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Research Article

Multiple Roles for the sRNA GcvB in the Regulation of Slp Levels in *Escherichia coli*

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The *Escherichia coli gcvB* gene encodes a small RNA that regulates many genes involved in the transport of dipeptides, oligopeptides, and amino acids (*oppA*, *dppA*, *cycA*, and *sstT*). A microarray analysis of RNA isolated from an *E. coli* wild-type and a Δ*gcvB* strain grown to midlog phase in Luria-Bertani broth indicated that genes not involved in transport are also regulated by GcvB. One gene identified was *slp* that encodes an outer membrane lipoprotein of unknown function induced when cells enter stationary phase. The aim of this study was to verify that *slp* is a new target for GcvB-mediated regulation. In this study we used RT-PCR to show that GcvB regulates *slp* mRNA levels. GcvB negatively controls *slp*::*lacZ* in cells grown in Luria-Bertani broth by preventing an Hfq-mediated activation mechanism for *slp*::*lacZ* expression. In contrast, in glucose minimal medium supplemented with glycine, GcvB is required for inhibition of *slp*::*lacZ* expression, and Hfq prevents GcvB-mediated repression. Thus, GcvB regulates *slp* in both LB and in glucose minimal + glycine media and likely by mechanisms different than how it regulates *sstT*, *dppA*, *cycA,* and *oppA*. Repression of *slp* by GcvB results in an increase in resistance to chloramphenicol, and overexpression of *slp* in a Δ*gcvB* strain results in an increase in sensitivity to chloramphenicol.

1. Introduction

The *E. coli* chromosome encodes ~100 small non-translated regulatory RNAs (sRNAs) [1]. A number of these sRNAs have been shown to function as regulators of outer membrane proteins and therefore play important roles in stress responses and virulence gene regulation [2–4]. In addition, most of these sRNAs regulate expression of target genes posttranscriptionally by base pairing with the target mRNAs [5]. The Hfq protein is required for regulation by this class of sRNAs [6]. In most cases, base pairing results in negative regulation of translational activity and altered stability of the target mRNA [5]. However, DsrA and RprA bind to *rpoS* mRNA, likely preventing formation of an inhibitory secondary structure that sequesters the ribosome-binding site, resulting in increased translation [7–9].

The *gcvB* gene encodes a nontranslated RNA of 206 nucleotides (nts) [10]. Transcription of the *gcvB* gene is activated by the GcvA protein when the cellular level of glycine is high and repressed by GcvA when the cellular level of glycine is limiting; repression requires an additional protein GcvR [10]. The production of GcvB is highest during the log phase of growth, and GcvB is not detectable in stationary phase cells [12]. In *E. coli*, GcvB regulates genes involved in the transport of amino acids and small peptides [10, 11, 14, 15]. GcvB also plays a role in acid resistance, although the precise mechanism is unknown [16, 17]. Recently it was shown that GcvB acts as a negative regulator of CsgD, the master regulator of curli and cellulose synthesis [18]. In *Salmonella enterica* serovar Typhimurium, GcvB has been validated to regulate more than 20 targets and possibly acts as a regulatory node in amino acid metabolism [19, 20]. GcvB represses many of its target mRNAs at the posttranscriptional level by an antisense mechanism, and repression requires the Hfq protein [14, 15, 20–22]. The sRNA RyhB regulates as many as 18 operons encoding 56 genes [23], and many other sRNAs are also predicted to regulate more than one target [24]. Thus, it is likely that GcvB regulates other genes in *E. coli*. To identify additional regulatory targets for *E. coli* GcvB, we compared RNA isolated from a wild type (WT) and an otherwise

Table 1: Strains and plasmids.

Strain or plasmid	Relevant genotype	Source or reference
Strain*		
GS162	WT	This laboratory
GS1132	GS162 Δ*gcvA*Δ*gcvB*	[10]
GS1144	GS162 Δ*gcvB*::ΩCM^r	[11]
GS1148	GS162 *hfq-1*::ΩCM^r	[12]
GS1149	GS162 Δ(*gcvAgcvB*)::Ω*aadA hfq-1*::ΩCM^r	[12]
Plasmid		
pGS554	Single-copy vector + WT *gcvB* with a constitutive promoter (p*gcvB*$^{2+}$)	[10]
pGS571	Multicopy vector + WT *gcvB* (p*gcvB*$^{3+}$)	[13]
pGS594	Single-copy vector + WT *gcvB* (p*gcvB*$^{+}$)	This laboratory
pGS609	Multicopy vector + WT *hfq* (p*hfq*$^{3+}$)	[14]
pGS631	pGS594 with a change in *gcvB* at bp +131 and +132 of TT to CC (p*gcvB* + 131CC)	[15]
pGS632	pGS594 with a change in *gcvB* at bp +142 and +143 of TG to CA (p*gcvB* + 142CA)	[15]
pGS633	pGS594 with a change in *gcvB* at bp +159 and +160 of TG to CC (p*gcvB* + 159CC)	[15]
pGS634	pGS594 with a change in *gcvB* at bp +142 and +143 of TG to CA and bp +159 and +160 of TG to CC (p*gcvB* + 142CA + 159CC)	[15]

*All strains also carry the *pheA905 thi araD129 rpsL150 relA1 deoC1 flbB5301 ptsF25 rbsR* mutations.

isogenic Δ*gcvB* strain grown to midlog phase in Luria-Bertani broth (LB) by microarray analysis [15]. One potential target identified was *slp*, encoding a carbon starvation-inducible outer membrane protein [25]. Although the function of Slp is unknown, a null *slp* mutant showed a modest increase in CM resistance and a strain that overproduced Slp was more sensitive to CM [26]. The EvgAS two-component system increases expression of *slp,* and the MarRA system downregulates *slp* expression [27, 28]. Our results confirm GcvB and Hfq also regulate *slp*::*lacZ* expression in both LB and glucose minimal (GM) supplemented with glycine. However, the mechanisms of regulation are different under the two growth conditions tested. GcvB appears to inhibit Hfq activation of *slp*::*lacZ* expression in LB, whereas Hfq appears to prevent GcvB from functioning to negatively regulate *slp*::*lacZ* in GM + glycine. The results confirm that *slp* is a new target for GcvB regulation and show that GcvB has multiple mechanisms for controlling gene expression.

2. Materials and Methods

2.1. Bacterial Strains, Plasmids, and Phage. The *E. coli* strains and plasmids used in this study are listed in Table 1 or are described in the text. The *λslp*::*lacZ* translational fusion was constructed by PCR as follows. A DNA fragment was synthesized using primer Slp-EcoRI 5′-GGGGTGGAAATA-GAAATACCATC that hybridized to *E. coli* DNA upstream of an *Eco*RI site that begins 288 base pairs (bps) upstream of the *slp* translation start site and primer Slp-SmaI 5′-CCT-AAACCCGGGGAGGATGAGTGCACCTTTTGTC with a *Sma*I site (underlined) and that hybridized to DNA beginning at codon 10 within the *slp* structural gene (Figure 1(a)). The amplified fragment was digested with *Eco*IRI + *Sma*I and the 320 bp *Eco*RI-*Sma*I fragment purified from a 1% agarose gel and ligated into the *Eco*RI-*Sma*I sites of plasmid pMC1403 [29], fusing the first 10 codons of the *slp* structural gene in frame with the 8th codon of the *lacZ* gene in pMC1403. The fusion was verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa. The intermediate plasmid was designated p*slp*::*lacZ*. A 5,771 bp *Eco*RI-*Mfe*I fragment from p*slp*::*lacZ* carrying the *slp*::*lacZ* fusion and *lacYA* genes was then ligated into the *Eco*RI site of phage *λ*gt2 [30]. The phage was used to lysogenize appropriate *E. coli* host strains as described previously [31]. Each lysogen was tested to ensure that it carried a single copy of the *λ* chromosome by infection with *λc*I90*c*17 [32]. All lysogens were grown at 30°C, since all fusion phage carry the *λc*I857 mutation, resulting in a temperature sensitive *λc*I repressor [30].

2.2. Media. The rich medium used was Luria-Bertani broth (LB) [33]. The defined medium used was Vogel and Bonner minimal salts [34] supplemented with 0.4% glucose (GM). GM medium was always supplemented with phenylalanine, glycine, and thiamine, since all strains carry the *pheA905* and *thi* mutations and glycine induces *gcvB* expression [10]. Agar was added at 1.5% for solid media. Supplements were added at the following concentrations (μg mL^{-1}): phenylalanine, 50; glycine, 300; thiamine, 1; chloramphenicol (CM), 3; ampicillin (AP), 50 for single-copy plasmids and 150 for multicopy plasmids.

2.3. Enzyme Assays. β-galactosidase assays were performed on cultures harvested at midlog phase of growth (OD_{600} ~ 0.5) using the chloroform/SDS lysis procedure [33]. Results are the averages of two or more assays with each sample done in triplicate.

2.4. CM Sensitivity Assay. CM sensitivity was tested by the agar disk diffusion method. Cells were grown overnight, and ~5×10^8 cells spread on Mueller-Hinton plates. CM disks

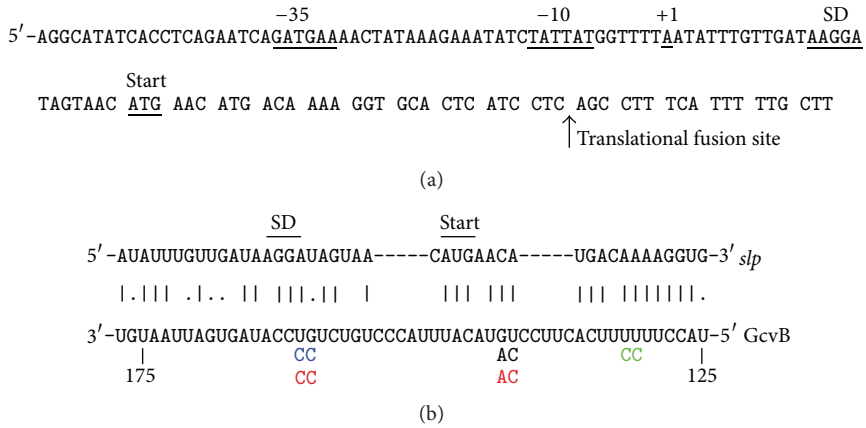

Figure 1: The *slp* control region sequence. (a) *slp*. The promoter −35 and −10 sequences, the +1 transcription initiation site, Shine-Dalgarno (SD) sequence, and translation start codon are underlined [25]. The translation fusion point at codon 10 of *slp* with codon 8 of *lacZ* is indicated with an arrow. The upstream fusion site occurs 288 bps upstream of the transcription start site (not shown). (b) Comparison of GcvB between nts +125 and +177 with *slp* mRNA. The AUG translation initiation site and SD sequence for *slp* are indicated above the sequence. Complementarity between GcvB and *slp* mRNA is indicated with lines between the sequences and GU bps with dots. Separate changes made to *gcvB* [15] are indicated below the sequence and are color coded.

(disk potency, 30 μg CM) were added to each plate and zones of inhibition measured after 18 hours incubation at 30°C. A total of eight measurements were made for each strain tested. Results were analyzed using the Student's *t*-test.

2.5. RNA Isolation and RT-PCR. Strains GS162 (WT), GS1144 (Δ*gcvB*), GS1144 [pGS594] (Δ*gcvB*/p*gcvB*$^+$), and GS1149 (Δ*gcvB*Δ*hfq*) were grown in 5 mL LB to an $OD_{600} \sim 0.5$. RNA was isolated from cell pellets by phenol extraction [35]. RNA samples were DNase I treated for 1 hour at 37°C, isolated by phenol/chloroform extraction, ethanol precipitated, and the RNA pellets resuspended in 30 μL of DEPC treated water. RNA was quantified using a NanoDrop ND-1000 Spectrophotometer. The Access RT-PCR kit (Promega, Madison, WI) was used with 0.5 μg of total cell RNA isolated from each sample in a 25 μL reaction. The *slp* primers were (sense primer) slp-RT 5′-CTCAGCCTTTCATTTTTGCTTGCC and (antisense primer) slp-RT 5′-CTGACTCACCGCATTGGTGTAG. The *ompT* control primers were (sense primer) ompT-RT 5′-GCTTCTACCGAGACTTTATCG and (antisense primer) ompT-RT 5′-GCTGTAGTCTGAAGTGTTATTATTG. The PCR fragment for *slp* was 515 bp and for *ompT* was 830 bp. Cycling conditions were 45°C for 45 minutes, 94°C for 2 minutes, 20 cycles of 94°C for 30 seconds, 49°C for 30 seconds, and 68°C for 2 minutes followed by 1 cycle of 68°C for 7 minutes. PCR products were run on 2% agarose gels and stained with ethidium bromide.

2.6. DNA Manipulation. Plasmid DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen, Santa Clara, CA). Vent DNA polymerase, restriction enzymes, and DNA ligase were from New England Biolabs, Inc. (Beverly, MA), and reactions were as described by the manufacturer.

3. Results

3.1. The slp Gene Is a Target for GcvB and Hfq Regulations. Microarray analysis of RNA isolated from an *E. coli* WT strain and an otherwise isogenic Δ*gcvB* strain grown in LB suggested that Slp, an outer membrane lipoprotein of unknown function [25], is negatively regulated by GcvB [15]. To confirm the microarray analysis that *slp* is regulated by GcvB, we carried out RT-PCR. WT strain GS162, the Δ*gcvB* strain GS1144, and GS1144 transformed with the single-copy plasmid pGS594 carrying WT *gcvB* (p*gcvB*$^+$) were grown in LB at 30°C to midlog phase of growth ($OD_{600} \sim 0.5$) and total cellular RNA isolated. Samples of 0.5 μg of RNA from each culture were used in an RT-PCR assay. We observed an increased level of DNA corresponding to the *slp* mRNA amplified from the Δ*gcvB* sample compared to the level of DNA amplified from either the WT or Δ*gcvB* complemented strain (Figure 2, compare lane 3 with lanes 2 and 4). The results agree with the microarray analysis indicating that GcvB negatively regulates *slp* mRNA levels in cells grown in LB. As a control, we added primers to the RT-PCR reactions to amplify *ompT* mRNA; *ompT* was not identified by microarray analysis to be regulated by GcvB. We observed roughly the same levels of amplified DNA corresponding to the *ompT* mRNA from each strain (Figure 2). These data confirm that GcvB does not regulate *ompT* mRNA levels in LB and that the increased *slp* mRNA levels observed in the Δ*gcvB* strain are due to the absence of GcvB.

3.2. Regulation of slp::lacZ in LB. To confirm that GcvB negatively regulates *slp*, we constructed a *slp*::*lacZ* translational fusion (Figure 1(a)). The fusion was cloned onto phage λgt2, and the fusion phage used to lysogenize the WT strain GS162 and the Δ*gcvB* mutant strain GS1144. The lysogens were grown in LB to midlog phase of growth ($OD_{600} \sim 0.5$), and the cultures assayed for β-galactosidase activity. Expression

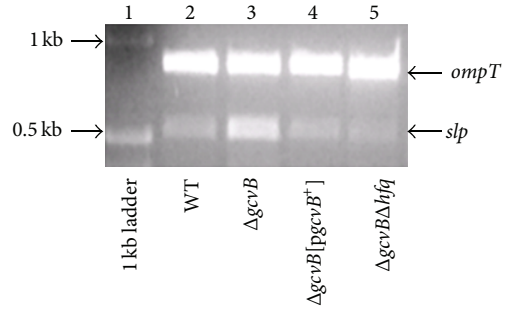

Figure 2: *slp* and *ompT* mRNA levels from WT (GS162), Δ*gcvB* (GS1144), Δ*gcvB*/p*gcvB*$^{+}$ complemented (GS1144 [pGS594]), and GS1149 (Δ*gcvB*Δ*hfq*) strains. The levels of *slp* and *ompT* mRNAs were determined by semiquantitative RT-PCR. Each reaction had 0.5 μg of total cellular RNA added for the RT reaction with 20 PCR cycles. The amplification product for *slp* was predicted to be 515 bps, and the amplification product for *ompT* was predicted to be 830 bps. When reverse transcriptase was omitted from the reactions, there was no product, indicating that no DNA was present in the RNA samples (data not shown).

of the *slp*::*lacZ* fusion in the WT lysogen was about 2-fold lower than in the Δ*gcvB* lysogen (Figure 3(a), lanes 1 and 2). Transformation of the WT lysogen with plasmid pGS594, a single-copy plasmid carrying WT *gcvB*, reproducibly reduced *slp*::*lacZ* expression compared to the nontransformed WT strain (Figure 3(a), compare lanes 1 and 5). Transformation of the Δ*gcvB* lysogen with pGS594, however, only partially restored the level of expression to that of the WT lysogen (Figure 3(a), compare lanes 1, 2 and 6). It is unknown why the single-copy plasmid failed to fully complement the Δ*gcvB* mutation. These results, however, support the microarray assay and RT-PCR data that GcvB has a negative role in *slp* expression.

The Hfq protein is required for regulation by sRNAs that base pair with target mRNAs [6]. Thus, the *λslp*::*lacZ* fusion was also used to lysogenize the Δ*hfq* mutant strain GS1148. The lysogen was grown in LB to midlog phase of growth and assayed for β-galactosidase activity. Expression of the *slp*::*lacZ* fusion in the Δ*hfq* lysogen was reduced compared to the level in the WT lysogen (Figure 3(a), compare lanes 1 and 3). Transformation of the WT lysogen and the Δ*hfq* lysogen with multicopy plasmid p*hfq*$^{3+}$ (pGS609) resulted in about a 2-fold increase in *slp*::*lacZ* expression compared to the WT lysogen (Figure 3(a), compare lane 1 with lanes 7 and 8). These results suggest that Hfq has a positive role in *slp* expression.

Two models could explain the pervious results for a positive role of Hfq in *slp*::*lacZ* expression. GcvB RNA could directly reduce *slp*::*lacZ* expression, and the role of Hfq could be to antagonize this repression. Alternatively, Hfq could act to directly activate *slp*::*lacZ* expression, and GcvB could function in some manner to block Hfq activation. To test which model, if either, is correct, we lysogenized the Δ*gcvB*Δ*hfq* double mutant GS1149 with *λslp*::*lacZ*, grew the lysogen in LB to midlog phase of growth, and assayed for β-galactosidase activity. We hypothesized that if the role of Hfq is merely to prevent GcvB repression, then *slp*::*lacZ* expression should be high in the double mutant without GcvB, whereas if GcvB functions to block Hfq activation, then *slp*::*lacZ* expression should be low in the double mutant without Hfq. Expression of *slp*::*lacZ* was reduced in the double mutant compared to WT (Figure 3(a), compare lanes 1 and 4). The results suggest that Hfq is required to elevate *slp*::*lacZ* expression.

Two additional experiments support a positive role for Hfq in *slp*::*lacZ* expression. If GcvB directly represses *slp*::*lacZ* rather than antagonize Hfq activation, we hypothesized that high levels of GcvB would superrepress *slp*::*lacZ* in the absence of Hfq. We transformed the Δ*hfq* lysogen with plasmids p*gcvB*$^{+}$ (pGS594), p*gcvB*$^{2+}$ (pGS554), and p*gcvB*$^{3+}$ (pGS571). p*gcvB*$^{+}$ is a single-copy plasmid that expresses *gcvB* from the native *gcvB* promoter, p*gcvB*$^{2+}$ is a single-copy plasmid that constitutively expresses *gcvB* [10], and p*gcvB*$^{3+}$ is a multicopy plasmid that overexpresses *gcvB* [13]. Expression of *slp*::*lacZ* in the three transformants was only slightly lower than in the WT lysogen (Figure 3(a), compare lane 1 with lanes 9, 10, and 11). The results suggest that GcvB's role is not to directly repress *slp*::*lacZ*.

We then transformed the Δ*gcvB* and Δ*gcvB*Δ*hfq* lysogens with multi-copy plasmid p*hfq*$^{3+}$ (pGS609) carrying WT *hfq*. If Hfq is a positive regulator of *slp*::*lacZ,* and GcvB functions to block Hfq activation, then *slp*::*lacZ* should be overexpressed in the presence of high levels of Hfq and in the absence of GcvB. Expression of *slp*::*lacZ* was more than 4-fold higher in the transformants than in the WT lysogen (Figure 3(a), compare lane 1 with lanes 12 and 13). The results are in agreement with Hfq positively regulating *slp*::*lacZ,* and the role of GcvB is to prevent activation by Hfq. Whether Hfq directly interacts with *slp* mRNA to increase stability or to increase translation is unknown, Hfq has been shown to be able to act alone as a translational repressor of mRNA [36, 37]. Thus, it is possible that Hfq alone could bind *slp* mRNA to increase expression. We have purified Hfq and are in the process of making *slp* mRNA and GcvB RNA to run *in vitro* gel mobility shift experiments to test whether Hfq binds *slp* mRNA and determine if GcvB competes with this binding. Also, Hfq could interact with an additional sRNA to regulate *slp* mRNA levels. We used the TargetRNA program [38] to search the *E. coli* chromosome for a possible sRNA that could base pair with the *slp* mRNA, but found no significant matches. However, if small or noncontiguous sequences are involved in the sRNA/*slp* mRNA interaction, the identification of this putative sRNA could have been missed in the search.

Many *E. coli* sRNAs that use base pairing to regulate gene expression require Hfq [6]. It was shown previously that GcvB repression of *oppA*, *dppA*, *cycA,* and *sstT* mRNAs is Hfq-dependent [11, 14, 15], and it is known that GcvB interacts with Hfq [39]. The same results were reported for *S. typhimurium* [19]. If the role of GcvB in *slp* regulation is to bind Hfq and prevent Hfq activation of *slp*, we predicted that in a Δ*gcvB*Δ*hfq* mutant, there would not be a significant increase in *slp* mRNA levels. We carried out RT-PCR on total RNA isolated from the Δ*gcvB*Δ*hfq* strain GS1149 grown in LB at 30°C to midlog phase of growth (OD_{600} ~ 0.5). As expected, we did not observe an increased level of DNA corresponding

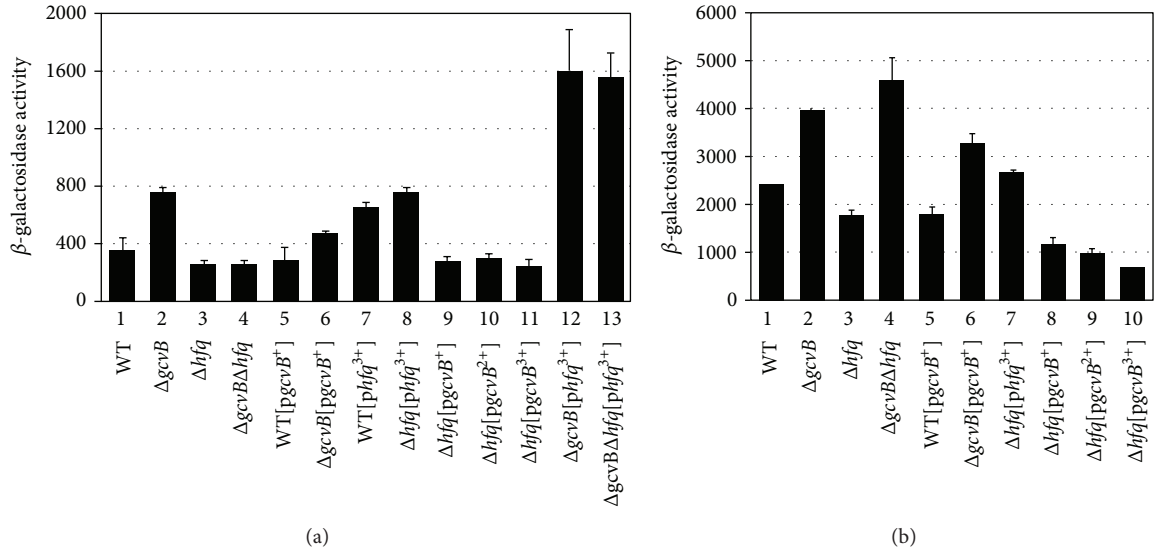

Figure 3: β-Galactosidase levels of λslp::*lacZ* lysogens. (a) WT (GS162), Δ*gcvB* (GS1144), Δ*hfq* (GS1148), and Δ*gcvB*Δ*hfq* (GS1149) λslp::*lacZ* lysogens and the same lysogens transformed with the indicated plasmids were grown in LB (+ AP for complemented strains) to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. (b) WT (GS162), Δ*gcvB* (GS1144), Δ*hfq* (GS1148), and Δ*gcv*Δ*hfq* (GS1149) λslp::*lacZ* lysogens or the same lysogens transformed with the indicated plasmids were grown in GM + glycine (+ AP for complemented strains) to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. Results are averages of two independent assays with each assay performed in triplicate.

to *slp* mRNA amplified from the Δ*gcvB*Δ*hfq* sample and possibly a decreased level, compared to the level of DNA amplified from the WT strain (Figure 2, compare lanes 2 and 5). We will use qRT-PCR to test if there is less *slp* mRNA in the Δ*gcvB*Δ*hfq* strain compared to the WT. The results, however, are in agreement with the β-galactosidase assays (Figure 3(a)) suggesting that Hfq is required for increased *slp* expression in LB.

3.3. Regulation of slp::lacZ in GM + Glycine Medium. Significant repression of *dppA*::*lacZ*, *oppA*::*phoA*, and *cycA*::*lacZ* fusions by GcvB was only observed in WT lysogens grown in LB, with little or no repression seen when the lysogens were grown in GM + glycine [10, 15]. Repression of an *sstT*::*lacZ* fusion by GcvB in a WT lysogen grown in LB was ~12-fold, and repression of the WT lysogen in GM + glycine was ~2-fold [14]. Because the *gcvB* gene is regulated over a 25-fold range in GM media [10], we tested if GcvB and Hfq are able to regulate *slp*::*lacZ* in GM medium. The lysogens used previously were grown in GM + glycine to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. Expression of *slp*::*lacZ* in WT was ~7-fold higher in GM + glycine compared to LB grown cells (Figures 3(a) and 3(b)). In addition, β-galactosidase levels in the WT lysogen were about 2-fold lower compared to the Δ*gcvB* lysogen (Figure 3(b), lanes 1 and 2). Transformation of the WT lysogen with the single-copy plasmid p$gcvB^+$ consistently reduced *slp*::*lacZ* expression compared to the nontransformed WT lysogen (Figure 3(b), compare lanes 1 and 5). Transformation of the Δ*gcvB* lysogen with p$gcvB^+$ reduced *slp*::*lacZ* expression, although full complementation was not observed (Figure 3(b), compare lanes 2 and 6). The results suggest that GcvB has a negative role in *slp*::*lacZ* expression in cells grown in GM + glycine as well as in LB.

Expression of *slp*::*lacZ* in the Δ*hfq* lysogen was consistently lower than in the WT lysogen (Figure 3(b), lanes 1 and 3). Transformation of the Δ*hfq* lysogen with multicopy phfq^{3+} restored *slp*::*lacZ* expression to slightly higher levels than in the WT lysogen (Figure 3(b), compare lanes 1 and 7). The results suggest Hfq is required for normal *slp*::*lacZ* expression in GM + glycine. However, expression of *slp*::*lacZ* in the Δ*gcvB*Δ*hfq* double mutant was 2-fold higher than in the WT lysogen (Figure 3(b), compare lanes 1 and 4). This is opposite of the result observed in LB (Figure 3(a), lanes 1 and 4) and suggests that GcvB and Hfq have different roles in GM + glycine compared to LB.

One model to explain the previous results is that GcvB negatively regulates *slp*::*lacZ* in GM + glycine, and the role of Hfq is to block GcvB repression rather than to activate *slp*::*lacZ* expression as in LB. To test this hypothesis, the Δ*hfq* lysogen was transformed with plasmids p$gcvB^+$ (pGS594), p$gcvB^{2+}$ (pGS554), and p$gcvB^{3+}$ (pGS571), the transformants grown in GM + glycine to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. We hypothesized that high levels of GcvB would superrepress *slp*::*lacZ* in the absence of Hfq. Expression of *slp*::*lacZ* in the transformants was reduced significantly compared to the WT lysogen (Figure 3(b), compare lane 1 with lanes 8–10). The results suggest that GcvB does negatively regulate *slp*::*lacZ* levels in GM + glycine, and the role of Hfq is likely to prevent GcvB repression.

3.4. The slp Leader Sequence. Most sRNAs affect translation of target mRNA molecules, although the precise

Table 2: GcvB alters sensitivity to CM.*

Relevant genotype	Disk potency (30 μg CM) Zone diameter (mm)	P value compared to the WT strain	GT (min) grown in:	
			LB	LB + CM
WT	22.19 ± 0.92		57 ± 3	198 ± 23
Δ*gcvA*Δ*gcvB*	23.31 ± 0.88	0.0107	58 ± 2	237 ± 30
WT[p*gcvB*$^{3+}$]	21.06 ± 0.56	0.0260	58 ± 2	170 ± 5

*WT (GS162), Δ*gcvA*Δ*gcvB* (GS1132), and WT transformed with p*gcvB*$^{3+}$ were grown overnight at 30°C (AP was added for the plasmid-carrying strain) and tested for CM sensitivity on Mueller-Hinton plates (see Materials and Methods). In addition, 50 μL of each overnight culture was used to inoculate 5 mL of LB with or without 3 μg mL^{-1} CM, the cultures grown at 30°C and generation times (GT) measured. Average inhibition zones and standard deviations from eight Mueller-Hinton plates and average generation times and standard deviations of 3 independent growth experiments are reported.

mechanism(s) remain(s) unknown. Thus, it makes sense that sRNAs with either known or predicted targets base pair to the 5′-untranslated region near or overlapping the translation initiation site (e.g., RyhB/*sodB* mRNA, DsrA/*rpoS* mRNA) [8, 24]. In addition, these target mRNAs usually have long 5′ untranslated regions that normally can fold into distinct secondary structures. The *slp* mRNA has a short untranslated region of 25 nts [25]. This sequence, including the first 20 nts of the coding region, does not fold into any distinct secondary structure by the mfold program [40, 41]. A comparison of the *slp* mRNA to GcvB RNA did not identify any region of significant complementarity between the two RNAs. However, small noncontiguous regions of complementarity between the two RNAs around the *slp* translation start codon and nts +125 to +177 of GcvB were identified (Figure 1(b)). Thus it is possible that GcvB/mRNA pairing is part of the mechanism employed by GcvB for negative regulation of *slp* mRNA in GM + glycine. Previously, we mutated nucleotides in GcvB predicted to disrupt base pairing with *cycA* mRNA [15]. Since these mutations also disrupt putative base pairing of GcvB with *slp* mRNA, we determined if there was an effect of the mutations on *slp*::*lacZ* expression. We transformed the Δ*gcvB* λ*slp*::*lacZ* lysogen with single-copy plasmids carrying WT *gcvB* or various mutations in *gcvB* between nts +125 and +177 that disrupt complementarity between GcvB and *slp* mRNA (Figure 1(b)). The strains were grown in GM + glycine (+ AP for the complemented strains) to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. However, none of the *gcvB* mutations predicted to alter base pairing between GcvB and *slp*::*lacZ* mRNA altered expression of *slp*::*lacZ* compared to WT GcvB (data not shown). Recently it was shown that a high degree of redundancy exists in the *E. coli* and *S. enterica* GcvBs [20, 22]. Thus, it is possible that more extensive changes are required to prevent GcvB repression or a few critical base pairing interactions necessary for repression were not changed in the mutational analysis. It is also possible that GcvB does not regulate *slp*::*lacZ* directly in GM + glycine media but regulates an additional factor necessary for repression.

3.5. GcvB Does Not Regulate slp Indirectly by Altering marR Expression. The *E. coli marRAB* multiple antibiotic resistance operon encodes two regulatory proteins. The *marR* gene encodes a repressor for the *marRAB* operon, and the *marA* gene encodes a transcriptional activator of genes in the Mar regulon [42, 43]. The operon is normally repressed by MarR and is induced by compounds such as tetracycline, CM, menadione, and salicylates, leading to increased antibiotic resistance [44, 45]. Expression of the *slp* gene is downregulated by the Mar system [27, 28]. If GcvB alters expression of the *marRAB* operon, it could regulate *slp* expression indirectly by altering MarRAB levels. The *marRAB* mRNA levels were not altered by the presence or absence of GcvB in a microarray analysis [15] or by the presence or absence of Hfq by microarray analysis [46]. Nevertheless, we constructed a *marR*::*lacZ* translational fusion to verify that the levels of the MarR gene product are not altered by GcvB. The λ*marR*::*lacZ* fusion was used to lysogenize WT strain GS162, Δ*gcvB* strain GS1144, and Δ*hfq* strain GS1148. The Δ*gcvB* and Δ*hfq* lysogens were then transformed with p*gcvB*$^{+}$ (pGS594) and p*hfq*$^{3+}$ (pGS609), respectively. The strains were grown in LB (+ AP for the transformants) and in GM + glycine (+ AP for the transformants) to midlog phase of growth (OD_{600} ~ 0.5) and assayed for β-galactosidase activity. GcvB and Hfq had no significant effect on *marR*::*lacZ* expression in LB or in GM + glycine medium (data not shown). The results suggest that GcvB regulates the *slp*::*lacZ* fusion by a mechanism that is independent of the Mar regulatory system.

3.6. Effects of GcvB Levels on CM Resistance. In addition to expression of *slp* being downregulated by the Mar system [27, 28], a null *slp* mutant showed a modest increase in CM resistance and a strain that overproduced Slp was more sensitive to CM [26]. We hypothesized that a Δ*gcvB* strain that overproduces Slp would be more sensitive to CM than a WT strain, and a strain carrying multicopy plasmid p*gcvB*$^{3+}$ that overproduces GcvB RNA would produce less Slp and be more resistant to CM. We tested the effect of CM on growth of WT (GS162), Δ*gcvA*Δ*gcvB* (GS1132), and WT transformed with plasmid p*gcvB*$^{3+}$ (pGS571). We used the Δ*gcvA*Δ*gcvB* strain since it carries a spectinomycin resistance cassette, whereas the Δ*gcvB* strain (GS1144) carries a CM resistance cassette. We showed previously that *dppA*::*lacZ* and *oppA*::*phoA* fusions are regulated similarly in a Δ*gcvB* strain and a Δ*gcvA*Δ*gcvB* strain [12]. In an agar diffusion disk assay, the Δ*gcvA*Δ*gcvB* strain showed a modest but statistically significant increase in CM sensitivity, and the WT (p*gcvB*$^{3+}$) strain showed a modest but statistically significant decrease in CM sensitivity relative to the WT strain (Table 2). Because the changes were small, we also tested the previous strains in a growth experiment. The generation times (GTs) of the 3 strains in

LB were essentially the same (Table 2). Thus, neither the Δ*gcvA*Δ*gcvB* allele nor plasmid p*gcvB*$^{3+}$ had any significant effect on growth rate. The presence of 3 μg mL^{-1} CM added to LB significantly increased the GTs of all 3 strains. In addition, we saw a consistent increase in the GT of the Δ*gcvA*Δ*gcvB* strain relative to WT and a consistent decrease in the GT of WT (p*gcvB*$^{3+}$) compared to the WT strain (Table 2). Although the differences in generation times are modest, in conjunction with the results from the diffusion disk assay, suggest that negative regulation of Slp levels by GcvB alters the sensitivity to CM.

4. Discussion

In both *E. coli* and *S. enterica*, genes shown to be regulated directly by GcvB are controlled by an antisense mechanism, and many are involved in the transport of amino acids and small peptides [10, 11, 14, 19, 20]. Since GcvB is only expressed in log-phase cells when nutrients are plentiful [12, 19], it likely controls the uptake of amino acids, peptides, and small toxic molecules under this condition to optimize cell growth. The *slp* gene encodes an outer membrane protein of unknown function [25]. Thus, Slp possibly does not fit the typical class of target genes controlled by GcvB. The localization of Slp in the outer membrane suggests a potential role in protecting stationary phase cells from environmental stress or facilitating nutrient availability in the periplasm. Since GcvB is not detected in stationary phase cells [12, 19], our results suggest that the role of GcvB is to keep Slp levels low in exponentially growing cells. The disappearance of GcvB in stationary phase would allow a more rapid induction of *slp* expression.

In LB grown cells, the mechanism of regulation of *slp*::*lacZ* by GcvB appears to be by sequestration of Hfq, which is required for full expression of *slp*::*lacZ*, rather than by an antisense mechanism (Figure 3(a)). In GM + glycine, however, GcvB negatively regulates *slp*::*lacZ*, and the role of Hfq appears to be to block repression by GcvB (Figure 3(b)). The ability of GcvB to repress *slp*::*lacZ* expression in the absence of Hfq suggests that GcvB might function alone or with a different chaperone to regulate this system. In *Staphylococcus aureus*, RNAIII functions to repress the synthesis of virulence factors without a requirement for Hfq, suggesting another protein chaperone [47, 48]. If GcvB regulates *slp* expression in GM + glycine without Hfq, it possibly does so in a way that is mechanistically different from the way it regulates other known target mRNAs [11, 12, 14, 15, 18, 20]. Verification of this model for regulation of *slp* would demonstrate that GcvB is a more versatile regulatory RNA than previously thought.

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