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* 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.

Received 21 August 2008

Revised 1 October 2008

Accepted 2 October 2008

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; Hersberg *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; Udekwi *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 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;

Abbreviations: DEPC, diethylpyrocarbonate; sRNA, small non-translated regulatory RNA; WT, wild-type.

A supplementary table listing genes shown to be significantly regulated in the WT versus the $\Delta gcvB$ strain by microarray analysis is available with the online version of this paper.

Robbins & Oxender, 1973; Russell, 1972; Wargel *et al.*, 1971). 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 λ *cycA::lacZ* translational 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 *cycA* translation start site and a downstream primer with an *SmaI* site that hybridized to DNA beginning at codon 11 within the *cycA* structural gene. The PCR-amplified DNA fragment was digested with *EcoRI* and *SmaI*, and the 337 bp *EcoRI*–*SmaI* fragment was ligated into the *EcoRI* and *SmaI* 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 *pcycA::lacZ*. A 5788 bp *EcoRI*–*MfeI* fragment from *pcycA::lacZ* carrying the *cycA::lacZYA* fusion was then ligated into the *EcoRI* site of phage λ gt2 (Panasencko *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 λ chromosome by infection with

λ d90c17 (Shimada *et al.*, 1972). All lysogens were grown at 30 °C, since all fusion phages carry the λ d857 mutation, resulting in a temperature-sensitive λ d repressor (Panasencko *et al.*, 1977).

Three λ *cycA::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 *pcycA*^{–8}::*lacZ*, *pcycA*^{–26}::*lacZ* and *pcycA*^{–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 λ gt2 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 carry 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 (μ g 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

Strain or plasmid	Relevant genotype	Source or reference
Strains*		
GS162	WT	Our laboratory
GS1144	GS162 Δ <i>gcvB</i> :: Ω CM ^r (referred to as Δ <i>gcvB</i>)†	Pulvermacher <i>et al.</i> (2008)
GS1148	GS162 <i>hfq-1</i> :: Ω CM ^r (referred to as Δ <i>hfq</i>)	S. C. Pulvermacher, unpublished results
GS1149	GS162 Δ (<i>gcvAgcvB</i>):: Ω <i>aadA hfq-1</i> :: Ω CM ^r (referred to as Δ <i>gcvAB</i> Δ <i>hfq</i>)	S. C. Pulvermacher, unpublished results
Plasmids		
pGS341	Single-copy vector + WT <i>gcvA</i>	Jourdan & Stauffer (1998)
pGS571	Multicopy vector + WT <i>gcvB</i>	Jourdan & Stauffer (1999)
pGS594	Single-copy vector + WT <i>gcvB</i> (<i>pgcvB</i> ⁺)	Our laboratory
pGS595‡	pGS594 with a -TGTTGTGTT- to -ACAAgAgAA- change of bases +79 to +87 (<i>gcvB</i> ^{+79ACAAgAgAA})	Pulvermacher <i>et al.</i> (2008)
pGS596	pGS594 with a -TGT- to -CCC- change of bases +71 to +73 in <i>gcvB</i> (<i>gcvB</i> ^{+71CCC})	Pulvermacher <i>et al.</i> (2008)
pGS602	pGS594 with a -TGT- to -AAA- change of bases +76 to +78 in <i>gcvB</i> (<i>gcvB</i> ^{+76AAA})	Pulvermacher <i>et al.</i> (2008)
pGS605	pGS594 with a -TT- to -AA- change of bases +65 and +66 in <i>gcvB</i> (<i>gcvB</i> ^{+65AA})	Pulvermacher <i>et al.</i> (2008)
pGS609	Multicopy vector + WT <i>hfq</i> (<i>phfq</i> ⁺⁺)	S. C. Pulvermacher, unpublished results
pGS619	pGS594 with a -TG- to -CC- change of bases +71 and +72 in <i>gcvB</i> (<i>gcvB</i> ^{+71CC})	Pulvermacher <i>et al.</i> (2008)
pGS631	pGS594 with a -TT- to -CC- change of bases +131 and +132 in <i>gcvB</i> (<i>gcvB</i> ^{+131CC})	This study
pGS632	pGS594 with a -TG- to -CA- change of bases +142 and +143 in <i>gcvB</i> (<i>gcvB</i> ^{+142CA})	This study
pGS633	pGS594 with a -TG- to -CC- change of bases +159 and +160 in <i>gcvB</i> (<i>gcvB</i> ^{+159CC})	This study
pGS634	pGS594 with a -TG- to -CA- change of bases +142 and +143 and a -TG- to -CC- change of bases +159 and +160 in <i>gcvB</i> (<i>gcvB</i> ^{+142CA+159CC})	This study

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

†CM^r, chloramphenicol resistant.

‡The *pgcvB*^{+79ACAAgAgAA} 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.

Fig. 1. Sequence of the region upstream of the *cycA* translation start site and the beginning of the *cycA* structural gene. The AUG translation initiation site and Shine-Dalgarno sequence (SD) are overlined. The -A- nucleotide of the translation initiation codon is designated +1. The predicted transcription start site (Gama-Castro *et al.*, 2008; Rudd, 2000) occurs at nucleotide -84 relative to the translation start site. The upstream fusion site (not shown) was made at position -296 to ensure that all *cycA* regulatory elements would be contained in the cloned fragment. The translational fusion site at codon 11 is indicated by a black arrow. Transcriptional fusion sites at -8, -26 and -34 relative to the translation initiation site are indicated by red arrows. A region of complementarity between GcvB and *cycA* mRNA is indicated with lines between the sequences. Individual changes made to *gcvB* are indicated below the sequence and are colour coded.

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 *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 ($OD_{600} \sim 0.5$) 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 ($\Delta gcvB$) were grown in 10 ml LB to $OD_{600} \sim 0.5$. 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.

RNA isolation and RT-PCR. Bacterial strains GS162 (WT), GS1144 ($\Delta gcvB$), GS1148 (Δhfq), GS1149 ($\Delta gcvABA\Delta hfq$) and GS1144 transformed with the single-copy plasmid pGS594 (WT *gcvB*) were grown in 5 ml of LB to $OD_{600} \sim 0.5$. 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'-GCCTCGCTGGCGGTGATAGTGCTG (sense) and *cycA*-RT 5'-CAGTTCAGCAGCCGCTTCTTACC (antisense). The *dksA* control primers were *dksA*-RT 5'-GGTGGAA-CATATCAGGAG (sense) and *dksA*-RT 5'-GTCGATGCACAGATCGGCTG (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 $\Delta gcvB$ 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 $\Delta gcvB$ strain (Supplementary Table S1). As expected, the WT strain showed a >250 -fold higher GcvB level than the $\Delta gcvB$ strain. In addition, *dppA* 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 $\Delta gcvB$ 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 Δ *gcvB* strain grown in LB, determined by microarray analysis

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

Gene	Gene product type
<i>gcvB</i>	RNA
<i>rfbAC</i> , <i>mdoB</i> , <i>adhE</i> , <i>gdhA</i> , <i>glf</i> , <i>ilvBN</i> , <i>ilvC</i> , <i>serA</i> , <i>ssuE</i> , <i>wbbH</i> , <i>gatDZ</i> , <i>srlD</i> , <i>lon</i> , <i>cysD</i>	Enzyme
<i>wbbJK</i>	Enzyme, predicted
<i>cycA</i> *, <i>sstT</i> , <i>dppABCDF</i> , <i>argT</i> , <i>livK</i> , <i>gatABC</i> , <i>srlB</i>	Transporter
<i>rfbX</i> , <i>yifK</i> , <i>emrY</i> , <i>yicO</i>	Transporter, predicted
<i>fimZ</i>	Regulator
<i>yafU</i> , <i>ybfB</i>	Membrane, predicted
<i>clpB</i> , <i>ibpA</i> , <i>dnaKJ</i> , <i>htpG</i> , <i>groS</i> , <i>hdeB</i>	Factor
<i>sfmH</i>	Structural component
<i>ydhU</i> , <i>ygeG</i>	Carrier, predicted
<i>slp</i> , <i>yddL</i>	Lipoprotein
<i>ykiB</i> , <i>ymgG</i> , <i>yncH</i> , <i>yedM</i> , <i>ypjC</i> , <i>ygcI</i> , <i>yqeK</i> , <i>ygeF</i> , <i>ygeI</i> , <i>ygeL</i> , <i>ygeM</i> , <i>yhaC</i> , <i>yrhA</i> , <i>yhiL</i> , <i>yibG</i> , <i>yjbM</i> , <i>yebB</i>	Unknown

*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 Δ *gcvB* 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 ($OD_{600} \sim 0.5$) and total cellular RNA was isolated. We observed an increased level of DNA corresponding to *cycA* mRNA amplified from the Δ *gcvB* 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 Δ *gcvB* 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 Δ *hfq* strain GS1148 and the Δ *gcvAB* Δ *hfq* double mutant GS1149 grown

Fig. 2. Expression of *cycA* and *dksA* from WT (GS162), Δ *gcvB* (GS1144), Δ *gcvB* complemented [GS1144(pGS594)], Δ *hfq* (GS1148) and Δ *gcvAB* Δ *hfq* (GS1149) 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.

in LB at 30 °C to mid-exponential phase ($OD_{600} \sim 0.5$). We observed an increased level of DNA corresponding to *cycA* mRNA amplified from the Δhfq and $\Delta gcvAB\Delta hfq$ 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 $\Delta gcvAB\Delta hfq$ 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 λ gt2, and lysogenized WT, $\Delta gcvB$, Δhfq and $\Delta gcvAB\Delta hfq$ strains. Next, we transformed $\Delta gcvB\lambda cycA::lacZ$ with the single-copy plasmid pGS594 ($pgcvB^+$) and transformed $\Delta hfq\lambda cycA::lacZ$ with the multi-copy plasmid pGS609 ($phfq^{++}$). These strains were grown in LB (with ampicillin for the complemented strains) to mid-exponential growth phase ($OD_{600} \sim 0.5$) and assayed for β -galactosidase activity. Expression of *cycA::lacZ* in the $\Delta gcvB$ 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 $\Delta gcvAB\Delta hfq$ 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 $\Delta gcvAB\Delta hfq$ 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 $\Delta gcvB$ 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 $\Delta gcvB$ 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 $\Delta gcvB$ 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 $\lambda dppA::lacZ$ and $\lambda oppA::phoA$ 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

Fig. 3. β -Galactosidase levels of $\lambda cycA::lacZ$ lysogens. WT (GS162), $\Delta gcvB$ (GS1144), Δhfq (GS1148), $\Delta gcvAB\Delta hfq$ (GS1149), $\Delta gcvB$ complemented [GS1144(pGS594)] and Δhfq complemented [GS1148(pGS609)] $\lambda cycA::lacZ$ lysogens were grown in either (a) LB or (b) GM + glycine (+ ampicillin for complemented strains) to mid-exponential phase ($OD_{600} \sim 0.5$) 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 Δ *gcvB* 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 Δ *hfq* and Δ *gcvAB* Δ *hfq* 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 Δ *hfq*-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 Δ *gcvB* λ *cycA::lacZ* lysogen with single-copy plasmids carrying either the WT *gcvB* gene or the *gcvB* mutant alleles. When the Δ *gcvB* λ *cycA::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

Fig. 4. β -Galactosidase levels of λ *cycA::lacZ* from WT (GS162), Δ *gcvB* (GS1144) and the Δ *gcvB* 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 (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.

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 *fhlA* 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 *cycA::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 $\Delta gcvB \lambda cycA::lacZ$ 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 ($OD_{600} \sim 0.5$) 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*, *oppA* and *sstT* regulation is not required for *cycA* repression. It is possible that GcvB does not act directly on *cycA* mRNA, but instead regulates an additional factor necessary for *cycA* 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 *cycA* to *lacZ*

Our failure to identify any base pairs in GcvB that are essential for regulation of *cycA::lacZ* prompted us to construct a series of transcriptional fusions of *cycA* to *lacZ* to determine whether GcvB and Hfq regulate *cycA* at the level of transcription or post-transcriptionally, and to determine which region of the *cycA* mRNA is required for regulation (Fig. 1). The transcriptional fusions were cloned into $\lambda gt2$ and the phage used to lysogenize WT, $\Delta gcvB$ 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 regulation by GcvB and Hfq with the $\lambda cycA^{-34}::lacZ$ and $\lambda cycA^{-26}::lacZ$ lysogens (Fig. 5, columns 1–6). However, there was a twofold increase in β -galactosidase levels in the $\lambda cycA^{-26}::lacZ$ lysogen compared with the $\lambda cycA^{-34}::lacZ$ lysogen. This result suggests that a region of *cycA* mRNA between nucleotides –26 and –34 is important for a GcvB- and Hfq-independent mechanism of regulation. In contrast, the $\lambda cycA^{-8}::lacZ$ lysogen showed twofold and 1.5-fold higher β -galactosidase levels in the $\Delta gcvB$ and Δhfq strains, respectively, compared with the WT (Fig. 5, columns 7–9). The results suggest that the region of *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 *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

Fig. 5. β -Galactosidase levels of *λcycA::lacZ* transcriptional fusions. WT (GS162), $\Delta gcvB$ (GS1144), and Δhfq (GS1148) $\lambda cycA^{-8}::lacZ$, $\lambda cycA^{-26}::lacZ$ and $\lambda cycA^{-34}::lacZ$ lysogens were grown in LB to mid-exponential phase ($OD_{600} \sim 0.5$) and assayed for β -galactosidase activity (Miller, 1972). Results are averages of two independent assays with each sample tested in triplicate.

produce altered regulation or an alternative regulatory intermediate is necessary for repression.

$\Delta gcvB$ allele increases D-cycloserine sensitivity

The *E. coli cycA* gene encodes a permease for transporting D-alanine, D-serine and glycine into the cell (Cosloy, 1973; Robbins & Oxender, 1973). The CycA 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 CycA levels, we hypothesized that a WT strain, with repressed levels of CycA, would be more resistant to D-cycloserine than a $\Delta gcvB$ strain, with derepressed levels of CycA. To test this hypothesis, the WT and $\Delta gcvB$ strains were grown in LB or LB + D-cycloserine at 30 °C. The presence of the $\Delta gcvB$ allele consistently resulted in earlier and more complete lysis of cells (Fig. 6). In separate experiments in which the $\Delta gcvB$ 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 $\Delta gcvB$ strain is due to increased levels of CycA 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

Fig. 6. Effects of D-cycloserine on growth of the WT strain GS162 and the $\Delta gcvB$ strain GS1144. Cells were grown overnight in LB at 30 °C, and 50 μ l samples were then used to inoculate 5 ml fresh LB or LB supplemented with 2.5×10^{-4} M D-cycloserine. Growth was monitored until cell lysis occurred. ○, WT strain; □, $\Delta gcvB$ strain, grown in LB. ●, WT strain; ■, $\Delta gcvB$ strain, grown in LB with D-cycloserine. Results are the averages of two independent experiments.

stress responses and virulence gene regulation (Argaman *et al.*, 2001; Gottesman, 2004; Romby *et al.*, 2006; Vogel & Papenfort, 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.

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Edited by: R. G. Sawers