Genetic analysis of the GcvA binding site in the *gcvA* control region

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The GcvA protein both activates and represses the *gcv* operon and negatively regulates its own transcription. GcvA binds to three sites in the *gcv* control region and to one site in the *gcvA* control region; each of these binding sites contains the conserved 5 bp DNA sequence 5'-CTAAT-3'. This report describes the role this DNA sequence plays in autoregulation and expression of *gcvA*. Through single base-pair mutations, the importance of three of these five basepairs in the autoregulation of *gcvA* expression is shown. Two of the *gcvA* control region mutations described cause a *gcvA::lacZ* fusion to be overexpressed at 9–24 times the wild-type level. The increase in expression is due in part to a complete loss of autoregulation and in part to a GcvA-independent mechanism. One of the mutants was shown by Western blot analysis to increase the intracellular concentration of GcvA. This high level of *gcvA* expression subsequently causes the loss of purine-mediated repression of a *gcvT::lacZ* fusion. However, overexpression of *gcvR* re-established purine-mediated repression of the *gcvT::lacZ* fusion, supporting the model for *gcv* regulation that suggests the need for a relatively constant GcvA to GcvR ratio for appropriate regulation of *gcv* expression in response to glycine and purine availability.

Keywords: *gcvA*, GcvA, glycine cleavage, autoregulation, DNA binding site mutations

INTRODUCTION

The glycine cleavage (GCV) enzyme system in *Escherichia coli* catalyses the oxidative cleavage of glycine to CO₂ and NH₃, and the transfer a C-1 methylene unit to tetrahydrofolate (Kikuchi, 1973) (Fig. 1). The resulting 5,10-methylenetetrahydrofolate is used as a C-1 donor in the biosynthesis of purines, methionine, thymine and other cellular components (Mudd & Cantoni, 1964). In *E. coli*, three of the proteins of the GCV enzyme complex are encoded by the *gcvTHP* operon which maps at 62.5 min on the chromosome (Plamann *et al.*, 1983; Stauffer *et al.*, 1994). The fourth protein, encoded by *lpd*, is not a part of the *gcv* operon and maps at 2.5 min (Steiert *et al.*, 1990).

Regulation of the *gcv* operon is complex and involves at least four proteins. PurR, a global regulator in *E. coli* involved in negative regulation of many purine and pyrimidine *de novo* biosynthesis genes (Kilstrup *et al.*,

1989; Rolfes & Zalkin, 1988), also represses *gcv* expression twofold in the presence of exogenous purines. *In vitro* studies have shown that PurR binds the *gcv* control region at a site overlapping the transcriptional start site of *gcvT*, the first gene of the *gcv* operon, and presumably interferes with transcription initiation by RNA polymerase (Wilson *et al.*, 1993a).

GcvR is also required for repression of *gcv* (Ghrist & Stauffer, 1995). Mutations in *gcvR* result in high constitutive expression of a *gcvT::lacZ* fusion and overexpression of *gcvR* results in superrepression of the fusion. Data suggest that repression is mediated through the GcvA protein (Ghrist & Stauffer, 1995). GcvR alone does not bind to the *gcv* promoter (A. Ghrist & G. Stauffer, unpublished data) and whether it is directly or indirectly involved in the repression mechanism has not been determined.

The leucine-responsive regulatory protein (Lrp) is another global regulator in *E. coli* and has been shown to activate or repress many genes involved in amino acid metabolism (Calvo & Matthews, 1994; Newman *et al.*, 1992). An *lrp* mutant containing a *gcvT::lacZ* fusion shows low, uninducible β -galactosidase synthesis (Lin *et*

Abbreviations: GCV, glycine cleavage; GM, glucose minimal; GMS, gel mobility shift; LM, lactose minimal; Lrp, leucine-responsive regulatory protein; TPEG, phenylethyl- β -D-thiogalactoside; wt, wild-type.

Fig. 1. The serine-glycine pathway in *E. coli*. The genes and the enzymes encoded are as follows: *serA*, 3-phosphoglycerate dehydrogenase; *serC*, 3-phosphoserine aminotransferase; *serB*, 3-phosphoserine phosphatase; *glyA*, serine hydroxymethyltransferase; *lpd*, lipoamide dehydrogenase; *gcv*, glycine cleavage enzyme system. THF, tetrahydrofolate.

al., 1992; Stauffer & Stauffer, 1994). *In vitro* studies identified multiple Lrp binding sites in the *gcv* control region from base -229 to -92 relative to the transcription initiation site (Stauffer & Stauffer, 1994). Lrp appears to play primarily a structural role in *gcv* regulation, bending the DNA (Stauffer & Stauffer, 1999).

GcvA, a member of the LysR family of transcriptional regulators (Schell, 1993), plays a dual role in *gcv* regulation by mediating a fivefold, PurR-independent repression of *gcv* expression in the presence of purines and a six- to sevenfold activation in the presence of glycine (Wilson *et al.*, 1993a, b). GcvA binds to three sites in the *gcv* control region from base -271 to -242 (site 3), from -242 to -214 (site 2) and from -69 to -34 (site 1) relative to the transcription initiation site (Wilson *et al.*, 1995). All three sites are required for GcvA-mediated repression, but only the upstream sites 2 and 3 are required for GcvA-mediated activation (Wilson *et al.*, 1995). GcvA also binds to a site in the *gcvA* control region from base -28 to +20 relative to the *gcvA* transcription initiation site, negatively regulating its own expression over an approximately threefold range (Wilson & Stauffer, 1994). A comparison of these four GcvA binding sites revealed a conserved 5 bp DNA sequence, 5'-CTAAT-3', shown to be important for GcvA binding (Wilson *et al.*, 1995) (Fig. 2a). Overexpression of *gcvA* results in constitutive expression of a *gcvT::lacZ* fusion in the absence of exogenous glycine and in the presence of exogenous purines (Ghrst & Stauffer, 1995), and thus it appears critical that GcvA levels are maintained within a narrow range for the cell

to respond appropriately to a glycine-inducing or purine-repressing signal. A model was previously proposed to explain how GcvA and GcvR might interact to regulate *gcv* expression, where GcvA homocomplexes function as activators and GcvA-GcvR heterocomplexes function as repressors (Ghrst & Stauffer, 1995; Wilson & Stauffer, 1994). This model proposes that the amount of each type of complex formed is likely to be due to the level of the possible coregulators (glycine and purines). High glycine levels would favour activator formation and high purine levels would favour repressor formation. This model also predicts that artificially raising the levels of either GcvA or GcvR would favour the formation of either activator or repressor complexes, respectively.

To define domains of the GcvA protein involved in activation and repression, a genetic selection was designed to screen for mutations in *gcvA* that produce proteins able to activate but unable to repress the *gcv* operon. One of the mutations isolated, however, was in the *gcvA* control region rather than in the structural gene and altered *gcvA* expression and subsequently *gcvT::lacZ* expression. The results are consistent with the model proposed above to explain how GcvA is involved in accomplishing activation and repression of the *gcv* operon based on GcvA being maintained at a relatively constant ratio with the GcvR protein.

METHODS

Strains and plasmids. The genotypes of strains and plasmids used in this study are listed in Table 1. All are laboratory strains or were constructed during this investigation.

Media. The minimal growth medium used was Vogel & Bonner (1956) minimal salts supplemented with 0.4% glucose (GM) or lactose (LM) and appropriate amino acids and antibiotics as needed. The complex medium used was Luria-Bertani broth (LB) (Miller, 1992). Supplements were added at the following concentrations: phenylalanine, 50 µg ml⁻¹; phenylethyl-β-D-thiogalactoside (TPEG), 2 mM; inosine, 50 µg ml⁻¹; glycine, 300 µg ml⁻¹; vitamin B₁, 1 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; ampicillin, 50 µg ml⁻¹ for single-copy plasmids and 100 µg ml⁻¹ for multicopy plasmids. Minimal medium was supplemented with phenylalanine and vitamin B₁ because all of the strains used in this study contain *pheA905* and *thi* mutations.

Enzyme assays. β-Galactosidase assays were performed as described by Miller (1992). All results are the means of two or more assays, with each sample done in triplicate. Protein concentrations were determined by the method of Lowry.

DNA manipulation. Isolation of plasmid DNA, restriction digestions, ligations, DNA sequencing and plasmid transformations were performed as described by Sambrook *et al.* (1989).

PCR mutagenesis and site-directed mutagenesis. Random mutagenesis of *gcvA* was performed using the PCR mutagenesis protocol (Zhou *et al.*, 1991). The upstream primer was complementary to the DNA template upstream of the *gcvA* gene (which retains its own promoter) and a unique *EcoRI* site, and the downstream primer was complementary to the DNA template distal to the *gcvA* gene and a unique *HindIII* site. Several independently mutagenized PCR products were

Table 1. *E. coli* K-12 strains and plasmids used in this study

Strain or plasmid	Relevant genotype*	Source or reference
Strain		
GS162†	wt	This laboratory
GS973†	<i>gcvA1</i> λ gcvT::lacZ	This laboratory
GS986†	<i>gcvA1 purR</i> ::Tn10 λ gcvT::lacZ	This laboratory
GS998†	<i>gcvA1</i>	This laboratory
GS1039†	<i>gcvA1 serA25 purR</i> ::Tn10 λ gcvT::lacZ	This laboratory
Plasmids		
pMC1403	<i>lac</i> fusion vector	Casadaban <i>et al.</i> (1980)
pGS272	pACYC177 derivative, <i>Sma</i> I site converted to <i>Eco</i> RI site in <i>kan</i> gene, <i>Kn</i> ^s Ap ^r	This laboratory
pGS311	Single-copy plasmid, Ap ^r , derived from plasmid pDF41‡	This laboratory
pGS334	1 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvR</i> fragment in pBR322, Ap ^r Tc ^r	This laboratory
pGS335	1.3 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvA</i> fragment in pGS272, Ap ^r	This study
pGS341	1.3 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvA</i> fragment from pGS335 in pGS311, Ap ^r	This study
pGS383	<i>Eco</i> RI to <i>Sca</i> I deletion in pGS334, Ap ^s Tc ^r	This laboratory
pGS468	1.3 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvA</i> + 13A fragment (T to A base change at +13) in pGS311, Ap ^r	This study
pGS469	1.3 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvA</i> + 15G fragment (A to G base change at +15) in pGS311, Ap ^r	This study
pGS470	1.3 kb <i>Eco</i> RI– <i>Hind</i> III <i>gcvA</i> + 16A fragment (T to A base change at +16) in pGS311, Ap ^r	This study

* Ap, ampicillin; *Kn*, kanamycin; Tc, tetracycline.

† These strains also carry *thi*, *pheA905*, Δ *lacU169*, *araD129* and *rpsL150* mutations.

‡ Plasmid pDF41 (Kahn *et al.*, 1979) was obtained from S. R. Kushner, University of Georgia, USA.

collected, digested with *Eco*RI and *Hind*III, purified from a low-melting-point agarose gel, ligated into the *Eco*RI–*Hind*III sites of the single-copy plasmid pGS311 and transformed into the *gcvA1* strain GS1039 lysogenized with a λ gcvT::lacZ translational fusion phage.

Site-directed mutagenesis of *gcvA* was performed using a PCR 'megaprimer' mutagenesis protocol (Sarkar & Sommer, 1990). Changes were introduced through downstream primers complementary to *gcvA* except at the position of the desired base change. PCR products were generated using a primer upstream of a unique *Eco*RI site and complementary to vector DNA outside of the *gcvA* insert. These PCR products were then used as the upstream megaprimers in another round of PCR synthesis against the same DNA template. The downstream primer was complementary to an internal segment of *gcvA* distal to the mutagenic primer and an internal *Mlu*I site. PCR products were cut with *Eco*RI and *Mlu*I, purified from a low-melting-point agarose gel and ligated into *Eco*RI–*Mlu*I-digested plasmid pGS341. The resulting plasmids (pGS468, pGS470 and pGS471) each contained specific base mutations that were verified by the complete DNA sequence analysis of each *Eco*RI–*Mlu*I fragment.

Construction of lysogens. Plasmids pGS468, pGS469 and pGS470 were digested with *Eco*RI and *Eco*RV and the 519 bp fragment from each digest, containing the 303 bp region upstream of *gcvA* and the first 72 codons of *gcvA*, were isolated from a low-melting-point agarose gel and ligated into the *Eco*RI–*Sma*I sites of plasmid pMC1403 (Casadaban *et al.*,

1980), forming an in-frame translational fusion to *lacZ*. The resulting plasmids were sequenced across the fusion junction, confirming that the correct reading frame had been maintained. Each plasmid was cut at a unique *Sal*I site at the end of the *lacZYA* segment, the ends filled in using T4 DNA polymerase and *Eco*RI linkers added using T4 DNA ligase. The plasmids were then digested with *Eco*RI and a 6686 bp *Eco*RI–*Eco*RI fragment, containing each mutant *gcvA* control region, the first 72 codons of *gcvA* and *lacZYA*, was isolated and cloned into the *Eco*RI site of λ gt2 (Panassenko *et al.*, 1977). In the resulting phages β -galactosidase synthesis is under control of the *gcvA* control region. The phages were used to lysogenize appropriate strains as described previously (Urbanowski & Stauffer, 1986). The lysogens were tested for the presence of a single copy of λ phage by infection with λ c190c17 (Shimada *et al.*, 1972). The effect of each of the mutations in the *gcvA* control region on *gcvA* expression was measured by β -galactosidase assay. As controls, a wild-type (wt) λ gcvA::lacZ fusion constructed previously in an identical manner (Wilson & Stauffer, 1994) was used to lysogenize appropriate strains and β -galactosidase activity was measured.

Gel mobility shift assays. Gel mobility shift (GMS) assays were performed based on the methods of Fried & Crothers (1981) and Garner & Revzin (1981). A 519 bp *Eco*RI–*Bam*HI fragment from each of the *gcvA*::lacZ fusion plasmids, containing 303 bp upstream of the *gcvA* transcription start site and the first 72 codons of *gcvA*, was ³²P-labelled at the unique *Eco*RI site using T4 polynucleotide kinase (Sambrook *et al.*,

1989). The assay (20 μ l final volume) was performed by incubating each labelled DNA fragment in DNA binding buffer (5 mM Tris/HCl, pH 7.5, 25 mM KCl, 0.5 mM EDTA, 2.5% glycerol, 0.5 mM DTT) plus 125 μ g BSA ml⁻¹ for 5 min at 37 °C and then adding 2 μ l volumes of twofold serial dilutions of GcvA (Jourdan & Stauffer, 1998) diluted in DNA binding buffer plus 125 μ g BSA ml⁻¹. Incubation was continued at 37 °C for 15 min, 2 μ l loading dye (0.1% xylene cyanol, 50% glycerol) was added and the samples were loaded and run on a 5% polyacrylamide/3% glycine gel.

Western blot analysis. Cells were grown in 1 ml GM plus ampicillin (50 μ g ml⁻¹) overnight at 30 °C, then diluted into 50 ml GM plus ampicillin and incubated at 30 °C until mid-exponential phase (OD₆₀₀ ~ 0.5). Cells were collected at 4 °C by centrifugation and the cell pellet was frozen overnight at -70 °C. The cell pellet was resuspended in 2 ml sodium phosphate buffer (50 mM sodium phosphate, 500 mM NaCl) and sonicated on ice until viscous and clear. Cytoplasmic and membrane fractions were separated by centrifugation and the supernatant was brought to 33% ammonium sulfate saturation. Precipitated protein was collected by centrifugation and resuspended in 120 μ l TEG buffer (50 mM Tris/HCl, pH 7.9, 0.5 mM EDTA, 5% glycerol). Protein concentration was determined using the Bio-Rad Protein Assay Kit II. Proteins were electrophoresed by SDS-PAGE, followed by transfer onto a nitrocellulose membrane as described by Sambrook *et al.*, (1989). The blotted proteins were reacted with primary antibody (Tetra-His Antibody; Qiagen) diluted according to the manufacturer's suggestions, followed by reaction with secondary antibody [alkaline-phosphatase-conjugated polyclonal sheep anti-mouse IgG (H+L); ICN Biomedicals] diluted according to the manufacturer's directions. Colorimetric detection was done according to the Qiagen protocol for Western blot immunodetection.

RESULTS AND DISCUSSION

Isolation of a *gcvA* mutant affecting purine repression of *gcv*

It was shown previously that GcvA functions to both activate and repress a *gcvT::lacZ* fusion (Wilson *et al.*, 1993a, b). To define amino acids in GcvA important for its repressor function, we attempted to isolate *gcvA* mutants that encoded proteins that could no longer repress *gcvT::lacZ* expression in the presence of purines but could activate expression in the presence of glycine. The following selection strategy was used to obtain such *gcvA* mutants. Strain GS1039 is a *serA gcvA* double mutant that requires serine for growth. This double mutant cannot grow on a GM plate supplemented with only glycine because in the absence of GcvA there are insufficient C-1 units available from the GCV enzyme system for the conversion of glycine into serine via the serine hydroxymethyltransferase reaction (Fig. 1). If wt *gcvA* is supplied *in trans*, growth on a GM plate supplemented with glycine occurs because GcvA, in the presence of glycine, activates expression of the GCV enzyme system resulting in cleavage of glycine and the production of C-1 units. Although strain GS1039 carries a *gcvT::lacZ* translational gene fusion on an integrated λ phage, when wt *gcvA* is supplied *in trans* this lysogen cannot grow on a LM plate supplemented with serine, inosine and TPEG (a competitive inhibitor of β -

galactosidase; Miller, 1992) because GcvA, in the presence of inosine, represses the fusion resulting in insufficient β -galactosidase expression to support growth on lactose. The presence of TPEG increases the stringency of the selective media. Lysogen GS1039 was transformed with a single-copy plasmid pool carrying PCR-induced random base changes in *gcvA* (including the promoter region) and plated on LB agar (appropriate antibiotics were added to all selection media). Colonies were patched to LM plates supplemented with serine, inosine and TPEG, and to GM plates supplemented with glycine. Our selection assumed that cells containing mutations in *gcvA* that alter the repressor function but not the activator function of GcvA would grow on both of the scoring media. One of about 1000 transformants tested was capable of growth on both of the scoring media. The transformant was single-colony-purified and the plasmid carrying the putative *gcvA* repressor mutation was isolated.

To determine the effects of the *gcvA* repressor mutation on *gcvT::lacZ* expression, the plasmid carrying the putative *gcvA* mutation was used to transform the *gcvA1* mutant strain GS986 which was lysogenized with a *gcvT::lacZ* fusion. Repression of the *gcvT::lacZ* fusion in strain GS986 is mediated through the plasmid-borne *gcvA*-encoded protein and not PurR. As a control, lysogen GS986 was also transformed with plasmid pGS341 which carries the wt *gcvA* gene. The transformants were grown in GM medium or GM medium supplemented with either glycine or inosine and assayed for β -galactosidase activity. GS986 transformed with the plasmid carrying the repressor mutation in *gcvA* displayed normal induction of *gcvT::lacZ* expression when grown in the presence of glycine, but in the presence of inosine there was an approximately twofold increase in *gcvT::lacZ* expression compared with that of the control strain GS986(pGS341) (data not shown). These results suggest that the plasmid carries the relevant *gcvA* repressor mutation allowing growth on the scoring plates.

The mutant *gcvA* gene from the plasmid was sequenced and a single base change from A to G at position +15 relative to the transcriptional start site was identified and designated +15G (Fig. 2b). This A to G transition occurs within the conserved 5 bp 5'-CTAAT-3' sequence common to all four known GcvA binding sites and is believed to be involved in binding of GcvA to DNA (Wilson *et al.*, 1995) (Fig. 2). The plasmid carrying the +15G *gcvA* mutation was designated pGS469.

The +15G mutation in the GcvA binding site causes overexpression of *gcvA::lacZ*

Overexpression of wt *gcvA* from the *tac* promoter results in a relief of purine-mediated repression of a *gcvT::lacZ* fusion (Ghrist & Stauffer, 1995). Because the +15G mutation also resulted in a relief of purine-mediated repression of a *gcvT::lacZ* fusion and lies in the putative autoregulatory GcvA consensus binding site, it was hypothesized that this mutant might over-

* Denotes the *λgcvA::lacZ* wt and mutant phages used to lysogenize strains GS998 and GS162.

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Fig. 3. Gel mobility shift assay for the binding of GcvA to wt and mutant *gcvA* DNAs. The 519 bp fragments containing the wt and mutant *gcvA* control regions were incubated with GcvA protein and the samples were run on a 5% polyacrylamide/3% glycine gel (see Methods). Each reaction was performed in a 20 μ l volume. (a) Lanes: 1–6, wt DNA; 7–12, *gcvA* +16A DNA; 1 and 7, no protein; 2–6 and 7–12, 5, 10, 20, 40 and 80 nM GcvA protein, respectively. (b) Lanes: 1–6, *gcvA* +13A DNA; 7–12, *gcvA* +15G DNA; 13–18, wt *gcvA* DNA; 1, 7 and 13, no protein; 2–6, 8–12 and 14–18, 5, 10, 20, 40 and 80 nM GcvA protein, respectively. The unbound DNA fragments are indicated. Complexes A and B are likely to represent GcvA bound to one half and the full 48 bp GcvA target sequence, respectively.

region are known to affect promoter strength (Record *et al.*, 1996), it should be noted that the changes reported here are a considerable distance from the +1 transcription initiation site. These changes may have generated a new promoter that is stronger than the wt promoter. However, the base changes do not create a recognizable promoter consensus sequence. Changing these bases may have altered the efficiency at which the mRNA is translated, or may have increased the stability of the mRNA. Finally, there could be an additional *E. coli* protein that regulates *gcvA* expression, or a protein that normally does not recognize the *gcvA* control region DNA but, when this DNA is mutated, now increases *gcvA* expression. We are developing *in vitro* assays and *in vivo* selections that should allow us to determine which of the above possible scenarios is most likely responsible for the altered *gcvA* expression in these mutants.

GcvA binding to the mutant *gcvA* control regions

GMS assays were performed to determine if the loss of autoregulation observed by changing bases in the GcvA binding site were due to an altered binding affinity of GcvA for DNA (Fig. 3). Purified GcvA protein used in all

GMS assays is given in monomer concentrations (Jourdan & Stauffer, 1998). At the lower concentrations of protein used in the GMS assay the wt *gcvA* fragment was shifted to two bands of slower mobility (Fig. 3a, lanes 2–3; Fig. 3b, lanes 14–16; complexes A and B). At the higher concentrations of protein used the fragment was shifted to multiple bands of slower mobility (Fig. 3a, lanes 4–6; Fig. 3b, lanes 17–18). It should be noted that complex B is the predominant band at any GcvA concentration used in the assay. Previous results from a DNase I footprint analysis of *gcvA* identified a single target site of about 48 bp (Fig. 2) (Wilson *et al.*, 1995). We believe that complex A and B indicate that the binding region is made up of two target sites and that GcvA binding to these two sites is cooperative. However, it is also possible that the two sites have essentially equal affinity for GcvA. The additional bands of slower mobility at the higher concentrations of GcvA used in the assay are likely to be due to non-specific binding, as DNase I footprint assays did not show protection other than the GcvA binding region previously identified (Fig. 2) (data not shown). The +16A fragment had a GMS pattern essentially the same as the wt (Fig. 3a, compare lanes 7–12 with lanes 1–6). The amount of GcvA protein required to bind and shift the +13A and +15G *gcvA*

Table 3. Effects of varying *gcvA* expression on *gcvT::lacZ* expression

All plasmids were assayed in strain GS986. Cells were grown in GM medium with the indicated supplements and assayed for β -galactosidase activity.

Plasmid	<i>gcvA</i> allele	β -Galactosidase activity (Miller units)		
		None	Glycine	Inosine
pGS341	wt <i>gcvA</i>	157 $\pm$ 10	732 $\pm$ 26	26 $\pm$ 1
pGS468	<i>gcvA</i> + 13A	942 $\pm$ 50	1148 $\pm$ 46	649 $\pm$ 13
pGS469	<i>gcvA</i> + 15G	182 $\pm$ 7	705 $\pm$ 23	54 $\pm$ 3
pGS470	<i>gcvA</i> + 16A	187 $\pm$ 11	791 $\pm$ 79	34 $\pm$ 2

fragments was essentially the same as the wt fragment (Fig. 3b, compare lanes 1–6 and 7–12 with lanes 13–18), suggesting that the overall affinity of GcvA for the mutant DNAs was not significantly altered. However, complex A present in the GMS pattern of the wt and +16A *gcvA* DNA, was not observed for the +13A and +15G *gcvA* DNAs. At the two highest concentrations of protein, there was essentially no difference in the GMS patterns between the +13A and +15G mutant and wt *gcvA* DNAs (Fig. 3b, compare lanes 5–6 and 11–12 with lanes 17–18). A DNase I footprint analysis of the +13A and +15G DNAs revealed that despite the slightly altered GMS pattern there was no significant difference in the DNase I protection observed between the mutant *gcvA* control regions and the wt control region (data not shown). Despite the similarity in the overall affinity of GcvA for the wt and mutant DNAs *in vitro*, GcvA either does not bind *in vivo* or when bound to these DNAs *in vivo* is unable to regulate *gcvA::lacZ* expression. The results also suggest that the 5'-CTAAT-3' sequence shown to be important for GcvA binding in the *gcv* control region (Wilson *et al.*, 1995) is not essential for GcvA binding in the *gcvA* control region.

Modifying the level of *gcvA* expression affects *gcv* regulation

Previous studies have shown that regulation of a *gcvT::lacZ* fusion is sensitive to the level of GcvA in the cell (Ghrist & Stauffer, 1995). Overexpression of *gcvA* leads to the loss of purine-mediated repression and constitutive expression of a *gcvT::lacZ* fusion in the absence of exogenously added glycine (Ghrist & Stauffer, 1995). Each of the mutants described above had either increased or decreased *gcvA::lacZ* expression. To determine the effects the altered levels of *gcvA* expression had on *gcvT::lacZ* expression, plasmids were constructed in which each mutant control region was translationally fused upstream of the *gcvA* structural gene (see Methods). The plasmids were then transformed into the *gcvA1 purR::Tn10* mutant strain GS986 that was lysogenized with a λ *gcvT::lacZ* fusion. The transformants were grown in GM medium and GM medium supplemented with either glycine or inosine and

assayed for β -galactosidase activity. The wt control transformant GS986(pGS341) showed a 4.7-fold glycine-mediated induction and a 6-fold purine-mediated repression of the *gcvT::lacZ* fusion (Table 3). Transformant GS986(pGS469), carrying the *gcvA* +15G mutation which caused an 8.7-fold overexpression of the *gcvA::lacZ* fusion (Table 2), resulted in a 2-fold loss of GcvA-mediated repression of the *gcvT::lacZ* fusion when grown in the presence of inosine (Table 3). The +15G base change had no significant effect on *gcvT::lacZ* expression when the lysogen was grown in GM medium or GM medium supplemented with glycine. Transformant GS986(pGS468), carrying the *gcvA* +13A mutation which caused a 22-fold overexpression of the *gcvA::lacZ* fusion (Table 2), resulted in constitutive expression of the *gcvT::lacZ* fusion under all growth conditions (Table 3). These results suggest that the ability of the *gcvT::lacZ* fusion to be regulated in response to normal physiological signals for activation and repression was altered by increased levels of GcvA. Transformant GS986(pGS470), carrying the *gcvA* +16A mutation which had no significant effect on levels of *gcvA::lacZ* expression (Table 2), showed essentially normal *gcvT::lacZ* expression compared to the wt *gcvT::lacZ* fusion (Table 3).

The +13A mutant control region increases intracellular levels of GcvA

We tested directly whether the +13A base change in the *gcvA* control region that caused *gcvA::lacZ* expression to increase 22-fold resulted in overproduction of the GcvA protein by Western blot analysis. The Tetra-His Antibody (Qiagen) reacts with four histidine residues present in a histidine-tagged protein. A sequence encoding six histidine residues (His₆) was added to the 3' end of the *gcvA* gene (*gcvA::His₆*) (Jourdan & Stauffer, 1998). Strain GS986 transformed with single-copy plasmids carrying the wt control region upstream of *gcvA::His₆*, the +13A control region upstream of *gcvA::His₆* and the vector only were grown as described, protein prepared and used in Western blot analysis (see Methods). The vector only protein preparation did not react with the Tetra-His Antibody at any

Fig. 4. Western blot analysis of the amount of GcvA expressed from the wt and +13A *gcvA* control regions. Lanes 1–3, 128, 64 and 32 µg crude extract protein, respectively, prepared from vector only transformants; 4–6, 128, 64 and 32 µg crude extract protein, respectively, prepared from +13A transformants; 7–9, 128, 64 and 32 µg crude extract protein, respectively, prepared from wt transformants.

of the protein concentrations used (Fig. 4, lanes 1–3). The GcvA–His₆ protein preparation under control of its native promoter gave a weak signal that increased as the protein concentration was increased (Fig. 4, lanes 7–9). The low level of GcvA–His₆ detected corresponds well with the low level of wt *gcvA::lacZ* expression in a GS162 wt strain (Table 2). The GcvA–His₆ protein preparation under control of the +13A control region displayed a strong reaction that increased as the amount of total protein increased (Fig. 4, lanes 4–6). The Western analysis agrees well with the *λgcvA::lacZ* +13A expression data (Table 2). Since it was shown previously that overexpression of *gcvA* from the *tac* promoter causes a loss of *gcvT::lacZ* repression (Ghrist & Stauffer, 1995), the overproduction of the GcvA protein in the +13A mutant could account for the loss of *gcvT::lacZ* repression observed in cells containing plasmid pGS468 (Table 3).

Overexpression of GcvR in mutant *gcvA* strains mediates the return of *gcvT::lacZ* regulation

Overexpression of *gcvR* results in superrepression of a *gcvT::lacZ* fusion and this repression is dependent on a functional *gcvA* gene (Ghrist & Stauffer, 1995). We hypothesized that if the signal for activation and repression of a *gcvT::lacZ* fusion is dependent upon the ratio of GcvA to GcvR in the cell, then the altered regulation of the *gcvT::lacZ* fusion observed with the

gcvA +13A and *gcvA* +15G mutations (Table 3) would be eliminated by overexpressing *gcvR* in the mutant *gcvA* backgrounds. To examine this hypothesis, strain GS973, a *gcvA1* mutant carrying a *λgcvT::lacZ* fusion, was transformed with plasmids pGS468 and pGS469 carrying the *gcvA* +13A and *gcvA* +15G mutations, respectively. These strains were then transformed with plasmid pGS383, a pBR322 derivative carrying wt *gcvR*, to produce two strains containing plasmids that overproduce both GcvA and GcvR. Two control strains were also constructed by transforming GS973 with plasmid pGS341, the single-copy plasmid carrying wt *gcvA*, and by transforming GS973 with both plasmids pGS341 and pGS383. The GS973(pGS341) transformant contained one plasmid-borne copy of *gcvA* and a chromosomal copy of *gcvR*. The GS973(pGS341)-(pGS383) transformant contained one plasmid-borne copy of *gcvA* and multiple copies of *gcvR*. These strains were grown in GM medium and GM medium supplemented with either glycine or inosine and assayed for β -galactosidase activity. The control strain GS973(pGS341) showed normal glycine-mediated activation and purine-mediated repression (Table 4). When both the single-copy *gcvA* plasmid pGS341 and the multicopy *gcvR* plasmid pGS383 were transformed into GS973, superrepression occurred under all growth conditions (Table 4). In the GS973(pGS469)(pGS383) double transformant, where *gcvA* was overexpressed about 8.7-fold and *gcvR* was overexpressed about 30-fold [based on the copy number of the vector (Bolivar *et al.*, 1977) and the observation that *gcvR* is constitutively expressed (Ghrist & Stauffer, 1995)], superrepression of *gcvT::lacZ* occurred under all growth conditions (Table 4). In the GS973(pGS468)(pGS383) double transformant, where *gcvA* was overexpressed about 22-fold and *gcvR* was overexpressed about 30-fold, the level of *gcvT::lacZ* expression was still repressed under all growth conditions. However, there was a significant increase in β -galactosidase activity in this transformant compared to the strain carrying a single copy of *gcvA*, or in the strain where *gcvA* was overexpressed only 8.7-fold. In addition, this transformant could mediate a 6.2-fold activation of *gcvT::lacZ* expression in the presence of glycine (Table 4), although the induced levels were 3.7-fold lower than the control strain with a single copy

Table 4. Effects of overexpressing *gcvR* on *gcvT::lacZ* expression in cells overproducing *gcvA*

All plasmids were assayed in strain GS973. Cells were grown in GM medium with the indicated supplements and assayed for β -galactosidase activity.

Plasmid	Relative levels of GcvA and GcvR	β -Galactosidase activity (Miller units)		
		None	Glycine	Inosine
pGS341	A ⁺ , R ⁺	163 ± 28	766 ± 110	7 ± 1
pGS341 + pGS383	A ⁺ , R ⁺⁺⁺⁺	7 ± 0	13 ± 1	1 ± 0
pGS468 + pGS383	A ⁺⁺⁺ , R ⁺⁺⁺⁺	33 ± 1	205 ± 10	3 ± 0
pGS469 + pGS383	A ⁺⁺ , R ⁺⁺⁺⁺	5 ± 1	13 ± 0	0.45 ± 0.1

of *gcvA* and *gcvR*. These results indicate that the loss of *gcvT::lacZ* repression caused by overexpression of *gcvA* can be overcome by proportionally overexpressing *gcvR* and suggest that the proper ratio of GcvA and GcvR, rather than their absolute levels, is critical for normal regulation of the *gcv* operon. We have designed a genetic screen to isolate mutations in the coding region of *gcvA* that would render the GcvA protein either unable to repress *gcv* in the presence of inosine or, alternatively, unable to activate *gcv* in the presence of glycine. One *gcvA* mutant isolated encodes a protein that no longer responds to GcvR for repression (A. D. Jourdan & G. V. Stauffer, unpublished data), supporting a model where GcvR and GcvA might interact. Careful analysis of this and additional mutants will provide insight into the mechanisms by which GcvA and GcvR are involved in activating and repressing the *gcv* operon.

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