repression an additional 2-fold (Figure 6, compare lanes 4 and 8 and lanes 9 and 13). The results suggest pairing of GcvB in the +73 to +82 region with \( \text{cycA} \) mRNA is also required for repression. The above results are in agreement with a model of genetic redundancy as a mechanism for \( \text{cycA} \) regulation by \( \text{E. coli} \) GcvB.

4. Discussion

In \( \text{E. coli} \) and \( \text{S. enterica} \), GcvB has been shown to regulate multiple genes involved in amino acid and peptide transport [1–6]. However, most changes in GcvB predicted to disrupt pairing with target mRNAs had no significant effect on GcvB-mediated repression [2–4]. For the \( \text{cycA} \) mRNA, GcvB shows 2 regions of complementarity (Figures 1(b) and 1(c)). In this study, we tested if either region of complementarity is able to independently repress \( \text{cycA-lacZ} \). The \( \text{gc}vB^{\text{H11}} \) allele produces a truncated GcvB of ~134 nts and would remove most of the region from nt +124 to +161 complementary with \( \text{cycA} \) mRNA (Figure 1(a)). The \( \text{gc}vB^{\text{H11}} \) allele showed better repression of \( \text{cycA-lacZ} \) than WT \( \text{gc}vB \) (Figure 4, lanes 3 and 4). The results suggest the region distal to terminator t1 is not necessary for repression of \( \text{cycA-lacZ} \) and possibly prevents full repression by GcvB. Mutations in \( \text{gc}vB \) in the +76 to +82 region that reduce complementarity with \( \text{cycA} \) mRNA (Figure 1(c)) result in a significant loss of repression in the presence of the \( \text{gc}vB^{\text{H11}} \) mutation (Figure 4, compare lane 4 with lanes 5–8), and a change at nts +71 to +73 that increases complementarity results in increased repression (Figure 4, compare lanes 4 and 9). These results suggest the region of complementarity preceding terminator t1 is responsible for repression in the \( \text{gc}vB^{\text{H11}} \) background. Of interest, these mutations do not alter GcvB repression in the full-length molecule [2]. The \( \text{gc}vB^{\Delta74:82} \) allele, which removes the region of GcvB preceding terminator t1 involved in repression in the \( \text{gc}vB^{\text{H11}} \) background (Figure 1(c)), also showed better repression of \( \text{cycA-lacZ} \) than WT \( \text{gc}vB \) (Figure 5(a), compare lanes 3 and 4). Thus, when the region of GcvB distal to terminator t1 is intact, the region of GcvB in the +74 to +82 region is not required for repression and appears to partially inhibit repression. Mutations in \( \text{gc}vB \) in the +131 to +160 region that do not alter GcvB repression in the full-length GcvB [2] result in a significant loss of repression in the presence of the \( \text{gc}vB^{\Delta74:82} \) mutation (Figure 5(a), compare lane 4 with lanes 5–10). These results suggest the region of complementarity following terminator t1 is responsible for repression in the \( \text{gc}vB^{\Delta74:82} \) background. Several of the mutations change the Con-II sequence (Figure 1(b)). However, other changes that result in a loss of repression fall outside of this region. Thus, although the Con-II sequence is likely involved in regulation of \( \text{cycA} \), additional sequence is also required. In many bacteria, multiple largely redundant sRNAs control identical target mRNAs [27, 28]. In addition, a single sRNA can regulate many genes [29, 30]. Although most sRNAs use one region for basepairing, a few use independent regions to basepair with different target mRNAs. For example, two regions of DsrA are necessary for full activity on the \( \text{hns} \)
and rpoS mRNAs [31, 32] and two different regions of FnrS basepair with different sets of target mRNAs [33]. The results in this study show that 2 regions of GcvB complementary with the same region of the cycA mRNA are able to independently basepair with the cycA mRNA and repress expression by an antisense mechanism. In addition, the results open the possibility that GcvB can bind simultaneously and repress two different mRNA molecules.

Of interest, none of the mutations in the presence of the t11 allele or the Δ+t74:82 allele resulted in a complete loss of GcvB repression of the cycA-lacZ fusion (Figures 4 and 5(a)). An examination of each mutant allele identified small regions that could still basepair with the cycA mRNA (not shown). If these small regions are involved in the repression observed, the results would suggest a high degree of flexibility in GcvB basepairing with target mRNAs. S. enterica GcvB also shows several redundant pairing regions with cycA, and in vitro experiments suggest several regions of GcvB independently inhibit translation initiation of cycA mRNA [6]. These results suggest genetic redundancy is a mechanism for regulation by GcvB.

Since many of the genes that respond to GcvB are involved in transport of small peptides and amino acids, we hypothesize this is a survival mechanism to turn down transporters under conditions that favor the presence of toxic molecules that are also transported by these systems [2]. Another class of genes regulated by GcvB is involved in acid resistance (unpublished results) [34], suggesting GcvB plays a role in E. coli survival at low pH. Both of these environmental stresses would be encountered as E. coli moves from an external environment into the GI tract. We hypothesize the functions of the genes regulated by GcvB are crucial to cell survival when cells colonize the GI tract and the redundancy in GcvB prevents accidental loss of regulation of these genes by mutation or possible changes in GcvB structure induced by environmental conditions.

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