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DNA Binding Sites of the LysR-Type Regulator GcvA in the *gcv* and *gcvA* Control Regions of *Escherichia coli*

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The GcvA protein is a LysR family regulatory protein necessary for both activation and repression of the *Escherichia coli* glycine cleavage enzyme operon (*gcv*) and negative regulation of *gcvA*. Gel shift assays indicated that overexpressed GcvA in crude extracts is capable of binding specifically to DNA containing the *gcv* and *gcvA* control regions. DNase I footprint analysis of the *gcvA* control region revealed one region of GcvA-mediated protection overlapping the transcription initiation site and extending from −28 to +20. Three separate GcvA binding sites in *gcv* were identified by DNase I footprint analysis: a 29-bp region extending from positions −271 to −242, a 28-bp region extending from −242 to −214, and a 35-bp region covering positions −69 to −34 relative to the transcription initiation site. PCR-generated mutations in any of the three GcvA binding sites in *gcv* decreased GcvA-mediated activation and repression of *gcv*.

The *Escherichia coli* glycine cleavage enzyme system (GCV) is a glycine-inducible, purine-repressible metabolic pathway that catalyzes the cleavage of glycine into CO_2 + NH_3 and transfers a one-carbon (C_1) methylene unit to tetrahydrofolate (9, 15). This activated methyl group, in the form of 5,10-methylenetetrahydrofolate, can then be used in the biosynthesis of purines, methionine, thymine, and other cellular components (17). Three of the four enzymes required for glycine cleavage, the T, H, and P proteins, are encoded by the *gcv* operon that maps at min 65.2 on the *E. coli* chromosome (20). Expression of the *gcv* operon involves a complex regulatory system consisting of at least three regulatory proteins acting at the *gcv* promoter.

Two regulators known to play a negative role in the control of *gcv* expression are the PurR and GcvA proteins (36). PurR is required for negative regulation of numerous genes involved in nucleotide metabolism (7, 10, 21, 34) and has been shown to decrease *gcv* expression twofold when cells are grown in the presence of exogenous purines or purines and glycine (36). In vitro studies have indicated that PurR binds to the *gcv* control region near the transcription initiation site for *gcvT*, the first gene in the *gcv* operon, and presumably interferes with the ability of RNA polymerase to initiate transcription (28, 36). A PurR-independent mechanism for purine repression of *gcv* is mediated by the GcvA protein. This LysR-type regulator (8, 25) is necessary for an additional fivefold decrease in *gcv* expression when cells are grown in the presence of purines and without glycine (36).

GcvA plays a dual role in the regulation of *gcv*, since it is also required for six- to sevenfold activation of *gcv* when glycine is included in the growth media (37). Whether GcvA responds directly to two different cellular metabolites or whether GcvA interacts with another protein is unknown but is under investigation. GcvA also negatively regulates its own promoter independently of glycine or purine supplementation.

Full induction of *gcv* in response to glycine also requires Lrp (12), a global regulator necessary for either activation or repression of many genes involved in amino acid metabolism (1, 18). An *lrp* mutant carrying a *gcvT-lacZ* fusion, in which β-galactosidase synthesis is under the control of the *gcv* regulatory region, has low, noninducible β-galactosidase levels (30). In vitro studies have identified multiple Lrp binding sites in the −92 to −229 region of *gcv* (30). Further studies are needed to determine whether Lrp-dependent activation of *gcv* is a result of structural changes in the DNA or whether Lrp interacts directly with RNA polymerase or other regulatory proteins.

An additional factor involved in *gcv* regulation is suggested by deletion analysis of the upstream region of *gcv* (30). When nucleotides −466 to −313 of *gcv* are deleted, a fourfold decrease from the fully induced levels of β-galactosidase synthesis encoded by a *gcvT-lacZ* fusion occurs. This decrease in activity appears to be independent of *gcvA*, *lrp*, or *purR*, since strains containing mutations at these loci still show diminished expression from the −466 to −313 *gcvT=lacZ* deletion fusion (30).

As described above, the regulation of *gcv* involves several regulatory proteins, of which the PurR and Lrp proteins have been shown to act directly at the *gcv* promoter region (30, 36). This report describes the overexpression of GcvA and identifies the GcvA binding sites in the *gcv* and *gcvA* control regions.

MATERIALS AND METHODS

Bacterial strains and plasmids used in this study. *E. coli* K-12 strain GS162 carries the *thi pheA905* Δ*lacU169 araD129 rpsL150* mutations. Strain GS1029 is isogenic to GS162 except for a Δ*gcvA*::Sp^r mutation in which the chromosomal *gcvA* gene has been deleted and replaced with a spectinomycin resistance marker (35). GS852 is also isogenic to GS162 except for an additional *purR*::Tn*10* mutation (10). Where indicated, some strains were lysogenized with a λ*gcvT-lacZ* phage, in which β-galactosidase synthesis is under the control of the *gcv* regulatory region (29), or with a mutated λ*gcvT-lacZ* phage (see below).

Plasmid pGS285, which expresses high levels of GcvA, was constructed as follows. Two primers were synthesized with an Applied Biosystems DNA synthesizer. The first primer (5′-CCGAATTCAGGAATAGCCATGTCTAAACG-3′) is complementary to the region of *gcvA* overlapping the initiation codon and includes an artificial Shine-Dalgarno sequence and an *Eco*RI cloning site. The second primer (5′-TCCCCCGGGCATGGTCGTACCCTACG-3′) is complementary to the region downstream of *gcvA* (opposite DNA strand) and includes an artificial *Sma*I cloning site. These two primers were used for PCR amplification of plasmid pGS267, which carries the *gcvA* gene (35). The resulting 964-bp PCR product was purified on low-melting-point agarose, cut with *Eco*RI and *Sma*I, and cloned into expression vector pTrc99A (Pharmacia, Piscataway, N.J.), forming plasmid pGS280. DNA binding assays using crude extracts containing either pGS280 or control vector pTrc99A were complicated by the presence of shifted bands that were later shown to be specific to the pTrc99A vector and

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likely due to the overproduced LacI protein. Cloning of the *gcvA* fragment into a high-copy-number plasmid with a strong *tac* promoter and no *lacI* gene appeared to be detrimental to the cells. Therefore, the *gcvA* insert was subcloned into pGS286, a single-copy vector constructed from pGS225 (37) in which the strong *tac* promoter and an ampicillin resistance gene had been cloned in and the resident kanamycin gene had been removed. The resultant plasmid, containing a highly expressed *gcvA* gene, was designated pGS285.

DNA manipulation. Isolation of plasmid DNA, restriction enzyme digestions, ligations, and plasmid transformations were done as previously described (22).

Media. The complex medium used was Luria broth (16). Vogel and Bonner minimal salts (33) supplemented with 0.4% glucose (GM) was the minimal medium used. Appropriate amino acids, antibiotics, and other supplements were added, as required, at the following concentrations: phenylalanine, 50 μg/ml; glycine, 300 μg/ml; inosine, 50 μg/ml; vitamin B_1, 1 μg/ml; ampicillin, 50 μg/ml.

Enzyme assays. β-Galactosidase assays were performed as described by Miller (16). Protein concentrations were determined by the method of Lowry et al. (13). All of the results shown are averages of two or more assays, with each sample done in triplicate. Values from different assays varied by less than 15%.

Site-directed mutagenesis and construction of lysogens. Mutagenesis of the GcvA binding sites identified in the *gcv* control region was performed by using the PCR megaprimer mutagenesis procedure as previously described (24), with minor modifications. The following mutant oligonucleotides were synthesized: site 1, 5′-GCATTTTTTAACCATAAGAGTTTGTGATGATCAATTTTACC-3′; site 2, 5′-CACTAAATCAAAAAATAATGGCAAACTAAAATATAATGC G-3′; and site 3, 5′-CCTCATTTGAAATAAAAGTTTTTCACCTCCGTTTTC GC-3′ (mutated nucleotides are underlined). Each mutant oligonucleotide was used for PCR amplification (denaturation at 94°C for 2 min, annealing at 37°C for 2 min, and extension at 55°C for 1.5 min for 30 cycles) with template plasmid pGS239 (29) containing the wild-type *gcv* control region on a 759-bp *Eco*RI-*Bam*HI fragment fused to the *lacZYA* genes of *lac* fusion vector pMC1403 (2). The upstream primer is complementary to vector or *lacZ* sequences. Unincorporated oligonucleotides were removed with QiaquikSpin (Qiagen, Chatsworth, Calif.), and the PCR products were isolated on low-melting-point agarose. These PCR products were then used as "megaprimers" in another round of PCR synthesis, again with plasmid pGS239 as the template. The downstream primer is complementary to vector or *lacZ* sequences. The PCR products were isolated on low-melting-point agarose and cut with *Eco*RI and *Bam*HI restriction enzymes, and the 759-bp fragments were recloned into plasmid pMC1403. The mutations were verified by DNA sequence analysis (23), and the resultant plasmids were designated pGS357 (site 1 mutant), pGS358 (site 2 mutant), and pGS359 (site 3 mutant). Plasmid pGS360 (both sites 2 and 3 mutated) was constructed in a similar manner, except that the template used for PCR amplification was mutant plasmid pGS359 and the primer was the mutant oligonucleotide directed to site 2.

Each mutant *gcvT-lacZYA* fusion was cloned into bacteriophage λgt2 (19), and the resultant phage were designated λ*gcvT-lacZ site 1*, λ*gcvT-lacZ site 2*, λ*gcvT-lacZ site 3*, and λ*gcvT-lacZ sites 2+3*. Appropriate strains were lysogenized with the mutant phage as previously described (32), and lysogens were tested for a single copy of the λ phage by infection with phage λ*c*I90*c*17 (27). The effects of the mutations on *gcv-lacZ* expression were measured by β-galactosidase assay.

Preparation of extracts. To prepare extracts for mobility shift and DNase I protection assays, strain GS1029λ*gcvT-lacZ*, transformed with either plasmid pGS285 (*gcvA*$^+$) or pGS286 (vector only), was inoculated into 700 ml of Luria broth containing 50 μg of ampicillin per ml and grown for 12 h at 30°C. Cells were harvested by centrifugation, and the pellets were resuspended in 2× DNA binding buffer (20 mM Tris [pH 7.5], 100 mM KCl, 2 mM EDTA, 10% glycerol, 2 mM dithiothreitol). The cells were sonicated on ice, and cellular debris was removed by centrifugation at 11,000 × *g* for 30 min at 4°C. The supernatant was brought to 33% ammonium sulfate saturation, and the precipitated proteins were collected by centrifugation at 11,000 × *g*. The pellets were resuspended in 2 ml of 2× DNA binding buffer and dialyzed extensively against the same buffer. After dialysis, particulates were removed by centrifugation and the extracts were brought to a protein concentration of 10 mg/ml. These samples were used as crude extracts for in vitro studies.

Gel mobility shift assay. The gel mobility shift assay used was based on the methods of Fried and Crothers (3) and Garner and Revzin (5). A 759-bp fragment from pGS239 (29), from nucleotides −466 to +293 of the *gcv* control region, was ^{32}P labeled at its unique *Eco*RI site by using the large fragment of DNA polymerase I. A 519-bp fragment from *gcvA-lacZ* fusion plasmid pGS265 (35), from nucleotides −303 to +216 of the *gcvA* control region, was also ^{32}P labeled at its unique *Eco*RI site. The gel mobility shift assay was performed by preincubating the labeled DNA in 1× DNA binding buffer (18-μl total volume) at 37°C for 5 min and then adding 2-μl volumes of twofold serial dilutions of crude extracts diluted in 1× DNA binding buffer. After incubating the tubes at 37°C for 15 min, 1 μl of loading dye was added and the samples were loaded onto a nondenaturing 5% polyacrylamide–3% glycine gel.

DNase I protection assay. The DNase I protection assay used in this study is a modified version of the method of Schmitz and Galas (26). The 759-bp fragment from pGS239 and the 519-bp fragment from pGS265 described above were ^{32}P labeled at the unique *Eco*RI site. The labeled DNA fragments were preincubated at 37°C for 5 min in 22.5-μl reaction mixtures containing 1× DNA binding buffer plus 125 μg of bovine serum albumin per ml. A 2.5-μl volume of diluted crude extract from cells carrying either *gcvA*$^+$-carrying plasmid pGS285 or control plasmid pGS286 was then added, and incubation was continued at 37°C for an additional 15 min before DNase I was added (1.5 μl of a 2-μg/ml solution of DNase I in 20 mM sodium acetate–32 mM $CaCl_2$). After 30 s, the reactions were stopped by adding 65 μl of stop solution (3 M ammonium acetate, 15 μg of sonicated calf thymus DNA per ml, 0.25 M EDTA) and samples were precipitated with ethanol. Pellets were suspended in sequencing loading buffer and loaded onto a 5% polyacrylamide–7 M urea sequencing gel alongside the Maxam and Gilbert A+G and C+T sequencing reactions (14) performed on the same labeled fragment.

RESULTS

Overproduction of GcvA. Experiments examining the regulation of a translational *gcvA-lacZ* fusion suggested that expression of *gcvA* is relatively low (only 3 Miller units) and also that *gcvA* is negatively autoregulated over a twofold range (35). Analysis of the DNA sequence of *gcvA* (35) indicated the presence of a perfect 7-bp inverted repeat at positions −33 to −16 relative to the translation initiation codon of *gcvA* and a poor presumed Shine-Dalgarno sequence (5′-AAAG-3′) adjacent to this inverted repeat. Although the significance of this potential hairpin structure in *gcvA* has not been examined, it is well documented that both secondary mRNA structures and the nucleotides used in the ribosome binding site can have a significant effect on translation efficiency (6). To increase the expression of *gcvA* and abolish the capacity for negative autoregulation, the *gcvA* coding sequence was PCR synthesized with deletion of all but 6 bp upstream from the translation start codon and introduction of an artificial Shine-Dalgarno sequence (5′-AGGA-3′). This sequence was fused to the strong *tac* promoter (see Materials and Methods), forming plasmid pGS285. When a *gcvA* mutant containing a *gcvT-lacZ* fusion was transformed with this plasmid, high constitutive expression of β-galactosidase was measured (data not shown), suggesting that pGS285 overproduces the GcvA protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of crude extracts containing this plasmid indicated that GcvA was less than 1% of the total cell protein (data not shown).

GcvA protein binds to DNA containing the *gcv* and *gcvA* control regions. To determine whether the overproduced GcvA protein in cell extracts was able to bind DNA containing the *gcv* control region, a gel mobility shift assay was performed. As shown in Fig. 1, addition of increasing amounts of total protein resulted in a shift of *gcv* DNA to a slightly higher band (lanes 3 to 6) and then, at the highest concentrations used, two additional major bands with extremely slow mobilities (lanes 7 and 8). Two minor bands also appeared at higher protein concentrations but were not due to binding of GcvA since they also appeared when a control extract prepared from a *gcvA* mutant transformed with a control (*gcvA* mutant) plasmid was used (lane 9). Since the buffer used in this assay is glycine based, gel shift patterns were not examined in the presence of additional glycine.

Overexpressed GcvA in crude extracts was also able to bind to DNA containing the *gcvA* control region. At lower concentrations of GcvA, a slightly shifted band appeared and, at higher concentrations, additional bands with slower mobility appeared (Fig. 2, lanes 2 to 8). Again, a minor shifted band was seen in lanes containing the GcvA extract and those containing the control extract, suggesting that some other factor, in addition to GcvA, is present that binds to DNA containing the *gcvA* control region.

Localization of the GcvA binding sites on the *gcv* and *gcvA* control regions. To identify which sequences of DNA are necessary for binding of GcvA to the *gcv* and *gcvA* control regions, DNase I footprinting assays were performed. As seen in Fig. 3a, addition of increasing amounts of cell extracts containing

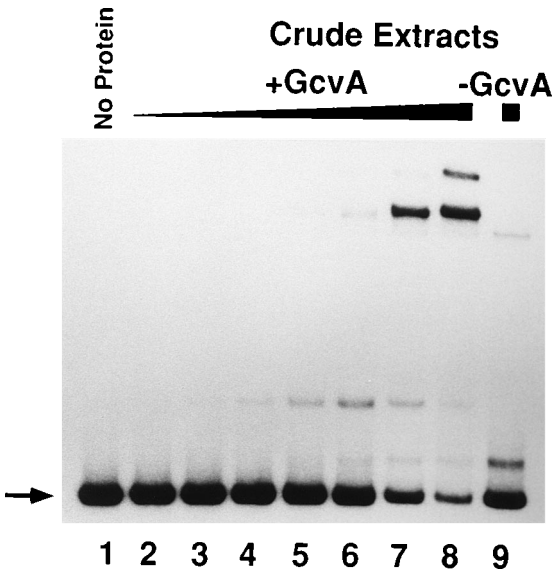

FIG. 1. Gel mobility shift assay for the binding of GcvA to *gcv* DNA. A 759-bp ^{32}P-labeled DNA fragment containing the *gcv* control region was incubated with twofold dilutions of crude extracts containing overexpressed GcvA, and the samples were run on a nondenaturing 5% polyacrylamide–3% glycine gel as described in Materials and Methods. Lane 1, DNA probe only; lanes 2 to 8, 0.8, 1.56, 3.10, 6.20, 12.5, 25, and 50 ng of protein from crude extract containing overexpressed GcvA added per 20-μl reaction mixture, respectively; lane 9, 50 ng of control (no GcvA) crude extract. The arrow denotes the unbound DNA fragment.

overproduced GcvA to reaction mixtures containing labeled *gcv* DNA resulted in protection from DNase I digestion in two distinct regions. The first region covers 57 bp, extends from about −271 to −214 in the *gcv* control region, and was seen at the lower concentrations of cell extracts. Because of the large size of this region and the various degrees of protection, it was assumed that this area of *gcv* protected by GcvA represents at least two distinct binding sites for GcvA, and these sites were designated sites 2 and 3. At higher protein concentrations, another protected site was seen closer to the transcription initiation site for *gcv*, and this site was designated site 1. To define the precise boundaries of site 1, a longer run of the same reaction mixtures as in Fig. 3a was done. As shown in Fig. 3b, the boundaries of GcvA-mediated protection of site 1 extend approximately 35 bp, covering the −69 to −34 region of *gcv*. Whether this represents a single GcvA binding site or a double binding site is uncertain. Addition of 10 mM glycine to the reaction mixtures had no effect on the degree of protection or on the pattern of DNase I digestion. No protection from DNase I digestion of *gcv* was seen when control extracts were used in the assay (Fig. 3a and b, lanes 8 to 11).

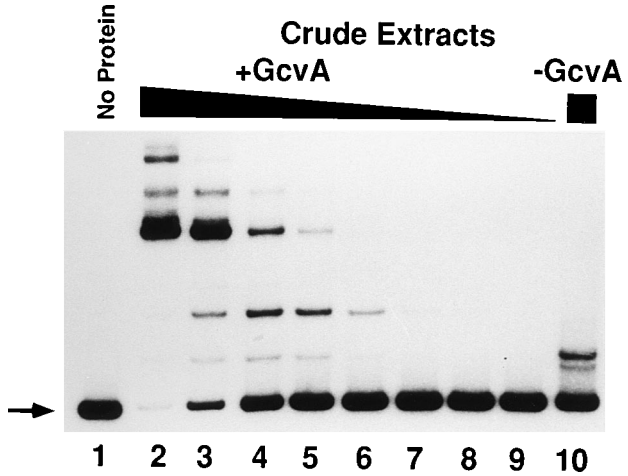

FIG. 2. Gel mobility shift assay for the binding of GcvA to *gcvA* DNA. A 519-bp ^{32}P-labeled DNA fragment containing the *gcvA* control region was incubated with twofold dilutions of crude extracts containing overexpressed GcvA, and the samples were run on a nondenaturing 5% polyacrylamide–3% glycine gel as described in Materials and Methods. Lane 1, DNA probe only; lanes 2 to 9, 100, 50, 25, 12.5, 6.20, 3.10, 1.56, and 0.8 ng of protein from crude extract containing overexpressed GcvA per 20-μl reaction mixture, respectively; lane 10, 100 ng of control (no GcvA) crude extract. The arrow denotes the unbound DNA fragment.

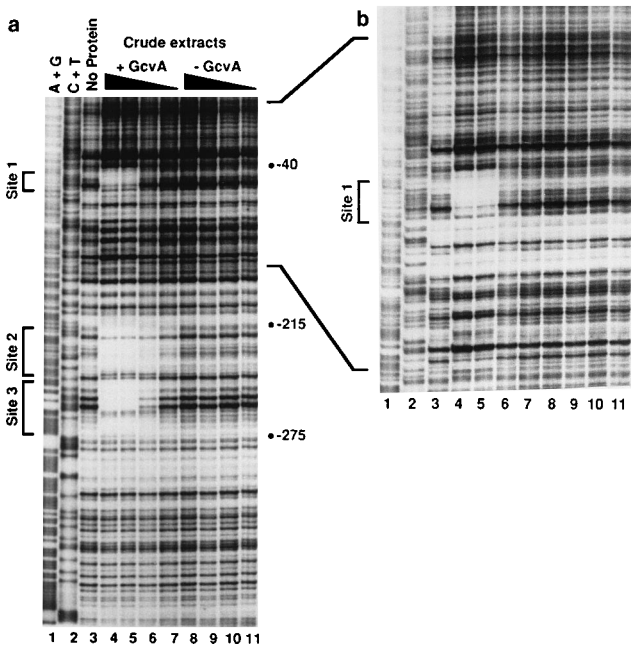

FIG. 3. Protection from DNase I digestion of the *gcv* control region by GcvA. A 759-bp ^{32}P-labeled DNA probe containing the *gcv* control region was incubated with twofold dilutions of crude extracts with or without overexpressed GcvA (see Materials and Methods) and digested with DNase I. The partial digestion products were run on a denaturing 5% polyacrylamide gel adjacent to the Maxam-Gilbert sequencing reactions of the labeled DNA probe. Lane 1, A+G; lane 2, C+T; lane 3, no protein; lanes 4 to 7, 500, 250, 125, and 62.5 ng of protein from crude extract containing overexpressed GcvA per 25-μl reaction mixture, respectively; lanes 8 to 11, 500, 250, 125, and 62.5 ng of control (no GcvA) crude extract per 25-μl reaction mixture, respectively. The three regions of the DNA protected by DNase I digestion are indicated by the brackets. a and b are short and long runs, respectively, of the digestion products.

DNase I protection assays were also performed with a ^{32}P-labeled DNA fragment containing the *gcvA* control region. As shown in Fig. 4, extracts containing overproduced GcvA were able to protect a large, 48-bp region of *gcvA* DNA from DNase I digestion. This region overlaps the *gcvA* transcription initiation site, extending from −28 to +20. Again, no protection from DNase I digestion of *gcvA* was seen when a control extract was used in the assay (Fig. 4, lanes 8 to 10).

Mutations in the GcvA binding sites of *gcv* affect binding by GcvA. The four GcvA binding sites identified by DNase I protection experiments were aligned and examined to determine a consensus binding site for GcvA (Fig. 5). The DNA binding sites for most LysR-type transcriptional regulators show some degree of dyad symmetry which contains a conserved T-N_{11}-A motif (25). Both sites 2 and 3 in the *gcv* control

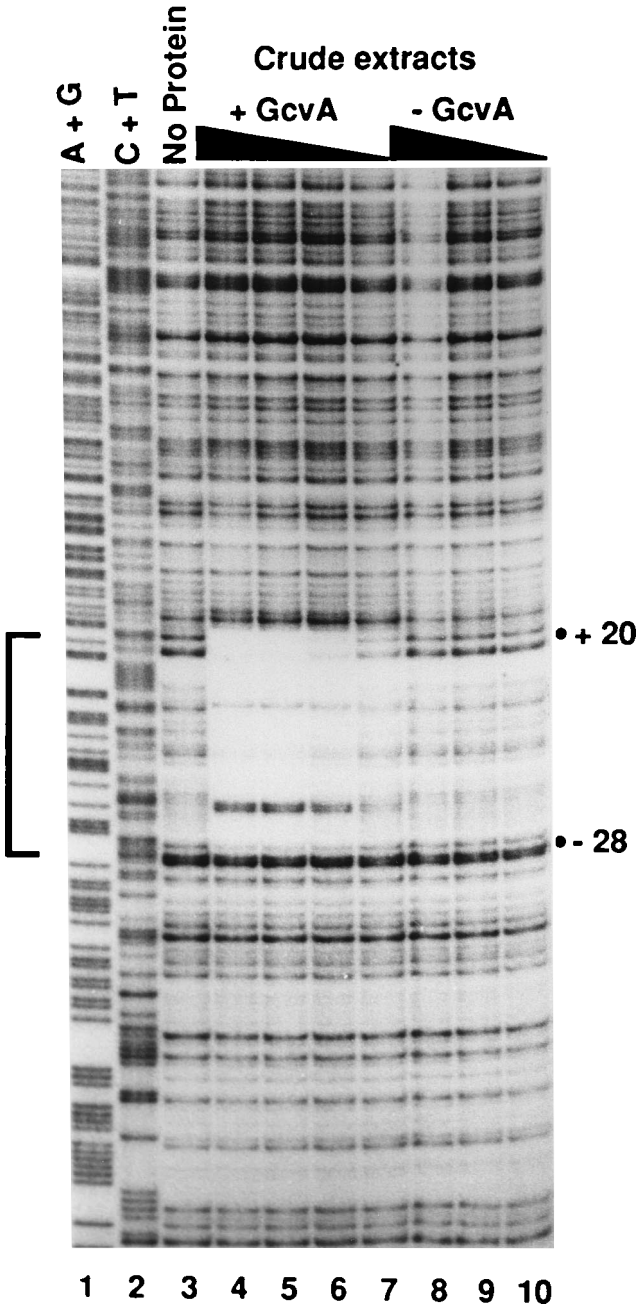

FIG. 4. Protection from DNase I digestion of the *gcvA* control region by GcvA. A 519-bp ^{32}P-labeled DNA probe containing the *gcvA* control region was incubated with twofold dilutions of crude extracts with or without overexpressed GcvA (see Materials and Methods) and digested with DNase I. The partial digestion products were run on a denaturing 5% polyacrylamide gel adjacent to the Maxam-Gilbert sequencing reactions of the labeled DNA probe. Lane 1, A+G; lane 2, C+T; lane 3, no protein; lanes 4 to 7, 500, 250, 125, and 62.5 ng of protein from crude extract containing overexpressed GcvA per 25-μl reaction mixture, respectively; lanes 8 to 10, 500, 250, and 125 ng of control (no GcvA) crude extract added per 25-μl reaction mixture, respectively. The region of the DNA protected by DNase I digestion is indicated by the bracket.

region have a conserved T-N_{11}-A sequence, but significant dyad symmetry is evident only in the site 2 binding site (5′-GCATTA-N_7-TAATGC-3′). The protected bases in the site 1, 2, and 3 binding sites do not show much homology to each other except for a conserved 5′-CTAAT-3′ region. Examination of the protected region of *gcvA* revealed the dyadic sequence 5′-ATTA-N_5-TAAT-3′ and also the conserved 5′-CTAAT-3′ sequence that was seen in the GcvA-protected regions of *gcv*.

gcv Site 3 5'- CCTCATTTGAAATAAACTAATTTCACCTC-3'

gcv Site 2 5'-TTTTCGCATTATATTTTCTAATGCCA-3'

gcv Site 1 5'-TTAACCATAAGCTAATGTGATGAT-3'

gcvA 5'-CAGATTAAAAAAACTAATAGGTT-3'

FIG. 5. Alignment of partial sequences of *gcv* and *gcvA* DNAs protected by GcvA from DNase I digestion. The T-N_{11}-A sequence found in most LysR-type binding sequences (25) is underlined, and the conserved 5′-CTAAT-3′ sequences found in *gcv* binding sites 1, 2, and 3 and the *gcvA* binding site are in boldface. It should be noted that the sequence 5′-TTAAC-3′ of the site 1 binding site may be outside the DNase I-protected region. Regions of dyad symmetry are indicated by arrows above each sequence.

To determine whether the conserved 5′-CTAAT-3′ sequence found in the regions protected by GcvA is important for GcvA binding and whether all three sites are required for regulation of *gcv*, site-directed PCR mutagenesis was used to mutate the 5′-CTAA-3′ sequence to 5′-AGTT-3′ in site 1, 2, or 3 of *gcv* (see Materials and Methods). These mutations conserve the nucleotide content and spacing of the wild-type sequence. In addition, a double mutant with changes at both sites 2 and 3 was constructed. DNA containing the mutated GcvA binding sites was ^{32}P labeled and used in DNase I footprinting assays with cell extracts containing overproduced GcvA. As shown in Fig. 6, when either site 2 or 3 was mutated, a decrease in binding affinity of GcvA for the altered site was seen compared with the wild-type sequence, with the effect being much greater for site 3 than for site 2. In addition, mutation of site 2 resulted in a slight decrease in GcvA binding at site 3 (lanes 8 to 10) but the converse was not true; mutation of site 3 had no effect on GcvA binding at site 2 (lanes 12 to 14). When site 1 was mutated, binding of GcvA to this site was prevented, although protection of sites 2 and 3 appeared to be unaffected (lanes 19 to 22). When both sites 2 and 3 were mutated, GcvA-mediated protection at these sites was abolished but protection of site 1 was similar to that seen with the wild-type template (lanes 15 to 18). It should be noted, however, that the degree of protection of site 1 in the site 2 and 3 double mutant varied slightly between experiments; sometimes a slight decrease in binding of GcvA to site 1 was noted. The cause for this variability is not understood.

Mutations in the GcvA binding sites of *gcv* affect regulation by GcvA. To test whether the altered binding affinities demonstrated in the DNase I footprint analysis correlated with diminished GcvA regulation of *gcv* in vivo, translational fusions of the mutated *gcv* DNA fragments to the *lacZ* gene were made and cloned into λgt2. These phages, designated λ*gcvT-lacZ site 1*, λ*gcvT-lacZ site 2*, λ*gcvT-lacZ site 3*, and λ*gcvT-lacZ sites 2+3*, were used to lysogenize both wild-type strain GS162 and *gcvA* deletion mutant GS1029. In these lysogens, β-galactosidase synthesis is under control of the mutated *gcv* regulatory region. As a control, strains GS162 and GS1029 were also lysogenized with the wild-type λ*gcvT-lacZ* phage constructed previously (29).

These lysogens were grown in GM with no supplements, with glycine, or with the purine nucleotide inosine and assayed for β-galactosidase activity. Wild-type lysogen GS162λ*gcvT-lacZ* normally shows a 6- to 7-fold increase in β-galactosidase expression when grown in the presence of glycine and a greater than 10-fold repression when grown in media containing pu-

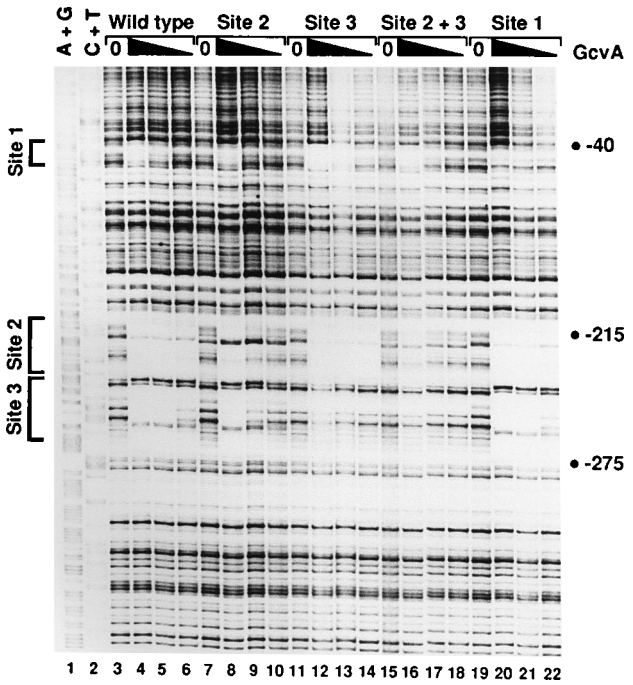

FIG. 6. Protection from DNase I digestion of wild-type and mutated *gcv* control region DNAs by GcvA. A 759-bp ^{32}P-labeled DNA fragment containing wild-type DNA (lanes 3 to 6), site 2-mutated DNA (lanes 7 to 10), site 3-mutated DNA (lanes 11 to 14), site 2+3-mutated DNA (lanes 15 to 18), or site 1-mutated DNA (lanes 19 to 22) was incubated with twofold dilutions of crude extract containing overexpressed GcvA (see Materials and Methods) and digested with DNase I. The partial digestion products were run on a 5% polyacrylamide gel alongside the Maxam and Gilbert sequencing reactions of the wild-type DNA probe. Lane 1, A+G; lane 2, C+T; lanes 3, 7, 11, 15, and 19, no protein; lanes 4, 8, 12, 16, and 20, 500 ng of protein from crude extract; lanes 5, 9, 13, 17, and 21, 250 ng of crude extract; lanes 6, 10, 14, 18, and 22, 125 ng of crude extract per 25-μl reaction mixture. The three regions of the wild-type fragment protected from DNase I digestion are indicated by the brackets.

rines (Table 1). As shown previously (36), both of these effects are mediated by GcvA; β-galactosidase levels of *gcvA* mutant GS1029λ*gcvT-lacZ* are no longer induced by glycine, and the severe inosine-mediated repression is relieved (only the twofold PurR-dependent repression is still seen). When GS162 was lysogenized with mutant phage λ*gcvT-lacZ site 2*, λ*gcvT-lacZ site 3*, or λ*gcvT-lacZ sites 2+3*, both activation by glycine and repression by inosine were abolished, with levels of β-galactosidase similar to those of the *gcvA* mutant and strain GS1029 lysogenized with the wild-type λ*gcvT-lacZ* phage. These results show that the 5′-CTAA-3′ in both sites 2 and 3 is necessary for GcvA-mediated activation and GcvA-mediated repression of *gcv*. This is not the case for the site 1 GcvA binding site.

When GS162 was lysogenized with phage λ*gcvT-lacZ site 1*, activation by glycine and repression by inosine still occurred, although the responses were significantly reduced compared with the wild-type control lysogen (compare lines 1 and 9). However, when *gcvA* mutant strain GS1029 was lysogenized with the λ*gcvT-lacZ site 1* phage, decreased β-galactosidase activity compared with that of the control lysogen was observed under all conditions (compare lines 2 and 10), indicating that some factor other than GcvA binding was affected by mutating this sequence of DNA. Since this region is close enough to the promoter to include bases that might be in contact with RNA polymerase, it is possible that this mutation affected the promoter for *gcv*. This could explain why glycine-induced activation of *gcv* was decreased. What it could not explain, however, is why inosine-mediated repression was partially relieved; instead of 12 Miller units of β-galactosidase activity or less (if the site 1 mutation simply caused a promoter down effect), the site 1 mutant had 28 Miller units. To determine how much of this repression was mediated by the PurR protein, *purR* mutant GS852 was lysogenized with either the λ*gcvT-lacZ site 1* phage or the wild-type phage and β-galactosidase activity was assayed. All repression measured in these *purR* lysogens must be due to the GcvA protein. An approximately twofold decrease in repression compared with GS852λ*gcvT-lacZ* phage was measured when GS852λ*gcvT-lacZ site 1* was grown in the presence of inosine (lines 11 and 12). These results suggest that the mutations present in the λ*gcvT-lacZ site 1* phage partially affected the ability of GcvA to act as a repressor of *gcv* when cells were grown in the presence of inosine.

DISCUSSION

Gel mobility shift assays indicated that extracts containing overexpressed GcvA were capable of binding to *gcv* and *gcvA* DNAs. Most LysR family members are able to bind to their target DNA without their coeffectors (reviewed in reference 25), and GcvA appears to be no exception. The binding of GcvA to *gcv* results in a complex shifting pattern consisting of three bands, two of which have extremely slow mobility (Fig. 1). Since the degree of shifting of these bands does not follow the pattern expected for a simple increase in molecular size with the binding of one and then two and three dimers (or tetramers) of GcvA, it is possible that a higher-order structure is formed upon binding of GcvA to *gcv*. Initially, we thought that since multiple dimers of Lrp can bind to *gcv* DNA (30), perhaps a large nucleoprotein complex is formed that retards the movement of the DNA through the gel. However, gel mobility shifts performed with crude extracts made from an *lrp* mutant transformed with the GcvA-overexpressing plasmid looked similar to those in Fig. 1 (data not shown). Studies with the *lac* repressor protein have shown that binding of the tetrameric *lac* protein to two *lac* operators on the same DNA fragment causes looping of the intervening DNA and a result-

TABLE 1. Mutations in the GcvA binding sites in *gcv* alter regulation of *gcv*

Strain	β-Galactosidase activity[a] of cells grown in GM[b] with addition of:		
	Nothing	Glycine	Inosine
GS162λ*gcvT-lacZ*	182	1,244	12
GS1029λ*gcvT-lacZ*	104	105	47
GS162λ*gcvT-lacZ site 3*	110	111	46
GS1029λ*gcvT-lacZ site 3*	110	105	51
GS162λ*gcvT-lacZ site 2*	103	100	44
GS1029λ*gcvT-lacZ site 2*	96	93	43
GS162λ*gcvT-lacZ sites 2+3*	94	96	33
GS1029λ*gcvT-lacZ sites 2+3*	95	98	39
GS162λ*gcvT-lacZ site 1*	171	668	28
GS1029λ*gcvT-lacZ site 1*	58	57	26
GS852λ*gcvT-lacZ site 1*	145	552	58
GS852λ*gcvT-lacZ*	170	1,104	26

[a] In Miller units (16).
[b] GM also contained phenylalanine and vitamin B_1.

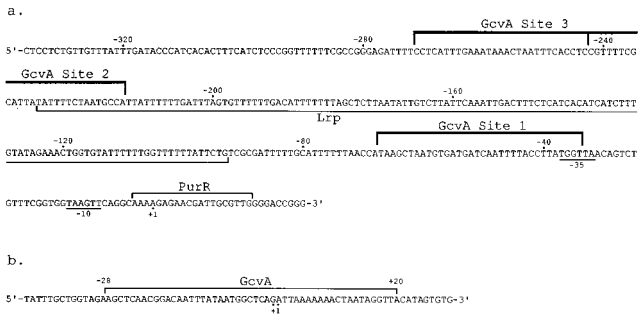

FIG. 7. (a) DNA sequence of the entire *gcv* control region (29). The transcription start site for *gcvT* is indicated as +1 (29). The −10 and −35 promoter sequence elements previously determined (29) are underlined. The regions of DNA protected from DNase I digestion by Lrp (30), PurR (36), and GcvA are indicated by brackets. (b) DNA sequence of the −42 to +30 region of *gcvA* (35). The entire region of *gcvA* DNA protected from DNase I digestion by GcvA is indicated by a bracket. The two transcription start sites are indicated as +1 (35).

ant decrease in mobility in gel shift assays (11). This looping also resulted in enhanced and diminished sensitivities to DNase I cleavage. It was apparent (Fig. 3b) that there exists such a pattern of enhanced and diminished DNase I cleavage between the site 1 and 2 GcvA binding sites. In later studies, however, the pattern was not as distinct (Fig. 6). The cause of this variation is uncertain, but the extracts used in the mutant analysis (Fig. 6) had gone through several freeze-thaw cycles. Perhaps this altered the ability of GcvA to self-associate in some manner. The question of whether binding of GcvA to its target sites in *gcv* results in looping of the intervening DNA will be pursued in future studies.

Gel mobility shift assays using overexpressed GcvA and DNA containing the *gcvA* regulatory region also resulted in a complex banding pattern (Fig. 2). This pattern is more consistent with the binding of one dimer (or tetramer) at lower concentrations of GcvA and then another at higher concentrations. Additional bands were seen at the highest concentration of protein used. It is not clear whether the bands with the slowest mobility are due to the binding of additional molecules of GcvA or whether they result from the binding of other factors present in the control extracts that are also able to bind to *gcvA* DNA. DNase I footprinting assays showed only a single, 48-bp site of protection on the *gcvA* DNA fragment (Fig. 4 and 7b). If this area represents two GcvA binding sites, then both appear to have the same affinity for GcvA since equal protection of both sites was seen at the lower protein concentrations. This site of protection overlaps the transcription initiation site for *gcvA*, suggesting that the negative autoregulation seen in vivo (35) operates by interference of GcvA with RNA polymerase at the *gcvA* promoter.

DNase I footprint analysis of *gcv* identified three GcvA binding sites at positions −271 to −243 (site 3), −242 to −214 (site 2), and −69 to −34 (site 1) (Fig. 3 and 7a). These three sites appear to have different affinities for GcvA, since binding at site 2 was seen at lower protein concentrations than binding at site 3 and binding at site 3 was seen at lower protein concentrations than binding at site 1. The upstream location of the site 2 and 3 GcvA binding sites is different from those of most LysR family members, in which DNase I-protected regions usually occur only near the −65 area (25). Perhaps the requirement for Lrp, with binding regions from −92 to −229 (30), necessitates the atypical location and may represent a novel mode of regulation for this LysR family member.

Alignment of sites 1, 2, and 3 in *gcv* with that protected by GcvA in the *gcvA* control region did not reveal much sequence similarity between sites, except for the conserved 5′-CTA AT-3′ region (Fig. 5 and 7). In this respect, GcvA may be similar to LysR family member TrpI of *Pseudomonas aeruginosa* (4), in which protein-protein interactions are more important for binding of the second regulatory protein to its target DNA than the actual DNA sequence itself. Alternatively, GcvA may be similar to the *E. coli* OxyR protein that is able to specifically recognize nonhomologous binding sites at different promoters (31).

Because of the diversity of the sequences of the target sites of GcvA, our initial mutational analysis focused on the conserved 5′-CTAA-3′ regions in sites 1, 2, and 3 of *gcv*. Mutation of this sequence to 5′-AGTT-3′ revealed that this sequence is important for binding of GcvA. Furthermore, some binding cooperativity between sites 2 and 3 might be involved. Although the binding of GcvA to site 2 did not appear to be affected when site 3 was mutated, the binding of GcvA to site 3 was at least partially dependent upon binding of GcvA to site 2 (Fig. 6). Mutations made in either site 2 or 3 resulted in total loss of GcvA-mediated activation and repression of *gcv* in vivo as measured by β-galactosidase expression from a *gcvT-lacZ* fusion (Table 1). It is interesting that mutation of the site 1 GcvA binding site, which is closest to the transcription initiation site for *gcv* and could conceivably bring GcvA and RNA polymerase in direct contact, had little effect on GcvA-mediated activation of *gcv* (Table 1, line 9). One model fitting the data presented is that binding of GcvA to site 3 is required for activation and repression of *gcv*, while site 2 stabilizes the binding of GcvA to site 3. The site 1 GcvA binding site might not be involved in activation of *gcv* by glycine but in some manner is necessary for repression of *gcv* by purines, perhaps by binding of some cofactor or other protein and interaction with GcvA at sites 2 or 3 to prevent transcription by RNA polymerase. A more refined mutational analysis of the *gcv* regulatory region and purification of GcvA and identification of its cofactors will provide additional data to aid in our understanding of the role of GcvA in the complex regulation of the *gcv* operon.

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REFERENCES

1. **Calvo, J., and R. G. Matthews.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466–490.
2. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **143:**971–980.
3. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6525.
4. **Gao, J., and G. N. Gussin.** 1991. Mutations in TrpI binding site II that differentially affect activation of the *trpBA* promoter of *Pseudomonas aeruginosa*. EMBO J. **10:**4137–4144.
5. **Garner, M. M., and A. Revzin.** 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *Escherichia coli* lactose operon regulatory system. Nucleic Acids Res. **9:**3047–3060.
6. **Gold, L.** 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57:**199–233.
7. **He, B., K. Y. Choi, and H. Zalkin.** 1993. Regulation of *Escherichia coli glnB*, *prsA*, and *speA* by the purine repressor. J. Bacteriol. **175:**3598–3606.
8. **Henikoff, S., G. W. Haughn, J. M. Calvo, and J. C. Wallace.** 1988. A large family of bacterial activator proteins. Proc. Natl. Acad. Sci. USA **85:**6602–6606.
9. **Kikuchi, G.** 1973. The glycine cleavage system: composition, reaction mechanism, and physiological significance. Mol. Cell. Biochem. **1:**169–187.
10. **Kilstrup, M., L. M. Meng, J. Neuhard, and P. Nygaard.** 1989. Genetic

evidence for a repressor of synthesis of cytosine deaminase and purine biosynthesis enzymes in *Escherichia coli*. J. Bacteriol. **171:**2124–2127.
11. **Kramer, H., M. Niemoller, M. Amouyal, B. Revet, B. von Wilcken-Bergmann, and B. Muller-Hill.** 1987. *lac* repressor forms loops with linear DNA carrying two suitably spaced *lac* operators. EMBO J. **6:**1481–1491.
12. **Lin, R., R. D'Ari, and E. B. Newman.** 1992. λ p*lac*Mu insertions in genes of the leucine regulon: extension of the regulon to genes not regulated by leucine. J. Bacteriol. **174:**1948–1955.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
14. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65:**499–560.
15. **Meedel, T. H., and L. I. Pizer.** 1974. Regulation of one-carbon biosynthesis and utilization in *Escherichia coli*. J. Bacteriol. **118:**905–910.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Mudd, S. H., and G. L. Cantoni.** 1964. Biological transmethylation, methyl-group neogenesis and other "one-carbon" metabolic reactions dependent upon tetrahydrofolic acid, p. 1–47. *In* M. Florkin and E. H. Stotz (ed.), Comprehensive biochemistry, vol. 15. Elsevier Publishing Co., Amsterdam.
18. **Newman, E. B., R. D'Ari, and R. T. Lin.** 1992. The leucine-Lrp regulon in *E. coli*: a global response in search of a raison d'etre. Cell **68:**617–619.
19. **Panasenko, S. M., J. R. Cameron, R. W. Davis, and I. R. Lehman.** 1977. Five hundred-fold overproduction of DNA ligase after induction of a hybrid lambda lysogen constructed *in vitro*. Science **196:**188–189.
20. **Plamann, M. D., W. D. Rapp, and G. V. Stauffer.** 1983. *Escherichia coli* K12 mutants defective in the glycine cleavage enzyme system. Mol. Gen. Genet. **192:**15–20.
21. **Rolfes, R. J., and H. Zalkin.** 1988. *Escherichia coli* gene *purR* encoding a repressor protein for purine nucleotide synthesis. J. Biol. Chem. **263:**19653–19661.
22. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
24. **Sarkar, G., and S. Sommer.** 1990. The "megaprimer" method of site-directed mutagenesis. BioTechniques **8:**404–407.
25. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
26. **Schmitz, A., and D. J. Galas.** 1979. The interaction of RNA polymerase and lac repressor with the lac control region. Nucleic Acids Res. **6:**111–137.
27. **Shimada, K., R. A. Weisberg, and M. E. Gottesman.** 1972. Prophage λ at unusual chromosomal locations. I. Location of the secondary attachment sites and properties of the lysogens. J. Mol. Biol. **63:**483–503.
28. **Stauffer, G. V., S. Fogerty, and L. T. Stauffer.** 1994. Characterization of the *Escherichia coli gcv* operon. Gene **142:**17–22.
29. **Stauffer, G. V., A. Ghrist, and L. T. Stauffer.** 1993. The *Escherichia coli gcvT* gene encoding the T-protein of the glycine cleavage enzyme system. DNA Sequence **3:**339–346.
30. **Stauffer, L. T., and G. V. Stauffer.** 1994. Characterization of the *gcv* control region from *Escherichia coli*. J. Bacteriol. **176:**6159–6164.
31. **Tartaglia, L. A., C. J. Gimeno, G. Storz, and B. N. Ames.** 1992. Multidegenerate DNA recognition by the OxyR transcriptional regulator. J. Biol. Chem. **267:**2038–2045.
32. **Urbanowski, M. L., and G. V. Stauffer.** 1986. Autoregulation by tandem promoters of the *Salmonella typhimurium* LT2 *metJ* gene. J. Bacteriol. **165:**740–745.
33. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
34. **Wilson, H. R., and C. L. Turnbough, Jr.** 1990. Role of the purine repressor in the regulation of pyrimidine gene expression in *Escherichia coli* K-12. J. Bacteriol. **172:**3208–3213.
35. **Wilson, R. L., and G. V. Stauffer.** 1994. DNA sequence and characterization of GcvA, a LysR family regulatory protein for the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol. **176:**2862–2868.
36. **Wilson, R. L., L. T. Stauffer, and G. V. Stauffer.** 1993. Roles of the GcvA and PurR proteins in negative regulation of the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol. **175:**5129–5134.
37. **Wilson, R. L., P. S. Steiert, and G. V. Stauffer.** 1993. Positive regulation of the *Escherichia coli* glycine cleavage enzyme system. J. Bacteriol. **175:**902–904.