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Isolation and Partial Characterization of *Escherichia coli* Mutants with Altered Glycyl Transfer Ribonucleic Acid Synthetases

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Isolates with mutations in *glyS*, the structural gene for glycyl-transfer ribonucleic acid (tRNA) synthetase (GRS) in *Escherichia coli*, are frequently found among glycine auxotrophs. Extracts of *glyS* mutants have altered GRS activities. The mutants grow with normal growth rates in minimal media when high levels of glycine are provided. No other metabolite of a variety tested is capable of restoring normal growth. The *glyS* mutants fail to make ribonucleic acid (RNA) when depleted of exogenous glycine in strains which are RC^{str} but do so when the cells are RC^{rel}. In contrast, biosynthetic mutants which are unable to synthesize glycine (*glyA* mutants) do not make RNA when deprived of glycine even if they are RC^{rel}; in this case, RNA is synthesized upon glycine deprivation only when the nucleic acid precursors made from glycine are provided in the medium. The level of serine transhydroxymethylase is unaltered in extracts of any of the *glyS* mutants, even though the level of charged $tRNA^{Gly}$ is at least 20-fold lower than that found in a prototrophic parent; this indicates that, if there is control over the synthesis of serine transhydroxymethylase, it is not modified by reduced levels of charging of the major species of $tRNA^{Gly}$.

Conditional mutations are expressed only under certain environmental conditions and therefore provide a useful approach for studying genetic changes in indispensable functions. The thermosensitive mutation has provided important information about the catalytic and regulatory properties of the amino acyl-transfer ribonucleic acid (tRNA) synthetases. For example, a thermosensitive mutation modifying valyl-tRNA synthetase completely blocks the acylation of $tRNA^{val}$ (10, 20) at the nonpermissive temperature, and, as a consequence of this defect, the synthesis of the enzymes needed for valine biosynthesis is derepressed (3, 30).

Other types of conditional mutations affecting amino acyl-tRNA synthetases have also provided important information concerning the role of these enzymes in regulatory processes. In several instances, mutational alterations in amino acyl-tRNA synthetases confer resistance to amino acid analogues [e.g., isoleucine (24), histidine (21), phenylalanine (12), and arginine (17)]; as a result of the mutational change, the first two have their respective biosynthetic operons derepressed. Strains with defective amino acyl-tRNA synthetases have also been found among histidine (20; G. Nass and F. C. Neidhardt, Bacteriol. Proc., p. 87, 1966), tryptophan (8, 18), isoleucine (Iaccarino and Berg, *unpublished data*), tyrosine (22), and serine (J. Katze, *personal communication*) auxotrophs. The respective biosynthetic operons are known to be derepressed in the first and not in the second, third, and fourth cases; in the fifth case, this question has not been examined.

In previous studies (4, 6; Pouwels, Yanofsky, and Berg, *unpublished data*), it was shown that the glycyl-tRNA synthetase (GRS) of *Escherichia coli* K-12 strains occurs in two forms. The two forms are coded for by the same genetic locus (*glyS*) but differ in certain physical and catalytic parameters [*see* above references and Folk and Berg (13)]. We felt it was important to obtain additional mutationally altered forms of the GRS for studies of structure-function relationships with this enzyme and to determine whether the GRS participates in the regulation of glycine biosynthesis.

To obtain mutants with altered glycyl-tRNA synthetases, we examined glycine auxotrophs. Presumably, GRS mutants with elevated K_m values for glycine might not be able to grow at the prevailing intracellular glycine levels. Mutants which require glycine but fail to grow with serine

are likely to have either a defective serine transhydroxymethylase (STH) or a defective GRS. In fact, both types have been prominent among our isolates of glycine auxotrophs. In this paper, we describe some genetic and physiological characteristics of the GRS mutants (*glyS* mutants).

MATERIALS AND METHODS

Bacterial strains. All bacterial strains described herein were derived from *E. coli* K-12 W3110 unless otherwise noted. The genealogy and the method of isolation of the mutants and their derivatives used in this work are summarized in Fig. 1; Fig. 2 shows the genetic map location of the relevant markers. The origin of strains or mutants other than those shown in Fig. 1 is given below. In the text, only relevant genes will be listed after the strain number.

Growth of bacteria. Bacteria were grown in flasks on a rotary shaker at 37 C unless otherwise noted. Usually, overnight cultures of bacteria were diluted 1/50 into fresh media and growth was followed by measuring the optical density of the culture at 590 nm with a Zeiss PMQII spectrophotometer. Auxotrophic strains (grown to an amino acid limit) were grown overnight on limiting glucose to an absorbancy $(A)_{590}$ of 0.1 and then supplemented with 0.2% glucose and allowed to grow until depletion of the amino acid occurred (usually at an A_{590} between 0.6 and 0.9). "Exponential" cultures were harvested in the late exponential phase of growth (A_{590} of 0.5 to 1.0).

Bacteria were harvested by centrifugation at 4 C, washed once with 0.15 M NaCl, and then frozen at −20 C, unless otherwise noted.

Isolation of mutants. Glycine-requiring strains were isolated after ultraviolet (UV) mutagenesis and penicillin selection. Several techniques were attempted, but the following gave the best results [modified from (14)]. An exponentially growing culture of bacteria in minimal medium (2×10^8 to 6×10^8 cells/ml) was irradiated with UV light to produce approximately 10^4 survivors/ml and then diluted 1/40 into fresh medium containing glucose and 5 mg of glycine per ml. The culture was incubated at 37 C for 18 to 20 hr and then a portion was centrifuged, washed, and resuspended in minimal medium with glucose but without glycine at a cell concentration of 10^8 bacteria/ml. The cells were incubated at 37 C for 90 min, penicillin G was added to a final concentration of 2,000 units/ml, and the culture was incubated without shaking at 37 C for 150 min. The cells were centrifuged and washed twice with minimal medium, and then after resuspension in minimal medium several dilutions were spread on minimal-glucose plates containing 5 mg of glycine per ml. After several days at 37 C, colonies were picked and tested for their glycine requirement on minimal-glucose plates with and without glycine.

Thymidine-requiring (Thy^-) strains were prepared by subculturing the Thy^+ strain twice in broth containing 20 μg of trimethoprim per ml and 200 μg of thymidine per ml, and then twice more in broth containing 20 μg of trimethoprim per ml and 50 μg of thymidine per ml (23).

Transductions. Phage P1kc was prepared by the confluent lysis technique and was used for transduc-

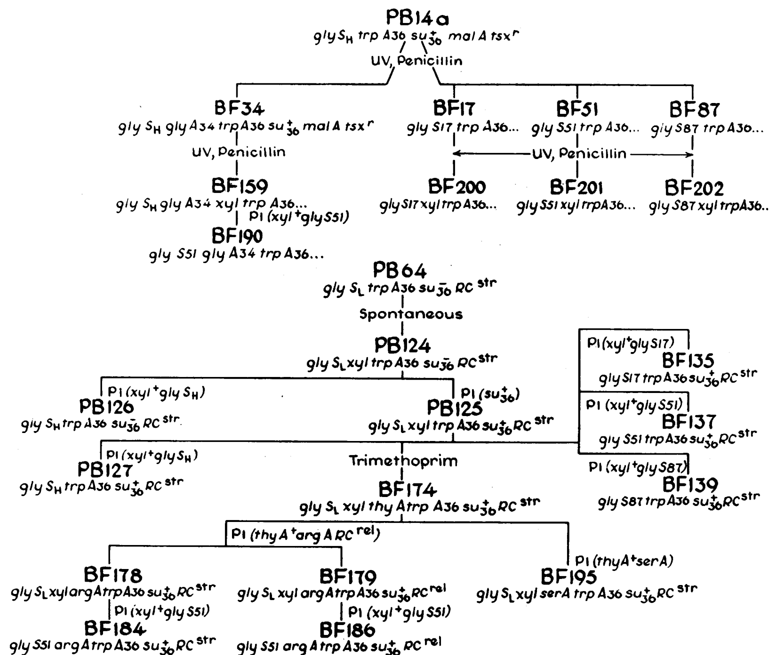

FIG. 1. *Genealogy of strains used in this work. Abbreviations of genetic markers follow the conventions proposed by Demerec et al. (7); explanatory comments concerning the abbreviations may be found in Taylor and Trotter (25).*

tion as described by Hill et al. (15). Multiplicities of infection were always between 0.2 and 2.

Reagents and media. ^{14}C-amino acids and ^{14}C-uridine were obtained from New England Nuclear Corp. *E. coli* B tRNA was purchased from Schwarz BioResearch. *d,l*,L-Tetrahydrofolic acid was obtained from Pierce Chemicals. Trimethoprim was a gift of G. Hitchings, Burroughs Wellcome & Co., Long Beach, N.Y. All other reagents were of the highest purity available from commercial sources.

The minimal medium is that described by Vogel and Bonner (29), supplemented with 0.2% glucose or other carbon source as noted. When used as a wash fluid, no carbon source was included.

The rich medium ("broth") contains 10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride per liter.

Preparation of cell extracts. A frozen cell pellet was suspended to a concentration of 20% (w/v) in 0.1 M potassium phosphate buffer (*p*H 7.0) with 0.01 M β-mercaptoethanol. Acid-washed glass beads were added and the suspension was sonic treated for 90 sec with a Mullard sonicator. The extracts were clarified by centrifugation at about 12,000 × *g* for 10 to 15 min. Sonic-treated material of strains containing GRS_L were kept at room temperature, as the GRS_L is inactivated at 0 C [P. Berg, *unpublished data; also, see* Folk and Berg (13)]. Extracts normally contained between 15 and 25 mg of protein per ml as determined by the method of Lowry et al. (19) with crystalline bovine plasma albumin as a standard.

Enzyme assays. GRS activity was assayed by measuring the rate of formation of ^{14}C-gly-tRNA by the procedure of Calendar and Berg (5). The reaction mixture (0.5 ml) contained 100 mM sodium cacodylate buffer (*p*H 7.0), 1 mM adenosine triphosphate (ATP; *p*H 7.0), 10 mM $MgCl_2$, 10 mM KCl, 4 mM reduced glutathione (*p*H 7.0), 100 μg of bovine plasma albumin, 1 mM ^{14}C-glycine (uniformly labeled, 3,500 counts per min per nmole), 20 to 25 A_{260} of tRNA and 0.1 to 1 unit of GRS. (One unit of enzyme activity is equivalent to the formation of 1 nmole of gly-tRNA in 10 min at 37 C.) The reaction mixture was incubated for 10 min at 37 C, unless otherwise noted, and processed as described by Calendar and Berg (5).

STH activity was assayed by a modification of the method of Taylor and Weissbach (26). The assay measures the rate of formation of ^{14}C-formaldehyde from the cleavage of ^{14}C-L-serine. The reaction mixture (0.4 ml) contained 75 mM potassium phosphate buffer (*p*H 7.4), 0.25 mM pyridoxal phosphate, 1.8 mM *d,l*,L-tetrahydrofolic acid (*p*H 7.4), 25 mM β-mercaptoethanol (*p*H 7.4), 0.25 mM ^{14}C-L-serine (uniformly labeled, 1,100 counts per min per nmole), and between 0.004 and 0.04 units of enzyme (1 unit equals the formation of 1 μmole of ^{14}C-formaldehyde per hr at 37 C). After a 5-min preincubation without serine at 37 C, serine was added and the reaction mixture was incubated for 15 min at 37 C; then, 0.3 ml of cold 1 M sodium acetate (*p*H 4.5) was added, samples were chilled, and 0.2 ml of 0.1 M formaldehyde was added, followed by 0.3 ml of 0.4 M 5,5-dimethyl-1,3-cyclohexanedione in 50% ethanol. The samples were heated for 5 min at 100 C and cooled and the precipitate was collected by filtration on Whatman GF/C glass-paper filters. The precipitates were washed with 30 ml of cold water, dried under a heat lamp, and then counted in a scintillation counter in a toluene-based liquid scintillation counting fluid.

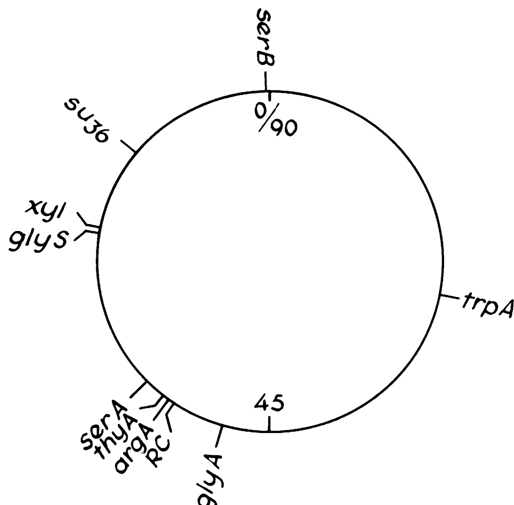

FIG. 2. *Genetic map indicating positions of relevant markers. Map distances are taken from Taylor and Trotter (25).*

Sucrose density gradient analysis of cell extracts. Appropriately diluted samples (0.05 ml) of cell extracts prepared by ultrasonic disruption were layered on preformed 5 to 20% sucrose gradients (4 ml) containing 10 mM sodium cacodylate (*p*H 7.0), 1 mM reduced glutathione, 1 mM ATP, and 10 mM $MgCl_2$ [conditions which stabilize the GRS_H and GRS_L activities (P. Berg, J. Kriss, and M. Dieckmann, *unpublished data*)]. Four gradients were centrifuged in different tubes in the same rotor at 61,000 rev/min for 255 min at 20 C. After piercing the bottom of the tubes with a pin, fractions were collected into tubes containing 0.03 ml of $MgCl_2$ (66 mM) and ATP (6.6 mM); after 3 hr at room temperature, samples were assayed by adding the remaining reaction components for the usual amino acyl-tRNA synthetase assay and then analyzing the reactions in the normal manner.

Determination of the RC phenotype. The RC phenotype of a strain was determined by comparing ribonucleic acid (RNA) synthesis (incorporation of ^{14}C-uridine into acid-insoluble material) in the presence of all of the required amino acids and in the absence of one required amino acid. The procedure is essentially that described by Alföldi, Stent, and Clowes (1).

Bacteria were centrifuged, washed, and resuspended into minimal medium containing 0.2% glucose and ^{14}C-uridine (20 μg/ml, 0.25 μc/ml). Samples of 1 ml were placed into tubes containing supplements and incubated at 37 C. Samples of 0.2 ml were removed, precipitated with 6% trichloroacetic acid (1 ml), filtered through membrane filters (Millipore Corp., Bedford, Mass.), washed with 5%

trichloroacetic acid, and dried. The filters were glued on planchets and counted in a gas flow counter.

RESULTS

Isolation and initial characterization of glycine-requiring mutants. It was our initial premise that a certain class of GRS mutants would be phenotypically Gly^-, i.e., require glycine for growth. For example, organisms with an altered GRS that has a lowered affinity for the amino acid might not grow at the prevailing intracellular concentration of glycine but would grow when the culture was supplemented with glycine. Accordingly, PB14a (Fig. 1 and 2) was irradiated with UV light and 70 Gly^- auxotrophs were selected for further study. (Although only *glyS* mutants derived from the $glyS_H$ wild type are classified in this paper, we also isolated *glyS* mutants starting with $glyS_L$ wild type. However, because it is difficult to work with GRS_L and mutant forms of this enzyme, no further studies were performed with these derivatives.)

All of the Gly^- mutants were assayed for both GRS and the enzyme specific for glycine biosynthesis, STH. Forty of the Gly^- isolates had altered GRS activities, 22 had markedly reduced or no STH activity, and 8 were unchanged in either activity. None of the isolates was altered in both activities.

The structural gene for GRS (*glyS*) is linked to *xyl* and is cotransduced with *xyl* at a frequency of approximately 80% (4). Six randomly chosen Gly^- Xyl^+ mutants with altered GRS activities were transduced to Gly^+ with phage P1 grown on JC411 (*xyl* $glyS_H$) to determine whether our *glyS* mutations were also linked to *xyl*. The appearance of a very heavy, spontaneously occurring Gly^+ background prevented determination of an accurate cotransduction frequency, but, by careful comparison of the types of colonies arising on transductant plates and background plates, a distinct class was found on the transductant plates. In this class, between 11 and 88% of the Gly^+ transductants were Xyl^-. Two Gly^+ Xyl^- transductants from each cross were purified by single-colony isolation and shown to have GRS activities identical to that of the wild-type donor.

One of the isolates with an altered STH activity (BF34) and three representatives of the third mutant class were also transduced to Gly^+ with P1 grown on JC411 (*xyl* $glyS_H$). In each of these cases, very few Gly^+ colonies arose on the plates without added P1, and none (<2%) of the Gly^+ transductants was also Xyl^-. When P1 prepared on SP-4 (*tyrA* $glyA^+$) was used to transduce BF34 ($tyrA^+$ *glyA*), 4% of the Gly^+ transductants were also Tyr^-. This is the linkage expected between *tyrA* and *glyA* (25).

Although mutants of the third class (those with normal GRS and STH activities) could be transduced to Gly^+ with P1 grown on a prototroph, no Gly^+ transductants were obtained when transduced with P1 grown on JC158 (*serA*); however, the same preparation of P1 readily transduced BF34 (*glyA*) to Gly^+.

We can, therefore, assign the 70 Gly^- isolates to three genetic classes: 40 *glyS* (GRS) mutants, 22 *glyA* (STH) mutants, and 8 *serA* mutants [*serA* is the structural gene for 3-phosphoglyceric acid dehydrogenase (28)]. Mutants in the other two steps of serine biosynthesis from 3-phosphoglyceric acid (transamination of phosphohydroxypyruvate to phosphoserine and hydrolysis of phosphoserine) were not found among our isolates.

Further characterization of the altered activities. The *glyS* mutants exhibited a range of GRS activities in vitro from approximately 50% to less than 1% of the parental $glyS_H$ strain. Three mutants, BF17 (*glyS17*), BF51 (*glyS51*), and BF87 (*glyS87*), having approximately 20, 50, and 1%, respectively, of the wild-type activity were chosen for further study. These were representative of the range of activities among the 40 mutants in this class. Table 1 summarizes the GRS and STH activities of these *glyS* mutants, a *glyA* mutant (BF34), the parental strain PB14a ($glyS_H glyA^+$), and two prototrophic strains in which the genetic region $glyS_L xyl$ of one (PB125) has been replaced by $glyS_H xyl^+$ by P1 transduction (PB127; Fig. 1 and 2).

The low GRS activity of extracts from each of the three classes of *glyS* mutants was not due to a diffusible inhibitor present in the extracts. When each mutant extract was mixed with the analogous extract prepared from the parental strain PB14a, there was less than 8% deviation from the additivity expected from summing the activities of the individual extracts. Also, there was no increase in activity after dialysis of the extracts.

Conceivably, an elevated level of endogenous glycine in the mutant extracts could give erroneously low values by reducing the specific activity of the ^{14}C-glycine used as substrate. However, no evidence of endogenous glycine could be detected by an isotope dilution experiment, and, as mentioned above, dialysis failed to raise the GRS activity.

GRS activity in extracts from $glyS_L$ strains decayed at 0 C but extracts from $glyS_H$ strains were quite stable under the same conditions; after inactivation, the GRS_L activity could be reactivated by incubation at 25 C. The low tem-

perature-induced inactivation was accompanied by a dissociation of GRS into subunits; in the presence of ATP and Mg^{2+}, the sedimentation coefficient of GRS_L was 6.8 at 20 C and 5.3 at 4 C, whereas GRS_H has a sedimentation coefficient of 6.8 at both temperatures (Berg, Kriss, and Dieckmann, *unpublished data*).

To determine whether our mutations in *glyS* caused analogous structural changes, sonic extracts of each of the mutants (BF17, BF51, and BF87) as well as of the wild-type parent (PB14a) were sedimented in sucrose gradients at 20 C. The GRS activities of BF17 and BF51 (arrow B in Fig. 3) sedimented identically and were very close to that of GRS_H; in contrast, the GRS activity of BF87 sedimented at 5, 3*S* or very close to that of the low-temperature form of GRS_L (arrow A). Thus, it appears that the mutation to produce *glyS87* promotes dissociation of the native protomer. It would be interesting to know whether this change in aggregation occurs after disruption of the cells or whether the enzyme is partly dissociated in vivo.

The unusual sensitivity of GRS_L to low temperature prompted us to examine the sensitivity of the mutant activities at different temperatures. The GRS_H and GRS_{51} activities were unaltered after several hours at 0 C, whereas the GRS_{17}, GRS_{87}, and GRS_L activities were lowered to 66, 45, and 33% of their initial values, respectively. At 45 C, GRS_{51} was not markedly different than the parental enzyme, but GRS_{17} and GRS_{87} were clearly more sensitive (Fig. 4). The GRS_L activity, under these conditions, was the most rapidly inactivated of all the activities.

Genetic characterization. As mentioned above, our *glyS* mutants readily reverted spontaneously to a Gly^+ phenotype and therefore selection for the transduction of *glyS*$^+$ was very difficult. Instead, transduction of a particular Gly phenotype has been scored in the following experiments as a nonselective marker.

TABLE 1. *Glycyl-tRNA synthetase (GRS) and serine transhydroxymethylase (STH) activities of various strains*

Strain	Relevant genotype	GRS activity[a]	STH activity[b]
PB125	$glyS_L$	7	3.8
PB127	$glyS_H$	133	4.4
PB14a	$glyS_H$	133	3.4
BF17	*glyS17*	24	4.0
BF51	*glyS51*	57	3.7
BF87	*glyS87*	1	4.2
BF34	$glyS_H glyA34$	139	<.05

[a] Nanomoles of gly-tRNA formed per 10 min per milligram of protein at 37 C.

[b] Micromoles of formaldehyde formed per hour per milligram of protein at 37 C.

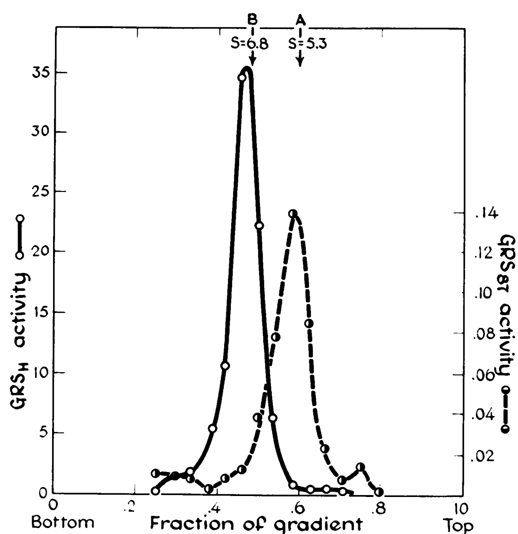

FIG. 3. *Sucrose gradient sedimentation of GRS_H, GRS_{87}, GRS_{17}, and GRS_{51}. The arrow at B indicates the positions of the peaks of GRS_{51} and GRS_{17}. The arrow at A indicates the position of the peak of the internal marker, isoleucyl tRNA synthetase, and the sedimentation position of GRS_L at 4 C. Symbols: solid line, GRS_H; dashed line, GRS_{87}.*

Phage P1 grown on BF17 (*glyS17 xyl*$^+$), BF51 (*glyS51 xyl*$^+$), or BF87 (*glyS87 xyl*$^+$) was used to transduce PB125 ($glyS_L$ *xyl*) to Xyl^+. The recipient bacteria were plated on a minimal medium containing xylose and glycine to select for Xyl^+; simultaneous acquisition of a Gly^- phenotype was scored by testing clones on minimal-xylose plates with and without glycine. With each mutant, the Gly^- phenotype was cotransduced with Xyl^+ at a frequency between 60 and 71% (Table 2).

Several transductants from each cross were purified by single-colony isolation and assayed for GRS activity. Every Gly^-Xyl^+ transductant had the GRS activity of its respective Gly^- parent. With one exception, all of the Gly^+Xyl^+ transductants, which represent transductants that received only the *xyl*$^+$ gene, had GRS activities characteristic of the recipient strain, PB125. One of eight Gly^+Xyl^+ transductants from the cross BF51 (*glyS51 xyl*$^+$) × PB125 ($glyS_L xyl$) exhibited an activity more characteristic of the donor than of the recipient strain. We believe that this transductant was probably plated as a Gly^-Xyl^+ but spontaneously became Gly^+ as the result of a secondary mutation that occurred prior to being scored. Because of such spontaneously occurring revertants, the true

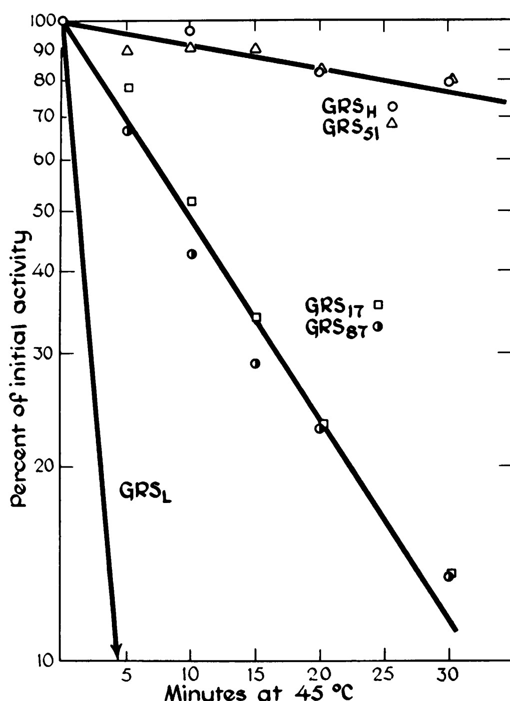

FIG. 4. *Thermal inactivation of GRS activities. Sonic-treated material of strains containing the various glyS alleles were placed at 45 C, and, at the noted times, appropriate samples were withdrawn and cooled to room temperature. Approximately 40 min after the first sample was removed, the extracts were diluted and the activities were assayed at room temperature for 20 min. After 5 min at 45 C, the GRS_L activity was 4% of the initial value. Symbols:* ○, GRS_H; △, GRS_{51}; □, GRS_{17}; ◑, GRS_{87}.

cotransduction of Gly^-Xyl^+ may be somewhat higher than the frequencies determined in the above crosses.

P1 grown on each of BF17 (*glyS17 xyl*$^+$), BF51 (*glyS51 xyl*$^+$), and BF87 (*glyS87 xyl*$^+$) was used to transduce BF200 (*glyS17 xyl*), BF201 (*glyS51 xyl*), or BF202 (*glyS87 xyl*) to Xyl^+; the ratio of the number of transductants which simultaneously acquired a Gly^+Xyl^+ phenotype to the total number of Xyl^+ transductants was scored on minimal-xylose plates with and without glycine. As expected in the homologous crosses (i.e., BF17 × BF200, BF51 × BF201, and BF87 × BF202), there were never more Gly^+Xyl^+ than would be expected from the transduction of Xyl^+ into cells that had spontaneously become Gly^+ (up to 10^{-3}). In heterologous crosses, the ratios of Gly^+Xyl^+ to Xyl^+ ranged from 0.002 to 0.07, suggesting that recombination between the various alleles had occurred.

Growth requirements. A more detailed study of the growth requirements of these strains showed that glycine is the only metabolite, of a variety tested, that can restore a normal growth rate. Without glycine, in minimal media, the *glyS* mutants grew with a doubling time greater than 8 hr, whereas the parental strain doubled approximately every 70 min (Table 3). Cell viability did not decrease in a culture of a *glyS51* mutant deprived of glycine for as long as 6 hr.

Adenosine, guanosine, thymidine, or histidine, all metabolites involved in glycine and one-carbon metabolism, did not support growth of BF51 (*glyS51*) singly or in combination. L-Serine, at a concentration as high as 1 mg/ml, did not significantly increase the growth rate. Strain BF51 had a doubling time of 95 min in a synthetic medium containing 18 of the amino acids (including serine and glycine) at a concentration of 40 μg/ml, tryptophan at 25 μg/ml, and tyrosine at 5 μg/ml. Removal of glycine from this medium increased the doubling time to more than 6 hr. Analogous results have been obtained with BF17 (*glyS17*).

In broth, even when 1 mg of glycine per ml was added, the *glyS* mutants grew approximately

TABLE 2. *Cotransduction frequency between glyS and xyl*

Donor		Recipient		Transductant		Cotransduction frequency
Strain	Genotype	Strain	Genotype	Xyl^+	Xyl^+ Gly^-	%
BF17	*xyl*$^+$*glyS17*	PB125	*xylglyS*$_L$	349	236	68
BF51	*xyl*$^+$*glyS51*	PB125	*xylglyS*$_L$	696	417	60
BF87	*xyl*$^+$*glyS87*	PB125	*xylglyS*$_L$	356	253	71

TABLE 3. *Growth rates of strains*

Strain	Doubling time (min)			
	Without glycine	With glycine (100 μg/ml)	Broth	Broth + 1 mg of glycine per ml
PB125	73			
PB127	75			
PB14a	67	66	32	32
BF17	>540	80	64	56
BF51	>480	89 70[a]	67	60
BF87	>600	78 69[a]	78	67
BF34	>600	67		

[a] Plus 1 mg of glycine per ml.

half as fast as the parent, PB14a (Table 3). Clearly, even in the presence of very high levels of exogenous glycine, the mutant activities apparently cannot function efficiently enough to allow rapid growth.

A curve relating the extent of growth to the amount of glycine provided in the medium shows that BF51 requires 54 μg of glycine per ml to reach an A_{590} = 1.0 (when glycine is the only supplement). [The *glyS* mutants continue growing very slowly (generation time between 8 and 17 hr) when deprived of glycine. The "extent of growth" in these measurements is that A_{590}, at which the tangential slope of the growth curve is approximately that of a 12-hr generation time, following exhaustion of glycine.] BF159 (*glyA34*), which lacks a functional STH, required only 23 μg of glycine per ml to reach an A_{590} = 1.0. BF190 (*glyA34 glyS51*), a double mutant, which contains a defective GRS and an inactive STH, required 35 μg of glycine per ml to reach an A_{590} = 1.0. Conceivably, in *glyS* mutants, some of the exogenous glycine is converted to serine and is then no longer available to overcome the defect in the GRS. This would explain why the double mutant, which cannot convert glycine to serine, utilizes the exogenous glycine more efficiently than does the *glyS* single mutant.

Effect of glycine deprivation on RNA synthesis. How does a block in the esterification of glycine to $tRNA^{Gly}$ affect the synthesis of RNA in RC^{rel} and RC^{str} strains?

Isogenic strains differing only by their RC allele were prepared by transducing a Thy^- derivative of PB125 to Thy^+Arg^- with P1 grown on NF59 (*argA* RC^{rel}) and assaying the transductants to determine which had received the donor RC^{rel} allele and which had retained the recipient RC^{str} allele. One of each type was then transduced to Xyl^+ with P1 prepared on BF51 (*glyS51* xyl^+), and Gly^- Xyl^+ transductants were collected. (We are indebted to G. Edlin for suggesting how to make the isogenic RC^{rel}, RC^{str} pair and for strain NF59.) BF184 (*glyS51 argA* RC^{str}) and BF186 (*glyS51 argA* RC^{rel}) were then assayed for the incorporation of ^{14}C-uridine into trichloroacetic acid-insoluble material in the presence of both arginine and glycine and in the presence of each amino acid alone.

Figure 5 shows that BF184 is stringent when starved for either glycine or arginine (a) and that BF186 is relaxed when starved for either amino acid (b). The same result was obtained when a similar pair of isogenic RC^{rel} and RC^{str} strains with the *glyS87* allele were tested for ^{14}C-uridine incorporation in the same manner.

When a pair of *glyA argA* RC^{rel} and *glyA argA* RC^{str} (BF265 and BF266, respectively)

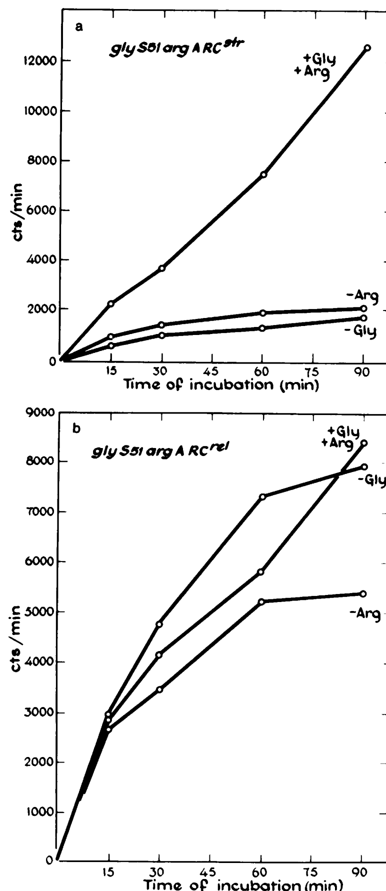

FIG. 5. *Synthesis of RNA in glyS strains which are* RC^{str} *or* RC^{rel}. *(a) BF184 (trpA36* su_{36}^+*glyS51 argA* RC^{str}*): arginine and glycine, when present, were at 100* μ*g/ml and 1 mg/ml, respectively. (b) BF186 (trpA36* su_{36}^+*glyS51 argA* RC^{rel}*): arginine and glycine concentrations were the same as in (a).*

with defective STH activities were prepared in a similar manner, the stringent strain stopped synthesizing RNA when deprived of glycine (both in the presence and absence of added histidine, methionine, adenosine, guanosine, thymidine, and sodium formate), but the relaxed strain continued RNA synthesis upon glycine deprivation only when histidine, methionine, adenosine, guanosine, thymidine, and sodium formate were included. (Each was not tested individually.) In the absence of these metabolites, the relaxed phenotype was not expressed upon glycine starvation (Fig. 6a, b, and c). When starved for arginine, in the presence of glycine, both strains exhibited a control of RNA synthesis that was dependent upon the allelic state of the RC locus; that is, the RC^{str} strain stopped synthesizing

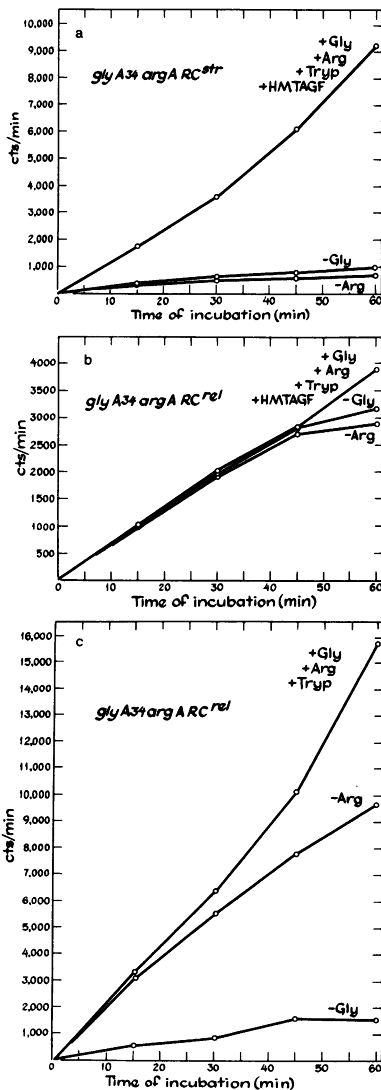

FIG. 6. *Synthesis of RNA in glyA strains which are RC^{str} or RC^{rel}. (a) BF265 (trpA36 su_{36}^-glyA34 argA RC^{str}): when present, concentrations were: tryptophan (tryp), 25 μg/ml; arginine (arg), 100 μg/ml; glycine (gly), 100 μg/ml; histidine (his), 50 μg/ml; methionine (met), 50 μg/ml; thymidine, 50 μg/ml; adenosine, 50 μg/ml; guanosine, 10 μg/ml; and sodium formate, 50 μg/ml. The latter six components are abbreviated HMTAGF on the figure. (b) BF266 (trpA36 su_{36}^- glyA34 argA RC^{rel}): same concentrations as in (a). (c) BF266: same as in (b), except that the compounds HMTAFG were omitted. (Bacterial densities differed between Fig. 5a and b and 6a, b, and c, so comparisons between strains of the absolute amounts of ^{14}C-uridine incorporated cannot be made.)*

RNA, and the RC^{rel} strain continued synthesizing RNA.

Control of the level of STH. The level of the valine biosynthetic enzymes appears to be regulated by the functional state of the valyl tRNA synthetase (9, 10, 30). It, therefore, was of interest to determine whether the level of the glycine biosynthetic enzyme, STH, is altered by a reduced ability for charging $tRNA^{Gly}$. First, we compared isogenic strains differing only by the *$glyS_H$* and *$glyS_L$* alleles (PB125 and PB127); these two strains have approximately a twofold difference in the in vivo level of gly-tRNA (13) and yet they have identical levels of STH activity under various growth conditions with and without glycine (Table 4).

With the *glyS* mutants, we could examine the same question under conditions where only 2 to 4% of the $tRNA^{Gly}$ is esterified in vivo (13). The parental strain, PB14a (*$glyS_H glyA^+$*), in exponential growth in minimal medium had STH activity that was not significantly different from that of each of the three *glyS* mutants deprived of glycine for 270 min (Table 4). Under these conditions, the level of gly-tRNA in the mutants was at least 20 times less than in PB14a. Here too, then, the level of STH was not dependent on the in vivo level of gly-tRNA.

One objection to using these *glyS* mutants to test for derepression is that the method of selection used to obtain the mutants (selection for glycine requirers) might have selected against those bacteria which can derepress their glycine biosynthetic pathway. If derepression occurred and the internal level of glycine increased, these cells might have grown in the absence of exogenous glycine and been killed by the penicillin treatment.

The isolation of a temperature-sensitive *glyS* mutant in our laboratory by M. Iaccarino and P. Reid permitted us to circumvent this objection. This strain, PB130, was isolated by ethyl methane sulfonate treatment and penicillin selection as a mutant which did not grow at 42 C but did grow at 30 C. Genetic and biochemical analysis showed that this strain is a *$glyS_{ts}$* mutant which produces a GRS with reduced activity (W. Folk, *unpublished data*). No apparent bias for a dependence upon exogenous glycine was introduced in the selection procedure.

We prepared a strain with the *$glyS_{ts}$* allele in a genetic background essentially identical to that of BF248 (and PB14a). A *$glyS_{ts}$ xyl^+* transductant of BF248 (BF249), when grown at 30 C in minimal media, has normal levels of gly-tRNA. A temperature shift to 42 C prevents further bacterial growth, and the level of gly-tRNA drops to 4%, whereas the level of gly-tRNA in the parental strain (*$glyS_H$*) growing at 42 C does not decrease (13). The level of STH after such a temperature shift remained unaltered (Table 4).

If the amount of gly-tRNA does not regulate

TABLE 4. *Levels of serine transhydroxymethylase in various strains*

Bacterial strain	Relevant genotype	Growth supplement	Growth stage	STH activity (μmoles per hr per mg of protein)
PB125	$glyS_L$		Exponential	3.7
PB125	$glyS_L$	Glycine (100 μg/ml)	Exponential	3.9
PB125	$glyS_L$	Glycine (1 mg/ml)	Exponential	3.6
PB127	$glyS_H$		Exponential	4.4
PB127	$glyS_H$	Glycine (100 μg/ml)	Exponential	4.4
PB14a	$glyS_H$		Exponential	3.4
BF17	*glyS17*	Glycine (30 μg/ml)	Glycine deprivation	4.2
BF51	*glyS51*	Glycine (30 μg/ml)	Glycine deprivation	3.7
BF87	*glyS87*	Glycine (30 μg/ml)	Glycine deprivation	3.7
BF249	$glyS_{ts}$	Tryptophan (25 μg/ml)	Exponential (30 C)	5.0
BF249	$glyS_{ts}$	Tryptophan (25 μg/ml)	At 42 C (90 min)	3.8
BF195	$glyS_L serA$	Glycine (50 μg/ml)	Glycine deprivation	4.0
BF195	$glyS_L serA$	Serine (40 μg/ml)	Serine deprivation	3.5

the synthesis of STH, is it controlled by an intermediate in the serine-glycine pathway? Strain BF195 (*serA* $glyA^+glyS_L$) is isogenic with PB125 except for the *serA* allele, which imposes a requirement for either glycine or serine. The *serA* locus is known to be the structural gene for 3-phosphoglyceric acid dehydrogenase, the first enzyme in the pathway of serine and glycine synthesis (28). This strain, when deprived of serine or glycine, maintained the same level of STH as its parental strain, PB125, in exponential growth (Table 4). If some intermediate in the serine biosynthetic pathway, which precedes the biosynthesis of glycine, regulated the synthesis of the last enzyme in the sequence, one would expect that depletion of such intermediates, or products in the mutant, would result in derepression of STH synthesis. Clearly, that is not the case. Thus, it appears that STH is made constitutively in this strain of *E. coli*. The only condition under which we have observed any change in the STH activity is with PB14a grown in a medium containing high levels of glycine and serine (1 mg/ml); in this case, the STH activity is reduced to about half the usual value. Since the change is small, we have not considered this as repression control, but additional studies are needed to explore this possibility.

DISCUSSION

Our premise that *E. coli* mutants with altered GRS could be isolated as glycine auxotrophs anticipated that certain changes in GRS would lower the overall catalytic activity at the normal intracellular concentration of glycine to the point that gly-tRNA would become growth-limiting. The growth of such mutants would be stimulated by increasing the endogenous pool of glycine. Such an increase could result from a derepression of a biosynthetic pathway or by supplementation with exogenous glycine.

In mutants with altered amino acyl-tRNA synthetases for valine (3, 30) and histidine (21; G. Nass and F. C. Neidhardt, Bacteriol. Proc., p. 87, 1966), some or all of the enzymes in the biosynthetic pathway for that amino acid are derepressed; the signal for the derepression is not known but the amino acyl-tRNA synthetase per se and its metabolic product, amino acyl-tRNA, are reasonable candidates. However, mutational alterations affecting the amino acyl tRNA synthetases for tryptophan (8) and arginine (17) do not cause derepression of the corresponding biosynthetic enzymes. Since *glyS* mutants of the type we describe can be isolated, we infer that *E. coli* is unable to elevate sufficiently the internal glycine pool by a regulatory response. And, indeed, by direct measurement, the level of STH, which quantitatively is the most important element for generating glycine in *E. coli*, is the same in mutant and wild-type strains; when mutant cells exhaust the exogenous glycine from the medium and approach stationary phase, there is no detectable change in the level of STH. Moreover, because a *glyS* mutant selected for its temperature sensitivity (and not auxotrophy) does not derepress its STH after a shift to nonpermissive temperature, we conclude that the level of STH is probably not regulated solely by the functional state of GRS or its product gly-tRNA. However, since other metabolically crucial compounds are generated by STH (through N5, 10-methylene-tetrahydrofolic acid), it may be that regulation of STH involves additional inputs. One argument favoring the view that STH is constitutive in *E. coli* is that strains defective in the synthesis of serine, when grown on glycine, depend on STH to generate serine and "C1" compounds from glycine but do not have altered levels of STH.

Paradoxically, extracts of some of the *glyS*

mutants have a higher GRS activity than extracts of prototrophic $glyS_L$ strains. The auxotrophic nature of the *glyS* mutants, then, is not explainable solely on the basis of their apparent GRS activities. Alternatively, the paradox may be due to some cryptic component of the GRS_L activity which permits it to function efficiently under conditions in which the mutant GRS activities cannot. This problem, though unresolved at present, is explored more fully in the accompanying paper (13).

RNA synthesis in the *glyS* mutants during glycine deprivation appears to be under the same type of control as described for other auxotrophs with lesions in genes coding for amino acid biosynthetic enzymes (11) or for amino-acyl tRNA synthetases (10, 11, 30). Such control is a function of the allelic state of the *RC* locus. When deprived of glycine, RNA synthesis continues in the RC^{rel} strain; it follows, then, that these mutants can synthesize glycine (and from it, the precursors for RNA synthesis) and that the auxotrophy is not a result of a block in glycine biosynthesis resulting from the *glyS* mutation.

As expected from the role of STH in the production of components crucial for nucleoside biosynthesis (glycine and "C1" fragments), bacteria lacking this activity, and starved for glycine in the absence of exogenously provided nucleoside precursors, exhibit a stringent phenotype regardless of the allelic state of the *RC* locus. When such nucleoside precursors are provided exogenously, RNA synthesis is under the control of the *RC* locus during glycine deprivation. The observation that a $Ser^- Met^-$ RC^{rel} strain starved for serine in the presence of nucleoside precursors exhibits a stringent response (2) poses an interesting problem. Is the difference in response between a $Ser^- RC^{rel}$ and a $Gly^- RC^{rel}$ due to the different enzymatic deficiencies (presumably the Ser^- had an alteration in an enzyme involved in serine biosynthesis) or to some other difference? This question is being investigated further.

Quite possibly, mutants with defective amino acyl-tRNA synthetases represent a significant fraction of certain classes of amino acid auxotrophs. On simple grounds, a high proportion of mutants with altered amino acyl-tRNA synthetases would be anticipated among auxotrophs for amino acids which have the shortest biosynthetic pathway; any operational procedure which selects or screens against mutations affecting the biosynthetic enzymes permits enrichment for activating enzyme mutants. Branched biosynthetic pathways offer good candidates; mutants with alterations in the structural genes of enzymes before the branch point may be eliminated by requiring auxotrophy for only one amino acid. For example, because four of the five enzymes used to synthesize isoleucine are also needed to synthesize valine (27), it is not surprising that a majority of the mutants which require isoleucine but not valine for growth have defective isoleucyl-tRNA synthetases and a minority have defective threonine deaminases (the one enzyme not common to the biosynthesis of both amino acids; Iaccarino and Berg, *unpublished data*). Similarly, a high proportion of *trpS* mutants have been found among tryptophan auxotrophs generated in strains made diploid for the genes governing the biosynthesis of tryptophan (16, 18). And recently, mutants with defective seryl-tRNA synthetases have been isolated among serine auxotrophs (J. Katze, *personal communication*). Other obvious examples for which amino acyl-tRNA synthetase mutants might easily be selected for as amino acid auxotrophs are alanine, glutamic, and aspartic acids and their amides, etc. However, if the biosynthetic pathway is derepressed as a result of defects in the corresponding amino acyl-tRNA synthetase, the isolation of such mutants as amino acid auxotrophs might not be possible.

Although we have not yet fully examined the properties of the various mutant GRS, it is quite clear that they provide interesting materials for the study of structure-function relationships of this enzyme. Although such mutants were selected on the basis of their requirement for higher levels of amino acid, it is not unlikely that the structural changes in the protein could have important consequences for other features of the catalytic and recognition properties of the enzyme. Such studies are now being undertaken.

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