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Genetic Characterization of a Highly Efficient Alternate Pathway of Serine Biosynthesis in *Escherichia coli*†

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There exists in *Escherichia coli* a known set of enzymes that were shown to function in an efficient and concerted way to convert threonine to serine. The sequence of reactions catalyzed by these enzymes is designated the Tut cycle (threonine utilization). To demonstrate that the relevant genes and their protein products play essential roles in serine biosynthesis, a number of mutants were analyzed. Strains of *E. coli* with lesions in *serA*, *serB*, *serC*, or *glyA* grew readily on minimal medium supplemented with elevated levels of leucine, arginine, lysine, threonine, and methionine. No growth on this medium was observed upon testing double mutants with lesions in one of the known *ser* genes plus a second lesion in *glyA* (serine hydroxymethyltransferase), *gcv* (the glycine cleavage system), or *tdh* (threonine dehydrogenase). Pseudorevertants of *ser* mutants capable of growth on either unsupplemented minimal medium or medium supplemented with low levels of leucine, arginine, lysine, threonine, and methionine were isolated. At least two unlinked mutations were associated with such phenotypes.

In *Escherichia coli*, the availability of serine and glycine is absolutely essential for the metabolism of several diverse classes of cellular components (Fig. 1). During growth on glucose, as much as 15% of the assimilated carbon in *E. coli* has been estimated to involve serine or its metabolites (19).

Early work established that in *E. coli* glucose is a precursor of serine (23). In a three-step pathway, the glycolytic intermediate, 3-phosphoglycerate, is converted to serine (18, 26, 27; Fig. 2). The *ser* mutants used in these early studies were deficient either in 3-phosphoglycerate dehydrogenase (3PGA-DH; *serA* gene product) or phosphoserine phosphatase (*serB* gene product). Serine auxotrophs lacking phosphoserine aminotransferase (*serC* gene product) were not identified until later (5, 9, 25). Because serine auxotrophs are readily isolated, it has generally been assumed that only one pathway of serine biosynthesis is used by *E. coli* (27).

In other organisms, there are several examples in which serine biosynthesis originates with threonine rather than with 3-phosphoglycerate. The obligate anaerobe *Clostridium pasteurianum* and a variety of mammalian liver extracts constitutively produce a threonine aldolase that converts threonine to acetaldehyde and glycine (6, 10). Glycine and a C_1 unit are then converted to serine. *Pseudomonas cepacia* and the soil bacterium *Arthrobacter* sp. convert threonine to glycine in two sequential steps (14, 31). Threonine is first oxidized to α-amino-β-ketobutyrate (AKB) in a reaction catalyzed by threonine dehydrogenase. The AKB is then cleaved by AKB lyase to produce acetyl coenzyme A and glycine. In both organisms, the glycine is subsequently converted to serine in a reaction catalyzed by serine hydroxymethyltransferase. Newman and co-workers isolated a pseudorevertant of an *E. coli* strain deficient in serine hydroxymethyltransferase (*glyA*) activity (8). Pseudorevertants are extragenically suppressed mutants that retain their original mutation. The pseudorevertant was capable of growth on glycine-free minimal medium provided that threonine was provided. After further biochemical studies, Newman and co-workers proposed that the pseudorevertants derive glycine from threonine via the same series of reactions used by *P. cepacia* (8, 17). These workers further proposed that in *E. coli* the pathway serves primarily as a route for threonine degradation (17, 21).

In this paper, we describe culture conditions that allowed all *ser* mutants to adapt physiologically to deriving serine via a second pathway that did not use any other *ser* gene products. The physiological adaptation required no mutational changes. Additionally, the isolation of pseudorevertants from *serA*, *serB*, and *serC* mutants is described. Through mutational analysis, we identified the genes encoding enzymes that are necessary for serine biosynthesis via this second pathway in *ser* mutants and pseudorevertants of *ser* mutants.

MATERIALS AND METHODS

Media. Basal medium was salts mix E of Vogel and Bonner (29). Solid medium contained 1.5% Bacto-Agar (Difco Laboratories). The following compounds were included when appropriate: DL-lactate (0.4%), glycerol (0.5%), glucose (0.4%), and amino acids (40 mg/liter) unless otherwise specified. All minimal media contained vitamin B_1 (1 mg/liter) and biotin (0.1 mg/liter). Medium that contained each amino acid (40 mg/liter) except serine and glycine was designated NoSG medium. Medium that contained leucine, arginine, lysine, threonine, and methionine (240 mg/liter of each amino acid) was designated LRKTM:240. Either L broth (12) or Bacto-Nutrient Agar (31 g/liter; Difco) was used as the complete medium. Minimal top agar contained 5.0 g of NaCl and 6.5 g of Bacto-Agar per liter. Antibiotics were added in the following amounts (per liter): ampicillin, 25 mg; tetracycline, 15 mg; and chloramphenicol, 30 mg.

Chemicals. The following were purchased from Sigma Chemical Co.: amino acids (L-isomers), CAPS buffer, *p*-iodonitrotetrazolium violet, hexadecyltrimethylammonium bromide, NAD^+, NADH, tetracycline hydrochloride, chloramphenicol, and DL-β-hydroxynorvaline. Ampicillin was

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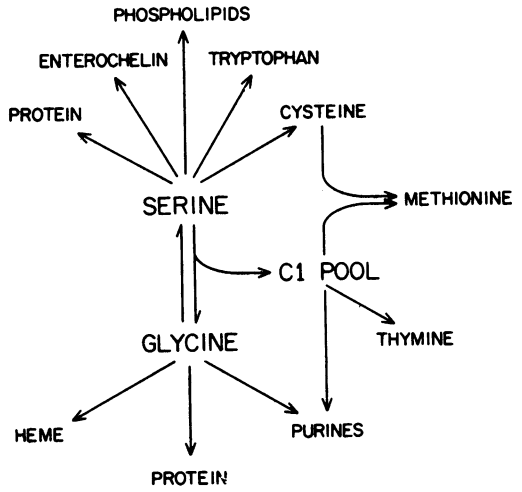

FIG. 1. Metabolites derived from serine and glycine.

purchased from Bristol Laboratories. Phenazine methosulfate was purchased from Calbiochem-Behring.

Strain constructions and genetic manipulations. Standard P1 transductions were done by the method of Miller (16). Strains containing the (*deoD-serB-trpR*)*37-1* deletion [designated Δ(*serB*)*37-1*] were constructed with P1 donor lysates propagated on strain SP516 (Table 1). Deletion mutants (recovered with approximately 40% linkage to *zjj*::Tn*10*) were identified as serine auxotrophs by replica plating. The *serA* and *gcv* mutations were transferred between strains by exploiting the linkage between *zgb*::Tn*10* and *serA* (approximately 20% linkage) and *gcv* (approximately 50% linkage). Donor lysates were made on GS415 and SP789 (Table 1). Transductants with lesions in *serA* were scored on the basis of their serine requirement. The *gcv ser* double mutants were scored as serine auxotrophs that could not grow on minimal glycine medium (20). The Gcv phenotype can only be observed in background strains harboring *ser* mutations (20). The *glyA* mutation was transferred between strains by linkage (approximately 40%) to *zfe-208*::Tn*10* with P1 lysates made on SP752 (Table 1).

Pseudorevertants of *ser* mutants (Table 1) were isolated in two successive steps. First-stage pseudorevertants were obtained by plating approximately 2×10^9 cells of a freshly grown overnight L broth culture on NoSG medium. Initially, the nutritional phenotype of the resulting pseudorevertants was elucidated as described by Davis et al. (7). Four pools of amino acids (10 mg/ml each) were made as follows: pool 1, Val, Leu, Ile, Pro, Trp, Lys, Arg, Asp, Thr, Cys, and Met; pool 2, Leu, Phe, Tyr, Trp, His, Asp, Asn, Glu, and Gln; pool 3, Leu, Phe, Tyr, Trp, His, Lys, Asn, Gln, Cys, and Met; pool 4, Ala, Val, Ile, Pro, Trp, Lys, Arg, Asp, Asn, Glu, and Thr. The ability of pseudorevertants to grow on minimal medium supplemented with one of the four amino acid pools was scored by streaking on four different types of medium. Pseudorevertants grew well on pool 1 and poorly on pool 4. Media with pools 2 and 3 failed to support the growth of first-stage pseudorevertants. Amino acids were eliminated one at a time from pool 1 media to further define the nutritional requirements of the pseudorevertants. Second-stage pseudorevertants were isolated from first-stage pseudorevertants by plating approximately 2×10^9 cells of a freshly grown overnight L broth culture on unsupplemented minimal medium.

Susceptibility to β-hydroxynorvaline inhibition. β-Hydroxynorvaline has long been known to function as a threonine antagonist in bacteria (4). Strains to be tested were grown overnight in 10 ml of L broth. The cells were washed with saline and diluted 1:100. Diluted cells (0.2 ml) were suspended in 2.5 ml of minimal top agar and poured onto minimal serine-glycine plates. β-Hydroxynorvaline (3 mg) from a concentrated stock solution was spotted onto a sterile filter paper disk placed in the center of the plate. After incubating at 37°C for 24 to 48 h, the plates were scored for the presence (or absence) of a zone of growth inhibition.

Threonine dehydrogenase assay. L-Threonine-dependent NADH formation was assayed in toluene-permeabilized cells by the method of M. Levinthal and M. Levinthal (unpublished data). In this assay, electrons from threonine are transferred via the NAD system and phenazine methosulfate to the terminal acceptor *p*-iodonitrotetrazolium violet, whose reduced form is measured colorimetrically. Cultures (10 to 50 ml) were grown in 250-ml flasks at 37°C with vigorous shaking. Cells grown in unsupplemented minimal medium were routinely assayed after growth had reached the stationary phase. In control experiments, threonine dehydrogenase activity was shown to be maximal, and the assay results were more reproducible under those conditions. For the same reason, cultures grown on LRKTM:240 medium were assayed when they had reached mid-log phase. Cells were washed once with saline and suspended in 1.5 ml of 0.1 M phosphate buffer (pH 7.0). Immediately prior to the assay, cells (1.5 ml) were made permeable by mixing for 2 min on a

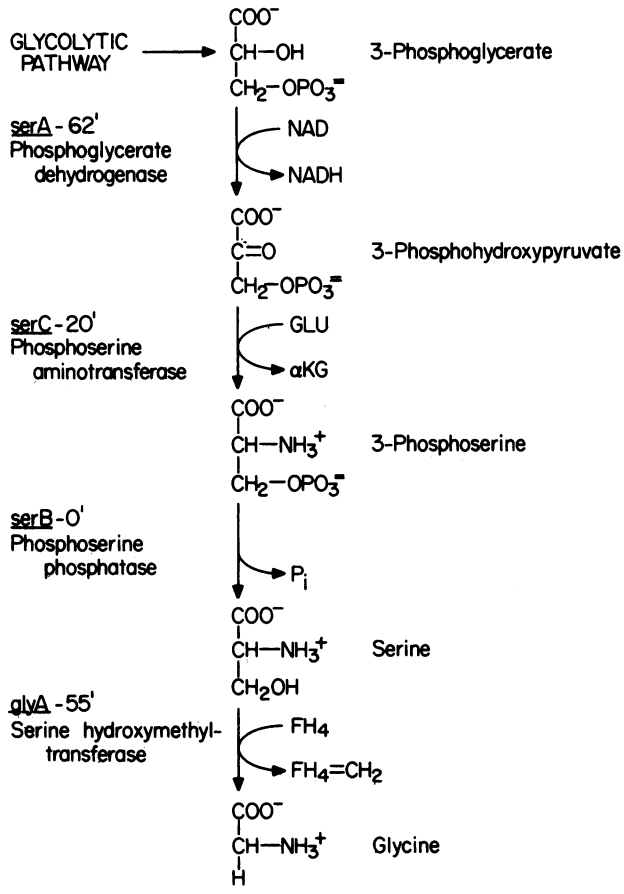

FIG. 2. Conventional pathway of serine biosynthesis.

TABLE 1. Bacterial strains used

Strain	Parent	Pertinent genotype	Source or reference
W3110		Prototroph	C. Yanofsky
37-1		Δ(*serB*)*37-1*	24
SP516		Δ(*serB*)*37-1 zjj*::Tn*10*	R. Somerville
SM20	W3110	*glyA*	R. Somerville
SP550	SM20	*glyA* Δ(*serB*)*37-1 zjj*::Tn*10*	This work
BW258		*zfe-208*::Tn*10*	B. Weiss
SP752	SM20	*zfe-208*::Tn*10 glyA*	This work
NK5031		Prototroph	N. Kleckner
SP361	NK5031	Δ(*serB*)*37-1*	2
GS400		*serA25 gcv*	G. Stauffer
GS415		*serA25 zgb*::Tn*10*	G. Stauffer
SP789	GS400	*serA25 gcv zgb*::Tn*10*	This work
SP783	W3110	*serA25 zgb*::Tn*10*	This work
SP790	W3110	*serA25 gcv zgb*::Tn*10*	This work
SP791	SP361	Δ(*serB*)*37-1 gcv zgb*::Tn*10*	This work
KL282		*serC13*	5
SP897	37-1	Δ(*serB*)*37-1 tdh*::*cat*	This work
SP902	SP783	*tdh*::*cat serA25 zgb*::Tn*10*	This work
SP903	KL282	*tdh*::*cat serC13*	This work
SP915	SM20	*tdh*::*cat glyA*	This work
SP668	SP361	Δ(*serB*)*37-1 crp*	R. Somerville
SP672	SP361	Δ(*serB*)*37-1 cya*	R. Somerville
SG52	SP361	Δ(*serB*)*37-1*, first-stage pseudorevertant	This work
SG480	SG52	Δ(*serB*)*37-1 tdh*::*cat*	This work
SG438	KL282	*serC13*, first-stage pseudorevertant	This work
SG481	SP783	*serA25 zgb*::Tn*10*, first-stage pseudorevertant	This work
SG460	SG52	Δ(*serB*)*37-1*, second-stage pseudorevertant	This work
SG471	SG460	Δ(*serB*)*37-1 gcv zgb*::Tn*10*	This work
SG484	SG460	Δ(*serB*)*37-1 tdh*::*cat*	This work
SG485	SG481	*serA25 zgb*::Tn*10*, second-stage pseudorevertant	This work
SG486	SG485	*serA25 zgb*::Tn*10 tdh*::*cat*	This work

vortex Genie mixer (Scientific Products) in the presence of 15 μl of 0.3% hexadecyltrimethylammonium bromide and 15 μl of toluene. The assay mixture contained, in a final volume of 1.0 ml, 125 μmol of L-threonine (pH 10.4), 100 μmol of CAPS buffer (pH 10.4), 10 μmol of NAD^+ (neutralized with 1 M CAPS buffer [pH 10.4]), 5 μg of phenazine methosulfate, 200 μg of *p*-iodonitrotetrazolium violet, 200 μg of gelatin, and permeabilized cells. The assay blanks contained no threonine. The reaction was started by adding L-threonine. The assay tubes, containing various amounts of permeabilized cells, were incubated at 37°C for 10 min. The reaction was stopped by adding 2 ml of 0.25 N HCl. The optical density at 520 nm was determined for both the blanks and the assay tubes after zeroing the spectrophotometer with water. The threonine-dependent change in absorbancy was converted to nanomoles of NADH produced by using a standard curve prepared with known amounts of NADH. Protein concentrations were determined by the method of Lowry et al. (13).

RESULTS

Overview. Mutational analysis has shown that a group of reactions, designated the Tut cycle (threonine utilization), constitute the second pathway of serine biosynthesis in *E. coli*. Threonine is converted to glycine and subsequently serine via the same pathway that functions in *P. cepacia* and *Arthrobacter* sp. (14, 31).

Phenotypes of serine and glycine mutants. All serine (*serA*, *serB*, or *serC*) and *glyA* mutants tested were able to grow on NoSG minimal medium provided that the medium contained elevated levels of leucine, arginine, lysine, threonine, and methionine (i.e., LRKTM:240). Overnight L broth cultures of serine mutants were washed, and dilutions were plated on either LRKTM:240 or minimal serine-supplemented medium. The viable-cell count determined for a culture on serine-supplemented medium was the same as that determined for a culture on LRKTM:240 medium (data not shown). Growth on LRKTM:240 medium was simply a physiological adaptive response, involving no mutational events. Amino acid analysis of spent culture medium after growth of a *serB* deletion mutant in LRKTM:240 showed that all of the threonine had been exhausted. The other amino acids were used to lesser and various extents (10 to 50%; data not shown). Threonine-dependent growth suggests that serine was derived from threonine. The other four amino acids most likely enhanced the growth of *ser* mutants either because they acted as inducers of necessary enzymes or because they spared a drain of some essential metabolites (see Discussion).

Serine auxotrophs, once adapted to growth in LRKTM:240 medium, displayed mass doubling times that were indistinguishable from those observed in serine- and glycine-supplemented medium (Fig. 3). Serine auxotrophs that were grown in L broth prior to inoculation into LRKTM:240 exhibited a carbon source-dependent variation in lag time. They rapidly entered log-phase growth in LRKTM:240 when lactate or glycerol was the carbon source. When glucose was the carbon source, the onset of log-phase growth in LRKTM:240 medium took three to four times longer to be observed. Serine or glycine auxotrophs were unable to grow on unsupplemented minimal medium with acetate or lactate as the carbon source. The addition of cyclic AMP (cAMP) (5 mM) or formate (0.005 or 0.05%) (as a potential source of C_1 units) did not shorten the lag period in glucose medium. Furthermore, serine auxotrophs deficient in adenylate cyclase (SP672) or the cAMP receptor protein (SP668) grew efficiently on LRKTM:240. It appears that growth on LRKTM:240 does not require the participation of the cAMP-*crp* system.

Isolation of Ser^+ pseudorevertants. Serine auxotrophs spontaneously gave rise to pseudorevertants (Table 2). First-stage pseudorevertants of *serB* deletion mutants as well as *serA* and *serC* point mutants were selected on NoSG medium. First-stage pseudorevertants of *serA* and *serB* mutants grew on serine-free medium supplemented with low levels of leucine, arginine, lysine, threonine, and methionine (40 μg of each amino acid per ml; LRKTM:40). The nutritional requirements of first-stage pseudorevertants were deduced as described in Materials and Methods. The requirement for threonine was absolute. Pseudorevertants did not grow on medium supplemented with leucine, arginine, lysine, and methionine. Any one, but not all, of the amino acids leucine, arginine, lysine, and methionine could be omitted from the medium without abolishing the growth of pseudorevertants. Pseudorevertants of the *serC* point mutant KL282 required isoleucine, valine, and cysteine in addition to LRKTM:40 (Table 2). Since we were not able to transfer the *serC* mutation of KL282 into other strain backgrounds, it was not possible to ascertain whether the requirement for the additional amino acids is typical of *serC* mutants or unique to the KL282 strain background. Second-stage pseudorevertants were isolated from first-stage pseudorevertants by plating

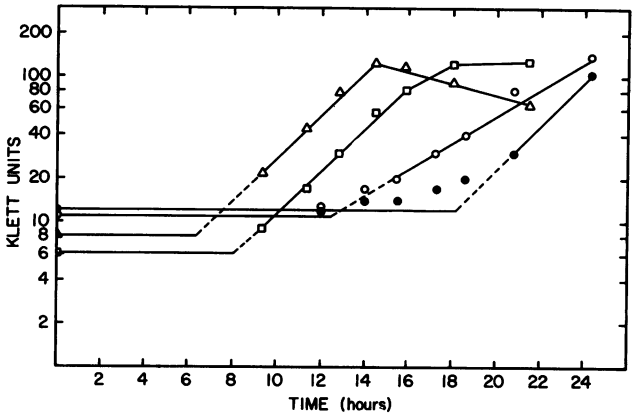

FIG. 3. Typical growth curves for a *serB* deletion mutant. SP361 [Δ(*serB*)*37-1*] was pregrown on L broth, washed once with saline, and inoculated into the minimal media at an initial density of approximately 10 Klett units, measured with a no. 66 filter. Growth was monitored by recording the Klett readings of a 15-ml culture grown in a sidearm flask at 37°C with vigorous aeration. Media: △, LRKTM:240 with lactate (0.4%) as the carbon source; □, LRKTM:240 with glycerol (0.5%) as the carbon source; ○, LRKTM:240 with glucose (0.4%) as the carbon source; ●, minimal serine-glycine medium with glucose (0.4%) as the carbon source.

large numbers of cells on unsupplemented minimal medium (Table 2).

Both first- and second-stage pseudorevertants had elevated levels of threonine dehydrogenase (Tables 2 and 3). First-stage pseudorevertants displayed increased resistance to the threonine analog β-hydroxynorvaline as determined by zone tests (Table 2). After 2 days of incubation, no zone of growth inhibition could be observed for first-stage pseudorevertants. Second-stage pseudorevertants were highly resistant to β-hydroxynorvaline, an indication that the level of intracellular threonine was sufficient to prevent the growth-inhibitory action of this compound.

It was not possible to use P1 transduction to transfer the mutations characteristic of first- or second-stage pseudorevertants from one strain to another. This suggests that at least two unlinked mutational events were responsible for the observed phenotypes.

Mutational analysis of the Tut cycle. The products of the *tdh*, *glyA*, and *gcv* genes were all essential for the formation of serine from threonine via the Tut cycle. Serine mutants bearing a second lesion in any one of the above loci could not grow on LRKTM:240 (Table 4 and below).

The *tdh* gene is available in cloned form in the multicopy plasmid pDR121. Strains harboring pDR121 have a 50- to 500-fold elevation of threonine dehydrogenase (P. D. Ravnikar, Ph.D. thesis, Purdue University, West Lafayette, Ind., 1985). The physical and kinetic parameters of threonine dehydrogenase from cells harboring pDR121 are identical to those of purified threonine dehydrogenase from *E. coli* (3; Ravnikar, Ph.D. thesis; P. D. Ravnikar and R. L. Somerville, submitted for publication). The *tdh* gene of pDR121, disrupted by insertion of the *cat* (chloramphenicol acetyltransferase) gene, was used to generate a chromosomal *tdh* null mutant by the procedure of Winans et al. (22, 30).

The *tdh*::*cat* null mutation was introduced into the chromosome of *serA*, *serB*, *serC*, and *glyA* strains by P1 transduction. None of the resulting double mutants were able to grow on LRKTM:240 medium (Table 4). Likewise, introduction of the *tdh*::*cat* mutation into first- and second-stage pseudorevertants, such as SG52, SG460, and SG485 (Table 1), abolished the ability of such strains to grow on LRKTM:40 or minimal medium. These strains displayed a nutritional requirement that was satisfied only by the addition of serine or glycine. Thus, a mutational block in *tdh* rendered *glyA* mutants and *ser* mutants and their pseudorevertants unable to grow on LRKTM medium.

A *glyA* mutant, SM20, grew in minimal medium supplemented with glycine or LRKTM:240. A Δ*serB* mutation was then introduced into the SM20 background, generating a Δ*serB glyA* double mutant that grew on minimal glucose medium only when both serine and glycine were added as supplements. The double mutant SP550 failed to grow on LRKTM:240 unless serine was added to the medium (Table 4). Additionally, no pseudorevertants of SP550 could be isolated. The introduction of a *glyA* mutation into SG460, a pseudorevertant of a *serB* deletion mutant (Table 1), led to a nutritional requirement that was satisfied only by glycine plus serine. All *ser glyA* double mutants required both serine and glycine for growth on minimal medium. We conclude that *glyA* mutants cannot convert the glycine derived from threonine to serine.

The glycine cleavage system (*gcv*) enables cells to derive C_1 units (as N^5,N^{10}-methylenetetrahydrofolate) from glycine (11). Introduction of a *gcv* mutation into *ser* mutants SP790 and SP791 or a pseudorevertant of a *ser* mutant, SG460, abolished the ability of such cells to grow on LRKTM:240 or minimal medium. A *ser gcv* double mutant could not convert glycine to serine owing to a lack of essential C_1 units.

DISCUSSION

Several lines of evidence support the theory that a second pathway of serine biosynthesis is available to *E. coli*. Previous workers have described *ser* or *gly* mutants of *E. coli* that can be suppressed by exogenous threonine (8, 19, 28) as well as pseudorevertants of *ser* and *glyA* mutants capable of growth on unsupplemented minimal medium (8, 25). In this work, we showed that all classes of *ser* mutants in addition

TABLE 2. Selection and phenotypes of pseudorevertants derived from *ser* parental strains[a]

Strain	Selection medium	Nutritional phenotype	Threonine dehydrogenase phenotype	Susceptibility to β-hydroxynorvaline
ser mutant		Growth on LRKTM:240	Low but detectable	Susceptible
First-stage pseudorevertant	NoSG	Growth on LRKTM:40	Elevated	Resistant
Second-stage pseudorevertant	Minimal medium	Growth on minimal unsupplemented medium	Elevated	Resistant

[a] Pseudorevertants were isolated as described in Materials and Methods. Both first- and second-stage pseudorevertants arose at an apparent frequency of 10^{-8}. Threonine dehydrogenase activities were routinely determined from mid-log cultures grown in LRKTM:240 medium with glucose as the carbon source. First- and second-stage pseudorevertants typically had an approximately 20-fold elevation of threonine dehydrogenase activity relative to their parent strain. Resistance to the threonine analog β-hydroxynorvaline was determined as described in Materials and Methods. A *ser* mutant typically exhibited a 25-mm zone of growth inhibition. Growth of first-stage pseudorevertants was slower within a 20-mm diameter of the disk containing β-hydroxynorvaline. However, within 48 h, no growth inhibition could be observed.

to *glyA* mutants could derive glycine and serine via the Tut cycle. Thus, the Tut cycle is not cryptic in *E. coli*; under appropriate culture conditions it becomes a major route to serine and glycine.

Genetic analysis of the operation of the Tut cycle in Ser^+ strains is hampered because (i) *gcv* mutations can only be scored in *ser* mutant backgrounds (20), (ii) *tdh* mutations can only be scored in *ser* or *glyA* mutant backgrounds (22; Ravnikar, Ph.D. thesis), and (iii) no AKB lyase-deficient mutants have yet been described.

Serine mutants and pseudorevertants with an additional mutation in *tdh*, *gcv*, or *glyA* were unable to use the Tut cycle (Table 4). These genes encode enzymes that catalyze a set of reactions that constitute the Tut cycle (Fig. 4). The Tut cycle is postulated to operate as follows. (i) Threonine (2 mol) is first oxidized to AKB by threonine dehydrogenase (the *tdh* gene product). (ii) AKB is cleaved to yield 2 mol of acetyl coenzyme A and 2 mol of glycine. (iii) The glycine cleavage system converts 1 mol of glycine to a C_1 unit (N^5,N^{10}-methylenetetrahydrofolate). (iv) The *glyA* gene product (serine hydroxymethyltransferase) catalyzes the condensation of a C_1 unit with a second glycine to produce 1 mol of serine. (v) The 2 mol of acetyl coenzyme A produced in the AKB lyase reaction are available for recycling into C_4 metabolism via the glyoxylate cycle. In essence, the Tut cycle converts 1 mol of threonine to serine and CO_2. Serine can therefore originate either from the C_3 pool of glycolytic intermediates or from the C_4 metabolites, as if it were a member of the aspartate family of amino acids. The consumption of threonine for serine production is consistent with our observation that serine mutants utilize all of the exogenous threonine during growth in LRKTM:240 medium. Energetically, the Tut cycle provides a very efficient alternative for the production of serine. In contrast to the conventional pathway of serine biosynthesis (which requires 1 mol of ATP), the Tut cycle, by virtue of the reduced nucleotide cofactors that are produced, has the potential to produce a net yield of 5 mol of ATP during aerobic growth (Fig. 4). Physiologically, mutants blocked in the conventional pathway to serine adapted very readily to growth on media that necessitated using the Tut cycle (Fig. 3).

It is not yet clear whether the operation of the glyoxylate cycle is critical to the function of the Tut cycle. It is reasonable to assume that via the glyoxylate cycle, a known means of utilizing acetate, *E. coli* conserves the C_2 units generated from α-amino-β-hydroxybutyrate. Alternatively, *E. coli* may excrete all or some of the C_2 units into the medium. If the C_2 units are not recycled, the net reaction of the Tut cycle is the conversion of 2 mol of threonine to 1 mol of serine and CO_2 and 2 mol of acetate.

TABLE 3. Typical threonine dehydrogenase levels observed in representative *E. coli* strains

Strain	Sp act[a] of threonine dehydrogenase in medium:		
	Minimal	Serine-glycine	LRKTM:240
Wild type	4.0 ± 2	ND	40.0 ± 14
ser mutant	ND	9 ± 3.6	47.0 ± 14
First- or second-stage pseudorevertant	ND	ND	710 ± 250

[a] Threonine dehydrogenase activities were determined as described in Materials and Methods. Specific activity, nanomoles of NADH per milligram of protein per minute. NK5031 and W3110 were assayed as representative wild-type strains. SP361 (*serB*) and SP783 (*serA*) were assayed as representative *ser* mutants. First- and second-stage pseudorevertants of both SP361 and SP783 were assayed. The determinations. ND, Not determined.

TABLE 4. Phenotypes of *ser* mutants and Ser^+ pseudorevertants[a]

Pertinent genotype	Tut cycle functional (growth requirements)	Pseudorevertants isolated	
		First stage (LRKTM:40)	Second stage (minimal medium)
glyA	+ (LRKTM:240)	ND	ND
serA	+ (LRKTM:240)	+	+
Δ*serB*	+ (LRKTM:240)	+	+
serC	+ (LRKTM:240)	+[b]	ND
Δ*serB glyA*	− (Ser and Gly)	−	
serA gcv	− (Ser)	−	
Δ*serB gcv*	− (Ser)	ND	
serA, *serB*, or *serC tdh*::*cat*	− (Ser)	−	
glyA tdh::*cat*	− (Gly)	ND	

[a] Strains are listed in Table 1. ND, Not determined.
[b] First-stage pseudorevertants of *serC* mutants required cysteine (10 μg/ml), isoleucine, and valine (40 μg/ml) in addition to LRKTM:40.

Serine auxotrophs utilized the Tut cycle when the culture medium contained elevated levels of leucine, arginine, lysine, threonine, and methionine. The fact that first- and subsequently second-stage pseudorevertants could be isolated indicates that sequential mutational events can render the Tut cycle constitutive on minimal medium. It appears that multiple unlinked regulatory mutations are necessary to allow growth of *ser* pseudorevertants on minimal medium. Considering the number of steps in the Tut cycle which must be operating at full efficiency on minimal medium (such as expression of serine hydroxymethyltransferase, threonine dehydrogenase, and the glycine cleavage system; Fig. 3), the necessity for multiple mutational events is not unexpected. Since the structural genes encoding the various required enzymes are widely distributed on the *E. coli* chromosome (1), it is not surprising that the regulatory mutations are unlinked.

At least one regulatory mutation in pseudoreversion leads to greatly elevated levels of threonine dehydrogenase. A second aspect of pseudoreversion appears to be an increase in the threonine pool size, as judged by the resistance of pseudorevertants to β-hydroxynorvaline (Tables 3 and 4). Pseudorevertants growing on unsupplemented minimal medium clearly were able to synthesize all of the threonine needed for isoleucine, serine, glycine, and C_1 units, as well as the threonine needed for protein synthesis. One potential mechanism for increasing the size of the threonine pool might be through a reduction in the activity of threonine deaminase, encoded by *ilvA*. Endogenously formed threonine could then be preferentially shunted into glycine and serine biosynthesis via the Tut cycle. Threonine deaminase levels in first- and second-stage pseudorevertants were unchanged relative to their parental strains (data not shown). Fraser and Newman (8) likewise failed to observe changes in threonine deaminase in a *glyA* mutant converting endogenous threonine to glycine. Thus, it seems that any change in the threonine pool size is not accompanied by changes in the expression of *ilvA*.

Although serine and glycine auxotrophs facilitated our genetic analysis of the Tut cycle, it is likely that this sequence of reactions contributes to serine biosynthesis even in serine prototrophs in which the conventional pathway of serine biosynthesis is available. During the growth of

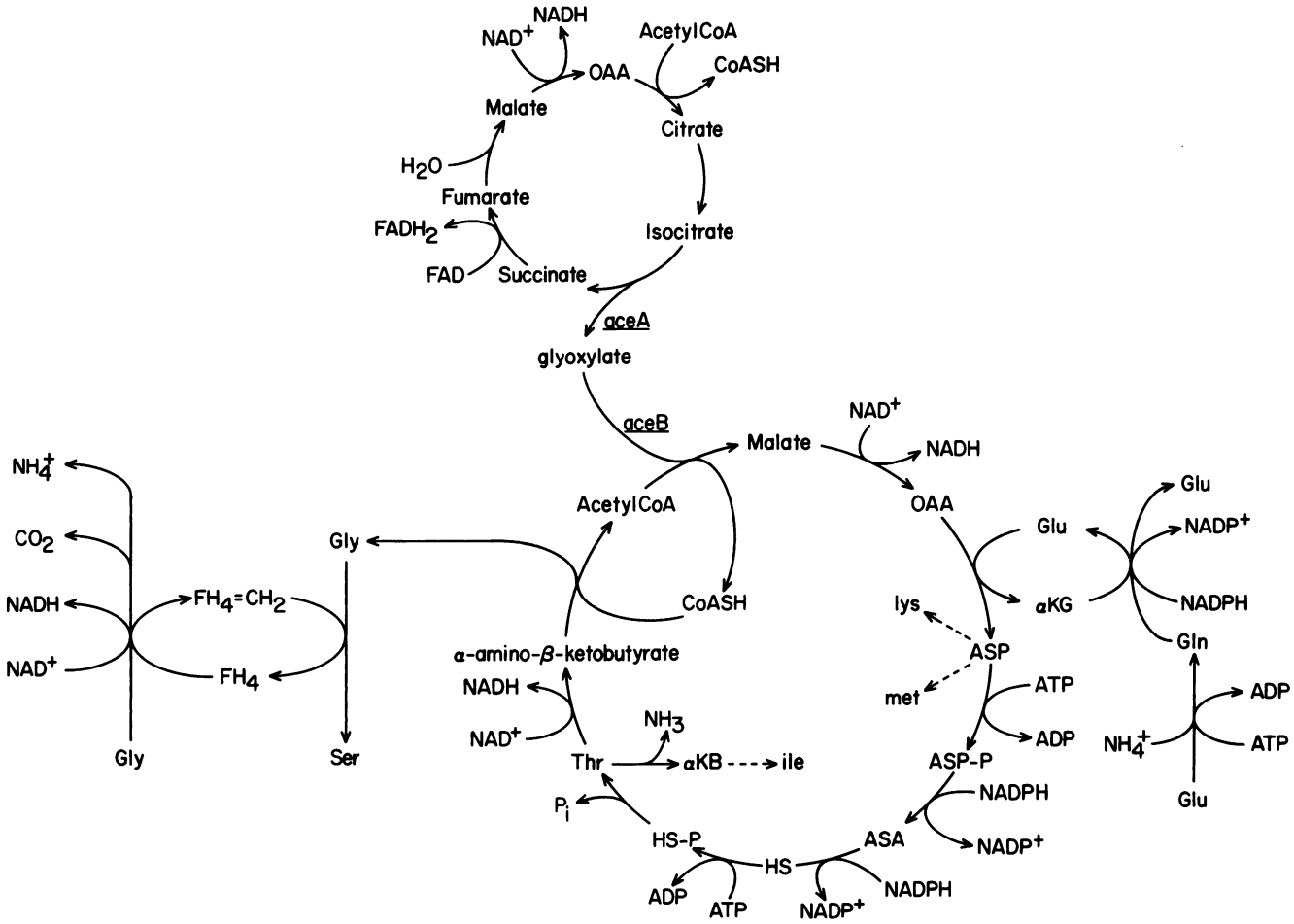

FIG. 4. Tut cycle. The final reaction of the Tut cycle is Thr + 3ATP + FAD + 2NAD$^+$→Ser + CO_2 + 3ADP + $FADH_2$ + 2NADH + 2H$^+$ + 3Pi. The final reaction of the conventional pathway is 3-phosphoglycerate + NH_4^+ + ATP→Ser + ADP + Pi. Abbreviations: *aceA*, gene for isocitrate lyase; *aceB*, gene for malate synthase; OAA, oxaloacetate; Glu, glutamate; Gln, glutamine; αKG, α-ketoglutarate; ASP, aspartate; ASP-P, β-aspartyl phosphate; ASA, aspartate β-semialdehyde; HS, homoserine; HS-P, homoserine phosphate; αKB, α-ketobutyrate; FH_4, tetrahydrofolate; FH_4=CH_2, N^5,N^{10}-methylenetetrahydrofolate.

E. coli on glucose, 3-phosphoglycerate is presumably abundant. However, during growth on carbon sources such as lactate or acetate, it may be more advantageous for the cell to derive serine from threonine via the Tut cycle, thereby sparing the 3-phosphoglycerate pool for gluconeogenesis. There is indirect evidence that the Tut cycle does operate and contribute to the serine pool in prototrophic strains. The threonine dehydrogenase levels of wild-type strains grown in LRKTM:240 medium are 10-fold greater than those in cells grown in unsupplemented medium. Furthermore, the specific activity of threonine dehydrogenase in such wild-type cells was essentially the same as that for a *serB* deletion mutant grown in LRKTM:240 medium (Table 3). Additionally, when *E. coli* K-12 is grown in the presence of elevated levels of leucine, isoleucine, threonine, and methionine with lactate as a carbon source, the organism has only 10% of the 3PGA-DH activity of control cells grown in minimal glucose medium (15, 19). Interestingly, the reduction in 3PGA-DH neither imposed a serine requirement nor reduced the intracellular serine concentration. It is likely that these growth conditions activated the Tut cycle, since the composition of the medium used by earlier workers is similar to that of the LRKTM:240 medium used in this study. All the *ser* or *glyA* mutants we tested could grow in medium having elevated levels of leucine, isoleucine, threonine, and methionine, suggesting that the growth of wild-type cells in this medium is accompanied by significant serine biosynthesis via the Tut cycle. Thus, a reduction in 3PGA-DH levels does not necessarily impose a nutritional requirement for serine. Our results, taken together with those of McKitrick and Pizer (15), suggest that the two pathways of serine biosynthesis may operate in a mutually exclusive fashion. Conditions which favor the operation of the Tut cycle may simultaneously repress the conventional serine pathway and vice versa.

Threonine must be present in culture medium for unsuppressed *ser* or *glyA* mutants to utilize the Tut cycle. Threonine is the precursor to serine, donating its carbon via glycine, when the Tut cycle is operating. Any one, but not all, of the other amino acids (leucine, arginine, lysine, and methionine) could be eliminated from the medium without abolishing the growth of a *ser* or *glyA* mutant. Among the 20 amino acids, only leucine, arginine, lysine, and methionine require either acetyl coenzyme A or succinyl coenzyme A

for biosynthesis. The growth-stimulatory effect of these amino acids probably involves either a sparing of potentially limited key metabolic intermediates (namely succinyl coenzyme A, α-ketoglutarate, and C_1 units) or an induction (17) of key enzymes.

Most of the early work directed toward defining the pathway of serine biosynthesis used cultures or cell extracts of cultures that had been grown in minimal medium with glucose as the carbon source. Most frequently the experimental design involved tracing ^{14}C-labeled glucose into serine. This strategy cannot discriminate between serine derived from triose phosphate via the conventional pathway (Fig. 2) and serine derived from threonine via the Tut cycle. The exchange of carbons between the intermediates of glycolysis and the tricarboxylic acid cycle catalyzed by phosphoenolpyruvate carboxylase and phosphoenolpyruvate carboxykinase results in a randomization of ^{14}C-labeled carbon from glucose.

Roberts and co-workers (23) observed that glycine is the precursor to serine when either fructose or acetate is the carbon source. If the early studies of serine biosynthesis had traced radiolabeled acetate or fructose into serine, it is possible that the role of the C_4 metabolites in serine biosynthesis would have received greater recognition. Roberts et al. (23) further noted that glycine biosynthesis appears to proceed via three different mechanisms, depending upon the growth conditions. The accumulated evidence of their work led them to conclude that "The 'normal' pathway [of glycine biosynthesis] depends upon what is defined as 'normal' medium." This point of view is fully supported by our results.

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