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Replacement of the *folC* Gene, Encoding Folylpolyglutamate Synthetase-Dihydrofolate Synthetase in *Escherichia coli*, with Genes Mutagenized In Vitro

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The folylpolyglutamate synthetase-dihydrofolate synthetase gene (*folC*) in *Escherichia coli* was deleted from the bacterial chromosome and replaced by a selectable Km^r marker. The deletion strain required a complementing gene expressing folylpolyglutamate synthetase encoded on a plasmid for viability, indicating that *folC* is an essential gene in *E. coli*. The complementing *folC* gene was cloned into the vector pPM103 (pSC101, temperature sensitive for replication), which segregated spontaneously at 42°C in the absence of selection. This complementing plasmid was replaced in the *folC* deletion strain by compatible pUC plasmids containing *folC* genes with mutations generated in vitro, producing strains which express only mutant folylpolyglutamate synthetase. Mutant *folC* genes expressing insufficient enzyme activity could not complement the chromosomal deletion, resulting in retention of the pPM103 plasmid. Some mutant genes expressing low levels of enzyme activity replaced the complementing plasmid, but the strains produced were auxotrophic for products of folate-dependent pathways. The folylpolyglutamate synthetase gene from *Lactobacillus casei*, which may lack dihydrofolate synthetase activity, replaced the complementing plasmid, but the strain was auxotrophic for all folate end products.

The *Escherichia coli folC* gene product, folylpolyglutamate synthetase (FPGS)-dihydrofolate synthetase, is an enzyme catalyzing two distinct reactions in the folate biosynthetic pathway. The dihydrofolate synthetase activity adds L-glutamate to dihydropteroate to form dihydrofolate. Dihydrofolate is reduced to tetrahydrofolate in the cell by dihydrofolate reductase. The FPGS activity of the *folC* product then adds a second and third glutamate in γ-linkage to tetrahydrofolates to form polyglutamates. A second enzyme catalyzes the addition of several more glutamates in α-linkage to the triglutamate (10, 12).

Ferone et al. reported the isolation of a *folC* mutant (W1485/I-21 [11] and SF2 and SF4 [5]) auxotrophic for methionine and stimulated by glycine in the absence of vitamin B_{12}. This mutant is leaky so that the cells have dihydrofolate synthetase and FPGS activity at levels 1 to 3% of the levels of the wild-type strain and possess 10 to 30% of normal total folate pools (11). Because the dihydrofolate synthetase activity is required for folate biosynthesis, a nonleaky mutant having no residual enzyme activity would have no folates. Such a mutant would require all of the folate end products (thymine, purines, glycine, methionine, and pantothenate), or it may be lethal, since folate is also required for synthesis of formyl methionine for initiation of protein synthesis. We wished to obtain a *folC* mutant which completely lacks FPGS-dihydrofolate synthetase activity to investigate its phenotype.

A number of methods for gene disruption and allele replacement in *E. coli* have been reported previously (3, 14, 15, 33). Double-stranded linear DNA fragments containing the *folC* gene with a Km^r cassette inserted into the coding sequence failed to transform a *recBC sbcB* strain to kanamycin resistance. In our hands these methods did not work even in the presence of a complementing wild-type plasmid. The recent report of the deletion of the *folA* gene encoding dihydrofolate reductase in a *polA thyA* strain by integration of recombinant plasmids containing the ColE1 origin and their subsequent segregation (14) suggested to us that constructing a deletion of *folC* by a similar method may be successful.

The SF4 mutant strain contains a *folC* gene with a point mutation causing an alanine-to-threonine amino acid change at codon 309 (16) and requires methionine for growth (11). Plasmid-encoded mutant *folC* genes which express as little as 1% of the wild-type activity of either FPGS or dihydrofolate synthetase complemented SF4 to methionine prototrophy (16, 17). The *Lactobacillus casei* FPGS gene, which has little or no dihydrofolate synthetase activity, can also complement SF4 (31). We have constructed a number of *folC* mutants on multicopy plasmids by in vitro mutagenesis, but the study of their properties was hampered by the background enzyme activity of the SF4 strain. In this report, we demonstrate the deletion of the chromosomal *folC* gene in the presence of a complementing gene on a plasmid temperature sensitive for replication. The complementing plasmid can then be replaced by a second plasmid containing a *folC* mutant gene, provided that the gene product expresses sufficient enzyme activity. This allows the study of many *folC* mutants synthesized in vitro in the absence of background FPGS-dihydrofolate synthetase activity encoded by the chromosome.

MATERIALS AND METHODS

Materials. [α-^{32}P]dATP (7,000 Ci/mmol), [α-^{32}P]dCTP (7,000 Ci/mmol), *para*-amino[carboxyl-^{14}C]benzoic acid (54 mCi/mmol), and L-[U-^{14}C]glutamic acid (10 mCi/mmol) were obtained from Amersham Corp. Tran^{35}S-label (methionine) was obtained from ICN Pharmaceuticals. Fluorography reagents (En^{3}Hance), Aquasol, and nylon membranes were from New England Nuclear. Nitrocellulose was obtained

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TABLE 1. Plasmids used in this study

Plasmid(s)	Description	Reference(s)
pUC19	Cloning vector; Amp^r	24
pH2A-1	pKC7 with *usg*, *folC*, and *dedD* genes on a 10-kb fragment; Amp^r Km^r	5
pCP1	pH2A-1 with deletion of 1-kb *Sph*I fragment inactivating Km^r gene; Amp^r	This study
pCP2	pCP1 with deletion of *folC* and *dedD*; Amp^r	This study
pCP3	pCP2 with Km^r gene from pUC4K in the *Stu*I site; Amp^r Km^r	This study
pAC3	pUC8 with 3.5-kb fragment containing *usg*, *folC*, and *dedD* genes; Amp^r	4
pPM103	pSC101 temperature sensitive for replication; segregates above 30°C; Tet^r	15, 20
pPMpac3	pPM103 containing 3.5-kb insert from pAC3; Tet^r	This study
pPMusg	pPMpac3 with deletion of *dedD* and *folC*; Tet^r	This study
pPMdedD	pPMpac3 with deletion of *usg* and *folC*; Tet^r	This study
pAC5	pUC9 with *folC* gene downstream of *lac* promoter; Amp^r	4
pAC6	pUC19 with *Pvu*II fragment containing *folC* gene from pAC5; Amp^r	This study
pPMfolC/amp	pPM103 containing pAC6 in the *Eco*RI site; Amp^r Tet^r	This study
pPMfolC	pPM103 with *folC* gene downstream of *lac* promoter; Tet^r	This study
pUGBgl	pUC19 with *usg* and linker to correct 3′ end of *usg*; Amp^r	6
pUC4K	pUC4 containing Km^r cassette; also Amp^r	Pharmacia
pGly, pIleu, pArg, pGlu, pVAl, pLeu	pUC19 with *dedD* gene and *folC* gene with a codon 309 mutation to indicated amino acid; Amp^r	16
pSF4	pUC19 with *folC* and *dedD* genes cloned from SF4 mutant strain; Amp^r	16
pGT3-8.1	pEMBL18+ with *L. casei* FPGS gene; Amp^r	31
pL2, pL4, pL5, pL6, pL8, pL10	pAC5 with 6-bp linker inserted at various sites; Amp^r	17

from Schleicher & Schuell. Tetrahydrofolate, folinic acid, adenosine, thymidine, ATP, methionine, glycine, glutamic acid, and the antibiotics kanamycin, tetracycline, and chloramphenicol were purchased from Sigma Chemical Co. Ampicillin and proteinase K were from Boehringer Mannheim. The Geneclean kit was purchased from Bio 101. Restriction endonucleases, RNases, and DNA modifying enzymes were purchased from Boehringer Mannheim, Pharmacia, or Bethesda Research Laboratories.

Bacterial strains, phage, plasmids, and media. Strain MM383 [*thyA polA12*(Ts) Rha$^-$ Lac$^-$ Str^r] (22) was a generous gift of J. Brunton, Mount Sinai Research Institute, Toronto, Canada. SF2 (*folC strA*) has been previously described (5). SF4 (*folC strA recA srlC*::Tn*10*) (5) was used as the recipient strain to verify insertion of the *folC* gene in some plasmid constructions. JF1754 (*hsdR lac gal metB leuB hisB*) (19) was used as a highly competent host in plasmid constructions. DR1984 (25) was the host strain used for maxicell studies. Phage P1 (*cml clr*100) (21) was a gift of M. Herrington, Concordia University, Montreal, Canada. The plasmids used in this study are listed in Table 1. Plasmid pPM103 was a gift of M. Toth, M.I.T.

E. coli strains were grown in 2YT or LB (21) medium supplemented with ampicillin (50 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), or chloramphenicol (12.5 μg/ml) where indicated. SF4 was grown on Vogel-Bonner minimal medium (32) supplemented with methionine (50 μg/ml) and glycine (50 μg/ml). Unsupplemented minimal medium solidified with 1.5% agar was used in complementation analysis. Strain MM383 was grown on 2YT medium supplemented with thymidine (200 μg/ml). The following folate end products were added to minimal medium for growth of the SF2Δ*folC* strain transformed with mutant derivatives of the *folC* gene: methionine, 50 μg/ml; glycine, 50 μg/ml; adenosine, 20 μg/ml; thymidine, 200 μg/ml; pantothenate, 1 μg/ml; and serine, 50 μg/ml.

Plasmid isolation, DNA modification and analysis, and bacterial transformation. Plasmid preparations were made by the alkaline lysis procedure (2). Restriction endonuclease digestions, nuclease digestions, and ligations were carried out according to the manufacturer's instructions. Genomic DNA was isolated from *E. coli* strains by the method of Driver and Lawther (8) except that the DNA was spooled onto a glass rod from an overlay of 2 volumes of ice-cold ethanol after phenol-chloroform extraction and the DNA was redissolved in TE buffer overnight with gentle agitation prior to digestion with restriction endonuclease. Southern blots were performed following transfer of DNA to nitrocellulose or nylon membranes and hybridizations as described by Southern (28) with the appropriate probes at 42°C in 50% formamide. DNA probes were labelled with [α-^{32}P]dCTP by the random primer procedure of Feinberg and Vogelstein (9) using the Klenow fragment of DNA polymerase I. Transformation was by the method of Hanahan (13), except for temperature-sensitive MM383 cells or when the strains were transformed with the pPM103-derived plasmids, in which instance the cells were grown at 30°C and the heat shock step was done at 37°C. Selection of integrants was done on YT plates supplemented with 25 μg of ampicillin per ml (on which cells were grown at 37°C) followed by streaking on YT plates containing 25 μg of kanamycin per ml. Although the pPM103 plasmid was reported to be temperature sensitive for replication (15, 20), we found that transformants grew in the presence of tetracycline at 42°C. The plasmid segregated at high frequency, however, when grown in the absence of selection at 37°C or higher (but at only low frequency at 30°C). In order to maintain the complementing pPM103-derived plasmids in the appropriate *E. coli* strains, the cells were grown in the presence of tetracycline at 30°C.

Transduction of the *folC* deletion to Pol$^+$ strains. The MM383Δ*folC*/pPMpac3a strain was lysogenized with P1 (*cml clr*100), and lysates were prepared by standard methods (21). Cells of *E. coli* SF2 and JF1754 containing the plasmid pPMpac3a were infected with the lysate, plated on kanamycin resistance-selective plates, and incubated at 30°C.

Enzyme extraction and assay. Cells were grown in 1-liter cultures of YT medium supplemented with 100 μg of ampicillin per ml. Cells were harvested by centrifugation and lysed by sonication in a Branson sonifier. Solid ammonium sulfate was added to 60% (wt/vol) to the cleared lysate (50 ml), and the precipitate was collected by centrifugation. This

fraction was resuspended in 5 to 10 ml of 50 mM Tris HCl, pH 7.5.

FPGS activity was measured by the incorporation of [^{14}C]glutamate (1 mM) into folate products as described previously (7, 27). Both 5,10-methylenetetrahydrofolate (100 μM) and 10-formyltetrahydrofolate (100 μM) were used as folate substrates. Dihydrofolate synthetase activity was measured with dihydropteroate (25 μM) as a substrate as previously described (5).

Incorporation of labelled *para*-aminobenzoic acid into cellular folates. 2YT medium was pretreated with activated charcoal and filtered to remove any folates or *para*-aminobenzoic acid. *para*-Amino[carboxyl-^{14}C]benzoic acid (54 mCi/mmol) was added to 20 ml of the medium (12,000 cpm/ml) at a concentration of 0.2 μM. The medium was inoculated with the appropriate *E. coli* strains and shaken for 24 h. The cells were washed twice by centrifugation and counted by using Aquasol scintillation fluid.

Analysis of plasmid-encoded gene products in maxicells. *E. coli* DR1984 was transformed with various plasmids and irradiated with UV light, and plasmid-encoded proteins were labelled with Tran^{35}S-label (methionine and cysteine) as described by Sancar et al. (26). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 13% polyacrylamide gels according to the method of Laemmli (18), and the gels were fluorographed.

RESULTS

Construction of plasmid pCP3. The construction of pCP3 is shown in Fig. 1. All plasmid constructs were verified by restriction enzyme analysis, including redigestion of pCP3 with *Stu*I (data not shown).

The gene products expressed from pCP3 were analyzed in maxicells (Fig. 2, lane 3). The pCP3 plasmid expressed the labelled protein products expressed by its parent plasmid H2al (lane 4) but did not express the products of the *folC* gene, the *dedD* gene, or the Tn*5* Km^r gene (kan 2 in Fig. 2), since these genes were deleted in the construction of pCP3. The *folC* product (molecular mass, 47 kDa) is shown in lanes 2, 4, and 5. The *dedD* product (35 kDa) is shown in lane 4 and is present as a very faint band in lane 2. The product of the Km^r gene from pUC4K (lane 1) appears to have the same mobility as the β-lactamase and is not visible as a discrete band.

Integration of pCP3 and deletion of chromosomal sequences. Our strategy for disruption of the chromosomal *folC* gene is shown in Fig. 3A. pCP3 contains a selectable Km^r marker replacing the deleted *folC* and *dedD* genes, and the marker is flanked by extensive *E. coli* genomic DNA sequences required for homologous recombination. pCP3 was used to transform the *polA*(Ts) strain MM383 to ampicillin and kanamycin resistance. We tried several times but were unable to isolate any plasmid from transformants in MM383, suggesting that all had the plasmids directly integrated into the chromosome. Integration of intact pCP3 into the chromosomes of Amp^r Km^r transformants was verified by Southern blot analysis (Fig. 3B). The pCP3 probe hybridized to a 9-kb and a 2.7-kb *Eco*RI fragment in the host strain (lanes 1 and 2). The recombination event could occur on either side of the deletion (Fig. 3A), and both possible integrants were obtained. Fig. 3B, lane 3, shows integrant 2, in which the hybridizing fragments are 2.7, 5, 5.7, and 8.3 kb. Integrant 1 is shown in lanes 4 to 6 with hybridizing fragments of 2.7 and 9 kb and two of 5 kb. Following growth of the integrants, designated MM383::pCP3, in the absence of ampicillin selection, only Amp^s and Km^s segregants, which were verified to be wild type by Southern blots (not shown), but no Amp^s and Km^r segregants, which would possess deletions the *folC* and *dedD* genes, were obtained.

The results described above suggested that *folC* is an essential gene and that viable MM383 derivatives with a deletion of the chromosomal *folC* gene would therefore require a complementing gene on a plasmid which could replicate in the *polA* strain. To examine all of the genes which could be affected by the deletion, we constructed recombinant plasmids containing the *folC* gene (pPMfolC) expressed from a *lac* promoter, the upstream gene (pPMusg), the *dedD* gene (pPMdedD), or all three genes (pPMpac3a) on DNA fragments inserted into a pPM103 vector which can replicate in the MM383 *polA* strain (Fig. 4 and 5). The structures of these plasmids were verified by restriction endonuclease analysis, and the plasmid-dependent expression of the appropriate gene products was verified by maxicell experiments (data not shown). Expression of *folC* by pPMpac3a and pPMfolC was further verified by complementation of strain SF4 to methionine prototrophy.

Strain MM383::pCP3 was transformed to tetracycline resistance with each of the plasmids described above. The Tet^r transformants were grown in the absence of ampicillin to screen for segregants of the integrated pCP3 plasmid. Wild-type segregants were obtained from all transformants, but Amp^s Km^r segregants were obtained only from the transformants containing the pPMfolC or pPMpac3a plasmids. These strains were designated MM383Δ*folC*/pPMfolC and MM383Δ*folC*/pPMpac3a, respectively. No Amp^s Km^r segregants were observed in the strains transformed with pPMusg or pPMdedD, even when the cells were grown and selected in rich medium supplemented with the folate end products methionine, glycine, thymidine, adenosine, and pantothenate. The Amp^s Km^r segregants containing pPMfolC grew normally, even though the *dedD* gene was deleted and not present on the complementing plasmid. No difference in phenotype was observed as a result of the deletion of the *dedD* gene.

The deletion of the chromosomal *folC* gene in the strain MM383Δ*folC*/pPMpac3 was verified by Southern blotting. Figure 3B, lane 7, shows MM383::pCP3 integrant 1 containing plasmid pPMpac3a. The plasmid-specific bands hybridizing to the probe are shown in lane 9. Lane 8 shows the corresponding segregant, MM383Δ*folC*/pPMpac3a, in which the pPMpac3a-specific bands are seen but the two 5-kb fragments of the integrant are absent. Also present in lane 8 are the 8.3- and 2.7-kb fragments expected in a segregant in which the chromosomal region containing the *folC* and *dedD* genes is replaced by the Km^r marker (Fig. 3A, bottom left).

Replacement of the *folC* gene with in vitro-generated mutant genes. One of our objectives in deleting the *folC* gene was to express mutant *folC* genes produced by in vitro mutagenesis in a strain with no chromosomally encoded background enzyme activity. Since all of our mutants were constructed in ColE1 replicons, it was necessary to transduce the chromosomal *folC* deletion into a PolA$^+$ strain by using bacteriophage P1 as described in Materials and Methods. We selected for Km^r in transductants of strain SF2 containing the pPMpac3a plasmid. We obtained no Km^r transductants if the recipient strain did not contain a complementing pPMpac3a plasmid. The loss of the chromosomal *folC* gene in these strains was verified by Southern blot analysis. For the blot shown in Fig. 3C the *Bam*HI-*Hin*dIII fragment of the plasmid pAC5 (Fig. 5) is used as a *folC*-specific probe. Lanes 1 and 2 show strain SF2Δ*folC*/pPMpac3a. The probe hybrid-

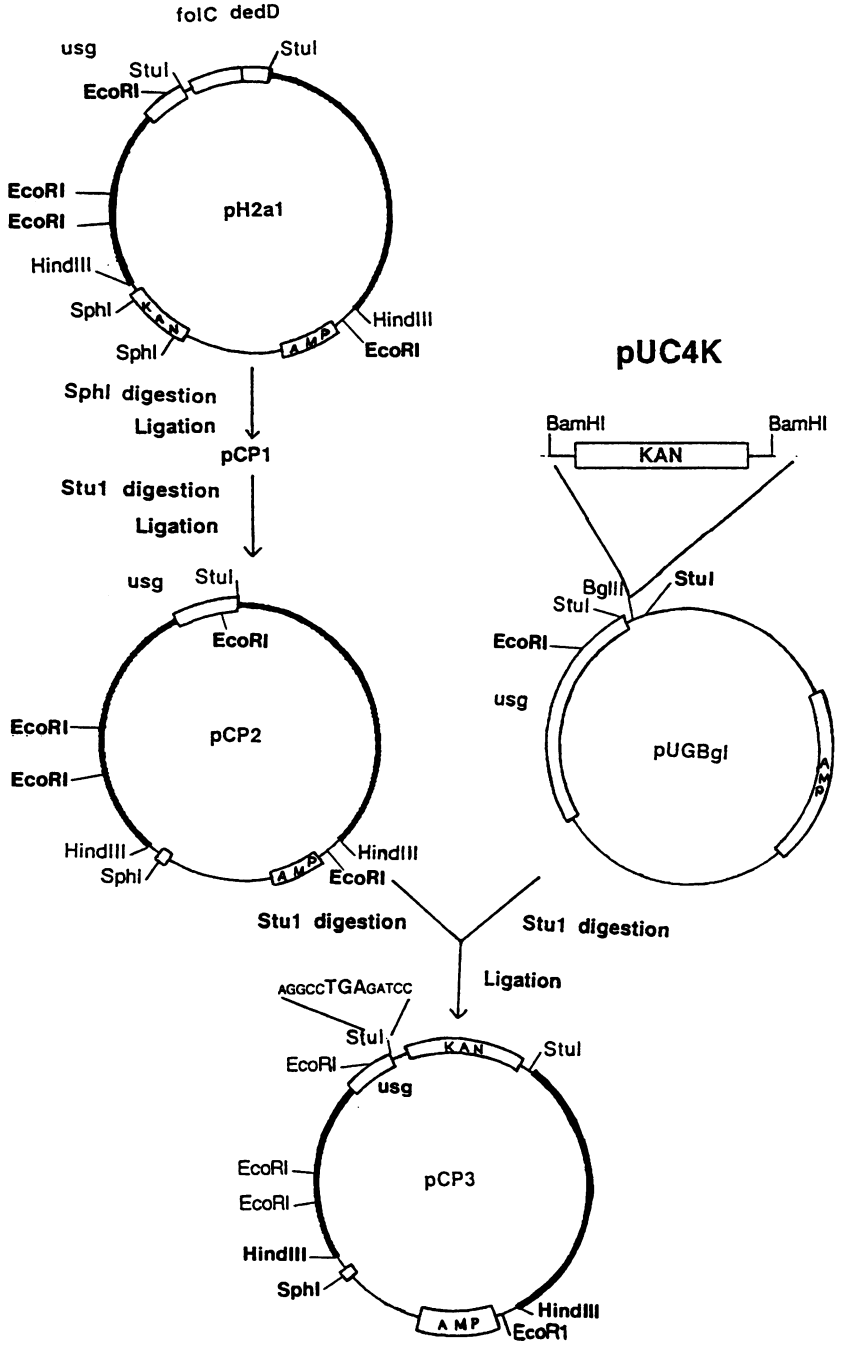


FIG. 1. Construction of pCP3. Plasmid pH2A-1 (5) was digested with *Sph*I and religated to form pCP1. This procedure removed a 1-kb fragment from the pKC7 vector portion of the plasmid and inactivated the kanamycin resistance gene. pCP1 was digested with *Stu*I and religated to form pCP2. This procedure removed a 2-kb DNA fragment containing the *folC* gene and the adjacent downstream gene (*dedD*) (23). The deletion also removed the last 5 bases, including the termination codon of the *usg*. A Km^r cassette which was inserted into pCP2 was constructed as follows: plasmid pUC4K (Pharmacia) was digested with *Bam*HI, and the 1.3-kb DNA fragment containing the kanamycin phosphotransferase gene was isolated from an agarose gel and ligated into the *Bgl*II site of pUGBgl (6). pUGBgl has a linker consisting of the *Bgl*II site flanked by *Stu*I sites, which contains DNA sequences to provide the proper termination for the upstream gene while allowing insertions into the *Bgl*II site (6). The kanamycin resistance cassette cloned into the *Bgl*II site of pUGBgl was excised by *Stu*I digestion so that it was flanked by these linker sequences. The DNA fragment was gel purified and ligated into the *Stu*I site of pCP2 to form pCP3. pCP3 was transformed into competent *E. coli* cells, and Km^r Amp^r colonies were selected. DNA sequences are as follows: ——, vector; ■, *E. coli*; □, open reading frames of specific genes. The DNA sequence between the *Stu*I and *Bgl*II-*Bam*HI sites adjacent to *usg* is shown. The TGA stop codon which is in frame with *usg* to provide the correct translation termination is highlighted.

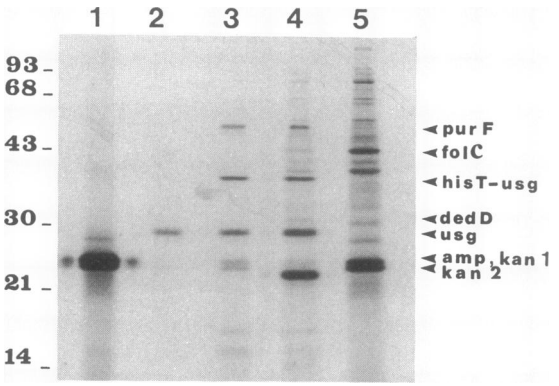


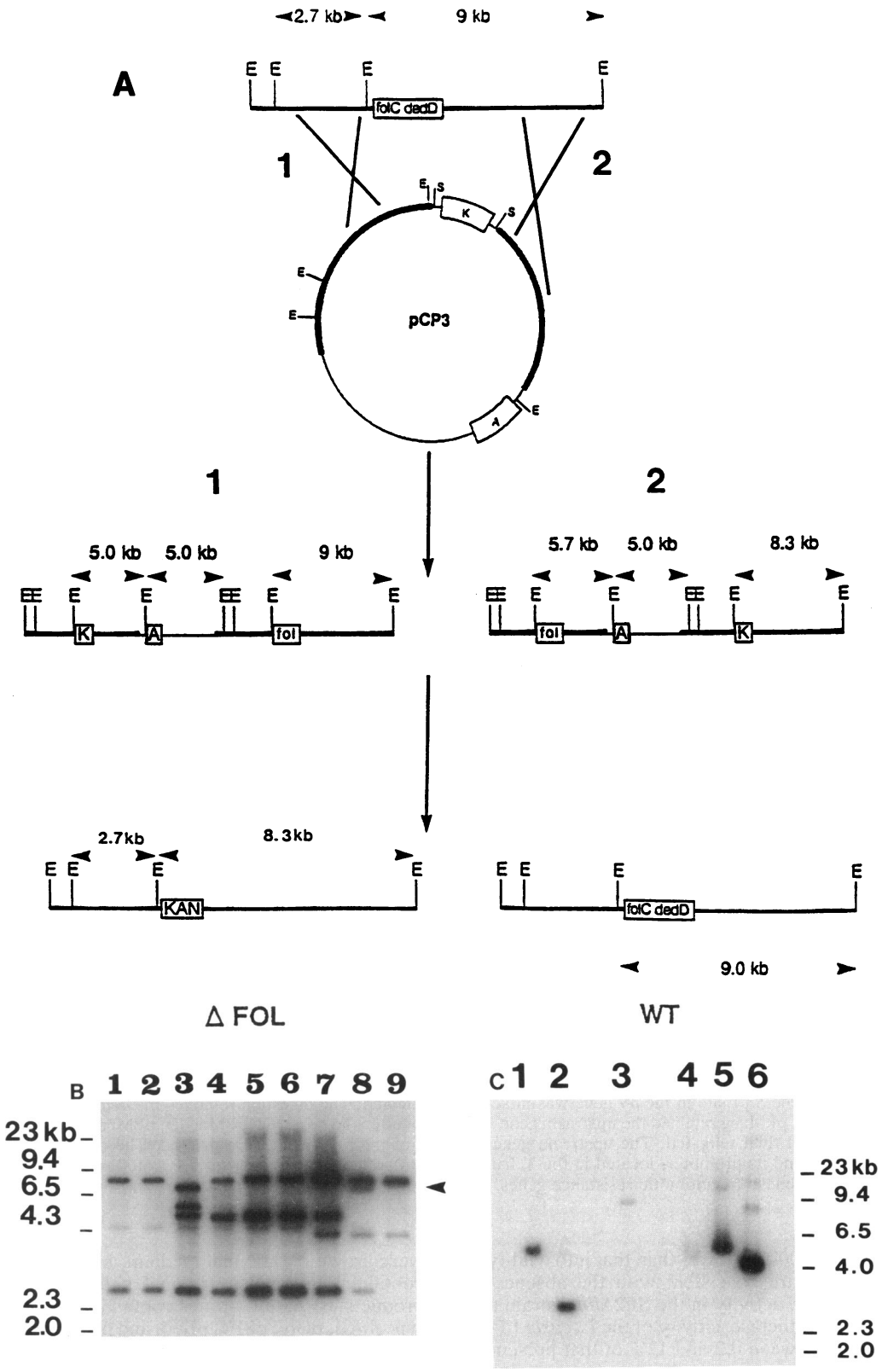
FIG. 2. Expression of plasmid-dependent proteins in maxicells. DR1984 cells were transformed with pUC4K (lane 1), pAC3 (lane 2), pCP3 (lane 3), pH2a1 (lane 4), and pAC5 (lane 5). All plasmids are described in Table 1. Sodium dodecyl sulfate-polyacrylamide gels of total cell extracts were autoradiographed to detect proteins labelled with Tran^{35}S-label (methionine and cysteine). The positions of the molecular weight markers are indicated on the left. The positions of identified gene products are shown on the right. kan 1, kanamycin resistance gene from pUC4K derived from Tn*903*; kan 2, kanamycin resistance gene, derived from Tn*5*, present in pH2a1; hisT-usg, product of a gene upstream of *hisT* (23). The sample in lane 5 was from a separate experiment with a higher background of labelled cellular proteins.

ized only to the 4.5-kb *Eco*RI and the 2.7-kb *Eco*RI-*Sma*I fragments corresponding to the pPMpac3a-encoded *folC* gene but not to the 9-kb chromosomal *Eco*RI fragment from the parental strain, SF2 (shown in lane 3). There are no *Sma*I sites within this chromosomal *Eco*RI fragment (23).

SF2Δ*folC*/pPMpac3a was transformed to ampicillin resistance with plasmids pUC19 and pAC5 (wild-type *folC* gene in pUC19) or plasmids containing mutated *folC* genes in pUC vectors (Table 1). pL2, pL4, pL5, pL6, pL8, and pL10 were derived from pAC5 and contain 2-amino-acid linker insertions at various points in the *folC* coding sequence (17). Plasmids pSF4, pGly, pIleu, pVal, pArg, pGlu, and pLeu contain point mutations in codon 309 of *folC* (the site of the mutation in SF4), and the DNA inserts also contain the entire *dedD* gene (16). Plasmid pGT3-8.1 contains the *L. casei* FPGS gene (30). All of the mutant plasmids tested, except pArg, pLeu, and pGlu, transformed strain SF4 to methionine prototrophy. The SF2Δ*folC*/pPMpac3a Amp^r transformants were grown at 37°C in the absence of tetracycline and then replica plated onto tetracycline-supplemented medium to screen for segregation of the pPMpac3a plasmid. Most of the colonies from pAC5-transformed cells were tetracycline sensitive, indicating that the pPMpac3a plasmid had segregated, but none of the pUC19-transformed cells were tetracycline sensitive. The pPMpac3a plasmid segregated from transformants containing the codon 309 point mutants when these were plated on complex medium. The SF2Δ*folC* strains containing pVal, pArg, pLeu, and pGlu grew more slowly than the wild type. SF2Δ*folC* strains transformed with the linker insertion mutants (all of which had <5% of wild-type FPGS or dihydrofolate synthetase activity) (17) were unable to segregate the pPMpac3a plasmid even when grown on complex medium.

In one experiment, the pPMpac3a plasmid segregated from SF2Δ*folC*/pGT3-8.1, (containing the *L. casei* FPGS gene). SF2Δ*folC*/pGT3-8.1 grew only on complex medium and could be stably maintained, but we have not been able to repeat the transformation and segregation experiment that produced this strain. The loss of both the chromosomal and pPMpac3a-encoded *folC* genes from the Tet^s strains from which pPMpac3a had segregated was verified by Southern blotting. Figure 3B, lanes 4 to 6, shows that the *folC*-specific probe hybridized only to 4- to 4.7-kb DNA fragments specific for the replacing plasmids and not to the 2.6-kb pPMpac3a plasmid-specific fragment (lane 2) or any chromosomal integrant. Lane 4 contained the same amount of DNA as lanes 5 and 6 did, but the *L. casei* FPGS gene-specific DNA hybridized only weakly to the *folC* probe. To verify that SF2Δ*folC*/pGT3-8.1 contained only the *L. casei* FPGS gene, extracts of the strain and controls were assayed for FPGS and dihydrofolate synthetase activity. Table 2 shows that SF2Δ*folC*/pGT8-1 has very high expression of FPGS activity, with sixfold-higher activity with 5,10-methylenetetrahydrofolate than with 10-formyl tetrahydrofolate, similar to the specificity of the enzyme purified from *L. casei*. The dihydrofolate synthetase specific activity is less than that of the wild-type *E. coli*, indicating that the chromosomal and pPMpac3a *folC* genes are absent. The apparent dihydrofolate synthetase activity of the *L. casei* FPGS may be due in part to traces of folate in the pteroate substrate. Table 3 shows incorporation of ^{14}C-*para*-aminobenzoic acid into the cells, which is another measure of folate biosynthesis and dihydrofolate synthetase activity. The incorporation of label into SF2Δ*folC*/pGT3-8.1 was 50-fold lower than that into the

FIG. 3. Integration of pCP3 into and segregation from the *E. coli* chromosome. (A) Above the pCP3 plasmid is the *Eco*RI restriction map of the *E. coli* chromosomal DNA in the vicinity of the *folC* gene. The region containing the *folC* and *dedD* genes which was deleted from the *E. coli* DNA in pCP3 is boxed. The recombination events (1 and 2) which gave rise to the integrants are shown. Immediately below the plasmid are the *Eco*RI restriction maps of the *E. coli* chromosomal DNA of integrants 1 and 2. The lowermost level shows the maps following segregation of the plasmid: the map at left shows the chromosomal DNA in which the *folC* gene is deleted and replaced by the Km^r marker; the map at right shows the wild-type segregant (WT). The *folC*, *dedD*, Km^r, and Amp^r genes are boxed. The sizes of the restriction fragments that hybridized to a probe of the linearized radiolabelled pCP3 plasmid are indicated above the restriction maps of the host strain; the integrants and the *folC*-deleted segregant are indicated by divergent arrowheads. The restriction fragment that hybridized to the *folC*-specific gene probe (the *Bam*HI-*Hin*dIII fragment of pAC5, shown in Fig. 5) is indicated below the restriction map of the wild-type segregant. (B) Southern hybridizations of wild-type, integrant, and segregant genomic DNA. Chromosomal DNA was digested with *Eco*RI and probed with pCP3 plasmid DNA. Lanes 1 and 2, MM383; lane 3, MM383::pCP3 integrant 2; lanes 4 to 6, MM383::pCP3 integrant 1; lane 7, MM383::pCP3/pPMpac3a (same as lane 6 but containing plasmid pPMpac3a); lane 8, MM383Δ*folC*/pPMpac3a; lane 9, pPMpac3a plasmid DNA (see Fig. 4 for restriction map). The arrowhead indicates the position of the 8.3-kbp band in lane 8 that is present only when the chromosomal *folC* is deleted. (C) Southern hybridizations of wild-type and *folC*-deleted strains containing complementing plasmids. Chromosomal DNA was digested with *Eco*RI (lane 1) or *Eco*RI and *Sma*I (lanes 2 to 6) and probed with polyacrylamide gel-purified and radiolabelled pAC5 insert DNA (Fig. 5), which is specific for the *folC* coding sequence. Lanes 1 and 2, SF2Δ*folC*/pPMpac3a; lane 3, SF2; lane 4, SF2Δ*folC*/pGT3-8.1; lane 5, SF2Δ*folC*/pSF4; lane 6, SF2Δ*folC*/pAC5. pPMpac3a is described in the Fig. 4 legend. No additional bands corresponding to the wild-type genomic fragment or to integrated plasmids were observed on a sixfold longer exposure of the autoradiogram.


A
2.7 kb
9 kb
folC dedD
1
2
pCP3
5.0 kb
5.0 kb
9 kb
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5.0 kb
8.3 kb
2.7kb
8.3kb
KAN
9.0 kb
Δ FOL
WT
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C
23 kb
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6.5
4.3
2.3
2.0
4.0

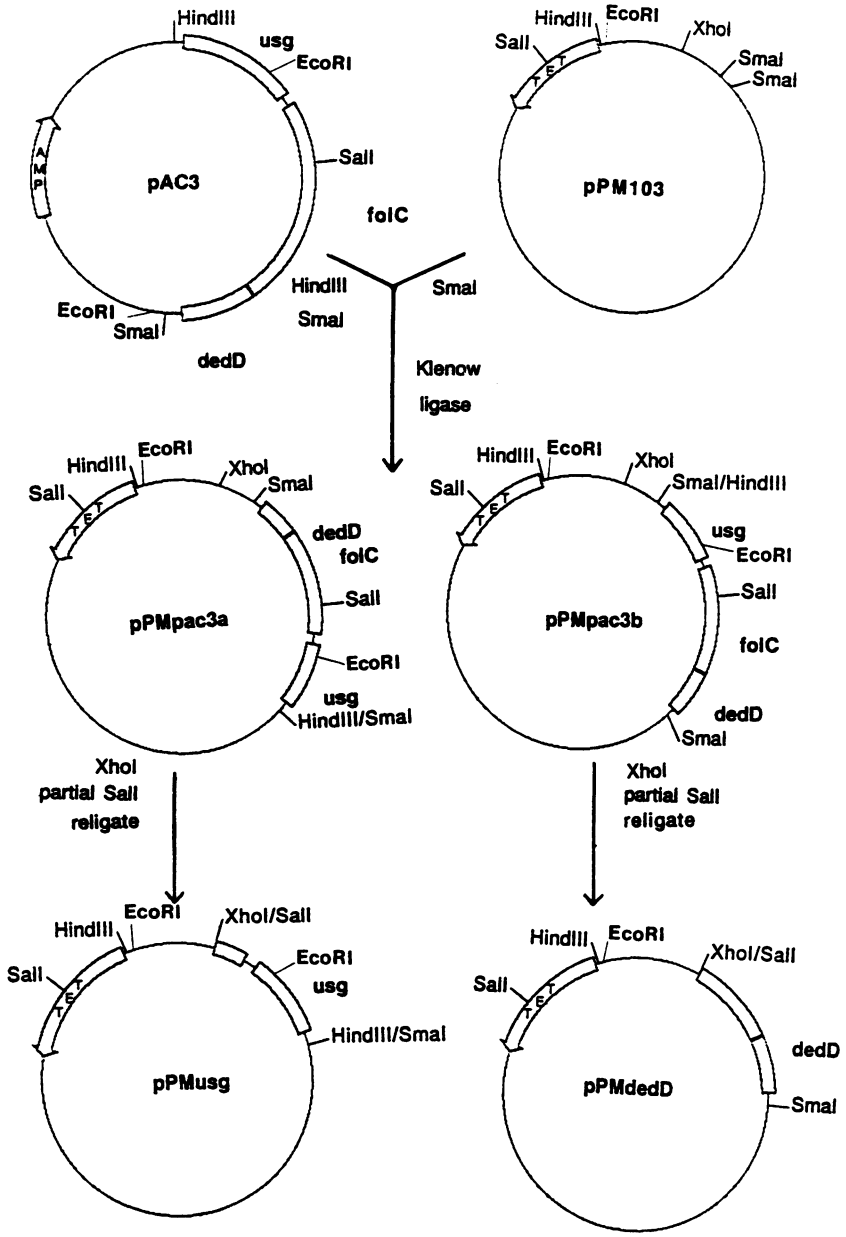

FIG. 4. Construction of plasmids in pPM103. Plasmid pAC3 was digested with *Sma*I and *Hin*dIII. The cohesive ends produced by *Hin*dIII were filled in with the Klenow fragment of DNA polymerase I, and the DNA was ligated to pPM103 digested with *Sma*I and dephosphorylated with calf intestine alkaline phosphatase. The ligated plasmids were used to transform *E. coli* JF1754 selecting for tetracycline resistance and ampicillin sensitivity. The resultant plasmid, designated pPMpac3, was obtained in two possible orientations, pPMpac3a and pPMpac3b. To construct pPMusg, pPMpac3a was digested with *Xho*I and partially with *Sal*I and then religated. Transformants which retained tetracycline resistance, indicating that the *Sal*I site in the *tet* gene was intact, were selected. The digestion removes the *dedD* gene and half of the *folC* gene. The religated plasmid pPMusg retained the upstream gene and its promoter. To construct pPMdedD, pPMpac3b was digested with *Xho*I and partially with *Sal*I and then religated. The upstream gene and half of the *folC* gene were deleted. The religated plasmid pPMdedD contained the *dedD* gene and its promoter, located in the 3′ half of the *folC* gene. DNA sequences are as follows: ——, vector; ▭, open reading frames of *E. coli* genes; ⇐, antibiotic resistance genes.

SF4 mutant strain and 500-fold lower than that into wild-type *E. coli*. These results are consistent with the absence of dihydrofolate synthetase activity in the SF2Δ*folC* strain and with a dihydrofolate synthetase activity of the *L. casei* FPGS gene product that is between 0.2 and 12% of that present in wild-type *E. coli* cells.

The SF2Δ*folC* strains containing mutant *folC* genes from which the complementing pPM103 plasmid had segregated were grown on minimal medium, both liquid and solidified, containing various nutritional supplements of folate end products. SF2Δ*folC* strains containing plasmids pAC5 (wild-type *folC*), pGly, pSF4, pIleu, and pVal were able to grow on minimal medium, although the growth rates of the last two were lower (16). SF2Δ*folC*/pArg produced microcolonies on solidified minimal medium but required methionine and glycine for growth in liquid medium. The SF2Δ*folC*/pGlu

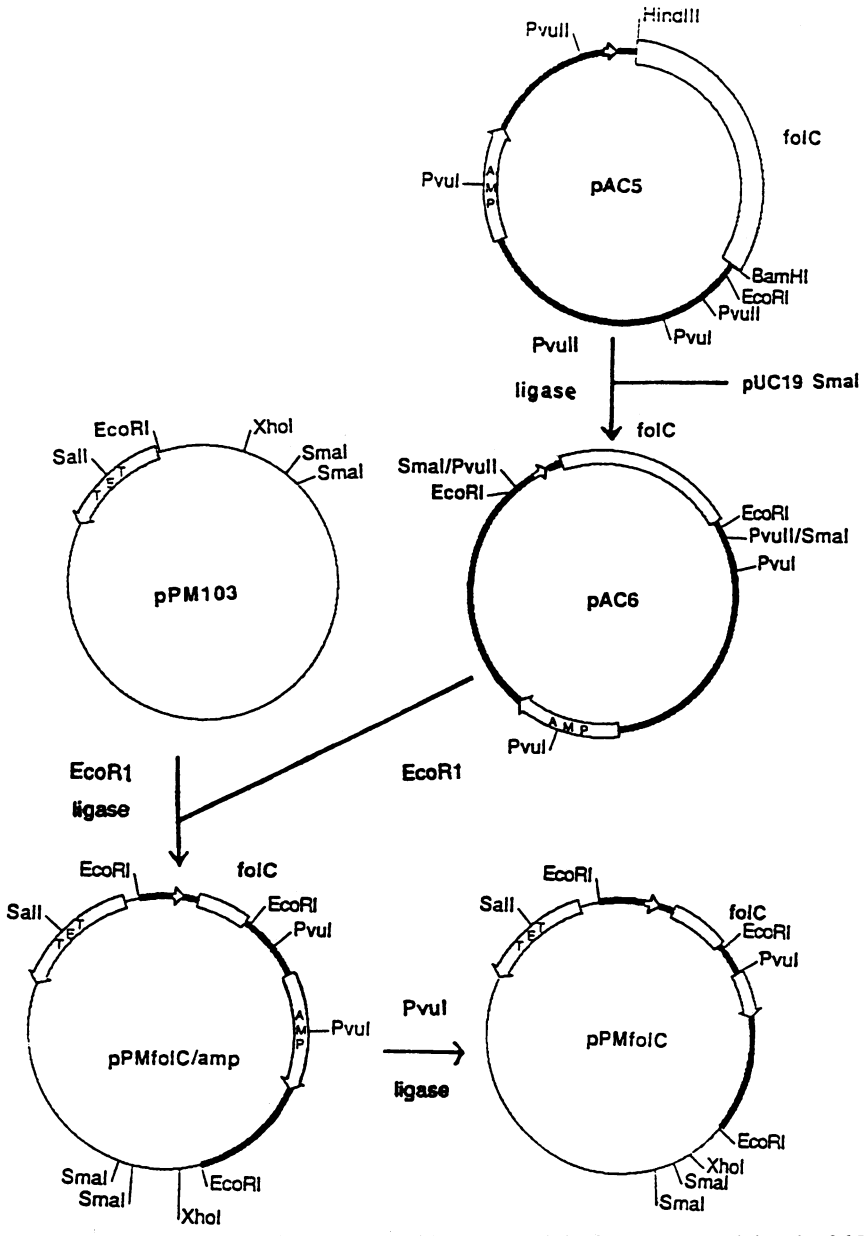

FIG. 5. Construction of pPMfolC. Plasmid pAC5 was digested with *Pvu*II, and the fragment containing the *folC* gene downstream of the *lac* promoter was isolated from an agarose gel and ligated to pUC19 digested with *Sma*I. The plasmids were isolated from Amp^r transformants, and those with the orientation in which the insert could be excised on an *Eco*RI fragment were selected. This plasmid, pAC6, was digested with *Eco*RI and ligated to pPM103 linearized with *Eco*RI. Tet^r transformants on *E. coli* JF1754 were selected and pooled, and their plasmid DNA was isolated. The plasmids from this pool were used to transform *E. coli* SF2 to methionine prototrophy, selecting for plasmids containing a functional *folC* gene. The only Tet^r colonies obtained were also Amp^r. Restriction analysis of the plasmids isolated from these transformants showed that they were a hybrid plasmid, pPMfolC/amp, caused by ligation of the two linearized plasmids and that the *Eco*RI digest of pAC6 had been a partial digest. pPMfolC/amp was digested with *Pvu*I and religated to delete the Amp^r gene. The resultant plasmid, pPMfolC, could replicate in the MM383 *polA* strain of *E. coli* and could complement the *folC* mutation in strain SF4. Transformants of pPMfolC were Tet^r and Amp^s. DNA sequences are as follows: ——, pPM103; ■, pUC19; □, open reading frames of *E. coli* genes; ⇐, antibiotic resistance genes. Open arrowheads indicate the *lac* promoter.

strain required supplements with methionine, glycine, thymidine, and adenosine for growth. The SF2Δ*folC*/pLeu strain and the SF2Δ*folC*/pGT3-8.1 strain required all of the folate end products (including pantothenate) for growth.

DISCUSSION

Our results indicate that the *folC* gene is an essential gene in *E. coli*. Segregants which deleted the chromosomal *folC* gene were not viable unless the deleted gene was complemented with a functional plasmid-encoded gene. Transduction of the chromosomal deletion by using the Km^r marker also required the presence of a complementing gene in the recipient strain. The presence of a *thyA* mutation in *E. coli* allows the deletion of the *folA* gene encoding dihydrofolate reductase (14), but deletion of *folC* was lethal even in a *thyA* strain. It was not sufficient to include the end products of folate-dependent biosynthetic pathways in the medium to

TABLE 2. FPGS and dihydrofolate synthetase in *E. coli* strains and transformants[a]

Strain/transformant	Relative sp act[b]		
	H_2Pte	5,10-CH_2-H_4Pteglu	10-CHO-H_4Pteglu
W1485[c]	1	1	1
SF2Δ*folC*/pPMpac3	1.3	1.1	5.3
SF2Δ*folC*/pAC5	180	134	139
SF2Δ*folC*/pGT3-8.1	0.12	1,438	248

[a] Experimental details are described in Materials and Methods.
[b] Abbreviations: H_2Pte, dihydropteroate; 5,10-CH_2-H_4Pteglu, 5,10-methylenetetrahydrofolate; 10-CHO-H_4Pteglu, 10-formyl-tetrahydrofolate.
[c] Typical specific activities of these extracts were 1, 1.8, and 5.9 nmol/h/mg with H_2Pte, 5,10-CH_2H_4Pteglu, and 10-CHO-H_4Pteglu, respectively.

complement the *folC* function, as it is in FPGS-deficient Chinese hamster ovary cells (29). The folate requirement for synthesis of formyl-methionyl-tRNA for the initiation of protein synthesis would not be satisfied by these supplements (11). Since the biosynthesis of folate requires dihydrofolate synthetase, the dihydrofolate synthetase activity of the *folC* gene product may be essential. We have not obtained any *folC* mutants which have dihydrofolate synthetase activity but no FPGS activity, so we could not determine whether dihydrofolate synthetase activity alone is sufficient for cell viability. The loss of FPGS alone may simply result in methionine auxotrophy (11).

A major application of the strains we have constructed is as recipient strains for mutant *folC* genes, synthesized in vitro, which allow examination of the mutants in the absence of a background activity from the chromosomal gene. We have constructed such strains as well as a strain in which the FPGS gene of *L. casei* replaced the *folC* gene. We will now be able to purify the mutant enzymes and study their properties. The mutant genes were all present in high copy number, and the enzymes were expressed as about 4% of soluble cellular protein, but in the strains complemented by the pGlu, pArg, and pLeu mutant genes, the enzyme-specific activity in the cells was much lower than that in the wild type (16). The lower the specific activity of the enzyme was, the more supplements of folate end products were required for growth. The decrease in enzyme activity in the IB1 (SF2 and SF4) strain resulted in a 70% decrease in intracellular folate pools (11). The level of enzyme activity was even lower in the SF2Δ*folC*/pArg, SF2Δ*folC*/pLeu, and SF2Δ*folC*/pGlu strains than in SF2, suggesting that folate pools may be lower in these strains, resulting in the lower growth rates and auxotrophies. The methionine biosynthetic pathway appears to be most sensitive to low folate, followed by glycine biosynthesis, while biosynthesis of nucleotides and pantothenate are the least sensitive. Studies to measure the levels of folate in the strains described here are in progress and will allow us to determine the folate concentrations which limit the growth of the cell and the functioning of each folate-dependent biosynthetic pathway.

TABLE 3. Incorporation of *para*-amino[carboxyl-^{14}C]benzoic acid into *E. coli* strains[a]

Strain	pmol of pABA incorporated/mg of cells[b]
W1485[c]	287
SF4	24
SF2Δ*folC*/pGT3-8.1	0.53

[a] Experimental details are described in Materials and Methods.
[b] The SF2Δ*folC*/pGT3-8.1 cells grew to 40% of the density of the other two strains; 1 ml of cells at an optical density (at 550 nm) of 1 was assumed to be 1 mg of cells. pABA, *para*-aminobenzoic acid.
[c] The wild-type cells incorporated 68% of the labelled pABA during the course of the experiment.

The strain containing the *L. casei* gene, SF2Δ*folC*/pGT3-8.1, expresses levels of FPGS activity that are 1.8-fold higher than those in SF2Δ*folC*/pAC5 with the *E. coli folC* gene in the same vector. However, SF2Δ*folC*/pGT3-8.1 is auxotrophic for all of the folate end products. This auxotrophy is probably due to extremely low levels of dihydrofolate synthetase activity in the *L. casei* enzyme. The incorporation of *para*-aminobenzoic acid, a measure of folate synthesis, was 0.2% that of the wild type in SF2Δ*folC*/pGT3-8.1. The dihydrofolate synthetase-specific activity of the *L. casei* enzyme (Table 2) was 1/10,000 that of the *E. coli* enzyme, but with the high expression of the *L. casei* gene, the total enzyme activity would be sufficient to account for the viability of SF2Δ*folC*/pGT3-8.1. Preliminary results from extraction and high-performance liquid chromatography of cellular folates suggest that SF2Δ*folC*/pGT3-8.1 does contain folates (30), favoring this interpretation. The *L. casei* gene in the strain may have undergone a mutation to give it some dihydrofolate synthetase activity, because we have not been able to repeat the experiment in which we constructed this strain. An alternate possibility is that the *E. coli* genome in the recipient SF2Δ*folC* that was transformed with pGT3-8.1 had undergone a second mutation, making it able to initiate protein synthesis without formyl methionine. Such a mutant has been reported in the literature (1), although it has been subsequently lost.

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