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The Effect of Perturbing Folate Metabolism on Translational Accuracy and Growth of *Escherichia coli*

Johnny Basso

A Thesis

in

The Department

of

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Presented in Partial Fulfillment of the Requirements
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ABSTRACT

The Effect of Perturbing Folate Metabolism on Translational Accuracy and the Growth of *Escherichia coli*

Johnny Basso
Concordia University, 1993

In this study two topics were investigated. The first topic dealt with the effect of perturbing tetrahydrofolate metabolism on translational accuracy. Suppression of an amber mutation located in the gene encoding the tail fiber of bacteriophage T4 was used as a measure of translational accuracy. Thymine-requiring mutants of *Escherichia coli* suppress nonsense and frameshift mutations of T4 phage. We proposed that these mutants make errors during translation because of elevated levels of reduced folates. I tested the effect of either mutational blocks or the inhibition of various steps in folate biosynthesis on suppression. Conditions which prevented the accumulation of N5-methyltetrahydrofolate inhibited suppression. Conditions which favoured an accumulation of N5-methyltetrahydrofolate permitted a thymine prototroph to suppress and inhibited suppression by a strain containing a suppressor tRNA. Additionally, I found that an accumulation in dihydrofolate affect the ability of a thymine-requiring strain and a strain containing a suppressor tRNA to suppress.

In the second part of this study I investigated the phenotypes associated with strains containing *folA* null mutations. Most *Escherichia coli folA* null mutants required

folate end products to grow on minimal glucose medium. Furthermore, the folA deletion mutation affected sensitivity to ultraviolet light, the level of resistance to gyrA and gyrB inhibitors and the expression of several genes.

Mutations in three genes affected these phenotypes. Mutations in the thyA gene partially suppressed the folate end product auxotrophy of a folA deletion mutant but not of a folA insertion mutant. A thyA mutawion also affected the level of resistance to gyrase inhibitors and the susceptibility to ultraviolet light. A pair of mutations which I refer to as M suppressed the folate end product auxotrophy of both types of folA null mutants, restored the level of resistance to gyrase inhibitors to wild type levels, and increased the sensitivity to ultraviolet light of wild type strains. One of the mutations affects the gyrB gene which encodes one of the subunits of DNA gyrase. The second mutation probably affects the uvrD gene which codes for DNA helicase II.

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ABBREVIATIONS USED

THF: tetrahydrofolate

DHF: dihydrofolate

DHFR: dihydrofolate reductase

DHFS: dihydrofolate synthetase

FPGS: folylpolyglutamate synthetase

pABA: para-aminobenzoic acid

M.I.C.: minimal inhibitory concentration

INTRODUCTION

Tetrahydrofolate (THF) metabolism is of interest for two reasons. First, a number of reactions in prokaryotes and eukaryotes involve the transfer of one-carbon units from one carbon substituted THF derivatives. These include the syntheses of glycine, histidine, methionine, adenine, thymidylate, pantothenate, and N-formylmethionyl-$tRNA^{fmet}$. Secondly, drugs directed against the pathway for the de novo synthesis of THF or THF dependent reactions are routinely used in the treatment of cancer (Douglas, 1987), some bacterial infections (Seydel, 1968), and some opportunistic infections associated with AIDS; such as pneumocystis carinii pneumoniae (Stein et al., 1991). Furthermore, abnormalities in THF metabolism have been associated with a variety of disorders in humans. These include colon carcinoma (Snell et al., 1988), schizophrenia (Waziri et al., 1990), psychoses (Waziri et al., 1984), and chromosomal breakage (Kashura, 1992).

In this study I examined the effect of perturbing folate metabolism on the physiology of Escherichia coli. Mutations and drugs affecting de novo folate metabolism and mutations affecting the synthesis of one carbon substituted THF derivatives were used. In the first part of this study I examined the effect of perturbing folate metabolism on translational accuracy. In the second part I examined the

diverse effects of null mutations in the folA gene, which codes for dihydrofolate reductase, on the physiology of E.coli.

In the following sections I will briefly review de novo THF biosynthesis, THF utilizing reactions, the means and consequences of perturbing folate metabolism, and the association of THF metabolism to various global responses.

THF Metabolism

THF is composed of a pteridine, p-aminobenzoate (pABA), and one or more L-glutamic acid units (Figure 1; Keagy, 1980). Reduced forms of THF, containing one carbon substitutions at the N5 and/or N10 position (Figure 1) are are a source of one-carbon units for the syntheses of some amino acids, purines, thymidylate, pantothenate, and N-formylmethionyl-$tRNA^{fmet}$.

E.coli can not use exogenously supplied folic acid and thus must obtain it from de novo biosynthesis. The pteridine moiety of THF is derived from the nucleotide GTP (Figure 2)(Katzenmeier et al., 1991). pABA is synthesized from chorismate which is also the precursor for aromatic amino acid biosynthesis (Pittard 1987). The enzyme dihydropteroate synthetase catalyzes the condensation of pABA and pteroate forming dihydropteroate (Brown and Williamson, 1987).

Figure 1. Structure of Tetrahydrofolate. One carbon units are addede at the N5 and/or N10 position.

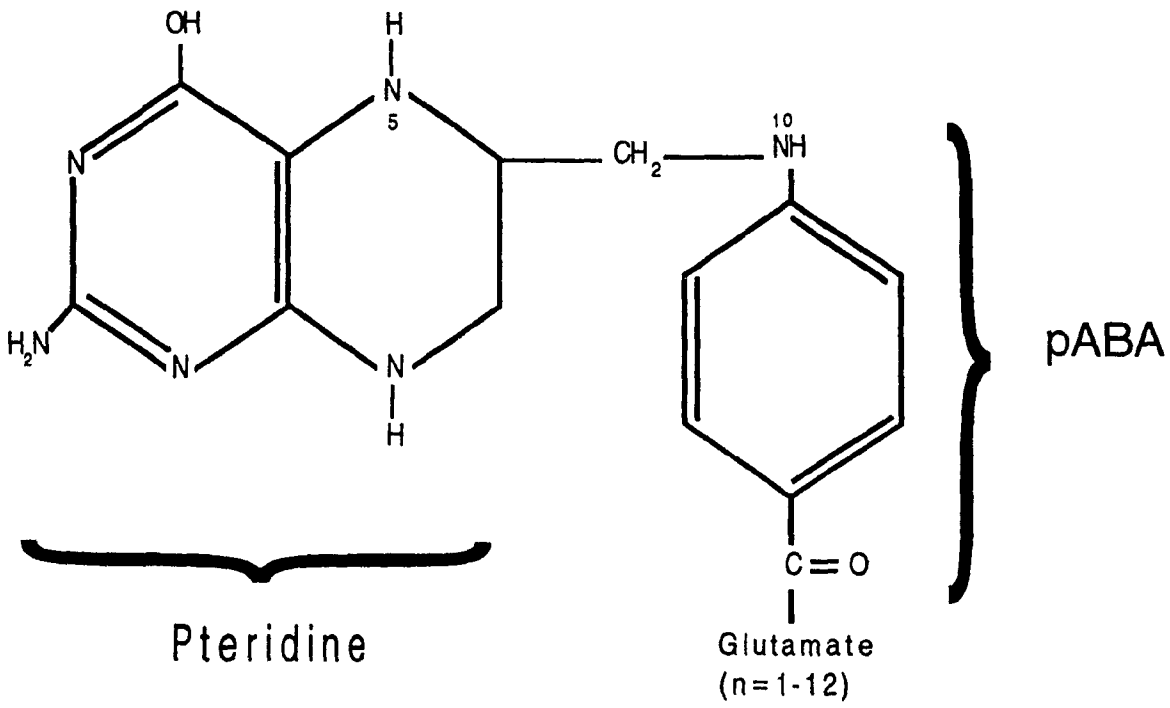
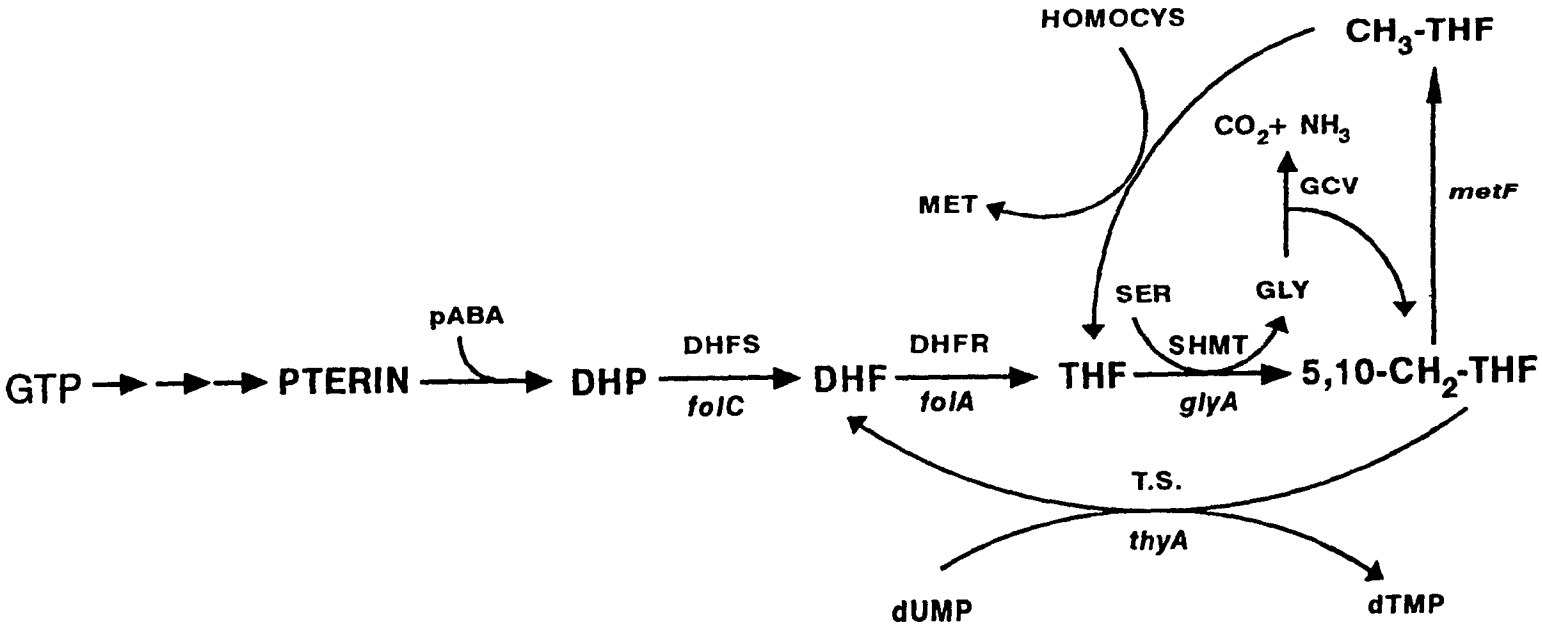

OH
H
N
5
N
CH2
10
NH
H2N
N
N
H
pABA
C=O
Pteridine
Glutamate
(n=1-12)

Figure 2. De novo THF and one carbon substituted THF synthesis:

A diagrammatic representation of de novo synthesis of folates and their interconversions into different reduction states. The abbreviations used are as follows: Pterin, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; pABA, para-aminobenzoic acid; DHP, 7,8-dihydropteroate; DHF, dihydrofolate; THF, tetrahydrofolate; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; GCV, glycine cleavage pathway; SHMT, serine hydroxytransmethylase; T.S., thymidylate synthase; ser, serine; gly, glycine; met, methionine; homocys, homocysteine; CH_3-THF, N5-methyltetrahydrofolate; N5,N10-CH_2-THF, N5,N10-methylenetetrahydrofolate.


HOMOCYS
CH3-THF
CO2+ NH3
GCV
metF
MET
SER
GLY
pABA
DHFS
DHFR
SHMT
GTP
PTERIN
DHP
DHF
THF
5,10-CH2-THF
folC
folA
glyA
T.S.
thyA
dUMP
dTMP

Dihydrofolate synthetase, encoded by the folC gene, then adds L-glutamate to dihydropteroate forming dihydrofolate (DHF) (Ferone et al., 1983). THF is synthesized from the NADPH dependent reduction of dihydrofolate by dihydrofolate reductase (DHFR) (Brown and Williamson, 1987), the protein product of the folA gene (Figure 2)(Singer et al., 1985).

Two pathways are present in E.coli for the synthesis of the primary one-carbon substituted THF derivative N5,N10-methyleneTHF (Figure 2). Serine hydroxymethyltransferase transfers a hydroxymethyl group from serine to THF forming glycine and N5,N10-methyleneTHF (Stauffer, 1987). This enzyme is encoded by the glyA gene. Alternatively, in the presence of THF the glycine cleavage pathway catalyzes the oxidative cleavage of glycine to ammonia, carbon dioxide and N5,N10-methyleneTHF (Stauffer, 1987). N5,N10-methyleneTHF can be reduced to N5-methylTHF by the metF gene product methyleneTHF reductase, or oxidized to N5,N10-methenylTHF by methyleneTHF dehydrogenase. N5,N10-methenylTHF can be further oxidized by methenylTHF cyclohydrolase to N10-formylTHF (Brown and Williamson, 1987). The dehydrogenase and cyclohydrolase activities are found within a protein composed of five different subunits (Dev and Harvey, 1978).

One carbon substituted THF occur in two forms, either as pteroylmonoglutamates or as poly-γ-glutamates (McGuire et al., 1980). Folylpolyglutamate synthetase (FPGS), encoded by folC, catalyzes the addition of 1-3 glutamic residues to

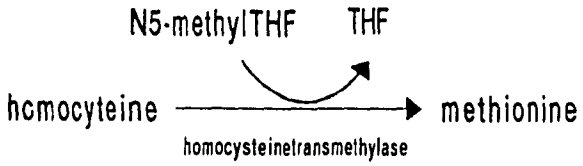
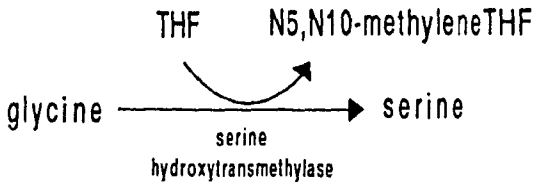
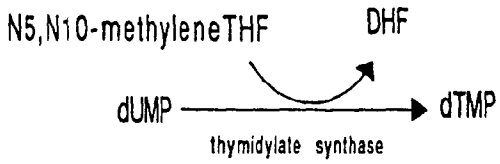
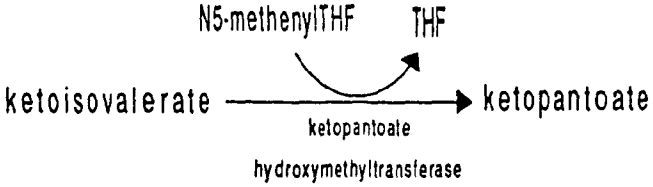
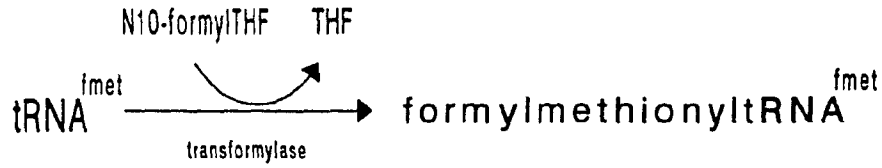
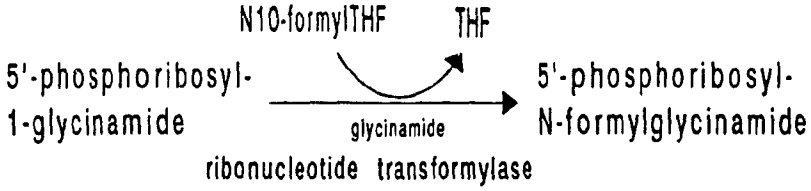
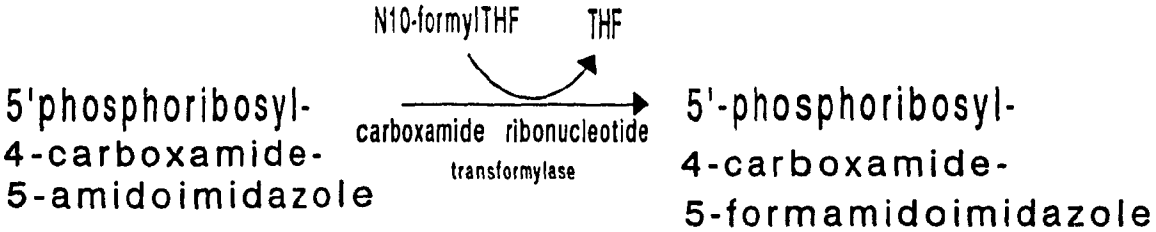
one carbon substituted THF derivatives (Ferone et al., 1983). THF with up to twelve glutamate residues has been isolated from E.coli, indicating that additional enzymes must be required for the formation of these higher polyglutamates (Brown and Williamson, 1987). The extent of polyglutamation affects the efficiency with which different one carbon substituted THF derivatives can be utilized (McGuire et al., 1980). Consequently, the different polyglutamation states could have a regulatory role.

One carbon substituted THF and biosynthesis

The reactions which use one carbon substituted THF derivatives as cofactors are shown in figure 3. In E.coli the last step in methionine biosynthesis is catalyzed by two isozymes of homocysteine transmethylase which are encoded by the metE and the metH genes (Cohen and Saint-Girons, 1987). The two isozymes differ in their co-factor requirement. The metE encoded enzyme requires polyglutamated ($n \geq 3$) N5-methylTHF. The metH encoded enzyme can use either monoglutamated or polyglutamated N5-methylTHF, but is not active unless vitamin B12 is provided (Cohen and Saint-Girons, 1987).

The purine ring of IMP, the precursor to AMP and GMP, is derived from 5-phosphoribosyl-α-1-pyrophosphate. Two carbon atoms of IMP can be obtained from N10-formylTHF.

Figure 3. THF cofactor requiring reactions.

Pathway	Reaction
methionine	
glycine	
thymidylate	
pantothenate	
formylmethionyl-tRNA^{fmet}	
purines	
5'phosphoribosyl-4-carboxamide-5-amidoimidazole	

The first THF dependent reaction involves the formylation by glycinamide ribonucleotide transformylase of the amino group of the glycine residue of 5'-phosphoribosyl-1-glycinamide producing 5'-phosphoribosyl-N-formylglycinamide and THF (Figure 3; Nygaard and Smith, 1993). This reaction can also be catalyzed by an isozyme of glycinamide ribonucleotide transformylase which uses formate instead of N10-formylTHF as the source of the carbon unit (Nygaard and Smith, 1993). The second reaction involves the formylation of the 5-amino group of 5'-phosphoribosyl-4-carboxamide-5-amidoimidazole forming 5'-phosphoribosyl-4-carboxamide-5-formamidoimidazole and THF (Figure 3; Neuhard and Nygaard, 1987).

The de novo synthesis of deoxythymidylate is carried out by thymidylate synthase, encoded by the thyA gene (Belfort, Maley, and Maley, 1983), which catalyzes the N5,N10-methyleneTHF dependent methylation of deoxyuridylate yielding deoxythymidylate and DHF (Molgaard and Neuhard, 1983). This is the only THF dependent reaction where the THF cofactor is oxidized to DHF.

Serine hydroxymethyltransferase, encoded by the glyA gene, catalyzes the transfer of a hydroxymethyl group from serine to THF forming glycine and N5,N10-methyleneTHF (Figure 3; Stauffer, 1987).

One carbon and one nitrogen atom of the imidazole ring of histidine are derived from the purine ring of ATP. The ATP used for histidine biosynthesis is regenerated from an

intermediate of the histidine biosynthetic pathway, 5'-phosphoribosyl-4-carboxamide-5-amidimidazole. This is also an intermediate of the purine biosynthetic pathway (Neuhard and Nygaard, 1987). The synthesis of purines from this intermediate requires N10-formylTHF (see purine synthesis above). Therefore, the THF requirement for histidine biosynthesis stems from the fact that the ATP required for its synthesis and its regeneration requires one carbon substituted THF.

The first step in the synthesis of pantothenate involves the transfer of the hydroxymethyl group from N5-methenylTHF to α-ketoisovalerate (Figure 3), an intermediate of valine biosynthesis forming ketopantoate and THF.

In most prokaryotes, N-formyl-methionyl-$tRNA^{fmet}$ is required for the initiation of protein synthesis. The formyl group or the formylmethionine residue is then removed from the protein post-translationally (Hershey, 1987). Transformylase transfers the formyl group from N10-formylTHF to the methionyl-$tRNA^{fmet}$ yielding THF and N-formylmethionyl-$tRNA^{fmet}$ (Hershey, 1987).

THF Metabolism and Global Responses

THF cofactors are required for a wide variety of reactions in E.coli. Cosequently, it is not surprising that several studies suggest an interplay of THF metabolism with

various global responses to different stresses. These include amino acid starvation, heat shock, and DNA damage. Each of these stimulates a global response; stringent response or general amino acid control, heat shock response, and SOS response respectively. These studies will be briefly reviewed in this section.

General amino acid control or the stringent response. General amino acid control has been most extensively studied in the yeast Saccharomyces cerevisiae. When yeast cells are starved for one of a group of amino acids they simultaneously induce the expression of the genes which encode the enzymes involved in the synthesis of this family of amino acids (Jones and Fink, 1982). The induction of these genes is made possible by a regulatory DNA sequence to which binds positive regulatory proteins whose synthesis requires amino acid starvation (Jones and Fink, 1982). Interestingly, this sequence is present in the upstream region of the yeast DFR1 gene encoding DHFR (Barclay et al., 1988) suggesting that DHFR is under general amino acid control in yeast.

Another study which demonstrated the induction of a folate metabolizing enzyme in response to amino acid starvation was done in Neurospora crassa. Regulation of serine hydroxymethyltransferase activity in N.crassa results from changes in the level of its messenger RNA (Robertson

McClung, et al., 1992). The levels of the serine hydroxymethyltransferase mRNA increase in response to amino acid starvation. This effect is dependent on the presence of a functional activating protein of the general amino acid control pathway (Robertson McClung et al., 1992).

In E.coli, amino acid starvation induces the stringent response which is the abrupt cessation of RNA accumulation (Cashel and Rudd, 1987). Induction of this response has been correlated with the accumulation of guanosine tetraphosphate. E.coli relA mutants which can not synthesize this nucleotide, do not induce the stringent response (Cashel and Rudd, 1987). Interestingly, E.coli cells grown in the presence of the antifolate drugs sulfathiazole or trimethoprim accumulate guanosine tetraphosphate (Rohlman and Mathews, 1990). This result suggests that inhibition of de novo THF metabolism induces the stringent response. Since C1-substituted THF is required for the syntheses of histidine, methionine, and glycine, the observed accumulation of guanosine tetraphosphate in response to the treatment of cells with antifolate drugs may be due to amino acid starvation.

The stringent response can also be induced by starving E.coli cells for a carbon source or a nitrogen source (Cashel and Rudd, 1987). This response is dependent on the relB gene product but is independent of the relA gene product (Cashel and Rudd, 1987). RelB mutants show an

increased resistance to some sulfonamides (Cashel and Rudd, 1987) suggesting that this type of stringent response is also influenced by THF metabolism.

Heat shock response. The expression of eighteen different proteins, most of which are chaperones, is increased when E.coli cells are exposed to an abrupt increase in temperature (VanBogelen, Kelley, and Neidhart, 1987). Chaperones are proteins which assist in protein folding and the assembly of multi-protein complexes. Recently, it was reported that a glyA mutant did not induce some of the proteins associated with the heat shock response and grew linearly rather than exponentially (Gage and Neidhart, 1993). Surprisingly these phenotypes could be complemented by the glyA gene when it was located on a low copy number plasmid, but not when it was on a high copy number plasmid (Gage and Neidhart, 1993). Gage and Neidhart (1993) suggested that the reason for this is that a high level of serine hydroxymethyltransferase may be toxic to the cell.

SOS response. When E.coli cells are exposed to DNA damaging agents, DNA synthesis is rapidly arrested, and the expression of genes involved in DNA repair is increased (VanBogelen, Kelley, and Neidhart, 1987). The most potent inducing agent of the SOS response is exposure to ultraviolet light (Walker, 1987). UV damage, predominantly

the formation of thymine dimers, can be repaired by photoreactivation which is catalyzed by DNA photolyase (Smith, 1977). The E.coli DNA photolyase contains N5-methylTHF as a prosthetic group (Payne et al., 1987). This suggests that THF may affect the activity of this enzyme and thus the sensitivity of E.coli to ultraviolet light exposure. Furthermore, I will demonstrate in this study that E.coli cells which lack DHFR show an increased sensitivity to ultraviolet light exposure.

Perturbing De Novo THF Biosynthesis in E.coli

Due to its central role in the physiology of the cell, several studies have investigated the effect of perturbing folate metabolism. In E.coli, two approaches have been used to perturb THF metabolism. These include the use of antifolate drugs, such as sulfonamides and trimethoprim, and genetic manipulations of genes encoding the enzymes required for the de novo synthesis of one carbon substituted THF.

Antifolate drugs. Sulfonamides, competitive inhibitors of dihydropteroate synthase, inhibit the growth of E.coli presumably because they prevent de novo THF biosynthesis Woods, 1962). The action of sulfanilamides is quite slow, requiring several cycles of cell division before they completely inhibit growth. The reasons for this may be that

very low levels of THF are required for growth, and folates are not broken down. Thus, blocking THF synthesis only becomes growth limiting when the folate pool is sufficiently diluted as a result of cell division.

Cells can grow in the presence of sulfathiazole if supplied with the folate end products (Woods, 1962)(glycine, methionine, a purine source, thymine, and pantothenate). This suggests that folates are essential only for the biosynthesis of these metabolites.

When grown in the presence of sulfathiazole cells undergo thymineless death (Then and Anghern, 1972) which occurs when cells are starved for thymidylate. This is due to the fact that the thymidylate synthase reaction is the only THF dependent reaction which turns over THF. In the presence of high levels of thymine (100µg/ml), sulfathiazole is bacteriostatic but not bactericidal. This result suggests that THF is mainly required for the synthesis of thymidylate. Consistent with this hypothesis, the folate end product requirement of Lactobacillus casei, which can not synthesize folates de novo, can be satisfied by thymine alone (Jukes and Broquist, 1963).

Several metabolites, which are not folate end products, antagonize the action of sulfonamides. These include tryptophan, arginine, lysine, and nicotinate (Martin, 1951; Teply et al., 1965). Therefore, folate metabolism is affected by metabolites of pathways which do not use

substituted THF cofactors.

The growth of E.coli in the presence of high levels of the DHF analogue trimethoprim has been extensively studied. DHFR is required for the de novo synthesis of THF and for the regeneration of THF from the DHF produced by the thymidylate synthase reaction (Molgaard and Neuhard, 1983). In minimal medium containing trimethoprim, wild type E.coli requires all folate end products to grow (Harvey, 1973). The model proposed by Harvey (1973) to explain this result is as follows. The thymidylate synthase reaction is the only known THF dependent reaction where the THF co-factor, N5,N10-methyleneTHF, is oxidized to DHF (Mollgaard and Neuhard, 1983). Consequently, cells grown in the presence of trimethoprim can not regenerate the THF used by the thymidylate synthase reaction and thus deplete THF needed for other reactions. Consistent with this hypothesis cells lacking thymidylate synthase activity, thyA mutants, are resistant to high levels of trimethoprim (Bertino and Stacey, 1966).

E.coli cells grown in minimal medium containing trimethoprim and all the folate end products grows at one third of the wild type growth rate (Harvey, 1973). The reduced growth rate is due to the inability of cells to synthesize N-formylmethionyl-$tRNA^{fmet}$ which is required for the initiation of protein synthesis (Harvey, 1973).

Mutations in genes encoding enzymes of THF metabolism.

The folC gene codes for the bifunctional enzyme folylpolyglutamate synthetase-dihydrofolate synthetase (FPGS - DHFS). This enzyme catalyzes the synthesis of DHF and the addition of two additional glutamate residues to one carbon substituted THF derivatives (Bognar et al., 1985; Ferone and Warskow, 1983). Strains containing a mutation in the folC gene require methionine or vitamin B12 to grow and their growth is improved by glycine. The requirement for methionine or vitamin B12 is believed to be due to the lack of FPGS activity. The strain cannot synthesize polyglutamated N5-methylTHF and consequently only the B12 dependent homocysteine transmethylase can function in methionine biosynthesis (see methionine biosynthesis above). Consistent with this hypothesis, the L. casei gene encoding FPGS activity complements the methionine and glycine auxotrophy of folC mutants of E.coli (Toy and Bognar, 1990).

It is not clear why the growth of folC mutants is stimulated by glycine. Possibly serine hydroxymethyltransferase, which catalyzes the synthesis of glycine from serine prefers polyglutamated THF as is the case in Clostridium cylindrospororum (Wright, 1955).

De novo synthesis of THF is reduced in folC mutants (Pyne and Bognar, 1992). This is consistent with the fact that DHFS is required for the de novo synthesis of THF.

The folC gene is essential in wild type E.coli strains,

unless the strain expressed FPGS activity from the L.casei gene (Pyne and Bognar, 1992). This gene also expresses a very low level of DHFS activity (1/10,000 as compared to the E.coli enzyme) (Pyne and Bognar, 1992). Consequently it is not clear whether DHFS, FPGS, or both are essential in E.coli.

The folA gene codes for DHFR (Smith and Calvo, 1980). This is the most extensively studied enzyme of folate metabolism because it is the target of different therapies against cancer (Douglas, 1987), parasitic infections (Jukes and Broquist, 1963), and bacterial infections (Seydel, 1968).

A strain of E.coli containing a point mutation in the folA gene was isolated and characterized by Singer et al. (1985). This mutant contains no detectable DHFR activity or protein, grows at a reduced growth rate and is auxotrophic for all folate end products. These results are consistent with the studies performed with the DHF analogue trimethoprim.

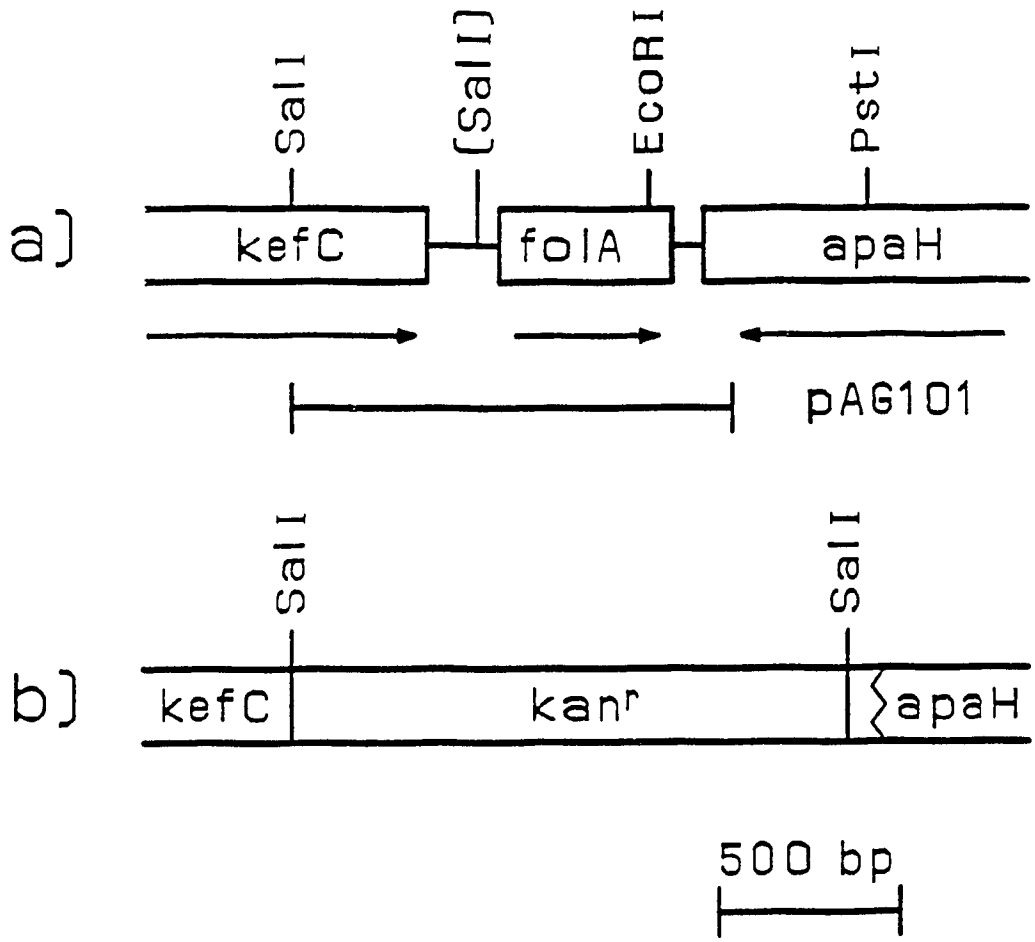
Recently, folA deletion mutants have been isolated using recombinant DNA techniques (Howell, 1988; Ahrweiler, 1988). In both cases the folA gene and some flanking sequences were deleted from a plasmid containing the folA gene. A kanamycin resistance cassette was inserted to provide a selectable marker. The engineered sequence was then substituted into the chromosome replacing wild type

folA sequences (Figure 4).

These folA deletions could only be introduced in a mutant thyA background (Ahrweiler and Frieden, 1988; Howell, Foster and Foster, 1988). Howell, Foster, and Foster (1988) suggested that this observation was analogous to the observation that Thy^- cells are resistant to trimethoprim, whereas Thy^+ cells are not. Krishnan and Berg (1993) demonstrated that the folA deletion constructed by Ahrweiler and Frieden (1988) could be introduced in a Thy^- or a Thy^+ background at an equivalent efficiency.

Conflicting reports as to the folate end product requirement of these deletion mutants have been made. Howell, Foster and Foster (1988) reported that their mutant required exogenous folate end products to grow. Ahrweiler and Frieden (1988) reported that their mutation did not confer a folate end product auxotrophy. Hamm-Alvarez, Sancar and Rajagopalan (1990) state that the folA deletion mutants constructed by Howell, Foster, and Foster (1988) and Ahrweiler and Frieden (1988) grew normally in the absence of folate end products. Krishnan and Berg (1993) reported that strains containing the deletion mutation constructed by Ahrweiler and Frieden (1988) had an absolute requirement for folate end products. These differences could result from secondary mutations which suppress the folate end product auxotrophy. These mutations could accumulate during the handling and storage of the strains.

Figure 4. The folA region. a) The positions of the coding regions of the kefC (Munro et al., 1991), folA (Smith and Calvo, 1980) and apaH (Blanchin-Roland et al., 1986; Mechulam et al., 1985) genes are shown by the boxes. The beginning of the folA coding sequence is nucleotide 49,483 on the E. coli sequence (Yura et al., 1992). The SalI site in parentheses is present in the wild type folA sequence but not in the trimethoprim resistant allele present on the plasmid, pCV29 (Smith and Calvo, 1982) used in the construction of the folA deletion (Howell, Foster, and Foster, 1988) The arrows indicate the directions of transcription of the three genes. The bar indicates the portion of this sequence which is present in plasmid pAG101. b) The structure of ΔfolA::kan. A kanamycin cassette was inserted into the SalI site in kefC. Approximately 1.1 kb were deleted by Bal31 digestion initiated at the EcoRI site in folA. Restriction analysis indicated that the SalI sites flanking kan were still present but that the PstI site was not (Howell, Foster and Foster, 1988). Southern analysis indicated that all the folA coding sequence was deleted (Howell, Foster, and Foster, 1988). The jagged line indicates the approximate end points of the deletion but we do not know how much remains of the kefC-folA sequences downstream of the SalI site in kefC.


a)
SalI
(SalI)
EcoRI
PstI
kefC
folA
apaH
pAG101
b)
SalI
SalI
kefC
kanr
apaH
500 bp

This was investigated in this study and will be discussed later.

To determine whether de novo folate metabolism was impaired in these deletion mutants, THF levels and the distribution of one carbon substituted THF derivatives in FolA$^+$ Thy$^+$ and Δfola::kan Thy$^-$ strains were compared (Hamm-Alvarez, Sancar, and Rajagopalan, 1990). Surprisingly the total THF level of a Thy$^-$, Δfola::kan strain was comparable to those observed in a Thy$^+$ FolA$^+$ strain. This result suggests that DHFR is not essential for de novo THF biosynthesis and that an alternative pathway must exist to reduce DHF. A dihydropteridine reductase activity which can reduce DHF to THF in vitro has been identified in E.coli extracts (Vasudevan, Paal, and Aramrego, 1992).

Another study compared the incorporation of the folate precursor pABA by different FolA$^+$ and FolA$^-$ strains containing the ΔfolA::kan mutation constructed by Howell, Foster and Foster (1988). Most ΔfolA::kan mutants incorporated less pABA than the isogenic FolA$^+$ strains (Basso and Herrington, submitted).

In this study, I extended the phenotypic characterization of strains containing the ΔfolA::kan mutation (Howell, Foster and Foster, 1988). I attempted to determine whether strain differences affected the phenotypes assosciated with the ΔfolA::kan mutation. I identified secondary mutations which suppressed the folate end product

auxotrophy associated with strains containing this deletion or a folA insertion mutation.

Folate metabolism and translational accuracy.

Thymine requiring strains of E.coli suppress nonsense and frameshift mutations of T4 phage (Herrington, Kholi, and Lapchack, 1984). Suppression occurs when a secondary mutation, which is present at a site in the chromosome different from the first mutation, somehow masks the phenotypic expression of the first mutation restoring the wild type phenotype. Suppression can result from mutations affecting genes whose products are required for the translation of mRNA into protein. This type of suppressor acts by inserting an amino acid in response to a premature termination codon, by substituting an amino acid for another one creating a functional protein or by correcting the reading frame. The suppressor strain permits a mutated codon to be translated incorrectly but beneficially. Mutations affecting several components of the translational apparatus have been identified as suppressor mutations. These include mutations in tRNA, tRNA modifying enzymes, ribosomal RNA, ribosomal proteins, release factors and elongation factors (Murgola, 1985; Herrington, 1989).

Two observations suggest that suppression by thyA mutants is due to a decreased translational fidelity. Thy$^-$

strains with a mutated rpsL ribosomal protein do not suppress (Herrington, Kholi, and Lapchak, 1984). This mutation confers streptomycin resistance and is known to increase translational fidelity (Yates, 1979). Furthermore, the aminoglycoside antibiotic kasugamycin, which reduces translational errors in vitro (Van Buul, Visser, and Knippenberg, 1984), inhibits suppression (Tiganos and Herrington, 1993). These results strongly suggest that the suppression event occurs at the level of the ribosome.

Since the thymidylate synthase reaction is the only THF dependent reaction where the THF cofactor is oxidized to DHF, it was proposed that Thy^- strains accumulate THF and thus mismodify some component of the translational apparatus (Cheung and Herrington, 1982). For instance tRNA's in Thy^- cells may be mismodified. These mismodified tRNA's would more readily slip through the ribosomal screens for non-cognate codon-anticodon interactions than do normally modified tRNAs resulting in occasional readthrough of nonsense codons and frameshifting (Cheung and Herrington, 1982; Herrington, Kholi and Faraci, 1986). To verify this model total THF and methylTHF levels of Thy^- and Thy^+ cells were compared. Under suppressing conditions, the level of total THF and methylTHF were higher in non-infected Thy^- cells than in Thy^+ cells (Tiganos, 1990).

In this study I have extended the characterization of the suppression phenomena of thyA mutants. I attempted to

determine whether suppression could be corelated to the elevated THF levels observed in Thy$^-$ cells and whether one folate species could be correlated to the suppressor phenotype. To do this I examined the effect of perturbing folate metabolism on the suppression efficiency of a thyA mutant. This was accomplished by using antifolate drugs directed against de novo THF biosynthesis as well as strains with either reduced or increased levels of enzymes involved in de novo folate metabolism.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The E.coli K12 strains used in this study are described in Table 1. Conjugations and P1cm mediated transductions were used to construct strains and for mapping (Miller, 1972). Most strain constructions are described in Table 2. P1cm was obtained from a lysogenic strain of E.coli provided by M. Belfort. The transposition vector λplacMu53 and the helper phage λpMu507 (Bremer, Silhavy, and Weinstock, 1985) were obtained from E.B. Newman.

Novobiocin and nalidixic acid resistant derivatives of MH581 were obtained by plating approximately 10^8 cells on LB plates containing 50μg/ml thymidine and 200μg/ml novobiocin or 20μg/ml nalidixic acid.

Wild type T4 was obtained from I. Takahashi. Mutant strains elp12 and M103, which have UGA and UAG mutations respectively in the lysozyme gene, were provided by J. Owen. JC1922 (Templin, Margossian, and Clark, 1978), which has a UAA mutation in the lysozyme gene, was provided A.J. Clark. The mutants opc23 (UGA mutation in the tail fiber protein gene 37), opc105 (UGA mutation in the tail fiber protein gene 34) and opc100 (UGA mutation in the major coat protein gene 23) were obtained from W. Wood.

Construction of apaH and folA insertion mutants. To isolate mutants with insertions in folA or apaH I used strain KL719, which contains F'101. This F' contains a segment of the E.coli chromosome spanning the region between 98 - 2 min. (uxuAB - panB). This region includes the apaH and folA genes. My strategy ensured that the only mutants isolated had insertions in F'101, thus simplifying the screening for folA and apaH insertions. A 10 ml culture of strain KL719 was infected with λplacMu53 and λplacMu507 (Bremer,Silhavy, and Weinstock, 1985). This results in the random insertion of λplacMu53 into the genome. To obtain those insertions which occured in the F'101, 10 ml of exponentially growing Y10 was added after a one hour incubation period. Strain Y10 was used as a recipient strain to recieve the F'101 by conjugation. After a one hour incubation period at 37°C samples of the mixture were plated on minimal medium containing kanamycin. Under these condition only Y10 cells which contain an F'101 with a λplacMu53 insertion, which complements the leucine auxotrophy of strain Y10 and confers kanamycin resistance because of the λplacMu53 insertion, grow. To obtain F'101 containing an insertion in either folA or apaH, approximately 10,000 kanamycin resistant Y10 conjugants were pooled in 10 ml of LB medium, grown for one hour and used as the donor in a conjugation mixture containing strain MH722 (ΔfolA::kan, leuB) as the recipient. After a one hour incubation period samples were plated on

appropriately supplemented minimal medium I selected for ampicillin resistant (from plasmid pRK11 of strain MH722) leucine prototrophs (from the F'101). Fifty conjugants were screened for sensitivity to sulfathiazole (1µg/ml) on supplemented minimal medium and for motility by stabbing into soft AB plates. Since folA⁻ strains were sensitive to sulfathiazole (this study) and this sensitivity was reversed by the F'101, sulfathiazole sensitivity of conjugants indicated that λplacMu53 was inserted in folA. Since apaH mutants are non-motile (Farr et al., 1989), apaH insertion mutants were identified by screening for conjugants where the F' factor failed to complement the non-motile phenotype of the ΔfolA::kan strain. Twenty eight percent of the conjugants were sulfathiazole sensitive and non-motile and 32% were non-motile but sulfathiazole resistant. The F' factor from a conjugant of each class was transferred by conjugation to strain MH718. Kanamycin (from the F'101 containing a λplacMu53 insertion) and streptomycin resistant (from the rpsL mutation of strain MH718) conjugants were selected. MH718 is recA and therefore recombination between genes on the F' and the chromosome is greatly reduced, thereby stabilizing the F'. P1cm phage grown on strain MH718/F'101 (folA::λplacMu53) and strain MH718/F'101 (apaH::λplacMu53) was used to transduce the mutations into strains MH429, MH581, MH293, and JF1754. Kanamycin resistant transductants were selected.

The folA gene from plasmid pAG101 complemented the folate end product auxotrophy of the folA insertion mutant MH581 (folA::λplacMu53) thus supporting the conclusion that this strain has an insertion in folA.

Plasmids and manipulations. Plasmid pJB11 contains the E.coli gyrB gene (2.7kbp) on a 3.4 kbp AvaI-EcoRI insert in pBR322 (Yamagishi et al., 1986; Kindly provided by J. Yamagishi). Plasmid pPL113 contains the yeast S. cerevisiae DFR1 gene in pUC8 (Lagosky, Taylor, and Haynes, 1987; Kindly provided by R.K. Storms). Plasmid pAG101 contains the E.coli folA gene on a 1.2 kbp BamHI insert in pUC8 (2)(Howell, Foster, and Foster, 1988; Kindly provided by E.E. Howell). The plasmid pBluescript (Alting-Meese and Short, 1989) was kindly supplied by F. Noubani. Plasmid pRK11 contains a temperature sensitive thyA allele on a 7 kbp HindIII-BamHI insert in pBR322 (Kurzman, 1989). Plasmid pAC5 contains the E.coli folC gene under the control of the lac promoter of pUC8 (Bognar, Osborne, and Shane, 1987; kindly supplied by A. Bognar). Plasmid pGT3-8.1 contains the L.casei gene encoding folylpolyglutamate synthetase activity inserted into pEMBL19 (Toy and Bognar, 1990; Kindly supplied by A. Bognar).

Plasmids were introduced into cells made competent by the $CaCl_2$ method (Schleif and Weinsink, 1981).

Media. AB medium (Cheung and Herrington, 1982), LB medium (Miller, 1972), K medium (Epstein and Kim, 1971), M9 minimal medium (Miller, 1972) and minimal medium A (Miller, 1972) have been described. Minimal medium was supplemented with any required amino acids at a final concentration of 50µg/ml each. Folate end product supplementation included methionine, glycine, and histidine at a final concentration of 50µg/ml each; adenine at 30µg/ml; thymidine at 50µg/ml and pantothenate at 1µg/ml (Howell, Foster, and Foster, 1988). Media were supplemented with 100µg/ml thymidine unless otherwise indicated. K medium was used to screen for the kefC phenotype. M9S was M9 minimal medium lacking $CaCl_2$ and supplemented with 1.0µM $FeSO_4$, 0.02% $MgSO_4$, 50µg/ml methionine, 50µg/ml tryptophan, 1µg/ml thiamine, 0.2% glucose and thymidine as indicated below.

JB medium consisted of M9S minimal medium supplemented with 0.001 mM $ZnCl_2$, 3µg/ml nicotinate, 10µg/ml histidine, 37µg/ml valine, 22.5µg/ml isoleucine, 30µg/ml adenine, 50µg/ml phenylalanine, and 50µg/ml tyrosine. Thymidine was supplemented for the growth of either Thy^+ or Thy^- strains at a final concentration of 30µg/ml in JB medium and 20µg/ml in AB medium for suppression assays, and at 100µg/ml for overnight cell growth. For optimal growth in minimal medium, strains SF4 and MH538 required the addition of methionine and glycine at a final concentration of 50µg/ml each.

TABLE 1. *E.coli* K12 Strains.

Strain	Genotype	Source and Reference[a]
AT2457	*glyA6 relA1 spoT1 thi-1*	CGSC
BW6165	Hfr P801 *ara-41 lacY1* or *lacY40* λind^- *xyl17 argE86::Tn10*	M. Singer (1)
CAG5052	KL227 *btuB3139::Tn10 metB1 relA1*	M. Singer (1)
CAG5055	KL16 *zed-3069::Tn10 thi-1 relA1*	M. Singer (1)
CAG12095	*zac-3051::Tn10*	M. Singer (1)
CAG12158	*pheA18::Tn10*	M. Singer (1)
CAG18501	*zie296::Tn10*	M. Singer (1)
CAG18431	*ilv500::Tn10*	M. Singer (1)
CAG18491	*metE3079::Tn10*	M. Singer (1)
CAJ64	Lac^- Su^+(UGA)	J. Friesen (2)
JF1754	F^- *hsdR lac gal metB leuB hisB436*	R.K. Storms (3)
JFF	F^- *hsdR lac gal metB leuB hisB436* Δ*folA::Kan*	This Study
JFFJ	F^- *hsdR lac gal metB leuB hisB436* Δ*folA::Kan zid501::Tn10*	This Study
JFFmu	F^- *hsdR lac gal metB leuB hisB436* *folA::*λ*placMu53*	This study
JW355	F^- *asnB32 relA1 thyA95 spoT1 zid501::Tn10 thi-1 deo33* λ^-	CGSC

Table Continued.

KL719	F'101 thr-1 ara-14 leuB6 Δ(gpt - proA)62 lacY1 tsx-33 supE44 galK2 λ^- rac^- hisG4 rfbD1 mgl-51 recA13 rpsL31 kdgK-51 xyl-5 mtl-1 argE3 thi-1	CGSC (4)
LH18	(F'lac-pro) thyA Δlac-pro ΔfolA::Kan	E.E. Howell (5)
LH18$^+$	(F'lac-pro) thyA Δlac-pro	E.E. Howell (5)
LH18-2	(F'lac-pro) thyA Δlac-pro thi hsd5 supE	This Study
LH18-3	(F'lac-pro) thyA Δlac-pro M^- thi hsd5 supE	This Study
LH18-4	(F'lac-pro) thyA Δlac-pro M^- thi hsd5 supE	This Study
LH18-5	(F'lac-pro) thyA Δlac-pro thi hsd5 supE ΔfolA::Kan M^-	This Study
LH18-9	(F'lac-pro) Δlac-pro thi hsd5 supE	This Study
LH18-13	(F'lac-pro) Δlac-pro ΔfolA::Kan M^- thi hsd5 supE	This Study
MH128	metB lysA24 rna	(7)
MH293	F^- hsdR lac gal metB leuB hisB436 thyA723	This Study
MH293F	F^- hsdR lac gal metB leuB hisB436 thyA723 ΔfolA::Kan	This Study

Table 1 Continued.

MH293A	F⁻ hsdR lac gal metB leuB hisB436 thyA723 apaH::λplacMu53	This Study
MH420	HfrC ΔlacZW4680A lysA24	(7)
MH429	metB rna thyA723	(7)
MH429F	metB rna thyA723 ΔfolA::Kan	This Study
MH429FM	metB rna thyA723 ΔfolA::Kan M⁻	This Study
MH429Fmu	metB rna thyA723 folA::λplacMu53	This Study
MH429A	metB rna thyA723 apaH::λplacMu53	This Study
MH442	thyA Lac⁻ Su⁺(UGA)	This Study
MH537	glyA6 relA1 spoT1 thi-1 nadB51::Tn10	This Study
MH538	metB rna thyA723 nadB51::Tn10 glyA6	This Study
MH581	metB rna	This Study
MH581F	metB rna ΔfolA::Kan	This Study
MH581FM	metB rna ΔfolA::Kan M⁻	This Study
MH581Fmu	metB rna folA::λplacMu53	This Study
MH581A	metB rna apaH::λplacMu53	This Study
MH718	ara Δ(lac - proAB) strA thi-1 (Φ80 lacZΔM15) recA::Tn10 λ	This Study
MH722	F⁻ hsdR lac gal metB leuB hisB436 thyA72 ΔfolA::kan λ pRK11	This Study

Table 1 Continued.

MH723	Hfr P0120 *hsdR lac gal argE::Tn10* Δ*folA::kan* pRK11	This Study
MHBF	*metB rna thyA723 metF::Tn10*	Z.Q. Shao (6)
NI748	*thr-1 leuB6 fhuA21 lacY1 supE44* λ^- *thyA6 malT49* λ^R *gyrB41 ilv-655 thi-1 deoC1*	CGSC (8)
NK6042	Δ(*gpt-lac*)5 *nadB51::Tn10 relA1 spoT1 thi-1* λ^-	CGSC
RC709Kan^R	*metF63 proA3 zij-3167::Tn10Kan*	E.B. Newman
SF4	*folC strA relA*	A. Bognar (9)
SF4$^+$	*strA relA recA zed-3069::Tn10*	This study
Y10	*ara-1 leuB6 thi-1 rfbD1 supE44*	L. Brakier-Gingras

a (1) Singer *et al.*, 1989; (2) Sambrook, Fan, and Brenner, 1967; (3) Storms, Holowachuck, and Friesen, 1981; (4) Low, 1968; (5) Howell, Foster, and Foster, 1988; (6) Basso, Tiganos, and Herrington, 1993; (7) Herrington *et al.*, 1991; (8) Gellert *et al.*, 1976; (9) Bognar *et al.*, 1985. CGSC: Coli Genetic Stock Center.

Table 2. Summary of Strain Constructions:

Strain Constructed	Cross[a]	Selected (Screened) Phenotype	Relevant[b] phenotype
JFF	P1CM/LH18 X JF1754	Kan^R	$FolA^-$ Thy^+ M^-
JFFJ	P1CM/JW355 X JFF	Tet^R	$FolA^-$ Thy^+ M^-
LH18-2	P1CM/MH420 X LH18	FEP^{+c} (Kan^S)	$FolA^+$ Thy^+ M^+
LH18-3	P1CM/CAG12095 X LH18-5	Tet^R (Kan^S)	$FolA^+$ Thy^- M^-
LH18-4	P1CM/MH420 X LH18-3	Thy^+	$FolA^-$ M^- Thy^+
LH18-5	P1CM/JF1754 X LH18	FEP^+, Kan^R	$FolA^-$ Thy^- M^-
LH18-9	P1CM/MH420 X LH18-2	Thy^+	$FolA^+$ Thy^+ M^+
LH18-13	P1CM/MH420 X LH18-5	Thy^+	$FolA^-$ Thy^+ M^-
MH293F	P1CM/LH18 X MH293	Kan^R	$FolA^-$ Thy^- M^-
MH429F	P1CM/LH18 X MH429	Kan^R	$FolA^-$ Thy^- M^+
MH537	P1CM/NK6042 X AT2457	Tet^R (Gly^-)	$GlyA^-$
MH538	P1CM/MH537 X MH429	Tet^R (Gly^-)	$GlyA^-$
MH581	P1CM/MH128 X MH429	Thy^+	Thy^+
MH581F	P1CM/LH18 X MH581	Kan^R	$FolA^-$ Thy^+ M^+
MH581FM	P1CM/JF1754 X MH581F	FEP^+	$FolA^-$ Thy^+ M^-
MH722	P1CM/LH18 X MH293/pRK11	Kan^R	$FolA^-$ Thy^{ts} M^-
MH723	BW6165 X MH722	Met^+,His^+,Leu^+,Thy^+	Hfr
$SF4^+$	CAG5055 X SF4	Met^+ Gly^+	$FolC^+$

a. P1cm mediated transductions were performed by growing P1cm on the donor strain and then using it to infect the

recipient strain (for example Plcm/LH18 (donor) X JF1754 (recipient)) and selecting either for a Tn10 (confers tetracycline resistance) located in the vicinity of the gene of interest, kanamycin resistance for the ΔfolA::Kan mutation or prototrophy. The last two strain constructions (where Plcm is not indicated) were conjugations where the first strain indicated was the donor and the second strain was the recipient (for example BW6165 (donor) X MH722 (recipient).

b. The $FolA^+$ phenotype indicates that the strain is kanamycin sensitive and did not require folate end products to grow. $FolA^-$ phenotype indicates that the strain is kanamycin resistant and required folate end products in an M^+ background. M^- indicates the presence of a pair of mutations which suppress the folate end product auxotrophy of $FolA^-$ strains. M^+ represents the wild type alleles of these genes. $GlyA^-$ indicates that the strains required glycine because of a mutation in the glyA gene. Thy^{ts} indicates the presence of a temperature sensitive allele of the thyA gene. The Hfr phenotype of strain MH723 was verified by testing the ability of this strain to transfer known markers to an F^- strain.

c. FEP^+ indicates that the strain does not require folate end products to grow. FEP^- indicates that the strain requires folate end products to grow.

Motility was tested on soft AB plates containing 0.5% Bacto-agar. ß-glucoside fermentation was screened on minimal medium plates containing 0.5% salicin, 0.02% bromthymol blue, 0.1% yeast extract, and 100µg/ml thymidine. ß-glucoside fermenters were orange whereas non-fermenters were grey.

When appropriate kanamycin (30µg/ml), tetracycline (12µg/ml) or ampicillin (100µg/ml) were added to media.

Suppression assays. Suppression was measured by two methods. First a fast assay for plaque formation was used (Cheung and Herrington, 1982). The mutant phage was judged to be suppressed if it formed at least 100 times more plaques on the Thy^- host as on the Thy^+ host.

Secondly, the burst sizes of the UGA mutant OPC105 and of wild type phage in different strains grown in liquid JB medium were determined. The burst size of the mutant phage was then expressed as a percentage of the wild type burst obtained on the same host grown under identical conditions. The mutation was said to be suppressed by a mutant strain if its relative burst size was significantly higher in that strain than in the isogenic wild type strain.

To determine burst sizes, cultures were grown at 37^0C in JB medium supplemented with the indicated amount of thymidine to either an O.D. 600 of 1.0 or 200 Klett units. Cell aliquots of 0.1ml were then added to 1ml of prewarmed

medium supplemented with 30µg/ml thymidine and containing approximately 2 X 10^8 wild type or mutant T4 phage particles. Following a 5 minute adsorption period, the samples were diluted to give 1000 infected cells/ml. Chloroform treated and non chloroform treated samples were taken 5 minutes after infection to measure the ratio of adsorbed to unadsorbed phage. Chloroform treated samples were taken after one hour following infection to measure total phage production. Phage numbers were determined by standard plaque assays done on AB plates. Strain CAJ64, which permits the growth of both the mutant phage, opc105, and wild type T4 phage was used as the plating bacteria.

Statistical analyses. Experimental data was compared by students paired t-test. Differences were considered significant only if the t value corresponded to a probability of greater than 95%.

Determination of antibiotic and potassium dichromate sensitivities. To test antibiotic or potassium dichromate sensitivity, a small volume (0.1ml or less) of novobiocin, oxolinic acid, nalidixic acid or potassium dichromate was placed on the side of a well of a 24 well plate (manufactured by Falcon). Ten µl of an overnight culture, grown in LB medium, was placed on the opposite wall. AB soft agar (1ml) containing thymidine (100µg/ml) was then

added to each well. Six concentrations of each compound were assayed; novobiocin ranged from 20 - 1280µg/ml; oxolinic acid ranged from 0.025 - 1.6µg/ml; nalidixic acid ranged from 0.5 - 16µg/ml; and potassium dichromate ranged from 0.025 - 2.4mM. The concentrations of the antibiotic or potassium dichromate were increased by two fold increments. The plates were incubated for approximately 16hrs at 37°C. The agar became turbid if the cells grew. The M.I.C. (minimum inhibitory concentration) was the lowest concentration of the antibiotic or potassium dichromate which prevented growth.

Determination of sulfathiazole sensitivity. Sulfathiazole sensitivity was tested by streaking the strains for single colonies on appropriately supplemented minimal plates. Six different concentrations of sulfathiazole ranging between 0.05 - 5µg/ml were used. The M.I.C. was the lowest concentration that prevented single colony formation during a 16 hr. incubation at 28°C.

UV sensitivity. To measure UV sensitivity, 5 ml cultures were grown in LB medium to a density between 2 - 3 X 10^8 cells/ml. Cultures were chilled on ice, spun down for 5 min. at 3400Xg and then resuspended in an equal volume of 1X minimal medium A salts containing 0.02% $MgSO_4$. The cultures were placed in glass petri dishes and exposed to a UV source

placed 10 cm above the plate. Aliquots (200 µl) were withdrawn at different time points and assayed for viable cells by spotting 10 µl aliquots of diluted samples on LB plates (six spots per plate).

Determination of frequency of T4 resistant cells. Approximately 10^{10} T4 phage were added to 0.1ml of an overnight culture. The mixture was incubated for 30 min. at 37°C to allow for cell killing, and then diluted and plated on LB medium to determine the number of viable cells. The proportion of T4 resistant cells in each culture was determined by comparing the number of viable cells from infected cultures to that from uninfected cultures.

Sources of chemicals. The majority of the chemicals utilized were reagent grade and were purchased from either Sigma Chemical Company, Fisher Scientific, or Canlab Scientific Products. Casamino acids, nutrient bro , tryptone, and yeast extract were obtained from Difco.

RESULTS:

Part I. Folates and translational accuracy:

We proposed that the ability of Thy$^-$ cells to suppress T4 nonsense mutations results from an alteration in folate pools (Basso, Tiganos, and Herrington, 1993). To determine whether altered THF levels corelated with the ability of Thy$^-$ cells to suppress I assayed suppression under conditions which would be predicted to decrease total folate, the level of reduced folates, and the level of N5-methylTHF. To verify the postulate that altered THF levels in Thy$^-$ cells affects translational accuracy I verified the effect of a thyA mutation on the misreading activity of a known suppressor tRNA.

To determine whether THF levels correlated with the ability of Thy$^-$ strains to suppress we wanted to measure folate levels and phage yields from cells grown under the same conditions. These experiments can only be done with cells growing in liquid medium. However, the medium which we routinely use for monitoring suppression by plating assays, AB medium, does not allow for suppression in liquid cultures (see below). Therefore, it was necessary to formulate a defined medium which would allow for suppression in liquid cultures.

Media dependent suppression. To develop this medium I used plating efficiency to monitor suppression of six T4 nonsense mutants. In AB medium all six of the phage mutants produced at least 100 times more plaques on the Thy^- strain MH429 than on the Thy^+ strain MH581, indicating that these mutations were suppressed by the Thy^- host. In contrast, none of the mutants were suppressed when suppression was assayed on M9S (Table 3). In addition to supplements required for the growth of the strains, M9S contained tryptophan which is required for T4 adsorption. The plating efficiency of wild type T4 on strains MH429 and MH581 was similar in both AB and M9S. This indicated that the inability of Thy^- strains to suppress in M9S was not due to a general effect on T4 growth.

I tested the effect on suppression of adding different supplements to M9S. Different T4 mutants required different supplements to be suppressed by the Thy^- strain MH429 (Table 3). Five of the six mutants tested were suppressed by the Thy^- strain MH429 when eight additional supplements were added to M9S (JB mediun; Table 3). The roles of the different supplements will be discussed later.

The T4 mutant, opc100, was suppressed in AB medium but was not suppressed in any of the media shown in Table 3 or in JB medium supplemented with any one additional amino acid which was not already present in JB medium; vitamin B12, pantothenate, biotin, or para-aminobenzoic acid.

Burst size determinations. Plating efficiency of phage mutants provides a relatively simple and sensitive way to detect suppression. However, it does not allow us to determine the suppression efficiency nor can we analyze the constituents of the suppressing cells. To do this it is necessary to monitor phage growth in liquid cultures.

I measured the burst size or the number of phage produced per cell in a single round of infection with the phage mutant opc105. This mutant has a mutation affecting gene 34 which codes for one of the tail fiber proteins. Each tail fiber contains two molecules of this protein. Since T4 phage with fewer than three tail fibers are not infective (Bloomfield, 1983), the number of infectious particles produced is a function of the amount of this protein that is made. Thus, the burst size of opc105 is indicative of the efficiency of suppression.

To determine whether the cells suppressed the burst sizes and the percent of the wild type burst size obtained with different strains and/or media were compared performing a student's paired t-test. Differences were considered significant if the t value corresponded to a p value of greater than 0.05. Suppressing conditions were defined as those which resulted in a significantly higher burst size and relative burst size on the Thy^- strain as compared to those obtained on the Thy^+ strain.

Table 3. Suppression in minimal media:

Medium	Additions[b]	Suppression[a] of Phage[a]					
		JC1922 UAA	Elp12 UGA	M103 UAG	OPC23 UGA	OPC105 UGA	OPC100 UGA
AB	None	+	+	+	+	+	+
M9s	None	-	-	-	-	-	-
JB	H,I,V,F,Y,Ade,Nic,Zn	+	+	+	+	+	-
1	H,Nic	+	-	-	-	-	-
2	H,I,V,Nic	+	+	-	-	-	-
3	H,I,V,Ade,Nic	+	+	+	-	-	-
4	H,I,V,F,Y,Ade,Zn	+	+	+	+	-	-

a. The plating efficiency of the phage mutants listed on strain MH429 was assayed semi-quantitatively using the fast phage assay. Suppression was assessed by the ability to form at least 100 times more plaques on the Thy^- host than on the Thy^+ host; + indicates suppression, - no suppression.

b. Additions indicates the different supplements added to M9S. Concentrations are given in the materials and methods (JB medium). Standard one letter symbols were used for the amino acid designations. Nic., ADE, and Zn refer to nicotinate, adenine and $ZnCl_2$ respectively.

In JB medium the burst size of opc105 on the Thy$^-$ strain MH429 was significantly higher than its burst size on the Thy$^+$ strain MH581 (Table 4). The burst of wild type phage was the same in both strains (Table 4) indicating that the difference in the burst size of opc105 is not due to a simple enhancement of phage production in the Thy$^-$ strain. The wild type burst sizes were considerably higher than those of the mutant, and were similar to published values for wild type T4 (for example, Mathews, 1965).

For comparison the burst size of opc105 and wild type T4 in strain CAJ64 which contains a mutated suppressor tRNA^{trp} were 19.4 and 71.7 respectively.

One of the phenotypes of thyA mutants is an enhanced frequency of mutagenesis (Kunz and Glickman, 1985), which could be mistaken for suppression if the mutant phage were reverting to wild type at a high frequency. I plated samples from the burst size experiments on strain MH581, which is nonpermissive for plaque formation by opc105. No wild type phage were detected in the bursts from either the Thy$^-$ or the Thy$^+$ strains, indicating that there is no detectable stimulation of reversion in the Thy$^-$ strain.

I also measured the burst sizes from cells grown in M9S and AB media (Table 4). As expected from plaque assays, Thy$^-$ cells did not suppress in M9S. Surprisingly, they did not suppress in AB medium either. This suggests that the physiology of cells grown on solid medium is different from

that of cells grown in broth. Possibly, a component of AB medium inhibits suppression. Cells grown on solid medium may cause the localized depletion of this component.

Amplification of suppression. Plaque formation by the mutant opc105 occurs only on the Thy^- host even though a relatively large burst is also obtained on the Thy^+ strain. To determine whether the difference in burst sizes observed was sufficient to account for this observation I assayed phage yields after multiple rounds of infection. Cells growing exponentially in JB medium were diluted to 10^7 cells/ml and infected at a multiplicity of infection of 10^{-5}. The infected cells were incubated at 37^0C for 16 hours. The yield of wild type phage was 6.0×10^4 and 6.6×10^4 per input phage on strains MH429 and MH581 respectively. In contrast, the yield of opc105 was 1.3×10^5 on strain MH429 and only 6.2 on the Thy^+ strain MH581. Cultures of strain MH581 infected with opc105 were not lysed whereas all other cultures were. Mutant phage produced on either strain did not form plaques on the non permissive host MH581, indicating that there was no detectable reversion of the mutant phage. Thus, the small difference in burst sizes of the mutant phage is amplified in later rounds of infection. The low yield of mutant phage on the Thy^+ strain suggests that the phage produced is not propagated.

Table 4. Burst sizes in different media:

Medium	Phage	Burst sizes on Strain[a]		% of WT T4 on	
		MH429 (Thy^-)	MH581 (Thy^+)	Thy^-	Thy^+
JB	opc105	19.3 ± 7.0	5.6 ± 3.2	6.9	2.0
	T4	280 ± 71.0	280 ± 84.0		
M9s	opc105	2.7 ± 0.5	4.8 ± 0.3	1.3	2.0
	T4	213 ± 42.0	238 ± 59.0		
AB	opc105	8.1 ± 3.4	4.5 ± 2.0	6.0	4.1
	T4	135 ± 27.0	110 ± 30.0		

a. The mean and standard deviation of 10 (JB medium) or 4 (AB and M9S media) independent determinations are given for the burst sizes (number of phage produced per cell).

Suppression and folate levels

To determine whether suppression by Thy$^-$ strains was correlated with folate levels, I examined the effect of perturbing folate metabolism on suppression.This was done by assaying suppression in the presence of antifolate drugs and in strains with either reduced or increased levels of folate biosynthetic enzymes.

Thymidine inhibits suppression. Folate levels of cells grown in JB medium are reduced by high concentrations of thymidine (Tiganos, 1990; Basso, Tiganos and Herrington, 1993). Consequently, I wanted to determine if thymidine inhibited suppression in burst size assays. Normally, for burst size assays 30 µg/ml thymidine was added both during growth of the cells and during the infection. Thymidine (150 µg/ml) added during cell growth markedly reduced the burst size of opc105 on the Thy$^-$ strain MH429 without affecting the burst size of T4 thus indicating that it inhibits suppression (Table 5). When 500 µg/ml thymidine was tested, the burst sizes of both the mutant and wild type phages were reduced. The effect on the wild type burst size was greater in the Thy$^+$ host than in the Thy$^-$ host (Table 5). Under these conditions, it is difficult to separate an effect on suppression from an effect on T4 production.

Table 5. Effect of thymidine on suppression:

Strain	Burst of opc105[a] Thy (µg/ml)			Burst of T4 Thy (µg/ml)			% of WT[b] Thy (µg/ml)		
	30	150	500	30	150	500	30	150	500
MH429	19.3	2.1	5.4	280	244	77	7.0	0.9	7.0
MH581	5.6	N.D.[c]	2.6	280	N.D.	21	2.0	N.D.	12.3

a. Phage infections were done in JB medium supplemented with 30µg/ml thymidine. Thymidine concentrations indicated were those present during cell growth. The data represent the average of three independent experiments. The standard deviation was approximately 30 percent of the average.
b. % of the wild type burst was determined by dividing the mutant burst by the wild type burst and multiplying by 100.
c. N.D. Not Determined.

Inhibition of suppression by antifolate drugs. Sulfanilamide drugs inhibit dihydropteroate synthetase an enzyme required for the de novo synthesis of THF (Figure 2; Thomas and Broquis, 1963). Trimethoprim inhibits the E.coli encoded DHFR (Bertino and Stacey, 1966) whereas aminopterin inhibits the T4 enzyme (Kaneko and Kuno, 1979). Growth in the presence of either sulfathiazole or trimethoprim reduces the level of de novo folate biosynthesis (Rholman and Matthews, 1990; Herrington, 1994). I tested the effect of these folate antagonists on the burst sizes of mutant and wild type phage (Table 6).

Sulfathiazole reduced the burst sizes of both the mutant and wild type phages. The relative burst sizes in the Thy^- and Thy^+ hosts were not significantly different, indicating that sulfathiazole treatment inhibited suppression. The same effect was observed whether sulfathiazole was present during the growth of the cells and not during infection, or if it was present only during infection (Table 6).

Aminopterin inhibited suppression only if it was present at the time of infection (Table 6). In contrast, 20µg/ml trimethoprim did not inhibit suppression (Table 6). Higher concentrations of trimethoprim could not be assayed since they inhibited cell growth.

Table 6. Effect of antifolate drugs on suppression:

Compound	Concentration (μg/ml)	Time[a] Added	Burst of[b] opc105 on		Burst of[b] T4 on		% of WT[c] Burst on	
			THY^-	THY^+	THY^-	THY^+	THY^-	THY^+
none			19.3	5.6	280	280	7.0	2.0
Sulfathiazole	5	B	1.0	0.9	30	36	4.0	2.4
Sulfathiazole	5	A	1.3	1.8	27	45	4.6	3.6
Aminopterin	2	B	18.6	N.D.[d]	232	N.D.	8.0	N.D.
Aminopterin	2	A	4.8	9.0	500	468	0.9	1.9
Trimethoprim	10	B	15.0	N.D.	172	N.D.	12	N.D.
Trimethoprim	10	A	18.0	N.D.	129	N.D.	16	N.D.

a. Antifolate drugs were added to give the final concentrations indicated. Cells were treated with the drugs during the 5 minute adsorption period (A) at the time of infection and then diluted 100 fold or treated during growth of cells (B).

b. Burst sizes were determined in JB medium supplemented with 30μg/ml thymidine. The data represents the average from three independent experiments. The standard deviation was approximately 30 percent of the average.

c. % of the wild type burst was determined by dividing the mutant burst by the wild type burst and multiplying by 100.

d. N.D. Not Determined.

A folA mutation inhibits suppression. Strain LH18 contains a folA null mutation (Howell, Foster, and Foster, 1988) and is also Thy^- since Thy^+ derivatives of this strain are not viable (Howell, Foster, and Foster, 1988). Strain LH18 did not suppress the T4 mutant opc105.

Since Thy^- dependent suppression can be affected by the strain background (Herrington et al., 1991), I asked whether a $FolA^+$ Thy^- derivative of LH18 could suppress. The relative burst size of opc105 was the same on this strain ($LH18^+$) as on our standard strain, MH429 (Table 7), indicating that suppression was prevented when the folA gene was deleted.

Effect of elevated expression from the folC gene. The folC gene encodes a bifunctional enzyme having dihydrofolate synthetase (DHFS) and folylpolyglutamate synthetase (FPGS) activity (Ferone et al., 1983). This gene has been cloned, and cells transformed with this plasmid (pAC5) contain 15 times the folC enzyme activity of wild type cells (Bognar, Osborne and Shane, 1987). Strain MH429 transformed with this plasmid no longer suppressed (Table 7).

In contrast to E.coli, the FPGS activity of L.casei is mediated by a monofunctional enzyme (Toy and Bognar, 1990). The gene coding for this activity has been cloned and expressed in E.coli. To determine whether inhibition of suppression by plasmid pAC5 is due to overexpression of DHFS

or FPGS activity I assayed suppression by strain MH429 transformed with a plasmid (pGTS-8.1) carrying the L.casei gene. This strain still suppressed indicating that it is the increased DHFS activity which inhibits suppression in MH429 transformed with pAC5 (Table 7).

Effect of glyA and metF mutation on suppression. Since N5,N10-methyleneTHF is the precursor to all other one carbon substituted THF derivatives, a reduction in its synthesis should decrease total folate pools. I assayed suppression by a glyA thyA double mutant (strain MH538) grown in the presence or absence of inosine, which represses the synthesis of the glycine cleavage enzymes (Stauffer, 1987). When strain MH538 was grown in the absence of inosine the burst size of wild type phage was the same as in the isogenic Thy$^-$ strain MH429, but the burst size of the mutant phage was significantly reduced, indicating that the glyA mutation interferes with suppression (Table 8). When grown in the presence of inosine the burst sizes of both wild type and mutant phage were reduced. The efficiency of suppression by the glyA thyA double mutant was significantly reduced by inosine (Table 8).

N5-methylTHF does not serve as a precursor for any other one carbon substituted THF derivative (Figure 2). Consequently, a metF mutant should have reduced levels of N5-methylTHF but not of other C1 substituted THF

derivatives, since these would still be provided from de novo synthesis. The burst size in strain MHBF (metF thyA) was much lower than in the Thy^- strain (Table 4) and was even lower than that observed in the Thy^+ strain (compare MH581 (Table 4) and MHBF (Table 8)). These results indicate that suppression by thyA mutants is a consequence of elevated N5-methylTHF levels.

Suppression by a folC mutant. Strain SF4 contains about 10% of wild type DHFS - FPGS activity due to a folC mutation (Bognar, Osborne and Shane 1987). This strain has an auxotrophic requirement for methionine because it can not form polyglutamated N5-methylTHF. Consequently, a folC mutant could accumulate monoglutamated 5-methylTHF. I asked whether this strain could suppress. Strain SF4 suppressed the mutant opc105 whereas the isogenic $FolC^+$ strain ($SF4^+$) did not (Table 7).

FPGS activity encoded by the L.casei gene inhibited suppression by strain SF4 (Table 7).

The methionine requirement of strain SF4 can also be satisfied by vitamin B12, since E.coli contains a B12 dependent homocysteine transmethylase activity which can use monoglutamated 5-methylTHF as a substrate. This activity could prevent the accumulation of 5-methylTHF. Vitamin B12 inhibited suppression by strain SF4 (Table 7).

Table 7. Suppression as a function of de novo folate metabolism:

Strain	Relevant Phenotype	Burst of[a] opc105	Burst of[a] WT T4	% of WT Burst
LH18	Thy^- $FolA^-$	2.7	280	0.9
LH18$^+$	Thy^- $FolA^+$	11.5	142	8.0
SF4	Thy^+ $FolC^-$	12.0	92	13.0
SF4(+B12)[b]	Thy^+ $FolC^-$	1.3	101	1.3
SF4$^+$	Thy^+ $FolC^+$	1.0	127	0.7
MH429	Thy^- $FolC^+$	19.6	202	8.1
MH429/pAC5[c]	Thy^- $FolC^+$	2.6	95	2.7
MH429/pGTS-8.1	Thy^- $FPGS^+$	8.3	114	7.3
SF4/pGTS-8.1	Thy^+ $FolC^-$ $FPGS^+$	0.2	60	0.3

a. Burst sizes were determined as described in the Materials and Methods. The data represent the average from three independent experiments. The standard deviation was approximately 30 percent of the average.

b. Vitamin B12 was added at a final concentration of 2pg/ml during cell growth.

c. Since plasmid pAC5 contains the folC gene under the control of the lac promoter, cells were grown in the presence of the gratuitous inducer IPTG at a final concentration of 1mM.

Table 8. Suppression as a function of different folate species:

Strain	Relevant Genotype	Burst of[a] opc105	Burst of[a] WT T4	% of WT Burst
MH429	Thy^-	19.0	280	7.0
MH538	Thy^- $GlyA^-$	4.4	282	1.6
MH538(+Inosine)[a]	Thy^- $GlyA^-$	0.3	64	0.5
MHBF	Thy^- $MetF^-$	0.3	318	0.1

a. Burst sizes were determined as described in the Materials and Methods. The data represent the average of at least three independent experiments. The standard deviation was approximately 30 percent of the average.

b. Inosine was added during the growth of the cells at a final concentration of 100µg/ml.

Effect of thyA mutation on Sup(UGA). We proposed that as a consequence of altered folate pools, thyA mutants mismodify some component(s) of the translational apparatus and therefore suppress nonsense mutations. To confirm this postulate I asked if a thyA mutation would affect the readthrough efficiency of a known suppressor tRNA. The effect of a mutant thyA allele on the suppression efficiency of the T4 mutant opc105 by strain CAJ64 was assayed under conditions which were permissive and non-permissive to thyA dependent suppression (Table 9). Strain CAJ64 contains a mutant $tRNA^{trp}$ (SupUGA) which misreads UGA nonsense codons. Consequently, if Thy^- cells usually mismodify $tRNA^{trp}$, mismodification of the mutant $tRNA^{trp}$ of strain CAJ64 may alter its misreading activity. This strain suppressed opc105 with an efficiency of 27%. This result is in good agreement with the reported suppressor efficiency of this strain (30%, Sambrook, Fan and Brenner, 1976). The suppression efficiency on a Thy^- derivative of this strain was drastically reduced (Table 9).

To determine if antisuppression by the thyA mutation was due to the ability of Thy^- cells to suppress, suppression by the thy^- derivative of strain CAJ64 was assayed in the presence of aminopterin.

Table 9. Effect of a thyA mutation on SupUGA:

Strain	Relevant Genotype	Condition[a]	Burst of OPC105	Burst of T4	% of WT Burst
CAJ64	SupUGA Thy^+	None	19.4	71.7	27
MH442	SupUGA Thy^-	None	0.1	53.0	0.2
CAJ64	SupUGA Thy^+	Aminopterin	69.2	84.3	82
MH442	SupUGA Thy^-	Aminopterin	67.0	192	35

a. Aminopterin was added only during phage growth at a final concentration of 2µg/ml.

Aminopterin, which prevents thyA dependent suppression, restored the suppression efficiency of MH442 (SupUGA Thy^-) to that observed for the Thy^+ strain grown in the absence of aminopterin. Aminopterin also enhanced the suppression efficiency of strain CAJ64 (Thy^+, SupUGA).

The burst size of wild type phage on strain CAJ64 was approximately one third of those observed with our standard strains. A normal burst size was obtained with the Thy^- strain when exposed to aminopterin (Table 9).

PART 2. Effect of folA null mutations on E.coli:

The folA gene encodes the enzyme dihydrofolate reductase which is required for the de novo synthesis of THF and its regeneration from the thymidylate synthase cycle. This is thus a key enzyme for THF metabolism in E.coli. I wanted to determine the effect of removing this activity on the growth of E.coli. To do so, I used a deletion mutation an constructed by Howell, Foster, and Foster (1988), and an insertion mutation which I constructed.

Growth of folA deletion mutants on minimal medium. There are conflicting reports as to whether strains containing a deletion spanning the kefC, folA, and apaH genes (ΔfolA::kan) require folate end products and whether they tolerate a wild type thyA gene (Howell, Foster, and Foster, 1988; Ahrweiler and Frieden, 1988; Hamm-Alvarez, Sancar, and

Rajagopalan, 1990; Krishnan and Berg, 1993). To determine whether this was due to strain differences I introduced the Δ*folA::kan* deletion (Howell, Foster, and Foster, 1988) into different strains and tested the growth of the resulting strains on minimal medium with and without folate end products. Furthermore I tested the ability of making different folA$^-$ strains Thy$^+$. Since the Δ*folA::kan* mutation extends into adjacent genes I also tested the growth properties of a *folA* insertion mutant which I constructed.

All Δ*folA::kan* mutants grew on minimal medium supplemented with folate end products.

In my hands, strain LH18 (Δ*folA::kan thyA*) grew slowly on minimal medium (Table 10). This strain has been reported not to grow (Howell, Foster and Foster, 1988) or to grow normally on minimal medium (Hamm-Alvarez, Sancar, and Rajagopalan, 1990).

Howell, Foster, and Foster (1988) reported that strain LH18 could not be made Thy$^+$. My inability to transduce this strain with a wild type *thyA* gene is consistent with their finding. Furthermore, I could not transform this strain with a plasmid carrying the wild type *thyA* gene. I was able to transform it with a plasmid containing a temperature-sensitive *thyA* allele (pRK11) (Kurzman, 1989) as long as the transformants were selected at the non-permissive temperature (42°C). These transformants were not viable at the permissive temperature (28°C).

I was able to introduce the Δ*folA::kan* mutation into both Thy$^+$ and Thy$^-$ derivatives of strains MH581 and JF1754. This result is consistent with those reported by Krishnan and Berg (1993). The Δ*folA::kan* Thy$^-$ derivative of strain MH581 grew slowly whereas the Δ*folA::kan* Thy$^+$ derivative did not grow on minimal medium. In contrast, both Δ*folA::kan* derivatives of strain JF1754 grew on minimal medium (Table 10).

Compensatory mutation(s). Strain MH581F (Δ*folA::kan* Thy$^+$) plated on minimal medium supplemented with thymidine gave rise to slow-growing colonies at a frequency of 1.2×10^{-6}. These mutants proved to be thymidine requiring. In contrast, no colonies were obtained when 10^{10} cells were plated on minimal medium lacking thymidine. These results indicated that in this background, *thyA* mutations partially suppressed the folate end product auxotrophy caused by the Δ*folA::kan* deletion and that other types of suppressors do not arise at a high frequency.

Δ*folA::kan* deletion mutants derived from strain JF1754 grew normally on minimal medium, suggesting that compensatory mutation(s) were present in this background. Strain JF1754 is auxotrophic for methionine, histidine, and leucine. To determine whether any of the mutations causing these auxotrophies suppressed the Δ*folA::kan* phenotype I constructed a prototrophic derivative of this strain (Met$^+$,

His$^+$, Leu$^+$, ΔfolA::kan strain MH723). This strain grew as well as JF1754 (FolA$^+$) on minimal medium. Therefore, strain JF1754 has genetic differences, other than these auxotrophies, which allow ΔfolA::kan mutants to grow on minimal medium.

The compensatory mutation(s) of strain JF1754 could be transferred, by P1cm mediated transduction, to strains which grew slowly or not at all by selecting for cells which formed colonies on minimal medium after an overnight incubation (see mapping section). I refer to this as the M$^-$ phenotype, and the resultant strains are listed in Table 10 as LH18M and MH581M derivatives. The growth of these strains was identical to that of strains derived from JF1754 (Table 10).

Effect of the inactivation of neighbouring genes by the folA deletion. The folA::kan deletion was constructed in such a way that part of the upstream gene, kefC and part of the downstream gene, apaH, were also deleted (Figure 4). Consequently, it is not obvious whether the phenotypes associated with the ΔfolA::kan mutation are due to the inactivation of folA, apaH, or kefC. The kefC gene product is involved in potassium retention (Epstein and Kim, 1971). E.coli strains with mutations in the kefC gene do not grow on minimal media containing 0.1- 0.5 mM potassium (Epstein and Kim, 1971). All the strains containing the folA::kan

deletion grew normally on K medium containing either 0.1 mM or 0.5 mM potassium and all the folate end products. Thus insertion of the kanamycin cassette in the kefC gene does not confer this kefC phenotype.

One phenotype associated with apaH null mutations is a lack of motility (Farr et al., 1989). MH581F (ΔfolA::kan) is non-motile whereas the FolA$^+$ derivative is motile suggesting that the apaH gene is non-functional in this strain. Consequently, I asked whether the inability of the ΔfolA::kan strains to grow on minimal medium was due to a lack of DHFR activity, diadenosine tetraphosphate hydrolase activity or both. To determine this I used two approaches. First, I tested the growth on minimal medium of strain LH18 transformed with either plasmid pAG101 which contains the E.coli folA gene (Howell, Foster, and Foster, 1988), or plasmid pPL113 (Lagosky, Taylor, and Haynes, 1987) containing the yeast DFR1 gene coding for DHFR. Both transformed derivatives of LH18 grew overnight on minimal medium, indicating that the plasmids complemented the folate end product auxotrophy of strain LH18 and that this auxotrophy was due to the lack of DHFR activity.

Growth of folA and apaH insertion mutants. The second approach which I used was to generate λplacMu insertion mutations of folA and apaH. Both Thy$^-$ and Thy$^+$ derivatives of MH581 containing the folA::λplacMu53 insertion did not

grow on minimal medium lacking the full complement of folate end products (Table 10). These strains did grow on minimal medium supplemented with the full complement of folate end products. These results suggested that the partial suppression of the folate end product auxotrophy by a Thy$^-$ allele in strain MH429F and LH18 (Δ*folA::kan thyA*) is dependent on the inactivation of one of the other genes.

The *folA* gene from plasmid pAG101 allowed *folA* insertion mutants derived from MH581 to grow in the absence of folate end products, thus indicating that the plasmid copy of the *folA* gene complemented the insertion mutation.

The *apaH*::λ*plac*Mu53 insertion conferred a folate end product auxotrophy in a Thy$^-$ but not in a Thy$^+$ background (Table 10). The auxotrophy could not be satisfied with any subset of the folate end products and could not be complemented by the *folA* gene of plasmid pAG101. This auxotrophy was surprising since strain MH429F/pAG101 was not auxotrophic even though the *apaH* gene is inactivated by the Δ*folA::kan*, and the plasmid does not carry complementary sequences to *apaH*.

Suppression of *folA* and *apaH* insertion mutations. Since the folate end product auxotrophy associated with the *folA::kan* deletion was suppressed in strain JF1754, I asked whether the compensatory mutation(s) would also suppress the *folA* and *apaH* insertion mutations. Introduction of the

folA::λplacMu53 and the apaH::λplacMu53 insertions in the JF1754 or MH581M background did not result in folate end product auxotrophy, indicating that the compensatory mutation(s) of strain JF1754 suppressed the growth phenotypes associated with these mutations (Table 10).

Sulfathiazole sensitivity of FolA⁻ strains. It is believed that the folate end product auxotrophy of folA mutants is due to a decreased potential for de novo folate biosynthesis (Howell, Foster, and Foster, 1988; Krishnan and Berg, 1993). To verify this postulate I compared the sensitivity of isogenic FolA⁻ (insertion and deletion mutants) and FolA⁺ strains to the dihydropteroate synthetase inhibitor sulfathiazole. Sensitivity to sulfathiazole was only observed on minimal medium without folate end products. Consequently, only FolA⁻ strains containing the compensatory mutations present in JF1754 were used. The M.I.C. of sulfathiazole of FolA⁻ (insertion and deletion mutants) and FolA⁺ strains were 0.1 and 5.0μg/ml respectively. No difference in sulfathiazole sensitivity was observed on minimal medium with all the folate end products. The compensatory mutation(s) of strain JF1754 did not affect the sensitivity to sulfathiazole since both FolA⁺ strains MH581 and JF1754 were as sensitive (M.I.C. of 5.0μg/ml).

Mapping of the suppressor mutations of JF1754. P1cm mediated

transductions were used to transfer the suppressor mutation(s) from strain JF1754 to strains LH18, MH581F, and MH429F. I selected for transductants which formed normal sized colonies after an overnight incubation on supplemented minimal medium with and without kanamycin. The selection without kanamycin permitted the growth of both FolA$^+$ and suppressor derivatives of the ΔfolA::kan strains whereas selection in the presence of kanamycin allowed only growth of the suppressor derivatives.

TABLE 10. Growth on minimal medium.

Genotype	Growth[a] of Strains Derived From				
	LH18	MH581	JF1754	LH18M	MH581M
Wild Type	+	+	+	+	ND
thyA	+	+	+	+	ND
Δ*folA::kan*	ND	-	+	+	+
Δ*folA::kan thyA*	s	s	+	+	+
*folA::*λ*plac*Mu53	ND	-	+	ND	+
*folA::*λ*plac*Mu53 *thyA*	ND	-	+	ND	+
*apaH::*λ*plac*Mu53	ND	+	ND	ND	ND
*apaH::*λ*plac*Mu53 *thyA*	ND	-	+	ND	+

a. The growth of the strains was tested on minimal medium containing the appropriate supplements to satisfy auxotrophic requirements but without the full complement of folate end products. + indicates that the strain grew overnight; s indicates that the strain required 3 days to grow,; - indicates that the strain did not grow after five days.

[b] ND, not determined (note that it is not possible to obtain a Δ*folA::kan* Thy$^+$ derivative of strain LH18).

Suppressor mutants were obtained at a frequency of approximately 10^{-3} FolA$^+$ transductants. This low frequency suggests that the suppressor phenotype is caused by at least two compensatory mutations affecting genes which I will refer to as m1 and m2. These genes cannot be separated by more than 2.2 minutes since this is the maximum amount of DNA which can be transferred by P1cm (Margolin, 1987).

Interrupted conjugations using BW6165 as a donor (earliest marker transferred Leu; latest marker transferred Pan) and JFF (ΔfolA::kan) as recipient were used to determine the approximate map position of the suppressor mutations. Mating mixtures were interrupted after 12, 15, 20, 25, and 30 minutes and plated on LB medium with tetracycline and kanamycin. Tetracycline selection permitted me to monitor the frequency at which a Tn10 located in the argE gene was transferred to the recipient strain. Kanamycin selection ensured that the recipient strain remained ΔfolA::kan. The number of conjugants obtained was approximately the same at all times (approximately 200). Fifty conjugants from each sample were picked onto LB plates containing kanamycin. All of the conjugants picked from the 12 and 15 minute conjugation time points grew. In contrast, 20%, 52%, and 80% of the conjugants obtained after 20, 25, and 30 min. transfers respectively, did not grow again when picked. This result suggested that the ΔfolA::kan mutation was lethal in the

JF1754 background if one or both of the compensatory mutations was removed and that these mutation(s) mapped near the 85 min. region.

A reciprocal conjugation using as a donor strain MH723, which is a Hfr derivative of JFF (ΔfolA::kan) with the same point of origin as BW6165, and strain LH18 as a recipient supported this map location. The conjugation mixture was plated on minimal medium with tetracycline and kanamycin. Folate end product prototrophs were obtained after 20, 25 and 30 min. conjugation periods (34, 86, and 134 colonies respectively). Thus the suppressing alleles of m1 and m2 were both transferred in this cross. These results indicate that the compensatory mutations of JF1754 are located between 82 and 87 minutes on the E.coli chromosome.

Fine mapping of the compensatory mutations of strain JF1754 was performed by using several tetracycline resistant strains with a Tn10 transposon at known locations as donors in P1cm mediated transductions with strains JF1754 and JFF as recipients (Table 11). Tetracycline resistant transductants were selected. I monitored two events in transductions with strain JFF (ΔfolA::kan). First, I tested transductants for growth on minimal medium without folate end products. Second, I estimated the number of non-viable transductants of strain JFF by comparing the number of tetracycline resistant colonies obtained with JFF to the number obtained with strain JF1754 (Table 11).

Two donor strains with Tn10s at map positions 83.0 and 86.25 min. gave very low numbers of transductants using strain JFF as the recipient (crosses 2 and 5, Table 11). This suggests that the m1 gene maps close to 83 min. and the m2 gene maps near 86.25 min.. Furthermore, these results suggested that strains which were Δfol*A*::kan $m1^+$ $m2^-$ or Δfol*A*::kan $m1^-$ $m2^+$ were not always viable.

All of the transductants obtained in cross 2 (Table 11) grew on minimal medium, suggesting that their genotype was the same as that of strain JFF (that is, Δfol*A*::kan $m1^-$ $m2^-$). In contrast, 66% of the viable transductants obtained in cross 5 (Table 11) did not grow on minimal medium. These transductants are probably Δfol*A*::kan $m1^-$ $m2^+$ and are not always viable. Viable recombinants require folate end products to grow.

Sixteen percent of the transductants in cross 4 (Table 11) were auxotrophs, which is consistent with the formation of viable Δfol*A*::kan $m1^-$ $m2^+$ recombinants. Approximately the same numbers of transductants were observed with both strains JF1754 and JFF in this cross (cross 4, Table 11). Co-transduction of the m1 or m2 genes with the Tn10 in this donor strain would be expected to be low. The appearance of non-viable transductants with strain JFF could be masked if the efficiency of transduction into strain JFF is substantially higher than that in strain JF1754. This appears to be the case. Transductions which did not involve

the transfer of a wild type allele of either of the compensatory mutations gave rise to two to ten times more transductants with strain JFF than with strain JF1754 (for example, cross 1, Table 11). Thus, my estimates of the number of non-viable transductants were probably low.

The location of the compensatory mutations was confirmed by performing reciprocal crosses. I used strain JFFJ, a transductant from cross 2 (Table 11) which did not require folate end products as a donor in P1cm mediated transductions. This strain was Δ*folA::kan* and presumably $m1^-$ $m2^-$ *zid::Tn10*. Strains MH581 and MH581F were used as recipients (Table 12). A higher transduction frequency was observed with strain MH581 than with strain MH581F (Table 12; compare cross 1 and 2). This result is consistent with the conclusion that Δ*folA::kan* $m1^-$ $m2^+$ recombinants are not always viable. Fifty tetracycline resistant MH581F recombinants were screened for the ability to grow on minimal medium in the absence of folate end product supplementation. All were auxotrophic for folate end products. These strains could be $m1^-$ $m2^+$ or $m1^+$ $m2^+$. Two recombinants generated from this cross (strains MH581FJ-1 and MH581FJ-2) and their parent strain MH581F were used as recipients in transductions using strain JF1754 as the donor (Table 12; cross 5 and 6). Folate end product prototrophs were selected on minimal medium containing kanamycin. Under these conditions only Δ*folA::kan* $m1^-$ $m2^-$ strains can grow.

Approximately 200 transductants were obtained with strains MH581FJ-1 and MH581FJ-2 whereas no transductants were obtained with the parent strain MH581F. This indicates that strains MH581FJ-1 and MH581FJ-2 are genotypically ΔfolA::kan $m1^-$ $m2^+$ and only require the $m2^-$ allele from strain JF1754 to become prototrophic. In contrast strain MH581 must obtain both mutant alleles and thus prototrophs are rare in this cross. P1cm grown on a recombinant obtained from cross 5 (Table 11) which did not require folate end products (JFF91; presumably ΔfolA::kan $m1^-$ $m2^-$ metE::Tn10) was used to transduce strain MH581F and MH581 to tetracycline resistance (Table 12; cross 3 and 4). Fewer tetracycline resistant transductants were obtained with strain MH581F than with strain MH581. This result supported the conclusion that ΔfolA::kan $m1^+$ $m2^-$ recombinants are not viable.

Strain JF1754 contains a gyrB mutation. One of the compensatory mutations (m1) found in JF1754 derivatives mapped very close to 83.0 min.. A gene located in this area is gyrB. The gyrB gene encodes one of the two subunits of DNA gyrase (Yamagishi et al., 1986). To determine whether one of the compensatory mutations is in the gyrB gene, I transformed ΔfolA::kan and $FolA^+$ derivatives of JF1754 and MH581 with plasmid pJB11 which contains the gyrB gene.

TABLE 11. Mapping of compensatory mutations of strain JF1754.

Cross	Donor	Location of Tn10 (Min)	Frequency[a] of transductants JF1754	JFF	%[b] Fep$^-$	%[c] non-viable
1	CAG12158	56.75	110	231	0	0
2	JW355	83.0	750	44	0	94
3	CAG18501	83.75	212	102	6	52
4	CAG18431	84.5	393	411	16	0
5	CAG18491	86.25	200	12	66	94

a. Number of tetracycline resistant transductants obtained per 10^7 cells.

b. %Fep$^-$ indicates the number of transductants which did not grow without folate end products. Whenever possible 50 Tet^R derivatives of strain JFF were tested for their ability to grow on minimal medium lacking folate end products.

c. I estimated the frequency of non-viable transductants of strain JFF by dividing the difference in the number of transductants obtained on strains JF1754 and JFF by number of transductants obtained on JF1754 and multiplying by 100.

Table 12. Mapping by reciprocal crosses of compensatory mutations of strain JF1754.

Cross	Donor (genotype[a])	Recipient (genotype[a])	Selected phenotype	No. of recombinants
1	JFFJ (ml^- $m2^-$ $FolA^-$)	MH581 (ml^+ $m2^+$ $FolA^+$)	Tet^R	326
2	JFFJ (ml^- $m2^-$ $FolA^-$)	MH581F (ml^+ $m2^+$ $FolA^-$)	Tet^R	94[b]
3	JFF91 (ml^- $m2^-$ $FolA^-$)	MH581 (ml^+ $m2^+$ $FolA^+$)	Tet^R	280
4	JFF91 (ml^- $m2^-$ $FolA^-$)	MH581F (ml^+ $m2^+$ $FolA^-$)	Tet^R	56
5	JF1754 (ml^- $m2^-$ $FolA^+$)	MH581F (ml^+ $m2^+$ $FolA^-$)	FEP^+ [c]	0
6	JF1754 (ml^- $m2^-$ $FolA^+$)	MH581FJ-1 (ml^- $m2^+$ $FolA^-$)	FEP^+	*200

a. The relevant genotype is given for each strain.

b. Fifty transductants were tested and found to be folate end product auxotrophs.

c. Selection was performed on minimal medium containing tetracycline and methionine.

I selected for ampicicillin resistant cells on LB medium containing 100 µg/ml ampicillin. Strains MH581 and its FolA$^-$ derivative could be transformed with the same efficiency. In contrast the efficiency with which the ΔfolA::kan derivative of strain JF1754 could be transformed was at least 1000 times less than that observed with the FolA$^+$ strain JF1754. Both strains could be transformed with pBLUESCRIPT at an equivalent efficiency. These results strongly suggest that expression of the wild type gyrB allele from pJB11 was lethal in the JF1754 ΔfolA::kan background.

If this were correct, plasmids retrieved from transformants of the ΔfolA::kan derivative would not contain a wild type gyrB allele. To determine whether plasmid from viable JFF/pJB11 and MH581F/pJB11 transformants contained a wild type gyrB gene, plasmid DNA was isolated from three JFF and three MH581F transformants and used to transform the gyrB mutant NI748. Ten colonies from each transformation was tested for resistance to 200µg/ml novobiocin. The gyrB mutation of strain NI748 prevents binding of novobiocin to the gyrB subunit and thus confers novobiocin resistance. Plasmid pJB11 complemented the mutant gyrB allele of NI748, thus making the strain novobiocin sensitive. Strains which were transformed with plasmid DNA isolated from either MH581F transformants were sensitive to novobiocin, indicating that the plasmids were probably pJB11. In

contrast, strains transformed with plasmid DNA isolated from JFF/pJB11 transformants were resistant to novobiocin. These results indicated that transformants obtained when strain JFF was transformed with pJB11 do not retain a wild type gyrB gene. Consequently one of the compensatory mutations of JF1754 is in gyrB.

Sensitivity of different strains to gyrase inhibitors. Since one of the compensatory mutations of strain JF1754 mapped to the gyrB gene, I compared the sensitivity of strains containing different combinations of ΔfolA::kan, and thyA to the gyrB inhibitor novobiocin and to the gyrA inhibitors oxolinic acid and nalidixic acid (Table 13). In the MH581 background the Thy^+ ΔfolA::kan mutant MH581F was the most sensitive and the thyA ΔfolA::kan mutant MH429F was the most resistant to the gyrase inhibitors. Similar results were obtained with strains from the LH background, but the ΔfolA::kan thyA mutant LH18 was more resistant to novobiocin and nalidixic acid than strain MH429F. I could not determine whether the thyA allele had an effect on the LH derivative because LH18 can not be made Thy^+ (Howell, Foster, and Foster, 1988)

To determine whether the effect of the ΔfolA::kan mutation on the sensitivity to gyrase inhibitors was due to the inactivation of the folA gene the following approaches were used. First I assayed the sensitivity to gyrase

inhibitors of LH18-2 (FolA$^+$, Thy$^-$) grown in the presence of 200µg/ml of trimethoprim, a DHFR inhibitor. Trimethoprim had no detectable effect on the level of sensitivity to novobiocin or nalidixic acid. Secondly, I assayed the sensitivity of a Δ*folA::kan* mutant containing either a plasmid with the *E.coli* FolA$^+$ gene (pAG101) or the heterologous yeast DFR1 gene (pPL113). The *E.coli* FolA$^+$ gene from plasmid pAG101 increased the susceptibility of strain LH18 to the three gyrase inhibitors but did not restore it to wild type levels (Table 13). In contrast, the yeast DFR1 gene from plasmid pPL113 had no effect on the susceptibility of strain LH18 to any of the gyrase inhibitors (Table 13). Both plasmids complemented the folate end product auxotrophy of strain LH18.

The M.I.C. of novobiocin and nalidixic acid of strain LH18 was restored to wild type levels by an F' factor containing the 98 - 2min. region of the *E.coli* chromosome (Table 12).

I also compared the sensitivity of strains derived from MH429 with λ*plac*Mu53 inserted in either the *folA* or *apaH* gene (MH429Fmu and MH429A respectively). The M.I.C. of novobiocin and nalidixic acid of both insertion mutants was the same as that of the wild type strain MH429 (160 and 2µg/ml respectively).

Effect of mutant thyA alleles on the sensitivity to gyrase inhibitors. Since a mutant thyA gene affected the level of resistance of ΔfolA::kan mutants to gyrase inhibitors I asked whether thyA also affected the level of resistance to gyrase inhibitors in a FolA$^+$ background. A thyA mutation decreased the sensitivity of both FolA$^+$ and ΔfolA::kan strains (Table 13). The increased resistance caused by the thyA mutation was not restricted to the thyA723 allele since six other Thy$^-$ derivatives (including two deletion mutants) of MH581 were as resistant as MH429 (results not shown).

Effect of M mutations on the M.I.C. of gyrase inhibitors. I asked whether the compensatory mutations which suppress the folate end product auxotrophy of FolA$^-$ mutants also suppressed the resistance of Δfola::kan mutants to gyrase inhibitors. The M.I.C.s of all three gyrase inhibitors were decreased in a Thy$^-$ background and increased in a Thy$^+$ background by the M mutations (Table 13; compare LH18-5 to LH18, MH429 to MH429FM and MH581 to MH581FM). The M mutations had no effect on the M.I.C. of gyrase inhibitors of FolA$^+$ Thy$^+$ strains (Table 13; compare MH581, LH18-9 and JF1754).

TABLE 13. M.I.C. of gyrase inhibitors.

Strain	Relevant Phenotype			M.I.C. of (μg/ml)b		
	Thy	Fol	M[a]	Nal	Oxo	Nov
LH18-9	+	+	+	0.5	0.2	80
LH18-2	-	+	+	2	0.2	160
LH18	-	-	+	8	0.8	1280
LH18-5	-	-	-	4	0.4	160
LH18-13	+	-	-	4	0.2	80
LH18/pAG101	-	+	+	2	0.4	640
LH18/pPL113	-	+	+	8	0.8	1280
LH18/F'101	-	+	+	2	ND	160
MH581	+	+	+	0.5	0.2	80
MH581F	+	-	+	0.5	0.05	10
MH581FM	+	-	-	0.5	0.2	40
MH429	-	+	+	2	0.4	160
MH429F	-	-	+	4	0.8	640
MH429FM	-	-	-	2	0.4	160
JF1754	+	+	-	0.5	ND	80

a. M^- strains contain mutant alleles which compensate for the folate end product auxotrophy of folA mutants. M^+ strains presumably contain wild type alleles.

b. Nal, nalidixic acid; Oxo, oxolinic acid; Nov, novobiocin.

The folA gene is essential in some gyrA or gyrB mutants. Since DNA gyrase is affected by ΔfolA::kan and thyA mutations I asked whether the ΔfolA::kan mutation could be introduced into strains containing different gyrA or gyrB alleles. I isolated independent novobiocin and nalidixic acid resistant derivatives of MH581. A P1cm lysate of strain LH18 was used to transduce these strains to kanamycin resistance. Approximately 200 kanamycin resistant transductants were obtained with strain MH581, two nalidixic acid resistant derivatives, and one novobiocin resistant derivative. A higher transduction frequency was obtained with one nalidixic acid and one novobiocin resistant derivative (796 and 540 kanamycin resistant transductants respectively). No kanamycin resistant transductants were obtained with two novobiocin and one nalidixic acid resistant derivatives. By using the same lysate, all strains could be transduced equally well to methionine prototrophy. This indicated that the transduction efficiency did not differ among these strains and that the different frequencies of kanamycin resistant transductants observed reflects the ability of these strains to tolerate the ΔfolA::kan mutation.

Strain LH18 could not be made Thy^+ (Howell, Foster, and Foster, 1988). Since ΔfolA::kan mutations can be introduced into most Thy^+ strains (Krishnan and Berg, 1993) this suggested that LH18 has a secondary mutation which prevents

it from being made Thy$^+$. I asked if strain LH18 contained a gyrB mutation. A P1cm lysate made on strain JW355 was used to transduce strain LH18 to tetracycline resistance. Strain JW355 contains a Tn10 which is 90% linked to gyrB. Thus, the majority of the tetracycline resistant transductants contain the gyrB allele of JW355. I transduced four tetracycline resistant LH18-9 derivatives to kanamycin resistance with a P1cm lysate made on strain LH18. Approximately 200 kanamycin resistant transductants were obtained. In contrast, none were obtained with strain LH18-9. These results suggest that strain LH18 contains a mutant gyrB allele which prevents it from being thyA$^+$ Δfola::kan.

UV sensitivity of different LH18 derivatives. Since one of the compensatory mutations of strain JF1754 mapped in the same area as the uvrD gene, I compared the UV sensitivity of strains containing different combinations of the Δfola::kan, thyA and M mutations. The Δfola::kan mutation greatly increased the UV sensitivity of all the LH18 derivatives (Figure 5A and C). The level of UV sensitivity of the different ΔfolA::kan mutants was similar in M$^+$ and M$^-$ strains. In an M$^-$ background, the thyA allele did not greatly affect UV sensitivity (Figure 5C). I could not determine the effect of the thyA allele in an M$^+$ background because strain LH18 can not be made Thy$^+$ (Howell, Foster, and Foster, 1988).

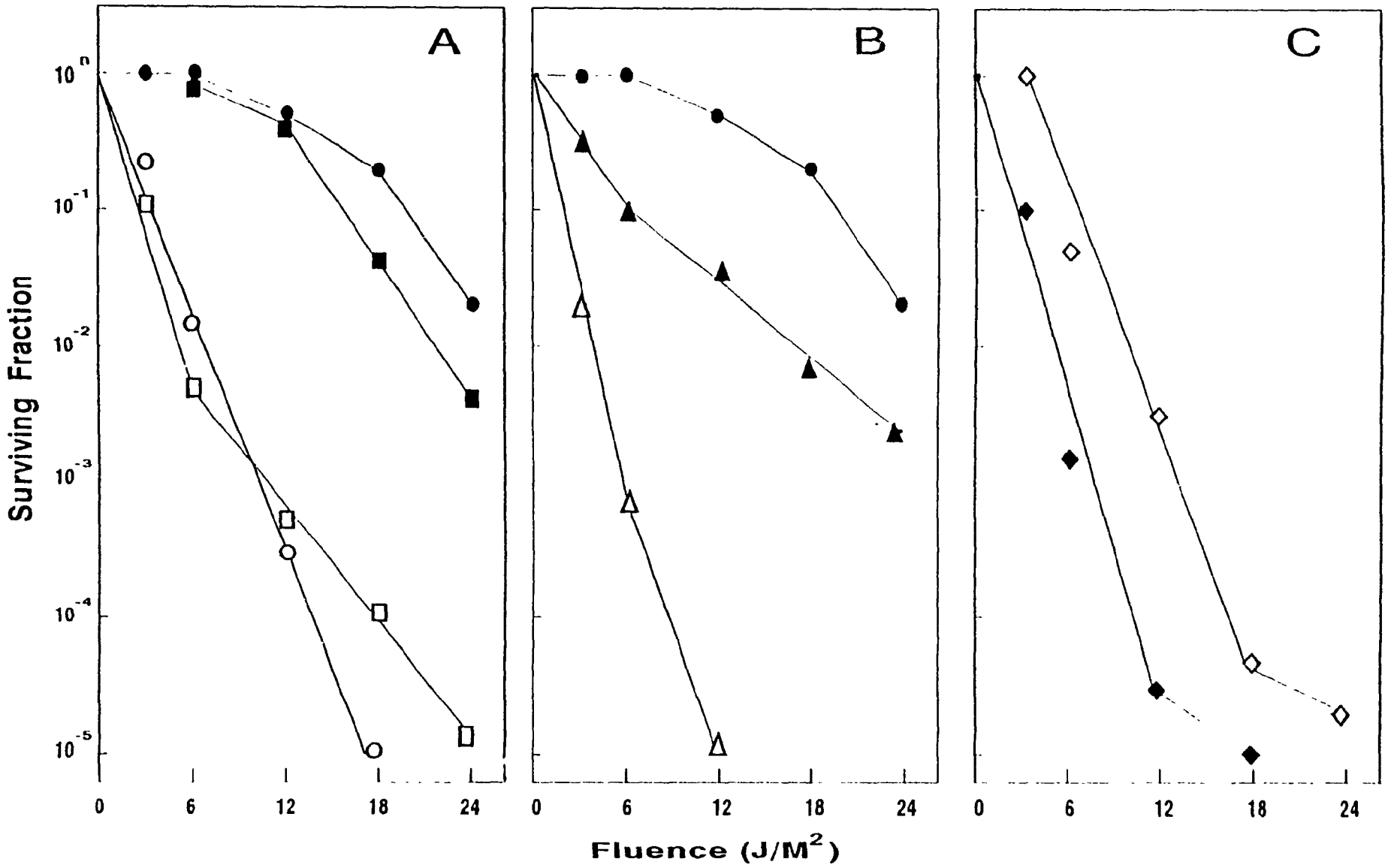
The E.coli FolA$^+$ gene of plasmid pAG101 made strain LH18 as UV resistant as the FolA$^+$ strain LH18-2. In contrast, the yeast DFR1 gene from plasmid pPL113 had no effect on the UV sensitivity of strain LH18 (Figure 5A). Thus the high UV sensitivity of ΔfolA::kan mutants is due to the lack of the E.coli DHFR protein.

The M mutations greatly increased the UV sensitivity of FolA$^+$ strains which is consistent with a uvrD phenotype (Figure 5B). This effect was more pronounced in a Thy$^+$ than in a Thy$^-$ background (Figure 5B).

The increased UV sensitivity of ΔfolA::kan strains suggests a decreased efficiency of DNA repair. To verify this I compared the frequency of spontaneous mutations between ΔfolA::kan and FolA$^+$ cells. I determined at what frequency these strains became resistant to T4 phage infection. The average frequencies (n=2) of T4 resistant colonies per 10^8 cells obtained with each strain were as follows: LH18-2 (Thy$^-$, FolA$^+$, M$^+$) 13; LH18(Thy$^-$, ΔfolA::kan, M$^+$) 35; LH18/pAG101 (Thy$^-$, ΔfolA::kan, FolA$^+$, M$^+$) 13; LH18-5 (Thy$^-$, ΔfolA::kan, M$^-$) 100.

Other phenotypes associated with folA mutants. The ΔfolA::kan, folA::λplacMu53 and thyA mutations repressed motility in the MH581 background.

Figure 5A-C. UV sensitivity of strains derived from LH18. ● LH18-2 (FolA$^+$, Thy$^-$, M$^+$); ○ LH18 (Δ<u>folA::kan</u>, Thy$^-$, M$^+$); ■ LH18/pAG101 (FolA$^+$, Thy$^-$, M$^+$); □ LH18/pPL113 (Δ<u>folA::kan</u>, DFR1$^+$, Thy$^-$, M$^+$); ▲ LH18-3 (FolA$^+$, Thy$^-$, M$^-$); △ LH18-4 (FolA$^+$, Thy$^+$, M$^-$); ◊ LH18-5 (Δ<u>folA::kan</u>, Thy$^-$, M$^-$); ♦ LH18-13 (Δ<u>folA::kan</u>, Thy$^+$, M$^-$).


A
B
C
Surviving Fraction
10^{0}
10^{-1}
10^{-2}
10^{-3}
10^{-4}
10^{-5}
0
6
12
18
24
Fluence (J/M^2)

The M mutations suppressed the non-motile phenotype of ΔfolA::kan mutants derived from strains MH581 and MH429 (Table 14). The non-motile phenotype of strain LH18 is not due to the ΔfolA::kan mutation, since the wild type derivative (LH18-9) is also non-motile (Table 14).

Although most E.coli strains do not ferment ß-glucosides they contain cryptic genes, that when expressed, permit the cell to utilize these sugars (Prasad and Shaeffer, 1974). Strains LH18-9 (M^+) and LH18-2 (thyA, M^+) fermented the ß-glucoside salicin (Table 14). The mutation responsible for this phenotype co-transduced 30% of the time with a Tn10 located at 83 min. (P1cm/JW355 X LH18-2). Thus LH18 derivatives probably have a mutation in the bglT gene which is thought to code for a regulatory protein of the bgl operon (Prasad and Shaeffer, 1974). The ΔfolA::kan mutant LH18 did not ferment ß-glucosides (Table 14), indicating that the ΔfolA::kan mutation interferes with the expression of either bglT or the bgl operon. The M mutations counteracted the effect of the ΔfolA::kan mutation. The folA gene of plasmid pAG101 did not allow strain LH18 to ferment ß-glucosides (Table 14).

The ΔfolA::kan mutation increased the level of resistance to potassium dichromate in a thyA background of LH18 and MH429 derivatives (Table 14).

TABLE 14. Other phenotypes of folA mutants.

Strain	Relevant Phenotype			Motility	ß-Glucoside Fermentation	M.I.C.(mM) of Chromate
	Fol	Thy	M			
LH18-9	+	+	+	-	+	0.3
LH18	-	-	+	-	-	1.2
LH18-2	+	-	+	-	+	0.3
LH18-5	-	-	-	-	+	0.3
LH18/pAG101	+	-	+	-	-	0.3
MH429	+	-	+	-	-	0.3
MH429F	-	-	+	-	-	0.6
MH429FM	-	-	-	+	-	0.3
MH581	+	+	+	+	-	0.3
MH581F	-	+	+	-	-	0.04
MH581FM	-	+	-	+	-	0.15
MH581Fmu	-	+	+	-	-	N.D.

N.D. Not Determined

In contrast, the Δ*folA::kan* mutation increased the sensitivity to potassium dichromate in the Thy^+ background of strain MH581 (Table 14). The *folA* gene of plasmid pAG101 made strain LH18 as sensitive as strain LH18-2 indicating that this phenotype is due to the inactivation of *folA*. The M mutations suppressed the high level of resistance to chromate of strains LH18 and MH429F as well as the high level of sensitivity of strain MH581F (Table 14). No difference in the sensitivity to molybdate and selenate was observed.

Δ*fola::kan* mutants form very small colonies on LB medium (Krishnan and Berg, 1993, this study). This phenotype is suppressed by the M mutations or $MgSO_4$ but not $MgCl_2$ supplementation (1mM). This result suggests that expression of the sulfate permease is repressed in Δ*folA::kan* mutants.

DISCUSSION

In this study I investigated the consequences of altering folate metabolism in E.coli. One part of my study monitored the effect of altering THF levels on the fidelity of translation. The second part examined the phenotypic consequences of a deletion spanning the kefC folA and apaH genes and a folA insertion mutation in different strains.

PART 1. Folates and translational accuracy:

Media dependent suppression. To study the physiological requirements for suppression, I formulated a defined medium which allowed Thy$^-$ cells to suppress T4 mutations in liquid medium. My results indicated that medium composition affected the ability of Thy$^-$ cells to suppress. A fairly complex medium (JB medium) was required to allow suppression of five of the six T4 phage mutants assayed and simpler media permitted the suppression of subsets of the phage mutants.

JB medium permitted me to determine the suppression efficiency at which a Thy$^-$ strain suppressed a tail fiber mutant (opc105) of T4. The suppression efficiency of opc105 by a Thy$^-$ strain (7%) was similar to that seen with ribosomal ambiguity mutants (ram) of E.coli (Olsson and Isaksson, 1980), but was much lower than that of strain CAJ64 (27%), which contains a suppressor $tRNA^{trp}$. The

suppression efficiency that I observed with strain CAJ64 was similar to published values (30%) (Sambrook, Fan, and Brenner, 1976). Note that although the net yield of mutant phage per cell on strain CAJ64 was as high as that observed with the Thy^- strain, the efficiency of suppression was higher because the yield of wild type phage on strain CAJ64 was lower.

JB medium allowed us to compare THF levels and their distribution in Thy^- and Thy^+ cells. A Thy^- strain grown in JB medium had higher total THF and N5-methylTHF levels than an isogenic Thy^+ strain (Tiganos, 1990; Basso, Tiganos, and Herrington, 1993). Some supplements of JB medium may contribute to the accumulation in THF levels observed by sparing THF for biosynthesis or by affecting the regulation of THF metabolism.

The syntheses of several of the supplements of JB medium are linked to THF metabolism. Adenine and histidine require N10-formylTHF for their syntheses. The aromatic amino acids and the THF precursor pABA are synthesized from a common precursor; chorismate (Pittard, 1987). Thus, the availibility of aromatic amino acids may increase the flux of chorismate towards pABA biosynthesis.

Teply, Axelrod, and Elvehjem (1943) observed that nicotinate rescued Lactobacillus arabinosus from the bacteriostatic effect of sulfathiazole. Furthermore, in JB medium, nicotinate supplementation (30µg/ml) prevents the

inhibition of suppression of the T4 mutant JC1922 by the THF antagonist sulfathiazole (J.Basso, unpublished observation). These results suggest that nicotinate affects folate metabolism.

The other supplements required for suppression could act to enhance the possibility of misreading a nonsense codon, or stabilize the product of the suppression event. For instance zinc chloride was required only for the suppression of tail fiber mutants (opc23 and opc105) and only at the time of infection (results not shown). Six molecules of zinc chloride per tail fiber are required for proper adsorption (Bloomfield, 1983). Sufficient zinc for phage with normal tail fibers is available as a contaminant in the water and in the chemicals present in M9S medium. If the tail fibers made as a result of suppression have a lower affinity for zinc, the amount present in M9S may not be sufficient for the formation of infective particles and thus the phage mutant would not seem to be suppressed. I suggest that zinc chloride is required to provide a functional suppressed product rather than for the suppression event itself.

I do not know what role valine and isoleucine supplementation serve.

thyA dependent suppression is a consequence of altered THF metabolism. To determine whether the ability of Thy$^-$ cells

to suppress is correlated to the observed elevation of THF levels I asked whether conditions which would be expected to lower these prevented suppression.

Thy$^-$ cells did not suppress when de novo THF synthesis was inhibited during cell growth by the dihydropteroate synthetase inhibitor sulfathiazole. Inhibition of dihydropteroate synthetase reduces total THF synthesis (Rohlman and Matthews, 1990; Herrington, 1993).

High levels of thymidine, which decrease THF and N5-methylTHF levels in uninfected cells (Tiganos, 1990; Basso, Tiganos and Herrington, 1993) prevented suppression by Thy$^-$ cells when assayed by plaque assays (Cheung and Herrington, 1982) or burst size assays (this study). Thymidine represses expression of the glyA gene (Stauffer, 1987) and thus decreases the level of serine hydroxymethyltransferase. All one carbon substituted THF derivatives are derived from N5,N10-methyleneTHF which is synthesized by serine hydroxymethyltransferase and the glycine cleavage pathway. Therefore, reducing serine hydroxymethyltransferase activity would reduce the level of one carbon substituted THF. A reduction of serine hydroxymethyltransferase activity by a mutation in the glyA gene also prevented suppression. These results indicate that conditions which reduce the level of one carbon substituted THF derivatives prevent suppression.

Suppression by a thyA mutant was prevented by a metF mutation. MetF mutants lack N5,N10-methyleneTHF reductase

activity, and therefore can not synthesize N5-methylTHF. The inability of a metF thyA double mutant to suppress strongly suggests that suppression is correlated to the elevated N5-methylTHF levels observed in a Thy^- strain.

The level of N5-methylTHF may also modulate translational accuracy by Thy^+ cells. The Thy^+ $FolC^-$ strain SF4 suppressed the T4 UGA mutant opc105 at an efficiency similar to that observed with the Thy^- strain MH429. The folC mutation of strain SF4 reduces the activity of both folC gene products, DHFS and FPGS. This strain is auxotrophic for methionine or vitamin B12 because of its inability to polyglutamylate N5-methylTHF (Ferone et al., 1983). Consequently, a folC mutant grown in the absence of vitamin B12 could accumulate monoglutamated N5-methylTHF because it cannot be used for methionine biosynthesis and is not converted to other THF derivatives (Dev and Harvey, 1982).

Conditions which permitted strain SF4 to use monoglutamated N5-methylTHF prevented suppression. This was accomplished either by providing FPGS activity from the heterologous gene (L.casei) or by providing vitamin B12. FPGS from L.casei polyglutamylates N5-methylTHF and thus would prevent its accumulation because it could now be used for methionine biosynthesis (Toy and Bognar, 1990). Similarly vitamin B12 supplementation would prevent the accumulation of monoglutamated N5-methylTHF since it could

now be used by the B12 dependent isozyme of homocysteine transmethylase for methionine biosynthesis.

In contrast to Thy$^-$ cells the level of THF in strain SF4 is lower that in wild type cells (Bognar, Osborne, and Shane, 1987). Consequently, suppression might not result from an elevation in the absolute levels of N5-methylTHF but rather from an increase in the relative amount of this THF derivative as compared to total THF levels.

Effect of a thyA mutation on the suppressor activity of SupUGA. Our model suggests that Thy$^-$ strains alter the fidelity of translation because they mismodify some component(s) of the translational apparatus. This proposal is supported by my observation that a thyA mutation can act as an anti-suppressor.

Wild type tRNA^{trp} inserts tryptophan at UGG codons and at a low frequency at UGA codons (Sambrook, Fan, and Brenner, 1976). However, as a result of a base substitution in the dihydrouridine stem (G24 to A24), the tRNA^{trp} of strain CAJ64 inserts tryptophan at UGA codons at a high frequency (Sambrook, Fan, and Brenner, 1976). When strain CAJ64 was made Thy$^-$ it no longer suppressed. This indicated that Thy$^-$ strains affect the accuracy of misreading by the tRNA^{trp} of strain CAJ64. This may be explained as follows. If Thy$^-$ strains misread UGA codons because they modify tRNA^{trp} differently than wild type cells, this modification

may be antagonistic to the base change in the $tRNA^{trp}$ of strain CAJ64 and thus reduce the level of misreading. Alternatively, if suppression by Thy^- strains is due to mismodified ribosomes, these may not as readily accept the $tRNA^{trp}$ of strain CAJ64.

DHF affects translational accuracy. Several results suggested that conditions which stimulate an accumulation of DHF either prior or post infection modulate translational accuracy. The antifolate drugs sulfathiazole and aminopterin prevented suppression when added at the time of infection. Furthermore, aminopterin increased the suppression efficiency of the T4 mutant opc105 by the Thy^- and Thy^+ derivative of strain CAJ64. Under these conditions, the THF content of the cells presumably does not decrease since the cells do not divide. T4 phage encodes both thymidylate synthase and DHFR and thus upon infection DHF is generated both from de novo synthesis by the cell and during phage directed thymidylate synthesis. Aminopterin specifically inhibits T4 DHFR and thus it would be predicted that infected cells would accumulate DHF. Sulfathiazole is metabolized by dihydropteroate synthetase to a DHF analogue (Swedberg, Castensson, and Skold, 1979). If this compound inhibits DHFR during T4 infection it would also cause an accumulation of DHF during T4 infection. Suppression was also prevented by a null mutation in the E.coli folA gene

encoding DHFR. Inhibition could not be attributed to a decrease in N5-methylTHF levels since a folA thyA double mutant has approximately ten times more N5-methylTHF than a Fol^+ Thy^+ strain (Hamm-Alvarez, Sancar, and Rajagopalan, 1990). However, this mutant would also accumulate DHF and thus inhibit suppression. This result is consistent with the interpretation I made concerning the inhibition of suppression by aminopterin.

Increasing expression from the folC gene inhibited suppression. Inhibition was due to increased DHFS activity rather than FPGS activity, since the L.casei gene encoding FPGS did not inhibit suppression by the Thy^- strain. If DHFS is rate limiting for the synthesis of DHF, increasing it could increase the flow through the de novo THF biosynthetic pathway and result in increased DHF levels.

How do THF and DHF levels affect the accuracy of translation? We proposed that the lack of thymidylate synthase activity in Thy^- cells perturbs THF metabolism, and that this results in the translational apparatus being mismodified, so that translation is less accurate.

The elevated levels of N5-methylTHF observed in Thy^- cells may reflect the accumulation of non-convertible forms of one carbon substituted THF derivatives as a result of the reduced turnover of N5,N10-methyleneTHF by the thymidylate synthase reaction. Furthermore, the reduced turnover of

N5,N10-methyleneTHF may also result in low DHF levels.

My results indicate that the accuracy of translation is affected by THF levels, specifically N5-methylTHF and DHF levels. An effect on translational stringency as a result of a defect in THF metabolism has been described in S.cerevisiae. Nonsense suppression by sup45 mutants of yeast is inhibited by an ade3 mutation (Song and Liebman, 1989). The ade3 gene encodes a trifunctional enzyme responsible for the interconversion of N5,N10-methyleneTHF to N5,N10-methenylTHF, N10-formylTHF, and N5-methylTHF (Song and Liebman, 1989).

My results do not permit me to say what component(s) of the translational apparatus are affected. THF levels may affect the modification of tRNA, ribosomal RNA, or ribosomal proteins. If Thy$^-$ cells mismodify tRNA, many different tRNA would have to be mismodified to enable Thy$^-$ strains to suppress all three nonsense codons and the wide variety of different T4 mutations reported to be suppressed. Alternatively, ribosomal RNA or ribosomal proteins may be mismodified. Presumably, as little as one mismodification could result in the suppression spectrum reported.

The elevated N5-methylTHF levels observed in Thy$^-$ cells may result in the over methylation of some component of the translational apparatus. This could occur if the level of N5-methylTHF is normally limiting for some methylation reactions. Alternatively, overmethylation may be the result

of a minor enzymatic activity which is catalyzed by an enzyme whose Km for methylTHF is high.

To my knowledge, no THF dependent methylation of the translational apparatus has ever been reported in E.coli. All the methyl-dependent modifications studied so far require the methyl group donor S-adenosylmethionine (SAM) (Bjork, 1987). The methyl group of SAM originates from N5-methylTHF (Cohen and Saint-Girons, 1987). Possibly high N5-methylTHF levels affect the synthesis of SAM. Alternatively, N5-methylTHF may be a competitive inhibitor of some SAM dependent methylations. In either case, this could result in the undermethylation of some component of the translational apparatus.

We do not know if T4 infection is required for the suppressor phenotype of Thy^- cells. Weak suppression of a UAG mutant of lambda by Thy^- cells has been observed (Basso, 1987). This suggests that T4 infection is not required for Thy^- cells to suppress. However, the observation that suppression could be inhibited post infection suggests that T4 infection contributes to the suppressor phenotype. Possibly T4 infection enhances the level and the spectrum of suppression. Modification of the translational apparatus could occur before or after T4 infection. T4 infection has been shown to result in the expression of T4 encoded enzymes responsible for the processing of tRNA's, ribosomal proteins and ribosome associated factors (Wilson and Abelson, 1972;

Smith and Haselkorn, 1969; Hsu and Weiss, 1969). Presumably, any of these may be affected by THF levels and thus alter translational accuracy. For instance Ribolini and Baylor (1975) reported the characterization of a T4D mutant which is a multi-nonsense suppressor. They proposed that suppression results from a mutation in a tRNA modifying enzyme. T4 dependent modifications may be additive to those resulting from the Thy$^-$ state of the cell.

My results suggest that the suppressor phenotype of thyA mutants is modulated by DHF levels established post infection. I propose that the translational apparatus is modified to a suppressor state prior to infection but its misreading activity is modulated following infection. DHF may directly or indirectly affect the stringency of misreading by mismodified tRNAs or ribosomal proteins. For instance treatments which presumably result in the accumulation of DHF, such as aminopterin and sulfathiazole, result in an increase in ppGpp pools (Rohlman and Matthews, 1990). The levels of this nucleotide influences the stringency with which cognate aminoacyl tRNA are selected (Gallant et al., 1982).

Future prospects.

To determine whether the folate utilizing enzymes encoded by T4 contribute to the suppressor phenotype of Thy$^-$ cells it would be of interest to assay the suppression of T4

nonsense mutants containing null mutations in the genes encoding these activities. Furthermore, to determine if T4 infection contributes to the folate imbalance required for suppression, folate levels of T4 infected Thy^- an Thy^+ cells should be compared. This could be achieved by high pressure liquid chromatography.

From my results it was not possible to determine what component of the translational apparatus is affected by the altered THF levels of Thy^- cells. Attempts at answering this question could include the following experiments. First since a mutant thyA allele affects the suppressor activity of Sup^{UGA} we could ask whether the modification of this tRNA is altered in Thy^- cells as compared to that of Thy^+ cells. This should be done with uninfected and T4 infected cells.

Alternatively in vitro translation experiments could be performed. Extracts from T4 infected and uninfected cells containing either ribosomes or tRNA from Thy^- and Thy^+ cells could be mixed to determine which of these components of the translational apparatus is mismodified. Since the most efficient suppression observed has been with T4 genes, a reporter gene fused to a T4 gene containing a nonsense mutation known to be suppressed would be ideal for these experiments.

PART 2. Effect of folA null mutations on E.coli.

There are conflicting reports concerning the growth properties of ΔfolA::kan mutants and their requirement for a mutant thyA allele (Howell, Foster, and Foster, 1988; Ahrweiler and Frieden, 1988; Hamm-Alvarez, Sancar and Rajagopalan, 1990, Krishnan and Berg, 1993). To determine whether this is a result of the different strain backgrounds or the accumulation of compensatory mutations, I compared the phenotypes of different strains containing the folA::kan deletion constructed by Howell, Foster, and Foster (1988).

Howell, Foster, and Foster (1988) reported that their strain, LH18, required folate end products to grow on minimal glucose medium but did not indicate the length of the incubation period when testing growth. In my hands this strain grew slowly in the absence of folate end products. I propose that this discrepancy results from the incubation time used to assess growth.

Howell, Foster, and Foster (1988) and Ahrweiler and Frieden (1988) claimed that their ΔfolA::kan mutations are lethal to cells which contain wild type thymidylate synthase activity. Consistent with the results reported by Howell, Foster, and Foster (1988), I was not able to introduce a Thy^+ allele in their strain. In contrast, Krishnan and Berg (1993) reported that the folA::kan deletion constructed by Ahrweiler and Frieden (1988) could be introduced into both

Thy$^+$ and Thy$^-$ strains and that both derivatives had an absolute requirement for folate end products. I observed that both the folA::kan deletion constructed by Howell, Foster, and Foster (1988) and a newly constructed folA insertion mutation could be introduced into some Thy$^+$ strains and that the folate end product requirement was dependent on the strain background. The reason for these discrepancies is discussed below.

Growth on minimal medium of folA null mutants. Some folA mutants are either not auxotrophic for folate end products (this study) or lose their initial auxotrophy (Ahrweiler and Frieden, 1988; Hamm-Alvarez, Sancar, and Rajagopalan, 1990) suggesting that some strains contain compensatory mutations or accumulate them with time. In this study I found that the folate end product requirement of folA mutants is affected by the nature of the mutation and by at least three different genes.

Most Thy$^-$ strains containing the folA::kan mutation grew slowly in the absence of folate end products (LH18 and MH429F), whereas a Thy$^+$ strain containing this mutation had an absolute requirement for folate end products (MH581F). I could readily select Thy$^-$ derivatives of strain MH581F by plating on minimal medium containing thymidine. This is analogous to the classical method of isolating Thy$^-$ cells by selecting for trimethoprim resistance in the presence of

thymine or thymidine (Bertino and Stacey, 1966). The mutational inactivation of thymidylate synthase may spare THF sufficiently to allow Δ*folA::kan* mutants to grow on minimal medium.

Surprisingly, both Thy$^-$ and Thy$^+$ derivatives of strain MH581 which had a *folA*::λ*plac*Mu53 insertion mutation (MH581Fmu and MH429Fmu) required folate end products. Since the *folA::kan* deletion extends into adjacent genes (Figure 4), suppression of the folate end product auxotrophy of Δ*folA::kan* mutants by a *thyA* mutation must require the inactivation of one of the other genes. This would suggest that the deletion affects a gene other than *folA* which influences folate metabolism. This gene might be *apaH* since a *thyA apaH* double mutant required all folate end products to grow on minimal medium indicating that *apaH* mutations affect THF metabolism.

The *folA::kan* deletion and the *folA* insertion mutations could be introduced at a comparable efficiency in Thy$^-$ and Thy$^+$ derivatives of strain JF1754. Furthermore, none of the FolA$^-$ derivatives of this strain required folate end products. Mutations mapped to two different loci (M$^-$) were shown to be responsible for suppression of the folate end product auxotrophy associated with a *folA::kan* and a *folA*::λ*plac*Mu53 mutation. The M mutations also suppressed the folate end product requirement associated with an *apaH*::λ*plac*Mu53 insertion mutation.

One of the compensatory mutations affects the gyrB gene which encodes one of the subunits of DNA gyrase. Modulation of DNA gyrase activity affect DNA topology and thus the level of expression of several different genes (Dlrica, 1984). The gyrB mutation of JF1754 could contribute to the suppression of the folate end product auxotrophy of folA mutants by increasing the expression of dihydropteridine reductase activity that is also able to reduce DHF (Vasudevan, Paal, and Armarego, 1992) or by permitting the expression of a cryptic gene encoding DHFR.

The second mutation was closely linked to the metE gene (86.25 min.). I think that this gene is uvrD which codes for a DNA helicase (Bachmann, 1990). This conclusion is based on the following observations. In a FolA$^+$ background, the M mutations increased UV sensitivity. This effect was more pronounced in a Thy$^+$ than in a Thy$^-$ background. The increased UV sensitivity of the Thy$^+$ strain is consistent with a uvrD phenotype (Maples and Kushner, 1982). These results also suggest that thymidylate synthase affects DNA helicase II activity.

THF metabolism in FolA$^-$ strains. The folate end product requirement of ΔfolA::kan strains was proposed to result from low THF levels (Howell, Foster, and Foster, 1988; Krishnan and Berg, 1993). However, when THF levels of the ΔfolA::kan mutants constructed by Howell, Foster, and Foster

(1988) and Ahrweiler and Frieden (1988) were compared to those of a FolA$^+$ Thy$^+$ derivative no reduction in total THF levels was observed (Hamm-Alvarez, Sancar, and Rajagopalan, 1991). The significance of this result is not clear since this group reports that LH18 grew normally in the absence of folate end products. Consequently their stock of this strain may have acquired a compensatory mutation(s).

We found that FolA$^-$ strains (deletion and insertion mutants) which grew optimally with folate end products incorporated less pABA than the isogenic FolA$^+$ derivatives (M.B. Herrington, unpublished results). No difference in the level of pABA incorporation was observed between FolA$^-$ and FolA$^+$ strains containing the compensatory mutations of strain JF1754 (M.B. Herrington, unpublished results). These results suggest that the folate end product requirement of FolA$^-$ strains is a consequence of impaired de novo THF biosynthesis.

Surprisingly, even though FolA$^-$ M$^-$ strains do not require folate end products they were much more sensitive to the dihydropteroate synthase inhibitor sulfathiazole. Since the level of pABA incorporation did not differ between FolA$^+$ and FolA$^-$ strains in an M$^-$ background, this suggests that the increased sensitivity to sulfathiazole is not a consequence of decreased THF or pABA levels. If sulfathiazole is metabolized into an inhibitory compound, FolA$^-$ strains may do this to a greater extent.

The folA::kan mutation affects DNA gyrase. Several results obtained in this study suggest that the inactivation of folA and some other gene affects DNA gyrase.

The thyA and folA::kan mutations altered the cell's sensitivity to gyrase inhibitors. In a Thy^+ background the folA::kan mutation increased the cell's sensitivity to gyrase inhibitors. In contrast, in a Thy^- background the folA::kan mutation greatly increased the cell's resistance to gyrase inhibitors. The thyA mutation by itself also increased the level of resistance to gyrase inhibitors. The effect of the ΔfolA::kan mutation on the sensitivity of cells to gyrase inhibitors was only partly due to the inactivation of folA since the $FolA^+$ gene of pAG101 only partially complemented this phenotype. Furthermore a folA insertion mutation did not affect the susceptibility to gyrase inhibitors.

The increased resistance to gyrase inhibitors may be partly due to the lack of DHFR protein rather than DHFR activity since this phenotype was partially complemented by the E.coli folA gene of plasmid pAG101 but not by the DFR1 gene of S.cerevisiae.

The M mutations partly suppressed the high level of resistance to gyrase inhibitors of folA::kan Thy^- mutants. This suggests that these mutations affect the suceptibility of DNA gyrase to inhibitors. I believe that one of the mutations affects the uvrD gene (see below). Consistent

with this hypothesis uvrD mutations increase the sensitivity of E.coli to gyrase inhibitors.

One of the genes deleted by the ΔfolA::kan mutation is essential in some Thy$^+$ strains containing mutations in the gyrA or gyrB gene.

The ΔfolA::kan mutation affected the expression of several genes, which is consistent with an alteration in DNA gyrase activity. The mutation repressed motility, expression of the bgl operon, and affected the level of chromate resistance. Furthermore, sulfate supplementation suppressed the small colony phenotype of ΔfolA::kan mutants, suggesting that the level of the sulfate transporter is low in ΔfolA::kan mutants.

The M mutations suppressed the above described phenotypes, suggesting that they generate near wild type gyrase activity in a ΔfolA::kan background.

DHFR protein affects sensitivity to UV. The ΔfolA::kan mutation greatly increased UV sensitivity in either an M$^+$ or an M$^-$ background. This phenotype was not affected by a mutant thyA gene. The high level of UV sensitivity of ΔfolA::kan mutants was suppressed by the E.coli FolA$^+$ gene from plasmid pAG101 but not by the DFR1 gene of S.cerevisiae. These results indicated that the effect of the ΔfolA::kan mutation on UV sensitivity is due to the inactivation of folA. Furthermore, since both plasmids

complement the folate end product auxotrophy of Δ*folA::kan* mutants (this study), these results indicate that the increased sensitivity to UV is a consequence of a lack of *E.coli* DHFR protein rather than DHFR activity. DHFR may regulate the expression of genes involved in DNA repair. Alternatively, if DHFR interacts with enzymes involved in DNA repair, its absence could affect their activity and thus increase UV susceptibility.

The increased sensitivity to UV of Δ*folA::kan* and M^- mutants is possibly a consequence of a reduced efficiency of DNA repair since the mutation frequency in Δ*folA::kan* and M^- mutants was higher than that of $FolA^+$ and M^+ strains.

Structural role of DHFR. The results presented in this study suggest that DHFR has a structural role which is affected by mutations in *thyA*, *gyrA*, *gyrB*, and the *uvrD* gene products as well as one of the genes inactivated by the Δ*folA::kan* deletion. I propose that DHFR interacts with thymidylate synthase, DNA gyrase, and DNA helicase. Further support for this model comes from the observation that some combinations of the *gyrB* and *uvrD* alleles of strain JF1754 are lethal in a Δ*folA::kan* background. These mutations may be lethal individually if the mutant gyrase or DNA helicase protein can not function properly in a strain lacking DHFR.

A complex which includes the bacteriophage T4 DNA and dNTP metabolizing enzymes, DHFR, thymidylate synthase, dCMP

hydroxymethyltransferase, dNDP kinase, DNA helicase, and DNA primase, is believed to be assembled in T4 infected E.coli cells (Mathews et al., 1988). MacDonald and Hall (1984) have described genetic evidence, similar to mine, suggesting an interaction between DHFR and enzymes involved in DNA replication. They report the isolation of FAR mutants (Folate Analogue Resistant) which were resistant to the DHF analogue pyrimethamine. Surprisingly some of the mutants isolated did not map within the DHFR gene. Some of the FAR mutations were mapped to the genes encoding DNA helicase, DNA primase, DNA polymerase, and dCMP hydroxymethyltransferase. Resistance was allele specific thus indicating that it was not due to a lack of enzyme activity. These results strongly suggested an interaction between DNA and dNTP synthesizing enzymes. Resistance to pyrimethamine as a result of mutations in either DNA primase or helicase could be suppressed by mutations in DHFR. Similarly, resistance as a result of mutations in DNA helicase could be suppresses by mutations in dCMP hydroxymethyltransferase. Since suppression was allele specific, this suggests that these enzymes directly interact with one another. Furthermore, mutations which affect the assembly of this complex increases the mutation frequency (Mathews et al., 1988). The increased mutation frequency and the increased resistance to an inhibitor which does not affect the mutant protein is similar to the phenotypes I

have observed with Δ*folA::kan* mutants.

Inactivation of an unknown gene affects phenotypes of Δ*folA::kan* mutants. Repression of ß-glucoside fermentation by the Δ*folA::kan* mutation was not complemented by the FolA$^+$ gene of pAG101 indicating that this phenotype is not due to the inactivation of *folA*. This phenotype may be due to the inactivation of the *apaH* gene which encodes a diadenosine hydrolase activity (Johnstone and Farr, 1991). ApaH null mutants accumulate diadenosine tetraphosphate a metabolite known to affect catabolite repression (Farr *et al.*, 1989). Incidently, the *bgl* operon is regulated via catabolite repression (Lin, 1987).

The increased resistance to gyrase inhibitors of Δ*folA::kan* mutants was only partially complemented by the FolA$^+$ gene of plasmid pAG101 suggesting that this phenotype is due to the inactivation of *folA* and some other gene. It is unlikely that inactivation of the *apaH* gene affects DNA gyrase since an *apaH*::λ*plac*Mu53 mutation had no effect on the level of resistance to gyrase inhibitors.

Future Prospects. An intriguing observation made during this study is that a combination of two mutations suppress the folate end product auxotrophy. One of these was found to affect the *gyrB* gene, whereas the other affects UV sensitivity. It would be of interest to determine whether

the second mutation affects the uvrD gene. This could be done by transforming M^- ΔfolA::kan mutants with a plasmid containing the uvrD gene, and verifying whether the resulting transformants require folate end products to grow on minimal medium.

The results obtained in this study suggest that DHFR may affect DNA gyrase. To further confirm this hypothesis the level of DNA supercoiling in $FolA^+$ and $FolA^-$ strains could be compared.

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