Cloning, Purification, and Properties of *Candida albicans* Thymidylate Synthase

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The thymidylate synthase (TS) gene was isolated from a genomic *Candida albicans* library by functional complementation of a *Saccharomyces cerevisiae* strain deficient in TS. The gene was localized on a 4-kilobase HindIII DNA fragment and was shown to be expressed in a Thy− strain of *Escherichia coli*. The nucleotide sequence of the TS gene predicted a protein of 315 amino acids with a molecular weight of 36,027. The gene was cloned into a T7 expression vector in *E. coli*, allowing purification of large amounts of *C. albicans* TS. It was also purified from a wild-type *C. albicans* strain. Comparison of several enzyme properties including analysis of amino-terminal amino acid sequences showed the native and cloned *C. albicans* TS to be the same.

Thymidylate synthase (TS) has been characterized from a wide variety of organisms and is a proven target in cancer chemotherapy (25). TS should be a good chemotherapeutic target in *Candida albicans*, a common fungal pathogen, since the product of the enzyme, dTMP, can only be synthesized de novo in yeasts; they lack thymidine kinase and are impermeable to thymine, thymidine, and dTMP (6). Effective inhibition of TS in yeasts would result in death, since these organisms are unable to produce their own dTMP or obtain it from the environment. 5-Fluorocytosine inhibits *C. albicans* and several other fungi both in vitro and in vivo (3). Furthermore, treatment of *C. albicans* with 5-fluorocytosine (9) results in 5-fluoro-dUMP accumulation and inhibition of TS, thus implicating this enzyme as a chemotherapeutic target in fungi.

Information obtained from the characterization of target enzymes in vitro has contributed to the design of new potential chemotherapeutic agents. Such studies are facilitated by the availability of large quantities of pure enzyme. Since low levels of TS are present in cultures of *C. albicans*, the cloning and overexpression of *C. albicans* TS in *Escherichia coli* was undertaken. We report the isolation of the *C. albicans* TS gene by functional complementation of a *Saccharomyces cerevisiae* strain deficient in TS. The gene was sequenced with about 400 base pairs (bp) of DNA 5′ to the gene and a short 3′-flanking region and expressed in *E. coli* by using a T7 expression vector. The purified enzyme from both *C. albicans* and *E. coli* was prepared, and its properties were examined to ensure that the cloned TS enzyme was expressed in *E. coli* was identical to the native TS enzyme of *C. albicans*.

**MATERIALS AND METHODS**

**Strains, plasmids, and media.** Table 1 contains a list of the strains used in this study. *S. cerevisiae* GY712, provided by Garret R. Taylor, Mt. Sinai Research Institute, Toronto, Ontario, Canada, was grown at 34°C in YPD broth (17) supplemented with 1.5 mg of KH₂PO₄ per ml and 100 μg of dTMP per ml. This strain is Tup− and thus permeable to exogenous dTMP (35). A genomic library prepared from *C. albicans* B792 (ATCC 36803) was obtained from Yigal Koltin, Tel-Aviv University, Tel Aviv, Israel. The library was prepared by ligating a SaU3A partial digest of genomic DNA, isolated as a pool of 8- to 10-kilobase-pair (kb) fragments, into the BamHI site of the low-copy-number *S. cerevisiae*- *E. coli* shuttle vector pYSK35 (Fig. 1). The *S. cerevisiae*- *E. coli* shuttle vector YEP13 (8) was used to detect the subcloned TS in *S. cerevisiae*. The *E. coli* vector pBS+ (Stratagene Inc., La Jolla, Calif.) was used for manipulations of DNA in *E. coli* and for production of single-stranded DNA templates. *E. coli* SF34 was prepared for this study by plating DH5α on minimal plates (1.5% agar containing Vogel-Bonner salts [32] and 0.2% glucose) with 15 μg of trimethoprim per ml and 70 μg of thymidine per ml. We assume SF34 to have a point mutation in the thyA gene; it had no detectable TS activity and required thymidine for growth. *E. coli* SS507 was prepared from BL21(DE3) by insertion of a transposon into the *E. coli* thyA gene. *E. coli* were routinely grown at 37°C in YT broth (21). Thymidylate auxotrophs were supplemented with 100 μg of thymidine per ml. High-level expression of the *C. albicans* TS gene was accomplished by using an expression plasmid derived from pET-3C (23). The expression system is more fully discussed in the Results and Discussion section.

**Plasmid manipulations.** Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and used as recommended by the supplier. DNA fragments were analyzed on agarose gels (0.7 to 1.0%) (19). Yeast transformations were performed as outlined by Sherman et al. (26). *E. coli* transformations were performed as recommended by Maniatis et al. (19).

**DNA preparations.** Plasmid DNA from *E. coli* was prepared by using a rapid alkaline lysis procedure (5) or by Triton X-100 lysis (15) followed by cesium chloride-ethidium bromide centrifugation. Yeast plasmid DNA was prepared from spheroplasts prepared as if for transformation, but instead lysed by addition of 2 volumes of 0.2 N NaOH containing 1% sodium dodecyl sulfate (SDS). After gentle mixing, 0.5 volume of chilled 2.7 M potassium acetate (pH 4.8) was added and gently mixed. Plasmid DNA was precipitated with 2 volumes of cold ethanol, phenol extracted, and reprecipitated from ethanol. Genomic DNAs were prepared by the method of Weeks et al. (34).

**DNA sequencing.** The DNA sequence was determined by the dideoxy-chain termination method (24) with the sequencing system from Bethesda Research Laboratories. Ordered deletion subclones were made by unidirectional digestion by
the method of Henikoff (13). Exonuclease III and S1 nuclease were obtained from Bethesda Research Laboratories.

Site-directed mutagenesis. Changes in DNA sequences were accomplished using oligonucleotide-directed mutagenesis (38). Oligonucleotides were prepared on a Biosearch 8600 DNA synthesizer.

TS assay. TS activity was determined spectrophotometrically (33) or by the tritium release assay (10). The spectrophotometric assay used the same reagents and concentrations as the tritium release assay, with 1 mM dUMP substituted for the tritiated dUMP. [6R,S]-H$_2$pteroylglutamate was obtained from Sigma Chemical Co., St. Louis, Mo., and [6S]-H$_2$pteroylglutamate was prepared as previously outlined (11). One unit of activity is equal to 1 nmol/min. For kinetic determinations, the standard error for individual determinations was less than 20%.

Purification of TS from C. albicans ATCC 11651. Cells were grown in 2% yeast extract, 2% proteose peptone number 3-5% glucose to an optimal density of ca. 30 at 550 nm. Cells were harvested by centrifugation. Nine 1-liter cultures grown at 37°C with vigorous shaking in 2-liter Erlenmeyer flasks yielded 144 g of packed cells. The cells were lysed, and TS was partially purified by ammonium sulfate fractionation (40 to 90%) and gel filtration on Ultogel AcA-54 (LKB Instruments, Inc., Rockville, Md.) as outlined for the yeast dihydrofolate reductase (1). Fractions containing TS were pooled from the AcA-54 column and further purified by one passage through an affinity column containing 10-formyl-5,8-dideazafolate-ethyl-Sepharose followed by concentration on DE-52-cellulose (22). Although the purpose of the chromatography on DE-52 is primarily to collect and concentrate the enzyme from the affinity column, a purer

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**TABLE 1. Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or relevant characteristic</th>
<th>Source or reference*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. cerevisiae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GY712</td>
<td>MATα leu2-3,112 tsp1-6</td>
<td>G. Taylor (31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>ATCC</td>
<td></td>
</tr>
<tr>
<td>ATCC 11651</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli K-12</td>
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<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F$^-$ endA1 $\text{hsdR17 (}$\text{rK}^{-}$\text{mK}$^{-}$\text{)}$</td>
<td>BRL</td>
</tr>
<tr>
<td>supE44 thi-1 $\lambda$ recA1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gyrA96 relA1 $\phi 80$dacZM15</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>supE thi $\Delta$ lac-proAB (F$^+$) traD36 proAB lac$^+$ ZM15</td>
<td>BRL</td>
</tr>
<tr>
<td>SF34</td>
<td>as DH5α, Thy$^+$</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli B</td>
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<td></td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>F$^-$ $\text{hsdS gal DE3}$</td>
<td>W. Studier (29)</td>
</tr>
<tr>
<td>SS5507</td>
<td>as BL21(DE3), thyA105::</td>
<td>S. A. Short (personal communication)</td>
</tr>
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</table>

* ATCC, American Type Culture Collection; BRL, Bethesda Research Laboratories, Inc.
preparation was obtained if the elution from DE-52 was modified from the conditions recommended by Rode et al. (22). After the affinity column had been disconnected, the DE-52 column was washed with 20 mM potassium phosphate (pH 7.5) containing 10 mM 2-mercaptoethanol (2-ME) and 20% glycerol, and TS was eluted from the column with a 20 to 60 mM potassium phosphate gradient. Eluted protein was further concentrated by ultrafiltration with YM10 membranes (Amicon Corp., Lexington, Mass.). A summary of the purification procedure is shown in Table 2.

**Purification of C. albicans TS from E. coli.** Cells were grown in 12 liters of Luria broth (21) containing Vogel-Bonner salts (32), 10 µg of tetracycline per ml, 10 µg of kanamycin per ml, and 0.04% Mazu 60 P (an antifoaming agent; Mazer Chemicals, Inc., Gurnee, Ill.) in a 16-liter fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N.J.) at 30°C. When the optical density of the culture was 600 nm was 1.25, the lac promoter was induced with 1.0% lactose for 4 h. The lac promoter transcribed the T7 RNA polymerase, which then transcribed the cloned C. albicans TS gene. The cells were centrifuged and washed with 50 mM potassium phosphate (pH 7.4) containing 1 mM MgCl2. Cell pellets (100 g [wet weight]) were suspended in 200 ml of 20 mM Tris hydrochloride (pH 7.4) containing 20 µg of lysozyme per ml, 1 mM EDTA, 10 mM 2-ME, and 100 µM phenylmethylsulfonyl fluoride and frozen at −70°C. The cells were thawed at 37°C, DNase and RNase (1 mg/ml in 100 mM MgSO4) were added to a final concentration of 1 µg/ml, and the suspension was centrifuged at 9,000 × g for 60 min. The supernatant was used; the pellet was suspended in the initial volume of lysis buffer, and the process was repeated. Both supernatants were combined and dialyzed overnight against 10 mM Tris hydrochloride (pH 7.4) containing 10 mM 2-ME and 100 µM phenylmethylsulfonyl fluoride. The dialyzed crude extract was loaded onto a Rapid Flow column (100 ml; Sepragen Corp., San Leandro, Calif.) containing Q-Sepharose (Pharmacia, Inc., Piscataway, N.J.). The column was washed with the dialysis buffer, and the protein was eluted with a 0 to 200 mM NaCl gradient in buffer. Tubes containing TS, as determined by SDS-polyacrylamide gel electrophoresis, were pooled and precipitated with 70% (NH4)2SO4. After centrifugation, the pellet was suspended in a minimal volume of water and dialyzed overnight against 50 mM potassium phosphate (pH 7.4) containing 10 mM 2-ME and 5% glycerol. The dialyzed sample was loaded onto a column (7.5 by 55 cm) containing Sephacryl S-300 (Pharmacia) and eluted with the same buffer minus glycerol. The peak tubes of enzyme activity were pooled, precipitated with 70% (NH4)2SO4, and dialyzed against 10 mM potassium phosphate (pH 7.4) containing 10 mM 2-ME and 5% glycerol. The purified protein was stored at −20°C after concentration with YM10 membranes. The purification of the C. albicans TS from E. coli is summarized in Table 2.

**DNA hybridization.** Electrophoretically separated restriction digestes were transferred to nitrocellulose as described by Fling et al. (12). The filters were treated with 5× Denhardt solution–5% SSPE (19)–0.3% (wt/vol) SDS–25% deionized formamide for 1 h at 37°C and then hybridized under the same conditions with labeled probe overnight at 37°C. Hybridized filters were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature for 15 min, with a minimum of three changes, and then at higher temperatures (37 and 42°C) and exposed overnight to X-ray film with intensifying screens. The HindIII-EcoRI fragment of pSS92-9 was nick translated by using a nick-translation kit (Bethesda Research Laboratories) and [α-32P]dATP (>600 Ci/mmol; Du Pont, NEN Research Products, Boston, Mass.) and used as the probe.

**Other procedures.** Amino acid analysis was done by Dave Klapper, University of North Carolina, Chapel Hill. Samples were desalted by high-pressure liquid chromatography on a model 214 TPS C8 steel column (VYDAC, Hesperia, Calif.) with 0.1% trifluoroacetic acid and acetonitrile. The amino acid sequence analysis was performed on a model 470A gas phase sequencer (Applied Biosystems, Foster City, Calif.) with in-line high-pressure liquid chromatography analysis. Amino acid analysis was performed by high-pressure liquid chromatography analysis of phenylisothiocyanate derivatives (14).

Protein concentrations were determined by the Coomassie blue method (27) or by the method of Lowry et al. (18) with bovine serum albumin as the standard.

SDS-polyacrylamide gel electrophoresis was done by the method of Laemmli (16) with 12% polyacrylamide gels. The purity of TS was estimated by using a model 2190 laser densitometer (LKB) and the Gelscan Program version 2190 (LKB) after staining and destaining the gels.

**RESULTS AND DISCUSSION**

**Isolation of the C. albicans TS gene.** S. cerevisiae GY712, which requires dTMP for growth, was transformed with a pYSK35 C. albicans genomic library, selecting directly for the ability to grow in the absence of dTMP. One transformant, containing plasmid pSS9-3, grew in the absence of exogenous dTMP, indicating the presence of a functional TS gene product.

pSS9-3 contains about 14 kb of C. albicans genomic DNA. A partial restriction map was determined (Fig. 1), and the three HindIII fragments were individually subcloned by ligation of a HindIII digest of pSS9-3 to HindIII-digested pBS* and HindIII-digested YEp13. To ascertain whether any of these plasmids contained an intact TS gene, the plasmids derived from YEp13 were transformed into GY712 and selected for the ability of this transformant to grow in the absence of dTMP. One HindIII fragment, when present in YEp13, was sufficient to relieve the dTMP auxotrophy of GY712. This 4-kb HindIII fragment was also cloned in both orientations in pBS* (Fig. 1, pSS65-2 and pSS92-9). An E. coli strain lacking a functional TS gene, SF34, was transformed with ampicillin resistance with pSS65-2 and pSS92-9. SF34(pSS65-2) and SF34(pSS92-9) were tested for their ability to grow without

### Table 2. Purification of C. albicans TS

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (ml)</th>
<th>Total protein (mg)</th>
<th>Total U (nmol/min)</th>
<th>Sp act (U/mg)</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td><strong>C. albicans</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Crude</td>
<td></td>
<td>ND*</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>Ammonium sulfate</td>
<td>124</td>
<td>5,654</td>
<td>ND</td>
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<td>ND</td>
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<td>AcA-54</td>
<td>425</td>
<td>3,782</td>
<td>1,122</td>
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<tr>
<td>Affinity column</td>
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<td>0.10</td>
<td>333</td>
<td>3,325</td>
<td>29.7b</td>
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<tr>
<td>TS cloned in E. coli</td>
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<td></td>
</tr>
<tr>
<td>Crude</td>
<td>310</td>
<td>5,400</td>
<td>1.2 × 10^6</td>
<td>220</td>
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<tr>
<td>Q-Sepharose</td>
<td>14</td>
<td>276</td>
<td>0.9 × 10^6</td>
<td>3,291</td>
<td>75.0</td>
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<tr>
<td>S-300</td>
<td>73</td>
<td>183</td>
<td>0.68 × 10^6</td>
<td>3,724</td>
<td>56.7%</td>
</tr>
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</table>

* ND, Value not determined.
+ Recovery from AcA-54.
# Recovery from crude lysate.

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exogenous thymidine. Only SF34(pSS92-9) was able to grow, indicating that the *C. albicans* TS is biologically functional in *E. coli* and that the gene as present on the 4-kb HindIII fragment does not contain all the genetic signals needed for expression in *E. coli*. In SF34(pSS92-9), the *C. albicans* TS gene was apparently transcribed by using some or all of the signals from the *lac* promoter present on the vector.

Further subclones were derived from pSS65-2 by deletion of the EcoRI, KpnI, and PstI fragments formed between sites in the vector multiple cloning region and sites within the *C. albicans* DNA. pSS65-2 was digested with each of the restriction enzymes, religated upon itself, and transformed into DH5α, selecting for ampicillin resistance. The *C. albicans* DNA remaining was removed from these truncated plasmids, blunt ended, and ligated to BamHI-digested YEp13 which had also been blunt ended. None of these constructs complemented the *dTMP* auxotrophy of GY712. Thus, all or part of the TS gene had been deleted, locating the gene near the end of the 4-kb HindIII fragment of pSS65-2 containing the PstI restriction site. Furthermore, the DNA sequence determined from the PstI deletion clone was found to be similar to the sequence near the 5' terminus of the *S. cerevisiae* TS gene (31).

**Sequencing and features of the *C. albicans* TS gene.** Using ordered deletions prepared from pSS65-2 and pSS92-9, we determined the DNA sequence for 1,413 bp of the 4-kb fragment of *C. albicans* DNA (Fig. 2). The initiation codon for *C. albicans* TS was found at position 413 and was followed by an opening reading frame of 498 bp encoding 315 amino acids with a predicted molecular weight of 36,027. The predicted molecular weight agreed with the observed molecular weight of the native protein (34,500) determined by SDS-polyacrylamide gel electrophoresis. The G+C content of the sequenced region, 33.2%, was in good agreement with the 35.1% G+C content that has been calculated for *C. albicans* (28). Codon usage showed a very strong preference for A or T in position 3. The position 3 preference for A or T was stronger in the *C. albicans* TS gene (77.7%) than in the *S. cerevisiae* TS gene (67.2%) (31) or in 64 other *S. cerevisiae* genes (61.3%) (20).

About 400 bp of DNA 5' to the TS gene was sequenced (Fig. 2). This DNA might be expected to contain transcriptional and translational signals for the expression of the gene. Control of the expression of genes in *Candida* species has not been as extensively studied as in *S. cerevisiae* and higher eucaryotes; however, it is expected that expression signals in *Candida* species will resemble those of *S. cerevisiae* and higher eucaryotes. CAAT (4) and TATA (7) boxes have been implicated in the initiation of transcription in eucaryotes. Several TATA-like sequences were present in the sequence 5' of the TS gene at positions 118, 179, and 364 (Fig. 2). A possible CAAT box was also present as TCAAT at positions 53 to 57 (Fig. 2). This sequence was present 67 bp upstream of the TATA box at 118. Interestingly, the same sequence is a part of the larger sequence CTCAATTTGA at positions 52 to 60 (Fig. 2), which is also found 5' of the *C. albicans* dihydrofolate reductase gene and is similarly spaced from an upstream TATA box (C. Richards, unpublished results). Many yeast mRNAs from *S. cerevisiae* have sequences that are complementary to the 3' terminus of *S. cerevisiae* 18S ribosomes and that may enhance the efficiency by which ribosomes bind to the mRNA (36). Two potential sites for such ribosome binding are present. ATG at positions 233 to 237 and AATGA at positions 352 to 356. A region of dyad symmetry containing the 4-bp palin-

FIG. 2. DNA sequence of *C. albicans* TS gene and flanking regions. The numbering is from the 5' end. The deduced amino acid sequence is shown. Upstream of the gene, possible TATA sequences appear boxed, a potential CAAT sequence is underlined, the region of dyad symmetry is indicated by arrows above the sequences, and possible sites for ribosome binding are marked by an asterisk (*) beneath the sequences. Downstream of the gene, possible transcription termination sequences are underlined.
The EcoRI-AvaI fragment of pBR322 (\textbullet\textcircled{1}) was used to generate the probe. Partial Sau3A digestion of pBR322 (data not shown) resulted in numerous fragments, at least two Sau3A restriction sites were ligated into the pYSK35 to form pSS9-3 and thus the 14 kb of C. albicans DNA in pSS9-3 is not contiguous in the *Candida* genome. These discrepancies between the restriction map of pSS9-3 and *Candida* genomic DNA were found for HindIII and EcoRI restriction digests but not for an *EcoRI-Clal* double restriction digest. The probe does hybridize as expected to the 1.8-kb *Clal-EcoRI* fragment of genomic *Candida* DNA.

### Expression of Candida TS in E. coli

The T7 expression system described by Studier and Moffat (29) was used to overproduce the *C. albicans* TS gene product (*E. coli*). Briefly, this system exploits the specificity of T7 RNA polymerase for its own unique promoters. The gene to be expressed is cloned immediately 3' to a T7 RNA polymerase promoter. In the absence of T7 RNA polymerase, this gene will not be transcribed. However, in the presence of T7 RNA polymerase, the gene will be very actively transcribed. *E. coli* SS507, derived from BL21(DE3) (29), is a λ lysogen carrying the RNA polymerase gene from T7 under the control of the inducible *E. coli* lac promoter such that in the absence of inducer, little or no T7 RNA polymerase is made by the cell. SS507 would yield preparations of *Candida* TS that are not contaminated with *E. coli* TS since the bacterial TS gene is interrupted by a transposon.

The T7 expression plasmid, pTX1927, is a modified version of pET-3C (23). Briefly, the *BglII-EcoRV* region of pET-3C containing the T7 promoter that precedes gene 10 in T7 DNA and a T7 transcription terminator was modified to contain new cloning sites 3' to the T7 RNA polymerase promoter such that genes to be expressed could be inserted.
as EcoRI-SsrI fragments (Fig. 3). The T7 promoter and T7 transcription terminator of pET-3C are unchanged. Other salient points of the plasmid are its high copy number and its ability to be maintained with tetracycline selection.

Placement of restriction sites before and after the gene was necessary to transfer the C. albicans TS gene into the expression plasmid. Oligonucleotide-directed mutagenesis was used to specifically insert an EcoRI (GAATTC) site after nucleotide position 414 and an SstI (GAGCTC) site after position 1377 (Fig. 1 and 2). Also, the adenine residue found at position 1149 was changed to a guanine, eliminating an EcoRI site in the gene while retaining the Glu codon. With these changes made, the TS gene was removed from pSS177-57 (Fig. 1) as an EcoRI-SsrI fragment, ligated into the expression plasmid to yield pTX1927 (Fig. 3), and transformed into E. coli SSS507. When the T7 RNA polymerase is supplied by a chromosomal lysogen under lac control, small amounts of T7 RNA polymerase will be made even under repressed conditions (29). This is apparently the case for SSS507, since uninduced cultures of SSS507(pTX1927) produce sufficient TS to eliminate the need for exogenous thymidine. Under optimally induced conditions, this strain contains approximately 20% of its soluble cellular protein as TS. This is less than that reported for this system (29) and may be due to the use in the C. albicans gene of codons that corresponded to rare tRNAs in E. coli.

Purification and properties of TS. TS was purified from wild-type C. albicans ATCC 11651 by gel filtration and affinity chromatography (Table 2) to a specific activity of 3,325 U/mg of protein. C. albicans TS expressed in E. coli SSS507(pTX1927) was purified to a specific activity of 3,724 U/mg, of protein by ion-exchange chromatography and gel filtration (Table 2). Both preparations were >95% pure as judged by SDS-polyacrylamide gel electrophoresis. The purified cloned enzyme which was concentrated by ultrafiltration lost activity during this procedure. However, once concentrated, the enzyme could be stored without further loss of activity (at least 6 months) at −80°C in 20 mM potassium phosphate buffer (pH 7.5) containing 20 mM 2-ME and 20% glycerol.

Amino-terminal amino acid analysis of the enzyme purified from C. albicans revealed the amino-terminal sequence of Thr-Val-Ser-Pro-Asn-Thr-Ala-Glu-Glu-Ala-Tyr-Leu-Asp-Leu-XXX-Lys-Arg-Ile-Ile-Asp-Glu-Gly . . . This exactly matched the first seven amino-terminal residues of the cloned enzyme expressed in E. coli. Both sources yielded TS protein that had had its amino-terminal methionine removed.

Kinetic characteristics of C. albicans TS prepared from C. albicans and E. coli varied less than twofold and were well within the ranges found for TS from other sources (25). The apparent kinetic constants for dUMP (using 200 μM [6R,S]-5,10-CH2-H4pteroylglutamate as the cosubstrate) was 2.5 μM for the enzyme produced in C. albicans and 3.1 μM for the enzyme produced in E. coli. The apparent kinetic constants for [6R]-5,10-CH2-H4pteroylglutamate was 38.4 μM for the enzyme prepared from C. albicans and 46.2 μM for the enzyme prepared from E. coli. In TS from many other sources (25), naturally occurring 5,10-CH2-H4pteroylglutamates show significantly lower Km values than the monoglutamate does. With the C. albicans TS, as the folate substrate was changed from [6R]-5,10-CH2-H4pteroylglutamate to [6S]-5,10-CH2-H4pteroylglutamate, the Km decreased several-fold to 6.5 μM, with an increase in Vmax of about 2.5- to 3-fold. TS from both sources exhibited competitive inhibition kinetics for the product, dTMP, versus dUMP. With 200 μM [6R,S]-5,10-CH2-H4pteroylglutamate as the cosubstrate, the apparent Km of dTMP for C. albicans TS was 15.9 μM, compared with 27.8 μM for the same enzyme expressed in E. coli. With 27 μM [6R]-5,10-CH2-H4pteroylglutamate as the cosubstrate, the apparent Km of dTMP for the cloned enzyme was 23.1 μM. This product inhibition is consistent with the same ordered reaction mechanism as found for TS from other sources (25).

The C. albicans TS gene has been isolated, its sequence has been deduced, and the gene has been overexpressed in E. coli. Properties of highly purified enzyme preparations were compared, showing the native and cloned enzymes to be the same. Although the overall amino acid sequence is similar to that of other mammalian TS, differences exist which encourage further studies to search for selective inhibitors as chemotherapeutic agents. Sufficient quantities of pure enzyme for further evaluation of C. albicans TS as a chemotherapeutic target can now be produced.

ACKNOWLEDGMENTS

Special thanks to Mary Fling for encouragement and helpful counsel during this project. We thank Yigal Koltin and Gareth Taylor for providing the Candida genomic library and the Saccharomyces host, respectively. We are also grateful to William Studier for providing the T7 expression system, to S. A. Short and W. Dallas for modifying the E. coli host and the expression plasmid used for overproducing the protein, to I. Dev for preparing the affinity column used for enzyme purification, and to P. Parker, M. Hanlon, and R. Tansik for technical assistance.

ADDENDUM IN PROOF

The nucleotide sequence data reported here will appear in the GenBank nucleotide sequence data base under the accession number J04230.

LITERATURE CITED