

## Characterization of the MetR Binding Sites for the *glyA* Gene of *Escherichia coli*

EVA LORENZ AND GEORGE V. STAUFFER\*

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

Received 14 July 1994/Accepted 4 May 1995

**Sequence analysis of the *glyA* control region of *Escherichia coli* identified two regions with homology to the consensus binding sequence for MetR, a *lysR* family regulatory protein. Gel shift assays and DNase I protection assays verified that both sites bind MetR. Homocysteine, a coregulator for MetR, increased MetR binding to the *glyA* control region. The MetR binding sites were cloned into the pBend2 vector. Although the DNA did not show any significant intrinsic bend, MetR binding resulted in a bending angle of about 33°. MetR-induced bending was independent of homocysteine. To verify that the MetR binding sites play a functional role in *glyA* expression, site-directed mutagenesis was used to alter the two binding sites in a  $\lambda$ *glyA-lacZ* gene fusion phage. Changing the binding sites toward the consensus MetR binding sequence caused an increase in *glyA-lacZ* expression. Changing either binding site away from the consensus sequence caused a decrease in expression, suggesting that both sites are required for normal *glyA* regulation.**

The *glyA* gene product of *Escherichia coli*, serine hydroxymethyltransferase, catalyzes the interconversion of serine, glycine, and one-carbon ( $C_1$ ) units and is the cell's major source of  $C_1$  units (19).  $C_1$  units are used in a variety of biochemical reactions, including the synthesis of purines, histidine, thymine, and methionine and the formylation of aminoacylated initiator tRNA (29). Thus, the serine hydroxymethyltransferase reaction plays a major role in cell physiology, and it is becoming evident that the *glyA* gene is highly regulated (5, 8, 14, 17, 34).

Previous work has shown that the addition of purines to the growth medium results in repression of the *glyA* gene (5, 32). PurR, a repressor protein for genes encoding enzymes used in purine nucleotide biosynthesis (10, 24), was shown to bind upstream of the *glyA* promoter and repress *glyA* expression (31).

MetR, a *lysR* family regulatory protein (26), is a positive regulator for the *metA* (15), *metF* (4), *metE* (36, 37), and *metH* (36, 37) genes. Homocysteine, a methionine pathway intermediate, serves as a coregulator for MetR-mediated regulation of these genes and has a negative effect on *metA* (15) and *metH* expression (36, 37) and a positive effect on *metE* expression (36, 37). However, the mechanism of homocysteine involvement in the regulation of any of these genes is unknown. MetR is also required for activation of the *glyA* gene, and homocysteine has a positive effect on this MetR-mediated regulation (22). Whether MetR is directly involved in the regulation of *glyA* or affects regulation indirectly is unknown.

Previous studies identified two possible MetR binding sites in the *E. coli* and *Salmonella typhimurium glyA* regulatory regions (21, 23, 33). In this paper, we present evidence confirming that MetR and homocysteine are required for activation of *glyA* and show that at least two MetR binding sites located upstream of the *glyA* transcription start site are required for normal regulation of the *glyA* gene.

## MATERIALS AND METHODS

**Strains and plasmids.** GS162 is  $\Delta$ *lacU169 pheA905 araD129 rpsL thi*, GS244 is  $\Delta$ *lacU169 pheA905 araD129 rpsL thi \Delta metR::Mu*, and JM107 is F' *traD36 lacI<sup>\Delta</sup>(lacZ)M15 proA<sup>+</sup>B<sup>+</sup>e14<sup>-</sup> (McrA<sup>-</sup>) \Delta(lac-proAB) thi gyrA96 (Nal<sup>r</sup>) endA1 hsdR17 (r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>-</sup>) relA1 supE44*. Plasmid vectors pBR322 (1), pMC1403 (3), and pBend2 (11) have been described previously. Plasmid pGS287 carries the *E. coli glyA* control region on a 265-bp *Bam*HI-*Hind*III fragment (nucleotides -261 to +4) (see Fig. 1) cloned into the *Bam*HI and *Hind*III sites of plasmid vector pBR322. The *Bam*HI and *Hind*III restriction sites for the *glyA* gene were generated by PCR. Plasmid pGS291 carries the two MetR binding sites on an 86-bp *Xba*I fragment (nucleotides -174 to -86) (see Fig. 1) cloned into the *Xba*I site of pBend2. The *Xba*I sites flanking the MetR binding sites were generated by PCR. The orientation of the insert was verified by sequencing as described by Sanger et al. (25). Plasmid pGS300 carries the *glyA* control region and about 200 bp of the *glyA* structural gene on an 813-bp *Eco*RI-*Bam*HI fragment (nucleotides -596 to +217) from pGS53 (20) cloned into the *Eco*RI and *Bam*HI sites of plasmid pBR322. Plasmid pGS310 carries the *E. coli glyA* control region on a 397-bp *Bam*HI-*Hind*III fragment (nucleotides -261 to +136) cloned into the *Bam*HI-*Hind*III sites of plasmid pBR322. The *Bam*HI and *Hind*III restriction sites were generated by PCR. Plasmids pGS312, pGS313, and pGS346 carry the point mutations -146T-147T (after mutation, the final sequence had changes to T at -146 and -147), -125T-126C, and -137G-148A, respectively (see Fig. 1), on 397-bp *Bam*HI-*Hind*III fragments (nucleotides -261 to +136) cloned into the *Bam*HI-*Hind*III sites of plasmid pBR322. The *Bam*HI-*Hind*III restriction sites were generated by PCR with pMC1403 mutant plasmids as templates (see "Mutant isolation" below).

**Media.** Luria broth and Luria agar were used as rich media (18). The glucose minimal medium (GM) used was described previously (30). GM was always supplemented with phenylalanine and thiamine except where indicated since most strains carry the *pheA905 thi* mutations. The following supplements were added at the concentrations indicated (in micrograms per milliliter): methionine, 200; phenylalanine, 50; thiamine, 1; DL-homocysteine, 100; X-Gal (5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside), 40.

**Gel retardation assay.** The gel retardation assay was based on the methods developed by Fried and Crothers (6) and Garner and Revzin (7). A 328-bp *FokI-NdeI* fragment that contains the entire *glyA* control region was <sup>32</sup>P labeled at the 5' terminus of the *NdeI* site with T4 polynucleotide kinase (13). A 20- $\mu$ l reaction mixture containing 6  $\mu$ l of labeled DNA (less than 3 ng) in Tris-EDTA buffer, 2  $\mu$ l of bovine serum albumin (BSA; 2 mg/ml), 2  $\mu$ l of H<sub>2</sub>O, and 10  $\mu$ l of 2 $\times$  DNA binding buffer (DBB; 1 $\times$  DBB is 10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 5% glycerol) was preincubated for 5 min at 37°C. Two microliters of the purified MetR protein at different dilutions (0.29 to 293 nM final concentration of dimer) (see Fig. 2) was added to each reaction mixture, and incubation was continued for an additional 15 min at 37°C. Where indicated, 1  $\mu$ l of homocysteine (20 or 200 mM solution of DL-homocysteine in H<sub>2</sub>O) was also added. One microliter of dye (0.1% xylene cyanol and 50% glycerol) was then added to each reaction mixture, and the samples were loaded onto a 5% polyacrylamide gel (1:30 bisacrylamide-acrylamide buffered with 10 mM Tris [pH 7.4], 0.38 M glycine, and 1 mM EDTA). Gels were prerun at 9 V/cm for 1 h before loading and then run at 12 V/cm. After electrophoresis, the gels were dried and analyzed by autoradiography.

**DNase I protection assay.** The DNase I protection assay was determined by

\* Corresponding author. Phone: (319) 335-7791. Fax: (319) 335-9006.

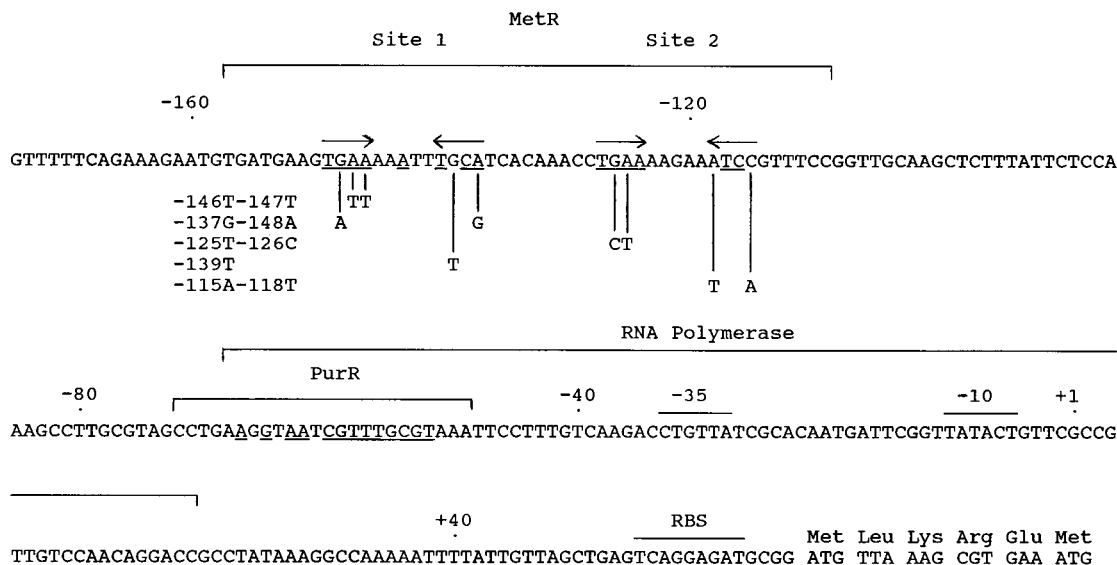


FIG. 1. The *E. coli glyA* control region. The site of transcription initiation (+1), the -35 and -10 regions of the *glyA* promoter, and the ribosome binding site (RBS) were determined previously (21, 23). The regions protected from DNase I digestion by bound PurR, RNA polymerase, and MetR are indicated by brackets. The consensus sequence for PurR is 5'-ACGCAAACGTTTGCCT-3', and the consensus sequence for MetR is 5'-TGAANNA/TNNTTCA-3'. The degenerate dyad symmetries in the *glyA* MetR binding sites are indicated with arrows above the sequence. Bases that match the consensus binding sites are underlined. Base changes in the MetR binding sites produced by *in vitro* mutagenesis are shown below the sequence.

the method of Schmitz and Galas (27) with the following modifications. The assay was carried out in a reaction volume of 25  $\mu$ l. A  $^{32}$ P-labeled 397-bp *Bam*HI-*Hind*III fragment from pGS310, pGS312, pGS313, or pGS346 containing the wild-type or mutant *glyA* control region (less than 12 ng) was preincubated for 5 min at 37°C in 1 $\times$  DBB plus 125  $\mu$ g of BSA per ml. Different dilutions of MetR protein (see Fig. 4 and 7) were added, and incubation was continued at 37°C for 15 min. A 1.25- $\mu$ l volume of a 0.003-mg/ml solution of DNase I in 20 mM sodium acetate (pH 6.0) and 32 mM CaCl<sub>2</sub> was added to each reaction mixture, and incubation was continued for 30 s at 37°C. The DNase I digestions were stopped by the addition of 5  $\mu$ l of DNase I stop solution (3 M ammonium acetate, 0.25 M EDTA, 15  $\mu$ g of sonicated calf thymus DNA per ml), and the samples were precipitated with ethanol. The partial DNase I digestion products were electrophoresed on denaturing 5% DNA sequencing gels alongside the Maxam and Gilbert (16) A+G and C+T sequencing reactions of the same fragments. After electrophoresis, the gels were dried and analyzed by autoradiography.

**Mutation isolation.** An 813-bp *Eco*RI-*Bam*HI fragment carrying the *glyA* control region and the beginning of the structural gene was cloned into the *Eco*RI-*Bam*HI sites of phage M13mp18 (31). Oligonucleotide-directed mutagenesis (12) was then used to change bases in the MetR binding sites of the recombinant phage. DNA sequencing (25) was used to screen for the desired mutations. Replicative-form DNA was isolated from each recombinant phage carrying a base change, and the 813-bp *Eco*RI-*Bam*HI fragment carrying the mutation(s) was isolated and ligated into the *Eco*RI-*Bam*HI sites of plasmid pMC1403, creating in-frame fusions of the 50th codon of *glyA* to the 8th codon of *lacZ*. The approximately 7-kb fragments carrying the mutant *glyA-lacZ* fusions and *lacY4* genes were isolated from the pMC1403 derivatives and ligated into phage  $\lambda$ gt2 as described previously (35). The wild-type phage and mutant *glyA-lacZ* phage were used to lysogenize strains GS162 and GS244. Lysogens were assayed for a single copy of the  $\lambda$  phage by infection with  $\lambda$ cI90c17 (28).

**Enzyme assay.**  $\beta$ -Galactosidase activity was assayed as described by Miller (18) by the chloroform-sodium dodecyl sulfate lysis procedure. All results are the averages of two or more assays in which each sample was done in triplicate.

**Cell growth and extract preparation.** The growth of cells and preparation of extracts for enzyme assays were as described previously (30).

**DNA manipulation.** The procedure for phage and plasmid DNA isolation, restriction enzyme digestion, DNA ligation, and polyacrylamide and agarose gel electrophoresis were as described previously (13). DNA sequencing was by the method of Sanger et al. (25) by use of the Sequenase version 2.0 kit from U.S. Biochemicals (Cleveland, Ohio) or the method of Maxam and Gilbert (16).

**PCR amplification.** PCRs were carried out under the following conditions: 10  $\mu$ l of 10 $\times$  buffer (10 $\times$  buffer is 500 mM KCl, 100 mM Tris HCl [pH 8.3], 15 mM MgCl<sub>2</sub>, 0.1% gelatin), 10  $\mu$ l of deoxynucleotide mix (2 mM of each deoxynucleoside triphosphate in 0.2 mM EDTA [pH 8.0]), 50 pmol of primer 1, 50 pmol of primer 2, 0.1  $\mu$ g of target DNA, 0.5  $\mu$ l of *Taq* DNA polymerase (Promega, Madison, Wis.), and deionized H<sub>2</sub>O in a final volume of 100  $\mu$ l. Cycling condi-

tions were as follows: 1 min of annealing at 57°C, 1 min of extension at 65°C, 30 s of denaturation at 94°C.

**Bending assay.** Fragments of equal length and with the MetR binding sites at circularly permuted locations were isolated by digestion of plasmid pGS291 with appropriate restriction enzymes, and the resulting DNA fragments were labeled with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP (13). The bending capability of each fragment was tested by electrophoresis in nondenaturing 5% polyacrylamide gels. After electrophoresis, the gels were dried and analyzed by autoradiography.

**Chemicals.** [ $\gamma$ - $^{32}$ P]ATP and [ $\alpha$ - $^{32}$ P]TTP were purchased from Amersham (Arlington Heights, Ill.). Nonradioactive chemicals were reagent grade and available commercially.

## RESULTS

**Gel retardation assay.** Sequence analysis of the *glyA* control region from *E. coli* and *S. typhimurium* identified two stretches of DNA with homology to known MetR binding sites and that were conserved in both organisms (23, 33). The more-upstream site (Fig. 1, site 1) is centered at bp -143, and the sequence 5'-TGAANNANNTGCA-3' has eight of nine nucleotides matching the consensus sequence 5'-TGAANNA/TNNTTCA-3'. The second site (Fig. 1, site 2) is centered at bp -121, and the sequence 5'-TGAANNGNNATCC-3' has six of nine nucleotides that match the consensus sequence. To verify that MetR protein binds to a DNA fragment carrying these sequences, we used a gel mobility shift assay (see Materials and Methods). As the concentration of MetR was increased from 0.29 to 4.6 nM, a DNA fragment carrying these sites was shifted to a single band of lower mobility (Fig. 2, band A1, lanes 8 to 12). As the concentration of MetR was increased from 4.6 to 9.2 nM, a second band was observed (Fig. 2, band B, lane 7), which was the prominent band at 36.6 nM (Fig. 2, band B, lane 5). The two bands correspond primarily to complexes of MetR dimers bound at MetR binding sites 1 and 2, respectively (see Fig. 1 and DNase I protection assay below). At higher concentrations of MetR (greater than 36.6 nM), a third band was observed, suggesting that a third binding site with a low affinity for MetR is also present (Fig. 2, band C,

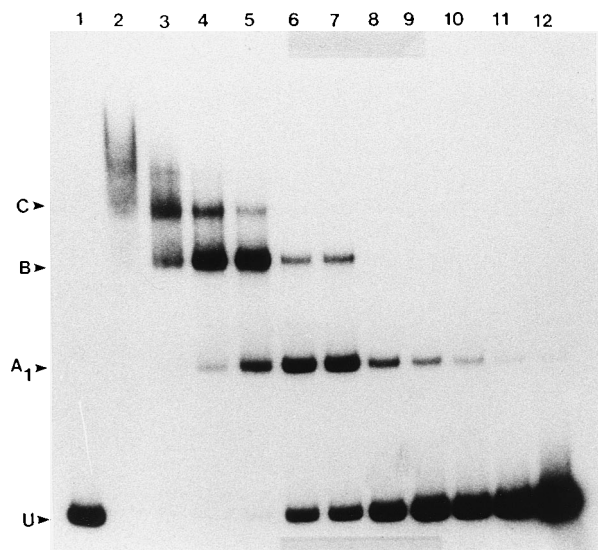


FIG. 2. Gel mobility shift assay of MetR binding to the wild-type *glyA* control region. A 328-bp  $^{32}\text{P}$ -labeled *FokI-NdeI* fragment carrying the *glyA* control region was incubated with dilutions of MetR, and the samples were run on a 5% polyacrylamide gel as described in Materials and Methods. Lanes: 1, no MetR; 2 through 12, 293, 146, 73, 36.6, 18.3, 9.2, 4.6, 2.3, 1.1, 0.57, and 0.29 nM MetR dimer, respectively. The positions of unbound (U) fragment and three gel-retarded bands (A<sub>1</sub>, B, and C) are indicated.

lanes 3 to 5). At a MetR concentration of 293 nM, discrete bands were not identified (Fig. 2, lane 2), suggesting that MetR was binding nonspecifically to the DNA fragment.

**Effects of homocysteine on MetR binding.** We determined whether homocysteine, the coregulator for MetR-mediated activation of *glyA* (22), has any effect on MetR binding to the *glyA* control region. The MetR concentrations used were 9.1 to 36.6 nM since these concentrations consistently produced signifi-

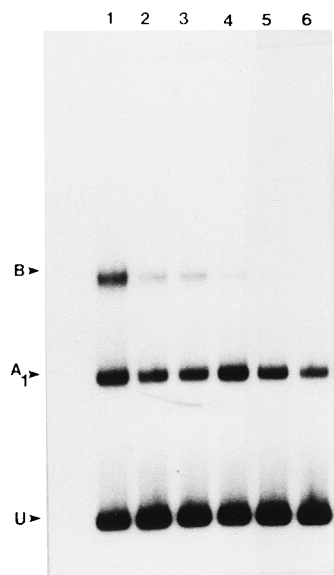


FIG. 3. Effect of homocysteine on MetR binding. A 328-bp  $^{32}\text{P}$ -labeled fragment carrying the *glyA* control region was incubated with dilutions of MetR and either with (lanes 1 to 3) or without (lanes 4 to 6) 10 mM homocysteine. Lanes: 1 and 4, 36.6 nM MetR; 2 and 5, 18.3 nM MetR; 3 and 6, 9.2 nM MetR. The positions of the unbound (U) fragment and bands A<sub>1</sub> and B are indicated.

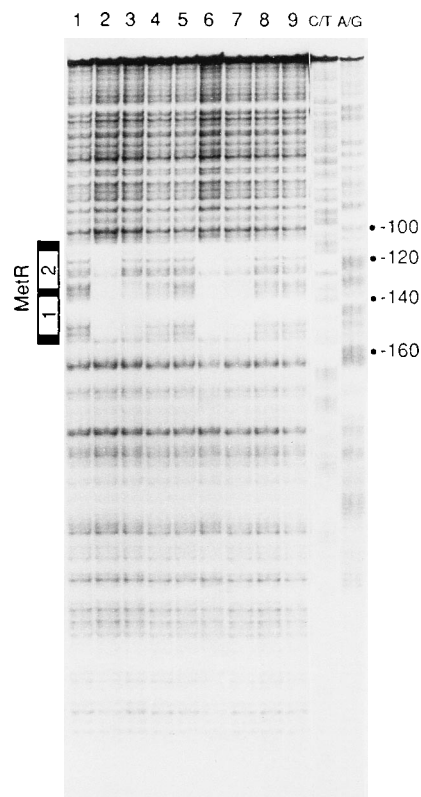


FIG. 4. Protection of the *glyA* control region from DNase I by MetR. A 397-bp 5'  $^{32}\text{P}$ -labeled DNA fragment carrying the *glyA* control region was incubated with dilutions of MetR as described in Materials and Methods and digested with DNase I. The partial digestion products were run on a sequencing gel alongside the A+G and C+T sequencing reactions (16) of the original DNA fragment. Lanes: 1, no MetR; 2 and 6, 146 nM MetR; 3 and 7, 73 nM MetR; 4 and 8, 36.6 nM MetR; 5 and 9, 18.3 nM MetR. In addition, lanes 6 to 9 had homocysteine added to a final concentration of 10 mM. The protected region is indicated, as well as the relative locations of MetR binding sites 1 and 2. The sequence is numbered on the right of the gel according to the nucleotide assignments shown in Fig. 1.

cant amounts of unbound fragment and bands A<sub>1</sub> and B. Homocysteine was added at a final concentration of 10 mM. Homocysteine resulted in a significant increase in MetR binding to the *glyA* control region (Fig. 3). Similar results were obtained with 1 mM homocysteine (data not shown), although 10 mM homocysteine was more effective than 1 mM.

**DNase I protection assay of the *glyA* control region.** To verify that MetR binds to sites 1 and 2 and that homocysteine increases its affinity to these sites, a DNase I footprint analysis was carried out (see Materials and Methods). As the MetR concentration was increased from 36.6 to 73 nM, a 25-bp region was protected from DNase I digestion, from nucleotides -155 to -135 (Fig. 4, MetR site 1). As the concentration of MetR was increased to 146 nM, a second region was protected from DNase I digestion from nucleotides -109 to -134 (Fig. 4, MetR site 2). These are the two sites predicted as MetR binding sites from a comparison of the *E. coli* and *S. typhimurium* sequences and are shown in Fig. 1.

We then tested whether homocysteine, the coactivator of MetR, affects binding of MetR to either site 1 or site 2. When homocysteine was added at a concentration of 10 mM, there was a small increase in protection of high-affinity site 1 at 73 nM MetR (Fig. 4, compare lanes 3 and 7, nucleotides -155 to -135). However, site 2 was also almost fully protected at a

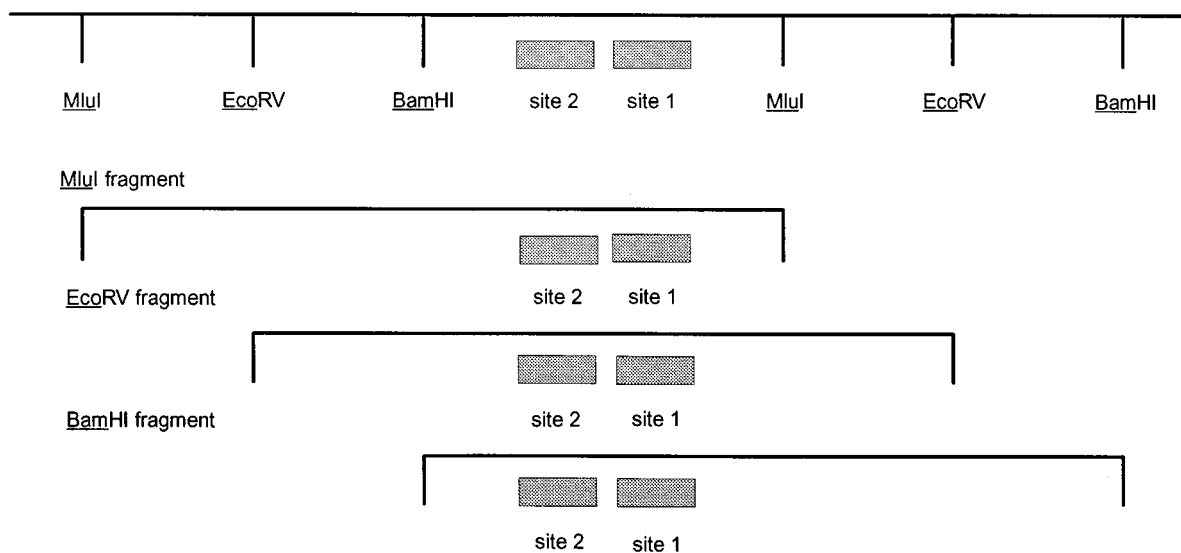


FIG. 5. Location of MetR binding sites 1 and 2 in the 207-bp *MluI*-, *EcoRV*-, and *BamHI*-generated fragments from plasmid pGS291. Only relevant restriction sites are indicated.

MetR concentration of 73 nM, the same concentration providing full protection of site 1. This concentration of MetR does not protect site 2 from DNase I digestion in the absence of homocysteine.

**Bending of the *glyA* control region.** When bound, DNA-binding proteins sometimes cause a conformational change in the DNA to exert either a positive or negative effect on gene expression (9, 38). Bending of DNA is one kind of conformational change often seen, presumably allowing appropriate protein-DNA and protein-protein interactions to occur. Bending occurs as a smooth curving of the DNA or as strong localized bends, such as kinks or junction bends (38). In addition, DNA sequences rich in AT allow conformational changes such as bending. Specifically, the sequence CA<sub>5/6</sub>T has been implicated in allowing DNA bending (39). The region spanning the two MetR binding sites in the *glyA* control region is rich in AT stretches, and the sequence GA<sub>5</sub>T is centered around the upstream MetR binding site 1 (Fig. 1). Therefore, we investigated whether a DNA fragment carrying the MetR binding sites has an intrinsic bend or whether MetR binding induces bending of the DNA. A DNA fragment carrying the two MetR binding sites was cloned into the pBend2 vector (see Materials and Methods), and three different enzymes were used to generate fragments of identical length (207 bp). *MluI* and *BamHI* were used to generate fragments with the MetR binding sites located at the left and right ends of the fragment, respectively, while *EcoRV* was used to generate a fragment with the MetR binding sites in the center of the fragment (Fig. 5). Gel electrophoresis of the DNA fragments showed no significant intrinsic DNA bending (Fig. 6). However, addition of MetR caused a change in DNA conformation, as shown by the altered mobilities of the different MetR-DNA complexes. MetR binding to the *EcoRV*-generated fragment, with the MetR binding sites in the center of the fragment (Fig. 5), resulted in the most slowly migrating species. This is the case when MetR is bound only to site 1 or when both MetR binding sites are occupied (Fig. 6, bands A<sub>1</sub> and B). These results suggest that the bending center is located in the region of the two MetR binding sites. It should be noted that the *MluI*-generated fragment migrated slightly faster than the *BamHI*- and the *EcoRV*-generated fragments. However, when only MetR binding site 1

is occupied, it appears that the difference in the mobility of the *MluI*-generated fragment and the *BamHI*-generated fragment is enhanced. Since MetR binding site 1 is closer to the end in the *MluI*-generated fragment, whereas MetR binding site 2 is closer to the end in the *BamHI*-generated fragment (Fig. 6), the results suggest that MetR binding site 1 is the center of bending for the singly occupied species.

By use of the formula  $\mu M/\mu E = \cos\alpha/2$  (11), where  $\mu M$  is the mobility of the DNA with the protein bound in the middle of the fragment and  $\mu E$  is the mobility of the DNA with the binding site located at the end of the DNA, the apparent bending angle for the *glyA* control region was determined. Apparent bending angles of about 22 and 30° were calculated for the *BamHI* and *MluI* fragments relative to the *EcoRV* fragment, respectively, with MetR bound at site 1. When both MetR sites were occupied, an apparent bending angle of about

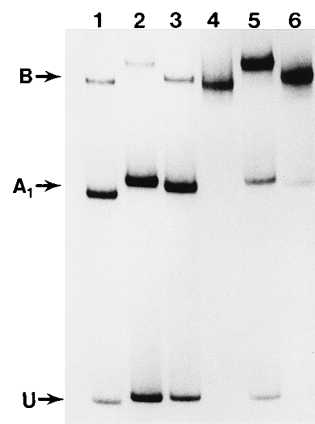


FIG. 6. Bending assay. The 207-bp <sup>32</sup>P-labeled *MluI* (lanes 1 and 4), *EcoRV* (lanes 2 and 5), and *BamHI* (lanes 3 and 6) fragments carrying MetR binding sites 1 and 2 of the wild-type *glyA* control region were incubated with MetR added at 73 nM. Lanes 4 to 6 also had homocysteine added at a final concentration of 10 mM. The samples were then electrophoresed on a 5% polyacrylamide gel. The positions of the unbound (U) fragment and the single (A<sub>1</sub>)- and double (B)-bound species are indicated.

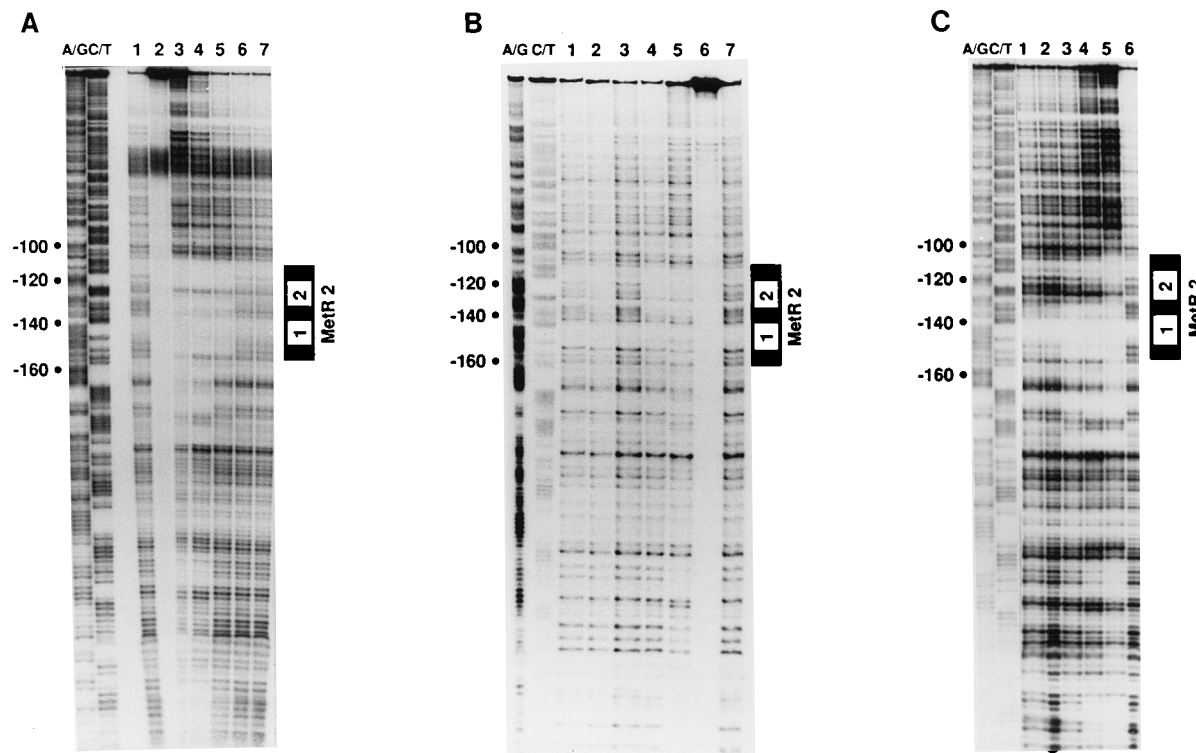


FIG. 7. Protection of the mutated *glyA* control regions by MetR. A 397-bp 5'  $^{32}$ P-labeled DNA fragment carrying the *glyA* control region with base pair changes in MetR binding site 1 or 2 was incubated with dilutions of MetR protein and digested with DNase I as described in Materials and Methods. The partial digestion products were run on a sequencing gel alongside the A+G and C+T sequencing reactions (16) of the original DNA fragment. (A) Site 1 mutant -146T-147T from plasmid pGS312. Lanes: 1, no MetR; 2, 374.4 nM MetR; 3, 187.2 nM MetR; 4, 93.6 nM MetR; 5, 46.8 nM MetR; 6, 23.4 nM MetR; 7, 11.7 nM MetR. (B) Site 1 mutant -137G-148A from pGS346. Lanes: 1, 11.7 nM MetR; 2, 23.4 nM MetR; 3, 46.8 nM MetR; 4, 93.6 nM MetR; 5, 187.2 nM MetR; 6, 374.4 nM MetR; 7, no MetR. (C) Site 2 mutant -125T-126C from pGS313. Lanes: 1, 11.7 nM MetR; 2, 23.4 nM MetR; 3, 46.8 nM MetR; 4, 93.6 nM MetR; 5, 187.2 nM MetR; 6, no MetR. Except for the lanes with no protein, all lanes had homocysteine added to a final concentration of 10 mM. The relative locations of MetR binding sites 1 and 2 are indicated. The sequence is numbered to the left of each gel according to the nucleotide assignment in Fig. 1.

33° was calculated for both fragments (Fig. 3), suggesting that MetR bound at both sites contributes to the overall bending of the DNA. It should be noted that the measurement of bending by gel electrophoresis is only an apparent bending angle, since the conformational changes of the DNA itself, such as the level of flexibility, influence the degree of bending (38).

**Role of homocysteine in DNA bending.** We used a gel mobility shift assay to determine whether homocysteine affects the degree of DNA bending as well as the affinity of MetR for DNA. The MetR concentration used was 18.3 nM, and homocysteine was added at a final concentration of 10 mM. Homocysteine increased MetR binding to the *glyA* control region but did not affect the degree of DNA bending (Fig. 6).

**Effects of mutations in MetR binding sites 1 and 2 on *glyA-lacZ* expression.** To determine whether MetR binding sites 1 and 2 are functionally important in the MetR-mediated activation of the *glyA* gene, we used site-directed mutagenesis to introduce mutations into both sites (see Materials and Methods). The nucleotide changes were confirmed by DNA sequencing and are shown in Fig. 1.

Initially, we used a DNase I footprint assay to determine whether MetR is able to bind to the mutant templates with base pair changes away from the consensus sequence (see Materials and Methods). At low concentrations of MetR protein (11.7 and 23.4 nM), site 1 and site 2 were unprotected from DNase I digestion for all mutant templates (Fig. 7). As the concentration of MetR protein was increased (46.8 to 187.2 nM), site 1 mutant -137G-148A (Fig. 7B) and site 2 mutant

-125T-126C (Fig. 7C) were essentially unprotected. These concentrations of MetR allow full protection of site 1 and site 2 of a wild-type template (Fig. 4). However, site 1 mutant -146T-147T still showed good protection from DNase I digestion at concentrations of MetR of 46.8 nM and higher (Fig. 7A). At concentrations higher than 187.2 nM, MetR bound and protected nonspecifically.

The altered *glyA-lacZ* fusions were cloned into  $\lambda$ gt2 as described above (see Materials and Methods). The mutant phage and the wild-type  $\lambda$ *glyA-lacZ* phage were used to lysogenize strains GS162 and GS244 (*metR*). The lysogens were grown in GM supplemented with D-methionine or D-methionine plus homocysteine, and  $\beta$ -galactosidase levels were measured. In the control 162 $\lambda$ *glyA-lacZ* lysogen, addition of the coactivator homocysteine increased *glyA-lacZ* expression slightly (Table 1). The small homocysteine-mediated increase in *glyA-lacZ* expression is likely due to sufficient homocysteine levels in the cell under the growth conditions used (36). In lysogen 244 $\lambda$ *glyA-lacZ*,  $\beta$ -galactosidase levels were reduced as a result of the absence of MetR, and homocysteine had no effect on enzyme levels.

Lysogens 162 $\lambda$ *glyA-lacZ*-146T-147T and 162 $\lambda$ *glyA-lacZ*-137G-148A carry base pair changes away from the MetR consensus sequence in site 1, and lysogen 162 $\lambda$ *glyA-lacZ*-125T-126C carries base pair changes away from the MetR consensus sequence in site 2 (Fig. 1). The changes result in significant decreases in *glyA-lacZ* expression with growth in GM plus D-methionine, and the addition of homocysteine

TABLE 1. Effects of mutations in MetR binding sites 1 and 2 away from the consensus sequence on *glyA-lacZ* expression in GS162 (wild type) and GS244 (*metR*)

Lysogen <sup>a</sup>	Mutation site	β-Galactosidase activity <sup>b</sup>	
		D-Methionine	D-Methionine + homocysteine
GS162λ <i>glyA-lacZ</i>	None (wild type)	3,600	4,000
GS244λ <i>glyA-lacZ</i>	None (wild type)	2,800	2,600
GS162λ <i>glyA-lacZ</i> -146T-147T	Site 1 down	1,800	1,700
GS244λ <i>glyA-lacZ</i> -146T-147T	Site 1 down	2,500	2,700
GS162λ <i>glyA-lacZ</i> -137G-148A	Site 1 down	1,900	1,900
GS244λ <i>glyA-lacZ</i> -137G-148A	Site 1 down	2,900	2,900
GS162λ <i>glyA-lacZ</i> -125T-126C	Site 2 down	1,600	1,800
GS244λ <i>glyA-lacZ</i> -125T-126C	Site 2 down	2,200	2,400
GS162λ <i>glyA-lacZ</i> -125T-126C-146T-147T	Site 1 and 2 down	2,600	2,000
GS244λ <i>glyA-lacZ</i> -125T-126C-146T-147T	Site 1 and 2 down	2,400	2,000

<sup>a</sup> All lysogens were grown in GM with the indicated supplements.

<sup>b</sup> Units of activity are Miller units (18). The standard deviations in all samples were less than 15%.

had no significant effect (Table 1). In lysogens 244λ*glyA-lacZ*-146T-147T, 244λ*glyA-lacZ*-137G-148A, and 244λ*glyA-lacZ*-125T-126C, where no MetR protein is produced, β-galactosidase levels were about 1.5-fold higher than those in the GS162 lysogens, and the addition of homocysteine had no significant effect. We also inactivated both sites 1 and 2 simultaneously. Lysogen 162λ*glyA-lacZ*-146T-147T-125T-126C, which carries mutations in both sites 1 and 2, had elevated levels of β-galactosidase compared with lysogens with mutations in site 1 or 2 alone, and the addition of homocysteine had no effect on *glyA-lacZ* expression (Table 1). We also lysogenized strain GS244 with phage λ*glyA-lacZ*-146T-147T-125T-126C. In GM plus D-methionine or GM plus D-methionine and homocysteine, β-galactosidase levels were comparable to those of strain GS162.

We also made mutations in sites 1 and 2 towards the MetR consensus binding sequence. Phage λ*glyA-lacZ*-139T carries a single change in site 1, creating a perfect match to the consensus MetR binding site (Fig. 1), and phage λ*glyA-lacZ*-115A-118T has two changes in site 2 towards the consensus sequence. The mutant phage and wild-type λ*glyA-lacZ* phage were used to lysogenize strains GS162 and GS244, the lysogens were grown in GM plus D-methionine or GM plus D-methionine and homocysteine, and β-galactosidase levels were measured. Changes in either site towards the consensus sequence increased *glyA-lacZ* expression about 2- to 2.5-fold compared with that of the control 162λ*glyA-lacZ* lysogen (Table 2). The coactivator homocysteine increased β-galactosidase levels in the site 1 up mutant but caused a slight decrease in the site 2 up mutant.

We also tested the effects of the mutations in the *metR* mutant strain GS244. β-Galactosidase levels in lysogen 244λ*glyA-lacZ*-139T were significantly reduced compared with those of the 162λ*glyA-lacZ*-139T lysogen, and homocysteine had no effect on the enzyme levels (Table 2). However, lysogen 244λ*glyA-lacZ*-139T consistently showed higher β-galactosidase levels than those of the control lysogen 244λ*glyA-lacZ* (Table 1). Lysogen 244λ*glyA-lacZ*-115A-118T also showed reduced levels of β-galactosidase compared with those of 162λ*glyA-lacZ*-115A-118T, and homocysteine had no effect on the enzyme levels (Table 2). These levels were not significantly different from those of the 244λ*glyA-lacZ* control lysogen.

## DISCUSSION

A DNA sequence search of the *E. coli* and *S. typhimurium* *glyA* genes identified two regions upstream of the *glyA* promoter with homology to the consensus binding motif for MetR and that are conserved in both organisms. In this paper, we present genetic and biochemical evidence that both sites bind MetR and that both sites are necessary for normal *glyA* gene expression in *E. coli*.

Gel shift assays showed that MetR binds to a DNA fragment carrying the *glyA* control region, resulting in the formation of three more slowly migrating complexes (Fig. 2, bands A1, B, and C). As the concentration of MetR was increased from 0.29 to 4.6 nM, band A1 was present in high amounts and was followed by a shift to band B. A DNase I footprint analysis showed that these bands correlate to the binding of MetR to two binding sites centered around bp -143 and -121 up-

TABLE 2. Effects of mutations in sites 1 and 2 towards the MetR consensus binding sequence on *glyA-lacZ* expression in strains GS162 and GS244

Lysogen <sup>a</sup>	Mutation site	β-Galactosidase activity <sup>b</sup>	
		D-Methionine	D-Methionine + homocysteine
GS162λ <i>glyA-lacZ</i>	None (wild type)	3,600	4,000
GS244λ <i>glyA-lacZ</i>	None (wild type)	2,800	2,600
GS162λ <i>glyA-lacZ</i> -139T	Site 1 up	7,200	8,300
GS244λ <i>glyA-lacZ</i> -139T	Site 1 up	3,300	3,400
GS162λ <i>glyA-lacZ</i> -115A-118T	Site 2 up	6,300	5,500
GS244λ <i>glyA-lacZ</i> -115A-118T	Site 2 up	2,600	2,800

<sup>a</sup> All lysogens were grown in GM with the indicated supplements.

<sup>b</sup> Units of activity are Miller units (18). The standard deviations in all samples were less than 15%.

stream of the *glyA* transcription start site (Fig. 4). Sequence comparison showed that site 1, the stronger binding site, has a higher match to the MetR consensus sequence (Fig. 1). It should be noted that there was a significant increase in the affinity of MetR for site 2 (band B) as the protein concentration was increased from 4.6 to 9.1 nM (Fig. 2). It is possible that MetR uses cooperativity in binding to the *glyA* control region. Only at high concentrations of MetR (greater than 293 nM) was band C present in significant amounts (Fig. 2). Additional quantitative DNase I footprint assays should help elucidate the mechanism of binding of MetR to sites 1 and 2 and whether a third MetR binding site exists with low affinity for MetR.

Homocysteine is a coregulator of MetR for several of the *met* genes (36) and the *glyA* gene (22). We determined whether homocysteine affects the binding of MetR to *glyA*. At a concentration of 10 mM, homocysteine significantly increased binding of MetR to the *glyA* control region (Fig. 3). Similar results were observed with 1 mM homocysteine, although 10 mM was more effective (data not shown). Although homocysteine does cause a minor increase in the binding of MetR to site 1, the major effect appears to be to increase the binding of MetR to the low-affinity site 2 (Fig. 4). Increasing MetR affinity for the *glyA* control region is a possible mechanism for the homocysteine involvement in the activation of *glyA*. However, further experiments are needed to define the precise role of homocysteine in *glyA* regulation.

The MetR binding sites for *glyA* are centered at bp -143 and bp -121 relative to the transcription start site, considerably further upstream than the MetR binding sites found for the *met* genes (2, 4, 15, 37). This would not allow a direct interaction between MetR and RNA polymerase unless DNA bending occurs. In *E. coli*, a variety of DNA-binding proteins have been reported to result in bending angles ranging from 25° for the MerR protein to 90° for the CAP protein (38), and bending of the DNA is required for appropriate gene expression. Gel retardation assays showed that MetR bound to sites 1 and 2 results in a bending angle of about 33° for the *glyA* control region. Homocysteine, the known coactivator for MetR, did not affect the degree of bending and appears to exert its primary effect on increasing MetR binding to the *glyA* control region. Whether the MetR-induced bending is required for *glyA* expression either by the conformational change in the DNA molecule itself or by facilitating an interaction between MetR and RNA polymerase or other regulatory proteins that could bind between the MetR and RNA polymerase binding sites requires further experimentation.

To demonstrate that both MetR binding sites 1 and 2 are functionally important in *glyA* activation, we tested the effects of mutations in sites 1 and 2 either towards or away from the MetR consensus sequence on the expression of a *glyA-lacZ* fusion phage. Mutations in either site away from the consensus sequence decreased *glyA-lacZ* expression when assayed with or without homocysteine (Table 1). Mutations in either site towards the consensus sequence increased *glyA-lacZ* expression (Table 2). The results show that both sites are necessary for normal regulation of the *glyA* gene.

To determine whether the effects of the mutations on *glyA-lacZ* expression are MetR dependent, the wild-type phage and mutant phage were also used to lysogenize the *metR* mutant strain GS244. If MetR is involved in the mechanism of decreased or increased expression of the *glyA-lacZ* fusion, then a *metR* mutation should result in similar levels of expression in the wild-type and mutant lysogens. However, the  $\beta$ -galactosidase levels in GS244 lysogens carrying mutations away from the consensus sequence were about 1.5-fold higher than those

in the GS162 lysogens (Table 1). It is possible that MetR bound to only one active site has a negative effect on *glyA* expression, since the levels increase when both sites 1 and 2 are inactivated.

We used a DNase I footprint assay to verify that the mutant templates with base pair changes away from consensus were altered in their affinity for MetR protein. Although site 1 mutant -137G-148A and site 2 mutant -125T-126C were altered in their ability to bind MetR, site 1 mutant -146T-147T was not significantly altered in its ability to bind MetR protein (compare Fig. 4 and 7). It should be noted that the -146T-147T base pair changes in site 1 are located in the center of the dyad symmetry (Fig. 1). It was shown previously for the MetR binding site for the *metH* gene that base changes located on the outer ends of the dyad symmetry required for MetR binding severely reduce the affinity of the DNA for MetR, whereas base changes in the center of the dyad symmetry do not greatly affect the affinity of the binding site for MetR but prevent normal activation of the *metH* gene (2). Since all three mutants with base pair changes away from the consensus sequence prevent normal MetR-mediated activation of *glyA*, the results are consistent with the hypothesis that the MetR recognition sequence consists of two functional domains, one for binding and one for activation (2).

#### ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GM26878 from the National Institute of General Medical Sciences.

We thank S. Adhya for pBend2.

#### REFERENCES

- Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crossa, and S. Falkow. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2:95-113.
- Byerly, K. A., M. L. Urbanowski, and G. V. Stauffer. 1991. The MetR binding site in the *Salmonella typhimurium metH* gene: DNA sequence constraints on activation. *J. Bacteriol.* 173:3547-3553.
- Casadaban, M. J., J. Chou, and S. N. Cohen. 1980. In vitro gene fusions that join an enzymatically active  $\beta$ -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.
- Cowan, J. M., M. L. Urbanowski, M. Talmi, and G. V. Stauffer. 1993. Regulation of the *Salmonella typhimurium metF* gene by the MetR protein. *J. Bacteriol.* 175:5862-5866.
- Dev, I. K., and R. J. Harvey. 1984. Role of methionine in the regulation of the synthesis of serine hydroxymethyltransferase in *Escherichia coli*. *J. Biol. Chem.* 259:4802-4806.
- Fried, M., and D. Crothers. 1983. Equilibrium and kinetics of *lac* repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* 9:6505-6525.
- Garner, M., and A. Revzin. 1981. A gel electrophoresis method for quantifying the binding of proteins to specific DNA regions: application to components of the *E. coli* lactose operon regulatory system. *Nucleic Acids Res.* 9:3047-3060.
- Greene, R. C., and C. Radovich. 1975. Role of methionine in the regulation of serine hydroxymethyltransferase in *Escherichia coli*. *J. Bacteriol.* 124:269-278.
- Hagerman, P. J. 1990. Sequence-directed curvature of DNA. *Annu. Rev. Biochem.* 59:755-781.
- 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.* 171:2121-2127.
- Kim, J., C. Zwieb, C. Wu, and S. Adhya. 1989. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene* 85:15-23.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. USA* 82:488-492.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Mansouri, A., J. B. Decter, and R. Silber. 1972. Studies on the regulation of one-carbon-metabolism. II. Repression-derepression of serine hydroxymethyltransferase by methionine in *Escherichia coli* 113-3. *J. Biol. Chem.* 247:348-352.

15. Mares, R. M., M. L. Urbanowski, and G. V. Stauffer. 1992. Regulation of the *Salmonella typhimurium metA* gene by the metR protein and homocysteine. *J. Bacteriol.* **174**:390–397.
16. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499–560.
17. Meedel, T. H., and L. I. Pizer. 1974. Regulation of one-carbon biosynthesis and utilization in *Escherichia coli*. *J. Bacteriol.* **118**:905–910.
18. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. 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. Florin and E. H. Stolz (ed.), *Comprehensive biochemistry*, vol. 15. Elsevier, Amsterdam.
20. Plamann, M. D. 1985. Characterization of the *Escherichia coli glyA* and *gcv* genes. Ph.D. thesis. University of Iowa, Iowa City.
21. Plamann, M. D., and G. V. Stauffer. 1983. Characterization of the *Escherichia coli* gene for serine hydroxymethyltransferase. *Gene* **22**:9–18.
22. Plamann, M. D., and G. V. Stauffer. 1989. Regulation of the *Escherichia coli glyA* gene by the *metR* gene product. *J. Bacteriol.* **171**:4958–4962.
23. Plamann, M. D., L. T. Stauffer, M. L. Urbanowski, and G. V. Stauffer. 1983. Complete nucleotide sequence of the *E. coli glyA* gene. *Nucleic Acids Res.* **11**:2065–2075.
24. 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.
25. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463–5467.
26. Schell, M. A. 1993. Molecular biology of the LysR family of transcriptional regulators. *Annu. Rev. Microbiol.* **47**:597–626.
27. Schmitz, A., and D. J. Galas. 1979. The interaction of RNA polymerase and *lac* repressor with the *lac* control region. *Nucleic Acids Res.* **6**:111–117.
28. Shimada, K., R. A. Weisberg, and M. E. Gottesmann. 1972. Prophage lambda at unusual chromosomal locations. I. Location of the secondary attachment sites and the properties of the lysogens. *J. Mol. Biol.* **63**:483–503.
29. Stauffer, G. V. 1987. Biosynthesis of serine and glycine, p. 412–418. In F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
30. Stauffer, G. V., and J. E. Brenchley. 1977. Influence of methionine biosynthesis on serine transhydroxymethylase regulation in *Salmonella typhimurium* LT2. *J. Bacteriol.* **129**:740–749.
31. Steiert, J. G., C. Kubu, and G. V. Stauffer. 1992. The PurR binding sites in the *glyA* promoter region of *Escherichia coli*. *FEMS Microbiol. Lett.* **99**:299–304.
32. Steiert, J. G., R. J. Rolfes, H. Zalkin, and G. V. Stauffer. 1990. Regulation of the *Escherichia coli glyA* gene by the *purR* gene product. *J. Bacteriol.* **172**:3799–3803.
33. Steiert, J. G., M. L. Urbanowski, L. T. Stauffer, M. D. Plamann, and G. V. Stauffer. 1990. Nucleotide sequence of the *Salmonella typhimurium glyA* gene. DNA sequence. **1**:107–113.
34. Taylor, R., H. Dickerman, and H. Weissbach. 1966. Control of one carbon metabolism in a methionine B12 auxotroph of *Escherichia coli*. *Arch. Biochem. Biophys.* **117**:405–412.
35. Urbanowski, M. L., and G. V. Stauffer. 1986. Autoregulation by tandem promoters of the *Salmonella typhimurium* LT2 *metJ* gene. *J. Bacteriol.* **165**:740–745.
36. Urbanowski, M. L., and G. V. Stauffer. 1989. The role of homocysteine in the *metR*-mediated activation of the *metE* and *metH* genes in *Salmonella typhimurium* and *Escherichia coli*. *J. Bacteriol.* **171**:3277–3281.
37. Urbanowski, M. L., L. T. Stauffer, L. S. Plamann, and G. V. Stauffer. 1987. A new methionine locus, *metR*, that encodes a *trans*-acting protein required for activation of *metE* and *metH* in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **169**:1391–1397.
38. van der Vliet, P. C., and C. P. Verrijzer. 1993. Bending of DNA by transcription factors. *BioEssays* **15**:25–32.
39. Wu, H. M., and D. M. Crothers. 1984. The locus of sequence-directed and protein-induced DNA bending. *Nature (London)* **308**:509–513.