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Cloning and Expression in *Escherichia coli* of the Gene for 10-Formyltetrahydrofolate Synthetase from *Clostridium acidiurici* ("*Clostridium acidi-urici*")

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The gene for 10-formyltetrahydrofolate synthetase (EC 6.3.4.3) from the purinolytic anaerobic bacterium *Clostridium acidiurici* ("*Clostridium acidi-urici*") was cloned into *Escherichia coli* JM83 with plasmid pUC8. A *C. acidiurici* genomic library was prepared in *E. coli* from a partial *Sau*3A digest and screened with antibody against the synthetase. Of 10 antibody-positive clones, 1 expressed a high level of synthetase activity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis demonstrated that the protein synthesized in *E. coli* had the same subunit molecular weight as the *C. acidiurici* enzyme. The gene was located on an 8.3-kilobase genomic insert and appeared to be transcribed from its own promoter. Analysis of genomic digests with a fragment of the synthetase gene indicated that one copy of the gene was present in the *C. acidiurici* chromosome.

Folic acid coenzymes function in metabolism as donors and acceptors of one-carbon compounds. The one-carbon units attached to the coenzyme tetrahydrofolate (THF) may undergo enzymatic oxidation or reduction, creating the variety of coenzymes required for nucleic acid and amino acid biosynthesis and vitamin metabolism. The enzymatic activities required for these transformations are present in both eucaryotic and procaryotic cells; however, the proteins responsible for these activities vary greatly in their organization and structure. In eucaryotes, 10-formyl-THF synthetase (EC 6.3.4.3), 5,10-methenyl-THF cyclohydrolase (EC 3.5.4.9), and 5,10-methylene-THF dehydrogenase (EC 1.5.1.5) activities are associated with a single trifunctional protein (4, 19, 23, 26), termed C_1-THF synthase (6). In procaryotes, monofunctional proteins catalyze these activities, with the known exception of a bifunctional cyclohydrolase-dehydrogenase in *Escherichia coli* (7) and *Clostridium thermoaceticum* (18). In *Clostridium acidiurici* ("*Clostridium acidi-urici*"), 10-formyl-THF synthetase is a monofunctional protein whose enzymatic and physical properties have been extensively characterized (11).

Like other eucaryotes, the yeast *Saccharomyces cerevisiae* contains the trifunctional C_1-THF synthase (20). Mutational, chemical modification, and proteolysis studies indicate that the active site for 10-formyl-THF synthetase activity is independent of the cyclohydrolase and dehydrogenase active sites (2, 12, 20). Antibody raised against the *S. cerevisiae* C_1-THF synthase has been shown to cross-react with the *C. acidiurici* 10-formyl-THF synthetase, suggesting a structural similarity between these proteins (25). The gene for the *S. cerevisiae* trifunctional protein has recently been sequenced in our laboratory (25a). The occurrence of 10-formyl-THF synthetase activity in the form of monofunctional and multifunctional proteins prompted us to examine the structural, functional, and evolutionary relationships of these enzymes. One approach to elucidating the relationship between the yeast trifunctional protein and the clostridial monofunctional protein is to compare the individual gene and protein sequences. In this regard, we report the cloning of the *C. acidiurici* gene for 10-formyl-THF synthetase and its expression in *E. coli*.

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MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and *E. coli* DNA polymerase I were purchased from Bethesda Research Laboratories. Protein assay reagent, goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase complex, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) molecular weight markers were obtained from Bio-Rad Laboratories. [γ-^{32}P]ATP was kindly provided by M. J. Chamberlin of this department. Other radiolabeled compounds were purchased from Amersham Co. Nitrocellulose BA-85 was purchased from Schleicher & Schuell. Ampicillin and DNase I were purchased from Sigma Chemical Co. DNA molecular weight markers were obtained from PL Biochemicals. All other reagents were commercial products of the highest grade available.

Bacterial strains, plasmids, and media. *C. acidiurici* 9a (ATCC 7906) was used as the source of chromosomal DNA. The organism was grown on the uric acid medium described by Rabinowitz (22) in distilled water with trace metals added as described by Gariboldi and Drake (8). The plasmid used for cloning was pUC8 (27), and *E. coli* strains JM83 and JM109 (28) were used as the cloning hosts. *E. coli* cells were grown on LB (16) or LB supplemented with ampicillin (LBA, 40 μg/ml) for selecting transformants.

Isolation and cloning of DNA. *C. acidiurici* chromosomal DNA more than 50 kilobases (kb) long was isolated as described by Graves et al. (9) and then partially digested with *Sau*3A and size-fractionated on a linear 5 to 20% sucrose gradient. The fractions containing DNA fragments with a range of 5 to 10 kb were pooled, and the DNA was ligated into *Bam*HI-digested and phosphatase-treated pUC8 by using T4 DNA ligase. The ligated plasmids were used to transform competent strain JM83 (16), and transformants were selected for ampicillin resistance. To determine whether clones were carrying pUC8 with a genomic insert, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (2% in *N*,*N*'-dimethylformimide) was added to the medium. Results

of trial transformations indicated that approximately 50% of the transformants contained pUC8 with genomic inserts (white colonies), and the average insert size was determined to be 5 kb. For recovery of plasmid DNA, cultures of *E. coli* were grown on LBA to stationary phase, and plasmid DNA was isolated by the alkaline lysis method described by Maniatis et al. (16). Large-scale preparations were usually purified by CsCl gradient centrifugation.

Preparation of antibody. 10-Formyl-THF synthetase was purified to homogeneity by heparin-agarose affinity chromatography (T. R. Whitehead, N. Lacayo, and J. C. Rabinowitz, Fed. Proc. **44**:1076, 1985), and antibody to the enzyme was prepared in New Zealand white rabbits. Purified synthetase (100 μg) was combined with Freund complete adjuvant and injected intradermally at several sites along the back. The animal was boosted intramuscularly 8 weeks later with 50 μg of the enzyme in Freund incomplete adjuvant and bled 2 weeks later.

Immunological screening of transformed bacteria. Transformants were screened with antibody for production of 10-formyl-THF synthetase as described by Helfman et al. (10) with minor modifications. Approximately 6,000 transformants were plated on each of two LBA plates (150 mm) and incubated at 37°C until the colonies were 1 to 2 mm in diameter. Since approximately 50% of the transformants contained genomic inserts, each plate contained approximately 15,000 kb of cloned *C. acidiurici* genomic DNA. The colonies were replica plated onto nitrocellulose filters and allowed to regrow on LBA plates to 1 to 2 mm in diameter. The colonies on the nitrocellulose filters were lysed by placing the filters in a chloroform atmosphere for 20 min. The filters were then placed in a solution of Tris-buffered saline (TBS; 50 mM Tris hydrochloride, pH 7.5, 150 mM NaCl) containing 3% bovine serum albumin (BSA), 5 mM $MgCl_2$, 1 μg of DNase I per ml, and 40 μg lysozyme per ml and agitated overnight at room temperature on a rotary shaker. The filters were rinsed with TBS and incubated for 1 h at room temperature with rabbit antiserum diluted in TBS-BSA. The diluted antibody was preabsorbed for 2 h at 4°C with a boiled lysate of strain JM83 transformed with pUC8. The filters were washed with TBS (three changes, 5 min each), followed by a 15-min wash with TBS containing 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS, and then three more washes with TBS. The filters were then incubated with goat anti-rabbit IgG complexed with horseradish peroxidase for 1 h at room temperature. The filters were washed as before and incubated in a solution of 4-chloro-1-napthol (0.5 mg/ml) and hydrogen peroxide (0.01%). Positive clones were identified by the purple stain. Purified synthetase (100 ng) was spotted onto each filter as a positive control. The colonies on the master plate corresponding to the positive clones on the nitrocellulose were picked, streaked on LBA agar for single colonies, and rescreened with antibody to ensure a positive reaction.

Amino acid sequencing and preparation of oligonucleotides. The amino-terminal portion of purified 10-formyl-THF synthetase (amino acids 1 through 38) was sequenced by Stanley C. Rall, Jr., Gladstone Foundation Laboratories, University of California, San Francisco, with a Beckman 890M sequencer. The phenylthiohydantoin-derivatized amino acids were identified by high-pressure liquid chromatography on a Beckman model 332 chromatograph equipped with an Ultrasphere ODS reverse-phase column. Oligonucleotides corresponding to the codons for amino acids 10 to 15 of the protein sequence were prepared with an Applied Biosystems model 380A DNA synthesizer. The 32-fold degenerate pool of oligonucleotides contained the sequences 5′-GARGCNCARATGAARCA-3′, where R is either purine and N is any nucleotide. The oligonucleotides were purified by silica gel thin-layer chromatography with a solvent system of *n*-propanol–ammonium hydroxide–water (55:35:10) (1).

DNA analysis. DNA was digested with restriction endonucleases and electrophoresed through agarose gels in 89 mM Tris–68 mM phosphoric acid–2 mM EDTA and stained with propidium iodide (1 μg/ml). For hybridization with radiolabeled probes, the DNA was transferred to nitrocellulose by the method of Southern (24). Oligonucleotides were labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol) by T4 polynucleotide kinase to a specific activity of $>10^6$ cpm/μg. For genomic blots, gel-purified DNA fragments were nick translated with *E. coli* DNA polymerase I to a specific activity of $>10^6$ cpm/μg. Hybridizations were carried out as described by Maniatis et al. (16).

Protein and enzyme analysis. *E. coli* strains carrying the vector or hybrid plasmid were grown in LBA to an A_{550} of 1.0. The cells were centrifuged at 5,000 × *g* for 10 min at 4°C and suspended in 50 mM Tris hydrochloride (pH 7.5)–10 mM KCl–10 mM 2-mercaptoethanol. The cells were broken with a French pressure cell at 10,000 lbs/in^2, followed by centrifugation at 30,000 × *g* for 30 min at 4°C. The supernatant fluid was recovered and used for enzyme assays and protein analysis. Proteins were analyzed by SDS-PAGE as described by Laemmli (13). The stacking gel was 4% and the resolving gel was 10% acrylamide. Immunoblot analysis was carried out after SDS-PAGE as described by Staben and Rabinowitz (25). 10-Formyl-THF synthetase activity was assayed as described by McGuire and Rabinowitz (17), except that ammonium formate, pH 8.0, was used in place of sodium formate. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 μmol of 10-formyl-THF in 1 min at 37°C. Protein concentration was estimated by the dye-binding assay of Bradford (3), with the commercial Bio-Rad reagent and BSA as the standard.

RESULTS

Immunological screening for *C. acidiurici* 10-formyl-THF synthetase. *E. coli* has been reported to lack 10-formyl-THF synthetase (7), and thus selecting for recombinants that complemented a mutation was not possible. Therefore, an antibody screening technique was used to identify transformed *E. coli* expressing the synthetase gene. We prepared a genomic library of *C. acidiurici* DNA in *E. coli* JM83 with plasmid pUC8. To avoid possible cross-reaction with other *E. coli* proteins, the antiserum was preabsorbed against a boiled extract of JM83(pUC8). Approximately 6,000 clones containing an average DNA insert of 5 kb were screened. Assuming that the size of the *C. acidiurici* genome is equivalent to that of *E. coli*, then at least seven genome equivalents of *C. acidiurici* DNA were screened (5). Initially, 12 transformed *E. coli* colonies reacted with the antibody. After secondary and tertiary screening with the antibody, 10 independent clones were found to be synthetase positive.

Enzymatic and protein analysis. The 10 clones that reacted positively with antibody were grown to mid-log phase in LBA, and crude extracts were prepared. 10-Formyl-THF synthetase activity was assayed in the crude extracts, and of the 10 antibody-positive clones, 4 [in addition to JM83, JM83(pUC8), and JM109(pUC8)] had no detectable activity, 5 had relatively low activity (<0.15 U/mg), and 1 expressed

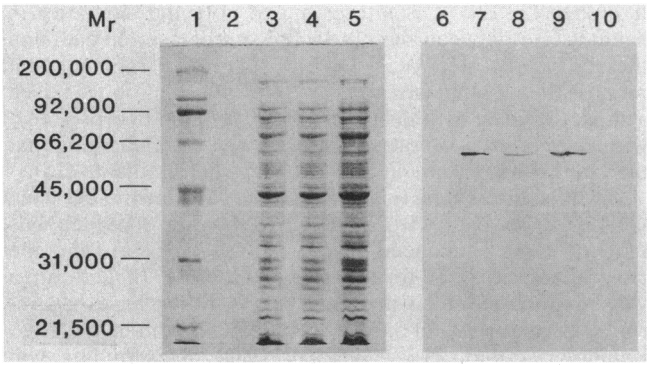

FIG. 1. SDS-PAGE and immunoblot analysis of crude extracts from JM83(pTW2) and JM83(pUC8). The Coomassie-stained gel is on the left, and a replica of the gel transferred to nitrocellulose and stained with antisynthetase antibody is on the right. Lanes 1 and 6, molecular weight markers; lanes 2 and 7, 0.1 μg of purified *C. acidiurici* synthetase; lanes 3 and 8, 25 μg of JM83(pTW2) crude extract; lanes 4 and 9, 25 μg of JM83(pTW2) crude extract plus 0.1 μg of *C. acidiurici* synthetase; lanes 5 and 10, 25 μg of JM83(pUC8) crude extract.

relatively high activity (1.62 U/mg). The plasmid in the *E. coli* clone expressing high activity was termed pTW2. The proteins in the crude extracts of JM83(pTW2) were subjected to SDS-PAGE and immunoblot analysis. The synthetase produced by JM83(pTW2) comigrated with purified *C. acidiurici* synthetase (Fig. 1, lanes 2 and 7, 3 and 8, M_r 60,000), and mixing purified synthetase with the crude extract of JM83(pTW2) produced only one immunoreactive band (lanes 4 and 9). The presence of enzyme activity and a comigrating protein in JM83(pTW2) indicated that the entire protein was being produced, and therefore the entire gene was present in pTW2. Immunoblot analysis of the clones with no detectable enzyme activity demonstrated the presence of immunoreactive bands with M_rs below 60,000 (data not shown), suggesting that the entire gene was not present in these plasmids.

To determine whether the synthetase gene was being transcribed from its own promoter or from the β-galactosidase promoter of pUC8, pTW2 was transformed into *E. coli* JM109, which requires an inducer such as lactose or isopropyl-β-D-thiogalactoside (IPTG) for expression of

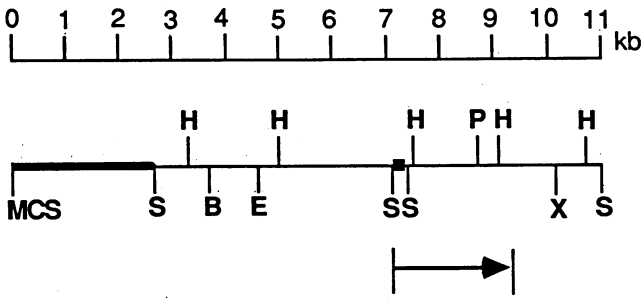

FIG. 2. Simplified restriction endonuclease map of plasmid pTW2 containing the synthetase gene. The thick line indicates the entire pUC8 DNA. Not all of the *Sau*3A sites are shown. The square indicates the site of hybridization of synthetic oligonucleotides prepared from codons for amino acids 10 to 15 of the protein. The arrow indicates the location of the synthetase gene and the direction of transcription. Abbreviations: B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sau*3A; X, *Xba*I; MCS, multiple cloning site.

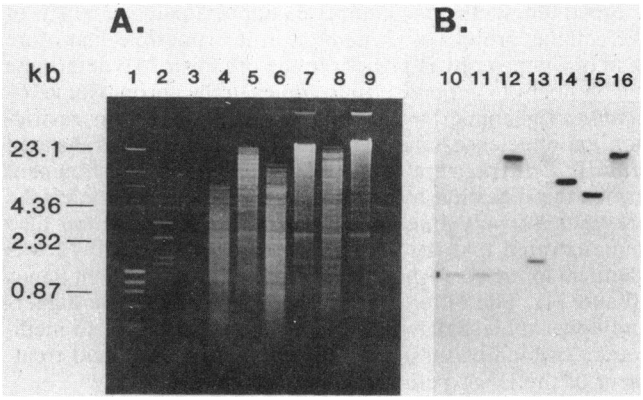

FIG. 3. Southern hybridization analysis of *C. acidiurici* genomic digests with the nick-translated 1.2-kb *Pst*I-*Hin*dIII fragment of pTW2. (A) Photograph of 0.8% agarose gel stained with propidium iodide. (B) Autoradiogram of DNA transferred to nitrocellulose and hybridized with the ^{32}P-labeled *Pst*I-*Hin*dIII fragment. Lane 1, molecular weight markers; lane 2, 0.4 μg of *Pst*I-*Hin*dIII-digested pTW2; lanes 3 and 10, 0.014 μg of *Pst*I-*Hin*dIII-digested pTW2. Lanes 4 through 9 and 11 through 16 contain 5 μg of *C. acidiurici* genomic DNA: lanes 4 and 11, *Pst*I-*Hin*dIII digest; lanes 5 and 12, *Eco*RI digest; lanes 6 and 13, *Hin*dIII digest; lanes 7 and 14, *Pst*I digest; lanes 8 and 15, *Hpa*II digest; lanes 9 and 16, *Bam*HI digest.

the β-galactosidase promoter (28). JM109(pTW2) was grown in LBA with and without IPTG (0.33 mM), and enzyme activity was determined in crude extracts. There was no significant difference in enzyme activity between the two cultures (4.43 U/mg with IPTG versus 4.11 U/mg without IPTG), indicating that the synthetase gene was being transcribed from its own promoter. Interestingly, the enzyme activity in JM109(pTW2) was about 2.5-fold higher than in JM83(pTW2). This may be due to differences in the copy number of pTW2 in the two strains. The synthetase constitutes about 2 to 4% of the cellular protein of *C. acidiurici* (21), and the activity in crude extracts is about 13 U/mg. Therefore, the activity in JM83(pTW2) was about eightfold lower than in *C. acidiurici*, and the activity in JM109(pTW2) was about threefold lower.

Restriction mapping and DNA hybridization. Plasmid pTW2 was characterized by restriction endonuclease digestion (Fig. 2). The *Sau*3A genomic insert was about 8.3 kb in size. A degenerate synthetic oligonucleotide pool was constructed corresponding to the codons for amino acids 10 to 15 of the amino acid sequence. The oligonucleotides were radiolabeled and used to probe the digested pTW2 plasmid DNA. The probe hybridized to the 2.5-kb *Hin*dIII fragment, as indicated by the square in Fig. 2 (data not shown). The subunit molecular weight of the synthetase is 60,000 (15); therefore, the size of the region coding for the enzyme should be approximately 2 kb. To determine the orientation of the gene with respect to the oligonucleotide hybridization site, pTW2 was digested with *Pst*I. This enzyme cuts once in the insert (site P, Fig. 2) and once in the multiple cloning site of pUC8 (MCS, Fig. 2). The resulting 8.7- and 2.3-kb fragments were separated by agarose gel electrophoresis, and the 8.7-kb fragment was recovered, ligated, and transformed into JM83. The resulting transformant had no detectable synthetase activity, indicating that the gene had been interrupted by removal of the 2.5-kb fragment. Therefore, the gene appears to encompass the region from approximately 7.2 to 9.2 kb on the map in Fig. 3. Initial DNA sequencing analysis confirmed this result.

Since the synthetase comprises approximately 2 to 4% of the cellular protein of *C. acidiurici*, it is possible that more than one gene copy is present in the organism. To determine the number of synthetase gene copies in the chromosome, *C. acidiurici* genomic DNA was digested with various restriction endonucleases and hybridized with the nick-translated *Hin*dIII-*Pst*I fragment of pTW2 (Fig. 3). Only one fragment from each digestion hybridized to the probe, indicating the presence of only one gene copy. The hybridization also demonstrated that the *Hin*dIII-*Pst*I fragment in pTW2 was identical to the corresponding genomic DNA fragment (lanes 10 and 11). The difference in intensity between the higher- and lower-molecular-weight bands is probably due to inefficiency of binding of small DNA fragments after acid treatment of the DNA before the Southern transfer.

DISCUSSION

The gene for 10-formyl-THF synthetase from *C. acidiurici* was cloned into *E. coli* with the vector pUC8, and positive clones were identified by antibody screening. The gene is present on an 8.3-kb genomic insert and appears to be transcribed from its own promoter. In *C. acidiurici*, the synthetase is a tetramer of four identical subunits (15). Enzymatic activity was present in the crude extract of JM83(pTW2), indicating that the active tetramer was being assembled. Immunoblot analysis of crude extract proteins separated by SDS-PAGE indicated that the subunit protein synthesized in *E. coli* was identical in molecular weight to the purified synthetase from *C. acidiurici*.

10-Formyl-THF synthetase constitutes approximately 2 to 4% of the cellular protein of *C. acidiurici* (21). Such a large amount of a single protein suggests that more than one gene copy for the synthetase may be present. To test this possibility, digests of *C. acidiurici* genomic DNA were probed with a radiolabeled fragment of the cloned synthetase gene. Single-band hybridization patterns over a range of 1.2 to 23 kb indicated that only one copy of the gene exists in the chromosome. Since pUC8 is a multicopy plasmid in JM83, one could expect a higher activity of the synthetase in the transformed *E. coli* than in *C. acidiurici*. The lower amount of activity observed may result from transcriptional or translational barriers, as well as protein or mRNA instability in JM83 and JM109.

10-Formyl-THF synthetase has been purified to homogeneity from only three clostridial species (14, 21) and *Peptococcus asaccharolyticus* (ATCC 14963), formerly "*Peptococcus aerogenes*" (17). The enzyme in each case has been found to be a tetramer of four identical 60,000-molecular-weight subunits. We have also found a 60,000-dalton protein in partially purified preparations of synthetase from *Bacteroides fragilis* and *Clostridium bifermentans* which cross-reacts with antibody raised against purified *C. acidiurici* synthetase (Whitehead et al., Fed. Proc. **44**:1076, 1985). In eucaryotic cells, however, the synthetase activity is associated with 5,10-methenyl-THF cyclohydrolase and 5,10-methylene-THF dehydrogenase on one trifunctional protein, termed C_1-THF synthase (19, 20, 23). The trifunctional protein is a homodimer of 100,000-dalton subunits. Trypsin digestion of purified C_1-THF synthase from *S. cerevisiae* results in conversion of the 100,000-dalton subunit to a 76,000-dalton species which retains synthetase activity but lacks cyclohydrolase and dehydrogenase activity (20). Mutational and chemical modification studies have also indicated that the active site for the synthetase is independent of the cyclohydrolase and dehydrogenase active sites (2, 12). It is interesting to note the similarity in subunit size between the clostridial synthetase (60,000) and the trypsin-digested yeast C_1-THF synthase fragment that retains the synthetase activity (76,000). Antibody raised against the yeast synthase has also been found to cross-react with *C. acidiurici* 10-formyl-THF synthetase, indicating that the two proteins have similar antigenic determinants (25).

It will be interesting to determine the relationship between the *S. cerevisiae* gene for C_1-THF synthase, which has recently been sequenced in our laboratory (25a), and the clostridial gene for 10-formyl-THF synthetase. In addition, a yeast mitochondrial isozyme of C_1-THF synthase has recently been purified in our laboratory (K. Shannon and J. C. Rabinowitz, Fed. Proc. **44**:1220, 1985). This finding will provide a unique opportunity to study the evolutionary relationships, structure, and organization of procaryotic, eucaryotic, and mitochondrial forms of 10-formyl-THF synthetase.

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