Amplification and Modification of Dihydrofolate Reductase in *Escherichia coli*

NUCLEOTIDE SEQUENCE OF *fol* GENES FROM MUTATIONALLY ALTERED PLASMIDS*

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Recombinant plasmids carrying the structural gene for *Escherichia coli* dihydrofolate reductase (*fol*) were mutagenized *in vitro* and *in vivo* and were used to transform a suitable recipient strain. Twenty-three transformants were isolated that were able to grow in the presence of high levels of the folate analog trimethoprim, and, in each strain, the resistance determinant was shown to be carried on the plasmid. Three of the strains produced dihydrofolate reductase with an increased K_i value for trimethoprim. DNA sequence analysis showed that the plasmids in these strains had mutations in *fol* which altered a conserved region of the polypeptide that forms part of the dihydrofolate-binding site. Two other strains had approximately 3-fold elevated dihydrofolate reductase levels, apparently resulting from plasmid copy number mutations. The remaining 18 strains had dihydrofolate reductase levels that were 10-30 times higher than those of the starting strain. Surprisingly, three of these strains had no discernible changes either in plasmid copy number or in the nucleotide sequence of the plasmid *fol* gene. Sequence analysis of the plasmids in 12 more of the strains revealed mutations in the promoter region adjacent to the *fol* gene. Most of these mutations occurred in the conserved sequences known as the Pribnow box and the −35 region and increased the homology of these sequences with the consensus *E. coli* promoter sequence. Strains carrying these plasmids produced a significant fraction of their total cell protein as wild type dihydrofolate reductase and should therefore be useful as sources of the purified enzyme.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, a cofactor required for the metabolism of amino acids, purines, and pyrimidines. Drugs that bind tightly to dihydrofolate reductase and

inhibit its action, such as the folate analogs methotrexate and trimethoprim, cause a depletion of tetrahydrofolate and, as a consequence, are toxic to cells. A number of these drugs are clinically important as antibacterial, immunosuppressant, and antineoplastic agents (1).

Cellular resistance to folate analogs is often observed after prolonged exposure of either bacterial or animal cells to these drugs, and there has been considerable interest in the mechanisms by which resistance develops (2–8). Much research has also been done on the structure and mechanism of action of dihydrofolate reductase and its inhibitors in the hope of designing drugs with higher potency and fewer side effects (9–12). Clearly, a rational approach to improved drug therapy requires knowledge of both the mechanisms by which cells acquire resistance to the drugs and the ways in which the drugs interact with the enzyme.

The *Escherichia coli* dihydrofolate reductase system is simple and well characterized and presents a unique opportunity to obtain detailed information relating to both these problems in a single organism. The enzyme can be purified in three steps (13) and both the amino acid sequence (14, 15) and the 3-dimensional structure are known (12). The gene coding for the enzyme (*fol*) has been cloned (16, 17), the nucleotide sequence has been determined (18), and the mRNA transcripts have been characterized (18). In this paper, we report the isolation and characterization of a large number of mutations on *fol* plasmids which lead to increased resistance to the antibiotic trimethoprim in *E. coli*. Among the strains isolated are overproducers which make 10–30 times more dihydrofolate reductase than previously reported cloned derivatives. These strains should facilitate physical studies on the enzyme because such experiments require relatively large amounts of pure enzyme.[1]

MATERIALS AND METHODS

Bacterial Strains, Media, and Chemicals—All strains were derived from JFM43 (C-600 rk⁻ mk⁺) or JFM65 (JFM43 *rpsL srl-1300::Tn10 recA-56*) (17, 19). All plasmids were derived from pJFM29 (19) by *in vitro* or *in vivo* mutagenesis. Media and chemicals were from sources described previously (18, 19) except that nitrocefin was a generous gift from Glaxo Pharmaceuticals (Great Britain). Minimal medium (20) was supplemented with either individual amino acids (50 μg/ml) or with 1% casamino acids. Brain heart infusion (Oxoid Ltd., Great Britain) was used as the rich medium.

Preparation of Plasmid DNA—Purified plasmid DNA was prepared as described (18, 21). Partially purified plasmid DNA was prepared as follows. Two drops of chloroform-saturated distilled water

were added to 2 ml of a stationary phase culture, and the cells were collected by centrifugation for 2 min at top speed in a Beckman Microfuge B and were washed with 1.5 ml of 10 mM Tris-HCl, pH 9.0, containing 1 mM EDTA. The cells were resuspended in 150 μl of 50 mM Tris-HCl, pH 9.0, containing 50 mM EDTA and 15% sucrose, and 50 μl of lysozyme (4 mg/ml) were added. The suspension was incubated for 15 min at room temperature and then for 30 min on ice, after which 150 μl of ice-cold distilled water was added. Following a further 5-min incubation on ice, the samples were heated for 15 min at 70 °C and then centrifuged for 15 min. DNA in the supernatant fluid was concentrated by ethanol precipitation.

Mutagenesis of Plasmid DNA—Purified pJFM29 DNA was mutagenized *in vitro* as described previously (22) except that incubation with hydroxylamine was at 70 °, 80 °, or 85 °C for 30 min. Plasmid mutations were generated *in vivo* as follows. Chloramphenicol (4.25 mg) was added to a 25-ml mid log phase culture of strain JFM161 (JFM43/pJFM29), and the culture was incubated for a further 3 h at 37 °C to allow for plasmid amplification. The cells were collected by centrifugation, washed, and treated with NTG (100 μg/ml in 0.1 M sodium citrate, pH 5.5) for 30 min at 37 °C (23). After washing twice with 0.1 M sodium phosphate, pH 7.0, the cells were resuspended in 20 ml of rich medium, divided into 10 aliquots, and incubated overnight at 37 °C. Partially purified plasmid DNA was isolated from each of these cultures.

Selection of Trimethoprim-resistant Mutants—Mutagenized plasmid pJFM29 DNA was used to transform strain JFM65 to ampicillin resistance using the procedure of Lederberg and Cohen (24). Trimethoprim-resistant transformants were selected directly by plating onto minimal medium containing 100 μg/ml of ampicillin and 20 μg/ml of trimethoprim. These procedures and all subsequent work with the resulting clones were carried out under P0 containment conditions as defined by the National Institutes of Health guidelines. Mutants were subcultured twice onto the same medium and examined for their auxotrophic growth requirements. The plasmids present in single colonies were examined on agarose gels as described previously (17).

Restriction Analysis of DNA Fragments—Purified plasmid DNA was digested with restriction endonucleases either as described previously (17) or as recommended by the manufacturer. Plasmid DNA was examined by agarose-gel electrophoresis as described (17). The sizes of the restriction fragments were calculated by comparison of their electrophoretic mobilities to those of standards of known size using a computer program (25).

Preparation and Assay of Enzymes—Cell extracts were prepared as described before (17) except that 100-ml cultures were used and the cells were disrupted by sonication using a Sonic Pen sonicator (Technic International, Inc.) (bursts (6 × 20 s) at maximum power). Dihydrofolate reductase was purified from 1-liter cultures by affinity chromatography using a Sepharose-methotrexate column (10), after which the enzyme was desalted on Sephadex G-75, concentrated by ultrafiltration through a Dia-flow UM10 membrane, and then stored at −20 °C until further use.

Dihydrofolate reductase activity was determined as described previously (10, 17). One unit of enzyme was defined as the amount of enzyme that will catalyze the transformation of 1 μmol of dihydrofolate/min at 30 °C. The concentration of dihydrofolate reductase was determined as described by Williams *et al.* (10). Trimethoprim-inhibition studies were carried out as before (17). β-Lactamase was determined by use of the chromogenic Cephalosporin compound 87/312 (nitrocefin) according to the manufacturer's instructions (26). One unit of β-lactamase activity is defined as the amount of enzyme that will hydrolyze 1 μmol of nitrocefin/min. Protein was determined by the Biuret method using bovine serum albumin as a standard (27).

DNA-Sequencing Techniques—DNA fragments were isolated from polyacrylamide gels, labeled at their 3′ ends with reverse transcriptase, and sequenced using a modification of Maxam and Gilbert's chemical cleavage method as described previously (18). The reaction products were separated by electrophoresis on polyacrylamide gels (80 × 40 × 0.04 cm) under conditions which allow unambiguous determination of the sequence up to 600 nucleotides from the labeled end (18). In cases where the bands on autoradiograms were ambiguous or otherwise difficult to read, the sequence was repeated. Sequencing gels were read at least twice to verify the correctness of the assignments.

RESULTS

Isolation of Trimethoprim-resistant Strains—*E. coli* strains that harbor recombinant plasmids containing the dihydrofolate reductase structural gene, *fol*, overproduce the enzyme (16, 17). These strains are able to grow on media containing 2 μg/ml of trimethoprim but not on media with 20 μg/ml of trimethoprim. To obtain mutants that further overproduce dihydrofolate reductase, it was decided to select for plasmid strains that were resistant to 20 μg/ml of trimethoprim.

Mutagenesis of the recombinant plasmid pJFM29 was carried out either *in vitro* by hydroxylamine treatment of purified plasmid DNA or *in vivo* by NTG[2] treatment of chloramphenicol-amplified cultures of strain JFM161 (JFM43/pJFM29). After transformation of strain JFM65 with plasmid DNA mutagenized by either method, mutants resistant to 20 μg/ml of trimethoprim were selected. Twenty-three independently derived mutants were isolated, 10 after hydroxylamine treatment and 13 after NTG treatment. All 23 mutants carried a plasmid of the same size as plasmid pJFM29, as determined by agarose-gel electrophoresis (not shown). Plasmid DNA was partially purified from each mutant and used to transform strain JFM65 to ampicillin resistance. All of these transformants were resistant to 20 μg/ml of trimethoprim.

Dihydrofolate Reductase and β-Lactamase Levels in Plasmid-containing Strains—Cell extracts were prepared from JFM65 derivatives carrying mutated plasmids and were assayed for dihydrofolate reductase and β-lactamase activities. β-Lactamase activity was determined to obtain a simple estimate of relative plasmid copy number in cultures of these strains. On the basis of these assays, the mutants fell into four groups. The results from some representative mutants are shown in Table I. The majority of the mutants (15/23) were designated as group A mutants. These strains produced normal amounts of β-lactamase but had 10–15 times more dihydrofolate reductase activity than the control strain JFM210. The three group B strains had dihydrofolate reductase levels similar to or somewhat higher than those strains in group A but had much lower β-lactamase levels. The three group C strains had dihydrofolate reductase levels similar to those of the control strain JFM210 and had slightly elevated β-lactamase levels. Finally, both dihydrofolate reductase and β-lactamase levels of the two group D strains were approximately 3-fold higher than the corresponding levels in the control strain.

Analysis of Dihydrofolate Reductases Coded by Derivative Plasmids—Preliminary experiments showed that cell extracts of the mutant strains were free of compounds or enzymes that might have interfered with the dihydrofolate reductase assay. Cell extracts were therefore used as the source of enzyme for kinetic analysis of the dihydrofolate reductases produced by the mutant strains. All of the group A and B enzymes that were examined had kinetic parameters similar to those of the control enzyme (Table II). However, the enzymes from the two group C strains tested had higher K_i values for trimethoprim. Since the inhibition studies were performed in the presence of saturating (90 μM) NADPH, the K_i values represent dissociation constants for the release of trimethoprim from the enzyme-NADPH-trimethoprim complex.

To confirm these results and to determine respective turnover numbers, dihydrofolate reductase was purified from cell extracts of strains JFM161, JFM202, JFM218, and JFM228. Enzyme kinetic studies showed that the Michaelis constants for dihydrofolate and the K_i values for trimethoprim were not significantly different from the values determined in the experiments using cell extracts (data not shown). Determination of the turnover number for these enzymes failed to reveal any differences in the maximum rate of catalysis that could account for the increased specific activity of dihydrofolate reductase that was observed in cell extracts of the mutant

[2] The abbreviations used are: NTG, *N*-methyl-*N*-nitro-*N*′-nitrosoguanidine; HA, hydroxylamine; bp, base pair.

TABLE I

Dihydrofolate reductase and β-lactamase levels in cell extracts

Strain[a]	Plasmid[b]	Mutagen	Dihydrofolate reductase	β-Lactamase
			Specific activity[c]	
Control				
JFM211	pBR322		0.001	8.0
JFM210	pJFM29		0.11	6.5
Group A				
JFM202	pJFM46	HA	1.2	7.6
JFM212	pJFM54	NTG	1.4	6.9
JFM218	pJFM60	HA	1.5	5.3
Group B				
JFM203	pJFM47	HA	1.6	0.36
JFM205	pJFM49	NTG	1.9	0.19
JFM228	pJFM70	HA	3.2	0.96
Group C				
JFM207	pJFM51	NTG	0.07	12.0
JFM213	pJFM55	NTG	0.24	8.9
JFM220	pJFM62	HA	0.08	13.0
Group D				
JFM217	pJFM59	NTG	0.34	18.0
JFM224	pJFM66	NTG	0.30	19.0

[a] Only representative strains are shown. All of the additional 12 mutants were in group A. All strains were JFM65 derivatives that carried the plasmids indicated.

[b] Except for pBR322, all plasmids were derivatives of pJFM29.

[c] Specific activities are expressed as units/mg of protein. Each value is the average from at least two cell-extract preparations.

TABLE II

Kinetic parameters for the interaction of dihydrofolate and trimethoprim with dihydrofolate reductase in cell extracts

Strain	Group[a]	K_m (dihydrofolate)	K_i (trimethoprim)
		μM	nM
JFM210	Control	0.73 ± 0.11	0.10 ± 0.02
JFM202	A	0.79 ± 0.17	0.096 ± 0.008
JFM212	A	1.1 ± 0.18	0.090 ± 0.009
JFM218	A	1.1 ± 0.13	0.085 ± 0.010
JFM228	B	0.72 ± 0.06	0.13 ± 0.02
JFM207	C	0.65 ± 0.12	0.19 ± 0.01
JFM220	C	0.71 ± 0.06	0.25 ± 0.03

[a] See Table I.

strains (not shown). In addition, each of the purified enzymes was subjected to co-electrophoresis with the control (JFM161) enzyme using the 2-dimensional electrophoresis system of O'Farrell (28, 29). In each experiment, a single spot corresponding to dihydrofolate reductase was observed, indicating that the control enzyme and those enzymes coded by the mutant plasmids could not be separated under these conditions (data not shown).

Agarose-Gel Electrophoresis of Derivative Plasmids—Plasmid DNA was isolated from seven group A mutants, all three group B mutants, and one group C strain. The plasmids were digested with restriction endonucleases and analyzed by agarose-gel electrophoresis (Fig. 1). Restriction analysis revealed that the location of the *Eco* RI and *Pst* I sites in each of these plasmids was identical with their location in the parent plasmid pJFM29. In contrast, digestion with *Sal* I showed that the plasmid pJFM70 was missing one of the three *Sal* I sites (marked by an *asterisk* in Fig. 2) that was present in all of the other plasmids.

DNA Sequence Analysis of Derivative Plasmids—The sequencing strategy for analysis of the mutant plasmids is shown in Fig. 2. The advent of 80-cm sequencing gels greatly facili-

tated this study because the entire structural gene (550 nucleotides) could be sequenced from a single-labeled fragment requiring only one set of sequencing reactions. An autoradiogram from one of these gels is shown in Fig. 3. The results of sequence analysis of *fol* genes from 18 of the mutant plasmids are shown in Fig. 4. The sequence of the parent plasmid (pJFM29) corresponded exactly to that previously determined for another wild type *E. coli* K12 *fol* gene (18). The mutations in the derivative plasmids were all derived by G:C → A:T transitions and fell into three categories.

1) *fol* promoter mutations. Most of these plasmids have an alteration at the fifth base of the Pribnow box (*position −8*, Fig. 4). With the exception of plasmid pJFM47 (from a group
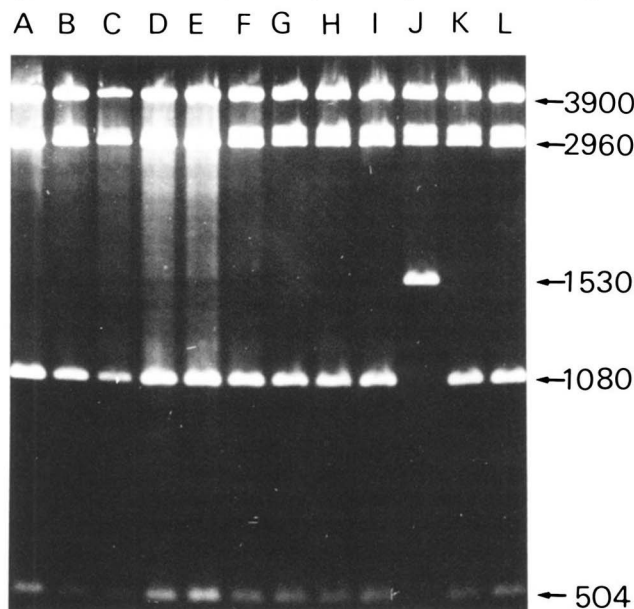

FIG. 1. **Agarose-gel electrophoresis of restriction fragments of pJFM29 derivatives.** Each of the plasmids pJFM29 (*A*), pJFM46 (*B*), pJFM47 (*C*), pJFM49 (*D*), pJFM51 (*E*), pJFM54 (*F*), pJFM60 (*G*), pJFM64 (*H*), pJFM65 (*I*), pJFM70 (*J*), pJFM71 (*K*), and pJFM72 (*L*) was digested sequentially with *Pst* I and *Sal* I. The resulting fragments were subjected to electrophoresis on a 2.0% agarose gel. The size of each fragment (base pairs) is as indicated. The *band* present in most *wells* just above the *2960-base pair band* is due to incomplete digestion. The *504-base pair fragment* was present in all digests except that of pJFM70.
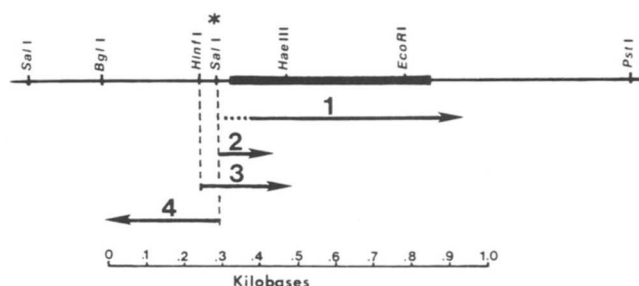

FIG. 2. **Sequencing Strategy.** The *bar* indicates the coding region for dihydrofolate reductase. The *arrows* indicate the approximate extent and direction of sequencing and point away from the labeled 3' ends. The following fragments were used: *1* and *2*, *Sal* I/*Pst* I, 1100 bp; *3*, *Hin*fI/*Hae* III, 233 bp; and *4*, *Sal* I/*Bgl* I, 293 bp. Each sequence was carried out with DNA fragments from the following plasmids: Sequence 1, pJFM29, 46, 49, 51, 54, 55, 56, 57, 60, 62, 63, 64, 65, 68, 69, 71, 72; Sequence 2, pJFM29, 46, 49, 51, 54, 55, 56, 71, 72; Sequence 3, pJFM29, 46, 47, 49, 51, 54, 55, 56, 57, 60, 62, 63, 64, 65, 68, 69, 70, 71; and Sequence 4, pJFM46, 56, 69. Thus, with the exception of plasmids pJFM47 and pJFM70, the entire promoter region and structural gene were sequenced in each case. The *asterisk* marks the *Sal* I restriction site that is missing in plasmid pJFM70.
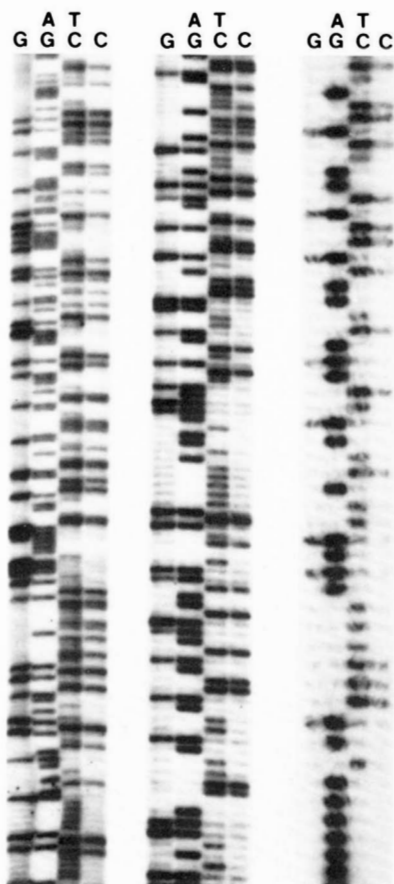
FIG. 3. **Autoradiogram of a sequencing gel.** Shown in the figure is the lower 25 cm of an 80-cm sequencing gel from which the sequence (550 nucleotides) of the dihydrofolate reductase gene of plasmid pJFM57 was determined. A 1.6-kilobase pair *Sal* I/*Pst* I fragment was used. The gel contained 5% acrylamide, 0.17% bisacrylamide, and a buffer containing 60 mM Tris, 60 mM boric acid, and 1 mM EDTA. Three sample loadings were applied, and electrophoresis was carried out at 2400 V for 13 h (first loading), followed by 2800 V for 8 h (second loading) and 3000 V for 5 h (final loading).
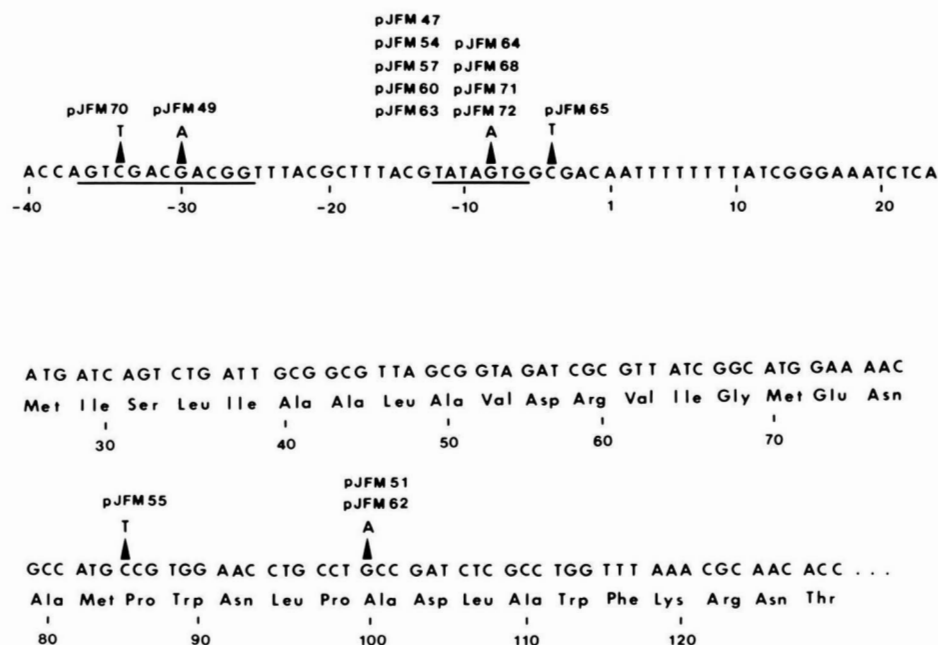
B mutant), all of these plasmids were from group A mutants. Two plasmids, pJFM49 and pJFM70, had mutations in the −35 region of the *fol* promoter (*positions* −34 and −30, Fig. 4). Both plasmids were derived from group B strains. The mutation in pJFM70 eliminates the *Sal* I restriction site located at this position, thus explaining the results of the agarose-gel electrophoresis experiments (Fig. 1). Plasmid pJFM∪5 (a group A mutant) had a mutation two base pairs downstream of the Pribnow box region (*position* −4, Fig. 4).

2) *fol* K_i mutations. The plasmids pJFM51, pJFM55, and pJFM62 had mutations in the dihydrofolate reductase structural gene. Strains with these plasmids did not overproduce the enzyme but instead produced a dihydrofolate reductase with an altered K_i for trimethoprim. The mutation at the first position of the proline 21 codon (*pJFM55, position 85*, Fig. 4) should change this amino acid to serine in the altered enzyme. The second mutation should change alanine 26 to threonine (plasmids *pJFM51 and pJFM62, position 100*, Fig. 4).

3) Mutations outside of *fol*. Plasmids pJFM46, pJFM56, and pJFM69 were classified as group A plasmids on the basis of enzyme assays. However, unlike the other group A plasmids analyzed, nucleotide sequence analysis did not uncover any alterations in either the promoter or the coding region of the *fol* genes on these plasmids.

DISCUSSION

On the basis of genetic and enzymological studies, previous workers (7, 8) postulated that there are two major mutational mechanisms by which *E. coli* cells may become resistant to trimethoprim. These mechanisms involve changes either in the synthesis of *fol* mRNA that result in overproduction of dihydrofolate reductase or in the structural gene itself whereby the resultant enzyme has a lower affinity for trimethoprim. The results presented here confirm both these mechanisms at the molecular level.

The experiments demonstrate that there are at least four possible types of plasmid mutations which lead to increased trimethoprim resistance in strains that carry a multicopy *fol* plasmid. These mutations involve alterations in the *fol* promoter sequence, the *fol* gene, plasmid copy number loci, or

FIG. 4. **Results of sequence analysis of mutant plasmids.** A portion of the noncoding strand of the wild type sequence from plasmid pJFM29 is shown, including the *fol* promoter (*underlined*) and the NH₂-terminal part of the coding sequence for dihydrofolate reductase. The nucleotide alterations that were observed in the various plasmids are indicated by *arrowheads*. Above each *arrowhead* are listed the plasmids in which that nucleotide change was observed. The numbering begins at the putative first nucleotide of *fol* mRNA; the nucleotide preceding this is designated −1. Plasmids pJFM46, pJFM56, and pJFM69 had no change in *fol* or in the 130 nucleotides upstream from the *fol* promoter. The *E. coli* promoter sequence is GTTGACAATTT-N 13-TATAATG (30).

another unknown site on the recombinant plasmid. Most of the group A and B plasmids (Table I) have mutations in the promoter region proximal to the *fol* gene. Eleven out of the 12 promoter mutations that were found occur within the conserved sequences commonly referred to as the −35 region and Pribnow box. All of these mutations resulted in greater homology of the *fol* promoter sequence with the consensus *E. coli* promoter sequence (30) (Fig. 4). These results suggest that the more nucleotides a promoter has in common with this sequence, the more efficient that region will be for initiation of mRNA synthesis by RNA polymerase. The DNA sequence data also indicate that there is an apparent mutational hot spot in the Pribnow box region (*position* −8, Fig. 4). There is no obvious explanation for the existence of this hot spot. The region did not contain any unusual sequences and there was no preponderance of HA- or NTG-derived mutations. Perhaps there was some intrinsic sequence-based instability of this part of the promoter sequence that made these nucleotides more accessible to reaction with the mutagens. One promoter mutation is located just downstream from the Pribnow box (*position* −4, Fig. 4). This region was not conserved in Rosenberg and Court's analysis of 45 *E. coli* promoter sequences (30). It may be noted, however, that this mutation breaks up a stretch of four consecutive G:C base pairs in a region of the DNA where melting must occur prior to mRNA initiation. It is conceivable that this alteration could facilitate initiation by lowering the stability of this region. Hybridization experiments using RNA isolated from representative strains carrying each of the four types of promoter mutations (Fig. 4) and a *fol*-specific DNA probe showed that *fol* mRNA was indeed overproduced in these strains.[3]

The group C plasmids (pJFM51, pJFM55, and pJFM62) all have mutations in the dihydrofolate reductase structural gene. The results indicate that strains which carry these plasmids produce approximately the same amount of enzyme as the control strain JFM210, yet they are resistant to high concentrations of trimethoprim. Kinetic analyses showed that the enzymes produced by two of these strains were somewhat less susceptible to inhibition by trimethoprim (Table II), thus presumably explaining why these mutants were more resistant to the folate analog. The mutations in these plasmids affect two amino acids (proline 21 and alanine 26) in a short highly conserved region of the enzyme. The region Pro 21-Trp 22-X 23-Leu 24-Pro 25-X 26-Asp 27 is conserved in dihydrofolate reductases from *E. coli*, *Lactobacillus casei*, and *Streptococcus faecium* (31). Examination of the 3-dimensional structure of the enzyme indicates that this sequence forms part of the lower and inner surfaces of the substrate-binding pocket, as represented in Fig. 1 of Ref. 12. Thus, it is not surprising that a change at proline 21 to serine affects the binding of the substrate analog trimethoprim to the enzyme. Although alanine 26 is not conserved in bacterial dihydrofolate reductases, it is adjacent to aspartic acid 27, which is thought to play a role in the catalytic mechanism of the enzyme (12). The change from alanine 26 to threonine could easily have an effect on trimethoprim binding because the threonine side chain is more bulky and hydrophilic than that of alanine and the change occurs directly adjacent to the active site. Exactly how these changes affect the structure of the enzyme and the mode of binding of dihydrofolate and trimethoprim is a matter for further investigation.

Finally, with respect to these mutants, it may be noted that

strains JFM207 and JFM220 have slightly higher than normal β-lactamase levels. This appears to be a real effect of these mutations on plasmid copy number because plasmid preparations from these strains consistently yielded more DNA than those of other strains. The two group D mutants, JFM217 and JFM224, had approximately 3-fold elevated levels of both dihydrofolate reductase and β-lactamase, suggesting that these strains contained three times more plasmid than the others. Several workers have reported the isolation of plasmid copy number mutants (32, 33). Throughout this work, it was assumed that the observed β-lactamase levels were an indication of plasmid copy number since β-lactamase is coded on the vector portion of the plasmid. It is clear, however, that this was not always the case. For example, the group B mutant JFM203 has the same *fol* mutation as group A mutant JFM218 (and comparable dihydrofolate reductase levels), but the β-lactamase levels are vastly different. Since the group B strains yielded similar amounts of plasmid as most of the other mutants in our standard plasmid isolation procedure, it seems likely that the low β-lactamase levels are caused by a second mutation that affects either the activity or production of that enzyme. A direct determination of plasmid copy number is necessary, therefore, to confirm that the group D mutants do indeed represent copy number mutants.

Three of the dihydrofolate reductase overproducers (strains JFM202, JFM214, and JFM227) had no detectable sequence alterations in the *fol* promoter or structural gene. As expected for a gene with a wild type nucleotide sequence, there was no detectable alteration in the kinetic parameters of the enzyme from the one strain that was analyzed (Table II). The overproduction of enzyme was apparently due neither to an increased plasmid copy number (Table I) nor to the insertion of a transposable element upstream from *fol* (Fig. 1). Moreover, an explanation based upon activation of an existing upstream promoter seems unlikely since these strains make large amounts of *fol*-specific mRNA that is indistinguishable in size from wild type mRNA.[3] Some aberrations in sequencing gel autoradiograms from these plasmids were observed in a region *110* base pairs upstream from the *fol* promoter.[4] Further work will be required to uncover the basis for these unusual results.

The initial aim of this study was to construct an *E. coli* strain that greatly overproduces dihydrofolate reductase. We have succeeded in isolating plasmid strains that produce up to 30 times more enzyme than previously reported cloned derivatives. These strains should be useful as plentiful sources of wild type *E. coli* dihydrofolate reductase. It is hoped that the availability of large amounts of purified dihydrofolate reductase will stimulate studies on the physical biochemistry and catalytic mechanism of the enzyme and will lead to important advances in our understanding of the mode of action of its inhibitors and in the design and analysis of additional folate analogs which may prove to be useful as antibiotics or anticancer drugs.

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[3] The intensities of bands on northern blots of these RNAs were approximately proportional to the dihydrofolate reductase levels given in Table I. The DNA probe was not in sufficient excess, however, to permit accurate quantitation of the relative mRNA levels (D. Smith, unpublished experiments.).

[4] These aberrations consisted of extraneous bands occurring in a 5-base pair stretch of the A + G track superimposed over a wild type nucleotide sequence. For further discussion, see Smith, D. (1982) Nucleotide sequence analysis of dihydrofolate reductase genes of wild type and trimethoprim-resistant mutants of *Escherichia coli* K12, Ph.D. dissertation, Cornell University.

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