Isolation and Characterization of an *Escherichia coli* Formate-Utilizing Mutant Carrying the *Clostridium acidiurici* 10-Formyltetrahydrofolate Synthetase Gene

Yingnian Shen

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfilment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

November, 1995

ISBN 0-612-05125-0

CONCORDIA UNIVERSITY

School of Graduate Studies

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By: **Yingnian Shen**

Entitled: **Isolation and Characterization of an *Escherichia coli* Formate-Utilizing Mutant Carrying the *Clostridium acidiurici* 10-Formyltetrahydrofolate Synthetase Gene**

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Master of Science

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ABSTRACT

Isolation and Characterization of an *Escherichia coli* Formate-Utilizing Mutant Carrying the *Clostridium acidiurici* 10-Formyltetrahydrofolate Synthetase Gene

Yingnian Shen

A *serA gcv* double mutant, CCSG32, was constructed from *Escherichia coli* K-12 *serA* strain MEW85. The *E. coli* strain CCSG32 was deficient in both 3-phosphoglycerate dehydrogenase (*serA*) and the glycine cleavage enzymes (*gcv*) so that one-carbon units could not be generated through the conversion of serine to glycine or the glycine cleavage pathway when bacteria were grown in glucose minimal medium. The *Clostridium acidiurici* 10-formyltetrahydrofolate synthetase gene was introduced into CCSG32 by transformation. Isolation of a series of spontaneous mutants resulted in strain GOF33 which showed normal growth in glucose minimal medium with glycine and formate. The mutations occurred on the chromosome DNA. Incorporation experiments with [^{14}C]formate demonstrated evidence that the external formate was utilized by GOF33 cells and that the biosynthesis of serine, methionine and histidine was dependent, possibly solely, on the external formate. These results indicate that the foreign 10-formyltetrahydrofolate synthetase gene was functioning in GOF33 cells and necessary for them to produce activated one-carbon units.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all those who helped me in fulfilling this work, especially to:

Dr. Elaine B. Newman, my supervisor, for giving me the opportunity to study and do researches in her laboratory, for her scientific guidance, support and encouragement. Dr. P. Gulick and Dr. R. Roy, who were on the committee for my programme, for their support and scientific guidance.

Dr. R. Storms, Dr. R. D'Ari and Dr. M. Herrington for valuable advices and discussion. Successive Graduate Program Directors Dr. B. S. Mangat and Dr. N. N. Kapoor, and their successive secretaries.

My labmates Dr. R.-T. Lin, Dr. Z.-Q. Shao, Dr. H.-S. Su, M. San Matano, J. Zhang, J. Garnon, X.-P. Feng and J. Moniakis for their help, support, love and friendship. All my friends, family members and relatives who encouraged me during those days.

Finally, Wei Shen, my husband, for his love, understanding, support, encouragement.

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ABBREVIATIONS

ADP: Adenosine diphosphate.

Amp: Ampicillin.

C_1: One-carbon.

cpm: Count(s) per minute.

(d)ATP: (Deoxy)adenosine triphosphate.

DNA: Deoxynucleic acid.

dpm: Disintegration(s) per minute.

dTTP: Decxythymidine triphosphate.

dUMP: Deoxyuridine monophosphate.

EMP pathway: The Embden-Meyerhof-Parnas pathway.

GCV: Glycine cleavage.

GOF: Growing on formate.

Kan: Kanamycin.

LB medium: Luria-Bertani medium.

Lrp: Leucine-responsive regulatory protein.

L-SD: L-serine deaminase.

NAD: Nicotinamide adenine dinucleotide.

pfu: Plaque-forming unit(s).

SAM: S-adenosylmethionine.

SHMT: Serine hydroxymethyltransferase.

TCA: Trichloroacetic acid.

THF: Tetrahydrofolate.

INTRODUCTION

1. Purpose of this study

One-carbon (C_1) units are very important in cellular metabolism. The biosynthesis of purines, thymine, histidine and methionine and the formation of the formyl group of fMet-$tRNA_f$ all need C_1 units (Figure 1; see Blakley, 1969, for review). In many organisms, C_1 derivatives of tetrahydrofolate (THF) can be interconverted into different oxidation states by three enzymes: 5,10-methylene-THF dehydrogenase, 5,10-methenyl-THF cyclohydrolase and 10-formyl-THF synthetase (Figure 1). However, Escherichia coli can carry out only two of these reactions. It has the dehydrogenase and cyclohydrolase activities, but does not have the synthetase activity (Dev and Harvey, 1978), and therefore can not make C_1 units from formate.

C_1 units in E. coli cells are mainly generated at a more reduced oxidation level, mainly through two pathways (Figure 2). One pathway is the conversion of serine to glycine. This reaction is catalyzed by serine hydroxymethyltransferase (SHMT) (Blakley, 1969). The other pathway is through the cleavage of glycine, catalyzed by the glycine cleavage (GCV) enzyme system (Newman and Magasanik, 1963). External formate can not be used as C_1 source by E. coli (Newman and Magasanik,

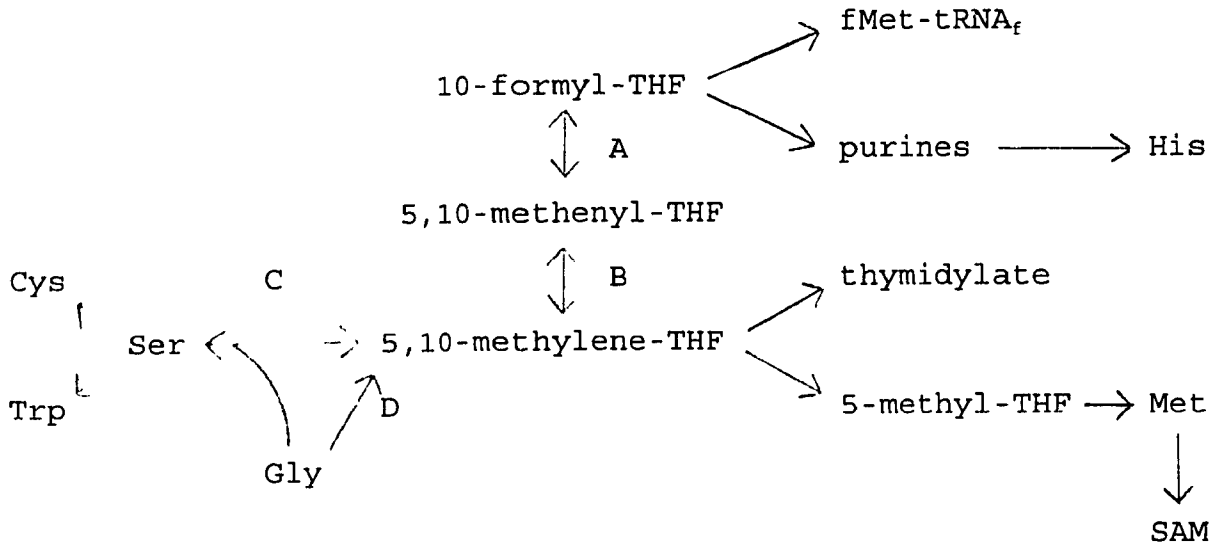


Figure 1. C_1 metabolism in E. coli. THF, tetrahydrofolate. SAM, S-adenosylmethionine. The enzymes shown here are: A, 5,10-methenyl-THF cyclohydrolase; B, 5,10-methylene-THF dehydrogenase; C, serine hydroxymethyltransferase; D, glycine cleavage enzymes.

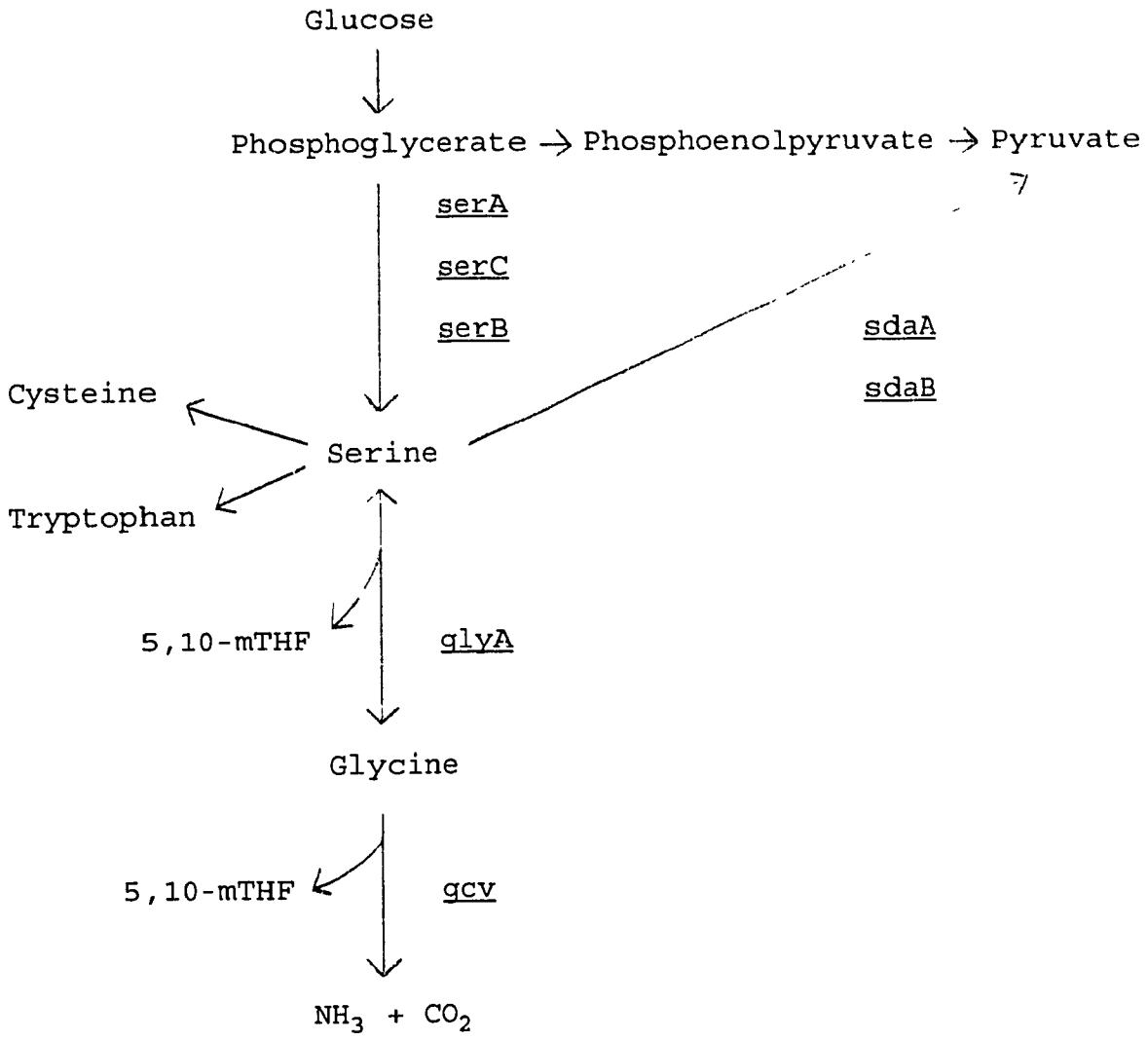


Figure 2. Biosynthesis of serine and glycine in E. coli. Designations of the genes: serA, 3-phosphoglycerate dehydrogenase; serC, 3-phosphoserine aminotransferase; serB, 3-phosphoserine phosphatase; sdaA and sdaB, L-serine deaminase; glyA, serine hydroxymethyltransferase; gcv, the glycine cleavage enzymes. 5,10-mTHF, 5,10-methylenetetrahydrofolate.

1963).

On the other hand, the level of the 10-formyl-THF synthetase activity in Clostridium acidiurici is high (Vogels and Van Der Drift, 1976). The gene coding for this enzyme has been cloned and characterized (Whitehead and Rabinowitz, 1986 and 1988).

This study is an attempt to introduce the C. acidiurici 10-formyl-THF synthetase gene to an E. coli mutant which can not produce C_1 units by itself. Such a study can answer a number of questions. First, will the introduction of the foreign synthetase gene permit the growth of the E. coli mutant when external formate is provided? External formate is necessary because E. coli produces formate only during anaerobic growth from pyruvate by pyruvate-formate lyase (Gottschalk, 1986). Second, if growth is possible, is it due to the expression of the foreign synthetase gene in E. coli cells? Third, if formate is used, into what compounds is it incorporated? Therefore, this study was intended to make contributions to the understanding of the C_1 metabolism. Moreover, it may lead to discovery of a less expensive route for industrial production of serine by microorganisms. Since serine is the precursor of tryptophan, it may also be valuable for the production of tryptophan.

2. Biosynthesis of one-carbon units in E. coli and its regulation

As mentioned above, in E. coli, C_1 units are mainly generated through two pathways, the conversion of serine to glycine and the cleavage of glycine. Serine is synthesized from 3-phosphoglycerate, which is derived from glucose through the Embden-Meyerhof-Parnas (EMP) pathway (Figure 2; see Stauffer, 1987, for review). The conversion of serine to glycine provides a major source of C_1 units and glycine for E. coli cells (Pizer, 1965; Newman et al., 1974). The cleavage of glycine is the second source of C_1 units. There is probably another unknown C_1 source in E. coli as indicated in a mutant defective in both SHMT and the GCV enzyme system (Newman et al., 1974).

2.1. Biosynthesis of serine

As shown in Figure 2, 3-phosphoglycerate is converted through three enzymatic reactions to form serine. The enzymes involved are 3-phosphoglycerate dehydrogenase (encoded by serA), 3-phosphoserine aminotransferase (serC) and 3-phosphoserine phosphatase (serB) (see Stauffer, 1987, for review).

The synthesis of serine from 3-phosphoglycerate is

subjected to feedback inhibition (Pizer, 1963). Inhibition of 3-phosphoglycerate dehydrogenase activity by L-serine, a noncompetitive inhibitor, is a major control point of both serine and glycine biosynthesis. Other known inhibitors are D-serine, glycine and leucine (McKitrick and Pizer, 1980). L-serine also inhibits, to a much lesser degree, 3-phosphoserine phosphatase.

The enzymes involved in the serine biosynthesis are constitutively synthesized since their levels are not affected by serine concentration of growth media and the synthesis of 3-phosphoglycerate dehydrogenase is not induced by its substrate 3-phosphoglycerate (McKitrick and Pizer, 1980). However, expression of the serA gene has been recently found to be regulated by the leucine-responsive regulatory protein (Lrp). Lrp has been shown to regulate the transcription of genes in the leucine/Lrp regulon (see Newman et al., 1992, for review). Repression of serA by leucine does not occur when the lrp gene, which encodes Lrp, is mutated (Lin et al., 1990). Therefore, Lrp is considered as an activator of the serA gene. Further, it has been shown that mutations in the promoter region of the serA gene affecting the Lrp binding also altered the expression of the serA gene (Zhang, 1994).

2.2. Conversion of serine to pyruvate and ammonia

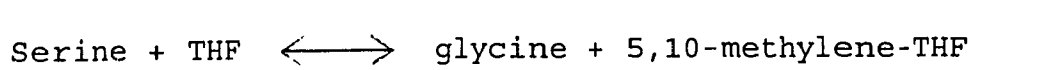
Serine can be degraded into pyruvate and ammonia by L-serine deaminase (Figure 2; Pardee and Prestidge, 1955). The physiological role of L-serine deaminase (L-SD) is not completely understood. It has been suggested that it may regulate the L-serine pool in cells to reduce the toxicity of high concentration of serine (Su, 1991). Also, the products of the reaction catalyzed by this enzyme, that is, pyruvate and ammonia, may be used to provide carbon and nitrogen sources from excess serine (Su et al., 1989; Su, 1991).

There are two kinds of L-SD, namely, L-SD1 and L-SD2, which are encoded by sdaA and sdaB respectively (Su et al., 1989; Su and Newman, 1991; Shao and Newman, 1993). L-SD1 and L-SD2 are very similar in biochemical properties, but the genes which code for them are regulated differently (Su and Newman, 1991; Su, 1991). sdaA is expressed in cells grown in both LB medium and glucose minimal medium (Su et al., 1989); while sdaB is expressed only in LB medium (Su and Newman, 1991). Environmental factors such as DNA damaging agents, anaerobic conditions and high temperatures affect the expression of the sdaA gene but not the sdaB gene (Newman et al., 1982; Shao, 1993). The expression of the sdaA gene is elevated in both lrp and ssd mutants; but these mutations have no influence on the sdaB gene (Newman et al., 1981; Lin et al., 1990; Shao, 1993). On the other hand, sdaB, but not

sdaA, is regulated by catabolite repression (Shao, 1993).

2.3. Formation of one-carbon units through the conversion of serine to glycine

The conversion of serine to glycine is catalyzed by SHMT (Figure 2). In this reaction, the ß-carbon of serine is transferred to THF to form 5,10-methylene-THF and glycine:



The reaction needs pyridoxal-5-phosphate as a cofactor (Schirch et al., 1985). The conversion of serine to glycine is the only glycine source in E. coli grown in glucose minimal medium (Pizer, 1965). The C_1 units produced through this pathway are then utilized in the biosynthesis of purines, thymidine, methionine and the formyl group of fMet-$tRNA_f$.

SHMT catalyzes a reversible reaction. It is important for the production of serine from glycine and 5,10-methylene-THF in E. coli mutants which are not able to produce serine from glucose. However, physiologically, it favours the direction from serine to glycine and 5,10-methylene-THF for the needs of glycine and C_1 units (Schirch et al., 1985).

SHMT has an apparent molecular weight of 170 kDa (Mansouri et al., 1972), and is a tetramer composed of four

identical subunits (Plamann et al., 1983a). The gene encoding the SHMT subunits, glyA, has been cloned (Stauffer et al., 1981) and sequenced (Plamann et al., 1983a).

Regulation of the glyA gene expression is complex. Products of C_1 metabolism such as serine, glycine, methionine, thymine and purines repress the synthesis of this enzyme (see Stauffer, 1987, for review). Intracellular concentration of glycine or C_1 products regulates the SHMT synthesis (Miller and Newman, 1974). When cells are grown in the presence of glycine at 3 mg/ml, the SHMT activity is reduced to 43%. Histidine, methionine, thymine, adenine, guanine and formate represses the SHMT activity of the cells grown in glucose minimal medium to 40%. Adenine and guanine account for a considerable proportion of the repressive effect (Miller and Newman, 1974). The SHMT activity is not regulated by the functional state of glycyl-tRNA synthetase or glycyl-tRNA (Folk and Berg, 1970).

The SHMT level is derepressed in E. coli metE and metF mutants under methionine limitation, but not in metA or metB mutants (Greene and Radovich, 1975; Mansouri et al., 1972). This supports the role of methionine in the production of glycine and C_1 units by controlling the SHMT synthesis. Purines or serine limitation also leads to significant derepression of SHMT (Miller and Newman, 1974). However, depression of SHMT is not observed during the thymine- or glycine-limited growth (Folk and Berg, 1970).

The synthesis of SHMT is also regulated by the metK, metJ and metR genes. The metK gene encodes S-adenosylmethionine synthetase. The metJ gene encodes a protein which represses the methionine biosynthesis genes when interacted with S-adenosylmethionine (Ahmed, 1973; Greene et al., 1973). The metK and metJ mutations prevent complete repression of SHMT synthesis (Meedel and Pizer, 1974; Greene and Rodovich, 1975). Moreover, the SHMT level is elevated in metK mutants. The glyA gene is positively controlled by the metR gene product (Plamann and Stauffer, 1989). This activation requires homocysteine which is an intermediate in the biosynthesis of methionine. Homocysteine acts as an inducer and S-adenosylmethionine acts as a corepressor in the SHMT synthesis. Therefore, SHMT is partially regulated as a methionine enzyme.

2.4. The glycine cleavage enzyme system

The GCV enzyme system catalyzes the cleavage of glycine to produce ammonia, carbon dioxide and 5,10-methylene-THF, thus, provides a secondary source of C_1 units (Figure 2). It has been found in a variety of organisms including animals (Motokawa and Kikuchi, 1974), plants (Walker and Oiver, 1986; Bourguignon et al., 1988) and bacteria (Klein and Sagers, 1966; Kochi and Kikuchi, 1974; Freudenberg and Andreesen,

1989). It is composed of four proteins, namely, the P-, H-, T- and L-proteins (Kikuchi, 1973). The P-protein is a glycine dehydrogenase catalyzing the decarboxylation of glycine and transfer of the remaining aminomethyl group to the lipoyl prosthetic group of the H-protein. The T-protein is an aminomethyltransferase catalyzing the release of ammonia from the H-protein and the transfer of the glycine methylene carbon to THF to form 5,10-methylene-THF. The L-protein is a lipoamide dehydrogenase catalyzing the reoxidation of the resulting dihydrolipoyl residue of the H-protein and the reduction of NAD^+.

In E. coli, the gcvT, gcvH and gcvP genes, encoding the T-, H- and P-proteins respectively, are located at 62.6 min on the E. coli chromosome as the gcv operon (Plamann et al., 1983b; Okamura-Ikeda et al., 1993; Stauffer et al., 1994). These three proteins have been purified and their genes have been sequenced (Stauffer et al, 1991, 1993 and 1994; Okamura-Ikeda et al., 1993). All these genes and proteins showed considerable sequence similarity to those characterized in other organisms. On the other hand, the L-protein in the E. coli GCV system was found to be encoded by the lpd gene which is located at 2.5 min (Steiert et al., 1990).

Glycine cleavage may function to balance the cellular requirements for glycine and C_1 units (Newman and Magasanik, 1963; Umbarger, 1977). If the requirement for C_1 units exceeds the requirement for glycine, part of the glycine

product made from serine by SHMT can be converted to additional C_1 units.

E. coli serine auxotrophs can grow on exogenous glycine because the C_1 units needed for the biosynthesis of serine can be obtained from the cleavage of glycine, and serine can be produced from glycine and C_1 units (Newman et al., 1974). However, serA gcv double mutants are unable to grow on glycine because they can not obtain the C_1 units needed for the biosynthesis of serine from glycine, and therefore require an exogenous supply of serine. A requirement for serine which can not be fulfilled by glycine (with glucose as carbon source) is used as a test for the GCV phenotype in a serA background.

The GCV enzyme system is induced by exogenous glycine and the induction is not blocked by the addition of adenine, methionine, or S-adenosylmethionine to the medium (Meedle and Pizer, 1974). Mutation in the glycine transport system, Cyc, shows GCV$^-$ phenotype in serA background and reduction in the induction of the GCV system by glycine (Ghrist and Stauffer, 1995). On the other hand, addition of a mixture of C_1 end-products, including adenine, guanine, methionine, histidine and thymidine, greatly decreases the gcv gene expression (Lin et al., 1992). The decrease is mostly due to the addition of purines.

So far, three regulatory proteins, Lrp, PurR and GcvA, have been shown to be involved in the regulation of the E.

coli GCV system.

Lrp is absolutely required for the induction of the gcv genes by glycine (Lin et al., 1992). lrp mutants are not able to use glycine as C_1 source, therefore, SHMT is responsible for the production of both glycine and C_1 units. In an lrp glyA double mutant, there is neither the SHMT activity nor the GCV enzyme activity, so that growth of such a mutant requires supplementation of C_1 source in medium (Lin et al., 1992). A certain sequence in the control region of the gcv operon is essential for the regulation by Lrp, which has been shown to be able to bind multiple sites upstream of the promoter of the gcv operon (Stauffer and Stauffer, 1994).

PurR, the purine repressor protein, represses the expression of the GCV system (Wilson et al., 1993b). Such repression occurs in the presence of purine when the GCV system is fully induced by glycine. PurR has been shown to be able to bind the control region of the gcv operon indicating that PurR is possibly a trans-acting factor in the GCV expression (Wilsen et al., 1993b).

The GCV enzyme system is also regulated by the gcvA gene which encodes a trans-acting regulatory protein showing similarity to the LysR family regulatory proteins. Mutation in gcvA results in the loss of the glycine-induced expression of the GCV enzyme system (Wilson et al., 1993a). Like Lrp, regulation by GcvA is through the upstream region of the gcv operon (Stauffer and Stauffer, 1994). The gcvA gene is

located outside the gcv operon region and has been cloned (Wilson and Stauffer, 1994). Interestingly, the gcvA gene is also responsible for the repression of the GCV system by purine (Wilson et al., 1993b). However, this repression is independent of the function of PurR.

3. Tetrahydrofolate carries activated one-carbon units at different oxidation levels

THF is a highly versatile carrier of activated C_1 units. It consists of three groups: a substituted pteridine, p-aminobenzoate and glutamate. The C_1 units carried by THF are bound to the N-5 or N-10 nitrogen atoms or to both. These C_1 units can exist in three different oxidation levels (Figure 1). The most reduced form carries a methyl group; the intermediate form carries a methylene group; and the most oxidized form carries a methenyl, formyl, or formamino group. The interconversion between 5,10-methylene-THF and 5,10-methenyl-THF is catalyzed by 5,10-methylene-THF dehydrogenase. The interconversion between 5,10-methenyl-THF and 10-formyl-THF is catalyzed by 5,10-methenyl-THF cyclohydrolase. The two catalytic activities in E. coli are associated as a bifunctional enzyme composed of two identical subunits (Dev and Harvey, 1978; D'Ari and Rabinowitz, 1991). The gene coding for this enzyme, folD, has been mapped to 12 min on the

E. coli chromosome (Rudd et al., 1990). It has been cloned and sequenced and the deduced amino acid sequence of this enzyme shows 40% to 45% identity to the dehydrogenase/cyclohydrolase domains of the trifunctional C_1-THF synthase from yeast, human and rat (D'Ari and Rabinowitz, 1991).

4. Use of one-carbon units in E. coli

In E. coli, the majority of C_1 units enter the folate pathway as 5,10-methylene-THF (Figure 1; Newman et al., 1974). 5,10-Methylene-THF is directly used in the biosynthesis of thymidylate from dUMP (Blakley, 1969). It can also be converted to 10-formyl-THF by 5,10-methylene-THF dehydrogenase/5,10-methenyl-THF cyclohydrolase to supply C_1 units for the biosynthesis of purines and the formyl group of fMet-$tRNA_f$ (Blakley, 1969). Moreover, it can be reduced to 5-methyl-THF for the biosynthesis of methionine (Cohen and Saint-Girons, 1987). Methionine can be further converted to S-adenosylmethionine which is the activated methyl group donor used in many methylation reactions such as DNA methylation (Mudd and Cantoni, 1964; Bickle, 1987).

5. 10-Formyltetrahydrofolate synthetase

10-Formyl-THF synthetase catalyzes the reaction:

$$\text{THF} + \text{ATP} + \text{formate} \longleftrightarrow \text{10-formyl-THF} + \text{ADP} + \text{Pi}$$

It has been found in a large number of diverse organisms including animals, plants, yeasts and bacteria (see MacKenzie, 1984, for review). Whitehead *et al*. (1988) investigated the distribution of this enzyme among different eubacteria. Examples of presence and absence of the activity were found in aerobic, facultative and anaerobic bacteria. The variation in detectable synthetase activity ranges from 0.001 U/mg in *Branhamella catarrhalis* to 13 U/mg in *C. acidiurici*, in which the enzyme constitutes approximately 2 to 4% of the cellular proteins (Rabinowitz and Pricer, 1962). *E. coli* and some enteric bacteria do not have this synthetase activity (Dev and Harvey, 1978; Whitehead *et al*., 1988) although they do have the 5,10-methylene-THF dehydrogenase activity. This suggests that, in organisms without the 10-formyl-THF synthetase activity, 10-formyl-THF is obtained independently of formate.

10-Formyl-THF synthetase activates formate into C_1 unit carried in 10-formyl-THF, and 5,10-methylene-THF dehydrogenase and 5,10-methenyl-THF cyclohydrolase catalyze the interconversion of activated C_1 units between different oxidation states. This pathway offers activated C_1 units for

the biosynthesis of purines, thymidylate, some amino acids and the formyl group of fMet-$tRNA_f$ (Blakley, 1969). On the other hand, some clostridia, for example, C. acidiurici, are able to use the activity of the reverse reaction of the enzyme to generate ATP from 10-formyl-THF during purine-fermentation (Vogels and Van Der Drift, 1976).

In all prokaryotes examined so far, 10-formyl-THF synthetase, 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase have been found as separate monofunctional proteins, except for Clostridium thermoceticum (O'Brien et al., 1973) and E. coli (Dev and Harvey, 1978) in which the dehydrogenase and cyclohydrolase activities are associated as a bifunctional enzyme. The bacterial monofunctional 10-formyl-THF synthetase is a tetramer of identical subunits, containing four nucleotide binding sites (Curthoys and Rabinowitz, 1971).

C. acidiurici has a single copy of the 10-formyl-THF synthetase gene, which has been cloned in E. coli (Whitehead and Rabinowitz, 1986). The nucleotide sequence of the gene has been determined (Whitehead and Rabinowitz, 1988). The gene contains an open reading frame of 1668 bases coding for a protein of 59.6 kDa. There are two potential transcriptional initiation sites at positions -10 and -35 upstream of the translation start codon. The synthetase gene was expressed in E. coli using its own promoter (Whitehead and Rabinowitz, 1986). However, in this case the activity in E.

coli was 1.62 U/mg protein, only one eighth of the activity in *C. acidiurici*.

In eucaryotes, the three enzymatic activities mentioned above are associated as a single trifunctional polypeptide named C_1-THF synthase (see MacKenzie, 1984, for review). C_1-THF synthase contains two domains: the cyclohydrolase/dehydrogenase domain is located at the amino terminus and the synthetase domain is at the carboxyl terminus (Paukert *et al.*, 1977; Appling and Rabinowitz, 1985). The genes coding for the enzyme have been isolated from yeast, rat and human and characterized (Shannon and Rabinowitz, 1988; Thigpen *et al.*, 1990; Hum *et al.*, 1988). The amino acid sequence of the synthetase domain of the *Saccharomyces cerevisiae* C_1-THF synthase is 48% identical to the *C. acidiurici* 10-formyl-THF synthetase (Whitehead and Rabinowitz, 1988). There are two isozymes of the C_1-THF synthase in *S. cerevisiae*, one found in mitochondria and the other found in cytoplasm (Shannon and Rabinowitz, 1986). Both of them are encoded by nuclear genes. Amino acid sequence analysis showed that they are structurally related although not identical.

MATERIALS AND METHODS

1. Bacterial strains, bacteriophages and plasmids

All the bacterial strains used and isolated in this study are derivatives of wild type E. coli K12. These strains are described in Table 1. λ bacteriophage lines and plasmids involved in this study are also listed in Table 1.

2. Conditions for growth of bacteria

Bacteria were grown in either nutrient Luria-Bertani (LB) medium or minimal medium. LB medium was composed of 0.5% NaCl, 0.5% yeast extract and 1% tryptone. Minimal medium contained 1.5% K_2HPO_4, 0.52% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.02% $MgSO_4$, 0.001% $CaCl_2$, 50 μg/ml L-isoleucine, 50 μg/ml L-valine, and 0.2% either glucose or lactose as carbon source. The medium is called either glucose minimal medium or lactose minimal medium according to the carbon source. Isoleucine and valine were included because the E. coli strains are derivatives of MEW1 which carries an ilv deletion (Lin et al., 1990). Minimal medium without carbon source is called NIV solution. The concentrations of the supplements in minimal medium were, unless otherwise indicated, 300 μg/ml for glycine, 100 μg/ml

Table 1. *E. coli* strains, bacteriophages and plasmids

	Description	Reference
E. coli strain		
CU1008	K-12 Δ*ilvA*	L. S. Williams
MEW1	CU1008 Δ*lacZ*	1
MEW85	MEW1 *serA*	2
CCSG32	MEW85 *gcv*::λp*lac*Mu9	This work
CCSG32Fgd	Faster growing derivative of CCSG32/pTW2	This work
MEW204	Faster growing derivative of CCSG32/p187	This work
MEW207	MEW204 mutant able to grow well on glucose minimal medium with serine	This work
GOF1-6	MEW207 mutant able to use formate as C_1 source	This work
GOF33	Faster growing derivative of GOF3	This work
GOFNP	GOF33 without p187	This work
MEW200	MEW1 *gcv*::λp*lac*Mu9	The author
Bacteriophage		
λ p*lac*Mu9	λ p*lac*Mu1, Kan^r	3
λ pMu507	λ *c*l ts857 *sam*7 MuA^+B^+	3
Plasmid		
pGS146	pACYC184, carrying *gcv*	4
pTW2	Carrying the 10-formyl-THF synthetase gene, Amp^r	5
pUC18	*lacZ* Amp^r	6
p187	pUC18, carrying the 10-formyl-THF synthetase gene	This work

References: 1, Lin *et al*., 1990; 2, Lin *et al*., 1992; 3, Bremer *et al*., 1985; 4, Stauffer *et al*., 1986; 5, Whitehead and Rabinowitz, 1986; 6, Yanisch-Perron *et al*., 1985.

for serine, and 500 μg/ml for formate. Sodium formate was used throughout this study except in one case, as indicated, ammonium formate was used. The concentrations of antibiotics were 100 μg/ml ampicillin (Amp) and 80 μg/ml kanamycin (Kan).

E. coli cells used for λ phage infection were grown in tryptone broth containing 1% tryptone and 0.05% NaCl.

For bacterial growth in liquid medium, cells were usually cultured in 20 ml of medium contained in a 250-ml flask and were agitated on a shaker. For growth on solid medium, 1.8% agar was added to LB medium or 0.8% gelrite to minimal medium. The temperature for growth was 37°C. The incubation time was usually 16 h unless otherwise indicated.

In liquid media, growth of bacteria was assayed by the turbidity of culture measured by Klett colorimeter with a No. 42 blue filter. Doubling times were determined from the logarithmic phase of the growth curves. On solid media, the growth was scored according to the size of colonies or the appearance of short streaks.

3. 10-Formyltetrahydrofolate synthetase assay

Bacteria were grown in liquid media till the absorbency at 550 nm was 1.0. Cultures were centrifuged at 5,000 x g at 4°C and cells were resuspended in buffer A (50 mM Tris-HCl, pH 7.5, 10 mM KCl, 10 mM ß-mercaptoethanol) and lysed by

sonication. Sonicated cells were centrifuged at 10,000 x g for 20 min at 4°C and supernatants were used for enzymatic activity assays. Protein concentration of cell crude extracts was determined by the dye-binding assay of Bradford (1976) with bovine serum albumin as standard.

10-Formyl-THF synthetase activity was assayed as described by McGuire and Rabinowitz (1978) and modified by Whitehead and Rabinowitz (1986). The assay was performed in 100 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 40 mM ammonium formate, 5 mM sodium ATP and 2 mM THF (CALBIOCHEM). The reaction was started by mixing 0.15 ml of buffer A-diluted cell extracts with 0.35 ml of a stock solution, prewarmed at 37°C for 1 min, which would bring Tris-HCl, KCl, $MgCl_2$, ammonium formate, sodium ATP and THF to their respective final concentrations. Reaction was then carried on at 37°C for exactly 10 min and stopped by addition of 1 ml of 2% perchloric acid to convert 10-formyl-THF to 5,10-methenyl-THF. The acidified reaction mixtures were incubated at 22°C for at least 10 min. Then, the absorbency at 350 nm was measured. Control assays were performed in the same way except that cell extract was not included. The ϵ_{350} of 5,10-methenyl-THF is 24,910 M^{-1}. One unit of activity is the amount of enzyme needed to produce 1 μmole of 10-formyl-THF in 1 min (equivalent to 1 International Unit).

THF used in this study gave rise to background absorbency at 350 nm of approximately 0.1 in control reactions, but the

synthetase activity determined was linear with the amount of enzyme added as long as the absorbency did not exceed 1.3 (data not shown).

4. Serine hydroxymethyltransferase assay

SHMT activity was measured by determining the formation of [^{14}C]formaldehyde from the cleavage of [^{14}C]serine by the method of Taylor and Weissbach (1965) with modifications. Reactions were done in 450-μl mixtures containing 8.3 mM potassium phosphate buffer, pH 7.5, 2.2 mM d,l,L-tetrahydrofolic acid, 8.3 mM pyridoxal phosphate, 0.025 μmole (27,000 cpm) 3-[^{14}C]-L-serine (ICN) and 100 μl of cell crude extract. The mixtures were incubated at 37°C for 5 min before the reactions were started by the addition of labelled serine. The reactions were carried out at 37°C for 9 min and stopped by the addition of 0.3 ml of cold 1 M sodium acetate, pH 4.5. After incubation on ice for 5 min, 200 μl of 0.1 M formaldehyde and 300 μl of 0.4 M 5,5-dimethyl-1,3-cyclohexanedine in 50% ethanol were added to the reaction mixtures. The mixtures were then heated at 98°C for 5 min followed by cooling at 22°C for 10 min. Precipitates were collected by filtration on filter paper and washed with water. The filter paper was then dried under a heat lamp. Radioactivity on the filter paper was measured in a liquid

scintillation counter. One unit of SHMT activity is the amount of enzyme to catalyze the formation of 1 μmole of [^{14}C]-formaldehyde per hour at 37°C.

5. DNA manipulation

Conditions for enzymatic reactions with restriction endonuclease, the Klenow enzyme and ligase were according to suppliers' instructions (BIO/CAN or Pharmacia). DNA fragments were purified from agarose gel with the GENECLEAN kit (BIO 101) after electrophoresis. Plasmid isolation, agarose gel electrophoresis of DNA, transformation of bacterial cells with plasmids, and other procedures concerning DNA manipulation were performed according to a laboratory manual (Sambrook *et al.*, 1989).

6. Subcloning the *C. acidiurici* 10-formyltetrahydrofolate synthetase gene for expression in *E. coli*

The insert of plasmid pTW2 is an 8.3-kb *C. acidiurici* genomic DNA fragment including the 10-formyl-THF synthetase gene (Whitehead and Rabinowitz, 1986). pTW2 was digested with *Hpa*II and the 4.5-kb fragment from the insert was purified by agarose gel electrophoresis. This fragment was further

digested with XbaI resulting a 3-kb fragment which contains the coding region of the 10-formyl-THF synthetase gene. The translation start site is 20-bp from the HpaII end of the 3-kb fragment. This fragment was end-filled with nucleotides by the Klenow enzyme, and inserted into the SmaI site in the vector pUC18 by T4 DNA ligase. After transforming CCSG32 cells with this construct, the plasmid was extracted and digested with EcoRI, which site is on the vector, filled with dATP and dTTP by the Klenow enzyme, and circularized again by T4 DNA ligase. The resulting plasmid was named p187 and used for transforming CCSG32 and other strains.

7. Isolation of *E. coli* mutants

7.1. Isolation of *serA gcv* double mutants

The *serA* strain MEW85 (Lin *et al.*, 1992) was grown in tryptone broth at 37°C for 24 h. Ten milliliters of the culture were mixed with 10^{11} pfu of each of λ *plac*Mu9 (Bremer *et al.*, 1985) and the helper phage λ pMu507. After incubation at 22°C for 30 min, 10 ml of tryptone broth containing 40 mM sodium citrate were added and the mixture was incubated at 37°C for 1 h with shaking. Cells were collected by centrifugation, washed twice in NIV solution, and resuspended

in 0.84% NaCl. One milliliter of the cell suspension was mixed with 10 ml of lactose minimal medium with glycine and ampicillin and incubated at 37°C for 4 h with shaking to kill GCV' cells which were able to grow in the presence of glycine. The gcv cells were subsequently washed in NIV solution twice and spread on plates of lactose minimal medium with serine and kanamycin. Colonies were checked for the ability of growing on lactose minimal medium with glycine and kanamycin. Isolates which could grow on the serine-containing medium but not on the glycine-containing medium were selected as putative serA gcv double mutants. MEW85 cells were transduced with the P1 lysates made from these isolates according to Miller (1972). serA gcv transductants were selected in the same way as mentioned above to confirm that the gcv phenotype was due to a single event of mutation.

7.2. Isolation of 10-formyltetrahydrofolate synthetase gene-containing serA gcv double mutants which grow well on LB medium

CCSG32/pTW2 cells were plated on LB medium containing kanamycin and ampicillin and grown at 37°C for 24 h. Larger single colonies were subcultured on the same medium as short streaks. After growing, spontaneous mutants which showed healthier growth were applied to another round of selection.

The same procedures were applied to CCSG32/p187 to screen for corresponding faster growing spontaneous mutants.

7.3. Isolation of 10-formyltetrahydrofolate synthetase gene-containing serA gcv double mutants which grow well in glucose minimal medium with serine

Cells of strain MEW204, a faster growing mutant derived from CCSG32/p187, were grown at 37°C for 24 h in liquid LB medium containing kanamycin and ampicillin. After growth, the cells were washed in NIV solution, then subcultured in liquid glucose minimal medium with serine, ampicillin and a combination of amino acids each at 50 μg/ml. There were four different combinations: 1) tryptophan, tyrosine, phenylalanine and histidine; 2) methionine, arginine, proline and alanine; 3) leucine, glycine, glutamic acid and glutamine; and 4) lysine, threonine, cysteine, aspartic acid and asparagine. After growing at 37°C for 27 h, the cells were plated on glucose minimal medium with serine and ampicillin. Single colonies were further streaked on the same medium with short lines. Spontaneous mutants showing faster growth were subjected to another round of selection.

7.4. Isolation of mutants using external formate as C_1 source

MEW207, an MEW204-derivative which can grow well in glucose minimal medium with serine, was grown in liquid glucose minimal medium with glycine, formate, ampicillin and 50 μg/ml methionine at 37°C for 52 h. The cultures were spread on LB-plates containing kanamycin and ampicillin. After incubation at 37°C, single colonies appeared on the plates. Cells of these spontaneous mutants, named GOF strains, were screened for faster growing mutants. The single colonies of the GOF strains grown on LB plates containing kanamycin and ampicillin were subcultured on glucose minimal medium with glycine and formate by streaking short lines. Faster growing isolates were applied to another round of selection.

8. [^{14}C] formate incorporation

Test strains were cultured in liquid test media containing non-radioactive formate and subcultured into the same corresponding media containing [^{14}C] formate (ICN) diluted with unlabelled formate to 50,000 dpm/μmole. The final concentration of formate in media was 500 μg/ml. The bacteria were then grown at 37°C for 18 h to reach full growth. The cultures were chilled on ice and the cells were harvested by

centrifugation. Proteins were isolated according to Revel and Magasanik (1958). Briefly, the cells were extracted with 5% ice-cold trichloroacetic acid (TCA) on ice for 15 min. Macromolecules were precipitated by centrifugation. Pellets were resuspended in 75% ethanol and heated at 80°C for 10 min. After centrifugation, the pellets were resuspended in 75% ethanol : ether (1 : 1) and incubated at 45°C for 10 min. Ethanol-ether-insoluble fractions were pelleted and extracted with 5% TCA in a boiling water bath for 10 min to solubilize nucleic acids. The hot TCA extraction was repeated once. Hot TCA-insoluble fractions which contained proteins were pelleted and washed subsequently in acidified ethanol (95% ethanol : 1 N HCl = 100 : 0.2) and ether. Aliquots of the protein fractions were hydrolysed by 4 mg/ml trypsin and the radioactivity was measured in a liquid scintillation counter.

The analysis of the incorporation of [^{14}C]formate into individual amino acid residues of bacterial proteins was done by the Department of Biochemistry, University of Toronto, Toronto. The protein fractions were hydrolysed in 6.0 N HCl at 107°C for 22 h and the radioactivity of different amino acids was analyzed with an amino acid analyzer.

RESULTS

1. Expression of the C. acidiurici 10-formyltetrahydrofolate synthetase gene in E. coli

1.1. Isolation of E. coli serA gcv mutants

To study if expression of the C. acidiurici 10-formyl-THF synthetase gene in E. coli can lead to utilization of external formate as C_1 source, a mutant in which C_1 units can not be generated internally in minimal medium is required. serA gcv mutants can serve this purpose since that the serA mutation blocks the biosynthesis of serine from glucose and the generation of C_1 units through the conversion of serine to glycine is prevented, and the gcv mutation confers the deficiency in the GCV pathway (see Introduction). To constructed this strain, an E. coli serA mutant, MEW85 (Lin et al., 1992), was infected with λ placMu9 (Bremer et al., 1985). If the phage DNA is integrated into the gcv operon in proper positions, the gcv genes will be disrupted. Therefore, cells which could grow on lactose minimal medium with serine but not on medium with glycine were selected, and P1 lysates were made from such cells. MEW85 was transduced with the P1 lysates to confirm the gcv mutation was due to a single event of

insertion of λ placMu9. Transformation of one of the transductants, CCSG32, with plasmid pGS146 which carries the gcv operon (Stauffer et al., 1986) caused the growth of CCSG32 on glucose minimal medium with glycine, indicating the gcv mutation in CCSG32 was complemented by the gcv operon in pGS146. Thus, the serA gcv double mutations were confirmed in CCSG32 and this strain was used for subsequent experiments.

CCSG32 could not grow on glucose minimal medium with glycine and formate. This indicates that CCSG32, like wild type E. coli, can not use external formate as C_1 source.

1.2. Introducing the C. acidiurici 10-formyltetrahydrofolate synthetase gene into E. coli serA gcv mutants

Plasmid pTW2 which contains the C. acidiurici 10-formyl-THF synthetase gene and the ampicillin-resistant gene (Whitehead and Rabinowitz, 1986) was introduced to CCSG32 cells by transformation. Transformants were selected on LB agar medium containing ampicillin. Colonies of the transformants were very small. The growth of subsequent streaks on LB plates looked unhealthy.

Spontaneous mutants with normal growth on LB medium were then to be isolated. Larger colonies of the transformants were streaked on LB plates. Those which showed healthier growth were selected and applied to another round of

selection. The phenotype of serA gcv, that is, growth on serine-containing glucose minimal medium but no growth on glycine-containing medium, was checked during selection. Finally strain CCSG32Fgd which contained the plasmid-borne C. acidiurici 10-formyl-THF synthetase gene and grew much better on LB plates than the original transformants was isolated.

1.3. The C. acidiurici 10-formyltetrahydrofolate synthetase gene was expressed in E. coli transformants

The 10-formyl-THF synthetase activity of the transformants was assayed on crude extracts of cells grown in liquid LB medium. The results are shown in Table 2. CCSG32, which did not have pTW2, showed no significant activity. CCSG32 transformed with pTW2 showed the 10-formyl-THF synthetase activity of 0.64 u/mg protein. CCSG32Fgd, which could grow better on LB plates, had about twice of the activity of CCSG32/pTW2.

Although CCSG32Fgd had 10-formyl-THF synthetase activity and showed better growth in LB medium, it could not grow on glucose minimal medium containing glycine and formate. This indicates that CCSG32 could not use external formate as its sole C_1 source. Moreover, no spontaneous formate-utilizing mutants derived from LB-grown CCSG32Fgd cultures were obtained by subculturing on glucose minimal medium with glycine and

Table 2. Growth and 10-formyl-THF synthetase activity of *E. coli* strains containing the *C. acidiurici* gene coding for the enzyme

Strain	Plasmid	Growth on LB plate	10-Formyl-THF synthetase activity (u/mg protein)
CCSG32	None	+++	0.005
CCSG32	pTW2	+	0.64
CCSG32Fgd	pTW2	++	1.20
CCSG32	p187	++	3.75
MEW204	p187	+++	6.45

To assay enzymatic activity, overnight liquid LB cultures were subcultured into liquid LB and incubated at 37°C until A_{600} reached 1.0. Crude cell extracts were prepared by sonication. Enzymatic activity was determined as described in Materials and Methods. One unit of activity is the amount of enzyme needed to produce 1 μmole of 10-formyl-THF in 1 minute at 37°C (equivalent to 1 International Unit). The data shown are the mean values from at least two experiments. +, unhealthy growth; ++, less unhealthy growth; +++, normal growth.

formate.

1.4. Isolation of E. coli strains with higher 10-formyltetrahydrofolate synthetase activity by subcloning the gene in E. coli

The inability of CCSG32Fgd to use external formate was probably due to the low 10-formyl-THF synthetase activity (1.2 u/mg protein vs. 13 u/mg protein in C. acidiurici, see Table 2 and Whitehead et al., 1988). To circumvent this problem, I then attempted to put the gene under the control of the stronger LacZ promoter to enhance the expression. pTW2 contained an 8.3-kb C. acidiurici genomic DNA fragment which carried the synthetase gene (Whitehead and Rabinowitz, 1986). The 3-kb HpaII/XbaI fragment contained the coding region. At the HpaII end of this 3-kb fragment, there were only 20 base pairs upstream the translation start codon of the gene. This fragment was treated with the Klenow enzyme to make ends blunt, and was inserted into the SmaI site of the vector pUC18. The EcoRI site on the vector was digested and filled by the Klenow enzyme to ensure the coding sequence of the synthetase gene was in-frame with the start codon for the ß-galactosidase. After transforming cells of CCSG32, the transformants were assayed for the 10-formyl-THF synthetase activity. One of the transformants showed 3.75 u/mg protein

of the synthetase activity (Table 2). This is three times that of CCSG32Fgd and nearly 30% of that of C. acidiurici.

The plasmid contained in this transformant was named p187. However, after checking the restriction enzyme map of the plasmid, I later realized that the coding sequence of the synthetase gene in p187 was in the opposite orientation to the LacZ promoter. The vector sequence upstream the synthetase gene might act as a promoter in p187 leading to the expression of the gene. The value of the enzyme activity, 3.75 u/mg protein, allowed me to try to isolate faster growing derivatives of CCSG32/p187 with possibly higher activity as demonstrated by the comparison of CCSG32 and CCSG32Fgd (0.64 vs. 1.2 u/mg protein). Such a screening resulted in the isolation of strain MEW204 in which 10-formyl-THF synthetase activity was 6.45 u/mg protein, almost 50% of that of C. acidiurici (Table 2). Therefore, MEW204 was used for subsequent experiments.

1.5. Isolation of E. coli serA gcv mutants with higher 10-formyltetrahydrofolate synthetase activity and normal growth on glucose minimal medium with serine

To test whether the transformed cells could use external formate, they must be grown in minimal medium, and thus should be able to grow in minimal medium at a reasonable rate.

Although MEW204 grew normally in LB medium, its growth in glucose minimal medium with serine was poor compared to strain CCSG32. It is possible that certain LB components, which are absent in the glucose minimal medium, could promote the growth of MEW204. Supplementation with casamino acids also improved the growth. Therefore, I tried to isolate spontaneous mutants which could grow at a reasonable rate in glucose minimal medium with serine as well as in LB medium. The selection was done on single colonies grown on glucose minimal medium with serine by subculturing them on the same medium by streaking short lines. The growth of the streaks was examined and better growing isolates were subjected to another round of selection. Finally, seven faster-growing strains were obtained. These strains still maintained the serA gcv phenotype. Among them, MEW207 is the fastest growing strain.

Plasmid of strain MEW207 was extracted and used to transform the original strain CCSG32. None of the transformants grew well on glucose minimal medium with serine. Therefore, the ability of MEW207 to grow well on minimal medium was likely due to changes on the chromosomal DNA of MEW204.

It was now possible to look at the expression of the C. acidiurici 10-formyl-THF synthetase gene in E. coli cells grown on minimal medium by measuring the enzymatic activity in cell extracts. Strain MEW207 demonstrated higher 10-formyl-THF synthetase activity when grown in glucose minimal medium

with serine than when grown in LB medium (3.53 vs. 2.10 u/mg protein, see Table 3). However, the activity of LB-grown MEW207 was only 33% of that of LB-grown MEW204 after mutation occurred (Table 3).

2. Isolation and characterization of E. coli mutants which could use external formate as C_1 source

2.1. Isolation of E. coli mutants which could use external formate

The aim of this study is to isolate mutants which can use external formate as C_1 source after being transformed with the C. acidiurici 10-formyl-THF synthetase gene. Although strain MEW207 had higher 10-formyl-THF synthetase activity and could grow well on glucose minimal medium with serine, yet it could not grow on minimal medium containing formate, ranging from 500 to 2,000 μg/ml, and glycine (Table 4). Glycine was necessary to be included in the minimal medium because the synthesis of serine in this situation was intended to rely on the reverse reaction of SHMT with glycine and C_1 units as substrates (see Introduction).

However, MEW207 showed delayed growth in some experiments in which the formate-containing medium was supplemented with

Table 3. 10-Formyl-THF synthetase activity of *E. coli* strains grown on LB medium or glucose minimal medium with serine

Strain	Plasmid	10-Formyl-THF synthetase activity (u/mg protein)	
		LB medium	Minimal medium with serine
CCSC32	none	0.005	0.021
MEW204	p187	6.45	N.D.
MEW207	p187	2.10	3.53

Crude cell extracts were prepared from bacteria grown on LB medium or glucose minimal medium with serine. 10-Formyl-THF synthetase activity was assayed as described in Materials and Methods. Data shown are mean values from at least two experiments. N.D., not determined.

Table 4. Growth of strain MEW207 in liquid glucose minimal media with various supplements

Supplement in medium	Growth		
	7 h	22 h	52 h
Ser	++	++	N.D.
Gly, formate (500 μg/ml)	-	-	-
Gly, formate (1000 μg/ml)	-	-	-
Gly, formate (2000 μg/ml)	-	-	-
Gly, formate, Met (50 μg/ml)	-	±	++
Gly, formate, C_1 end-products	-	±	++

Strain MEW207 was grown on liquid glucose minimal medium with serine at 37°C for 16 h. Cells were washed and resuspended in liquid glucose minimal medium and subcultured in liquid glucose minimal medium with various supplements as well as ampicillin. The subcultures were incubated at 37°C for up to 52 h. At 22 h, fresh ampicillin was added again. The bacterial growth was measured with a Klett colorimeter. -, no growth; ±, light growth; ++, heavy growth; N.D., not determined. Serine was at 100 μg/ml; glycine 300 μg/ml; formate 500 μg/ml unless otherwise indicated. C_1 end-products are a mixture of adenine, guanine, histidine and methionine with final concentrations at 40, 40, 20 and 20 μg/ml respectively.

methionine or C_1 end-products (Table 4). To determine whether the growth was due to reverse mutation, the phenotype of the isolates from the cultures was examined. The 52-h cultures grown in glucose minimal medium with glycine, formate, and methionine or C_1 end-products were plated on LB plates. Single colonies from the plates were tested for growth on glucose minimal medium, glucose minimal medium with glycine and glucose minimal medium with serine. All the isolates could grow on glucose minimal medium with serine but not on glucose minimal medium or glucose minimal medium with glycine. This indicates that these isolates still kept the phenotype of serA gcv mutants. Twenty-three isolates derived from the C_1 end-products-containing medium showed slight growth on glucose minimal medium with glycine and formate even without supplementation of the C_1 end-products. This indicates that the growth was not due to the supplementation but due to spontaneous mutation. Since these isolates could grow on glucose minimal medium with glycine and formate but not on glucose minimal medium with glycine only, formate in the medium must be essential for their growth, that is, they could use external formate. These isolates were named GOF for growing on formate. Six of them, GOF1 to 6, were selected for further studies.

2.2. Growth properties of the *E. coli* formate-utilizing strains

The GOF strains were isolated from solid glucose minimal medium with glycine and formate. I then tested their growth properties in the corresponding liquid medium. Strain GOF1 which showed better growth on plates was selected for the test. A pre-culture of GOF1 in liquid glucose minimal medium with serine derived from cells grown on LB plates was subcultured in liquid glucose minimal media with various supplements. The growth curves (Figure 3) indicate that GOF1 could grow in liquid glucose minimal media with glycine and formate (whether the sodium or ammonium salt) and that GOF1 could not grow on glucose minimal medium without supplement, or with glycine or formate alone. In other words, in liquid glucose minimal media, GOF1 could grow in the presence of glycine and formate, instead of serine. However, the doubling time in the serine-containing medium was 1.5 h, while in the glycine/formate-containing media it was 3.0 h for the ammonium salt of formate and 3.6 h for the sodium salt of formate (Figure 3).

The effects of different concentrations of formate or glycine in liquid glucose minimal media on the growth of strain GOF1 were also tested. Variations in the concentrations of formate ranging from 200 to 3,000 μg/ml or concentrations of glycine ranging from 100 to 500 μg/ml did

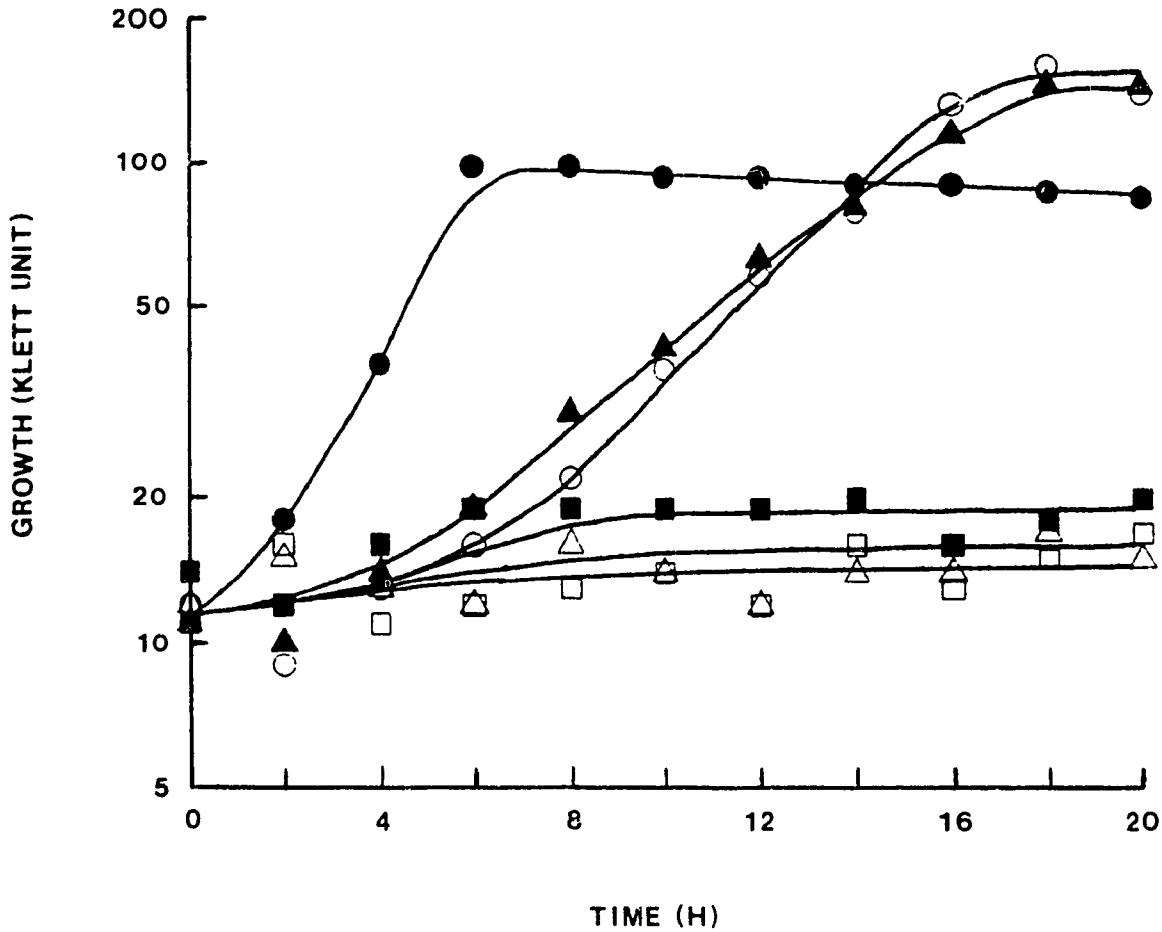


Figure 3. Growth of strain GOF1 in glucose minimal media. An overnight culture in liquid glucose minimal medium with serine was washed in minimal medium and 0.5% subcultured into glucose minimal medium with serine (●), glycine (□), ammonium formate (△), glycine and sodium formate (▲), or glycine and ammonium formate (○), or with no supplements (■). The subcultures were incubated at 37°C with shaking and the turbidity was measured with a Klett colorimeter every 2 h. Concentrations of serine, glycine and formate were 100, 300 and 500 μg/ml respectively.

not have significant influence on the growth of GOF1 (not shown). The phenotype of these liquid cultures was tested to be still serA gcv indicating the growth was not due to reverse mutation or contamination.

2.3. Isolation of a faster-growing E. coli formate-utilizing mutant

Strains GOF 1 to 6 could grow on glucose minimal medium with glycine and formate with relatively slow rates (Figure 3). Plates with streaks of inocula of these strains demonstrated nearly visible growth 12 h after inoculation and light growth at 24 h. Spontaneous faster growing mutants derived from these GOF strains should be isolated. This was achieved by selecting better growing derivatives of GOF1 to 6 subcultured on agar plates of glucose minimal medium with glycine and formate. Three rounds of screening were performed resulting in the isolation of strain GOF33, originated from strain GOF3, which showed light growth on solid glucose minimal medium with glycine and formate 12 h after inoculation and full growth at 24 h. Strain GOF33 still kept the phenotype of serA gcv mutants.

2.4. Mutations in chromosomal DNA mediated the ability of the GOF strains to utilize external formate

Mutations must have occurred in the original strain CCSG32/p187 that finally gave rise to the ability of GOF strains including GOF33 to be able to utilize external formate. It was not clear whether plasmid or chromosomal DNA carried these mutations. Plasmid-curing and re-transformation with plasmid could answer this question. Since p187 carried an ampicillin-resistance gene, I used LB medium without ampicillin to isolate GOF33 cells which spontaneously lost their plasmids. Plasmid-cured isolates, named GOFNP, were no longer able to grow on glucose minimal medium with glycine and formate (Table 5). Transformation of these plasmid-free cells with pTW2 or p187 showed that p187 could restored the ability of the cells to grow well on glucose minimal medium with glycine and formate (Table 5). Therefore, there was no difference between p187 and the plasmid carried by GOF33 cells. Further, the plasmid in GOF33 cells, named pGOF, was isolated and introduced into the original CCSG32 cells by transformation. Neither pGOF nor p187 could make CCSG32 cells to grow on glucose minimal medium with glycine and formate (Table 5). Taken together, these experiments indicate that the mutations on the chromosomal DNA but not on the plasmid enabled the cells to grow well on glucose minimal medium with glycine and formate when the 10-formyl-THF synthetase gene is

Table 5. Effects of different plasmids on the growth of *E. coli* strains GOFNP and CCSG32 in various media

Strain	Glucose minimal medium with				LB medium
	No addition	Gly	Gly and formate	Ser	
GOFNP	-	-	-	+	+
GOFNP/pTW2	-	-	±	+	+
GOFNP/p187	-	-	+	+	+
CCSG32/pGOF	-	-	-	+	+
CCSG32/p187	-	-	-	+	+

Bacteria were subcultured from LB plates onto different solid media as indicated and grown at 37°C for 72 h. When a strain carried a plasmid, ampicillin was included in the media. +, normal growth; ±, light growth; -, no growth.

present.

2.5. Incorporation of external [^{14}C]formate into bacterial proteins

If external formate was used by the GOF cells, the carbon from formate would be incorporated into bacterial proteins since the biosynthesis of some amino acids needs C_1 units (see Introduction). [^{14}C]formate was used to test this possibility. Bacteria were grown in either glucose minimal medium with glycine and [^{14}C]formate or glucose minimal medium with serine and [^{14}C]formate. Proteins were extracted from these cells and assayed for their radioactivity. Results showed that considerable amount of ^{14}C was incorporated into the proteins of GOF33 cells, whereas only trace amount of radioactivity was detected in proteins from the plasmid-cured GOF33 cells which did not contain the 10-formyl-THF synthetase gene (Table 6). There was more incorporation in GOF33 cells grown in the glycine-containing medium than in the serine-containing medium probably due to the fact that serine in the medium was a C_1 source as well (see Introduction). Also, little radioactivity was found in proteins of strain CCSG32 as well as strain MEW200 (isolated by the author) which was a gcv mutant and could not use external formate.

Incorporation of ^{14}C into individual amino acid residues

Table 6. Incorporation of ^{14}C into proteins of bacteria grown on glucose minimal media containing [^{14}C]formate

Strain	The 10-formyl-THF synthetase gene	Radioactivity of bacterial proteins (dpm)	
		Gly and [^{14}C]formate	Ser and [^{14}C]formate
GOF33	Yes	2587	1098
GOFNP	No	-	82
CCSG32	No	-	115
MEW200	No	68	51

Bacteria were grown in liquid glucose minimal media with supplements as indicated at 37°C for 18 h with full growth. The specific radioactivity of [^{14}C]formate in the media was 50 dpm/nmole, and its concentration was 500 μg/ml. The concentration of serine used here was 400 μg/ml. Bacterial proteins were extracted as described in Materials and Methods and their radioactivities were measured with a liquid scintillation counter. Data shown represent the radioactivity in proteins from 0.2 ml of bacteria culture. They are mean values from 3 to 4 experiments except that of GOFNP and CCSG32 which are from only one test. Note that GOFNP and CCSG32 did not grow in glucose minimal medium with glycine and formate.

in the proteins was analyzed by the Department of Biochemistry, University of Toronto, Toronto. The [^{14}C]-incorporated proteins were hydrolysed with HCl and the radioactivity of different amino acids was measured by an amino acid analyzer. The specific radioactivity of serine, methionine and histidine from GOF33 cells grown in glucose minimal medium with glycine and [^{14}C]formate was very high, roughly equal to the specific radioactivity of [^{14}C]formate in the medium, that is, 50 dpm/nmole (Table 7). The radioactivity of lysine and alanine was much lower than 50 dpm/nmole. When GOF33 was grown in glucose minimal medium with [^{14}C]formate and non-radioactive serine, there was still [^{14}C]-incorporation into serine residues in proteins, although only 14% of that of the cells grown in the glycine-containing medium (Table 7). On the other hand, there was a 50% decrease in incorporation for methionine; the decrease for histidine was very little; and [^{14}C] was not incorporated into lysine or alanine. There was almost no incorporation in CCSG32 (Table 7). These incorporation data constitute a direct evidence that external formate was utilized by GOF33 cells.

2.6. Serine hydroxymethyltransferase activity

SHMT is an important enzyme in the E. coli C_1 metabolism. Strain GOF33 resulted from a series of mutant selections. It

Table 7. Incorporation of ^{14}C into amino acid residues in proteins of *E. coli* strains GOF33 and CCSG32 grown in [^{14}C]formate-containing media

Amino acid	Specific radioactivity (dpm/nmole)		
	GOF33 (Gly, formate)	GOF33 (Ser, formate)	CCSG32 (Ser, formate)
Serine	55.0	7.8	1.5
Methionine	51.9	26.0	< 1.0
Histidine	45.0	43.2	< 1.0
Lysine	9.5	< 1.0	< 1.0
Alanine	6.0	< 1.0	< 1.0

Protein samples were prepared as described in Table 6. The supplements to the glucose minimal media are indicated in brackets. [^{14}C]formate in media was 50 dpm/nmole and 500 μg/ml. Extracted proteins were hydrolysed with 6 N HCl at 107°C for 22 h. The radioactivities of amino acids in protein lysates were analyzed with an amino acid analyzer. Data shown are from one experiment.

was interesting to see if there were any changes in the activity of SHMT. The SHMT activity was assayed on crude cell extracts. When the cells were grown in glucose minimal medium with serine, the SHMT activity of strains CCSG32, MEW207, GOF33 and GOFNP was similar to each other, ranging from 1.4 u/mg protein in CCSG32 to 1.9 u/mg protein in GOF33 (Table 8). However, there was a 63% increase, from 1.9 to 3.1 u/mg protein, of the SHMT activity in strain GOF33 when it was grown in the glucose minimal medium with glycine and formate compared to the growth in the minimal medium with serine (Table 8).

Table 8. Serine hydroxymethyltransferase activity of *E. coli* strains

Strain	SHMT activity (u/mg protein)	
	Ser	Gly and formate
CCSG32	1.4	-
MEW207	1.7	-
GOFNP	1.8	-
GOF33	1.9	3.1

Cell extracts were made from bacteria grown in liquid glucose mimimal media with supplements as indicated. The SHMT activity was assayed by the method of Taylor and Weissbach (1965) with some modifications. 3-[^{14}C]-L-serine was used as substrate. One unit of the SHMT activity is defined as the amount of enzyme to form 1 μmole of formaldehyde per hour at 37°C. Note that only GOF33 could grow in glucose minimal medium with glycine and formate. Data shown are the mean values from two experiments.

DISCUSSION

There are two routes in E. coli to generate C_1 units: the conversion of serine to glycine by SHMT and the glycine cleavage pathway (Figure 1, see Introduction). E. coli can not use external formate since it does not have the 10-formyl-THF synthetase activity to convert formate to activated C_1 units. This work was designed to provide E. coli with a foreign gene coding for 10-formyl-THF synthetase activity and determine the metabolic effects of this addition to its genome.

To do this, I attempted to introduce the C. acidiurici 10-formyl-THF synthetase gene into an E. coli serA gcv mutant which could not make its own C_1 units and investigate whether E. coli could use external formate as C_1 source. The serA mutation caused deficiency in the first pathway by blocking serine biosynthesis from glucose. Therefore, C_1 units could not be produced through the conversion of serine to glycine in serA mutants grown in glucose minimal medium with glycine. On the other hand, the gcv mutation caused deficiency in producing C_1 units by the GCV enzymes. Such a strain could grow in rich medium but not in glucose minimal medium with glycine. However, if the added 10-formyl-THF synthetase gene was functional, the strain should be able to grow in glucose minimal medium with glycine and formate. Glycine was

necessary to be included in the medium because the formation of serine was intended to rely on the reverse reaction of SHMT with glycine and activated C_1 units (Figure 1 and 2) External formate was also needed since E. coli produces formate only during anaerobic growth (Gottschalk, 1986).

The use of formate was indeed possible, not by the original strain but by a derivative. I introduced the C. acidiurici 10-formyl-THF synthetase gene to an E. coli serA gcv double mutant, and by selecting a series of spontaneous mutants, I finally obtained strain GOF33 (serA gcv, containing the 10-formyl-THF synthetase gene) which could grow in glucose minimal medium with glycine and formate. Incorporation of [^{14}C]formate showed evidence that the external formate was indeed utilized by GOF33.

1. Isolation of a 10-formyltetrahydrofolate synthetase gene-transformed E. coli mutant which could use external formate was successful

The C. acidiurici 10-formyl-THF synthetase gene carried in an 8.3-kb genomic DNA fragment was introduced to the E. coli serA gcv double mutant CCSG32 by transformation with plasmid pTW2 carrying the fragment. Expression of the synthetase gene was shown by the fact that enzyme activity could be determined. The enzyme activity in CCSG32/pTW2 was

measured to be 0.64 u/mg protein compared to 0.005 u/mg protein in CCSG32 (Table 2). But the growth of the transformants in nutrient LB medium was impaired. The reason for the poor growth is not clear, but is probably due to the presence of the insert in the plasmid pTW2 since that transformation of MEW1, the parent strain of MEW85 (serA), with pTW2 also caused poor growth of the transformants, whereas strain CCSG32 cells transformed with the vector alone (pUC18) had normal growth in both LB medium and minimal medium (data not shown). The impact of the foreign DNA fragment could be reduced by occurrence of mutation(s) as in the case of CCSG32Fgd which was a spontaneous mutant derived from CCSG32/pTW2 and could grow faster in LB medium. Interestingly, CCSG32Fgd expressed even higher 10-formyl-THF synthetase activity (1.2 u/mg protein, see Table 2). However, CCSG32Fgd was not able to use external formate because it could not grow in glucose minimal medium with glycine and formate. Neither could any formate-utilizing mutants, either spontaneous or induced, be isolated from CCSG32Fgd (data not shown). Multiple mutations might be necessary to overcome a number of blockages that prevented the E. coli cells to utilize external formate.

The 10-formyl-THF synthetase activity in CCSG32Fgd was 1.2 u/mg protein, much less than the activity in C. acidiurici which is 13 u/mg protein. Therefore, first, I tried to overexpress the synthetase gene in E. coli by a strong

promoter, that is, the lacZ gene promoter carried in the plasmid vector pUC18. Due to a mistake in subcloning, the coding region of the gene was put in a position downstream the lacZ promoter but in the wrong, opposite orientation. However, the subclone, CCSG32/p187, and its faster growing spontaneous mutant, MEW204, demonstrated respectively 3.75 and 6.45 u/mg protein of the 10-formyl-THF synthetase activity (Table 2). This unexpected expression of the gene was possibly driven by the vector sequence upstream the coding region of the synthetase gene. The synthetase activity was considerably higher in MEW204, almost 50% of that in C. acidiurici. But, again, neither CCSG32/p187 or MEW204 was able to grow in glucose minimal medium with glycine and formate.

The genetic codons for amino acids are conserved in almost all organisms although there is bias in the usage of these codons (Ycas, 1969). E. coli is widely used for production of heterogenous proteins, both prokaryotic and eucaryotic, when foreign DNA containing undisrupted coding sequences and E. coli-originated promoters and ribosome binding sequences are provided. In prokaryotes, ribosomes of both Gram-positive bacteria and Gram-negative bacteria, to which E. coli belongs, recognize the Shine-Dalgarno sequence for binding and initiation of translation. Moreover, many promoters from Gram-positive bacteria are active and functioning in E. coli. Indeed, a number of genes from the

Gram-positive clostridial bacteria have been reported to be successfully expressed in E. coli using their own promoters. These genes include those coding for lactate dehydrogenase (Contag et al., 1990), acetoacetyl-coenzyme A:acetate/butyrate:coenzyme A-transferase (Cary et al., 1990) and acetoacetate dehydrogenase (Petersen and Bennett, 1990) in Clostridium acetobutylicum, glucose isomerase in Clostridium thermosulfurogenes (Lee et al., 1990), and endo-1,4-ß-glucanase in Clostridium josui (Fujino et al., 1990). Therefore, expression of the C. acidiurici 10-formyl-THF synthetase gene in E. coli is possible, but codon usages should be considered.

Brinkmann et al. (1989) reported that overexpression of the E. coli dnaY gene, which encodes the rare arginine codon $tRNA_{AGA,AGG}$, was able to alleviate difference in preference of codon usage between E. coli and human genes, and could increase the yields of the foreign gene products in E. coli. In the case of C. acidiurici 10-formyl-THF synthetase, 16 out of the 17 arginine residues are coded by codon AGA and one by codon CGU (Whitehead and Rabinowitz, 1988). However, codon AGA is rarely used for E. coli proteins. I introduced the dnaY gene cloned in high expression vector into CCSG32/pTW2 and CCSG32/p187, but it did not help to increase the 10-formyl-THF synthetase activity in these strains and improve their growth (data not shown). Perhaps, the codon usage for other amino acid residues, for example, leucine and

isoleucine, should be considered, or, it might be that codon usage was just not the limiting factor in this case.

Although MEW204 could grow well in LB medium, its growth in glucose minimal medium with serine was poor. Addition of casamino acids into the minimal medium improved the growth of MEW204. However, one can not include amino acids in the minimal medium when testing if cells can use external formate since some amino acids, for example, methionine, histidine and tryptophan, are related to C_1 metabolism. The purpose of this study is to test if _E. coli_ can use external C_1 units in minimal medium with only glycine and formate. Therefore, I then attempted to isolate spontaneous mutants which could overcome the poor growth in minimal medium. MEW207 was the result of such a selection. The 10-formyl-THF synthetase activity of MEW207 was lower than that of MEW204 when the cells were grown in LB medium (2.10 vs. 6.45 u/mg protein, see Table 3), but when grown in glucose minimal medium with serine the synthetase activity of MEW207 remained relatively higher (3.53 u/mg protein, see Table 3), 27% of the activity of _C. acidiurici_. The nature of the spontaneous mutation(s) which caused the faster growth in glucose minimal medium with serine is difficult to speculate. Also, it is not clear how the expression of the foreign 10-formyl-THF synthetase gene is regulated in different media.

At this point, I tried to screen for isolates which can use external formate as C_1 source. MEW207 was not able to

grow in glucose minimal medium with glycine and formate; however, it showed delayed growth in one experiment when the medium was supplemented with methionine or C_1 end-products (Table 4). The growth was not due to the serA or gcv reverse mutation since isolates from these cultures were not able to grow on glucose minimal medium or glucose minimal medium with glycine. In fact, later tests showed that these isolates could grow in glucose minimal medium with glycine and formate even without supplementation of methionine or C_1 end-products. This indicates that new spontaneous mutations occurred in these isolates that led to the growth in the glucose minimal medium with glycine and formate. The growth of these GOF mutants, as represented by strain GOF1, was slower in the glycine and formate-containing medium than in the serine-containing medium (Figure 3). Again, a better-growing derivative, GOF33, was obtained through isolating spontaneous mutants.

Although the 10-formyl-THF synthetase activity of the GOF mutants were not determined in this study, the following experimental observations may indicate the synthetase was active in the GOF strains. First, GOF1 could grow in glucose minimal medium with glycine and formate but not in the medium with glycine only (Figure 3). This means that external formate, which is a direct substrate for 10-formyl-THF synthetase, was necessary for the growth. Second, by using [^{14}C]formate in glucose minimal medium with glycine and

formate, radioactivity could be traced to the cellular proteins and their composing amino acids in GOF33 (Table 6 and 7). Therefore, the external formate was actually utilized by the GOF cells. Finally, the 10-formyl-THF synthetase gene was necessary for the growth since the plasmid-cured cells could not grow in glucose minimal medium with glycine and formate (Table 5). It seems likely then that an external formate-utilizing E. coli serA gcv strain, that is, GOF33, has been isolated. The property of this strain to utilize external formate was conferred by the activity of the C. acidiurici 10-formyl-THF synthetase gene introduced to E. coli by transformation.

2. External formate was utilized as C_1 source by the 10-formyltetrahydrofolate synthetase gene-transformed E. coli serA gcv mutants

Addition of formate was essential for the growth of GOF33 in glucose minimal medium with glycine. Since GOF33 was serA gcv, no C_1 units could be produced in glucose minimal medium supplemented with glycine only (Figure 2). Therefore, it is reasonable to think that formate in the minimal medium was taken as C_1 source for GOF33. It was most likely that formate was used to synthesize 10-formyl-THF by 10-formyl-THF synthetase produced by the C. acidiurici gene. Then, 10-

formyl-THF could be converted to 5,10-methenyl-THF or 5,10-methylene-THF by 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase. These three compounds are activated C_1 donors in E. coli C_1 metabolism (Figure 1, see Introduction).

Addition of [^{14}C]formate in glucose minimal medium with glycine and formate demonstrated incorporation of ^{14}C into the protein fraction of the strain GOF33 (Table 6). When individual amino acid residues were considered, serine, methionine and histidine showed nearly the same specific radioactivity as that of the formate in the medium (Table 7). Since the biosynthesis of these amino acids needs C_1 units (Figure 1), this is a strong indication that the carbons derived from C_1 units in these amino acids were solely from the external formate. In the case of serine, the incorporation was likely due to the reverse reaction of SHMT, in which glycine and 5,10-methylene-THF were used to synthesize serine (Figure 2). The use of the reverse reaction of SHMT was also previously reported from this laboratory (Garnon, 1992). Since ^{14}C was incorporated into histidine in GOF33, it is reasonable to speculate, although not determined in this study, that this carbon had been incorporated into nucleic acids as well since the carbon 2 of the imidazole ring of histidine comes from the carbon 2 of the purine ring, both of the carbons are thought to be originated from C_1 (Blakley, 1969).

The extent of incorporation of ^{14}C into lysine and alanine were less than 20% (Table 7). It was low compared to that for serine, methionine and histidine whose synthesis are related to C_1 units. It should be noted that the fractions for lysine and histidine were very close to each other in the amino acid analysis, so that there might be cross contamination of radioactivity between these two amino acids. The incorporation of ^{14}C into tryptophan and cysteine could not be determined due to the limitation of the method for amino acid analysis used in this study. For the other amino acids, either there was no incorporation, or the poor resolution in the amino acid analysis did not allow to tell the exact rate of incorporation for an individual amino acid.

Interestingly, when GOF33 was grown in glucose minimal medium with [^{14}C]formate and, instead of glycine, serine, the radioactivity was still incorporated into bacterial proteins although less than in the glycine-containing medium (Table 6). External formate is not essential for the growth of E. coli serA gcv strains grown in the presence of serine since C_1 units can be produced through the conversion of serine to glycine by SHMT (Figure 1). Nevertheless, formate in the medium was assimilated in this case. At least three amino acid residues, serine, methionine and histidine, were shown to contain the ^{14}C derived from external formate (Table 7). The rate of incorporation into histidine was 86%, very close to the rate of 90% in the glycine-containing medium. It was

likely that 10-formyl-THF, the most direct C_1 source for the synthesis of histidine, derived mainly from formate through 10-formyl-THF synthetase but not from serine through SHMT (Figure 1). In the case of methionine, the incorporation was 52% in glucose minimal medium with serine and [^{14}C]formate. Therefore, it can be speculated that both serine and formate contributed equally in providing C_1 source for the synthesis of methionine via 5,10-methylene-THF (Figure 1). There was even a considerable amount, that is, 16%, of ^{14}C incorporated into serine itself. This is not surprising since serine could be synthesized by the reverse reaction of SHMT from glycine and 5,10-methylene-THF which might partly derived from external formate as mentioned (Figure 1). The reaction from serine to glycine should be favoured to meet the need for glycine by the cells. Taken together, it could be concluded that when GOF33 was grown in glucose minimal medium with formate and serine, the C. acidiurici 10-formyl-THF synthetase gene was also functioning and contributed significantly to C_1 source for cellular metabolism. However, the data I obtained here were not enough to indicate if there was any difference in the expression of this foreign gene or other E. coli genes involved in the C_1 metabolism.

3. Multiple mutations were required for the 10-formyltetrahydrofolate synthetase gene-transformed E. coli serA gcv strain to utilize external formate

Isolation of GOF33 was achieved through successive spontaneous mutations after the E. coli serA gcv strain CCSG32 was transformed with plasmid p187 carrying the 10-formyl-THF synthetase gene. First, MEW204, a derivative of CCSG32/p187 which grew faster on LB medium and had higher 10-formyl-THF synthetase activity was obtained. Then, I isolated an MEW204-derived mutant, MEW207, which showed better growth in glucose minimal medium with serine. From MEW207, GOF mutants which could grow in glucose minimal medium with glycine and formate were isolated. Finally, a faster-growing derivative of GOF mutant, GOF33, was obtained. All the mutations occurred on chromosomal DNA because when the plasmid-cured GOF33 was transformed with the original plasmid p187, it re-obtained the property of faster growth in glucose minimal medium with glycine and formate (Table 5).

What genes were affected by these mutations may be speculated according to the pathways of the C_1 metabolism in E. coli. The genes involved in converting 10-formyl-THF to 5,10-methenyl-THF and then to 5,10-methylene-THF, namely, the genes encoding 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase, were possibly essential for providing with activated C_1 units (Figure 1, see

Introduction). Therefore, enhanced expression of these genes might be expected in the mutants.

Since GOF33 carried the serA mutation, the biosynthesis of serine possibly relied on the reverse reaction of the glyA gene product SHMT as previously discussed. The activity of SHMT was slightly higher in GOF33 when grown in glucose minimal medium with glycine and formate than in the medium with serine (Table 8). Glycine at 3,000 μg/ml inhibits glyA (see Introduction). However, in this study, the concentration of glycine in media was less than 500 μg/ml. One might speculate that GOF33 probably had a mechanism of induction of the glyA gene in the glycine and formate-containing minimal medium to elevate the SHMT activity to produce sufficient serine by the reverse reaction of the enzyme.

Besides serine, the biosynthesis of histidine and methionine also relied on the external formate since there was also more than 90% of incorporation of ^{14}C for both amino acids (Table 7). This implies that the enzymes needed for the biosynthesis of these amino acids from 10-formyl-THF were active and that the genes encoding these enzymes were functioning and their expression might even be elevated (see Figure 1).

Expression of other genes involved in the metabolism of C_1 units and related amino acids and nucleic acids were possibly also altered to establish a new balance of these compounds after the introduction of the foreign 10-formyl-THF

synthetase gene. Expression of some genes might be elevated through activation or derepression; Others might be inhibited. Besides glyA-encoded SHMT, I also checked the activity of L-serine deaminase (L-SD) which is encoded by sdaA and sdaB and converts serine to pyruvate (Figure 2, see Introduction). The activity of this enzyme only slightly decreased when cells were grown in glucose minimal medium with glycine and formate compared to cells from the medium with serine (data not shown). It seems that mutations in GOF33 did not affect the expression of the genes encoding L-SD.

Genetically, the mutations can be mapped if each step of mutant-generation resulted in a single change on the chromosomal DNA, therefore, the genes involved can be cloned and defined. The mutations could be either dominant or recessive. The cloning strategies may include complementation by E. coli genomic DNA library clones, positioning cloning, chromosome walking, and transposon or λ tagging. The defined mutations will help us understand in depth the regulation of C_1 metabolism in prokaryotes.

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