JOURNAL OF BACTERIOLOGY, July 1990, p. 3799–3803
0021-9193/90/073799-05$02.00/0

Vol. 172, No. 7

Regulation of the *Escherichia coli glyA* Gene by the *purR* Gene Product

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The purine regulon repressor protein, PurR, was shown to be a purine component involved in *glyA* regulation in *Escherichia coli*. Expression of *glyA*, encoding serine hydroxymethyltransferase activity, was elevated in a *purR* mutant compared with a wild-type strain. When the *purR* mutant was transformed with a plasmid carrying the *purR* gene, the serine hydroxymethyltransferase levels returned to the wild-type level. The PurR protein bound specifically to a DNA fragment carrying the *glyA* control region, as determined by gel retardation. In a DNase I protection assay, a 24-base-pair region was protected from DNase I digestion by PurR. The *glyA* operator sequence for PurR binding is similar to that reported for several *pur* regulon genes.

Serine hydroxymethyltransferase (SHMT), the *glyA* gene product, catalyzes the conversion of serine to glycine and a one-carbon (C_1) unit. This reaction is the major source of glycine and C_1 units for the cell (11, 12). Although several compounds (serine, glycine, methionine, purines, thymine, and folates) are known to affect the expression of the *glyA* gene (1, 9, 21), no single compound completely activates or inhibits expression of the gene. Instead, a cumulative effect is observed in the growth medium with the addition or removal of these compounds (9, 21). Thus, the regulatory control mechanisms for this gene are complex and poorly understood at this time.

Recently, the MetR protein was identified as being the methionine component involved in *glyA* regulation (14). The MetR protein positively controls the expression of the *glyA* gene and requires homocysteine, an intermediate in methionine metabolism, as the coactivator.

Here we show that the PurR protein, a regulatory protein in purine nucleotide synthesis (5, 16, 17), is a purine component involved in the regulation of the *glyA* gene, and we identify the binding site of the PurR protein in the *glyA* promoter region.

MATERIALS AND METHODS

Bacterial strains. Strains and plasmids used in this study are listed in Table 1.

Lysogenic strains R100 and R300 were cured of λRRO (*purF-lacZ*) prophage by transduction with P1 bacteriophage from strain GS751 (Δ*galK*::Σ*tet-50*) (26). Transductants were selected on Luria agar plates containing tetracycline (3 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) (40 μg/ml). Transductants that were negative for β-galactosidase activity (white on X-Gal plates) were presumed to be cured of the λ prophage. This was verified by showing that the strains could be relysogenized with a second λ phage.

To construct a *metR purR* double mutant, P1 phage grown on strain GS849 (*purR*::Tn*10*) was used to transduce strain GS244 (*metR*). Transductants were selected on Luria agar plates containing tetracycline (10 μg) and then spotted on glucose minimal medium (GM) plates containing phenylalanine (50 μg/ml), thiamine (1 μg/ml), L-methionine (50 μg/ml), and 6-mercaptopurine (2 mM). One tetracycline-resistant, 6-mercaptopurine-resistant colony was saved and designated GS924.

Media. Luria broth and Luria agar were used as rich media (10). GM was made as previously described (23). Inosine was added as a purine supplement at a concentration of 100 μg/ml.

Growth of cells and extract preparation for SHMT assay. Cell growth and crude extract preparation for enzyme assays were as previously described (22).

SHMT assay. SHMT activity was measured as previously described (24). All assay results reported are averages from at least three separate trials done in triplicate. Protein determination was by the Lowry method, with bovine serum albumin as the standard (7).

Extract preparation for gel retardation and DNase I protection assays. Protein extracts for gel retardation and DNase I protection assays were prepared from strains R300C and R303(pRRM127). Cultures (250 ml) of cells were grown overnight in GM plus inosine. Kanamycin (20 μg/ml) was added to the medium for the growth of R303(pRRM127). The cells were collected by centrifugation and suspended in 2 ml of 2× DNA-binding buffer (2× buffer is 10 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol) (26). The cell suspensions were sonicated, and cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 4°C. The extracts were assayed for protein content by the method of Lowry (7). Portions of the extracts were placed in polypropylene tubes and stored at −70°C until further use.

Gel retardation assay. The gel retardation assay was based on the methods of Fried and Crothers (2) and Garner and Revzin (3). A 368-base-pair (bp) *Fok*I DNA fragment, which includes the entire *glyA* control region, was isolated from pGS54 (M. D. Plamann and G. V. Stauffer, Abstr. Genet. Soc. Am. **97**:586, 1981). The fragment was labeled with ^{32}P at the 5′ termini and digested with *Nde*I, and a 341-bp fragment carrying the *glyA* control region was isolated. The labeled DNA was added to 20-μl reaction mixtures at a final concentration of less than 10^{-9} M. The reaction mixtures

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TABLE 1. *E. coli* strains and plasmids

Strain or plasmid	Genotype or description[a]	Source
Strains		
R100	Δ(*argF-lac*)*169*(λRRO)	16
R300	Δ(*argF-lac*)*169*(λRRO) *purR300*	16
R303	R300 *recA*(Mu1$^+$)	17
R100C	R100 cured of λRRO	This study
R300C	R300 cured of λRRO	This study
GS244	Δ*metR*::Mu[b]	26
GS849	*purR*::Tn*10*	P. Nygaard
GS924	GS244 *purR*::Tn*10*	This study
Plasmids		
pRRM127	15.5-kb purR$^+$ Km$^+$ fragment	17
pPR1002	3.8-kb *PstI purR*$^+$ fragment in pMS421 Sp^r	17

[a] λRRO is *purF-lacZ*. kb, Kilobase.
[b] Strain GS244 also carries *pheA905*, *thi*, *araD129*, *rpsL*, and Δ*lacU169* mutations.

TABLE 2. Effect of *purR* mutation on SHMT activity

Strain	Relevant genotype	Sp act[a] in:			
		GM	GM + inosine	GM + L-methionine	GM + inosine + L-methionine
R100C	Wild type	36	22	19	8
R300C	*purR*	63	46	47	48
R300C (pPR1002)	*purR purR*$^+$	30	22	21	12
GS244	*metR*	23	8	27	10
GS924	*metR purR*	45	25	42	26

[a] Expressed as nanomoles of HCHO generated per-milligram of protein per minute. Cells were grown in GM with the indicated supplements. D-Methionine (50 μg/ml) was added as a limiting source of methionine for strain GS244 since the *metR* mutation results in methionine auxotrophy. Phenylalanine (50 μg/ml) and thiamine (1 μg/ml) were also added to the medium for growing GS244.

contained 1× DNA-binding buffer plus 125 μg of bovine serum albumin per ml. The assay mixtures were preincubated for 5 min at 37°C before protein was added. A 2-μl volume (1 μg of protein) of control extract from R300C or 2 μl of the PurR-enriched extract (1 μg to 30 ng) from R303(pRRM127) in a twofold dilution series was added to each assay mixture and incubated at 37°C for 15 min. A 1-μl volume of dye mix (0.1% xylene cyanole–50% glycerol in water) was added to each reaction mixture. The reaction mixtures were immediately loaded onto a 5% polyacrylamide gel (bisacrylamide-acrylamide buffered with 10 mM Tris hydrochloride [pH 7.4]–0.38 M glycine–1 mM EDTA) (1:30). The gel was prerun at 9 V/cm for 1 h, and samples were loaded while the gel was running at 9 V/cm. At the termination of the run, the gel was dried and the DNA fragments were detected by autoradiography.

DNase I protection assay. A modified version of the method of Schmitz and Galas was used for the DNase I protection assay (19). The 5′ ^{32}P-labeled *NdeI-FokI* DNA fragment described above was used for this assay. The labeled fragment was incubated at 37°C for 5 min in 100 μl of 1× DNA-binding buffer containing 125 μg of bovine serum albumin per ml. Protein (5 μg) from either the control extract from R300C or the PurR extract from R303(pRRM127) was added, and the mixtures were incubated for an additional 15 min at 37°C. A 6-μl volume of a DNase I solution (2.5 μg/ml dissolved in 20 mM sodium acetate (pH 7.0)–32 mM $CaCl_2$) was added, and incubation was continued for 30 s. The reactions were terminated by the addition of 25 μl of DNase I stop mix containing 3 M ammonium acetate, 0.25 M EDTA, and 15 μg of sonicated calf thymus DNA per ml. The samples were precipitated with ethanol, collected by centrifugation, dried, and suspended in sequencing dye mix. The DNase I digestion products were run adjacent to a sequence of the ^{32}P-labeled *NdeI-FokI* fragment obtained by the Maxam and Gilbert sequencing method (8). After electrophoresis, the gel was dried and autoradiographed.

RESULTS

Previous experiments have shown that purine limitation increases expression of the *glyA* gene approximately twofold (1). To determine if this regulation is mediated through the *purR* gene product, we measured SHMT activity in a wild-type strain (R100C), a *purR* mutant strain (R300C), and the *purR* mutant strain transformed with the low-copy-number plasmid pPR1002, which contains the wild-type *purR* gene (17). The strains were grown in GM either with or without inosine (100 μg/ml). In strain R100C, purine supplementation resulted in about a 40% decrease in SHMT activity (Table 2). The *purR* mutant, strain R300C, had elevated levels of SHMT activity compared with levels in the wild-type strain, but purine supplementation still resulted in about a 30% decrease in SHMT activity. The SHMT levels in the *purR* mutant transformed with the *purR*$^+$ plasmid, pPR1002, were comparable to those of the wild-type strain under all growth conditions.

Purine supplementation might be expected to have a sparing effect on C_1 units (4), leading to a stimulation of methionine synthesis. Therefore, we tested to determine if the decrease in SHMT activity observed in the *purR* mutant during purine supplementation is mediated through methionine regulation. Strains R100C and R300C were grown in GM supplemented either with L-methionine or with L-methionine plus inosine. In strain R100C (*purR*$^+$), L-methionine supplementation resulted in a 2-fold decrease in SHMT activity and inosine–L-methionine supplementation resulted in a 4.5-fold decrease in SHMT activity (Table 2). In strain R300C (*purR*), L-methionine supplementation resulted in a 1.4-fold decrease in SHMT activity, whereas inosine–L-methionine supplementation did not result in a further decrease in activity. These results suggest that the decrease in SHMT activity in the *purR* mutant during purine supplementation may be mediated through methionine regulation.

The MetR protein, with homocysteine as coactivator, has been shown to be the methionine component involved in the expression of the *glyA* gene (14). A *metR* mutant strain, GS244, was grown in GM supplemented with either D-methionine (a limiting source of methionine) or D-methionine–inosine to determine the degree of repression of SHMT in the absence of the MetR activator. Inosine supplementation of the *metR* mutant resulted in the greatest fold decrease in SHMT levels (threefold; Table 2), and this regulation was insensitive to the addition of methionine. The greater range of regulation by inosine in the *metR* mutant than in the wild type indicated to us that MetR-mediated activation influences the ability of PurR to repress the *glyA* gene. The addition of L-methionine alone or with inosine did not significantly affect the SHMT levels.

To examine the effect of PurR on expression of the *glyA*

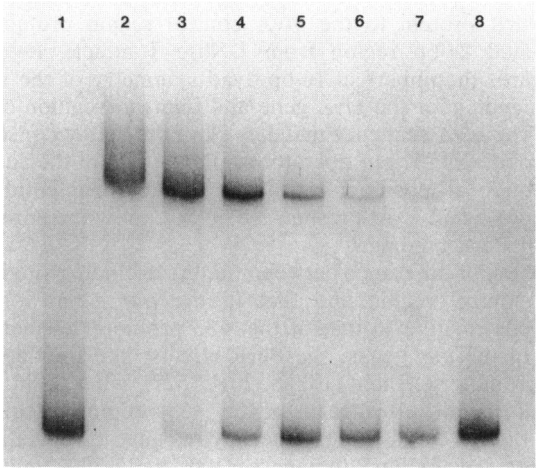

FIG. 1. Gel retardation assay for binding of PurR protein to the *glyA* control region. Crude cell extracts were prepared from strains R303(pRRM127) (*purR purR*$^+$) and R300C (*purR*). The extracts were incubated with a ^{32}P-labeled DNA probe containing the *glyA* control region to allow specific protein-DNA complexes to form. Lane 1, 1-μg of R300C (*purR*) extract; lanes 2 through 7, twofold dilutions of R303(pRRM127) (*purR purR*$^+$) extracts ranging from 1 to 0.03 μg; lane 8, DNA probe only.

gene in the absence of the influence of the MetR activation system, we constructed a *metR purR* double mutant (GS924) and compared the effects of purine supplementation in this strain and the parent *metR* strain, GS244. SHMT levels in strain GS924 were elevated and were only poorly regulated by inosine compared with the threefold repression observed in parent strain GS244 (Table 2).

Regulation of genes in the *pur* regulon by the PurR repressor has been shown to involve the binding of the PurR protein to specific operator sites within the *pur* promoters (17). A ^{32}P-labeled DNA fragment containing the *glyA* control region was used in a gel retardation assay to determine if the *purR* gene product binds to the *glyA* control region. Extracts were prepared from strains R303(pRRM127) (which overproduces the PurR protein) and R300C (a *purR* mutant). The PurR-enriched extract was able to bind to the labeled probe, resulting in a shift in the mobility of the DNA fragment (Fig. 1). Serial dilutions of the PurR-enriched extract showed decreasing amounts of DNA probe that shifted. No shift of the DNA probe was observed when extract from the *purR* mutant strain R300C was used.

DNase I protection assays were done to determine the specific binding site for the PurR protein. The same ^{32}P-labeled DNA fragment carrying the *glyA* control region that was used in the gel retardation assay was used in the DNase I protection assay. The DNase I footprint showed that about a 24-bp region was protected by the binding of the PurR repressor (Fig. 2). The bottom endpoint of the protected region could not be precisely determined because of an absence of bands in this region in the unprotected lane. The 24-bp region was chosen because PurR was shown previously to bind to and protect this length of DNA (17). This protected region extends from 15 to 38 bp upstream of the −35 promoter sequence for the *glyA* gene. This was the only site on this DNA fragment at which DNase I protection was observed.

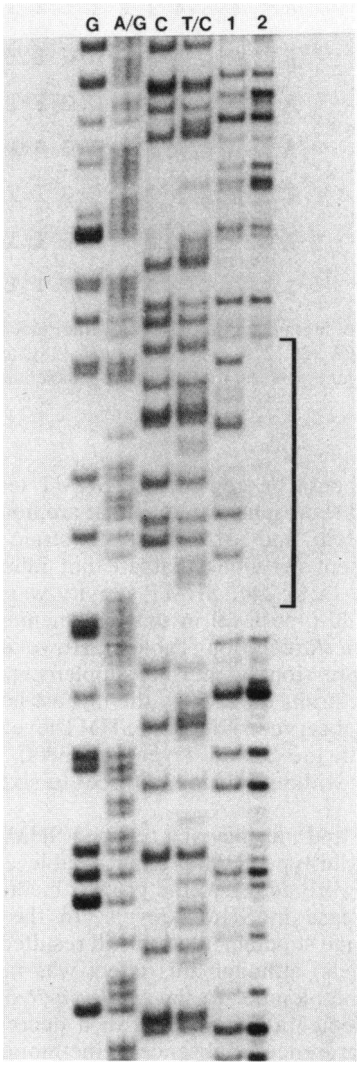

FIG. 2. Protection from DNase I digestion of the *glyA* control region by PurR. A ^{32}P-labeled DNA probe containing the *glyA* control region was incubated with crude cell extract either from R300C (*purR*) or from R303(pRRM127) (*purR purR*$^+$). The mixtures were subjected to partial DNase I digestion and then run adjacent to the Maxam-Gilbert sequencing reactions (lanes G, A/G, C, and T/C) (8) of the labeled probe. Lane 1, R300C extract; lane 2, R303 (pRRM127) extract. The DNase I-protected region is indicated by the bracket.

DISCUSSION

The *purR* gene product was shown to negatively regulate expression of the *glyA* gene in *Escherichia coli*. Regulation of the *glyA* gene by PurR, however, was over a narrow twofold range (compare strains R100C and R300C, Table 2). The MetR activator protein plus homocysteine was shown previously to regulate the *glyA* gene over only a threefold range (14). Because the products of the SHMT reaction are used in a number of metabolic pathways (e.g., purine, methionine, and thymine synthesis) (11, 12, 21), the narrow range of regulation by the PurR protein would ensure adequate levels of C_1 units and glycine for use in other pathways in the presence of high levels of purines.

To more accurately evaluate the effect of the PurR repressor on *glyA* expression, the SHMT levels in strains GS244

	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
purEK	A	C	G	C	A	A	C	C	G	T	T	T	T	C	C	T
purF	A	C	G	C	A	A	A	C	G	T	T	T	T	C	T	T
purL	A	C	G	C	A	A	A	C	G	G	T	T	T	C	G	T
purMN	T	C	G	C	A	A	A	C	G	T	T	T	G	C	T	T
glyA	A	G	G	T	A	A	T	C	G	T	T	T	G	C	G	T
Consensus	A	C	G	C	A	A	A	C	G	T	T	T	$^{G}/_{T}$	C	$^{G}/_{T}$	T

FIG. 3. DNA sequence comparison of the PurR-binding sites for *pur* genes (*purEK* [25, 27], *purF* [17], *purL* [18], and *purMN* [20]) and the *glyA* gene. Bases matching the consensus sequence are underlined.

and R300C should be considered. SHMT levels in GS244 (*metR*) should represent activities that are not influenced by the MetR protein, and SHMT levels in strain R300C (*purR*) should represent activities that are not influenced by the PurR protein. In GS244, SHMT activity was reduced relative to the wild-type level in unsupplemented medium because of the *metR* mutation (Table 2). However, the greatest degree of repression by purine supplementation was observed in this strain. In R300C, the highest levels of SHMT activity were observed. When the SHMT levels in GS244 are compared with the SHMT levels in R300C grown in GM either with or without inosine, a three- to sixfold difference is observed.

Methionine and inosine each reduced SHMT levels about 40% in the wild-type R100C strain (Table 2). When both supplements were added to the growth medium, there was an 80% decrease in SHMT levels. In the *purR* mutant (R300C), inosine supplementation still resulted in a decrease in SHMT levels, although this effect was not seen in the presence of methionine. In the *metR purR* double mutant, inosine supplementation resulted in a decrease in SHMT levels in the presence or absence of methionine. Additional studies will be necessary to understand the residual repression by inosine in the double mutant.

A gel retardation assay showed that the PurR protein binds to the *glyA* control region to produce a single band with altered mobility (Fig. 1). This band was reduced in intensity by serial dilution of the PurR protein extract. Although the stoichiometry for binding of PurR to the *glyA* control region cannot be determined from this assay, we assume that if more than one type of complex binds, the K_m for binding is similar, since only one retarded band is observed for any one of the dilutions.

A DNase I protection assay (Fig. 2) showed that binding of the PurR protein to the *glyA* control region protects an estimated 24-bp region from DNase I attack. Figure 3 compares the imperfect 16-bp dyad symmetry of the PurR-binding sites for the *glyA* gene and four *pur* regulon operators. The *glyA* sequence matches all of the highly conserved nucleotides of the *pur* operator sequence except the adenine nucleotide at position 2 and the cytidine nucleotides at positions 5 and 7. At present, we do not know the corepressor for PurR binding.

The major difference between the PurR-binding site in the *glyA* control region and that in the *pur* genes is their locations relative to that of the −35 promoter element. In each of the *pur* genes, the PurR-binding site overlaps the −35 promoter element (17, 18, 20, 25, 27), whereas in *glyA*, this site is 18 bp upstream of the −35 promoter sequence (Fig. 4). This could result in the narrow range of regulation of the *glyA* gene by PurR (compare R100C and R300C, plus and minus inosine; Table 2) compared with a 28-fold range of regulation for the *purF* gene (16). Results reported by Lanzer and Bujard (6) support the idea that the location of the repressor-binding site in the promoter region affects the level of repression. Using various promoter-*lac* operator combinations constructed in vitro, they showed that the position of the operator (Lac repressor binding sequence) within the promoter sequence greatly affects the repression of promoter activity. When the operator was placed upstream of the −35 promoter element (−38 to −59), repression was 50- to 70-fold less than when the operator was located between the −35 and −10 promoter elements. Their kinetic data suggest different mechanisms of repressor action, depending on the position of the operator within a promoter sequence.

It is possible that the PurR protein negatively regulates the *glyA* gene by blocking the MetR protein from binding to its target site rather than blocking RNA polymerase from binding to the *glyA* promoter. This is unlikely, however, since the MetR-binding site in the *glyA* control region is located 83 to 117 bp upstream of the −35 promoter region (unpublished results) and does not overlap the PurR-binding site.

It is interesting to note that when the DNA sequence of the *E. coli glyA* gene is compared with that of the *Salmonella typhimurium glyA* gene (J. G. Steiert, M. L. Urbanowski, L. T. Stauffer, M. D. Plamann, and G. V. Stauffer, Sequence, in press), the 16-bp PurR-binding site and its location relative to the −35 region are completely conserved. This conserved region is located adjacent to a poorly conserved region. Since purine supplementation represses the *S. typhimurium glyA* gene, the mechanism of *glyA* regulation by purines in *S. typhimurium* most likely is similar to that reported here for *E. coli*.

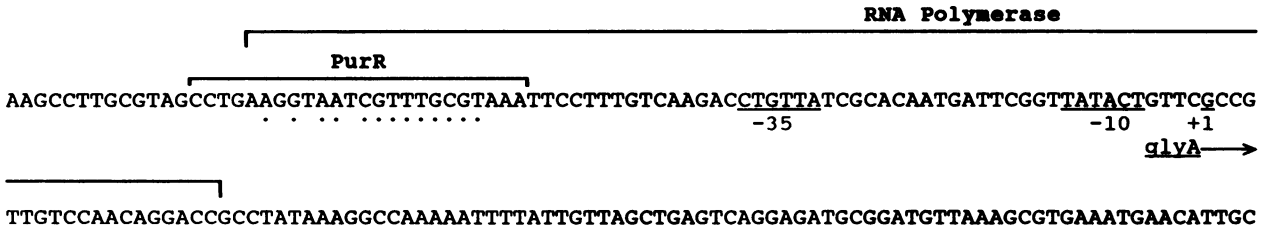

FIG. 4. DNA sequence of the control region for the *glyA* gene (13). The region protected from DNase I digestion by PurR protein (Fig. 2) and the region protected from DNase I digestion by RNA polymerase (RNAP) (13) are indicated by brackets. Bases in the PurR-binding region that match the consensus sequence are indicated by dots. The transcription start site (⟶) for *glyA* is indicated as +1, and the −10 and −35 promoter sequence elements are underlined (13, 15).

ACKNOWLEDGMENTS

We gratefully thank P. Steiert for assistance in SHMT assays and P. Nygaard for strain GS849.

This investigation was supported by Public Health Service grant GM-26876 from the National Institute of General Medical Sciences.

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