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Characterization of <u>Escherichia</u> <u>coli</u> K12 Mutants That Can

Use Glycine as Sole Source of Carbon and Energy


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A Thesis

in

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of

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ABSTRACT

CHARACTERIZATION OF ESCHERICHIA COLI K12 MUTANTS THAT CAN
USE GLYCINE AS SOLE SOURCE OF CARBON AND ENERGY

James Garnon

Mutant strains of Escherichia coli K12 were isolated
that are able to use glycine as their sole source of carbon
and energy. These were designated GOG. The parent strain of
the GOG strains is the pleiotropic mutant KEC9 (ssd) that has
high levels of L-serine deaminase and can grow on L-serine as
the sole carbon source. Results indicate the GOG strains
utilize glycine by way of serine hydroxymethyltransferase,
encoded by ıe glyA gene, and L-serine deaminase. Four GOG
strains studied had high levels of serine hydroxymethyl-
transferase activity. The selected mutations of three of the
GOG strains mapped to the glyA locus; the mutation of the
remaining GOG strain mapped roughly to 17 minutes. The GOG
strains are unable to grow on glycine as sole carbon source
when compounds known to decrease glyA transcription (purines
and methionine) are present. Derivatives were selected that
overcame this inhibition. Analysis showed that at least some
of these strains may be of use for L-serine production.

ACKNOWLEDGEMENTS

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INTRODUCTION

1. Objectives

The approach to this work was the selection of mutants of <u>Escherichia</u> <u>coli</u> K12 that are able to utilize exogenous glycine as their sole carbon and energy source. The major objective in undertaking this approach is that the selection may produce mutants with increased levels of serine hydroxymethyltransferase (SHMT), which can assimilate glycine. This enzyme has been studied extensively. However, the regulation of <u>glyA</u> (which encodes SHMT) is complex and requires further investigation. Obtaining mutants with altered SHMT activity will aid in unravelling the nature of the regulation of its synthesis.

A second major objective of this work was to explore the possibility that selection of mutants with increased SHMT activity could result in a strain that cverproduces L-serine. SHMT catalyses the reversible reaction of L-serine to glycine, and therefore can synthesize L-serine from glycine.

There is a need for an efficient microbial production of L-serine that can yield an inexpensive supply of this amino acid. L-Serine is required for laboratory and pharmaceutical use. Potentially, it can be an inexpensive source of another

costly amino acid, tryptophan, which can be added to livestock feed to increase its nutritive value.

There is a considerable amount of research into microbial production of L-serine. Fermentation has the potential to provide an inexpensive source of L-serine. It also has advantages over organic synthesis because there are fewer problems with byproducts and L-stereo isomers of amino acids can be specifically produced. Glycine is usually the substrate employed in research into the microbial production of L-serine. This is because glycine is an inexpensive substrate and is converted enzymatically to L-serine in a one-step reaction, catalyzed by SHMT.

Most of the research into microbial production of L-serine focuses on the methylotrophic bacterium (i.e. Pseudomonas; for examples see 18, 29, 51, 64, 67). The SHMT reaction requires two substrates to form L-serine, one-carbon unit (C_1) and glycine. A methylotrophic bacterium presents an immediate advantage in L-serine production research in being able to use exogenous methanol or methane as its C_1 source.

The work presented in this thesis follows a less common approach to L-serine production in that the organism used in the study was E. coli K12. The reason for using this organism is that its metabolism and genetics are well known, with a vast number of manipulative tools available, offering a greater control in the development of an L-serine producing strain. Although, E. coli may be unable to use an exogenous

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C_1 source, its glycine cleavage reaction (GCV) may be adequate to supply C_1 units. In addition, although much is understood about this organism, there still are some mysteries in its L-serine, glycine and C_1 metabolism, and exploring them can be done while researching into L-serine production. To understand what is known and the approach used to isolate strains useful for L-serine production, an overview of the relevant metabolism will now be presented.

2. Interconversion of Glycine and L-Serine: Serine Hydroxymethyltransferase

The metabolism in which SHMT is involved is depicted in Figure 1. The SHMT enzyme catalyses the reversible conversion of L-serine and tetrahydrofolate (THF) to glycine and 5,10-methylenetetrahydrofolate (methyleneTHF). The SHMT enzyme is encoded by the glyA gene and is constitutively synthesized. The enzyme is 170,000 daltons and is a tetramer of identical subunits (57). It requires two cofactors for its reaction, tetrahydrofolate (THF) and pyridoxal-5-phosphate (PLP). THF is the active folate molecule which functions as a carrier of one-carbon units (3). PLP is tightly bound to the active site of SHMT (49). PLP forms a Schiff base with glycine in the reaction of SHMT (3, 49).

Physiologically, the SHMT reaction is usually directed towards glycine and methyleneTHF production (49, 57). Because the reaction is reversible, the enzyme can also catalyse the

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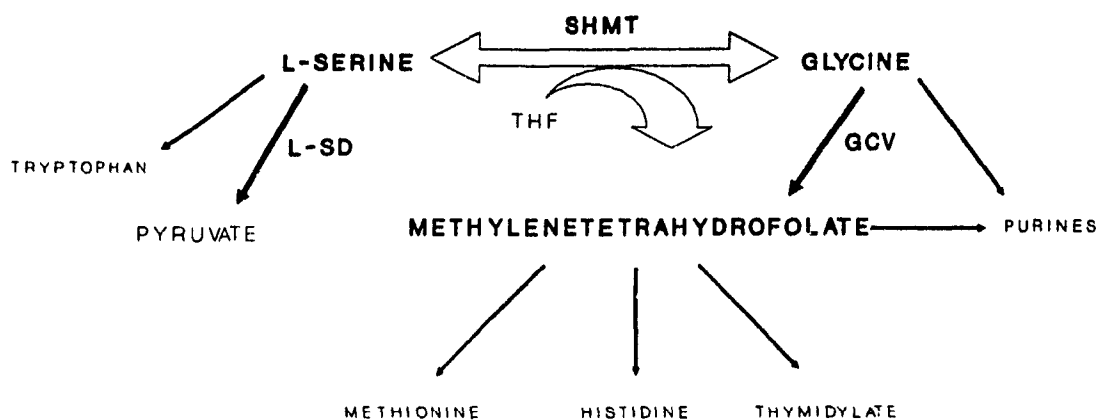
Figure 1. Serine/glycine pathway.
Abbreviations SHMT, serine hydroxymethyltransferase;
GCV, glycine cleavage system, L-SD, L-serine deaminase;
THF, tetrahydrofolate.

conversion of glycine to L-serine formation. That it is physiologically reversible can be deduced from the phenotype of a serine-glycine auxotroph (27, 32). This strain cannot form its L-serine from the usual phosphorylated biosynthetic pathway and therefore requires exogenous L-serine when growing on glucose minimal medium. The fact that L-serine may be replaced by glycine indicates that L-serine can be made from glycine through the SHMT enzyme.

SHMT is used to supply the sole source of glycine when cells are growing in glucose minimal medium. Glycine is a precursor of purines, heme-containing compounds, and is a component of proteins (57). In addition, SHMT is the major source of methyleneTHF, a one-carbon unit (C_1), essential for many important biosynthetic pathways, leading to compounds termed C_1 metabolites (30). The enzyme accounts for between 60 and 75 percent of the C_1 required by the cell (5). The other source of C_1 is from glycine through the GCV pathway. There is also evidence for an additional minor source(s) of C_1, for which L-serine may serve as donor (33). It is considered that the cell uses SHMT to form glycine and C_1, and uses the GCV pathway if its need for C_1 increases over its need for glycine (33, 57).

As stated, a serine-glycine auxotroph can grow on glucose minimal medium if it is supplemented with glycine (32). This indicates that it is deriving not only L-serine from the exogenous glycine by SHMT, but also all its required

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C_1 by the GCV pathway. A mutant lacking SHMT activity can obtain all required C_1 from glycine, through the GCV pathway (33). This is important if one wants to use SHMT to produce L-serine from glycine. The cell can obtain its required C_1 from the cleavage of glycine by GCV pathway, supplying the C_1 not only for its normal metabolic functions, but in addition, to combine with glycine to form the L-serine.

3. Alternative Source of One-Carbon Units: Glycine Cleavage Pathway

The GCV pathway is the second predominant source of C_1 in E. coli. Since growth on glycine would require use of SHMT enzyme in the direction of L-serine synthesis, the only other significant source of C_1 units in glycine-grown cells would presumably be the GCV pathway. In addition, a change in regulation of GCV may also occur in the selection of E. coli mutants able to grow on glycine as sole carbon source. This is particulary important because an adequate supply of C_1 units from the GCV pathway is needed for establishing an L-serine producing strain.

Thus, the C_1 unit is an important factor in L-serine production. The C_1 (methyleneTHF) unit consists of a methyl group carried by the cofactor tetrahydrofolate (THF). It is required directly in the synthesis of L-serine from glycine. Additionally, it is required by several other biosynthetic reactions, requiring methylation, leading to the C_1

metabolites. Since there is a varied demand for C_1 units in different growth conditions, C_1 metabolism should be under strict con_rol.

The GCV pathway was originally demonstrated in E. coli by Meedel and Pizer (26). However, this pathway has not been studied extensively in E. coli. The GCV enzyme system has been studied in considerable detail in Diplococcus glycinophilus (47) and Pisum sativum (4). The GCV system was found to consist of a pathway of four enzymes. Evidence indicate that this may be the case for E. coli as well (57). Many mutations of the GCV genes have been isolated, and all map to 62 minutes on the E. coli chromosome (42). In addition, a plasmid presumably carrying all the genes encoding the GCV pathway from E. coli has been isolated (56). Both of these results support the suggestion that the GCV system comprises an operon in E. coli.

The GCV reaction carries out the cleavage of glycine to methyleneTHF, CO_2 and NH_3. This pathway is induced by exogenous glycine (26). It was estimated that the induced level of GCV activity is only one-tenth that of the normal levels of SHMT (26). In addition, there is conflicting evidence that it is repressed by C_1 metabolites as well (31, 57).

There may be other sources of C_1 units in minimal medium grown cells. A mutant of E. coli, AT2046, which had no SHMT activity synthesized only 40 percent of C_1 from glycine via

the GCV pathway (33). As well, in a glycine-requiring mutant, some radioactive glucose was converted into C1 units (26). This indicates there is at least a third means to synthesize C_1 units growing in minimal medium. Evidence suggest that L-serine is the precursor for this additional source of C_1 (33).

4. Regulation of Serine Hydroxymethyltransferase Levels

The SHMT enzyme has been studied extensively. Regulation of the SHMT enzyme appears to involve repression of its synthesis. Many factors that regulate the enzyme have been implicated. However, in the majority of the results, the mechanism of their action and specific regulatory proteins that carry out the regulation are either not known or uncertain.

As stated, the SHMT reaction converts L-serine to two important compounds, glycine and methyleneTHF (see Figure 1). The cell requires glycine for protein synthesis and it also enters the pathway to purine biosynthesis. MethyleneTHF is the C_1 unit that enters a variety of pathways leading to important products. These include methionine, purines (adenine and guanine), thymidylate, histidine, L-serine and folates (14, 24, 27, 52, 57, 65). The compounds that require C_1 in their biosynthesis (i.e. C_1 endproducts) are collectively called C_1 metabolites. Therefore, formation of all the C_1 metabolites and glycine relies heavily on the activity of

SHMT. It is not a surprise that they play a part in regulating SHMT activity in the cell.

Since the enzyme forms compounds that branch off down pathways to many important metabolites, SHMT may be considered the first enzyme of these pathways. From experimental evidence, it is apparent that SHMT is under complex regulation. Studies suggest that the regulation affects the synthesis of SHMT. There is no evidence that there is feedback control on the enzyme itself, as is usual for the first enzyme of a pathway (24). For instance, L-serine feedback inhibits the first enzyme, 3-phosphoglycerate dehydrogenase, of the phosphorylated pathway of L-serine biosynthesis (41). However, Schirch (49) has found that SHMT shows high affinity for methyltetrahydrofolate, a major folate metabolite, and postulates that this may be evidence of a feedback control.

It was found that a mixture of C_1 metabolites represses the expression of the glyA gene, a phenomenon often referred to as C_1 repression (9, 14, 24, 27, 52, 65). As is often the case, when supplied with a compound exogenously, the bacteria are relieved from spending metabolic resources in forming that compound itself. As a result, since C_1 units are not drained into the C_1 pathways when the C_1 metabolites are supplied exogenously, the C_1 pool of the cell will conceivably increase. On the addition of purines, thymidine, and methionine, incorporation of C_1 units into these products is

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reduced by 95 percent (6). It is either the increase in the C_1 pool or the C_1 metabolites themselves that regulate SHMT activity (6, 27).

The repression is cumulative with a mixture of C_1 metabolites, where full repression depends on the combination of compounds (52). This suggests that the regulation of SHMT by the various C_1 metabolites works not through a common regulatory protein, but rather, an agglomeration of regulatory proteins. Therefore, not all the regulation of SHMT levels can be explained by a monitor of levels of C_1 units alone.

An effect on SHMT levels by C_1 metabolites can be shown by two methods: first, by the addition of excess C_1 metabolites, singly or in mixtures, to the growth medium, and second, by diminishing a C_1 metabolite through starvation of a particular C_1 metabolite auxotroph. Such studies have yielded much information about the regulation of SHMT synthesis. A brief overview of these studies follows.

L-Serine appears to have some effect on the synthesis of SHMT, albeit not much (6, 27). For instance, addition of L-serine results in some repression of SHMT synthesis in Salmonella typhimurium (closely related to E. coli) (52). However, addition of excess L-serine in E. coli cultures does not significantly alter SHMT levels (6, 27). When serine-glycine auxotrophs of E. coli and S. typhimurium were starved for L-serine, no significant derepression in SHMT synthesis was seen (27, 52), although some derepression was observed in

other studies (6). Hence, though these studies show some effect of L-serine, it is not a major factor in SHMT regulation.

Addition of methionine, purines (adenine and guanine), thymine, histidine, and formate to cultures results in repression of synthesis of SHMT by 40 percent (27, 65). Of the C_1 metabolites, only purines significantly decrease SHMT levels when tested alone (6). Addition of adenosine results in a two-fold reduction of SHMT activity (6). In a purine-auxotroph starved for purines, SHMT is derepressed three-fold (6, 27, 52). The starvation of methionine auxotrophs for methionine also derepresses SHMT synthesis. One study found an 18-fold derepression of SHMT synthesis in a methionine auxotroph starved for methionine (24). An increase in SHMT activity was observed in methionine-limited growth of methionine auxotrophs. metE (6, 14, 24) and metF (6) of E. coli. However, no change in SHMT levels was observed for metA mutants similarly starved (14). In addition, it was observed that during methionine limitation, SHMT does not respond to purines, and vice-versa. This suggests there is a common corepressor or repressor from the methionine and purine pathways (6).

Evidence indicate that regulatory proteins of pathways that involve C_1 units (i.e. pathways leading to C_1 metabolites) may act on the glyA gene. Implicated in the regulation picture are the gene products of metJ, metK, metR, and purR,

that code for products that regulate their respective pathways of methionine and purine biosynthesis. Mutations of these genes results in a phenotype that includes altered regulation of SHMT synthesis (14, 26, 46, 53, 58). This indicates that regulatory proteins of the methionine and purine pathways also regulate the expression of the glyA gene (14, 26, 46, 53, 58). It was found that the product of the purR, a repressor of the genes coding for enzymes of the purine pathway, represses glyA expression (2-fold) and binds to the operator region of this gene (58). Even though there is evidence that regulatory processes controlling other metabolic pathways influence glyA expression, the results indicate that this is only part of the regulation of SHMT synthesis (14, 53). If this is so, and giving that several other conditions affect SHMT activity levels, the regulation of glyA gene expression is complex indeed.

Strains carrying the metK mutation lack S-adenosyl-methionine synthetase, and show a two-fold derepression of SHMT (26). Methionine is the precursor of S-adenosyl-methionine (SAM), a one-carbon donor for many methylation reactions (30). This may indicate that SAM, not methionine directly, is a regulatory molecule that may repress SHMT synthesis. Evidence indicates that regulation of SHMT synthesis by the methionine and purine pathway operate by a common repressor or corepressor (6). SAM is a product of both purine (adenosine) and methionine biosynthesis, both of which

have regulatory ties with SHMT, which may make SAM the key regulatory molecule (26, 53).

Of the C_1 metabolites, purines appear to play the strongest role in regulation of SHMT synthesis (6, 27). As was shown, purine is formed not only from C_1 but also from glycine, and therefore depends on SHMT for two of its precursors.

Many researchers categorize the C_1 metabolites and glycine together when discussing SHMT regulation (57). However, glycine synthesis does not require C_1, and therefore, glycine should not be considered a product of C_1 metabolism.

Although low concentrations of glycine do not affect SHMT, it was found that SHMT was repressed 43 percent by high concentration (3000 μg/ml) of glycine (9, 27, 33). It was suggested that high concentrations of glycine are required for an effect on SHMT because some strains of E. coli are relatively impermeable to glycine (27). Experimental evidence suggests that glycine regulates SHMT by repression and that this is distinct from repression by C_1 metabolites (27). It was also suggested that much of the experimental evidence for C_1 repression on SHMT can be attributed to glycine itself (27). For instance, when E. coli is grown in medium supplemented with purines, repression of SHMT is observed (6, 27). As stated above, purines have the most significant affect on SHMT levels. As is common with many metabolites, addition of the purines exogenously causes E. coli to stop

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synthesizing the purines. As a consequence, the bacterium does not have to use its C_1 in purine production, resulting in an increase in the C_1 pool. Although regulation of glyA by the purR repressor (58) may explain purine repression, the increase in C_1 may also be involved. Since glycine is also required for purine synthesis, it also would be spared. The increase in the glycine pool due to the exogenous supply of purines may also be involved in the repression of SHMT levels (27). It is probable that it is a combination of regulatory effectors responding to the supply of C_1 metabolites and glycine that regulate the level of SHMT activity.

The glyA gene has been cloned and sequenced (44, 55). The sequence shows a 22 bp palindrome centered about the transcription start site (43). Such structures are common in bacterial operators and often the binding site of a regulatory protein (43). This may be the site of action of a repressor that may regulate part of the C_1 repression or glycine repression. Another site in the operator region has been shown to be the binding site of the purR repressor (58). Although, evidence clearly indicate that there are regulatory proteins that bind to and control glyA expression, more study is required to understand all components of the regulation of SHMT levels.

5. Scheme for the Selection of Mutants with Increased Levels of Serine Hydroxymethyltransferase: Use of L-Serine Deaminase

Study of the regulation of SHMT levels, as well as the intricate webs of the C_1 metabolism in which this enzyme finds itself, is of great interest. A commonly used means to study the regulation of a system is through the interruption of that regulation, accomplished by the isolation of mutants. A procedure was devised that selects for mutants with increased SHMT activity. These mutants will not only unveil aspects of the regulation in C_1 metabolism, but in addition, may be of use in the development of an L-serine producing strain. To understand the scheme L-serine deaminase will be introduced, for it is used as a tool in the selection procedure.

5.1. L-Serine Deaminase Reaction

The L-serine deaminase (L-SD) enzyme is encoded by the sdaA gene in E. coli K12 (61). The enzyme catalyses the deamination of L-serine to form pyruvate and ammonium. The pyruvate can then be routed to the tricarboxylic acid (TCA) cycle to be used for energy.

Even though the L-SD reaction forms pyruvate, E. coli K12 cannot grow with L-serine as its sole carbon source. However, it can grow if the L-serine medium is supplemented with glycine and leucine, which induce L-SD activity (37, 39). It indicates that through the increase in L-SD activity by

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glycine/leucine induction, the cell forms enough pyruvate for metabolic needs.

5.2. Use of Increased Levels of L-Serine Deaminase for the Selection of Mutants with Increased Serine Hydroxymethyltransferase Activity

Mutations in either of two genes, ssd (35, 36) and lrp (20), allow E. coli to use L-serine as a carbon source. These mutants have elevated levels of L-SD activity. Their ability to grow on L-serine results from their increased conversion of L-serine to pyruvate, which can then be catabolized for their carbon and energy requirements. Although SHMT can convert glycine to L-serine, neither the ssd nor lrp mutations allows use of glycine as sole carbon and energy source.

For this work, the KEC9 strain was used. This strain carries the ssd mutation (located near 87 minutes on the E. coli linkage map) (2). It has greatly increased levels of L-SD activity, allowing it to grow with L-serine as the sole carbon and energy source, without the addition of glycine and leucine (35, 36).

Since the ssd mutation allows growth on L-serine as the sole carbon source, then it should theoretically use glycine, assimilated through the SHMT enzyme to L-serine, as a sole carbon source for growth. However, the KEC9 strain could not grow on glycine at all. If the ssd mutation is not by itself enough to allow growth on glycine, then it may be possible to

select for a mutant of the KEC9 strain that could grow on glycine. This was the key to the approach used in this project to isolate a strain with presumably increased levels of SHMT activity. Such a mutant probably would have an improved rate of conversion of glycine to L-serine for growth on glycine to be possible, a useful property for an L-serine producing strain. Mutants (designated GOG) of KEC9 that are able to grow on glycine as the sole carbon and energy source were isolated and described in this thesis.

MATERIALS AND METHODS

1. Strains Used

All strains used in this project were derivatives of
E. coli K12. The strains and plasmids used are listed in
Table 1, along with relevant characteristics.

2. Media Used

Minimal medium: This medium consisted of 0.58% K_2HPO_4,
1.26% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.2% $MgSO_4.7H_2O$ and 0.001% $CaCl_2$
dissolved in distilled water, with pH7. Carbon sources used,
glucose, L-serine and glycine, were added at 0.2%. Since most
of the strains used have an _ilvA_ mutation, minimal medium also
included isoleucine (50 μg/ml) and valine (50 μg/ml), unless
specified otherwise.

Luria broth (LB): This rich medium consisted of 0.5%
yeast extract, 0.5% NaCl, and 1% bactotryptone dissolved in
distilled water.

Agar Plates: To the medium was added 0.8% gelrite (for
minimal medium plates) or 1.8% agar (for LB plates), which was
dissolved and autoclaved before pouring plates.

Concentrations of other additions, as stated in the
text: ampicillin (50μg/ml), chloramphenicol (40μg/ml),
kanamycin (80μg/ml). rifampicin (100μg/ml), tetracycline

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Table 1.   List of Strains and Plasmids

Strain or Plasmid	Genotype and Relevant Characteristics	Source
E. coli K-12		
CU1008	E. coli K-12 ilvA	L. S. Williams
MEW1	reference strain CU1008 . ΔlacZ	Newman et al., 1985
KEC9	CU1008 ssd	Newman et al., 1982
GOG1	KEC9 ssd gog-1 (gog designating a mutation conveying the ability to Grow On Glycine)	This work
GOG2	KEC9 ssd gog-2	This work
GOG3	KEC9 ssd gog-3	This work
GOG4	KEC9 ssd gog-4	This work
GOG1T	GOG1 zzp::Tn10 55 min	This work
GOG2T	GOG2 zzp::Tn10 55 min	This work
GOG3T	GOG3 zzp::Tn10 55 min	This work
GOGC1	GOG1 derivative able to grow on glycine with C_1 metabolites	This work, M. San Martano
MH537	glyA537 nicB::Tn10	J. Basso
MEWG	MEW1 glyA537 nicB:Tn10	This work
KECG	KEC9 ssd glyA537 nicB::Tn10	This work
MEW28	MEW1 sdaA::Cm^r	Su et al., 1989
CAG5051	HfrH nadA::Tn10	Singer et al., 1989
CAG5052	KL227 btuB3139::Tn10	Singer et al., 1989

Cont.

CAG5054	KL96 trpB::Tn10	Singer et al., 1989
CAG5055	KL16 zed-3069::Tn10	Singer et al., 1989
CAG18481	zzp::Tn10 55 min	Singer et al., 1989
MH2923	F+ Mucts 62 hp1-1 araD (Mud5005)	Groisman and Casadaban, 1986

Plasmids

pGS29	pBR322 plasmid harbouring the wild-type glyA gene	Stauffer et al., 1981
pGOG1	mini-Mu plasmids (kan^r) able to complement glyA isolated from GOG1	This work
pGOG3	mini-Mu plasmid (kan^r) able to complement glyA isolated from GOG3	This work
pGOG4	mini-Mu plasmid (kan^r) able to complement glyA isolated from GOG4	This work

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(25μg/ml), glycine (300μg/ml), histidine (20μg/ml), leucine (20μg/ml), methionine (20μg/ml), L-serine (100μg/ml), tryptophan (20μg/ml), adenine (40μg/ml), guanine (40μg/ml), B12 (10ng/ml), niacinamide (10μg/ml), thiamine (1μg/ml). All our strains carrying glyA mutation (MEWG, KECG) also carry a nicB::Tn10. Therefore, in all experiments (cloning and mapping) in which they were used, exogenous niacinamide and tetracycline were added. Strains carrying pGS29 were grown in the presence of ampicillin.

3. Procedures for Selection of Mutants

3.1. Method for Selecting Mutants Able to Grow on Glycine

Strain KEC9 was inoculated from a plate and grown overnight in glucose minimal medium. Five mls of this culture were centrifuged and the resulting pellet was washed three times with buffer to eliminate traces of glucose. The pellet was then resuspended in a small volume of buffer and spread on a glycine (0.2%) minimal medium plate. The plates were incubated at 37°C. Colonies appeared after some five days of incubation. Four independent mutants, originating from different cultures, were chosen for study. The first mutants selected using this method were isolated by M. Corriveau, who did not characterize them further.

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3.2. Method for Selecting Mutants Able to Overcome Inhibition of C_1 Metabolites

A strain that can grow on glycine minimal medium (GOG1) was inoculated in glucose minimal medium. Five mls of this culture were centrifuged and the resulting pellet was washed three times with buffer to eliminate traces of glucose. The pellet was then resuspended in a small volume of buffer and spread onto a glycine (0.2%) minimal medium plate with the following C_1 metabolites added: adenine (40 μg/ml), guanine (40 μg/ml), methionine (20 μg/ml), and histidine (20 μg/ml). The plates were incubated at 37°C until colonies appeared. This selection was done in collaboration with Marc San Martano.

4. Enzyme Assays

4.1. Serine Hydroxymethyltransferase Assay

The serine hydroxymethyltransferase (SHMT) activity was assayed by a coupled reaction of the SHMT enzyme with methylenetetrahydrofolate dehydrogenase (methyleneTHF dehydrogenase). As suggested by R. E. MacKenzie, this assay was based on the SHMT coupled reaction assay devised by Schirch et al (48), with modifications based on an assay of methyleneTHF dehydrogenase devised by R. E. MacKenzie et al (22).

The basis of this assay is as follows. SHMT cleaves L-serine to two products, glycine and methylenetetrahydro-

folate (methyleneTHF). The coupled enzyme, methyleneTHF dehydrogenase converts methyleneTHF to methenyltetrahydrofolate (methenylTHF), with the reduction of NADP to NADPH. The product methenylTHF equilibrates with formyltetrahydrofolate (formylTHF). The assay is terminated with 1 ml of TCA (7 %). Acidification not only stops enzymatic reactions, but also shunts formylTHF back to methenylTHF and destroys NADPH, which would affect absorption readings since it absorbs at 340 nm. The net result allows us to quantitate the amount of methenylTHF formed in the coupled reaction by its absorption at 350 nm. Specific activity of SHMT is expressed as nanomoles of methenylTHF formed/ mg protein/ hour, using the molar absorption coefficient (ϵ) for methenylTHF at 350 nm of 24900 M^{-1} cm^{-1} (22).

Assay tubes contained the following components in 1 ml of 50 mM KPO_4 buffer pH7.3: L-serine (10 μg/ml), tetrahydrofolate (0.4mM, CalBiochem), pyridoxal-5-phosphate (0.05mM), NADP (0.2mM), mercaptoethanol (0.2M), methylenetetrahydrofolate dehydrogenase (0.01 units, Sigma, yeast typeIII). The tubes were kept on ice until commencement of the assay. To each, 10 μl of cell extract (preparation shown below) was added. The control for the assay had the same ingredients as above, except for the exclusion of L-serine. Tubes were then incubated at 30°C for 10 minutes, after which the assay was terminated by the addition of 1 ml of TCA (7 %). After 10 minutes, during which time the tubes

were centrifuged at 6000 rpm for 3 minutes, the absorbance of the supernatant was measured at 350 nm on a spectrometer.

Assays were done on crude extracts. Cells were grown overnight in glucose minimal medium in a shaker at 37°C and subcultured in fresh medium. Cultures in mid-exponential phase were chilled on ice and the cells pelleted by centrifugation at 6000 rpm. The pellet was resuspended in cold 50 mM KPO_4 buffer at 5 g/ml wet weight pellet. Cell extracts were made by sonication (3 X 30 seconds) on ice.

Protein concentrations of the cell extracts was measured by the method of Lowry et al. (21)

4.2. L-Serine Deaminase Assay

The L-serine deaminase assay measured the amount of pyruvate produced from the deamination of L-serine based on the method by Pardee and Prestidge (39) modified by Isenberg and Newman (17). The L-serine deaminase was assayed on whole cells treated with toluene. Activity is expressed as nanomoles keto acid (pyruvate) produced by 0.1 ml of 100 Klett units (filter no. 54) suspension of cells in 35 minutes.

5. Genetic Transfer Experiments

5.1. Conjugation

Conjugations were done according to Miller (28). The recipient strain was made resistant to rifampicin for counterselection by the method in Miller (28). The Hfr

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strains used were constructed by Singer et al (50). These strains are convenient for conjugation because each has a Tn10 insertion located roughly 20 minutes following its origin of transfer. This insert makes it easy to select for exconjugants that have received at least 20 minutes of donor chromosome, at the same time supplying a marker relative to which mutations can be mapped.

Cultures were gown overnight in LB medium. Conjugation started with mixing 50 μl of a Hfr strain with 250 μl of recipient in 2 mls LB medium and incubating at 37°C for a half hour. Following conjugation, the conjugation mixture were shaken for an hour at 37°C. Then, 100 μl of the mixed cells were spread on LB plates with tetracycline and rifampicin added, and incubated at 37°C overnight. A number of exconjugants were then streaked on glucose minimal medium plates before screening. This step was required because cells often have problems growing after being transferred directly from LB to plates containing L-serine or glycine as carbon sources. The exconjugants were then screened on appropriate plates. Because the Hfr strains carry various markers, the following additions were required in the minimal medium plates: niacinamide, thiamine, vitamin B12, methionine, tryptophan, and leucine.

5.2. Transduction

Transductions using P1 phage were done according to Miller (28).

5.3. Transformation

The transformation procedure was done according to Maniatis et al (23).

6. Growth Experiments

6.1. Growth Rate Determination

Cells were grown in glucose minimal medium to exponential phase. When a growth rate was to be determined in glycine minimal medium, the cells were pelleted and washed three times with sterile minimal medium to remove traces of glucose. Cells were inoculated in side-arm flasks containing the appropriate medium to a turbidity on a Klett colorimeter (red filter) of 20. Flasks were placed in a shaker at 37°C. For glucose minimal medium cultures, turbidity readings using the Klett were taken at 30 minute intervals; for glycine minimal medium, the interval was larger.

6.2. Effect of Various Supplementations on Growth on Glycine

Cells were grown in glucose minimal medium. The cells were washed three times with sterile minimal medium, and resuspended in a small amount of minimal medium. To each

glycine minimal medium flask in any series of test flasks (at concentrations given in Section 2), a small, equal volume of the cell suspension was added. The cultures were placed in a shaker at 37°C, and the turbidity due to growth was observed at intervals thereafter.

7. DNA Techniques

7.1. Mini-Mu Cloning Procedure

The procedure used was the *in vivo* cloning system developed by Groisman *et al* (15, 16). The Muc ts and mini-Mu (Mud5005) are carried by strain MH2923, of which a Mu lysate is prepared. A strain to be used as the donor is first made lysogenic for Muc ts followed by transduction in mini-Mu (Mud5005) by selection for kanamycin-resistance. The cloning step involves the temperature induction of this strain into Mu lytic growth. The lytic growth for Mu involves Mu replication through random transpositions, about a hundred rounds, around the E. coli chromosome. In this case, the mini-Mu element is also inserted randomly throughout the chromosome. Mu packaging starts at a Mu insert and proceeds until the phage head is filled, at a length of around 39 kb, regardless of what DNA is follows the Mu insert. The Muc ts has been cut down and the mini-Mu is only 8 kb, leaving room in the phage head for larger stretch of bacterial DNA.

For the cloning procedure to work, packaging must consist of a fragment of E. coli chromosome, which can be up

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to 20 kb, possibly carrying the gene of interest. Surrounding this fragment should be a mini-Mu element and a second Mu element, either mini-Mu or Mucts. The phage is then used to transduce another strain selecting for kanamycin. Within the cell, the Mu homologous sequences flanking the bacterial fragment undergo recombination, resulting in the formation of a mini-Mu plasmid that carries the bacterial sequence, as can be selected by kanamycin resistance. Concurrent with the selection for the plasmid with kanamycin, selection of the cloned gene of interest is carried out, such as through complementation of a mutation of the gene of interest.

7.2. Plasmid Isolation

Isolation of plasmids was done following the miniprep methods of Maniatis et al (23).

7.3. Plasmid analysis

DNA restriction digests and agarose gel electrophoresis was done following methods in Maniatis et al (23).

8. Pigment Analysis

8.1. Pigment Isolation

Cultures that developed a brown colour during growth were centrifuged at 6000 rpm to pellet out cells. The resulting cell pellet was slightly brown, but most of the brown pigment stayed in the supernatant. Ammonium sulfate

(final concentration of 70 %) was dissolved in one ml of the brown supernatant. The resulting suspension was centrifuged at 10,000 rpm, and most of the pigmented material was found in the pellet. The pellet was resuspended in 0.2 ml of 50 mM potassium phosphate buffer.

Several observations suggest that a component of the pigment substance is a protein. The material was precipitated by mixing an equal volume of chloroform with the buffer suspension of the pigment substance. After centrifugation at 10,000 rpm, a thick, whitish material was observed at the solvent interface, and the aqueous phase, which still contained the brown pigment was carefully removed. Chloroform extraction was repeated until no material was visible at the interface.

To obtain an absorption spectrum of the pigment, the solution containing the separated pigment molecule was diluted 1/10 in water and read on a spectrometer. The absorption was measured at various wavelengths.

8.2. Protein Analysis

The ammonium sulfate precipitated pigmented material was loaded, and electrophoresed on a 7.5 % polyacrylamide gel. SDS-page gels were prepared according to the instructions provided with the Bio-Rad Mini PROTEAN II dual slab cell. Bands were visualized by staining with Coomassie blue (23).

9. L-Serine Production Analysis in Resting Cells

Cells from glucose minimal medium cultures, at stationary phase, were pelleted, washed, and resuspended in an equal volume of minimal medium with 0.2% glycine (and formate (50 μg/ml) sometimes). Since the cells are already at a high density, there is little, if any, growth in such conditions (i.e. resting cells). Suspensions were shaken at 37°C and samples (1 ml) taken at various times of incubation up to 36 hours. The samples were spun at 10,000 rpm for 10 minutes (this step was repeated 3 times). T! supernatant was then tested for L-serine via paper chromatography.

Chromatography was done using equilibrated phenol (25), which adequately separated the closely migrating L-serine from glycine. A quantity (15 μl) of the sample was spotted onto the paper (60cm by 30cm), along with various standard⁻, and run for about 36 hours. The amino acids were visualized using ninhydrin.

L-Serine was further quantified by a microbiological assay using a strain that is an L-serine and glycine auxotroph (glyA serA tet^r Amp^r). This indicator strain requires both L-serine and glycine for growth. The test culture consisted of glucose minimal medium, with glycine (300 μg/ml), niacinamide (10 μg/ml), tetracycline, and ampicillin added, to which the indicator strain was inoculated. Growth of this test culture requires the presence of L-serine. Various amounts of L-serine (0 to 100 μg/ml) was added to test

cultures, and the turbidity (using a Klett) of the cultures
(at stationary phase) after incubation at 37°C (for 23 hours)
was determined. A standard curve of the turbidity of the
cultures with a given amount of L-serine was constructed. To
quantitate L-serine in the supernatant from the production
process, the supernatant (1 ml) was added to the test cultures
(which has no L-serine added), and the turbidity of the
resultant culture was determined. This value, when read off
the standard curve, gives the corresponding amount of L-serine
present in 1 ml of supernatant from the production process.

10. Relevant Terms

Growth on glycine, or growth in glycine minimal medium,
refers to growth in minimal medium with glycine as the carbon
source. GOG phenotype refers to the ability to grow on
glycine.

1. Selection of Mutants Able to Use Glycine as Sole Carbon and Energy Source

Escherichia coli K12 cannot use either L-serine or glycine as carbon source. Mutations in either of two genes, ssd and lrp, confer the ability to use L-serine as carbon source. However, neither mutation allows the use of glycine as carbon source. If the use of glycine as a carbon source uses the same enzymes as needed for growth on L-serine as part of the pathway, then it may be possible to isolate mutants able to grow on glycine from either the KEC9 (ssd) or MEW26 (lrp) strain.

Spontaneous mutants of KEC9 able to grow on glycine were selected. This was done by plating overnight glucose grown cells on glycine (0.2%) minimal medium plates and incubating at 37°C until colonies appeared.

Four independent mutants were selected from glycine minimal medium plates for further studies. These strains were designated GOG for their characteristic of Growth On Glycine. The designations GOG1 (gog-1), GOG2 (gog-2), GOG3 (gog-3), and GOG4 (gog-4) are used to identify the four GOG mutants studied.

2. Test for Growth on Various Media

The GOG strains were tested for the ability to grow on various media and the results are summarized in Table 2. The GOG mutants were able to grow on minimal medium with glycine as sole carbon source, albeit slowly. In 3 days at 37°C, streaks of the mutants on glycine minimal medium were as well-grown as those in one day on glucose minimal medium. Although this may be relatively slow growth, it represents a significant change in phenotype since neither the parent strain, KEC9, nor the laboratory reference strain, MEW1, could grow on this medium at all. Although growth of the GOG strains on glycine is slow, various supplements can enhanced growth significantly; this will be described below.

If the GOG mutants acquired a new mutation permitting growth on glycine, they should still carry the original ssd mutation. In that case, they should still grow on L-serine as sole carbon source, unless the gog mutation interfered with that. In fact, all 4 GOG mutants grew on L-serine minimal medium, as well as their parent, KEC9.

Several bacteria able to use L-serine and glycine as carbon sources occur in nature and the possibility that a contamination was inadvertently selected should be ruled out. Since the parent strain, KEC9, carries a deletion in ilvA, and requires isoleucine in the medium, if the GOG mutants are in fact derived from KEC9, they also should require isoleucine. This was tested by plating on glucose minimal medium with and

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Table 2. Growth of GOG mutants on various media

Strain	Relevant genotype	Glucose minimal medium (- IV)[a]	Glucose minimal medium	Serine minimal medium	Glycine minimal medium
MEW1	Reference	-	+	-	-
KEC9	ssd	-	+	+	-
GOG mutants	ssd gog	-	+	+	+

[a] Minimal medium without addition of isoleucine and valine.

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without isoleucine and valine. No growth could be seen on plates in which isoleucine was omitted, indicating that the GOG strains carried the _ilvA_ marker as did their parent strain. Growth on glucose minimal medium (with isoleucine and valine) appeared normal, showing that the mutations selected do not result in a nutrient requirement.

Finally, it is known that KEC9 can use L-serine not only as its sole carbon and energy source, but also as its sole nitrogen source. It was also shown that in _E_. _coli_, glycine can serve as nitrogen source by its conversion to L-serine (34). The nitrogen is supplied, supposedly from deaminating L-serine, which yields ammonia. If the GOG mutants growing on glycine convert their glycine to L-serine, then they also may be able to use glycine as a nitrogen source. In addition, since the GCV also forms NH_4 from glycine, some of the nitrogen will come from this reaction (4, 26, 47). This was tested in glycine minimal medium in which the nitrogen source, ammonium sulfate, was left out. The GOG strains were inoculated into this medium and grew about as well as when ammonium sulfate is present. This means that the GOG strains can use glycine not only as their sole carbon and energy source, but also as their sole nitrogen source.

3. Necessity for L-Serine Deaminase for Growth on Glycine

The selection for the GOG mutations was done from the pleiotropic mutant KEC9 because it carries the ssd mutation. The reason is that the KEC9 strain has high L-SD activity levels that furnish it with the ability to grow on L-serine. Theoretically, the KEC9 strain should be able to grow on glycine by using the serine hydroxymethyltransferase (SHMT) enzyme to convert glycine to the usable L-serine. However, KEC9 does not grow on glycine. The acquisition of a gog mutation provides the KEC9 strain with this ability.

To determine whether L-SD activity is required for the growth of the GOG mutants on glycine, an experiment was conducted to remove the L-SD activity from the GOG mutants. This was accomplished by the introduction of an insertion mutation in the structural gene for L-SD, sdaA::Cm^r, constructed by Su (61), which confers chloramphenicol resistance. Mutation of the sdaA gene eliminates L-SD activity, but does not result in a nutritional requirement. P1 phage was grown on the strain carrying the sdaA::Cm^r and used to transduce sdaA::Cm^r into the GOG mutants by selection for chloramphenicol resistance.

The transductants of the GOG mutants that had become chloramphenicol resistant, indicating they carried sdaA::Cm^r, were tested for the GOG phenotype. The results are summarized in Table 3. The transductants were able to grow on glucose minimal medium, but unable to grow when isoleucine and valine

Table 3. Removal of L-serine deaminase activity from GOG strains

Recipient	Donor	Selected marker	Growth on glucose	Growth on serine	Growth on glycine
GOG1	MEW22	sdaA::Cm^r	+	−	−
GOG2	MEW22	sdaA::Cm^r	+	−	−
GOG3	MEW22	sdaA::Cm^r	+	−	−
GOG4	MEW22	sdaA::Cm^r	+	−	−

was omitted, which showed that they carry the _ilvA_ marker present in the GOG strains.

The transductants were then tested for their ability to grow on L-serine minimal medium. It was found that they had lost the ability to grow on L-serine as the sole energy source. This supported the hypothesis that the GOG strains have lost L-SD activity by the introduction of _sdaA_::Cm^r.

Finally, it was tested whether the introduction of the _sdaA_ mutation affected the GOG strains' ability to grow on glycine. The results, as shown in Table 3, showed that the transductants were unable to grow on glycine minimal medium plates. This experiment confirmed that L-SD activity in the GOG mutants is required for growing on glycine. This indicates that the high L-SD activity established by the _ssd_ mutation is necessary for the GOG mutants' ability to use glycine as a sole carbon source.

4.   Mapping the Mutations Responsible for Growth on Glycine

4.1.   An Experiment to Determine Whether the _gog_ Mutations Map
       Near _glyA_

This experiment was done to determine the location of the mutation(s) that give the GOG strains the ability to grow on glycine. It seemed probable that the _gog_ mutations affect the activity of SHMT, which catalyses the conversion of glycine, with the addition of a C_1 unit, to L-serine. This is supported by the previous experiment that indicated that the

GOG phenotype requires an increased level of L-SD activity furnished by the ssd mutation. An increased conversion of glycine to L-serine may furnish enough substrate to the high L-SD activity and thus permit growth. Since the SHMT enzyme is encoded by the glyA gene, an experiment was conducted to inquire whether the gog mutations lie near the glyA locus.

To answer the question, transductions were carried out using the GOG mutants as recipients and P1 phage grown on strain CAG18481, which carries a Tn10 insert at 55 minutes, near the glyA locus. Tetracycline-resistant transductants were selected on glucose minimal medium plates containing tetracycline and should therefore carry the Tn10 insert with the wild-type glyA gene cotransduced with it in the majority of cases. The tetracycline-resistant transductants (100 of each, 70 for GOG4) were then screened on glycine minimal medium plates (plus tetracycline) for the ability to grow on glycine. If the gog mutation mapped close to the Tn10 marker, cotransduction of the adjacent wild-type gog gene with the Tn10 would result in the loss of its ability to grow on glycine (i.e. GOG⁻).

The results of this transduction are presented in Table 4. The linkage represents the percentage of the transductants that lost the ability to grow on glycine when they received the piece of chromosome linked to the Tn10 of CAG18481. All the transductants were still able to grow on glucose minimal medium and L-serine minimal medium plates. This indicates

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Table 4. Transduction frequency of gog to Tn10 at 55 min

Recipient	Donor	Selected marker	Unselected marker[a]	Frequency of unselected marker (%)[b]
GOG1	CAG18481	Tn10::55min	GOG⁻	35
GOG2	CAG18481	Tn10::55min	GOG⁻	31
GOG3	CAG18481	Tn10::55min	GOG⁻	27
GOG4	CAG18481	Tn10::55min	GOG⁻	0

[a] GOG⁻ refers to the inability to grow on glycine.
[b] Frequency of tetracycline resistant transductants losing ability to grow on glycine m. m. 100 tetracycline resistant transductants (70 for GOG4) were screened for each cross.

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that the ssd mutation, which maps at 87 minutes (2), was not affected by the transduction.

Linkage of three of the gog mutations, gog-1, gog-2, and gog-3 to the Tn10 marker was observed. The linkage values were similar for each of these mutations. This may indicate that these mutations all represent one class of gog mutations. The linkage to the Tn10 suggests that this class of gog mutations maps near the glyA locus. However, the experiment does not determine whether these mutations map within the glyA gene, or the regulatory region of the glyA gene. This will be tested in the next experiment.

The gog-4 mutation showed no linkage to the Tn10 marker. Although only 70 transductants were screened, these should be sufficient to determine whether a mutation allowing growth on glycine maps in glyA. Thus, this experiment shows that the gog-4 mutation does not map within the glyA gene. It is possible that gog-4 represents a mutation within a regulatory gene that controls the expression of the glyA gene.

4.2. Fine Mapping gog-1,-2,-3 to glyA

The previous experiment showed that mutations gog-1, gog-2, and gog-3 map near the glyA locus. However, it was not shown that they actually map within the glyA gene itself. This was tested in the following experiment.

A strain was constructed that contained both the ssd mutation and a glyA point mutation. The strain was designated

KECG.

The basis of the experiment lies with the KECG strain's high level of L-SD activity coupled with its lack of SHMT enzyme, resulting in the inability to grow on glucose minimal medium lacking glycine. Selection for glycine-independent transductants of KECG using phage grown on the GOG mutants should accomplish the transfer of the functional glyA gene from the GOG mutants to replace the mutated glyA gene of the KECG strain. If in fact the gog mutation is within the glyA gene, then the glycine-independent KECG transductants would gain more than just a functional glyA gene, but rather, an altered glyA gene caused by the gog mutation. Since the KECG strain has high L-SD activity, if the glycine-independent KECG transductants also received the gog mutation, they also should gain the ability to grow on glycine as sole carbon source. Therefore, scoring the glycine-independent KECG transductants for the ability to grow on glycine (i.e. GOG$^+$) will give the linkage of the gog mutations to the glyA gene.

P1 phage was grown on the GOG mutants and used to transduce the recipient, KECG. Following these transductions, the cells were washed to remove traces of glycine and plated on glucose minimal medium plates without glycine to select for glycine-independence. Niacinamide, L-serine, and tetracycline were also included in the medium. Transductants which grew after a few days were scored on appropriate plates.

The result of this experiment is shown in Table 5. It

Table 5. Mapping the gog mutations to glyA

Recipient[a]	Donor	Selected marker	Unselected marker[b]	Frequency of unselected marker (%)[c]
KECG	GOG1	glyA$^+$	GOG$^+$	100
KECG	GOG2	glyA$^+$	GOG$^+$	100
KECG	GOG3	glyA$^+$	GOG$^+$	100
KECG	GOG4	glyA$^+$	GOG$^+$	0

[a] Relevant genotype of KECG: ssd glyA
[b] GOG$^+$ refers to the ability to grow on glycine.
[c] Frequency of glycine-independent transductants also
   gaining ability to grow on glycine (100 scored for each).

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is apparent that all the glycine-independent KECG transductants that received a functional glyA gene from GOG1, GOG2, and GOG3 were able to grow on glycine minimal medium plates. One hundred transductants from each cross were screened. This would indicate that this class of gog mutations are very closely linked to the glyA gene. This experiment does not tell us whether the mutations map within the regulatory portion of the glyA gene, or rather, within the coding region of the glyA itself. A small possibility exists that the mutations lie within a highly linked, adjacent gene. The fact that these gog mutations affect SHMT activity, as will be shown below, may suggest that the mutations lie within the glyA gene.

When GOG4 was the donor of the functional glyA gene, none of the glycine-independent KECG transductants were able to grow on glycine. This supports the conclusion from the earlier experiment that the gog-4 mutation does not lie in the glyA region.

4.3. Further Verification That gog-1,-2,-3 Map to glyA
4.3.1. Reconstruction of the GOG Phenotype From KEC9

In the previous experiment, it was shown that replacement of the glyA allele in the GOG strains resulted in loss of the GOG phenotype. This does not mean that the ability to grow with glycine results entirely from a change at glyA since secondary mutations might arise during the GOG

selection. The following experiment was conducted to verify that the GOG phenotype of GOG1, GOG2, and GOG3, is the result solely of mutation(s) mapping at or very near the glyA locus.

To accomplish this, the gog mutations were transferred into the parent strain, KEC9, to see whether the GOG phenotype could be reconstituted. GOG strains with a Tn10 (at 55 minutes) closely linked to the gog mutation were used. These strains still possessed the GOG phenotype, but were tetracycline resistant due to the Tn10. These strains were designated GOG1T, GOG2T and GOG3T, for convenience.

Transductions were done in which phage grown on the GOGT strains were used to infect the KEC9 strain. The cells were plated on glucose minimal medium containing tetracycline to select for the transfer of the Tn10 insert from the GOGT strains. The tetracycline resistant transductants were then scored for the GOG phenotype. The results are presented in Table 6.

These results show that a proportion of KEC9 that received the Tn10 insert also received the ability to grow on glycine (i.e. GOG$^+$). This is strong confirmation that only mutations mapping in or near glyA (along with the original ssd mutation) are required for the expression of the GOG phenotype.

From the results, it is observed that the linkage of the Tn10 to the gog mutation mapping within glyA differ somewhat from the linkage seen in Table 4. This probably reflects that

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Table 6.   Transfer of the gog mutations mapping to glyA
           into KEC9

Recipient	Donor	Selected marker	Unselected marker[a]	Frequency of unselected marker (%)[b]
KEC9	GOG1T	Tn10::55min	GOG$^+$	34
KEC9	GOG2T	Tn10::55min	GOG$^+$	52
KEC9	GOG3T	Tn10::55min	GOG$^+$	48

[a] GOG$^+$ refers to the ability to grow on glycine.
[b] Scored 50 transductants.

not as many transductants (i.e. 50) were screened in the experiment and this would give a poorer linkage value.

4.3.2. Reconstruction of the GOG Phenotype From MEW1

A prior experiment showed that the GOG strains' ability to grow on glycine requires L-SD activity. However, the experiment did not determine that there has to be the elevated level of L-SD activity resulting from the ssd mutation present in the GOG strains.

It is known that various growth conditions lead to an increase in L-SD activity. One of these is by growth with a combination of glycine (at 300 μg/ml) and leucine (at 150 μg/ml) in glucose minimal medium, which increases transcription from sdaA, resulting in a higher level of L-SD activity (37, 39). With this supplementation, MEW1, our reference strain, can grow on L-serine minimal medium (37). The level of L-SD activity when induced with glycine and leucine is not as high as that found in the KEC9 strain. However, the following experiment shows that the level is sufficient to allow growth on glycine if a gog mutation is introduced into the MEW1 strain.

This experiment was done in a similar fashion as in the last experiment. Transductions were done in which phage grown on the GOGT strains described above were used to infect MEW1. Transductants that received the Tn10 insert were selected by tetracycline resistance. The tetracycline resistant

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transductants were then scored on glycine minimal medium plates with leucine added at 150 μg/ml. Since these plates had both glycine and leucine present, this should induce L-SD synthesis in MEW1.

The results are shown in Table 7. It was observed that a proportion (about the same as the last experiment) of the MEW1 transductants that received a Tn10 from the GOGT strains also were able to grow on glycine when leucine was also provided (i.e. GOG$^+$). When leucine was left out, none of the transductants could grow on glycine. These results provide evidence that a high level of L-SD activity, which can be induced by supplemental glycine and leucine, is necessary for the growth on glycine, but show that the activity need not be as high as that found in the KEC9 strain.

4.4. Mapping the gog-4 Mutation

The results stated above showed that although some of the gog mutations map to glyA, the gog-4 mutation did not. To find out where the gog-4 mutation is located on the E. coli chromosome, it was mapped by conjugation.

To counterselect the Hfr strains, the GOG4 strain was made rifampicin resistant. The Hfr strains used in the conjugation experiment were part of an Hfr mapping kit devised by Singer et al (50). Each Hfr strain of the kit has its origin of transfer at a different map location, and a Tn10 insert transferred about 20 minutes following the origin of

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Table 7. Transfer of the gog mutations mapping to glyA
into MEW1

Recipient	Donor	Selected marker	Unselected marker[a]	Frequency of unselected marker (%)[b]
MEW1	GOG1T	Tn10::55min	GOG$^+$	44
MEW1	GOG2T	Tn10::55min	GOG$^+$	60
MEW1	GOG3T	Tn10::55min	GOG$^+$	48

[a] GOG$^+$ refers to the ability to grow on glycine minimal medium with supplemental leucine.
[b] Scored 50 transductants.

transfer. This allows for easy selection of the exconjugants receiving at least 20 minutes of the Hfr DNA. In addition, the insert allows screening the gene being mapped against the Hfr Tn10 in a rough analogy to cotransduction mapping.

The result of the mapping experiment is given in Table 8. The origin of transfer and the corresponding Tn10 of each Hfr used are diagrammed in Table 8 as well. The results were established through scoring the percentage of the exconjugants which received the Tn10 insert and, concurrently, lost the ability to grow on glycine minimal medium (i.e. GOG⁻). To interpret the results, the direction of transfer must be considered. In the CAG5051 cross, there is 91 percent linkage between gog-4 and the Tn10 at 17 minutes. Although conjugations are not exact, the high linkage observed between the Tn10 of CAG5051 and gog-4 indicates that the gog-4 mutation is not far from the Tn10 position. The percentage decreases the further a Tn10 is from 17 minutes: CAG5054 Tn10 at 27 minutes has 76 percent linkage, and CAG5055 Tn10 at 42 minutes has 16 percent linkage. The CAG5052 cross is probably mapping the ssd mutation, also required to grow on glycine, which maps at about 87 minutes. CAG5051 is certainly not mapping the ssd mutation since its direction of transfer is away from that gene.

Therefore, this conjugation shows that GOG4 has a mutation (gog-4) at around 17 minutes (+/- 5 minutes) that is required to gro ' on glycine. However, the experiment does

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Table 8. Hfr mapping of *gog-4* of the GOG4 mutant

Hfr Strain	Structure of Hfr [a]	Selected marker	Unselected [b] marker	Frequency of [c] unselected marker (%)
CAG5051	96 ← ↗ 17'	Tn10 17min	GOG⁻	91
CAG5052	89' ↗ ↘ 2	Tn10 89min	GOG⁻	12
CAG5054	27 ↗ ↘ 45'	Tn10 27min	GOG⁻	76
CAG5055	61.5' ↘ ↗ 42'	Tn10 42min	GOG⁻	16

a
Arrows indicate the start of transfer Opposite end indicates Tn10

b
GOG⁻ refers to the inability to grow on glycine

c
Percentage of GOG4 ex-conjugants losing the ability to grow on glycine
50 exconjugants scored from each cross

not rule out the possibility that the GOG4 mutant carries a secondary gog mutation selected for growth on glycine.

5.   Serine Hydroxymethyltransferase Levels in GOG Strains

Results up to this point strongly suggest that SHMT activity has been altered by the gog mutations, at least in the case of the mutations mapping to the glyA gene (mutants GOG1, GOG2, and GOG3). To test this conclusion, SHMT activity of the GOG mutants grown in glucose minimal medium was assayed.

The results of the SHMT assays on the GOG mutants are shown in Table 9. Controls were included to compare relative values of SHMT activities. There was no detectable SHMT activity observed in the MEWG strain, which has a point mutation in the glyA gene. Low SHMT activity was observed in both the reference strain, MEW1, and the parent strain of the GOG strains, KEC9 strain. This level represents the norm for SHMT activity for this experiment since all the strains used are E. coli with the same genetic background as these.

MEW1 strain carrying the pGS29 plasmid, which harbours the wild-type E. coli glyA gene on a multicopy pBR322 plasmid showed an SHMT level about 10 times higher than MEW1. This level is not as high as Stauffer et al (55) described for a strain carrying this plasmid (20-fold). An explanation may be that the SHMT assay conducted by Stauffer et al was done on an E. coli strain with a different genetic background. Another

**Table 9. Levels of serine hydroxymethyltransferase
in GOG mutants**

Strain	Relevant Genotype	SHMT Activity (units)[a]
MEW1	reference strain	39
KEC9	GOG parent	33
MEWG	glyA	ND[b]
MEW1pGS29	pGS29	406
GOG1	gog1	482
GOG2	gog2	466
GOG3	gog3	439
GOG4	gog4	152

[a] Units of SHMT activity are in nmoles methenylTHF/mg
protein/hour. Data represent an average of two assays.
[b] Not detectable.

reason could be that since a different assay was used, the differences in assay conditions affects the measurement.

Assay of the GOG strains whose gog mutation map to the glyA gene (GOG1, GOG2, GOG3), showed very high levels of SHMT activity, about 12-fold higher than the parent strain. Within the limits of the assay, the levels of SHMT activity for each of these GOG mutants are approximately the same, which may suggest that a common regulation of glyA is being interrupted. These SHMT activities appear even higher than the level observed in the strain harbouring the pGS29 multicopy plasmid carrying the glyA gene.

The SHMT assay results of the GOG4 mutant differed from the results of the other GOG mutants. The level of SHMT activity was still relatively high compared to the parent strain, but only about four times greater. Thus, the level of SHMT activity in GOG4 is lower than that observed in the mutants GOG1, GOG2, and GOG3.

It seems likely that the GOG mutants ability to grow on glycine stems from their high SHMT (and L-SD) activities. However, a KEC9 (ssd) strain carrying glyA plasmid pGS29 also has high SHMT and L-SD activities but cannot grow on glycine minimal medium, even though it does grow on L-serine minimal medium. Hence, either the SHMT activity in the GOG strains is underestimated in the assay used here, or some factor other than SHMT and L-SD was also altered in the GOG mutants and was essential for growth on glycine.

These results are indeed to be taken with certain reservations. With the intention to increase the ease of the SHMT assay, a simple assay was devised that can be run on crude cell extracts. The assay is the SHMT – methyleneTHF dehydrogenase coupled reaction, described in the methods. From interpretation of the data obtained, this assay presented certain disadvantages. For instance, the SHMT level of MEW1 carrying plasmid pGS29 is lower than that described in published work (55). It seems likely that the level of the coupled enzyme, methyleneTHF dehydrogenase, was below saturation (methyleneTHF dehydrogenase was ordered from Sigma, and has relatively low activity). This would limit the assay, the effect being more pronounced at higher levels of SHMT activity. This would suggest that the estimates of SHMT activity of the GOG mutants presented here are underestimated.

Although there are apparent problems with the data, for this work, the relative SHMT levels are of prime importance. From many assays, the relative values of SHMT in the GOG mutants, and the controls, were consistent to what has been presented. Marc San Martano is presently working on measuring the SHMT activity of the mutants using the more reliable, although more complicated, radioactive SHMT assay. He has shown results similar to those presented here, but confirmed that the real values for the mutants are even higher. For instance, he found 28-fold increase in SHMT level in MEW1 with pGS29 plasmid (control), and a 60-fold increase in SHMT level

in GOG1 (M. San Martano, personal communication).

6. L-Serine Deaminase Levels in GOG Strains

The GOG mutants were selected from the KEC9 strain because that strain has high L-SD activity that would probably be needed for growth on glycine. It was previously shown that the GOG mutants grow on L-serine and that growth on glycine (and L-serine) is arrested when L-SD activity is removed by introduction of the sdaA mutation. To verify directly whether the GOG mutants have the high level of L-SD activity expected from their ssd mutation, an assay of L-SD activity was performed on the GOG mutants, results given in Table 10. The level of L-SD activity in all the GOG mutants was high and not significantly different from KEC9, as would be expected if they carry the ssd mutation.

7. Growth Experiments on GOG Mutants

The growth of the GOG mutants is quite slow in liquid glycine minimal medium, showing an apparent doubling time (a.d.t = time for the turbidity to double) of between 8-10 hours. The growth may be quite slow, but is significant compared to the parent strain, KEC9, and the reference strain, MEW1. These strains could not grow at all in glycine minimal medium, even after a week's incubation. The growth rate of the GOG mutants on glucose minimal medium is slightly lower than that of MEW1 (a.d.t. 84 vs 63 min). However, this is not

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Table 10. Levels of L-serine deaminase in GOG mutants

Strain	Relevant Genotype	L-SD Activity (units)[a]
MEW1	reference strain	22
KEC9	ssd	270
GOG1	ssd gog1	220
GOG2	ssd gog2	218
GOG3	ssd gog3	270
GOG4	ssd gog4	262

[a] Units of L-SD activity are in nmoles keto acid/0.1ml cells (at 100 Klett units)/35 minutes. Data represent an average of two assays.

very different from the rate of the parent strain, KEC9 (a.d.t. 78 min), due to its _ssd_ mutation. The GCG strains can grow on L-serine minimal medium as well as KEC9. Hence, the _gog_ mutations do not significantly affect growth on glucose or L-serine minimal medium, but do allow growth in glycine minimal medium, though not at a high rate.

To see whether I could select a population of cells that grew more quickly in glycine minimal medium, I tried sequentially subculturing the GOG mutants in liquid glycine minimal medium. After 12 sequential subcultures, the cells maintained their slow growth on glycine minimal medium.

7.1. Conditions That Aid GOG Mutants Growing on Glycine

To explore whether there is a limiting factor in glycine minimal medium, such as insufficient synthesis of an amino acid or vitamin, which limits the growth of the GOG mutants, various supplementations were tested. Cells were inoculated evenly, and lightly, in the various media. Turbidity of the cultures was recorded at intervals. Figure 2 gives a summary of this experiment. Only supplements that gave results of interest are shown. The experiment was carried out on GOG1 (which may be representative of the strains carrying a _gog_ mutation in _glyA_) and GOG4.

It takes the GOG mutants about 5 days to reach maximal growth in glycine minimal medium. In these experiments, I use a reduction in the time to reach turbidity over about 200

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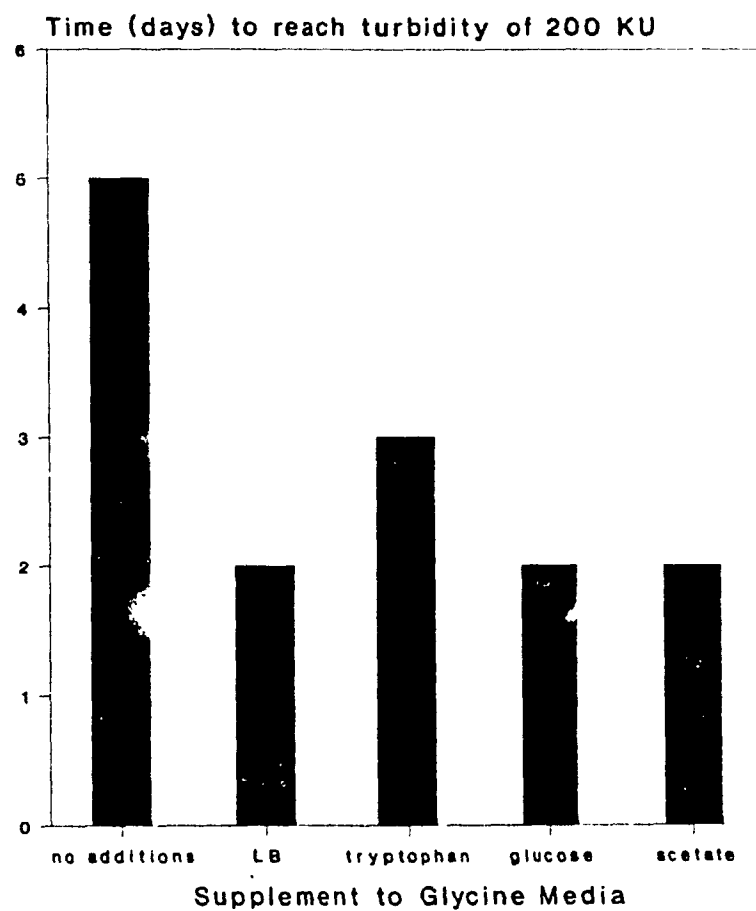
Figure 2. Effect of addition of various substances upon
growth on glycine by the GOG mutants. Concentrations
of the supplements LB (5 ul Luria broth/ml), tryptophan
(40 ug/ml), glucose (0.001%), acetate (0.01%)

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Klett units (KU) as an indicator of the extent of which an added supplement aids growth on glycine. However, this does not reveal whether a change in growth is due to a shortening of a lag phase or an increase in the growth rate.

Addition of small amounts of glucose (0.001 %), or acetate (0.01 %) to glycine minimal medium gave fully grown cultures in 2 days. This was much less time than the 5 days required to reach fully grown in glycine minimal medium alone. Control cultures with 0.001 % glucose or 0.01 % acetate with no glycine showed no detectable growth throughout the experiment, meaning there was not sufficient carbon source to support significant growth.

Addition of Luria broth (LB), 5μl LB/ml medium, to glycine minimal medium, cultures reached fully grown in 2 days. A control flask with the same concentration of LB but with no glycine did not show any growth throughout the experiment.

In cultures with tryptophan (40 μg/ml) added, cultures were fully grown in 3 days. The same concentration of tryptophan without glycine showed no detectable growth throughout the experiment. Tryptophan may be the factor in LB that aids growth.

Hence, it is apparent that although the GOG strains grow slowly in glycine minimal medium, growth can be enhanced by additions of certain substances. This may indicate that the supplements that showed a positive effect are supplying a

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factor or factors that the GOG strains require for reasonable growth on glycine.

7.2. Conditions That Inhibit GOG Mutants Growing on Glycine

7.2.1. Effect of C_1 Metabolites on GOG Phenotype

C_1 units are used in many biosynthetic pathways. These include pathways that form adenine, guanine, thymidylate, methionine, and histidine, called the C_1 metabolites. When the C_1 metabolites are added as supplement to a medium, or in various combination of C_1 metabolites, the C_1 pool does not have to be used for their synthesis. This presumably results in an increase in the C_1 pool. The cell compensates by decreasing the expression of the glyA gene, which codes for SHMT, the major source of C_1. It also may decrease the expression of the GCV pathway, the second means of C_1 production, though there is debate about this (26, 31, 57).

To see the effect of the C_1 metabolites on the GOG phenotype, the GOG mutants were tested for their ability to grow on glycine minimal medium with adenine (40 μg/ml), guanine (40 μg/ml), and methionine (20 μg/ml). The result is shown in Figure 3. In this medium, the GOG strains did not grow, even after over a week's incubation. The controls that had no C_1 metabolites grew in 5 days, as before. Thus C_1 metabolites prevents the growth of GOG strains on glycine.

61
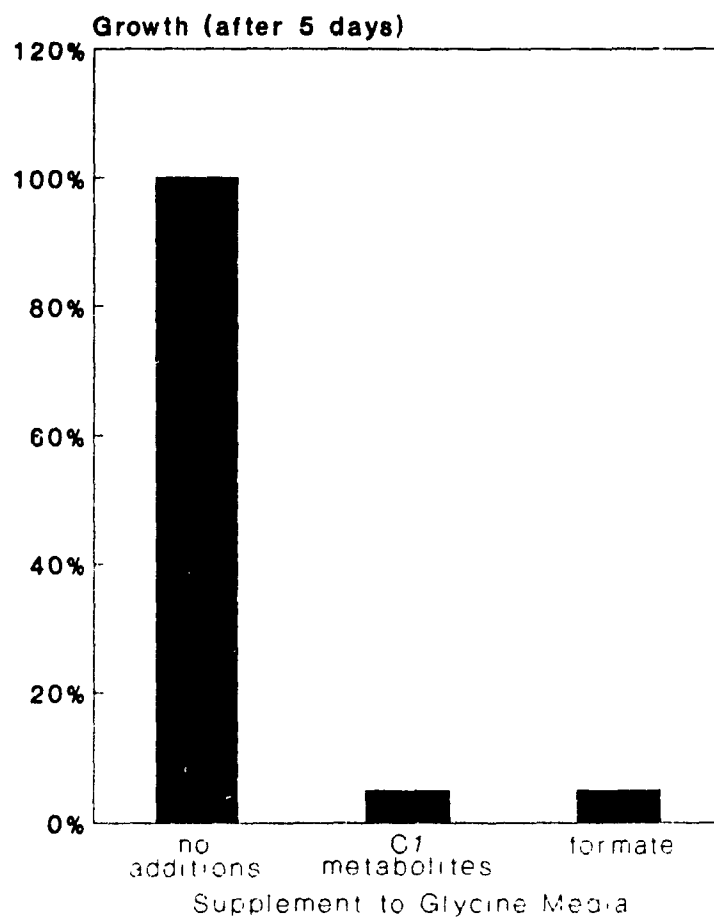
Figure 3   Effect of C1 metabolites upon growth on glycine
   by the GOG mutants   C1 metabolites include adenine(40ug/ml),
   guanine (40ug/ml), methionine (20ug/ml), formate (500ug/ml)

7.2.2. Effect of Formate on GOG Phenotype

Formate is a one-carbon molecule. In some organisms, it can be a source of C_1 (13). Effect of formate on the ability of the GOG mutants to grow on glycine was tested. The result is shown in Figure 3. Formate at 50 μg/ml or 500 μg/ml did not affect the growth of GOG mutants in glucose minimal medium. Formate at 50 μg/ml did not affect the growth of GOG mutants in glycine minimal medium. At 500 μg/ml, however, the formate prevented the growth of the GOG mutants on glycine completely. The pH of the medium was 7 at the beginning and end of incubation, showing that growth was not prevented by change in pH. This may indicate that formate increases the C_1 pool, which then prevents growth on glycine, as was shown above.

8. Cloning glyA from the GOG Mutants

8.1. Cloning of the Mutant Genes from GOG1, GOG2, and GOG3

To determine the location in the sequence of the glyA gene that is altered by the gog mutations of strains GOG1, GOG2, and GOG3, cloning of the mutant genes was undertaken. Eventually, DNA sequencing could establish the exact position and nature of the genetic change of each mutation. This could allow the delimitation of a regulatory region of the glyA gene. However, it is also possible that the mutations map within the glyA coding region, which could possibly alter SHMT structure and hence activity. Cloning the gog mutant genes

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might also aid in L-serine production.

To clone the gog mutations mapping to glyA, the mini-Mu procedure by Groisman et al (15, 16) was used. To commence, the GOG strains were made lysogenic for both the mini-Mu element and Mucts helper phage. The resulting GOG Mucts/mini-Mu strains were induced into lytic phase by heat induction and phage was collected. The phage was used to transduce MEWG that is glyA-deficient. The premise was since these gog mutations map within glyA, which results in raised SHMT activity, they should, when cloned, complement a strain deficient in glyA. However, if it turns out that the gog mutation does not map within, but very close to it, then this selection should still suffice, for selection for the glyA gene will also select for adjacent regions.

Clones that complemented the glyA mutation were obtained from the GOG1 and GOG3 strains, but unfortunately not from GOG2. The strains were resistant to kanamycin, indicating that they carried the mini-Mu vector. The plasmids were all around 20 kb. The plasmids studied were designated pGOG1 and pGOG3 selected from GOG1 and GOG3, respectively. Restriction analysis is incomplete because of the size of the plasmids, but clearly showed restriction sites found in mini-Mu. This indicates that each plasmid had a single mini-Mu vector flanking a bacterial DNA insert of about 12 kb.

As was done by Stauffer et al (55) when subcloning the wild-type E. coli glyA gene, the pGOG1 and pGOG3 plasmids were

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cut by EcoRI and SalI, which should isolate the glyA gene on a 3.0 kb fragment. This was ligated to an EcoRI/SalI digested pBR322 plasmid and a subclone carrying the glyA gene was selected in MEWG, by complementation of its glyA mutation. The clones were resistant to tetracycline, indicating that they carried the pBR322 vector. Plasmids which complemented the glyA mutation were analyzed. They were found to be around 7 kb, which should be the size if a 3.0 kb fragment was inserted in the pBR322 vector. These plasmids have not been characterized further.

The pGOG1 and pGOG3 mini-Mu plasmids were transformed into a KEC9 strain to see whether the plasmid, which may carry the gog mutations, allows the strain to grow on glycine. However, neither KEC9 pGOG1 nor KEC9 pGOG3 could grow on glycine minimal medium, though they grew on glucose minimal medium. The lack of growth on glycine could possibly be due to an additional nutrient requirement of the clones on glycine rather than the inability to utilize glycine. In any case, if the gog mutant gene has in fact been cloned, it does not suffice to allow growth on glycine.

8.2. Cloning of the Mutant Gene from GOG4

It would also be desirable to obtain a clone of the gog-4 mutant gene. It might be possible to obtain a clone of the gog-4 mutant gene by direct selection for growth on glycine, but several attempts failed. Since there was no

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other discernable selection for a clone of gog-4, I decided to clone a gene that would complement a glyA-deficient strain. As was done above, the GOG4 strain was lysogenized with mini-Mu and Mucts, and phage collected from this strain used to infect strain MEWG, which is deficient in glyA. One would expect this procedure to select for a plasmid carrying the wild-type glyA gene of GOG4, but not the gog-4 mutation that was located in the 17 minute region.

The result was very peculiar. Kanamycin resistant (conveyed by mini-Mu) clones did arise on glucose minimal medium plates; i.e. a gene complementing glyA was transferred. It was noticed that, of the 17 clones on the plate, about half (10/17) of the colonies were slightly brown. This phenomenon was not observed when cloning glyA from the other GOG mutants, nor was it reported by Stauffer et al (55) when cloning the wild-type glyA gene.

On rich medium (LB), these brown colonies released large amounts of a brown substance, which spread out from the colonies until the whole plate was dark brown. Since this is not to be expected when cloning of a wild-type glyA gene, it seems that the selection for complementation of a glyA mutation might have also resulted in cloning a mutant gene from GOG4. Either this mutant gene is linked to glyA, or is the mutant gene gog-4, that in some manner able to complement a glyA mutation. This statement will be discussed further in the discussion. Hence, this would then mean that about half

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of the clones carried the normal glyA gene, whereas the other half of the clones carried the mutant gene from GOG4. The plasmids (which were about 20 kb) of the brown clones were tentatively called pGOG4.

To test whether the brown clones were indeed our strains, and not contaminants, the clones were tested for the ilvA mutation, present in our strains. The results showed that the brown clones required isoleucine and valine to grow on glucose minimal medium, confirming that they still had the ilvA mutation. This would indicate that the brown clones were not contaminants.

A strain deficient in glyA (MEWG), while carrying the pGOG4 plasmid, grows on glucose minimal medium without glycine, indicating that the pGOG4 plasmid really does complement the glyA deficiency. However, this strain took longer to grow than the usual E. coli strain. Growth in glucose minimal medium, when supplemented with glycine, was faster than when glycine was not added. This appears to indicate that although the pGOG4 plasmid can complement the glyA deficiency sufficiently to allow growth on glucose without the addition of glycine, the complementation is not complete.

The pGOG4 clones were tested for growth on glycine minimal medium plates, at 28°C (since residue Mucts may be present). Some (about 1/3) of the brown clones isolated grew well on glycine; the other brown clones slight growth. Of the

brown clones studied, it was observed that production of brown pigment by the brown clone was unpredictable in the growth conditions used; this could explain the variability in growing on glycine. None of the white clones selected grew on glycine. Whatever gene pGOG4 may carry, it is remarkable that it allows growth on glycine in a host that does not carry the ssd mutation. This is not the case for pGOG1 and pGOG3. It is quite startling that pGOG4 did not confer ability to grow on glycine to MEW1 or to KEC9 when transformed with the plasmid, which were however tested at 37°C.

9. Serine Hydroxymethyltransferase Levels in Clones Carrying Plasmids of the gog Mutations

SHMT activity was measured in extracts of MEWG strains carrying the mini-Mu plasmids. The results are presented in Table 11. SHMT activities of the plasmid-carrying strains were much higher than the reference strain, MEW1, and had a level close to that seen in the MEW1 pGS29 strain carrying a clone of the normal glyA gene. What is surprising is that they are not higher than the levels seen in the GOG mutants carrying only one chromosomal copy of the same mutated gene. Thus, the GOG1 mutant showed 482 units but MEWG pGOG1 showed 394. Admitting that the assay presented difficulties, and that this work should be repeated using the Folk and Berg assay (9), it is still clear that the many plasmid-borne copies of the gene did not produce higher SHMT than a single

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Table 11.   Levels of serine hydroxymethyltransferase in
            strains carrying pGOG plasmids

Host	Plasmid	SHMT Activity (units)[a]
MEW1	none	39
MEWG	none	ND[b]
MEW1	pGS29	406
MEWG	pGOG1	394
MEWG	pGOG3	366
MEWG	pGOG4	426

[a] Units of SHMT activity are in nmoles methenylTHF/mg
    protein/hour.
[b] Not detectable.

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genomic copy.

The level of SHMT activity pGOG4 clone is also similar to the level found in a strain carrying a clone of the wild-type glyA gene. This result indicates that the pGOG4 plasmid, which results in brown pigment production, carries a gene that causes high levels of SHMT activity to be formed, even in a strain without a functional genomic glyA gene.

10. Characterization of the Brown Pigment Released by Strain Carrying the pGOG4 Plasmid

As stated, the MEWG strain carrying the pGOG4 plasmid released large amounts of a brown substance when growing in rich (LB) medium. An LB culture of this clone, usually light yellow, becomes nearly pitch black when incubated overnight at 37°C in a shaker. There is no obvious cell lysis. Centrifugation of the cultures pelleted the cells, leaving the brown product(s) in the supernatant. This indicates that the substance is released from the cells. Because this pigment may have a connection to the ability to grow on glycine, it was studied further.

Certain culture conditions change the releasing of the brown pigment. Addition of phenylalanine (100 μg/ml) to LB cultures of MEWG/pGOG4 resulted in darker brown cultures. Addition of $FeSO_4$ (0.1 %) to LB culture resulted in a dark pink colour, which eventually turned dark brown. Finally, although brown pigment was usually released by LB cultures

(observed with several different batches of LB media), occasionally no pigment was seen. This may indicate that some unidentified variable of the growth condition, such as pH or temperature, affected pigment production.

The brown product(s) could be reduced by addition of a strong reducing agent, sodium dithionite, and became colourless, the culture then regaining the light yellow of LB. It could then be reoxidized with oxygen (by shaking) or by an oxidation agent, potassium thiocyanate, and became dark again. This indicates the pigment is an oxidation/reduction compound, and is characteristic of a quinone molecule (40).

Acetone caused the pigmented substance to agglutinate and precipitate, and this precipitate remained insoluble even in aqueous solution. The pigmented product was precipitated out of the LB supernatant with 70 % ammonium sulfate, giving a dark brown pellet upon centrifuging. A large amount (0.5 mg/ml, wet weight of pellet) of this substance was released in cultures. The brown pellet was resuspended in buffer. Mixing this suspension with chloroform resulted in the precipitation of a thick, white material at the solvent interface. The chloroform did not precipitate the pigment compound, which remained in the aqueous phase. This shows that the pigment compound is soluble in an aqueous solution, but not in chloroform. Therefore, the pigment is associated with an ammonium sulfate-insoluble compound, and can be disassociated by chloroform extraction. These observations may suggest that

the released substance is a protein carrying a pigment non-covalently attached was released by the pGOG4 clones.

Polyacrylamide gel electrophoresis showed that the brown substance consists of two strong bands, visualized by staining with Coomassie blue, of molecular weights estimated at 30,000 and 50,000 daltons. There were a few other very faint bands suggesting slight cell lysis had occurred. The result may indicate that the protein is composed of two subunits. The brown pigment was observable on the gel at the dye front, showing up as a brown band overlapping the blue marker dye. Since the brown pigment migrates fast in the electric field on electrophoresis, it indicates that the pigment is a small, charged molecule, such as a quinone molecule.

The brown pigment absorption spectrum, shown in Figure 4, was determined in UV and visible range. As usual for all organic compounds, there is strong absorption in the lower UV, which peaks around 190 nm. This strong absorption makes interpretation of the spectrum difficult. The pigment (oxidized) absorbency shouldered at about 250-300 nm (i.e. broad peak that merges with the absorbency of organic substances peaking at 190 nm), and the absorption diminished slowly going up the visible spectrum, which would account for its brown colour. Reduction of the pigment with sodium dithionite resulted in a change in the absorption patterns, where the peak shoulders at 350 nm. Above 370 nm, the reduced form absorbs much less than oxidized. As a consequence, there
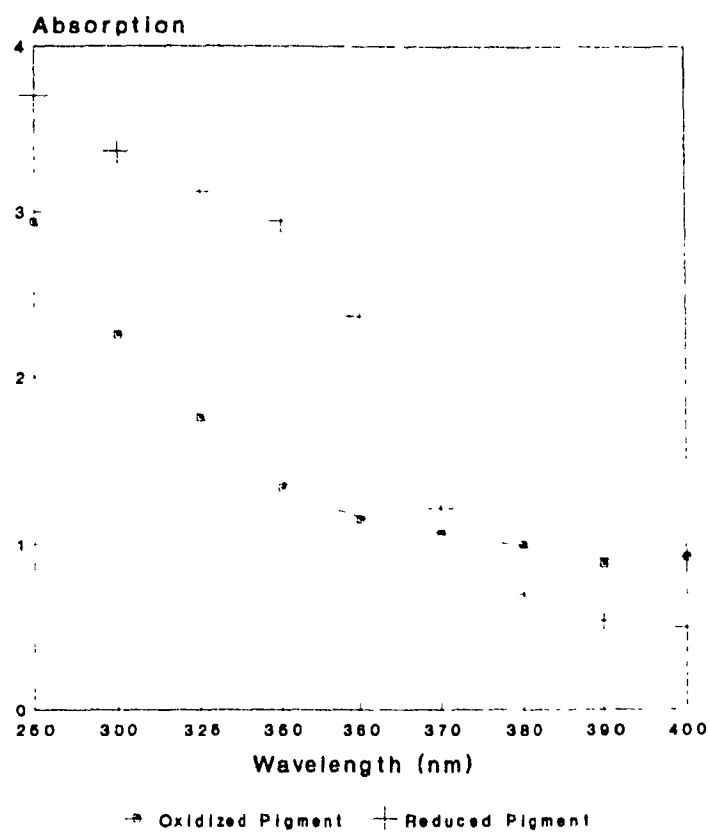
Figure 4. Pigment absorption spectrum. Pigment oxidized by oxygen and reduced by sodium dithionite.

is lower absorption in the visible spectrum, which would explain the lost of a visible colour upon reduction.

A hypothesis to explain the peculiar results found with pGOG4 will be presented in the discussion.

11. Isolation of Mutants of the GOG Strain Able to Overcome C_1 Repression

It was shown that the GOG mutants were incapable of growing on glycine minimal medium with the addition of the C_1 metabolites adenine, guanine and methionine (see Results Section 7.2.1). It should be possible to select for a mutant able to grow on glycine in the presence of C_1 metabolites.

In collaboration with Marc San Martano in our lab, the GOG1 mutant was plated heavily on glycine minimal medium with C_1 metabolites adenine, guanine, methionine, and histidine. Colonies, which arose from spontaneous mutation, grew on the selection plates. The new mutants, tentatively given the label GOGC1, were able to grow on glycine minimal medium without or with the addition of the C_1 metabolites mentioned. Marc San Martano has mapped the location of the selected mutation at 17 minutes.

12. Production of L-Serine Utilizing the GOG Strains

The underlying goal for this work is the construction of a strain that can produce L-serine in a commercially competitive process. The GOG mutants appear to have a higher carbon assimilation flow from glycine to L-serine. This makes them valuable in L-serine production studies.

With the assistance of Marc San Martano, the supernatant of resting cell cultures derived from glycine minimal medium-grown cells was tested for amino acids by paper chromatography. No L-serine was detected.

To try to obtain L-serine excretion, the L-SD enzyme, which degrades the L-serine, was removed from the strains. This was done by the introduction of the sdaA mutation (61), the mutation in the structural gene of L-SD, into the GOG mutants. The resulting strains lost the ability to grow on glycine because of the loss of L-SD activity. Resting cell cultures were tested but L-serine was not detected.

The lack of L-serine excretion may be due to the cell's deficiency in C_1 units; required, along with glycine, to form L-serine. Because of this, the derivative of GOG that can grow on C_1 metabolites (GOGC1) was tested for L-serine production. The mutation of GOGC1 may overcome the repression by C_1, and allow high levels of C_1 in the cell. GOGC1 was grown in glucose minimal medium, washed, and placed in glycine minimal medium (+/- formate) and incubated 48 hours. The supernatant was then tested as in the previous experiments.

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Chromatographic analysis (Figure 5) showed L-serine production from GOGC1 in glycine minimal medium with formate (50 μg/ml) added. Ninhydrin showed spots migrating parallel (i.e. same R_f) to the L-serine standard. These spots were stained red, typical of L-serine. The other amino acids usually stain purple or light brown. Marc San Martano estimated the amount of L-serine excreted into the medium by bioassay of about 0.01 mg/ml (from 2 mg/ml glycine in 48 hours). The L-SD activity was not removed from GOGC1 (by introduction of a sdaA mutation) meaning that a quantity of L-serine produced will have been lost. This result makes the possibility of L-serine production promising.
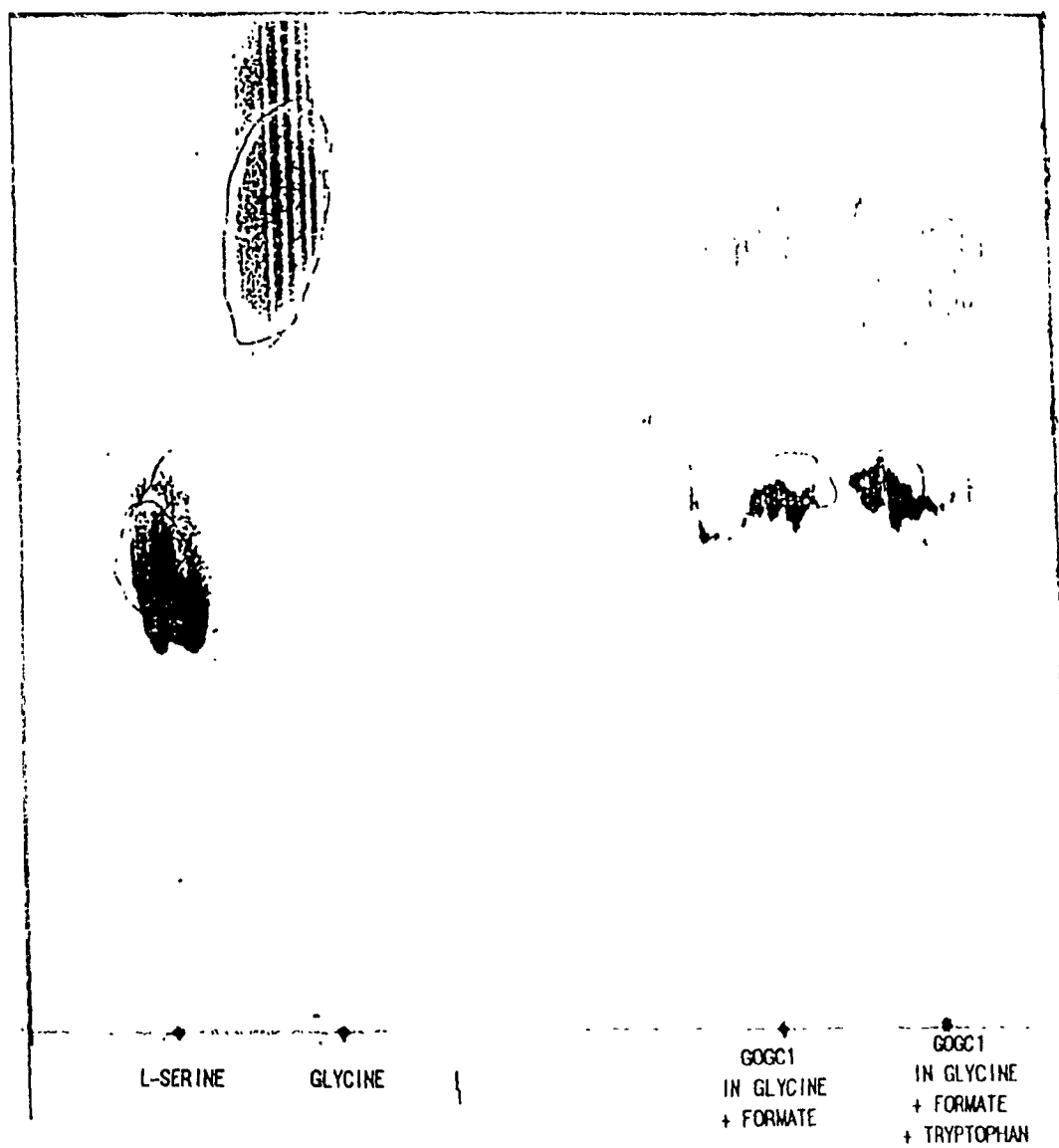
Figure 5. Chromatography to test for L-serine production by the GOGC1 strain. Supernatant (5µl) of various cultures was run on chromatography and stained with ninhydrin. Supernatant came from cultures of GOGC1 that were incubated 48 hours in glycine (0.2%) minimal medium. Formate (50µg/ml) and tryptophan (20µg/ml) added to cultures as indicated. Standard L-serine (µl of 20%) and glycine (1µl of 20%)was also run.

DISCUSSION

1. **Scheme for the Selection of Mutants with Increased Serine Hydroxymethyltransferase Activity**

   The scheme of this project was to obtain strains of *Escherichia* *coli* K12 that could grow on glycine as sole carbon and energy source. This selection would probably put strong pressure on the bacterial population to select for mutants with increased levels of serine hydroxymethyltransferase activity so that glycine can be assimilated for growth. As a consequence, this selection is valuable in obtaining mutants that may be the predecessors of an L-serine producing strain. Strains which were able to grow on glycine, using it as their sole source of carbon and energy, were isolated, and were designated GOG.

2. **Mechanism of the Effect of Various Substances Upon Growth on Glycine**

   The GOG mutants were able to grow on glycine minimal medium, but the growth was quite slow. The possibility exists that it is not the GOG strains' ability to assimilate glycine that is limiting, but rather that an important factor was limiting. If the strains are utilizing the SHMT reaction for

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conversion of glycine to L-serine, which then can be metabolically routed to energy production, then C_1 will also be required along with glycine. Therefore, one possible limiting factor is the supply of C_1 units.

As the results in Figure 2 show, various substances can enhance the ability of GOG mutants to grow on glycine. The positive effect of glucose and acetate on growth on glycine may indicate that a small initial energy source, which cannot be supplied by glycine, is required. This may indicate the need to synthesize certain proteins required for the utilization of glycine. For instance, the GCV pathway, known to be induced by exogenous glycine (26), and of extreme importance for its supply of C_1 units, would need to be transcribed upon the transfer of the GOG mutants into glycine minimal medium. The GOG strains GOG1, GOG2, and GOG3 transferred from glucose to glycine minimal medium (data not shown) showed a long lag phase of about 15 hours before beginning exponential growth. This suggests that glucose-grown cells do not have certain components required to start growing on glycine. Such a lag phase is seen in cultures transferred from glucose medium to lactose medium, and was deduced to require the induction of the lac operon (13). The addition of small amounts of glucose to the lactose cultures reduced the length of the lag phase, by supplying the initial energy source for synthesis of the lac proteins.

Hence, there may be an induction of protein(s) upon

transfer to glycine minimal medium. Although this may be so, it does not fit entirely with the experiment in which repeated subculturing of the strain in glycine minimal medium was done. Each subsequent culture took the same length of time to fully grow. This may be explained by the fact that the subcultures were done from stationary phase cells, in which most of the glycine may have been used up, and certain enzymes required for growth on glycine (i.e. GCV) were decreased to uninduced levels. Another possibility is glucose or acetate may be precursors for the synthesis of certain metabolites that limit growth on glycine.

A low concentration of Luria broth (LB), although not by itself enough to support detectable growth, enhanced growth on glycine of the GOG strains to what one would expect if glycine was being used efficiently. Further experiments indicated that tryptophan, which would be a component of LB, is a key substance in the ability to grow on glycine. Could it be that tryptophan, which is synthesized from L-serine, is limiting when GOG strains are growing on glycine? It is possible that high level of L-SD activity is deaminating the L-serine before sufficient amounts can be routed to tryptophan synthesis. This assertion is probably incorrect because the parent strain, KEC9, has the same L-SD levels but does not show a tryptophan requirement.

Exogenous tryptophan represses the tryptophan biosynthetic pathways, where it acts as a corepressor. It is

also required in attenuation control. Tryptophan also feedback inhibits the first enzyme of its pathway, anthranilate synthetase (66). As a result, L-serine, which would normally be routed to tryptophan synthesis, would be spared, and then could be used as an additional energy source. Although this may explain some increase in the rate of growth on glycine, it does not appear possible that the slight additional L-serine could result in the significant affect on growth on glycine that was seen.

Alternatively, tryptophan, and possibly LB, may supply an energy source as do glucose and acetate. Tryptophan is degraded by tryptophanase (13), which can route it to the energy pool. Alternatively, as suggested by glucose and acetate, tryptophan, or a component of LB, may serve as a precursor that is limiting in cells grown on glycine.

The most probable explanation is that tryptophan plays a regulatory role, perhaps increasing enzymatic activities required for growth on glycine. Although not done, it would be beneficial to assay SHMT, and GCV activities, in the mutant strains grown under these conditions, to see whether they are affected.

Although most discussion up to this point suggests a metabolic problem to explain the GOG mutants' slow growth on glycine, another possibility may exist. It was found that several strains of E. coli are quite impermeable to glycine and that glycine may enter the cell solely by diffusion,

resulting in a small glycine pool (6, 32). If this is so, the slow growth on glycine may be due to the slow, and rate-limiting, uptake of glycine. It is likely that the slow uptake of glycine plays a part in limiting growth on low concentrations of glycine. However, the high concentration of glycine used should circumvent this limitation. Thus, there is probably an additional limiting factor(s) as suggested above. However, it does not rule out the possibility that the supplements that aid growth on glycine do so by increasing the rate of glycine uptake.

It was also found that certain conditions prevented the growth of the GOG strains on glycine, as shown in Figure 3. The addition of the C_1 metabolites adenine, guanine, and methionine would relieve the cell from forming these compounds, and would conceivably result in an increased availability of C_1 units (6). This increase in the C_1 pool could supply GOG with the limiting factor when growing on glycine. However, there was no growth on glycine when the C_1 metabolites are added. It is known that SHMT synthesis is repressed by C_1 metabolites (6, 27) and since results indicate that the SHMT activity is important for growth on glycine, decreased SHMT activity could account for the observed results. However, this would be so if the regulation of glyA by C_1 metabolites is still operational, and had not been disrupted by the gog mutations. It seems more probable that growth on glycine does not occur because the synthesis of the

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GCV enzymes is inhibited by C_1 repression, which may leave the cell with no way to produce C_1 units. However, it is uncertain whether the GCV system is regulated by the availability of C_1 units (26, 31, 57).

If C_1 is limiting, then supplementation of some form of C_1 may be needed for efficient L-serine production. However, adding supplemental C_1 may work against us by repressing SHMT (if the mutations did not knock out this regulation), or the GCV pathway. This was the reason why the mutant, GOGC1, that overcame the inhibition of growth on glycine by C_1 metabolites was selected. GOGC1 may permit elevated levels of C_1 in their cells, which would be beneficial for L-serine production.

The last piece of interesting results from this experiment was the effect on growth on glycine with the addition of formate. As stated, it would be an advantage for L-serine production to add an exogenous, inexpensive source of C_1. This is why much of the research into a means of L-serine production is done on methylotrophic bacteria that can utilize methanol and formate as an exogenous supply of C_1. It is not known whether E. coli can use any exogenously supplied substance as a C_1 source. Formaldehyde is a possible C_1 source since methyleneTHF is formed spontaneously from it in the presence of THF (3). However, formaldehyde is toxic to E. coli. Formate is not known to be used as a source of C_1 by E. coli. It is known that formate can be used in the synthesis of purines by some unknown formylating enzyme (5,

32), and this would presumably spare C_1, resulting in an increased pool. The result of this experiment was that formate (500 μg/ml) stopped GOG mutants from growing on glycine, even though the same amount did not affect their growth on glucose. At first, this may appear obscure. However, comparing with the results observed with the addition of C_1 metabolites, formate may increase the C_1 pool resulting in repression of SHMT (or GCV), which may prevent growth on glycine. If this is so, feeding formate (at least to GOGC1) at the stage of L-serine production may be beneficial.

3. Hypothesis on the Genetic Alteration Caused by the gog Mutations

A priori, the gog mutations that permit utilization of glycine for growth would presumably affect SHMT activity encoded by the glyA gene. Alternatively, it may be that normal level of SHMT is sufficient for growth on glycine, and there is another limiting factor. For instance, because C_1 units are required along with glycine for growth, maybe this is limiting, since only glycine is added exogenously. If this was true, then it is possible that the gog mutations result in a larger C_1 pool by altering C_1 metabolism. It is also possible that E. coli cannot grow on glycine, not because of a limiting factor, but because the concentration of glycine used prevents growth, due to toxicity. These possibilities will be discussed further below.

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3.1. Identity of the Mutations gog-1, gog-2, and gog-3

The most direct means to alter the SHMT activity would be through the alteration, or mutation, of the gene that encodes the SHMT enzyme, the glyA gene. From the mapping experiments (Tables 4-8), the map position on the E. coli linkage map was determined. A summary of the relevant mutations is shown in Figure 6. It was found that three of the gog mutations, gog-1, gog-2, and gog-3, do map near glyA. These results indicate that these mutations may be located in the glyA gene itself. The similarities in map position of the gog mutation and SHMT activity of the GOG1, GOG2, and GOG3 mutants suggests that they carry similar mutations in one gene, even though they were independently derived. Although this is probable, it was not examined further. The fourth mutation, gog-4, did not map to the glyA region. Since the gog-4 mutation is evidently different from the other gog mutations studied, it will be discussed later.

The strains whose gog mutations mapped to glyA all showed increased levels of SHMT activity (Table 9). Figure 7 diagrams the results. The level of SHMT activity observed in these GOG strains was about 12 times that found in the reference strain, MEW1, and in the parent strain, KEC9. That they have elevated levels of SHMT activity indicates that the glyA gene of the GOG strains has become derepressed. The high linkage of these gog mutations to the glyA gene possibly suggests that they map in a regulatory site for this gene,

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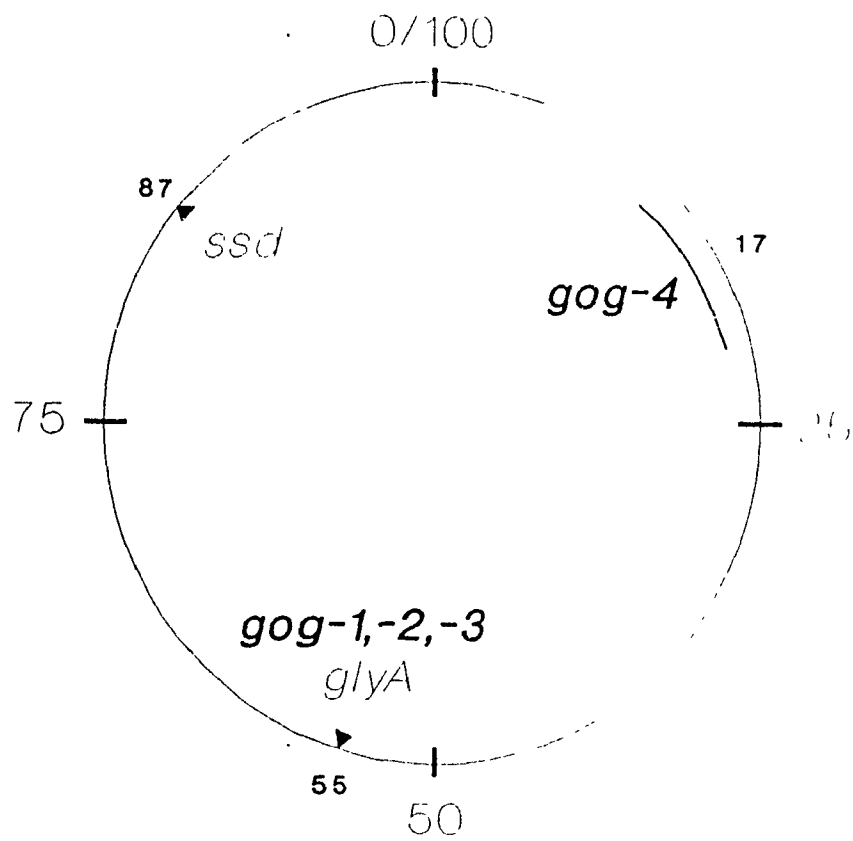
Figure 6. Map position of *gog* mutations on *E. coli* map (divided in minute)
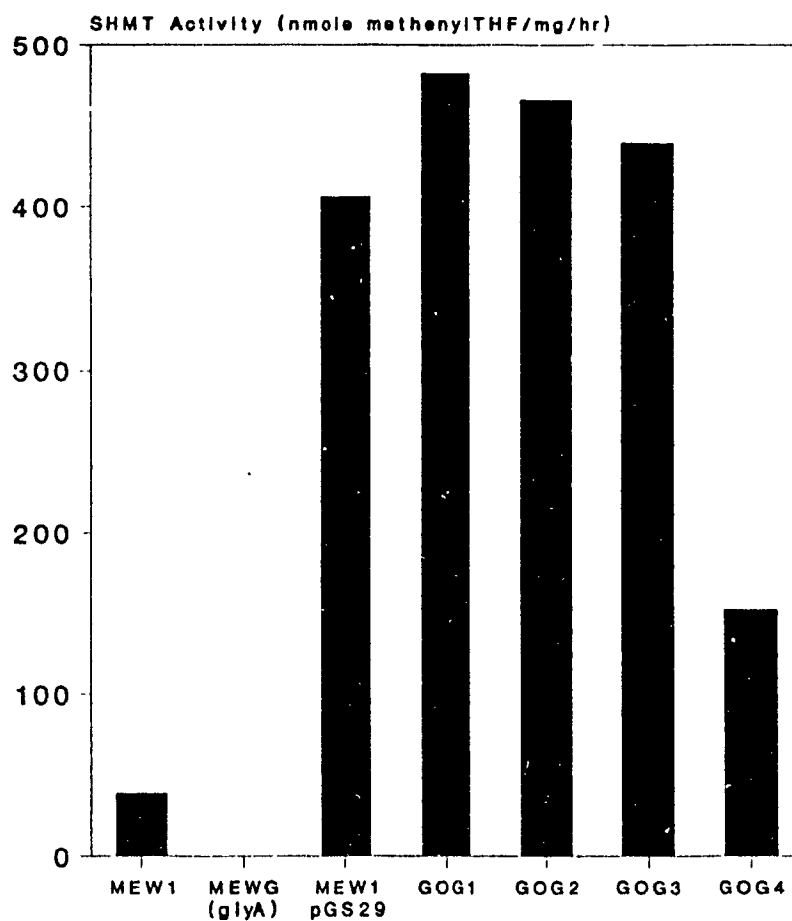
Figure 7. Comparative levels of serine hydroxymethyltransferase in the GOG mutants and other strains.

resulting in the derepression of SHMT synthesis.

The results indicates that utilization of glycine is accomplished by this increased level of SHMT activity in these GOG mutants. This is supported by experiments (Table 3 and 7) which show that elevated L-SD activity is required for the GOG strains to grow on glycine. From this, it is deduced that glycine is assimilated in a pathway that incorporates high levels of the activities of SHMT and L-SD, shown in Figure 8. The C_1 units would presumably come from the GCV reaction by the cleavage of glycine.

As mentioned, the results suggest that gog-1, gog-2 and gog-3 map in the glyA gene. Since these mutations result in elevated SHMT activity levels, it suggests that they interfere with glyA regulation (i.e. lie in its operator region). However, it is possible that they lie in the glyA coding region, altering the conformation of the SHMT enzyme, (i.e. altering the Km for its different substrates). In addition, although the results indicate that the mutations lie in the glyA gene, it is still possible that the mutations map to a regulatory gene(s) very closely linked to glyA that conveys growth on glycine, by indirectly altering glyA transcription. There have been mutations in other genes which alter the regulation of SHMT synthesis. These mutations are metJ (89 minutes), metK (64 minutes), metR (86 minutes) and purR (36 minutes) (14, 26, 46, 53, 58), but none of these mutations map (2) near glyA. Mutations in genes coding for enzymes of
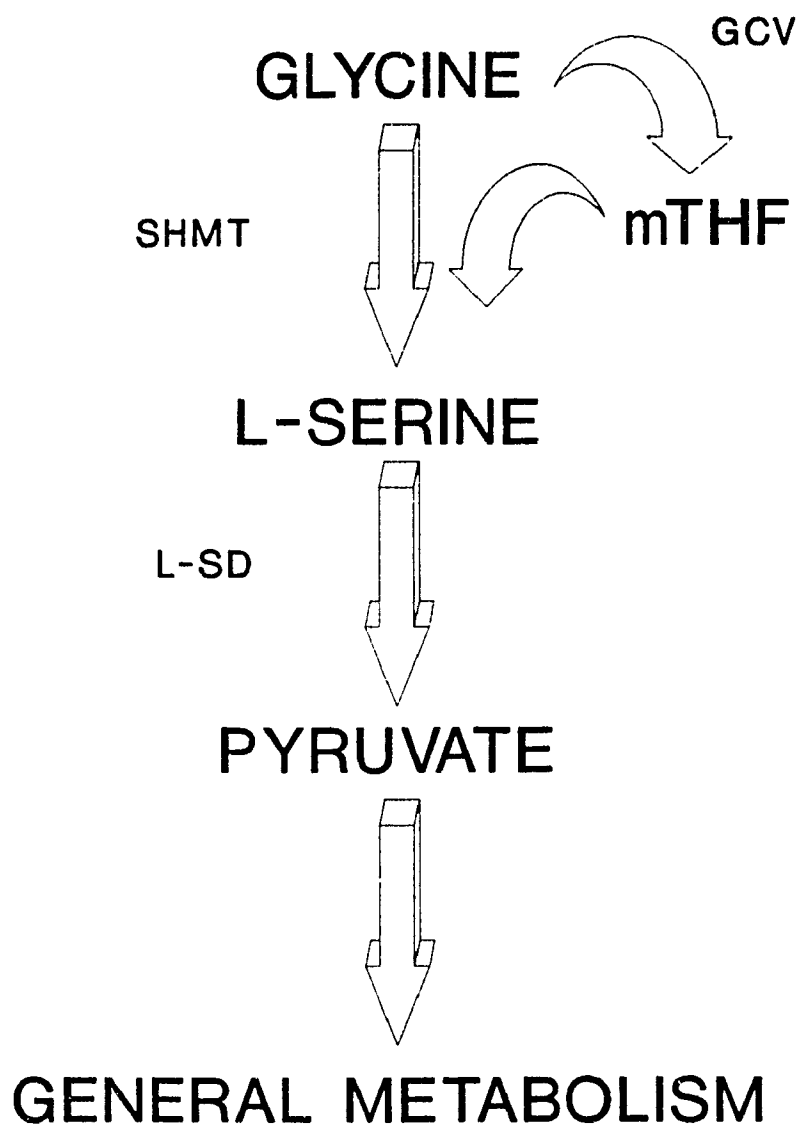
Figure 8 Pathway for glycine assimilation used by GOG mutants when growing on glycine

certain pathways (i.e. metE, metF, purE etc.) results in elevated SHMT activity when these auxotrophs are starved for the product of that pathway (6, 14, 24, 27, 52), but again, these genes do not map near glyA (2). Also, the GOG mutants are not auxotrophic for any C_1 metabolite. It seems then that these gog mutations are interrupting an unique regulatory locus (or loci) linked to glyA.

Mutations near glyA have been reported that resulted in altered SHMT levels. Plamann and Stauffer (42, 45) isolated a mutation of E. coli that was 97 percent linked to glyA that resulted in a reduction in SHMT activity to 30 percent. It was found that the mutant's glyA gene was regulated (by C_1 metabolites) as the parent strain, which indicates that the operator was not affected. The mutation mapped in the nontranslated region distal to the glyA structural gene, 35 bp after the translation stop codon. As deduced from the glyA sequence, this region after the translation stop site codes for several stable stem-loop structures (44). It was suggested that these stem-loop structures in the glyA mRNA function to give stability to the mRNA, and that mutation of them results in lower SHMT levels due to decreased glyA mRNA stability (45). It was also suggested that there is still a possibility that the stem-loop structures may be sites for DNA/RNA binding proteins (45). Since such a mutation results in a reduction in glyA expression, it does not appear that the gog mutations are related, for they cause higher levels of

SHMT activity.   Although, it is possible that the _gog_ mutations increases _glyA_ mRNA stability, which may result in elevated SHMT levels.   In addition, if a regulatory protein does bind to these stem-loop structures, increasing the affinity of binding of an activator (by the _gog_ mutation) could result in elevated SHMT activity.

In addition, Stauffer _et al_ (54) isolated two mutants of _Salmonella typhimurium_ that had increased levels of SHMT activity resulting from mutations linked to _glyA_.   These mutants were selected by a scheme analogous to the one presented in this thesis.   Their intention was to isolate mutants of _S. typhimurium_ that may have a specific alteration in the regulation of SHMT synthesis, resulting in increased SHMT activity.   The scheme made use of a mutant (_glyA951_) that had only 40 percent SHMT activity (54).   In the presence of C_1 metabolites (adenine, guanine, methionine, thymine, serine), repression of the already reduced SHMT activity prevented growth on glucose minimal medium.   They then selected for mutants that could grow on glucose medium (without glycine); such mutants would presumably have increased SHMT activity. Hence, although our selective pressure may be much more extreme, the schemes are similar.   Of the mutants isolated, one had 7-fold increased SHMT levels and its _glyA_ gene showed the normal response to C_1 metabolites.   A second mutant had 2-fold increased SHMT levels and its _glyA_ gene's response to C_1 metabolites was reduced.   Both were cotransducible with _glyA_

(29 and 4 percent, respectively).

These mutations were isolated from S. typhimurium, but because this bacterium is genetically similar to E. coli, the mutations may be similar to the gog mutations. However, the levels of SHMT in these mutants were lower than what was found in the GOG mutants (though, this could be because of differences in the regulation of SHMT in S. typhimurium). Also, the mutations isolated from S. typhimurium have a much lower linkage to the glyA gene than did the gog mutations being discussed here. It was suggested by the authors that this indicates that their mutations mapped to adjacent genes that code for proteins that regulate SHMT synthesis (54).

Hence, the gog mutations, gog-1, gog-2, and gog-3, which are highly linked to the glyA gene, do not appear to have been reported in previous literature. If these gog mutations do mutate the glyA operator region, one can think of several possibilities to explain the alteration in SHMT activity caused by the mutations. SHMT synthesis from glyA is under several kinds of regulation. For instance, the C_1 metabolites, where purines and methionine play prominent roles, repress the expression of the glyA gene. Effector proteins that govern C_1 regulation proba ly bind to the upstream region of the glyA gene. A mutation in this region that hinders such binding could result in a derepressed glyA.

In the sequence of the glyA gene, there is a palindrome in the operator region that may be the binding site of a

regulatory protein, and therefore, may represent a possible control site of glyA expression (43). In addition, the purR repressor was shown to bind to another site in the glyA operator region (58). Altering these regions may cause a change in expression of the glyA gene; SHMT activity should increase if the binding of a repressor is interfered with. Through sequencing of the gog mutations mapping to glyA, which have been cloned, the exact genetic alteration can be ascertained.

As the mutant isolated from S. typhimurium (54), which showed elevated levels of SHMT activity due to loss of C_1 repression, the gog mutations may be interrupting C_1 repression of glyA. However, if all, or some, of the C_1 regulation, has been interrupted, then the glyA gene of the GOG strains would not be repressed by C_1 metabolites. It was shown that the GOG strains are prevented in growing on glycine in the presence of C_1 metabolites. This indicates that at least some of the C_1 repression of glyA remains intact in the GOG mutants. However, there is a possibility that C_1 metabolites represses GCV expression (26, 31, 57), and this would also result in growth inhibition. However, since SHMT assays were not done in these conditions, it is not known whether the glyA gene is under normal control with respect to C_1 metabolites. Such an assay is of prime importance for future work on this project.

Another possibility is that the gog mutations mapping to

glyA interrupt repression by glycine. It has been shown that high concentrations of glycine (3000 μg/ml) repress SHMT by 40 percent (27). The glycine minimal medium used has a glycine concentration of 2000 μg/ml. Hence, this level of glycine may repress a normal glyA gene. The gog mutations may have altered the glyA gene such that it is not repressed by glycine. This results in the enhanced level of SHMT activity, which may not be lowered when the mutants are growing on glycine. If regulation of the glyA gene by glycine has been affected in the mutants, a simple assay of SHMT activity of strains grown in glycine minimal medium, and grown in glucose minimal medium with glycine added at high concentrations, would show it.

3.2. Identity of the Mutation gog-4

The situation with the GOG4 mutant is quite different. Of the four GOG mutants studied, this one gave the most unexpected, and intriguing, results. However, this makes it the most difficult to explain. It can utilize glycine for growth like the other GOG strains. It has an increased level of SHMT activity, although the increase appears not to be as high as seen with the other GOG strains. It still requires high levels of L-SD activity resulting from the ssd mutation. This indicates that the utilization of glycine involves its conversion to L-serine, as proposed for the gog mutations mapping to the glyA gene (see Figure 8). Finally, results

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show that the gog-4 mutation maps to a genetic position different from the other gog mutations, located roughly around 17 minutes (+/- 5 minutes) (see Figure 6), far from the glyA locus located at 55 minutes (2).

Since SHMT activity has increased significantly, being about 4-fold greater than that of the reference strain, it seems that the gog-4 mutation maps in a gene that regulates SHMT activity. Again, known genes that regulate glyA expression, metJ (89 minutes), metK (64 minutes), metR (86 minutes), and purR (36 minutes) (2) do not map near gog-4. Possibly, there is an unknown regulatory gene that produces a repressor protein, and the gog-4 mutation stops the production of this hypothetical repressor protein. It is known that C_1 metabolites (and glycine) repress glyA transcription. A repressor protein(s) that would carry out this regulation has been proposed, but such a protein has not been demonstrated (7, 43, 57). It is suggested that a product(s) of the purine and/or the methionine pathways acts as a repressor or corepressor of SHMT synthesis, however, this appears to be only part of the regulation (6, 14, 26, 46, 53). Hence, the gog-4 mutation may be interrupting a new regulatory protein of the glyA gene. A possible binding site for the regulatory protein has been pointed out in the operator region of the glyA gene (43), near which the mutations gog-1, gog-2, and gog-3 may map.

There is a known gene near gog-4 that may be involved.

The _gog-4_ mutation maps roughly around 17 minutes by conjugation (which is not precise). However, since the mapping experiment (Table 8) showed a high linkage (91%) between _gog-4_ and the Tn10 at 17 minutes (Hfr CAG5051), it is likely to be very close to 17 minutes. However, there is a low probability that the _gog-4_ mutation maps in _purE_ at 12 minutes (2). A _purE_ mutant (purine auxotroph), when starved for purines, shows increased SHMT levels (6). However, the GOG4 mutant is not a purine auxotroph. Nonetheless, it is possible that the _gog-4_ mutation affects the transcription of _purE_ such that it decreases purine levels, which in turn increases SHMT levels without disrupting metabolism enough to make the cell a purine auxotroph.

That the _gog-4_ mutation interrupts a regulatory gene of the _glyA_ gene may not be supported by all the results. The attempt to clone the mutant _gog-4_ gene by complementation of a strain deficient in the _glyA_ gene, which at first did not appear promising, gave a twist in the story. Since the GOG4 strain appears to carry a normal _glyA_ gene, then the experiment should have accomplished merely the cloning of this normal _glyA_ gene. This may be the case for half of the clones selected. The other half released a brown pigment. Such a phenomenon had not been reported in the literature in connection with cloning the wild-type _E. coli glyA_ gene by Stauffer _et al_ (55).

Why did half of the clones produce brown pigment? Two

possibilities to explain this result can be suggested. The brown clones may carry both glyA and a linked mutant gene responsible for pigment production while the white ones carry only glyA. Alternatively, the brown clones may carry an entirely different gene that complements glyA in some other way and also results in pigment formation.

In Salmonella typhimurium, whose genetic map is similar to E. coli, there is a gene not far (less than 1 minute) from the glyA gene that was designated pig (for pigment), for the cells formed brown colonies (59). Although such a gene has not been reported in E. coli, the genetic maps of these two bacteria are very similar so that a strong possibility exists that there is a pig gene in E. coli. Thus, the GOG4 mutant may have another mutation near glyA that affects the expression of the pig gene.

Evidence suggests that glyA is part of an operon of genes concerned with C_1 metabolism (44, 45, 54, 57). As stated, the sequence of the E. coli glyA gene shows probable stem-loop structures at the distal end (after the stop codon) of the gene (44). The sequence of these structures had very high homology to sequences of repetitive intercistronic palindromes found in other procaryotic operons (44, 57). In addition, as mentioned above, Stauffer et al (54) selected for a mutation of S. typhimurium 29% linked to glyA that resulted in a 7-fold increased in SHMT activity. It was suggested that this mutation results in deregulation of an operon of which

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glyA is part. However, because of the somewhat low linkage between the mutation and glyA, the authors did reflect this would represent a large operon. If GOG4 does have a secondary mutation adjacent, but not very linked, to glyA, in addition to gog-4 at 17 minutes, then it may be similar to the mutation isolated from S. typhimurium, for both strains had several fold increases in SHMT activity.

Hence, there is a possibility that the glyA gene is part of an operon of genes involved in C_1 metabolism. The pig gene itself may be involved in C_1 metabolism. If there is an operon, then the mutation near glyA may have been selected for increased synthesis of SHMT, and this mutation also affected expression of other genes (i.e. pig gene) in that operon. If there is a mutation mapping near glyA that alters expression of glyA, then the gog-4 mutation at 17 minutes need not be affecting glyA activity, but rather some other aspect of C_1 metabolism required for growth on glycine. If this is correct, then it is probable that both the gog-4 mutation near 17 minutes and the hypothetical mutation near glyA would be required for growth on glycine by the GOG4 mutant.

However, the results of the mapping experiment may contradict this suggestion, since results show that the gog-4 mutation maps to around 17 minutes (see Figure 6), remote from the glyA gene. Nonetheless, it is still possible that the GOG4 strain carries two mutations that were selected by demanding growth on glycine. The conjugation results may give

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an indication of a mutation required for growth on glycine that maps around 55 minutes. Referring to Table 8, the CAG5055 cross shows a linkage of 16 percent to 42 minutes. This could be mapping a second mutation around 55 minutes. However, there is certainly a gog-4 mutation that maps at around 17 minutes because of the high linkage to a Tn10 at 17 minutes and the fact that CAG5054 direction of transfer is away from the 55 minute region. Mapping by transduction to a Tn10 at 55 minutes (see Table 4) indicates that the GOG4 mutant carries no gog mutation around glyA. Although, since only 70 transductants were scored, it is possible that a mutation lies in a gene on the other side of glyA, opposite that of the Tn10 insert.

There is another possibility why the mapping experiments may not have shown a second hypothetical mutation required to grow on glycine and linked to glyA. The gog-4 mutation may be lethal (on glucose and glycine minimal medium used in the experiment), unless there is the hypothetical mutation near glyA. As is inherent in mapping experiments, the mutation has to be removed; but if the mutation near glyA is removed, then this would leave the gog-4 mutation alone, and the cell would die (i.e. they will not be scored).

Hence, the mapping experiments do not entirely rule out that another mutation, which causes pigment production (when cloned), maps near the glyA gene of the GOG4 strain (in addition to gog-4), which may have been selected for the

ability to grow on glycine. It is possible that both mutations are required for GOG4 to grow on glycine. If this is the case, then the gog-4 mutation may not be derepressing glyA, rather, it may be altering related C_1 metabolism required for this mutant to grow on glycine. The hypothetical mutation near glyA may be deregulating the whole proposed operon, of which glyA and the pig gene are part. Such a mutation would be similar to one isolated from S. typhimurium described above (54). This mutation is in a gene 26 percent linked to glyA and causes a seven-fold increase in SHMT activity (54). Hence, this mutation selected from S. typhimurium may be similar to the proposed mutation in GOG4. The low linkage to glyA could explain why it was not mapped to a Tn10 at 55 minutes. However, if the pGOG4 plasmid has both the glyA gene and the hypothetical mutation, they cannot be that far apart (no more than about 0.5 minutes since the bacterial DNA insert carried by the plasmid vector is around 20 KB in length). An additional alteration of C_1 metabolism by gog-4 could explain why the GOG4 mutant can grow on glycine even with only a four-fold increased in SHMT activity.

A second possible explanation for the phenomenon is that the plasmids do carry the gog-4 mutation (i.e. carry an insert of a region close to 17 minutes). If this is so, the cloned gog-4 gene causes pigment production, or an adjacent gene does, by amplification on mini-Mu (a multicopy plasmid). If

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this has indeed occurred, since a pGOG4 plasmid can complement a glyA-deficient strain, then it would indicate that the plasmid encodes SHMT activity, as the SHMT assay shows. However, there is a small possibility that residual SHMT activity from the mutant glyA gene (point mutation) was induced by a pGOG4 gene. However, this is unlikely since the pGOG4 clone has quite high SHMT activity. Thus, it is possible that the gog-4 mutation has resulted in switching on a gene which codes for an enzyme with SHMT-like activity. On this line, it is possible that the gog-4 mutation maps in a second cryptic glyA gene.

A suggestion that E. coli K12 has a second SHMT enzyme is not preposterous. There have been data supporting an existence of a second SHMT activity in some organisms. It has been shown that Saccharomyces cerevisiae has two SHMT isoenzymes, one cytoplasmic and one mitochondrial (68). Further, the methylotrophic bacterium, Pseudomonas AM1, has been shown to have two SHMT isoenzymes (38). It was shown that these isoenzymes have similar enzymatic properties, such as similar requirement for the cofactors THF and pyridoxal-5-phosphate. However the regulation of their activities and their molecular weights differ. It was observed that one of its SHMT iscenzyme predominates when Pseudomonas AM1 is grown on methanol or methane, whereas the other isoenzyme predominates when it is grown on succinate.

An example where E. coli possesses a duplicate enzyme

activity differing in regulation is L-SD (62). The commonly known L-SD (encoded by sdaA) is expressed constitutively, whereas the second L-SD activity is expressed mainly in rich medium.   E. coli also has three aspartokinases and two homoserine dehydrogenases (13).

An E. coli enzyme, ketopantoate hydroxymethyltransferase (KHMT), has been studied and was shown to be similar to SHMT in its mechanism of action (1). For instance, the KHMT enzyme also requires THF and pyridoxal-5-phosphate as cofactors and it carries out a reaction resembling the reaction SHMT catalyses. A second SHMT-like enzyme could be physiologically concerned with L-serine/glycine interconversion, or this may be a side reaction from another similar enzyme, such as KHMT. The study of KHMT did not suggest that KHMT could do anything to L-serine. However, it is known that SHMT, because of the reactive cofactor pyridoxal-5-phosphate, has several side reactions, such as the cleavage of threonine and allothreonine (49).

A second SHMT enzyme can explain why the GOG4 mutants can grow on glycine even though they do not show the large increase in SHMT activity as observed with the other GOG mutants. It may be that the second SHMT enzyme has different kinetics, possibly with different Km for the various substrates.   Hence, it may run physiologically towards L-serine synthesis, in contrast with the known SHMT enzyme. SHMT is usually assayed by converting L-serine to glycine.

The activity in the reverse direction may be much higher, and this would be beneficial for L-serine production. This also would explain the incomplete complementation of a glyA-deficient strain by the pGOG4 plasmid.

If the results of GOG4 mean that E. coli has a second SHMT enzyme encoded by gene which gog-4 mutates, then it would indicate that this second glyA gene is regulated differently from the known glyA gene. In any case, this gene would not be transcribed in significant amounts when E. coli is grown on glucose minimal medium. This can be concluded because a glyA-deficient strain has no detectable SHMT activity and therefore requires supplemental glycine to grow. Like Pseudomonas AM1 (38), the hypothetical E. coli isoenzymes of SHMT may be induced on different carbon sources. The gog-4 mutation would then have removed restraints on transcription of the second glyA gene, allowing production of the elevated level of SHMT activity observed in the GOG4 strain. There is some evidence that suggests that E. coli has a third means of producing C_1 units, other than SHMT and GCV, and that the C_1 donor is L-serine (33). This may be the function of a second SHMT activity.

It is apparent that the true nature of the GOG4 strain is still obscure and more study will be required to support or disprove these hypotheses. Additional mapping experiments are required. In addition, a more detailed analysis of the pGOG4 plasmid is required.

4. Thoughts on the Brown Pigment Released by Clones Carrying the pGOG4 Plasmid

The production of a brown pigment by pGOG4 clones (carrying plasmids constructed from GOG4) when grown in rich medium was very i: teresting, and probably important. Whatever idea one may suggest about the pGOG4 plasmid, the facts remain that it complements glyA, results in increased SHMT levels, and lastly, results in the release of large amounts of the brown pigment.

Pigment analysis revealed that the pigment is a small, charged molecule. This molecule was connected noncovalently to an ammonium sulfate-insoluble substance. A solution of this substance was agglutinated with acetone, precipitated with chloroform, found to be of high molecule weight with polyacrylamide gel electrophoresis, and stained with Coomassie blue. These observations suggests that the substance is a protein. The polyacrylamide gel electrophoresis experiment revealed that the protein is probably a dimer, but it is also possible that the protein had degraded into two parts.

It was found that the pigment-protein was released by cultures at around 0.5 mg/ml wet weight. This is an extremely high amount of protein being released by the bacterium. Significant cell lysis is ruled out as an explanation for this large amount of external protein. This is deduced from the fact that only two bands (apart from a few faint bands) were found on the polyacrylamide gel, and not a smear that would

occur from the soup of proteins released in lysing cells. By itself, the release of large amounts of proteins by this strain of E. coli is of interest. For if, by chance, it was to be a high quality protein (i.e. the essential amino acids are in high proportion) then it could be used in the microbial manufacture of single-cell protein, which can be used as a food supplement.

E. coli rarely release proteins into the culture medium. There have been instances where E. coli has released protein, and these proteins originated usually from the periplasmic region, though outer membrane proteins have on rare occasion been released. For instance, when β-lactamase was overproduced in E. coli, most of this enzyme as well as other periplasmic proteins were released into the medium (11). It was suggested this was due to an increased permeability of the outer membrane (11).

In other reports, the release of protein was attributed to protein-leaky mutants. For instance, mutants of E. coli were isolated that released periplasmic proteins, where 90 percent of the cells' alkaline phosphatase was released (19). Another group working with a protein-leaky mutant that released periplasmic proteins gave evidence to indicate that the purE gene is involved in causing the release of protein (63). They suggest that accumulation of metabolic intermediates due to the defect in the purE enzyme accounts for this.

Hence, the brown protein may be a periplasmic protein, or enzyme, that is being overproduced by the pGOG4 clones. It is possible the plasmid causes the strain to become protein-leaky. It is unknown whether the strain is leaky to other periplasmic proteins, as is the case of protein-leaky mutants studied (11, 19, 63), or whether the strain specifically releases the brown protein. The polyacrylamide gel showed two bands of equal intensity, which appear to indicate two subunits of the brown protein. However, a few light bands were also visible that may indicate that other periplasmic proteins were being released, but in much lower amounts such that they were not, or barely, detectable in the experiment. Because of the high concentration of the released brown protein, it may be suggested that the protein being released is being overproduced from a gene encoded on the pGOG4 plasmid. This would be similar to the strain reported to be releasing ß-lactamase, which was overproduced through genetic amplification on plasmids (11).

As stated above, studies indicate the involvement of the purE gene in the releasing of proteins by protein-leaky E. coli mutants (63). A mutation of the purE gene would result in a purine auxotroph. It is known that purines play a role in regulating SHMT synthesis (6, 27). Hence, alteration of purE may be the link between high level of activity of SHMT level caused by the gog-4 mutation and the release of the brown protein in the clone holding the pGOG4

plasmid. Although neither the GOG4 mutant nor the pGOG4 clone was a purine auxotroph, they may still have an alteration in their purine metabolism.

The pigment molecule attached to the protein may be a cofactor. It was observed that pGOG4 clones could grow somewhat on glycine minimal medium. Could this released protein be involved in conferring ability to use glycine?

From chemical analysis, the pigment is an oxidation-reduction compound, with characteristics of a quinone (40). There are several quinoid molecules that are coloured when oxidized and become colourless when reduced. For instance, riboflavin is yellow/orange when oxidized, and colourless when reduced (12).

There are enzymes that have the quinone, pyrrolo-quinoline, as cofactor (8). These enzymes are usually located in the periplasmic space, as suggested for the brown pigment protein. Most are involved in primary oxidation steps, for instance glucose dehydrogenase. As well, methanol dehydrogenase, which methylotrophs use to oxidize methanol to formaldehyde (i.e. C_1 source), has this quinone cofactor (8). However, absorption spectra of various pyrroloquinoline enzymes (8) did not match the spectrum derived from the brown pigment.

Another oxidation-reduction compound is tetrahydrofolate. Tetrahydrofolate (THF) is the reduced form and is pale yellow. The oxidized form is dihydrofolate (DHF)

and is darker yellow. Prolonged oxidation produces folate (60), which is even darker. When the brown pigment is diluted, it also takes on a yellow colour. Is it possible that the pigment is THF, the cofactor bound to the SHMT enzyme and used in many C_1 transfer reactions?

The absorption spectra (60) of THF (reduced) and DHF (oxidized), which is shown in Figure 9, are similar to the corresponding spectra of the reduced and oxidized forms of the brown pigment (see Figure 4). This suggests that the pigment molecule associated with the released protein is THF or one of its C_1 derivatives.

Hence, the released protein may be part of C_1 metabolism. If this protein is produced from a mutant gene (i.e. pig gene) linked to glyA, then it may suggest that these genes are part of a proposed operon concerned with C_1 metabolism.

Alternatively, if a second SHMT activity has been established by the gog-4 mutation, its reaction characteristics and regulation may be favourable to our aim of L-serine production. The released protein, which may have an associated THF derivative molecule, may even be the second SHMT-like enzyme. This statement is speculative, but it merits consideration for future studies.

Although, there are many enzymes with associated THF (or related cofactor) in E. coli. Another possibility for the pigment protein is phenylalanine hydroxylase (3). This enzyme
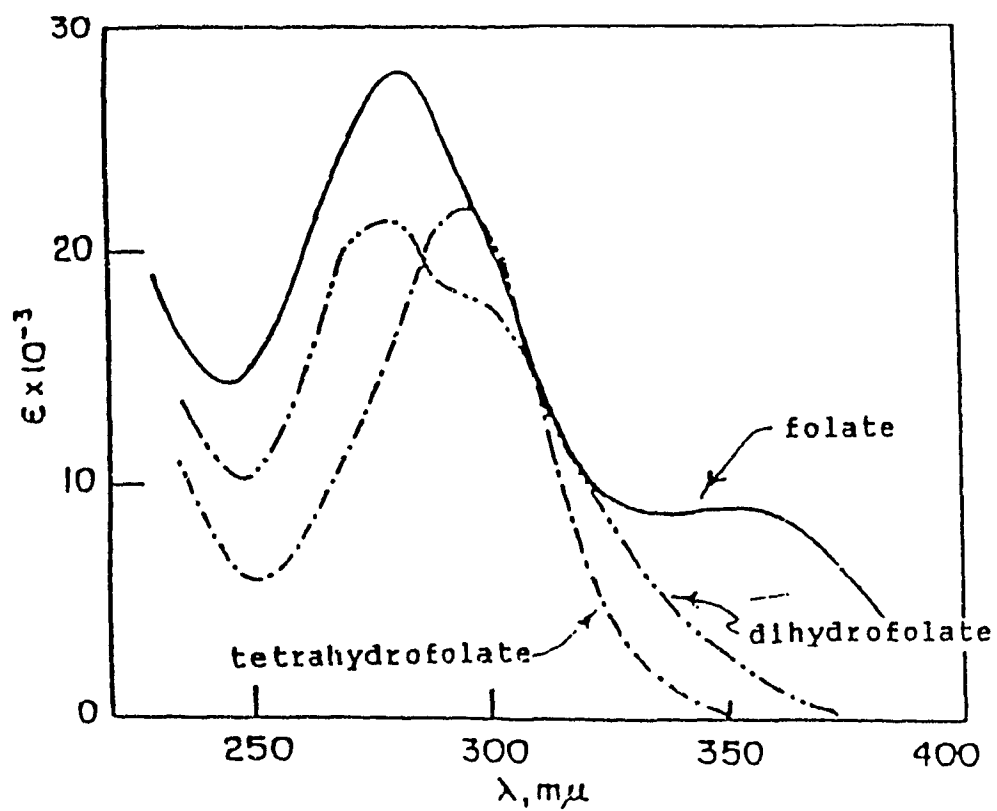
Figure 9. Absorption spectrum of tetrahydrofolate, dihydrofolate, and folate (60).

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hydroxylates phenylalanine. It was observed that the release of the brown pigment was induced by phenylalanine. Phenylalanine hydroxylase has a cofactor, tetrahydrobiopterin, which is chemically similar to THF (3). In addition, this enzyme has one iron per subunit, which is required for its activity (3). This could explain the pink culture when iron was added to the culture, which may bind to the pigment causing a shift in absorption.

Whatever the underlying mechanism, the pGOG4 clone results in overexpression of SHMT activity as well as a brown pigment protein, which is possibly involved in C_1 metabolism.

5. Growth on Glycine by GOG Strains Unveils a Paradox

The GOG strains' ability to grow on glycine reveals a paradox. The results suggest that they can grow because a pathway for glycine assimilation (for use as sole energy source) was opened up by increasing the activities of both the SHMT and L-SD enzymes (see Figure 8). However, the KEC9 pGS29 strain, with high SHMT activity due to the plasmid-carried glyA gene, and high L-SD activity due to the ssd mutation, cannot grow on glycine, although it can grow on L-serine. Hence, just the fact that a strain has elevated levels of SHMT and L-SD activities does not guarantee that it can use glycine as a sole source of carbon and energy.

What do the GOG strains have that strain KEC9 pGS29 does not? The answer may lie in the SHMT assay experiment that

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showed that at least three of the GOG strains have higher SHMT activity than that seen in any other strain. This extreme amount of SHMT activity may be enough to explain the ambiguity. This may not be the exact answer, at least for GOG4, which doesn't have as high SHMT activity as seen in the other GOG strains. There may be another prerequisite needed to grow on glycine. For instance, the gog mutations may also alter other reactions in C_1 metabolism, resulting in an increased supply of methyleneTHF (such as increasing GCV activity), needed in the SHMT reaction along with glycine. The brown pigment released by a strain carrying the pGOG4 plasmid supports this if it is an enzyme of C_1 metabolism. If this is so, then the GOG4 strain required two changes to grow on glycine, an increase in SHMT activity, and some other modification of C_1 metabolism.

The GOG strains' ability to grow on glycine may be for another reason, in addition to their enhanced ability to assimilate glycine. Growth on glycine may require that they detoxify glycine. It may be that other strains cannot grow on glycine because their growth is inhibited by the high concentrations of glycine. This detoxification of glycine would probably be by the drain of glycine through the increased levels of SHMT and L-SD. Hence, the GOG strains have overcome a certain threshold of glycine toxicity, and can grow.

However, the most probable explanation for ability of

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the GOG mutants to grow on glycine is that regulation of $glyA$ expression has been altered. As stated, high concentrations of glycine repress SHMT synthesis (27), which would be detrimental to growing on glycine. Repression of SHMT levels by glycine could be through glycine directly. However, this repression could work through the C_1 units (or C_1 metabolites in which these C_1 units would enter) following the cleavage of glycine to C_1 by the GCV pathway. If the repression of the $glyA$ gene by glycine has been disrupted in the GOG strains, then growth on glycine will not cause repression of their high level of SHMT activity. The cloned genes of the pGS29 plasmid are wild-type $glyA$ genes, with normal controls. Hence, in the case of a strain holding the pGS29 plasmid (harbouring the $glyA$ gene), its high SHMT activity may be repressed in glycine minimal medium to a level that cannot support growth.

Hence, the gog mutations are evidently disrupting a predominant regulatory mechanism of $glyA$ transcription. It is apparent that further pursuit into the gog mutations will give deep insight into the complex regulation of SHMT synthesis, as well as C_1 metabolism as a whole.

6. Use of Mutants Selected for L-Serine Production

The mutants isolated in this work may be useful for L-serine production. However, modifications of the strains will still be required to construct a commercially viable L-serine producing strain.

Serine analysis of resting cultures of the GOG strains did not show L-serine production (at least not significant). This may be because as in any fermentation research, culturing conditions must be explored for optimum production of a product. Alternatively, the isolation of derivatives of the GOG strains may be required.

Such a derivative (GOGC1) of GOG1 was selected (see Section 11 of the Results). This derivative overcame the inhibition of growth on glycine by a mixture of C_1 metabolites. This indicates this derivative can overcome repression exerted by an increased internal C_1 pool. Hence, this derivative shows promise in L-serine production, for it is now possible to increase the supply of C_1 units to achieve a better yield of L-serine. Marc San Martano (personal communication), who is working on this project, has found that the new mutation in GOGC1 maps to 17 minutes. Hence, it may be related to gog-4, which also maps around 17 minutes.

Serine analysis on resting cultures of GOGC1 showed promising results. A spot appeared on the chromatographic sheet (see Figure 5) that migrated parallel with L-serine and stained red with ninhydrin as does L-serine.

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The experiment suggests that the formate (50 μg/ml) added was required for the excretion of the L-serine. There is a question whether $\underline{E}$. $\underline{coli}$ can use formate as a C_1 source since the enzyme formyltetrahydrofolate synthetase was not detected in $\underline{E}$. $\underline{coli}$ (5). As discussed above, formate may enter purine synthesis (5, 32), and as a result, spare C_1 from entering into purine synthesis (5, 32). The final outcome of formate may still be an increase in the C_1 pool. Hence, these strains may be able to overproduce L-serine, but their C_1 source may be limiting and an exogenous source of C_1 may be required for detectable L-serine excretion. As stated, the ability to supply C_1 to GOGC1 makes this strain a strong candidate as a potential L-serine producer.

The amount of L-serine produced was not great (0.01 mg/ml from 2 mg/ml glycine). Yields should improve by exploring optimal conditions for L-serine production. Methylotrophic bacteria have shown production of, for instance, 24 mg/ml (7), and 11 mg/ml (18), of L-serine from 100 mg/ml glycine. However, the supply of C_1 units in methylotroph research is supplied by the addition of methanol. In addition, such concentrations of glycine (100 mg/ml) used in fermentations with methylotrophs have not been tried, and may give better yields.

Using our derivatives of $\underline{E}$. $\underline{coli}$, the supply of C_1 will come from cleavage of glycine by the GCV system (and possibly through formate addition). Hence, we may expect at most a 50

percent conversion of glycine to L-serine (i.e. two glycine molecules to form one L-serine molecule). If a good exogenous source of C_1 cannot be found for E. coli, it may be worthwhile to supply the strains with a means to use such a source. For instance, giving the mutants plasmids holding genes from other organisms, such as methanol dehydrogenase (to use methanol as a C_1 source) or formyltetrahydrofolate synthetase (to use formate as a C_1 source) should aid in L-serine production.

With continued fermentation studies on the mutants isolated, yields in L-serine production may improve. The detection of some L-serine production indicates a potential for these mutants for a commercial application in L-serine production. As well, coupling L-serine production to tryptophan production, by for instance overproducing tryptophan synthetase on a multicopy plasmid, has potential for development of an efficient tryptophan production system.

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