Construction of a fol Mutant Strain of Escherichia coli for Use in Dihydrofolate Reductase Mutagenesis Experiments

PATRICIA M. AHRWEILER and CARL FRIEDEN*

Department of Biochemistry, Washington University Medical School, St. Louis, Missouri 63110

Received 25 September 1987/Accepted 13 April 1988

A strain of Escherichia coli with the fol gene deleted and a kan gene inserted in its place was created for use in cloning and isolation of mutant dihydrofolate reductase. Southern blot analysis and dihydrofolate reductase enzyme assays confirmed the Δfol::kan genotype. A thyA mutation accompanied the fol deletion and is required for survival of a dihydrofolate reductase-deficient strain.

Dihydrofolate reductase (DHFR) is a small (18,000-dalton) protein of known tertiary structure which contains no disulfide bonds and has been the subject of extensive structural (3, 16, 21), kinetic (6; J. R. Appleman, M. Kuehl, E. E. Howell, J. Kraut, and R. L. Blakley, Fed. Proc. 46:2122, 1987; J. R. Morrison and S. R. Stone, Fed. Proc. 45:1751, 1986; M. H. Penner and C. Frieden, J. Biol. Chem., in press.), and folding (14, 20; J. J. Onuffer and C. R. Matthews, Fed. Proc. 46:2021, 1987.) studies. We planned to do site-directed mutagenesis on Escherichia coli DHFR for protein-folding studies but were faced with the following problems: (i) introduction of genetically engineered, plasmid-based E. coli DHFR sequences into the E. coli host may be followed by recombination between the mutant plasmid and chromosomal DHFR genes, and (ii) during purification of mutant proteins, contamination by a wild-type protein is often unavoidable, making interpretation of structural and functional studies more difficult. A strain apparently devoid of DHFR activity has been reported (18), but the nature of the mutation was not characterized, and the possibility existed of reversions or production of low levels of DHFR enzyme below the detection limits of the assay.

To circumvent these difficulties, we constructed a strain of E. coli with the fol gene deleted entirely, using the strategies of Jasim and Schimmel (10) and of Winans et al. (22). When a cloned fragment of E. coli chromosomal DNA with a kan gene replacing the fol gene is transformed into strain JC7623, transformants with a kan' phenotype should have the fol gene deleted from the chromosome by a reciprocal recombination event. In this report, we show by Southern blot analysis and enzyme assays that we have deleted the fol gene in a mutant strain of E. coli and that this deletion is lethal unless accompanied by inactivation of the thyA gene. We suggest that the thyA mutation is required for a DHFR-deficient strain to maintain the balance of folate cofactors in the cell and therefore to survive.

Bacterial strains and plasmids used are listed in Table 1. Strains AB1157, JC7623, and Pl vir were from P. Shen, and strain WB439 was from W. Barnes (Washington University Medical School, St. Louis, Mo.). pCV29 was a gift from D. Smith (Cornell University, Ithaca, N.Y.), and pBTAH2 was kindly provided by G. Maley (New York State Department of Health, Albany). All restriction and DNA modification enzymes were purchased from New England Biolabs, Inc., Beverly, Mass., except T4 DNA ligase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). All other biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo., except where stated. [α-32P]dATP was purchased from ICN Biochemicals, Irvine, Calif. Bacteria were grown in LB medium (11) at 37°C. Ampicillin, kanamycin, and trimethoprim were added at 100, 50, and 20 μg/ml, respectively, for selection purposes. Trimethoprim selection was done on minimal medium plates supplemented with the auxotrophic requirements of the strain. PA414 required thymine supplemented at 50 μg/ml for growth in minimal medium and for optimal growth rates in rich broth.

**Plasmid construction.** Creating the Δfol::kan mutation requires a cloned fragment of the E. coli chromosome encompassing the fol region with the desired mutations introduced. The steps utilized in the construction of this plasmid, pPA19, are shown in Fig. 1. The 8.3-kilobase (kb) BamHI insert of pCV27 was subcloned into pBR322 lacking the EcoRI site and was renamed pCV29. pCV29 was linearized near the center of the DHFR coding sequences with EcoRI. The DNA was subjected to BAL 31 to create a deletion approximately 1 kb in size (11). The digested ends were filled in with the Klenow fragment of DNA polymerase 1, and the blunt ends were ligated to produce pCVΔfol. pCVΔfol was partially digested with Sall (the SalI site of pBR322 is not shown in the figure), and then a kanamycin resistance (Kan') gene cartridge (Pharmacia, Inc., Piscataway, N.J.) with Sall ends was ligated to the linearized plasmid. A Kan' Amp' Tmp' clone was selected and named pPA19. Restriction analysis of the 8.3-kb BamHI insert of pPA19 revealed that the region assigned to the fol gene by Smith and Calvo (19) had been deleted from the E. coli chromosomal insert and that a Kan' gene cartridge had been inserted approximately in its place (data not shown).

**Strain construction.** Strain JC7623 was transformed with 2 to 3 μg of the gel-purified 8.3-kb BamHI fragment recovered from pPA19. Selection of JC7623 transformants on L plates supplemented with kanamycin produced 33 Kan' colonies. These colonies were further screened for DHFR activity by their inability to grow on M9 plates (11) supplemented only with the auxotrophic requirements of AB1157 and JC7623 (histidine, proline, arginine, leucine, threonine [20 μg/ml each], and thiamine [2 μg/ml]). Cells lacking DHFR activity have an auxotrophic requirement for metabolites which utilize folate cofactors in their biosynthesis (glutamine, methionine, adenine, pantothenic acid, and formylmethionyl-tRNAf). Formylmethionyl-tRNAf cannot be supplied exogenously, but Harvey (8) has shown the E. coli lacking formylmethionyl-tRNAf grows at a reduced rate, with un-
formylated methionyl-tRNA\textsubscript{f} utilized as the initiating amino acid in protein synthesis.

One Kan\textsuperscript{r} colony was also DHFR\textsuperscript{−} and was renamed JC7623A\textsubscript{fol}. Both DHFR enzyme assays and Southern blot analysis demonstrated the absence of any detectable DHFR activity or DHFR-coding sequences in this clone (data not shown). A P\textsubscript{1} vir lysate from this strain was used to transduce AB1157 to Δ\textsubscript{fol}:kan by the method of Miller (12). Several attempts to transduce AB1157 to Kan\textsuperscript{r} with the above-described lysate were made, as very few colonies per transduction were obtained (1 to 6 Kan\textsuperscript{r} colonies per transduction), and most of these Kan\textsuperscript{r} colonies were DHFR\textsuperscript{+}. In four transductions, 15 Kan\textsuperscript{r} colonies were screened for DHFR activity, and 1 colony was found to be both Kan\textsuperscript{r} and DHFR\textsuperscript{−}. This Δ\textsubscript{fol}:kan clone was renamed PA414.

Strain PA414 was repurified on L plates supplemented with kanamycin, and cultures grown from single colonies were subjected to DHFR enzyme assays and Southern blot analysis to verify the deletion of the \textit{fol} gene. DHFR enzyme assays of PA414 mini-cell-free Brj lysates (7) produced no detectable DHFR activity. DHFR activity was measured as previously described (15; Penner and Frieden, in press) on a Gilford 240 spectrophotometer. When dihydrofolate (FH\textsubscript{2}) was omitted from the assay mix, a nonspecific NADPH oxidase activity produced NADP\textsuperscript{+} at rates comparable to those in the DHFR assay of the parent strain AB1157.

Southern blots (Fig. 2) were done by the method of Maniatis (11), with the following exceptions. The agarose gel was treated sequentially with 2 volumes of 0.5 N NaOH–1 M NaCl (two times for 15 min each), and then two volumes of 1 M ammonium acetate–0.02 N NaOH (two times for 15 min each) (17), and the second solution was used in the transfer of DNA onto Nytran membranes (Schleicher & Schuell, Inc., Keene, N.H.). Hybridizations were done by the method of Church and Gilbert (4).

Deletion of the \textit{fol} gene and insertion of the \textit{kan} gene in PA414 were clearly established by Southern blot analysis. Probing with the [\textalpha-\textsuperscript{32}P]dATP-labeled (5) 445-base pair (bp) Draf-BamHI DHFR fragment produced signals corresponding to the DHFR gene in the 8-kb bands for the BamHI-digested pCV29 and AB1157 DNA and a 6-kb band for the BamHI-Xhol-digested pCV29 and AB1157 (wild-type) DNA, but produced no signal for the pPA19 or PA414 (Δ\textsubscript{fol}:kan) lanes (see Fig. 1 for restriction maps of these regions). The labeled Kan\textsuperscript{r} gene hybridized with the 8-kb inserts of BamHI-digested pPA19 and PA414 DNA and two smaller (2.5- and 3.5-kb) bands for the BamHI-Xhol-digested pPA19 and PA414 DNA. No signal appeared for the pCV29 or AB1157 controls (data not shown).

The 5' end of the deletion in the AB1157 chromosome was not determined exactly. Probing with a 575-bp SnaBI-BamHI fragment of pTY1 that includes 60 bp 5' to the DHFR-coding sequences produced faint bands for pPA19 and PA414 (data not shown) which were not present in Southern blots examined with the shorter probe. The 5' end of the deletion is therefore likely to be within 60 bp 5' to the DHFR initiation codon and 90 bp into the coding sequence.

\textbf{Relationship of \textit{thyA} and \textit{fol}.} It has been observed that an inactive \textit{thyA} (thymidylate synthetase) gene is required for the survival of a \textit{fol} deletion mutant (9; Elizabeth Howell, University of Tennessee, personal communication). PA414 was screened for a Thy\textsuperscript{−}\textsuperscript{ phenotype} by determining its dependence on thymidine supplemented to minimal medium for growth. According to Neuhard and Nygaard (13), the

\begin{table}[h]
\centering
\begin{tabular}{lll}
\hline
Strain or plasmid & Genotype or description & Reference or source \\
\hline
\textit{E. coli} & F\textsuperscript{−}his-4 thr-1 leu-6 thi-1 lacY1 galK2 ara-1 xyl-5 mtl-1 proA2 argE3 rps-31 tsx-38 supE44 $\lambda$ & P. Shen\textsuperscript{a} \\
AB1157 & AB1157 thyA & This study \\
AB1157 (thyA) & AB1157 recB21 recC22 sbcB15 & 9 \\
IC7623 & AB1157 thyA Δ\textsubscript{fol}:kan & This study \\
PA414 & AB1157 thyA Δ\textsubscript{fol}:kan & W. Barnes\textsuperscript{a} \\
WB439 & F\textsuperscript{−} endA1 hsdR17 (r\textsubscript{k} m\textsubscript{k} supE44 thi-1 $\lambda$ recA1 gyrA96 relA) & \\
\hline
\textbf{Plasmids} & & \\
pCV27 & pBR322 with 8.3-kb fragment of \textit{E. coli} chromosome encompassing \textit{fol} gene inserted at BamHI site; Amp\textsuperscript{r} Tmp\textsuperscript{r} & 18 \\
pCV29 & pCV27 with no pBR322 EcoRI site & This study \\
pCVΔfol & pCV29 with DHFR gene deleted (1 kb) & This study \\
pPA19 & pCVΔfol with Kan\textsuperscript{r} gene replacing DHFR gene; Amp\textsuperscript{r} Kan\textsuperscript{r} & This study \\
pBTAH2 & pBR322 with 1.2-kb HindIII insert of \textit{E. coli thyA} gene & 2 \\
pTY1 & pBR322 with 1.1-kb BamHI insert of DHFR gene under control of overproducing promoter; Amp\textsuperscript{r} Tmp\textsuperscript{r} & 14 \\
\hline
\end{tabular}
\caption{Bacterial strains and plasmids}
\end{table}
FIG. 2. (A) Southern blot analysis of fol gene deletions. The first eight lanes show a BamHI digestion of chromosomal and plasmid DNAs. The last four lanes show a BamHI-XhoI digest of chromosomal and plasmid DNAs. Approximate sizes of fragments detected in kilobases are given. The wild-type DHFR+ parent strain AB1157, the DHFR− pCV27 plasmid, and the DHFR− Kan′ pPA19 plasmid were used as controls. The Kan′ probe is the labeled Kan′ gene cartridge used in the construction of plasmid pPA19. (B) Derivation of the DHFR probe. The BamHI insert of pTY1 is represented in the diagram. DHFR-coding sequences are shown as an open box. The arrow represents the 445-bp DraI-BamHI fragment used as a probe for the presence of the DHFR gene in chromosomal or plasmid DNA.

The thymine requirement of a thyA mutant is 150 μM. Strain PA414 did not grow normally on glucose minimal medium plates until the concentration of thymine supplemented reached 150 μM, so it appeared that PA414 is also a thyA mutant.

The Δfol::kan phenotype of PA414 was transduced into both wild-type and a thyA mutant of AB1157 (12) to test the requirement of a thyA inactivation with a fol deletion. When thymine was supplemented to the kanamycin-supplemented L selection plates (50 μg/ml), the frequency of transduction into the AB1157 (thyA) strain was 20-fold greater than that into the wild-type AB1157 strain. When thymine was not added, both strains produced very few transductants as noted above. Attempts to transform PA414 (Δfol thyA) to thyA+ with pBHAT2 (2) (the E. coli thymidylate synthetase gene subcloned into pBR322) were also unsuccessful. These results confirm the idea that a Fol− Thy+ phenotype is lethal and that a fol deletion mutant must also be thyA to be viable. The low frequencies of transformation and transduction to Kan′ Δfol observed in the construction of PA414 are most likely due to the selection of two mutation events, thyA inactivation and fol deletion.

The folA gene (1 min) and the thyA gene (61 min) are not genetically linked on the E. coli K-12 linkage map (1). Their relationship must therefore be biochemical rather than genetic. Derivatives of tetrahydrofolate (FH4), N5,N10-methylene-FH4, and N5-methyl-FH4, are used in the biosynthetic pathways of purines, pyrimidines, glycine, and pantothenic acid, and methionine (23). FH4 is regenerated from every biosynthetic pathway except dTMP synthesis by thymidylate synthetase, which generates FH3. DHFR regenerates FH4 by catalyzing the reduction of FH2 to FH4. In the absence of DHFR activity, the cell would die from the lack of FH4-derived cofactors utilized for biosynthetic pathways or from the accumulation of FH3 in the cell. However, if thymidylate synthetase were also inactive, no FH4 would be produced and the reaction catalyzed by DHFR would not be crucial.

If the Δfol thyA mutant does maintain the FH4-derived cofactor pool intact in the cell, these double mutants should not have an auxotrophic requirement for the nutrients utilizing FH4-derived cofactors in their biosynthesis. This is exactly what we observed for PA414 (Δfol thyA::kan), which required only thymine supplemented to minimal medium for growth. This is in agreement with the strategy of Miller (12) for the production of a thyA mutant, which calls for artificially rendering the cell a fol mutant with trimethoprim treatment, supplementing the medium with thymine, and selecting for surviving cells which have acquired a thyA mutation. The Δfol and thyA mutations together are complementary and are not lethal.

Utility of strain PA414. The PA414 strain produced from the above manipulations is well suited as a host for cloning, mutagenesis, or protein production from vectors containing mutant DHFR genes. The strain has no detectable DHFR activity, and the DHFR coding sequences are deleted from its chromosome and replaced with a Kan′ gene, according to Southern blot analysis. The growth rate of the strain is comparable to that of the parent strain when thymine is supplemented to the media. Strain PA414 was readily transformable by pTY1 at about 1/10 the efficiency of the parent strain AB1157 and stably maintains this and other supercoiled plasmids at a copy number comparable to that of AB1157 (determined by Birnboim-Dolys plasmid minipreps [11] assayed on agarose gels). The growth rate of the PA414.pTY1 strain is somewhat reduced, and there is a 3-h time lag before the PA414.pTY1 culture starts its exponential growth phase. However, the culture reaches a saturation density and produces active DHFR enzyme 500-fold above wild-type levels. The strain has a thyA mutation, which creates an auxotrophic requirement for thymine but does not affect the utility of the strain.

We thank Ping Shen and Toni Kazic for technical assistance. This work was supported by Public Health Service grant DK13332 from the National Institutes of Health and by The Monsanto Company.

LITERATURE CITED
dihydrofolate reductase from *Escherichia coli*. Biochemistry 26: 4085–4092.