Role of the sRNA GcvB in regulation of cycA in Escherichia coli

Sarah C. Pulvermacher, Lorraine T. Stauffer and George V. Stauffer

Department of Microbiology, University of Iowa, Iowa City, IA 52242, USA

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 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 sRNA of 206 nt that regulates SsrT, 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;
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 lacZ::lacI transcriptional fusion was constructed by PCR synthesis of a DNA fragment using an upstream primer with an EcoRI site that hybridized to DNA beginning 296 bp upstream of the cyA translation start site and a downstream primer with an Smal site that hybridized to DNA beginning at codon 11 within the cyA 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 cyA structural gene in-frame with the eighth codon of the lacZ gene 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 pycA::lacZ. A 5788 bp EcoRI–MfeI fragment from pycA::lacZ carrying the cyA::lacZ 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 lac chromosome by infection with λd90::17 (Shimada et al., 1972). All lysogens were grown at 30 °C, since all fusion phages carry the λd857 mutation, resulting in a temperature-sensitive λd repressor (Panasenko et al., 1977).

Three λcyA::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 cyA fragments, generating plasmids pycA-18::lacZ, pycA-26::lacZ and pycA-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.

Media. The complex medium used was LB (Miller, 1972). The defined medium used was Vogel and Bonner minimal salts (Vogel & Bonner, 1956) supplemented with 0.4% glucose (GM). GM medium was always supplemented with phenylalanine and thiamine, since all strains carried the pheA905 and thi mutations. Glycine was always added to GM, since gcvB expression is activated by glycine in the growth medium (Urbanowski et al., 2000). Agar was added at 1.5% for solid media. Supplements were added at the following concentrations (μg ml−1): phenylalanine, 50; thiamine, 1; ampicillin, 50; glycine, 300; X-Gal, 40; d-cycloserine was added at 2.5 × 10−4 M.

Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</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::ΔCM' (referred to as ΔgcvB)</td>
<td>Pulvermacher et al. (2008)</td>
</tr>
<tr>
<td>GS1148</td>
<td>Δhfp-1::ΔCM' (referred to as Δhfp)</td>
<td>S. C. Pulvermacher, unpublished results</td>
</tr>
<tr>
<td>GS1149</td>
<td>Δ(gcvAgcvB)::ΔaadA hfp-1::CM' (referred to as ΔgcvABΔhfp)</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>
</tbody>
</table>
| pGS594            | Single-copy vector + WT gcvB (pgcvB+)
|                  | $p$GS594 with a -TGTGTGTGTGTT- to -ACAAgAgAA- change of bases +79 to +87 (gcvB+$^{79}$ACAAgAgAA) | Pulvermacher et al. (2008) |
| pGS596            | $p$GS594 with a -TGT- to -CCC- change of bases +71 to +73 in gcvB (gcvB+$^{71}$CCC) | Pulvermacher et al. (2008) |
| pGS602            | $p$GS594 with a -TGT- to -AAA- change of bases +76 to +78 in gcvB (gcvB+$^{76}$AAA) | Pulvermacher et al. (2008) |
| pGS605            | $p$GS594 with a -TT- to -AA- change of bases +65 and +66 in gcvB (gcvB+$^{65}$AA) | Pulvermacher et al. (2008) |
| pGS609            | Multicopy vector + WT hfp (hfp+)$^*$ | Pulvermacher et al. (2008) |
| pGS619            | $p$GS594 with a -TG- to -CC- change of bases +71 and +72 in gcvB (gcvB+$^{71}$CC) | Pulvermacher et al. (2008) |
| pGS632            | $p$GS594 with a -TG- to -CA- change of bases +142 and +143 in gcvB (gcvB+$^{142}$CA) | Pulvermacher et al. (2008) |
| pGS633            | $p$GS594 with a -TG- to -CA- change of bases +159 and +160 in gcvB (gcvB+$^{159}$CC) | Pulvermacher et al. (2008) |
| pGS634            | $p$GS594 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+$^{142}$CA+$^{159}$CC) | Pulvermacher et al. (2008) |

*All strains also carry the pheA905, thi, araD129, rpsL150, relA1, deoC1, flbBS301, ptsE25 and rbsR mutations.
†CM', chloramphenicol resistant.
‡The pgcvB+$^{79}$ACAAgAgAA allele was previously designated pgcvB$^{con-7}$ (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’ (Jourdan & Stauffer, 1998), replacing the WT gcvA EcoRI fragment, was inserted into the RI and HinIII sites of the single-copy plasmid vector pGS341 (Jourdan & Stauffer, 1998), resulting in the WT gcvA 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 (OD_{600} ~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 OD_{600} ~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 labelled 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. The background-corrected median signal intensity, median background, and median signal corrected for local background were imported into Microsoft Excel from ScanArray Express.

**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 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, dpaA and sstT 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

Genes with increased and decreased mRNA levels in the ΔgcvB strain are in bold and normal type, respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product type</th>
</tr>
</thead>
<tbody>
<tr>
<td>gcvB</td>
<td>RNA</td>
</tr>
<tr>
<td>rfbAC, mdoB, adhE, gdhA, glf, ilvBN, ilvC, serA, ssuE, wbbH, gatDZ, strD, lon, cysD</td>
<td>Enzyme</td>
</tr>
<tr>
<td>wbbJK</td>
<td>OppA levels are controlled</td>
</tr>
<tr>
<td>cycA, sstT, dppABCDF, argI, livK, gatABC, strB</td>
<td>Membrane, predicted</td>
</tr>
<tr>
<td>rfbX, yjK, emrY, yicO</td>
<td>Factor</td>
</tr>
<tr>
<td>fimZ</td>
<td>yafU, ybfB</td>
</tr>
<tr>
<td>clpB, ibpA, dnaKJ, htpG, groS, ldeB</td>
<td>Carrier, predicted</td>
</tr>
<tr>
<td>sfmH</td>
<td>ydhU, ygeG</td>
</tr>
<tr>
<td>ydhU, ygeG</td>
<td>Enzyme, predicted</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 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., 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

![Fig. 2. Expression of cycA and dksA from WT (GS162), ΔgcvB (GS1144), ΔgcvB complemented (GS1144[pGS594]), Δhfq (GS1148) and ΔgcvBΔhfq (GS11149) strains. The levels of cycA and dksA mRNAs were determined by RT-PCR. Microarray results predicted cycA mRNA to be negatively regulated and dksA mRNA not to be regulated by GcvB. The amplification product for cycA was predicted to be 992 bp, and the amplification product for dksA was predicted to be 354 bp.](http://mic.sgmjournals.org)
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 φgt2, and lysogenized WT, ΔgcvB, Δhfq and ΔgcvABΔhfq strains. Next, we transformed ΔgcvBΔcycA::lacZ with the single-copy plasmid pGS594 (pgcvB$^{+}$) and transformed ΔhfqΔcycA::lacZ with the multi-copy plasmid pGS609 (phfq$^{++}$). These strains were grown in LB (with ampicillin for the 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 λppA::lacZ and λoppA::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
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::phaA, GcvB does not regulate cycA::lacZ in GM + glycine medium. In contrast, expression of cycA::lacZ in the Δhfq and ΔgcvBAΔhfq 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 ΔgcvBcycA::lacZ lysogen with single-copy plasmids carrying either the WT gcvB gene or the gcvB mutant alleles. When the ΔgcvBcycA::lacZ lysogen was transformed with the single-copy plasmid carrying the gcvB+::1316CC, gcvB+::142CA, gcgb+::159CC or gcgb+::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 flhA 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.

![Fig. 4. β-Galactosidase levels of i$cycA$::lacZ from WT (GS162), ΔgcvB (GS1144) and the ΔgcvB strain transformed with a single-copy plasmid carrying either the WT gcvB gene or a mutated gcvB allele. The numbers of the mutant alleles in columns 4–12 indicate the first nucleotide changed for each specific mutation relative to the gcvB +1 transcription initiation site. The strains were grown in LB (with ampicillin for complemented strains) to mid-exponential phase (OD600 ~0.5) and assayed for β-galactosidase activity (Miller, 1972). Results are averages of two independent assays with each assay performed in triplicate.](http://mic.sgmjournals.org)
Our failure to detect any mutations in GcvB that result in loss of repression of \(\text{cycA::lacZ}\) prompted us to test the region of GcvB from +62 to +88 shown to be important for regulation of \(dppA::\text{lacZ}\), \(oppA::\text{phoA}\) and \(sstT::\text{lacZ}\) expression (Pulvermacher et al., 2008; S. C. Pulvermacher and others, unpublished results). We transformed the \(\Delta\text{gcvB::cycA::lacZ}\) lysogen with single-copy plasmids carrying mutations in \(\text{cycB}\) between nucleotides +62 and +88 that disrupt complementarity between GcvB and the \(dppA::\text{lacZ}\), \(oppA::\text{phoA}\) and \(sstT::\text{lacZ}\) mRNAs, and result in a significant loss of GcvB repression of \(dppA::\text{lacZ}\), \(oppA::\text{phoA}\) or \(sstT::\text{lacZ}\) (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 \(dppA::\text{lacZ}\), \(oppA::\text{phoA}\) and \(sstT::\text{lacZ}\) expression. This is the region of the \(\text{cycA::lacZ}\) intermediate.

### Transcriptional fusions of \(\text{cycA}\) to \(\text{lacZ}\)

Our failure to identify any base pairs in GcvB that are essential for regulation of \(\text{cycA::lacZ}\) prompted us to construct a series of transcriptional fusions of \(\text{cycA}\) to \(\text{lacZ}\) to determine whether GcvB and Hfq regulate \(\text{cycA}\) at the level of transcription or post-transcriptionally, and to determine which region of the \(\text{cycA}\) mRNA is required for regulation (Fig. 1). The transcriptional fusions were cloned into \(\lambda\)gt2 and the phage used to lysogenize WT, \(\Delta\text{gcvB}\) and \(\Delta\text{hfq}\) strains. The strains were grown in LB to mid-exponential phase and assayed for \(\beta\)-galactosidase activity. We did not observe any significant regulation by GcvB and Hfq with the \(\lambda\text{cycA}^{-34}::\text{lacZ}\) and \(\lambda\text{cycA}^{-26}::\text{lacZ}\) lysogens (Fig. 5, columns 1–6). However, there was a twofold increase in \(\beta\)-galactosidase levels in the \(\lambda\text{cycA}^{-26}::\text{lacZ}\) lysogen compared with the \(\lambda\text{cycA}^{-34}::\text{lacZ}\) lysogen. This result suggests that a region of \(\text{cycA}\) mRNA between nucleotides −26 and −34 is important for a GcvB- and Hfq-independent mechanism of regulation. In contrast, the \(\lambda\text{cycA}^{-8}::\text{lacZ}\) lysogen showed twofold and 1.5-fold higher \(\beta\)-galactosidase levels in the \(\Delta\text{gcvB}\) and \(\Delta\text{hfq}\) strains, respectively, compared with the WT (Fig. 5, columns 7–9). The results suggest that the region of \(\text{cycA}\) 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 \(\text{cycA}\) 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 \(\Delta\text{gcvB}\) allele increases \(\text{d-cycloserine}\) sensitivity

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

### 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 the transport of amino acids and peptides 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.

ACKNOWLEDGEMENTS

This work was supported by Public Health Service Grant GM069506 from the National Institute of General Medical Sciences.

REFERENCES


Simultaneous exploitation of Serine, and Glycine in Escherichia coli.


Edited by: R. G. Sawers