Role of the sRNA GcvB in regulation of cycA in *Escherichia coli*  
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In *Escherichia coli*, the *gcvB* gene encodes a small non-translated RNA that regulates several genes involved in transport of amino acids and peptides (including *sstT*, *oppA* and *dppA*). Microarray analysis identified *cycA* as an additional regulatory target of GcvB. The *cycA* gene encodes a permease for the transport of glycine, D-alanine, D-serine and D-cycloserine. RT-PCR confirmed that GcvB and the Hfq protein negatively regulate *cycA* mRNA in cells grown in Luria–Bertani broth. In addition, deletion of the *gcvB* gene resulted in increased sensitivity to D-cycloserine, consistent with increased expression of *cycA*. A *cycA::lacZ* translational fusion confirmed that GcvB negatively regulates *cycA* mRNA expression in Luria–Bertani broth and that Hfq is required for the GcvB effect. GcvB had no effect on *cycA::lacZ* expression in glucose minimal medium supplemented with glycine. However, Hfq still negatively regulated the fusion in the absence of GcvB. A set of transcriptional fusions of *cycA* to *lacZ* identified a sequence in *cycA* necessary for regulation by GcvB. Analysis of GcvB identified a region complementary to this region of *cycA* mRNA. However, mutations predicted to disrupt base-pairing between *cycA* mRNA and GcvB did not alter expression of *cycA::lacZ*. A model for GcvB function in cell physiology is discussed.

INTRODUCTION

The *Escherichia coli* chromosome encodes >80 small non-translated regulatory RNAs (sRNAs) that function as regulators in response to different growth conditions such as varying osmolarities, temperature, oxidative stresses and iron levels (Argaman et al., 2001; Gottesman, 2004; Hershberg et al., 2003; Majdalani et al., 2005; Storz et al., 2005; Vogel & Papenfort, 2006; Wagner & Flardh, 2002; Wassarman, 2002). These sRNAs have been shown to regulate expression of target genes by one of two main modes of action. Most act by base pairing with trans-encoded mRNA targets to modulate translation and/or stability. Examples of sRNAs identified that regulate targets by this mechanism include SgrS, MicA, MicC, MicF, RhyB, OxyS and Spot42 (Argaman & Altuvia, 2000; Chen et al., 2004; Kawamoto et al., 2006; Masse & Gottesman, 2002; Moller et al., 2002; Rasmussen et al., 2005; Schmidt et al., 1995; Udekwu et al., 2005). In most cases, base pairing results in negative regulation of translational activity and greater instability of the target mRNA (Gottesman, 2004). However, some sRNAs activate translation of mRNAs. For example, DsrA and RprA pair with *rpoS* mRNA and prevent the formation of an inhibitor structure that blocks *rpoS* mRNA translation (Lease & Belfort, 2000; Majdalani et al., 1998, 2002). It is unclear how extensive the base-pairing between an sRNA and a target mRNA must be, but research indicates that one or two regions of 8–9 bp is sufficient for regulation (Gottesman, 2004). The remaining sRNAs function by protein sequestration. Examples of sRNAs identified that regulate by this mechanism include CsrB, CsrC and 6S RNA (Liu et al., 1997; Wassarman & Storz, 2000; Weilbacher et al., 2003).

The *E. coli* *gcvB* gene encodes an RNA of 206 nt that regulates SstT, OppA and DppA levels, the serine transport system and the oligopeptide and dipeptide periplasmic binding proteins, respectively (Pulvermacher et al., 2008; Urbanowski et al., 2000; S. C. Pulvermacher and others, unpublished results). The *Salmonella typhimurium* GcvB RNA has also been shown to regulate OppA and DppA levels, as well as several other genes involved in the transport of polar and branched amino acids (Sharma et al., 2007). The sRNA RyhB regulates at least six different target mRNAs, and many other sRNAs are also predicted to regulate more than one target (Masse & Gottesman, 2002). We compared RNA isolated from a wild-type (WT) and an isogenic *gcvB* deletion strain grown to mid-exponential phase in Luria–Bertani broth (LB) by microarray analysis to identify additional regulatory targets. One potential target identified was *cycA*, which encodes a permease for glycine, D-alanine, D-serine and D-cycloserine (Cosloy, 1973;...
In this study, we show that cycA mRNA is a target for both GcvB and Hfq regulation. A model for the role that GcvB plays in cell physiology is discussed.

**METHODS**

Bacterial strains, plasmids and phages. The E. coli strains and plasmids used in this study are listed in Table 1 or described in the text. The lgcvB::lacZ translational fusion was constructed by PCR synthesis of a DNA fragment using an upstream primer with an EcoRI translation start site and a downstream primer with an Smal site that hybridized to DNA beginning at codon 11 within the cycA structural gene. The PCR-amplified DNA fragment was digested with EcoRI and Smal, and the 337 bp EcoRI–Smal fragment was ligated into the EcoRI and Smal sites of plasmid pMC1403 (Casadaban et al., 1980), fusing the first 11 codons of the cycA structural gene in-frame with the eighth codon of the lacZYA genes in pMC1403 (Fig. 1). The cloned sequence was verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa. The intermediate plasmid was designated pycvA::lacZ. A 5788 bp EcoRI–MfeI fragment from pycvA::lacZ carrying the cycA::lacZYA fusion was then ligated into the EcoRI site of phage λgt2 (Panasenko et al., 1977), and the phage used to lysogenize E. coli host strains as described previously (Urbanowski & Stauffer, 1986). Each lysogen was tested to ensure that it carried a single-copy of the i chromosome by infection with λc9017 (Shimada et al., 1972). All lysogens were grown at 30 °C, since all fusion phages carry the λc857 mutation, resulting in a temperature-sensitive λcI repressor (Panasenko et al., 1977).

Three λcycA::lacZ transcriptional fusions were constructed in a similar manner using the upstream primer with an EcoRI site that hybridized to DNA beginning 296 bp upstream of the translation start site and downstream primers that had HindIII sites and hybridized to DNA beginning at position −8, −26, or −34 relative to the translation initiation codon (Fig. 1). Following digestion with EcoRI and HindIII, the DNA fragments were gel-purified and ligated into the EcoRI and HindIII sites of plasmid pgcvB-lacZ+50 (Urbanowski et al., 2000), replacing the gcvB fragment with the cycA fragments, generating plasmids pycvA::lacZ, pycvA−26::lacZ and pycvA−34::lacZ. The cloned sequences were verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa. Each transcriptional fusion was subcloned into λgt2 as an EcoRI–MfeI DNA fragment, as described for the translational fusion above.

**Table 1. Strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS162</td>
<td>WT</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>GS1144</td>
<td>ΔgcvB::ΩCM' (referred to as ΔgcvB)†</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>GS1148</td>
<td>hfg-1::ΩCM' (referred to as Δhfg)</td>
<td>S. C. Pulvermacher, unpublished results</td>
</tr>
<tr>
<td>GS1149</td>
<td>Δ(gcvAgcvB)::ΩaadA hfg-1::ΩCM' (referred to as ΔgcvAhfg)</td>
<td>S. C. Pulvermacher, unpublished results</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGS571</td>
<td>Multicopy vector + WT gcvB</td>
<td>Jourdan &amp; Stauffer (1999)</td>
</tr>
<tr>
<td>pGS594</td>
<td>Single-copy vector + WT gcvB (gcvB+* )</td>
<td>Our laboratory</td>
</tr>
<tr>
<td>pGS595†</td>
<td>pGS594 with a -TGTTGGTGTGTT- to -ACAAGAGAA- change of bases +79 to +87 (gcvB+79ACAAGAA)</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>pGS596</td>
<td>pGS594 with a -TGT- to -CCC- change of bases +71 to +73 in gcvB (gcvB+71CCC)</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>pGS602</td>
<td>pGS594 with a -TGT- to -AAA- change of bases +76 to +78 in gcvB (gcvB+76AAA)</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>pGS609</td>
<td>Multicopy vector + WT hfg (hfg+* )</td>
<td>S. C. Pulvermacher, unpublished results</td>
</tr>
<tr>
<td>pGS619</td>
<td>pGS594 with a -TG- to -CC- change of bases +71 and +72 in gcvB (gcvB+71CC)</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>pGS631</td>
<td>pGS594 with a -TT- to -CC- change of bases +131 and +132 in gcvB (gcvB+131CC)</td>
<td>This study</td>
</tr>
<tr>
<td>pGS632</td>
<td>pGS594 with a -TG- to -CA- change of bases +142 and +143 in gcvB (gcvB+142CA)</td>
<td>This study</td>
</tr>
<tr>
<td>pGS633</td>
<td>pGS594 with a -TG- to -CC- change of bases +159 and +160 in gcvB (gcvB+159CC)</td>
<td>This study</td>
</tr>
<tr>
<td>pGS634</td>
<td>pGS594 with a -TG- to -CA- change of bases +142 and +143 and a -TG- to -CC- change of bases +159 and +160 in gcvB (gcvB+142CA+159CC)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*All strains also carry the pheA905, thi, araD129, rpsL150, relA1, deoC1, fthB350, ptsE25 and rbsR mutations.

†CM', chloramphenicol resistant.

‡The pGCVB+79ACAAGAA allele was previously designated pGCVB+4017. (Pulvermacher et al., 2008). The nucleotides in lower-case type are WT bases at those positions. Numbers of the mutant alleles indicate the first nucleotide changed for each specific mutation relative to the gcvB +1 transcription initiation site.
Site-directed mutagenesis of gcvB. Plasmid pGS571 carries the WT gcvB gene on a 406 bp EcoRI–HindIII fragment cloned into the EcoRI and HindIII sites in plasmid pGS272 (Jourdan & Stauffer, 1999). Using plasmid pGS571 as template, the PCR ‘megaprimer’ mutagenesis procedure (Sarkar & Sommer, 1990) was used to create nucleotide changes in the gcvB gene (Fig. 1). Base changes were verified by DNA sequence analysis at the DNA Core Facility of the University of Iowa. The changes made in gcvB were predicted by the mfold program to leave the GcvB secondary structure intact (Mathews et al., 1999; Zuker, 2003). The 406 bp EcoRI–HindIII fragments carrying the gcvB mutations were then subcloned into the EcoRI and HindIII sites of the single-copy plasmid vector pGS341 (Jourdan & Stauffer, 1998), replacing the WT gcvB gene. The constructs were verified as correct by PCR amplification of the gcvB gene and DNA sequence analysis of the PCR product at the DNA Core Facility of the University of Iowa.

β-Galactosidase assays. β-Galactosidase assays were performed on mid-exponential-phase cells (OD600 ~0.5) using the chloroform/SDS lysis procedure (Miller, 1972). Results are the averages of two or more assays with each sample done in triplicate.

Microarray analysis. Bacterial strains GS162 (WT) and GS1144 (ΔgcvB) were grown in 10 ml LB to OD600 ~0.5. Cells were immediately added to one-fifth volume of stop solution (95% ethanol/5% acidic phenol). The samples were centrifuged at 2000 g for 5 min and cell pellets were frozen at −80 °C. Total cellular RNA was isolated from cells by phenol extraction (Ledeboer et al., 2006) and DNase I-treated for 1 h at 37 °C, and RNA pellets were resuspended in 30 μl diethylpyrocarbonate (DEPC)-treated water. RNA was quantified using a NanoDrop ND-1000 spectrophotometer. Microarray analysis was performed by the University of Iowa DNA Core Facility using Affymetrix genome 2.0 arrays. Microarrays hybridized to labeled probe pools were scanned and quantified using a Packard Scientific 4000XL spotted array scanner and the accompanying ScanArray Express software. The median signal intensity, median background, and median signal corrected for local background were imported into Microsoft Excel from ScanArray Express. The background-corrected median signal intensity for each gene was normalized to the total signal for each dye.

RNA isolation and RT-PCR. Bacterial strains GS162 (WT), GS1144 (ΔgcvB), GS1148 (Δhfq), GS1149 (ΔgcvABΔhfq) and GS1144 transformed with the single-copy plasmid pGS394 (WT gcvB) were grown in 5 ml of LB to OD600~0.5. Total cellular RNA was isolated from cells by phenol extraction (Ledeboer et al., 2006) and DNase I-treated for 1 h at 37 °C, and RNA pellets were resuspended in 30 μl DEPC-treated water. RNA was quantified using a NanoDrop ND-1000 spectrophotometer.

The Access RT-PCR kit (Promega) was used with 0.5 μg total cellular RNA isolated from each sample in a 25 μl total reaction volume. The cycA primers were cycA-RT 5’-GCTCGCGCCTGAGGTGATTGCATGCTG (sense) and cycA-RT 5’-CTGGTGACAGACCATCGTCTTACC (antisense). The dksA control primers were dksA-RT 5’-GTGGAAC- CATATCGAGG (sense) and dksA-RT 5’-GTCGATGCACA-TGGCAGT (antisense). The PCR fragment for cycA was 992 bp and that for dksA was 354 bp. Cycling conditions were 1 cycle of 45 °C for 45 min and 94 °C for 2 min, then 15 cycles of 94 °C for 30 s, 50 °C for 1 min, and 68 °C for 2 min, followed by 1 cycle of 68 °C for 7 min. Primers for both targets were used in the same PCR. PCR products were run on a 2 % agarose gel and stained with ethidium bromide.

Procedures and enzymes. The procedures for plasmid DNA purification, restriction enzyme digestion, etc., were as described by the manufacturers. Restriction enzymes, Vent DNA polymerase and other DNA modifying enzymes were from New England Biolabs.

RESULTS AND DISCUSSION

The cycA gene is a target for GcvB and Hfq regulation

In E. coli and S. typhimurium, GcvB negatively regulates a number of genes involved in transport of amino acids, dipeptides and oligopeptides (Pulvermacher et al., 2008; Sharma et al., 2007; Urbanowski et al., 2000). We carried out a microarray analysis of RNA isolated from WT and an otherwise isogenic ΔgcvB strain grown in LB. Expression of a total of 69 genes was either increased or decreased about threefold or greater in response to the presence or absence of GcvB (Table 2 and Supplementary Table S1). To validate the microarray data, we initially looked at the GcvB levels in WT and the ΔgcvB strain (Supplementary Table S1). As expected, the WT strain showed a >250-fold higher GcvB level than the ΔgcvB strain. In addition, dppA and ssrT mRNAs, both shown by lacZ translational fusions to be negatively regulated by GcvB in E. coli ~10-fold (Pulvermacher et al., 2008; Urbanowski et al., 2000; S. C. Pulvermacher and others, unpublished results), showed 10-fold and 8.6-fold lower mRNA levels in the WT versus the ΔgcvB strain, respectively (Supplementary Table S1). The results suggest that the microarray approach is valid for revealing GcvB-regulated mRNAs. It should be noted,
### Table 2. Genes significantly regulated (about threefold or greater) in the WT versus ΔgcvB strain grown in LB, determined by microarray analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product type</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>gcvB</td>
<td>RNA</td>
<td>Regulator</td>
</tr>
<tr>
<td>rfbAC, mdoB, adhE, gdhA, g1f, ilvBN, ilvC, serA, ssaE, wbbH, gatDZ, srlD, lon, cysD</td>
<td>Enzyme, predicted</td>
<td></td>
</tr>
<tr>
<td>wbbJK</td>
<td></td>
<td>Transporter</td>
</tr>
<tr>
<td>cyaA*, sstT, dppABCDF, argT, livK, gatABC, srlB</td>
<td>Membrane, predicted</td>
<td></td>
</tr>
<tr>
<td>rfbX, yijK, emrY, yicO</td>
<td></td>
<td>Factor</td>
</tr>
<tr>
<td>fimZ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>yafU, yfbB</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>clpB, ibpA, dnaKJ, htpG, groS, hdeB</td>
<td></td>
<td>Structural component</td>
</tr>
<tr>
<td>sfnH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ydhU, ygeG</td>
<td></td>
<td>Carrier, predicted</td>
</tr>
<tr>
<td>slp, yidd</td>
<td></td>
<td>Lipoprotein</td>
</tr>
</tbody>
</table>

*The genes underlined have been shown by independent methods to be regulated by GcvB in *E. coli* or *Salmonella* (Sharma et al., 2007; Urbanowski et al., 2000; S. C. Pulvermacher and others, unpublished results).

However, that oppA mRNA, known to be regulated fourfold by GcvB (Urbanowski et al., 2000), did not show significant differences in mRNA levels by microarray analysis. It is possible that OppA levels are controlled primarily at the translational level rather than at the level of oppA mRNA (Tao et al., 1999; Urbanowski et al., 2000).

Genes shown to be regulated by GcvB by independent methods are all involved in the transport of amino acids and peptides (Pulvermacher et al., 2008; Sharma et al., 2007; Urbanowski et al., 2000). The microarray analysis suggested that cycA mRNA, encoding a permease involved in transport of glycine, D-alanine, D-serine and D-cycloserine, is also negatively regulated by GcvB approximately fourfold (Supplementary Table S1). To confirm the results of the microarray analysis that showed that cycA mRNA is regulated by GcvB, we carried out RT-PCR. WT strain GS162, the ΔgcvB strain GS1144, and GS1144 transformed with the single-copy plasmid pGS594 carrying WT gcvB were grown in LB at 30 °C to mid-exponential phase (OD600 ~0.5) and total cellular RNA was isolated. We observed an increased level of DNA corresponding to cycA mRNA amplified from the ΔgcvB sample compared with the level of DNA amplified from either the WT or the gcvB-complemented strain (Fig. 2, compare lane 3 with lanes 2 and 4). These results agree with the microarray results, indicating that GcvB negatively regulates cycA mRNA levels in cells grown in LB. As a control, we amplified the dksA mRNA. Microarray results predicted there to be no difference in the dksA mRNA levels between the WT and the ΔgcvB strain. We observed roughly the same levels of DNA corresponding to the dksA mRNA from each strain (Fig. 2). These data confirm that GcvB does not regulate dksA mRNA levels in LB and that the differences in intensities of the cycA mRNA levels shown in Fig. 2 are due to the presence or absence of GcvB.

Many sRNAs that use base-pairing to regulate gene expression require Hfq (Sledjeski et al., 2001; Storz et al., 2005; Valentin-Hansen et al., 2004; Zhang et al., 1998). It has been shown previously that GcvB interacts with Hfq in *E. coli* (Zhang et al., 2003), and that Hfq is required for GcvB regulation of oppA, dppA and sstT mRNAs (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results). The same results were reported for *S. typhimurium* (Sharma et al., 2007). To determine whether Hfq plays a role in GcvB regulation of cycA, we also carried out RT-PCR on total RNA isolated from the Δhfq strain GS1148 and the ΔgcvBΔhfq double mutant GS1149 grown
in LB at 30 °C to mid-exponential phase (OD_{600} ~0.5). We observed an increased level of DNA corresponding to cycA mRNA amplified from the Δhfq and ΔgcvABΔhfq samples compared with the level of DNA amplified from the WT strain (Fig. 2, compare lane 2 with lanes 5 and 6). These results suggest that Hfq also negatively regulates cycA mRNA levels in cells grown in LB. Furthermore, the level of DNA observed in the ΔgcvABΔhfq double mutant was not significantly altered compared with the level in either single mutant. Thus, it is likely that GcvB and Hfq are part of the same regulatory mechanism for cycA expression in LB.

**GcvB and Hfq repress cycA::lacZ in cells grown in LB**

To confirm that GcvB and Hfq negatively regulate cycA expression, we made a translational fusion of cycA with lacZ, cloned this fusion into phage λgt2, and lysogenized WT, ΔgcvB, Δhfq and ΔgcvABΔhfq strains. Next, we transformed ΔgcvBλcycA::lacZ with the single-copy plasmid pGS594 (pGcvB+) and transformed Δhfq::cycA::lacZ with the multi-copy plasmid pGS609 (phfq++). These strains were grown in LB (with ampicillin for complemented strains) to mid-exponential growth phase (OD_{600} ~0.5) and assayed for β-galactosidase activity. Expression of cycA::lacZ in the ΔgcvB strain was about twofold higher compared with the activity in the WT strain (Fig. 3a, compare columns 1 and 2). Repression of cycA::lacZ was restored in the gcvB-complemented strain (Fig. 3a, column 5), confirming that cycA is an additional regulatory target of GcvB. Expression of cycA::lacZ in the Δhfq strain was ~2.5-fold higher than the activity in the WT strain (Fig. 3a, compare columns 1 and 3). Repression of cycA::lacZ was restored in the hfq-complemented strain (Fig. 3a, column 6), confirming that cycA is also regulated by Hfq. Expression of cycA::lacZ in the ΔgcvABΔhfq strain was about twofold higher than in the WT strain (Fig. 3a, compare columns 1 and 4). Since the levels of cycA::lacZ expression are not significantly different in the ΔgcvABΔhfq double mutant compared with each single mutant, the results suggest that GcvB and Hfq work through the same mechanism.

Microarray analysis suggested a difference in cycA mRNA levels in the WT compared with the ΔgcvB strain of about fourfold (see Supplementary Table S1). In addition, the RT-PCR assay suggested more than a twofold difference in cycA mRNA levels in the ΔgcvB and Δhfq strains compared with the WT strain (Fig. 2, compare lane 2 with lanes 3, 5 and 6). Thus, there does not appear to be a direct correlation between the level of cycA::lacZ expression and the level of cycA mRNA in the ΔgcvB and Δhfq strains. It is possible that part of the discrepancy is due to differences in stabilities of the cycA mRNA in the WT versus the gcvB and hfq mutants. Alternatively, it is possible other factors are involved in determining the final level of CycA.

**Hfq represses cycA::lacZ in cells grown in GM+glycine**

Repression of ΔoppA::lacZ and ΔoppA::phoA fusions by GcvB was only observed in a WT background when the lysogens were grown in LB, with no significant repression observed when the lysogens were grown in GM + glycine medium (Urbanowski et al., 2000). Since gcvB is known to be regulated over a 25-fold range in different GM media (Urbanowski et al., 2000), we hypothesized that there are genes in E. coli that respond to changing levels of GcvB in different GM media. Thus, we tested whether cycA::lacZ was regulated by GcvB in GM + glycine medium. The

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**Fig. 3.** β-Galactosidase levels of ΔcycA::lacZ lysogens. WT (GS162), ΔgcvB (GS1144), Δhfq (GS1148), ΔgcvABΔhfq (GS1149), ΔgcvB complemented [GS1144(pGS594)] and Δhfq complemented [GS1148(pGS609)] ΔcycA::lacZ lysogens were grown in either (a) LB or (b) GM + glycine (+ ampicillin for complemented strains) to mid-exponential phase (OD_{600} ~0.5) and assayed for β-galactosidase activity (Miller, 1972). Results are averages of two independent assays with each assay performed in triplicate.
cycA:: lacZ lysogens were grown in GM + glycine (+ ampicillin for complemented strains) to mid-exponential phase of growth and assayed for β-galactosidase activity. Expression of cycA:: lacZ in the ΔgcvB strain was not significantly different compared with the activity in the WT strain and repression of cycA:: lacZ was not increased in the gcvB-complemented strain (Fig. 3b, compare columns 7, 8 and 11). Thus, like dppA:: lacZ and oppA:: phoA, GcvB does not regulate cycA:: lacZ in GM + glycine medium. In contrast, expression of cycA:: lacZ in the Δhfq and ΔgcvABΔhfq strains was about twofold higher than the activity in the WT strain (Fig. 3b, compare columns 7, 9 and 10). Repression was restored in the Δhfq-complemented strain (Fig. 3b, column 12). Thus, in GM + glycine, Hfq regulates cycA:: lacZ expression by a mechanism independent of GcvB. Whether Hfq acts alone on cycA:: lacZ expression or acts with a second sRNA in GM + glycine is unknown. A search of the E. coli chromosome using the TargetRNA program (Tjaden et al., 2006) examining 50 nt upstream and 20 nt downstream of the AUG translation start site for cycA did not identify any significant matches with any sRNA. However, if the complementarity between the putative sRNA and cycA is small, or not a perfect match, it could have been missed.

Genetic analysis of gcvB

Since the mechanism of GcvB repression appears to be by base-pairing to its target mRNAs and blocking ribosome binding (Pulvermacher et al., 2008; Sharma et al., 2007), we hypothesized that GcvB/mRNA pairing is also the regulatory mechanism employed by GcvB for negative regulation of cycA mRNA levels. We identified a region in GcvB from nucleotides +124 to +161 that is complementary to the cycA mRNA overlapping the translation start site for cycA (Fig. 1). This is different from the region of GcvB from nucleotides +62 to +88 complementary to the dppA, oppA and sstT mRNAs previously shown to be important for regulation of these genes (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results). Thus, if complementary pairing is part of the mechanism that GcvB uses to regulate cycA:: lacZ, then different regions of GcvB must be involved in regulation of the cycA mRNA and the dppA, oppA and sstT mRNAs. We constructed a set of mutations in gcvB in the region that is complementary to cycA mRNA from nucleotide +124 to +161 (Fig. 1), and transformed the ΔgcvBΔcycA:: lacZ lysogen with single-copy plasmids carrying either the WT gcvB gene or the gcvB mutant alleles. When the ΔgcvBΔcycA:: lacZ lysogen was transformed with the single-copy plasmid carrying the gcvB^+131CC, gcvB^+142CA, gcvB^+159CC or gcvB^+142CA +159CC allele, there was normal repression of the cycA:: lacZ fusion (Fig. 4, compare column 1 with columns 4–7). We have not tested transformants with these four mutant alleles by Northern blotting to determine the levels of GcvB RNA. However, since all four transformants show normal regulation of cycA:: lacZ, we assume that the mutations do not significantly alter the stability or levels of GcvB produced. For oppA, dppA and sstT, single-base-pair changes in gcvB also had little effect, and only multiple changes resulted in a significant loss of repression (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results). Thus, if GcvB directly regulates cycA, our results suggest that disrupting only a few of the predicted base-pairing interactions does not alter regulation and more extensive changes are necessary to disrupt GcvB regulation. However, it has been shown for the sRNA SgrS and its target mRNA ptsG and the sRNA OxyS and its target mRNA fliA that a single nucleotide change in either the sRNA or the target mRNA results in a complete loss of regulation (Altuvia et al., 1998; Kawamoto et al., 2006). It is possible that one or a few bases are critical for GcvB repression of cycA, and the mutations made in this study have not changed the appropriate base(s). Furthermore, if Hfq binds both GcvB and cycA mRNA to form a repression complex, the ability of Hfq to form this complex may be more important than a long stretch of complementarity between GcvB and cycA mRNA. We have purified Hfq to test its ability to bind these two RNAs singly and in combination.
Our failure to detect any mutations in GcvB that result in loss of repression of cya::lacZ prompted us to test the region of GcvB from +62 to +88 shown to be important for regulation of dppA, oppA and sstT expression (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results). We transformed the ΔgcvB::cycA::lacZ lysogen with single-copy plasmids carrying mutations in gcvB between nucleotides +62 and +88 that disrupt complementarity between GcvB and the dppA, oppA and sstT mRNAs, and result in a significant loss of GcvB repression of oppA, dppA or sstT (S. C. Pulvermacher and others, unpublished results). These changes have been shown previously by Northern analysis to not alter the level of GcvB in the cell, and many of the changes result in altered regulation of dppA::lacZ, oppA::phoA and sstT::lacZ expression (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results).

The transformants were grown in LB with ampicillin to mid-exponential phase (OD600 ~0.5) and assayed for β-galactosidase activity. All of the mutations tested in gcvB between nucleotides +62 and +88 showed normal repression of the cycA::lacZ fusion (Fig. 4, columns 8–12). These results show that the region of GcvB required for dppA::lacZ, oppA::lacZ and sstT::lacZ regulation is not required for cyaA repression. It is possible that GcvB does not act directly on cyaA mRNA, but instead regulates an additional factor necessary for cyaA regulation. If so, then the regions of GcvB from +62 to +88 and +124 to +161 analysed in this study are unlikely to control the levels of this regulatory intermediate.

Transcriptional fusions of cyaA to lacZ

Our failure to identify any base pairs in GcvB that are essential for regulation of cya::lacZ prompted us to construct a series of transcriptional fusions of cyaA to lacZ to determine whether GcvB and Hfq regulate cyaA at the level of transcription or post-transcriptionally, and to determine which region of the cyaA mRNA is required for repression (Fig. 1). The transcriptional fusions were cloned into pgt2 and the phage used to lysogenize WT, ΔgcvB and Δhfq strains. The strains were grown in LB to mid-exponential phase and assayed for β-galactosidase activity. We did not observe any significant repression by GcvB and Hfq with the λcyaA−34::lacZ and λcyaA−26::lacZ lysogens (Fig. 5, columns 1–6). However, there was a twofold increase in β-galactosidase levels in the λcyaA−26::lacZ lysogen compared with the λcyaA−34::lacZ lysogen. This result suggests that a region of cyaA mRNA between nucleotides −26 and −34 is important for a GcvB- and Hfq-independent mechanism of regulation. In contrast, the λcyaA−8::lacZ lysogen showed a twofold and 1.5-fold higher β-galactosidase levels in the ΔgcvB and Δhfq strains, respectively, compared with the WT (Fig. 5, columns 7–9). The results suggest that the region of cyaA mRNA from nucleotide −8 to nucleotide −26 relative to the translation start codon is required for GcvB and Hfq regulation. This is the region of the cyaA mRNA that shows complementarity to GcvB (Fig. 1). A more complete mutational analysis will be required to determine whether large regions of complementarity must be removed to produce altered regulation or an alternative regulatory intermediate is necessary for repression.

A ΔgcvB allele increases d-cycloserine sensitivity

The E. coli cyaA gene encodes a permease for transporting D-alanine, D-serine and glycine into the cell (Cosloy, 1973; Robbins & Oxender, 1973). The CyaA permease also transports D-cycloserine (Russell, 1972; Wargel et al., 1971), an analogue of D-alanine that interferes with cell wall synthesis (Chopra & Ball, 1982). If GcvB negatively regulates CyaA levels, we hypothesized that a WT strain, with repressed levels of CyaA, would be more resistant to D-cycloserine than a ΔgcvB strain, with derepressed levels of CyaA. To test this hypothesis, the WT and ΔgcvB strains were grown in LB or LB + D-cycloserine at 30 °C. The presence of the ΔgcvB allele consistently resulted in earlier and more complete lysis of cells (Fig. 6). In separate experiments in which the ΔgcvB strain was transformed with multi-copy plasmid pGS571 (pgcvB+), resistance to D-cycloserine was restored to that of the WT strain (data not shown). These results suggest that the increase in sensitivity to D-cycloserine in the ΔgcvB strain is due to increased levels of CyaA and increased transport of D-cycloserine.

Role of GcvB in cell physiology

Most sRNAs have been found to control the expression of outer-membrane proteins and are therefore involved in
stress responses and virulence gene regulation (Argaman et al., 2001; Gottesman, 2004; Romby et al., 2006; Vogel & Pappenfort, 2006; Wagner & Darfeuille, 2005). The above results, as well as previous data, have established that GcvB provides for tighter regulation of genes involved in transport of these small molecules under conditions where these molecules are in excess (Pulvermacher et al., 2008; Sharma et al., 2007; Urbanowski et al., 2000). However, the reason why GcvB functions as a negative regulator of genes involved in transport of these small molecules is less clear. It is known that these transport systems are responsible not only for transport of amino acids and peptides to provide nutrients, but also for transport of toxins and antibiotics (Hiles et al., 1987; Smith & Payne, 1990). A variety of natural antibiotics couple toxic moieties to peptide carriers for transport into the cell (Payne, 1986), and many antibiotics with intracellular targets are actively accumulated by solute-transport systems (Chopra & Ball, 1982). E. coli naturally inhabits environments that differ in nutrient and toxin concentrations. If conditions that favour relatively high concentrations of amino acids and small peptides also favour the presence of small toxic compounds, the decreased expression of transport systems for these small molecules by GcvB could provide a protective mechanism, preventing transport of toxic compounds into the cell.

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