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Functional Analysis of Mutations in a Eukaryote-Conserved Surface Loop in Thymidylate Synthase of *Saccharomyces cerevisiae*

Edith M. Munro

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ABSTRACT

Functional Analysis of Mutations in a Eukaryote-Conserved Surface Loop in Thymidylate Synthase of *Saccharomyces cerevisiae*

Edith M. Munro

Thymidylate synthase (TS) provides the sole *de novo* source of the DNA precursor thymidylate (dTMP) in almost all organisms, and is one of the most conserved enzymes known. One salient feature of the eukaryotic version of the enzyme is the occurrence of two peptide inserts, absent in the prokaryotes, in surface loops peripheral to the core structure. The biological function of these inserts is unknown, but they have been shown in yeast TS to contribute to the structural integrity of the enzyme (Munro *et al.*, 1999). More precisely, the removal of one of the inserts (EUK1) leads to reduced affinity for both substrates as well as decreased enzyme activity, while removal of the other (EUK2) completely abolishes enzyme activity.

In this study we further analyzed effects of deletion and point mutations in the loop (Loop 2) that harbours EUK2. Gel filtration chromatography indicated that inactive deletion mutants that mimic prokaryotic versions of this loop fail to form stable dimers. Activity could not be restored when a phenylalanine residue, presumed to be buried by the conformation of the normal eukaryotic loop, was substituted by a polar residue which even more closely mimicked the prokaryotic TS. Point mutations in EUK2 that substituted one or the other of two conserved tyrosines with phenylalanine partially affected enzyme activity; complementation of TS-deficient *E. coli* appeared to be gene-dosage dependent and tritium release activity was drastically reduced both in *E. coli* and

yeast. Surprisingly however, yeasts that were auxotrophic for dTMP did not suffer any discernible deleterious effects when complemented by the single tyrosine/phenylalanine mutants, even when the mutant genes were expressed from single copy plasmids. A mutant with both tyrosines replaced by phenylalanine did not complement in *E. coli*, complemented in a yeast TS-knockout strain only when overexpressed, but showed heteroallelic complementation in a yeast strain that has a mutation in the active site of TS. All mutations introduced in Loop 2 so far dramatically reduce enzyme activity as determined by tritium release assays.

These results indicate that Loop 2, despite its peripheral location, is highly sensitive to modification and contributes to the structural integrity of the protein. In contrast, prokaryotic TS (from *E. coli*) was functionally expressed in a yeast TS-knockout strain, suggesting that although EUK1 and EUK2 are essential for structural integrity they do not provide an additional essential biological function. Moreover, our results suggest that the enzymatic impairment of Loop 2 is offset by a factor present in *S. cerevisiae* cells but not in *E. coli*.

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Finally I want to express my appreciation of the excellent teaching faculty of Concordia, who throughout my undergraduate and graduate years trained me to become, I hope, a competent scientist.

I dedicate this thesis to my family.

To my mother, Lucie Calegari

To my brothers, William and Erik

To my husband, Gary, and my sons, David and Christopher.

And in memory of my father, Jacques Grados

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LIST OF ABBREVIATIONS

A: Adenine

ADH: Alcohol dehydrogenase

Arg: Arginine

Asn: Asparagine

Asp: Aspartate

Bp: Base pairs

BrdUMP: Bromodeoxyuridine monophosphate

BSA: Bovine serum albumin

C: Cysteine or cytidine

Cys: Cysteine

Cyt c: Cytochrome c

D: Aspartate

dCMP: Deoxycytidine monophosphate

DHF: Dihydrofolate

DNA: Deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

DPM: Disintegrations per minute

dTMP: Deoxythymidine monophosphate

dTTP: Deoxythymidine triphosphate

dUMP: Deoxyuridine monophosphate

EDTA: Ethylenediaminetetraacetic acid

F: Phenylalanine

FdUMP: Fluorodeoxyuridine monophosphate

FOA: Fluoroorotic acid

G: Glycine or guanine

GFP: Green fluorescent protein

Gly: Glycine

IdUMP: Iododeoxyuridine monophosphate

K: Lysine

kDA: kiloDalton

KO: Knock-out

Lys: Lysine

MTHF: N^5, N^{10}-methylenetetrahydrofolate

N: Asparagine

PCR: Polymerase chain reaction

Phe: Phenylalanine

PMSF: Phenylmethylsulfonylfluoride

R: Arginine

rUDP: Uridine diphosphate

S: Serine

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser: Serine

T: Threonine

T: Threonine or thymine

THF: Tetrahydrofolate

Thr: Threonine

TS: Thymidylate synthase

Tyr: Tyrosine

1. INTRODUCTION

Thymidylate synthase (TS), an enzyme common to almost all organisms, provides the DNA replication machinery with the vital precursor thymidylate (dTMP) by reductive methylation of deoxyuridine monophosphate (dUMP). Because the TS pathway is the sole *de novo* source of dTMP, thymidylate synthase plays a significant and central role in DNA replication and the cell division cycle. Furthermore, since the only fate of dTMP is to become incorporated into DNA, TS is a choice target for the design of chemotherapeutic drugs against cancer as well as species-selective drugs against proliferative agents (Harrap *et al.*, 1989).

For these reasons TS has been for over 40 years the subject of extensive study that includes the elucidation of its enzymatic mechanism, the solving of three-dimensional structures, the design and testing of inhibitive substrates, and more recently, the investigation of its regulation and role in the cell division cycle.

1.1. The Thymidylate Synthase Pathway

Thymidylate synthase converts dUMP to dTMP by reductive methylation of the substrate. This reaction requires the cofactor N^5,N^{10}-methylenetetrahydrofolate (MTHF) which serves both as the methyl donor and the reductant. The products of the reaction are 7,8-dihydrofolate and dTMP; the latter is subsequently phosphorylated by thymidylate kinases to produce dTTP for incorporation into newly synthesized DNA. The by-product of the TS reaction, dihydrofolate, must be reconverted to methylenetetrahydrofolate in

order to replenish cellular stores of this one-carbon donor to supply thymidylate synthase as well as other metabolic pathways. MTHF is regenerated by two enzymes: first dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate, which is then methylated by serine transhydroxymethylase with the concomitant conversion of serine to glycine (Figure 1.1). Another source of MTHF is provided by the glycine cleavage pathway which methylates THF by breaking down glycine.

The principal source of the substrate dUMP is the reduction of uridine diphosphate (rUDP) by ribonucleotide reductase. Other sources of dUMP come from the deamination of deoxycytidine monophosphate (dCMP) and phosphorylation of deoxyuridine, although not all organisms, yeast included, possess the kinase that mediates this last reaction.

1.2. The Catalytic Mechanism

Studies of the catalytic mechanism of TS were initiated in the1970's, and developed in the 80's and 90's largely by the collaborative efforts of the labs of Daniel Santi and Robert Stroud, with significant contributions of several others. The availability of solved crystal structures of wild type or mutant TS, bound with substrate and cofactor or their analogs made the elucidation of the mechanism possible. Particularly useful were the crystal structures of ternary complexes of mutant TS bound to substrate or cofactor analogs that trap otherwise transient intermediates (Matthews *et al.*, 1990a,b, Montfort *et al.*,1990, Kamb *et al.*, 1992a,b, Finer-Moore *et al.*, 1993, Fauman *et al.*,1994). The presently accepted catalytic mechanism (reviewed by Stroud and Finer-Moore, 1993, and

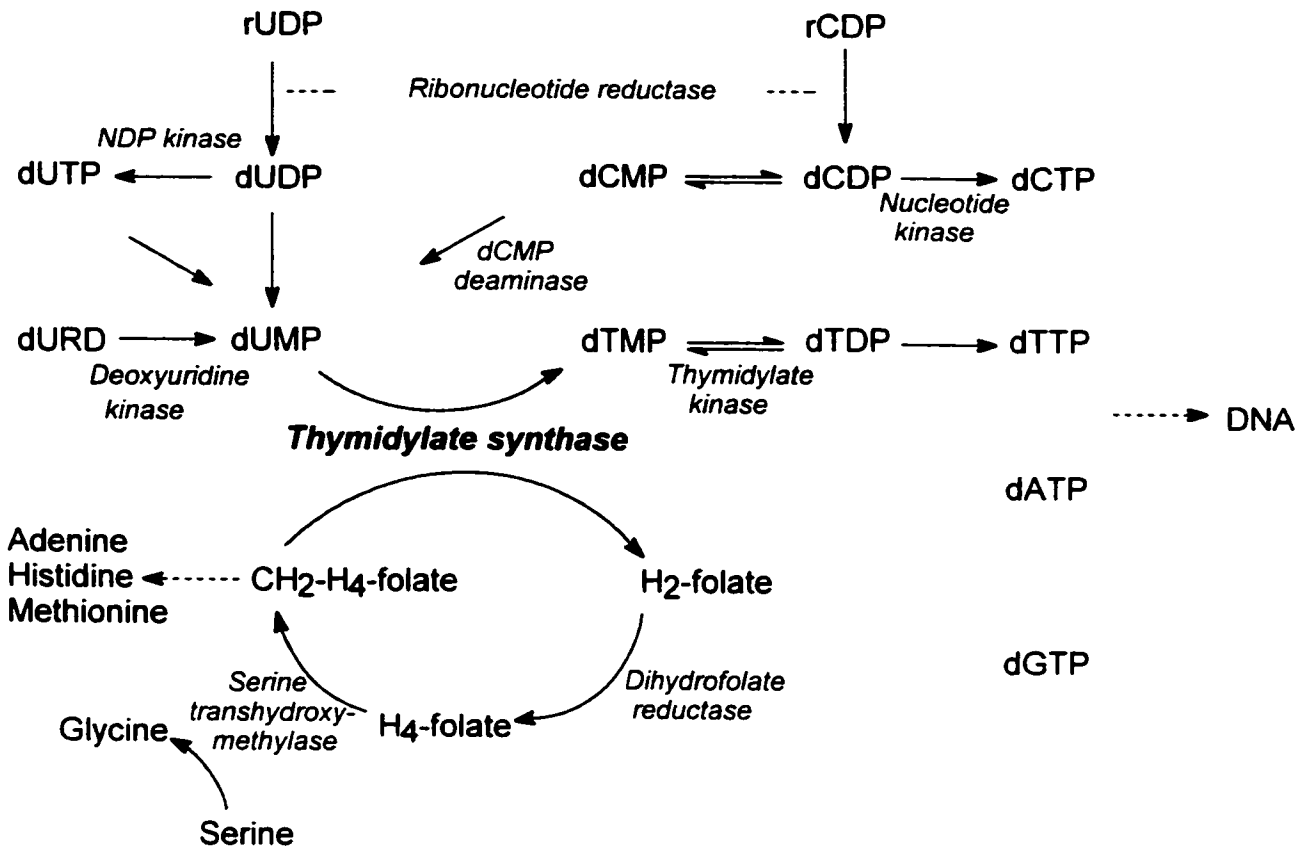


Figure 1.1. Pathways of thymidylate synthase and other enzymes of pyrimidine deoxyribonucleotide biosynthesis.

Carreras and Santi, 1995) occurs in three main steps: 1) the cofactor and substrate are separately activated by the enzyme following binding, 2) a methylene group is transferred from the cofactor to the substrate and 3) a hydride is transferred from the cofactor to reduce the methylene group.

Activation of the substrate dUMP occurs by a nucleophilic attack of its C-6 carbon by the invariant cysteine in the enzyme's active site (Cys185 in yeast). All mutations made at this position abolish enzyme activity, with the exception of serine, which imparts partial activity to the *E. coli* TS but nonetheless inactivates the *L. casei* enzyme (Michaels *et al.*, 1990, Climie *et al.*, 1990). The cysteine thiol binds covalently to C-6 of dUMP, causing delocalization of an electron pair that results in a transient enolate (Figure 1.2, Intermediate I) poised to react with the activated cofactor.

Upon binding to the active site, the imidazolidine ring of CH_2H_4folate is opened and forms an iminium ion; the positive charge and the one-carbon unit are favoured to be at position N-5 (Carreras and Santi, 1995, and references therein). Condensation of the enolate and iminium ions via C-5 of dUMP and the methylene group at N-5 results in an intermediate ternary complex where substrate and cofactor are covalently bound to the enzyme and to each other (Intermediate II).

It is currently thought that the breakdown of this ternary complex is initiated by the abstraction of a proton at C-5 of the pyrimidine ring. Although no suitable base has been identified that would assist the removal of this proton, it has been argued that this process is facilitated by an ordered water molecule (Matthews *et al.*, 1990b, Fauman *et al.*, 1994) or by an invariant tyrosine (Tyr115 in yeast) acting in concert with a water molecule (Liu

Figure 1.2. The catalytic mechanism of thymidylate synthase.

In the currently accepted model, the reaction is initiated with the nucleophilic attack of C-6 of dUMP by the sulphur atom of the conserved active site cysteine, which simultaneously loses a proton. The new bond between the cysteine and C-6 causes delocalization of an electron pair between C-6 and C-5, with the consequent formation of an enolate thought to be stabilized by a nearby water molecule, and activation of C-5 (I). Upon binding to the enzyme, the imidazolidine ring of the cofactor opens, with a concomitant proton capture at N-10, resulting in the formation of an iminium ion. The thiolate anion and iminium cation condense at C-5, producing the steady-state covalent ternary complex (II). The abstraction of a proton at C-5 ensues, presumably aided by a proximal ordered water molecule, since this hydrogen is poorly acidic. Deprotonation is concurrent with electron movement to, and elimination of, tetrahydrofolate. The TS inhibitor, FdUMP, blocks dTMP formation at this step since the fluorine at C-5 cannot be abstracted and thus prevents the breakdown of the ternary complex. The final step of the reaction is a hydride transfer from C-6 of the cofactor to the transferred methylene (III). Electron movement to the enzyme cysteine follows, coupled with the disengagement of the product and formation of a double bond between C-6 and C-5.

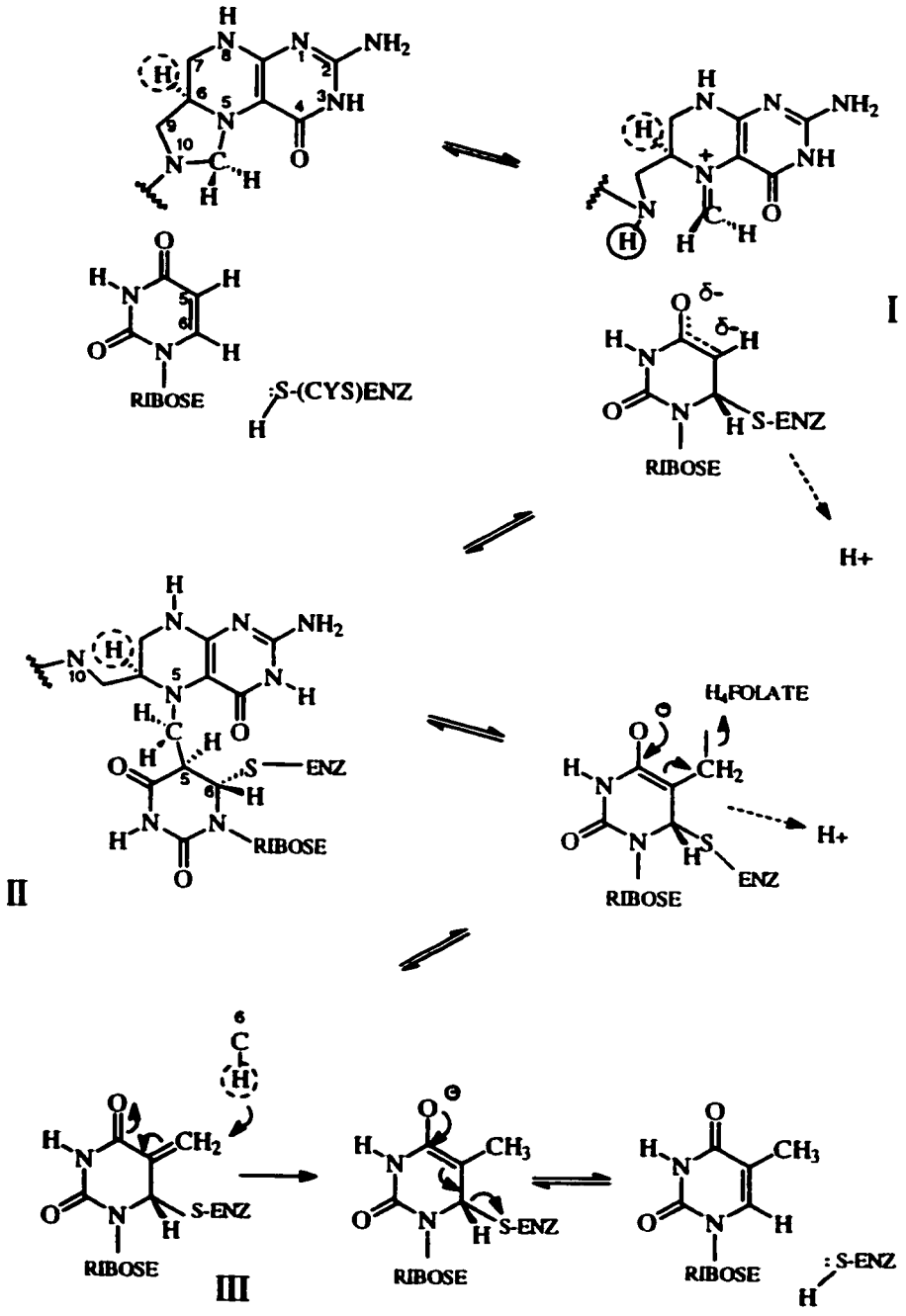
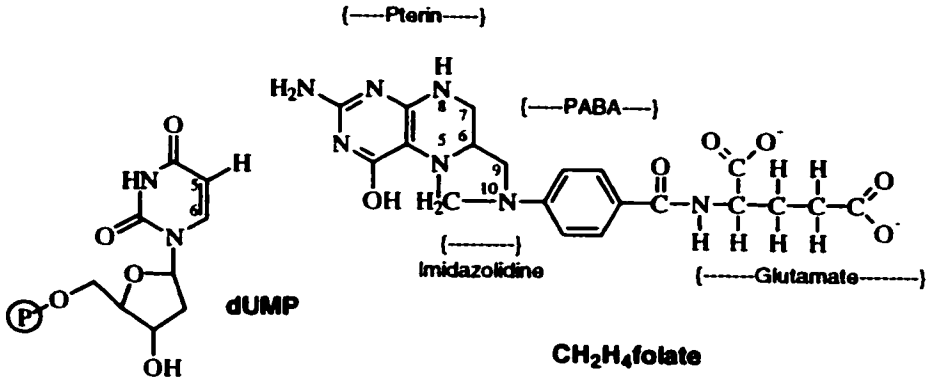

RIBOSE
S-(CYS)ENZ
I
S-ENZ
H+
II
H4FOLATE
ENZ
III
CH2
CH3

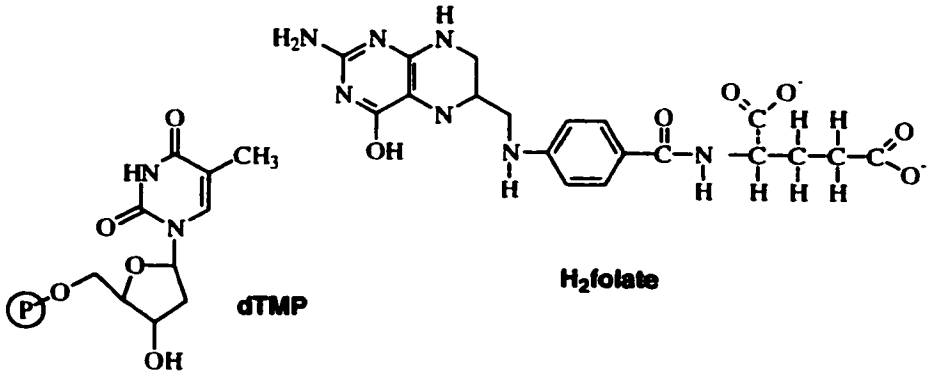
Figure 1.3. Ligands of thymidylate synthase.

The substrate dUMP differs from the product dTMP in that it has a hydrogen at C-5 whereas dTMP is methylated at that position. The substrate analog FdUMP has a fluorine at C-5, which results in blocking the TS catalyzed reaction at the first step (see mechanism, Figure 1.2). FdUMP is a potent inhibitor of TS used clinically as an anti-cancer drug. The cofactor, CH_2H_4folate, transfers its one-carbon unit from the imidazolidine ring, and then reduces the transferred methylene by donating a hydride from C-6 of the pterin ring, forming the by-product, H_2folate. The cofactor analog, CB3717 (10-propargyl-5,8-dideazafolate, sometimes abbreviated as PDDF) has a quinazoline ring substituted for the pterin ring, and a propargyl group at N-10. CB3717 was initially designed as a TS inhibitor to be used clinically as an anti-proliferative agent since it prevents dTMP synthesis by its inability to form a covalent bond with dUMP. Both analogs have been extremely useful in elucidating the TS mechanism as they form stable covalent complexes that are considered to mimic the intermediates of the reaction and are readily crystallized.



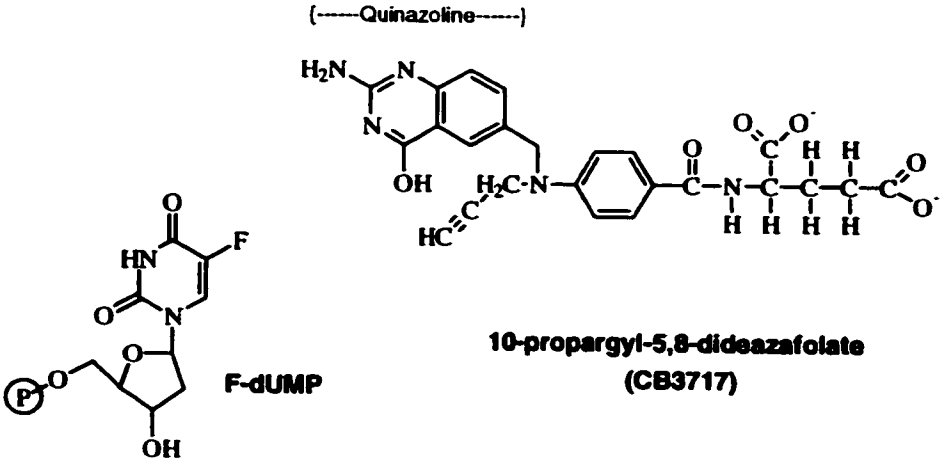
dUMP

CH_2H_4folate



dTMP

H_2folate



F-dUMP

10-propargyl-5,8-dideazafolate
(CB3717)

et al., 1999). Concomitantly, H_4folate disengages from the pyrimidine ring by β-elimination, and the transfer of the one-carbon unit is complete (Intermediate III).

The final step is the stereospecific transfer of a hydride from H_4folate that reduces the transferred methylene, followed by β-elimination of the enzyme at C-6. Release of the resulting product, dTMP is thought to be driven by the displacement of an ordered water molecule by the acquired methyl group at C-5 (Fauman *et al.*, 1994).

1.3. Assays of Thymidylate Synthase

Several useful assays have been developed that make possible the comparison of mutant variants of TS to the wild type enzyme. These include complementation of TS deficiency, catalytic assays, and direct binding assays.

Escherichia coli strains with deletions in their *thyA* gene coding for TS (Thompson *et al.*, 1987, Belfort and Pedersen-Lane, 1984) can be propagated because they are permeable to thymidine and possess kinases that convert the nucleoside to thymidylate; these strains are thus convenient for testing complementation by mutant TS genes. Cross-species complementation in *E. coli* has been used with *Lactobacillus casei* (Climie *et al.*, 1990, Schellenberger *et al.*, 1994), *Saccharomyces cerevisiae* (Munro *et al.*, 1999) and even human TS (Kupiec *et al.*, 1996). Although the complementation assay is simple and convenient, results can be affected by the expression system used and thus decreased activity is not always detected nor are subtle differences between mutants. However, trimethoprim can be used to discriminate between mutants with different levels of activity, since mutants with low activity may escape inhibition by this compound (Belfort

et al., 1984). The complementation assay in *E. coli* can detect as little as 0.002 U/mg in protein extracts (Climie *et al.*, 1990b).

Several enzymatic assays exist for measuring TS activity and kinetics. A spectrophotometric assay can be used that measures increase of Abs_{340} with increasing formation of the reaction by-product dihydrofolate (Pogolotti *et al.*, 1986); this assay is sensitive to 10^{-4} U/mg but is preferably employed with purified protein in order to avoid interfering absorbance of crude extracts.

The most sensitive assay, the tritium release assay (Roberts, 1966), can reportedly detect activity as low as 10^{-8} U/mg. This assay measures the release of tritium into solvent from the C-5 position of radiolabeled substrate [5-^{3}H]dUMP or decrease in $^{3}H/^{14}C$ ratio after evaporation of solvent if using [2-^{14}C,5-^{3}H]dUMP (Pogolotti *et al.*, 1979). Tritium release however, is known to also occur in the absence of cofactor as a result of proton exchange with water, albeit it at a much slower rate (10^{5}-fold decreased k_{cat}) than the cofactor-dependent reaction. The two mechanisms of tritium release can be distinguished by quantitating dTMP formation by HPLC (Kunitani and Santi, 1980).

A spectrophotometric assay that is useful for determining dUMP-binding ability is the cofactor-independent dehalogenation assay of Br-dUMP or I-dUMP to produce dUMP, which occurs at a much slower rate than dTMP synthesis (Garrett *et al.*, 1979). Several direct binding assays are available that measure, spectrophotometrically, binding of FdUMP (Santi *et al.*, 1974; Donato *et al.*, 1976), 5-NO_2dUMP (Wataya *et al.*, 1980) and pyridoxal-5'-phosphate (Santi *et al.*, 1993).

1.4. The Structure of Thymidylate Synthase

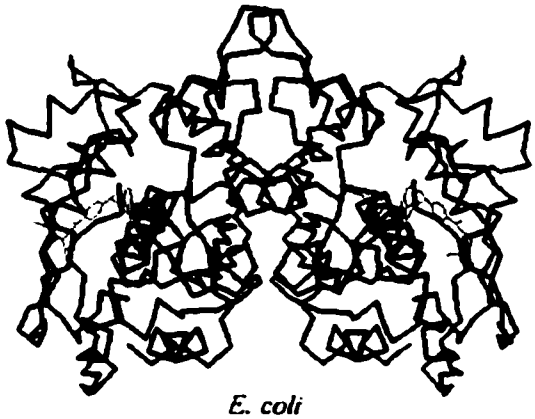
Even before three-dimensional structures of TS were solved, it was expected that the conformation of TS would be well conserved because of the high homology between the known amino acid sequences (now numbering about 30). In the last 15 years, many TS proteins have been crystallized, both for prokaryotic and eukaryotic organisms, and the expected conservation of overall fold has been confirmed. The prokaryotic structures that have been solved are those of *L. casei* (Hardy *et al.*, 1987; Finer-Moore *et al.*, 1993), *E. coli* (Matthews *et al.*, 1990a,b; Perry *et al.*, 1990; Montfort *et al.*, 1990, Kamb *et al.*, 1992a,b; Fauman *et al.*, 1994), phage *T4* (Finer-Moore *et al.*, 1994), *Bacillus subtilis* (Stout *et al.*, 1998); the solved eukaryotic structures include human TS (Schiffer *et al.*, 1991), *Leishmania major* TS (Knighton *et al.*, 1994), rat TS (Sotelo-Mundo *et al.*, 1999) and TS of *Pneumocystic carinii* (Anderson *et al.*, 2000).

The overall fold of TS is remarkably similar for these widely divergent species and the core structures of the enzymes are highly superimposable, even between prokaryotes and eukaryotes (Figure 1.4). Some regions of variability however, induce subtle differences within the enzyme core or more conspicuous ones on the periphery (discussed later).

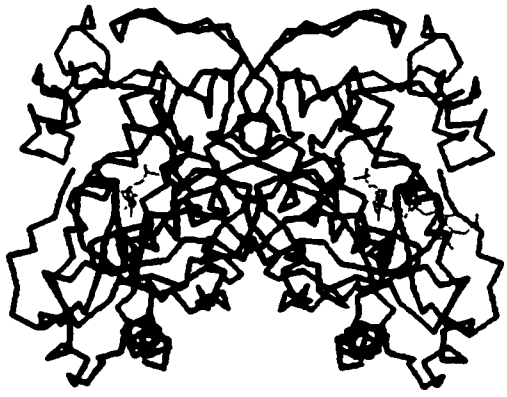
The conformation of TS is defined by a scaffold of β-strands and helices linked by intervening turns, coils and loops. The enzyme is an obligate dimer of two identical subunits of 30 to 35 kiloDaltons. The dimer has two active sites, each of which requires the participation of both subunits. Six β-strands mostly in anti-parallel alignment form a

Figure 1.4. Three-dimensional structures of TS from rat, *E. coli* and *P. carinii*. The atomic coordinates were obtained from the Brookhaven Protein Data Bank, access numbers 1RTS, 2TSC and 1CI7, and the structures were generated with RasMol Version 2.6 software (R. Sayle, 1994). The subunits are shown in contrasting colors, red and blue, while ligands are indicated as green stick figures. Loop 1 and Loop 2 are shown in light blue and black, respectively. The overall fold of TS is well conserved between prokaryotes (*E. coli*, middle structure) and eukaryotes (rat, top, and *P. carinii*, bottom). Loop 2 is distinctly different however; in the prokaryotes it projects toward the dimer interface, while in the eukaryotes it is folded back thus capping its own subunit. In eukaryotes, Loop 1 and Loop 2 contain inserts that are absent in the prokaryotes, making the loops longer.

Rat



E. coli



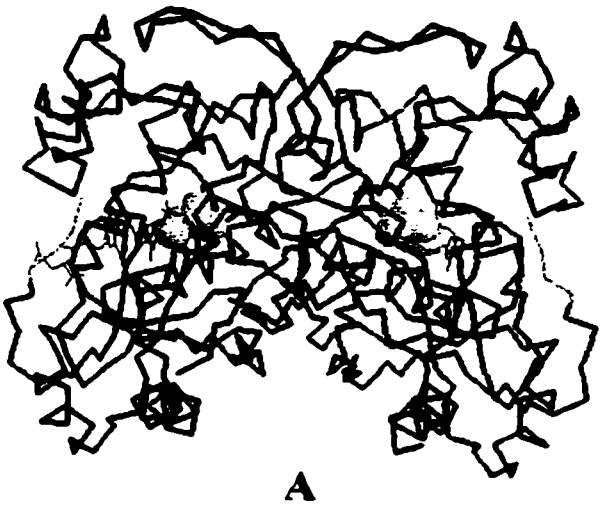
P. carinii

β-sheet that is apposed against the corresponding β-sheet of the opposite subunit, forming the dimer interface (Figure1.5). This "β-sandwich" conformation is atypical in two ways: 1) the β-sheets exhibit an uncommon right-handed twist relative to each other, accommodated by a bulge that disrupts the plane of contact (Matthews *et al.*, 1989), and 2) 13 of the 25 contacts between these buried sheets are hydrophilic and participate in electrostatic or hydrogen bonding, in contrast to most stacked β-sheets which usually contain a higher proportion of hydrophobic residues. This tight association between the subunits may partly explain why loss of structure and dissociation occur concomitantly when TS is treated with denaturants (Gokhale *et al.*, 1996).

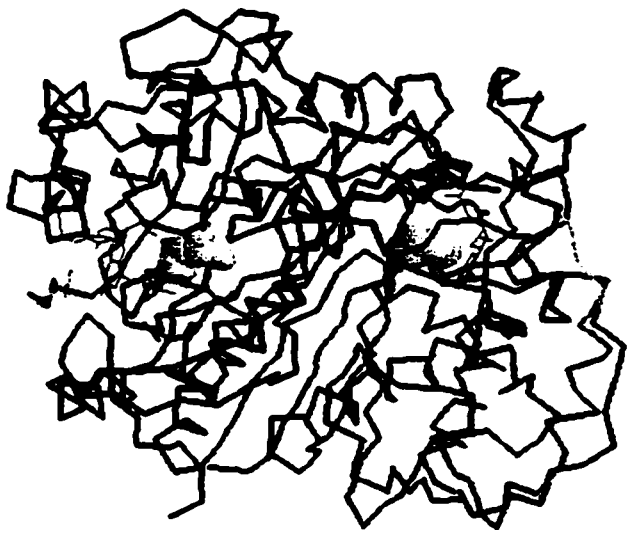
The 9-11 helices are mostly amphipathic, but the largest is completely buried and forms the hydrophobic core of the enzyme. Three of the helices, including the large hydrophobic helix, form part of the active site cavity while the remaining boundaries are delimited by the interface β-sheet and by several coils. The active site contains an extensive hydrogen bond network of side chains, backbone atoms and ordered water molecules that coordinately assist in substrate binding and catalysis (see section on catalytic mechanism).

Binding of ligands occurs in an ordered *bi-bi* reaction sequence (Danenberg & Danenberg, 1978, 1979) with dUMP binding first followed by the cofactor, and release of dihydrofolate followed by dTMP. The phosphate moiety of dUMP helps to anchor the substrate by hydrogen bonding with a conserved serine and four conserved arginines, (Ser205, Arg204, Arg260, Arg155', Arg156' in yeast) two of which are contributed across the dimer interface by the opposite subunit (Hardy *et al.*, 1987; Morse *et al.*, 2000).

Figure 1.5. Structure of *P. carinii* TS liganded with dUMP and a cofactor analog. **A.** Symmetric view with ligands shown in red stick form and catalytic cysteine in yellow (spacefill). Main features shown are: Loop 1 (red); Loop 2 (black); carboxy-terminal (yellow); folate-binding loop (magenta). **B.** The structure from **A** was rotated to show the alignment of the helices that flank Loop 2 (shown in black).



A



B

The pyrimidine ring of dUMP provides a stacking surface for the pterin ring of the cofactor, while the glutamate tail interacts electrostatically with a base-rich region (corresponding to residues 55-62 in yeast) known as the folate-binding loop (Finer-Moore *et al.*, 1990; Kamb *et al.*, 1992a). The carboxy terminal of the enzyme, a flexible tail-like coil, undergoes a large conformational change triggered by binding of the cofactor (Kamb *et al.*, 1992b). This conformational change results in capping the active site cleft, thus sequestering it from bulk solvent, and also properly orients the ligands to facilitate the transfer of the carbon unit from the cofactor to the substrate (Montfort *et al.*, 1990; Perry *et al.*, 1993). Comparisons of structures of liganded and unliganded TS show that the terminal valine undergoes a displacement of 4 angstroms. This well conserved residue can be mutated with some retention of activity (Climie *et al.*, 1992) but its deletion inactivates the enzyme (Carreras *et al.*, 1992) and alters the orientation of the ligands (Perry *et al.*, 1993).

TS can bind folates that are monoglutamylated or polyglutamylated. *In vivo*, glutamyl transferase lengthens the glutamate tail so that the population of folates typically contains 4 to 7 glutamyl residues. In *E. coli* and *L. casei*, the binding affinity of folates increases with the number of glutamyl residues; for example, the binding affinity of hexaglutamyl folate is 200 fold that of monoglutamyl folate for *E. coli* TS (Friedkin *et al.*, 1975), and 414 fold for *L. casei* TS (Matthews *et al.*, 1987). For porcine TS however, binding affinity is higher for tetraglutamyl folate (167 fold increase over monoglutamyl folate) than for hexaglutamyl folate (only 67 fold increase) (Lu *et al.*, 1984). For human TS, binding affinity peaks for tetraglutamyl folate (119 fold increase)(Sikora *et al.*, 1988). These numbers indicate a general preference for polyglutamylated folates among the

species compared, but suggest that binding affinity of folates varies between TSs of different species.

Although the pyrimidine ring of dUMP usually forms part of the folate binding site by creating a stacking platform for the pterin ring, Kamb *et al.* (1992b) have shown by X-ray crystallography that polyglutamylated folates are able to bind in the absence of dUMP, presumably because of their increased binding affinity compared to monoglutamylated forms. In the same report they showed that dUMP is able to bind even after folate has taken occupancy. The binding of dUMP following cofactor binding and the ensuing conformational change suggests that the "open" and "closed" conformations may be in equilibrium. Alternately, it is speculated that dUMP may enter the active site through the opposite side of the cavity, as X-ray structures are indicative that there is sufficient space to allow the passage of the nucleotide.

Several studies have demonstrated intersubunit communication between the two active sites of the TS dimer and asymmetry of the TS reaction. The earliest indication of this was that the removal of the C-terminus residue by carboxypeptidase A on one subunit resulted in loss of activity at both catalytic sites (Aull *et al.*, 1974). More recently, Bradshaw and Dunlap (1993) showed that when the catalytic cysteine of one active site was chemically modified, dUMP binding and dTMP synthesis were enhanced at the other site. Early kinetic studies indicated that the two active sites bind dUMP with different affinities (Lockshin and Danenberg, 1979), and several studies have shown that binding of folate at one site makes the second site accessible for dUMP or FdUMP binding (Galivan *et al.*, 1976; Danenberg & Danenberg, 1979; LaPat-Polasko *et al.*, 1990). More recently, X-ray structures have revealed that the TS subunits are

asymmetrical when liganded, and in 1994 Dev *et al.* presented data suggesting that cofactor only binds significantly at one site. In 1995 Maley *et al.* demonstrated that heterodimers with only one functional active site have specific activities that are equivalent to those of the wild type homodimers. Together these findings led to the proposal that TS is an "alternate-sites" enzyme whereby catalysis at one site is coupled to substrate binding at the other. In 1999 Anderson *et al.* presented strong structural evidence of a "half-the-sites" reaction mechanism for TS. The X-ray structure of TS from *P. carinii* bound to dUMP and a cofactor analog revealed an asymmetric dimer with dUMP and cofactor analog bound at one site, while the second site was occupied by dUMP only. Furthermore, the structure revealed that binding of both ligands at one site causes a conformational change that abrogates binding of the cofactor analog at the other site.

1.5. Regulation, Localization and Role in Cellular Events

The DNA metabolism pathway must be precisely regulated to meet several critical cellular requirements: 1) that DNA precursor levels be adequate when cells undergo a burst of DNA synthesis during S phase, 2) that deoxyribonucleotide pools be balanced so as to limit base misincorporation leading to fatal mutations, and 3) that DNA synthesis be temporally coordinated with the cell division cycle. Since thymidylate synthase provides a vital precursor for the synthesis of DNA, it follows that its regulation must be closely coordinated in compliance with these conditions.

The cell-cycle dependent regulation of TS expression in *S. cerevisiae* was reported in the 1980's (Storms *et al.*, 1984; Greenwood *et al.*, 1986; McIntosh *et al.*, 1988). It was shown that regulation of the enzyme is coupled to DNA synthesis since activity and TS-specific transcripts both peak during S phase. A rapid decrease of activity following S-phase (Greenwood *et al.*, 1986) suggested that TS may be regulated by a post-translational mechanism as well. Although elucidation of the mechanism underlying this inactivation remains elusive thus far, a number of studies point to several possibilities. Garvey and Santi (1985) reported that a eukaryote-specific peptide sequence in the TS domain of the bifunctional dihydrofolate reductase-thymidylate synthase protein of *Leishmania tropica* was susceptible to attack by several proteases *in vitro*, suggesting cleavage as a possible mode of inactivation of TS. Bures *et al.* (1991) showed that small molecules such as pyridoxal phosphate and glyceraldehyde-3-phosphate bind to and inhibit human TS activity *in vitro*, suggesting another possible means of repressing the enzyme. Recent evidence revealing that TS is subject to phosphorylation, both *in vitro* and *in vivo* (Samsonoff *et al.*, 1997), implicates another intriguing and perhaps most likely strategy for post-translational regulation of TS activity. Finally, allosteric interactions with other proteins in a multienzyme complex (discussed later) has been proposed as another possible mechanism of controlling the activity of TS and other dNTP synthesizing enzymes (Reddy and Fager, 1993, and references therein).

In addition to dTMP synthesis, TS may have other cellular roles. Human TS protein has been shown to bind to its own mRNA (Chu *et al.*, 1991; Keyomarsi *et al.*, 1993) which led to postulating that this "translational detainment" was a means of modulating TS expression levels. A more recent study (Chu and Allegra, 1996) corroborated that

human TS binds its own transcript *in vitro* and *in vivo*, and showed that in addition it binds other mRNAs (of the *c-myc* oncogene and p53 tumour suppressor gene) implicating potential involvement of TS in the regulation of the cell cycle.

Since TS is part of the DNA synthesizing pathway, it is appropriate to briefly outline a few aspects of the growing evidence that enzymes involved in DNA metabolism (DNA replication and dNTP biosynthesis) physically associate to form a complex termed "replitase" (reviewed by Reddy and Fager, 1993; references therein). The possibility that such a complex exists was first advanced for prokaryotic systems (in which it is termed "replisome") in studies of *E. coli* infected with phage T4. Although attempts to isolate such a complex have been unsuccessful, other lines of evidence, genetic and biochemical, strongly support its existence. For example, early genetic studies showed that a mutation in the phage T4 gene for dCMP hydroxymethylase could be suppressed by a mutation in the gene for DNA polymerase, suggesting interaction between the enzymes of DNA replication and those of deoxynucleotide metabolism (Chao *et al.*, 1977). In 1988 Mathews *et al.* reported that phage T4 TS is part of a complex of viral enzymes that are kinetically coupled for efficient dNTP synthesis. Further, Wheeler *et al.* (1992) showed by affinity chromatography that several phage T4 enzymes of DNA replication associate with enzymes of dNTP biosynthesis.

In eukaryotes, evidence supporting the association between enzymes of DNA replication and those of dNTP biosynthesis has been demonstrated with mammalian cells. Several enzymes of dNTP biosynthesis, including thymidylate synthase, were reported to cosediment in sucrose density gradients with DNA polymerase-α from nuclear extracts of rat liver cells (Baril *et al.*, 1974). Some of these enzymes were later shown to colocalize

in karyoplasts of Chinese hamster embryo fibroblasts (CHEF/18) (Reddy and Pardee, 1980). In 1983, Ayusawa *et al.* reported that complex formation was apparently species-specific; i.e. in contrast to endogenous mouse thymidylate synthase, heterologous TS from human DNA did not cosediment with other DNA synthesizing enzymes endogenous to transfected mouse cells.

The existence of replitase is strongly supported by the demonstration of substrate channeling *in vivo* and *in vitro* by numerous studies that made use of radiolabeled substrates. These studies showed that the radioactivity from exogenous nucleotides (for example ^{3}H-dUrd) is incorporated directly into new DNA without dilution by large unlabeled dUMP and dTTP pools present in the cellular milieu (reviewed by Reddy and Fager, 1993). Another phenomenon which supports the occurrence of replitase is the "cross-inhibition" of enzymes. In 1983, Reddy and Pardee reported that inhibitors of ribonucleotide reductase, DNA polymerase-α and DNA topoisomerase II all inhibited thymidylate synthase. This inhibition occurred in intact cells but not in crude extracts, suggesting interaction between these enzymes through a structured complex.

The validity of the replitase concept is dependent upon the colocalization of the enzymes of dNTP metabolism with those of DNA replication, i.e. in the nucleus or nuclear membrane. Localization studies have been contradictory, some placing ribonucleotide reductase and thymidylate synthase in the cytoplasm (Engstrom *et al.*, 1984; Kucera and Paulus, 1986; Johnston *et al.*, 1991) while others indicate that they reside in the nuclear vicinity. There is evidence that ribonucleotide reductase is located in karyoplasts of CHEF/18 cells (Reddy and Pardee, 1982) and in the nuclear extracts of rat liver cells (Youdale *et al.*, 1984). Thymidylate synthase has been shown to localize to the

nuclear periphery in yeast (Poon and Storms, 1994). In rat liver cells, TS associates with the nuclear region as well as the mitochondria (Samsonoff *et al.*, 1997). In the two latter studies, TS is also dispersed throughout the cytoplasm when overexpressed. Despite these conflicting results that might be caused by the use of different cells and techniques, a model has been proposed whereby a complex of DNA replicating enzymes and dNTP synthesizing enzymes is situated at the nuclear membrane (Reddy and Fager, 1993), with the possibility that the latter group faces the perinuclear side and the DNA replicating enzymes face the nuclear side.

1.6. Species-Specific Structural Variations in TS

Comparisons of amino acid sequences and three-dimensional structures of TS reveal that it is one of the most conserved enzymes known. The core structure of the protein is highly superimposable from one species to another. Nonetheless, as more structures are solved more differences between species are becoming apparent. These differences, some subtle, others more striking, presumably evolved to satisfy species-specific requirements. For example, the *Leishmania major* TS (Knighton *et al.*, 1994) retains a striking similarity with other TSs despite being covalently linked to dihydrofolate reductase to form the bifunctional protein that exists in protozoans and plants.

Some regions of the TS amino acid sequences that show high variability correspond to surface loops and coils which appear to interact in a minimal way with the core structure, and which mediate some of the structural variations. The *L. casei* enzyme contains a large insert of 38 residues in one of its surface loops (Hardy *et al.*, 1987); the

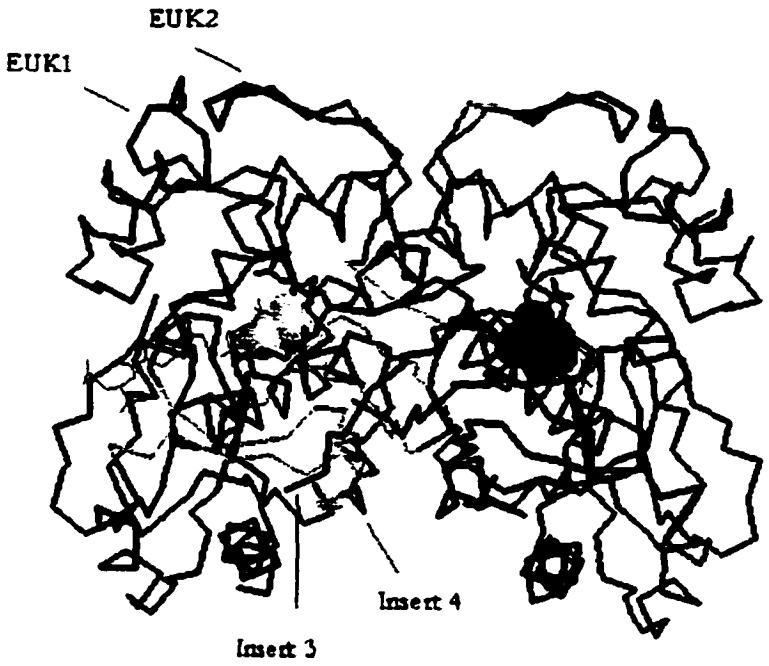
3D structure shows that this insert, whose function is unknown, assumes a helical conformation which is accommodated without structurally deforming the enzyme core with respect to other structures. Phage T4 TS contains three inserts and one deletion unique to the viral enzyme, all of which lie on one side of the protein surface; this region is speculated to be a putative site of intermolecular interactions specific to the phage TS (Finer-Moore *et al.*, 1994). The sequences of fungal TSs contain a small insert of 3 residues (two of which are prolines) that is absent in other organisms. The crystal structure of *P. carinii* (Anderson *et al.*, 2000) has revealed that this insert, located in the interface β-sheets, causes a distortion that pulls conserved helices away from the active site, enlarging the catalytic cleft by about 20% compared to other TSs. This small insert is also present in yeast TS. In addition, the fungal and yeast sequences contain a larger insert located between two β-strands: the conformation of this insert is highly disordered in the *P. carinii* structure (Figure 1.6) and its function is unknown. All eukaryotes contain two additional inserts, absent in prokaryotes, in loops located on the TS surface (discussed in the next section). Another structural dissimilarity between the TS proteins of two different species is illustrated by crystal structures of rat TS and human TS, both bound with dUMP and a cofactor inhibitor (Sotelo-Mundo *et al.*, 1999; Phan *et al.*, 2001). While the human TS was in the expected "closed" conformation, the carboxy terminal of the rat TS retained the "open" conformation.

These structural variations, together with exhaustive studies of mutations that are tolerated by the enzyme (Climie *et al.*, 1990; Michaels *et al.*, 1990; Kupiec *et al.*, 1996), indicate that despite its highly conserved sequence and structure TS exhibits a high degree of plasticity that accommodates minor and even major modifications.

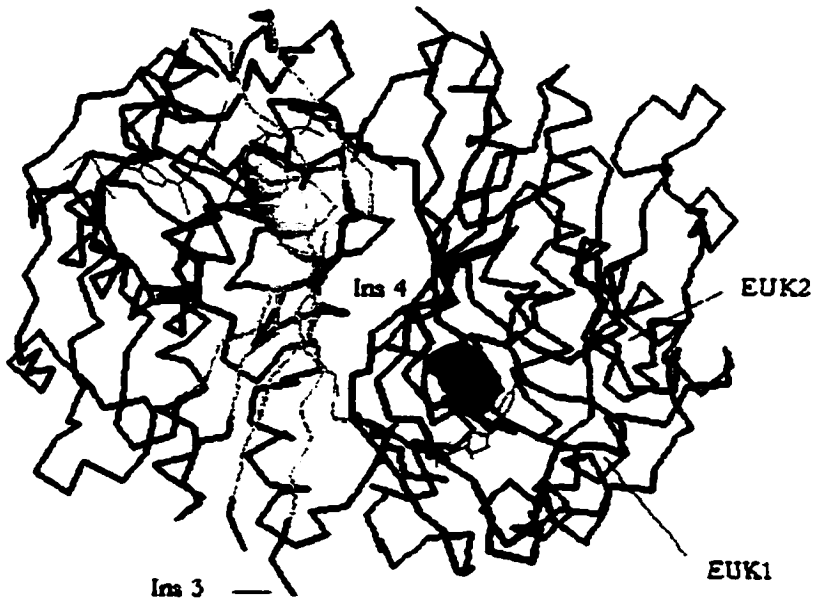
Figure 1.6. Inserts in TS of *P. carinii.*

A. The fungal TS contains several insertions that are absent in the prokaryotes. Inserts "EUK1" and "EUK2", located in surface loops "Loop 1" and "Loop 2", are present in all the eukaryotes. A small insert of 3 residues, two of which are prolines (labeled "insert 4") is specific to fungi. Also specific to these organisms is a larger insert, shown in red and labeled "insert 3". This insert is disordered in the crystal structure, hence some of it is missing in the diagram (atomic coordinates obtained from the Brookhaven Protein Data Bank, access number 1CI7). **B**. Rotation of structure in **A** to show the twist in the β-sheets that form the dimer interface (shown in yellow and dark blue).

A



B



1.7. Function of Loops

While α-helices and β-sheets are the secondary structural elements which by and large define the three-dimensional conformations of proteins, the less ordered features, namely the random coil and loop regions, play important roles. In addition to the obvious function of connecting secondary structures and individual strands of β-sheets, loop regions make a variety of other contributions to protein function or structure. In many proteins loops participate in substrate binding and/or the formation of catalytic sites (Branden and Tooze, 1991). In some cases their flexibility and mobility enables loops to close over active sites and thus sequester the substrates (Gerstein and Chothia, 1991), or to serve as hinges between structural motifs, allowing these to shift and meet conformational requirements for substrate binding or catalysis.

While α-helices and β-sheets form the relatively evolutionarily stable hydrophobic cores of proteins, loops are mostly found at the surface (Leszczynski and Rose, 1986) and show tendencies of higher variability in sequence composition and length, and as such may be regarded as units of evolutionary exchange. The fact that this variability has little or no effect on the spatial disposition of core secondary elements among homologous proteins would lead to speculate that core structures should be somewhat insensitive to alterations of surface loops, be it by mutagenesis or chemical modification. This has been shown in several studies. For example, in one study mouse dihydrofolate reductase was rearranged at the gene level, in order to fuse the natural carboxy and amino termini and generate new ones by cleaving a surface loop, without any impairment of enzyme function or activity (Buchwalder *et al.*, 1992). In another, two surface loops of myosin

were trypsin-digested separately, and while this did affect some kinetic parameters, the enzyme was still able to function (Bobkov *et al.*, 1996).

Because surface loops are less constrained by interactions with other parts of the protein, they are more adaptable to change and thus amenable to acquiring new properties. In thymidylate synthase, two surface loops contain variable stretches and exist as distinct prokaryotic or eukaryotic forms (Figure 1.7). All the eukaryotic sequences reveal inserts of unknown function within these loops (for convenient reference we have arbitrarily designated these loops and inserts as Loop 1, EUK1 and Loop 2, EUK2). Part of Loop 1, the longer of the two loops, lies in the vicinity of the active site. It was shown previously that alteration of this loop by deleting the eukaryotic insert and mimicking the corresponding loop of *E. coli* affected enzyme activity and binding of both substrates (Munro *et al.*, 1999).

Loop 2, located at the dimer interface, is almost closed at the base by an aspartate/arginine salt bridge. These residues are conserved in all species with the exception of phage T4 in which arginine is replaced by glycine. The loop interrupts two adjacent α-helices but nonetheless allows classical α-helical hydrogen bonding across the interface of the helices, which are more or less aligned along a common longitudinal axis (Matthews *et al.*, 1990a). The upstream helix contains a conserved tyrosine (Tyr115 in yeast) which is implicated in catalysis (discussed in section 1.2) and hence the precise orientation of this helix is probably critical. The downstream helix leads into the coil region that provides the conserved arginines (Arg155, 156) to the active site of the opposite subunit.

Figure 1.7. Alignment of amino acid sequences for Loop1 & 2 region.

The sequences span residues 86-140, numbering based on yeast sequence, of thymidylate synthases of various organisms (reported by Carreras and Santi, 1995), as well as yeast mutants (Munro *et al.*,1999). 1, *Saccharomyces cerevisiae*; 2, *Pneumocystis carinii*; 3, human; 4, mouse; 5, rat; 6, *Daucus carota*; 7, *Arabidopsis thaliana* I; 8, *Arabidopsis thaliana* II ; 9, *Cryptococcus neoformans*; 10, *Herpesvirus saimiri*; 11, *Herpesvirus atales*; 12, *Varicella zoster* virus; 13, *Leishmania major/tropica*; 14, *Leishmania mexicana amazonensis*; 15, *Crithidia fasciculata*; 16, *Trypanosoma brucei*; 17; *Trypanosoma cruzi*; 18, *Plasmodium falciparum*; 19, *Plasmodium chabaudi*; 20, *Candida albicans*; 21, *Paramecium tetraurelia*; 22, *Toxoplasma gondii*; 23, *Escherichia coli*; 24, *Bacillus subtilis*; 25, *Bacillus subtilis* phage Φ3T; 26, coliphage T4; 27, *Lactobacillus casei*; 28, *Staphylococcus aureus* transposon *Tn*4003; 29, *Lactobacillus lactis*. All eukaryotic thymidylate synthases known to date have inserts of 12 and 8 residues respectively (shown in italics) in surface loops designated '1'and '2'. In mutant TSΔEUK1, the underlined yeast sequence (left) was deleted and replaced with the *E. coli* sequence, EWADEN. The second yeast sequence underlined (right) is the deletion in mutants TSΔEUK2a and TSΔEUK2b; in TSΔEUK2b it was substituted by the *E. coli* sequence, TPDGR.

	LOOP 1	LOOP 2
	.....	.....
Eukaryotes:		
1-	VKIWDGNGSREYLDKMGFKDRKV	GDLGPVYGFQWRHFGAKYKTCDDDYTGQGIDQLK
2-	IHIWDANGSREYLDSIGLTKRQE	GDLGPIYGFQWRHFGAEYIDCKTNYIGQGVDQLA
3-	VKIWDANGSRDFLDSLGFSTREE	GDLGPVYGFQWRHFGAEYRDMESDYSGQGVDQLQ
4-	VRIWDANGSRDFLDSLGFSARQE	GDLGPVYGFQWRHFGAEYKDMDSDYSGQGVDQLQ
5-	VRIWDANGSRDFLDSLGFSARQE	GDLGPVYGFQWRHFGADYKDMDSDYSGQGVDQLQ
6-	VNIWEGNGSREYLDSIGLTDREE	GDLGPIYGFQWRHFGARYTDMHADYSGQGFDQLL
7-	IHIWDGNASREYLDGIGLTEREE	GDLGPVYGFQWRHFGAKYTDMHADYTGQGFDQLV
8-	IRIWDGNASRAYLDGIGLTEREE	GDLGPVYGFQWRHFGAKYTDMHADYTGQGFDQLL
9-	VGIWDGNGSKEFLEKVGLGHRRE	GDLGPVYGFQWRHFGAEYTDPDGDYKGKGVDQLQ
10-	VHIWDANGSRSFLDKLGFYDRDE	GDLGPVYGFQWRHFGAEYKGVGRDYKGEGVDQLK
11-	VHIWDANGSRSYLDKLGLFDREE	GDLGPVYGFQWRHFGAEYQGLKHNYGGEGVDQLK
12-	IHIWDIYGSSKFLNRNGFHKRHT	GDLGPIYGFQWRHFGAEYKDCQSNYLQQGIDQLQ
13-	IHIWDGNGSREFLDSRGLTENKE	MDLGPVYGFQWRHFGADYKGFEANYDGEGVDQIK
14-	IHIWKGNGSREFLDSRGLTENTE	MDLGPVYGFQWRHFGAEYRGLEANYDGEGVDQIK
15-	IHIWDGNGSREFLDSRGLTENKE	MDLGPVYGFQWRHFGADYKGFDANYD EGVDQIK
16-	IHIWDGNGSRAFLDSRGLTDYDE	MDLGPVYGFQWRHFGADYISSKVDSEGKGVDQIP
17-	VHIWDDNGSRRFLDSRGLTEYEE	MDLGPVYGFQWRHFGAAYTHHDANYDGQGVDQIK
18-	VRIWEANGTREFLDNRKLFHREV	NDLGPIYGFQWRHFGAEYTNMYDNYENKGVDQLK
19-	VRIWEANGTREFLDNRKLFHREV	NDLGPIYGFQWRHFGAEYTDMHADYKDKGVDQLK
20-	VKIWEGNGSREFLDKLGLTHRRE	GDLGPVYGFQWRHFGAEYKDCDSDYTGQGFDQLQ
21-	VKIWEGNGTREYLDTIGLQHRQE	HDLGPVYGFQWRHFGAKYKDCQTDYSNQGVDQVK
22-	VKIWDKNVTREFLDSRNLPHREV	GDIGPGYGFQWRHFGAAYKDMHTDYTGQGVDQLK
Prokaryotes:		
23-	VTIWDEWADEN	GDLGPVYGKQWRAWP TPDGRHIDQIT
24-	VRIWNEWADEN	GELGPVYGSQWRSWR GADGETIDQIS
25-	VHIWDQWKQED	GTIGHAYGFQLGKKN RSLNGEKVDQVD
26-	KTVWDENYENQAKDLGYHS	GELGPIYGKQWRDFG GVDQII
27-	NHIWDEWAFEKWVKSDEYHGPDM*GDLGLVYGSQWRAWH	TSKGDTIDQLG
28-	NNIWNEWAFENYVQSDDYHGPDM*GNLGNVYGKQWRDWE	DKNGNHYDQLK
29-	VKYWGEWGIGD	GTIGQRYGATVKKYNI IG
Yeast Mutants:		
ΔEUK1	VKIWDEWADEN	GDLGPVYGFQWRHFGAKYKTCDDDYTGQGIDQLK
ΔEUK2a	VKIWDGNGSREYLDKMGFKDRKV	GDLGPVYGFQWREFG GIDQLK
ΔEUK2b	VKIWDGNGSREYLDKMGFKDRKV	GDLGPVYGFQWRHFG TPDGRGIDQLK

*38 amino acid insert not shown

Comparisons of crystal structures of TS reveal that the conformation of Loop 2 is strikingly different in prokaryotes and eukaryotes. In prokaryotes, the loop projects toward the dimer interface and makes some contacts with its counterpart on the opposite subunit. In the eukaryotes, the loop is longer by eight residues and is folded back upon its own subunit (Figure 1.3), making several contacts that involve a pair of conserved tyrosines in the EUK2 insert.

The altered conformation of the eukaryotic Loop 2 raises several questions: 1) is this conformation a structural requirement of eukaryotic TS that corresponds to other differences within the core structure, 2) is it mediated by and dependent on the presence of the eukaryotic insert, and 3) does it serve a eukaryote-specific biological function relating to the involvement of TS with the cell division cycle (discussed in section 1.5).

In this study, we continue to investigate the effect of mutations in Loop 2. We made a set of mutants with deletions and substitutions, especially targeting the conserved tyrosines in EUK2, and assessed their effect on complementation activity and expression in a prokaryotic and eukaryotic background, as well as their effect on enzymatic activity. As well, we tested complementation in yeast by a prokaryotic TS (from *E. coli*) a fully functional enzyme that does not contain the EUK2 insert. Our results indicate that Loop 2 is highly sensitive to modifications, suggesting that it plays a considerable role in maintaining structural integrity. The functional expression of the *E. coli* TS in a TS-deficient yeast strain indicates that the EUK1 and EUK2 inserts do not have a biological role that is essential for enzyme function. Eukaryotes may have acquired these inserts early in their evolution, perhaps as introns that eventually lost their excision sequences,

without affecting the core structure of the protein. Subsequent mutations may then have rendered the inserts indispensable for enzyme structure and/or function.

2. MATERIALS AND METHODS

2.1. Generation of Mutants

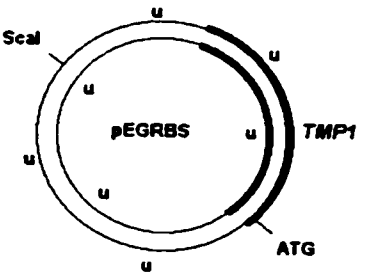
Site-directed mutagenesis was carried out with a Quant-Essential mutagenesis kit (Quantum Biotechnologies), based on the method described by Slilaty *et al.* (1990). Plasmids harbouring the *TMP1* wild type or mutant alleles were transformed into strain RZ3210 (*dut- ung-*) to generate uracil-containing DNA. DNA extractions were performed using a Wizard Miniprep kit from Promega, and the plasmids were then linearized with *Sca*I restriction endonuclease. These plasmids contain a unique *Sca*I site within the ß-lactamase gene for ampicillin resistance. The linear DNA was heat denatured and annealed with two primers. One primer is a "closing"primer which spans 20 bases on either side of the *Sca*I site (5'-CTGTGACTGGTGAGTACTCAACCAAGTCAT-3') while the other primer contains the mutation of interest. After the annealing period, both primers are extended by addition of T7 DNA polymerase, followed by ligation, resulting in a fully regenerated and closed plasmid (Figure 2.1). Since both primers are complementary to the same strand, and since the mutagenic primer is added in 5 times excess of the closing primer, any closed plasmid that is generated is likely to contain the mutation as well. There is a selection against wild type DNA in the transformation process since linear DNA transforms poorly, as well as in the DNA replication stage since a *dut+ ung+* bacterial strain which destroys uracilated DNA is used as host.

For screening purposes, each mutagenic primer contained in addition to the desired mutation a second, silent mutation which created a new restriction enzyme site or

Figure 2.1. Strategy for site-directed mutagenesis.

1. Uracilated DNA of pEGRBS-WT was generated in a *dut-ung- E. coli* strain.
2. pEGRBS-WT was linearized with *Sca*I.
3. The linear fragment was heat denatured and annealed with mutagenic and closing primers.
4. The second strand was synthesized *in vitro* with DNA polymerase.
5. The reaction product was transformed into a *dut+ung+ E. coli* for propagation (and destruction of the non-mutagenic strand).

1.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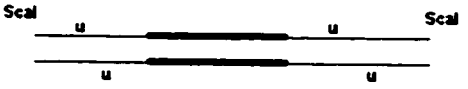
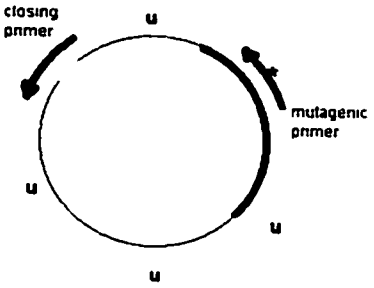
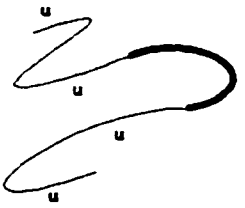
pEGRBS
TMP1
ATG

2.

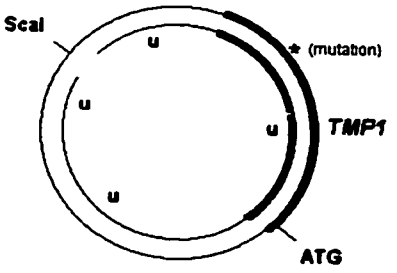

ScaI
ScaI

3.

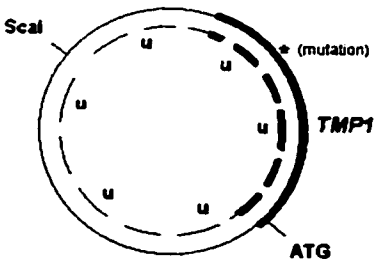

closing primer
mutagenic primer



4.


ScaI
(mutation)
TMP1
ATG

5.


ScaI
(mutation)
TMP1
ATG

removed an existing one. The mutations generated and their corresponding primer sequences are summarized in Table 1. The screening strategies and restriction enzyme analyses are shown in Table 4 and Figure 3.3 (Results section).

All mutants except for *tmp1-E2a/K117* were made in a pUC-derived plasmid (pEGRBS-WT) described previously (E. Munro, Master's Thesis). The *tmp1-E2a/K117* mutant was made using as template pEG89-EUK2a, a pEMBLY derivative described previously (E. Munro, Master's Thesis). Oligonucleotides were purchased from Biocorp.

2.2. Subcloning *TMP1* Alleles into a Yeast Centromere Plasmid

Three of the mutants, *tmp1-F126*, *tmp1-F133* and *tmp1-FF*, were moved from pEGRBS into a YCp50 vector. For *tmp1-F133*, a 370 base pair *Pvu*II fragment within *TMP1* and encompassing the mutation was exchanged with the corresponding fragment of wild type *TMP1* in the YCp50 derivative, pEG50-WT (Figure 2.2). The construct was verified by restriction analysis with *Age*I. The presence of the *tmp1–F133* mutation and the correct orientation of the cloned *Pvu*II fragment were confirmed by *Age*I fragments of 1784 and 7876 base pairs.

The *tmp1-F126* and *tmp1-FF* alleles were placed into pEG50-WT by a different strategy since using the small *Pvu*II blunt-ended fragment had been difficult. First the *Bgl*II/*Bam*HI fragment of pEGRBS encompassing the mutation was exchanged with the corresponding *Bgl*II/*Bam*HI fragment of pTL830, a pUC derivative that contains the wild type *TMP1*gene and promoter. The *tmp1-F126* and *tmp1-FF* mutations were screened in the pTL830 constructs with their specific *Apo*I and *Age*I sites respectively.

Table 1

Description of mutants generated by site-directed mutagenesis of plasmid pEGRBS-WT and derivatives

Mutant Plasmid	Mutation	Parent Template	Screen Site [a]	Primer Sequence 5' to 3' [b,c]
pEGRBSΔloop2	Deletion: 121 to138, Insert: EL	pEGRBS-WT	+*Sac*I	AACCTGTTTCAATTG***GAGCT*** ***C***CCATTGAAATCCGTA
pEGRBS-FY	Y126F	pEGRBS-WT	+*Bsp*MI +*Apo*I[d]	ATAGTCGTCATC*GCAGGT*CT TG***AATT***TAGCACCAAAATG
pEGRBS-YF	Y133F	pEGRBS-WT	+*Age*I	TTGATCAATACCTTG*ACCGG* ***T*****AAA**GTCGTCATCGCACGT
pEGRBS-FF	Y126F Y133F	pEGRBS-FY	+*Age*I	TTGATCAATACCTTG*ACCGG* ***T*****AAA**GTCGTCATCGCACGT
pEGRBS-FS	Y126F Y133S	pEGRBS-FY	+*Age*I	GATCAATACCTTG*ACCGGT*A **GA**GTCGTCATCGCAGG
pEGRBS-SF	Y126S Y133F	pEGRBS-FF	-*Apo*I	GTCGTCATCGCAGGTCTT**AG** **A**TTTAGCACCAAAATG
pEGRBS-FYF	Y126F Y133F Insert Y130	pEGRBS-FF	+*Dra*I	GTAAAGTCGTCATC**GTA**GCA GGT*TTTAAA*TTTAGG
pEGRBS-Lma	K125D T128G C129F D130E D131A D132N T134D Q136E	pEGRBS-WT	+*Bst*BI	CAATTGATCAATACC**CTC**TC **CATC**ATAA**TTCGC*TTCGAAC*** **CC**CTTATA**ATC**AGCACCAAA ATGCCTC

[a] Restriction enzyme sites added (+) or removed (-) by the mutagenic primer, with respect to the parent template

[b] Bold type indicates mutated codons that alter an amino acid

[c] Bases in italics denote an added or deleted restriction site containing a silent mutation

[d] *Apo*I was used for screening in pEGRBS plasmids, *Bsp*MI was used in yeast shuttle vectors where the *Apo*I sites were too abundant

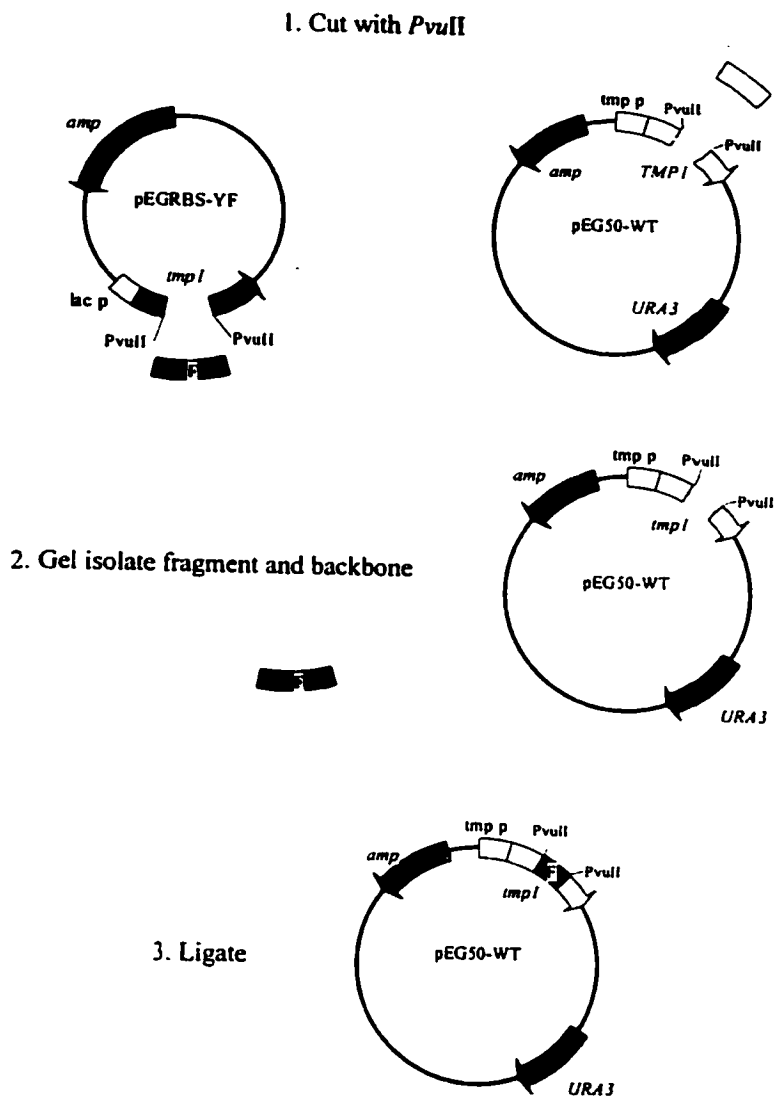


Figure 2.2. Construction of pEG50-YF. pEGRBS-YF and pEG50-WT were cut with *Pvu*II. The 370bp fragment of pEGRBS-YF and backbone of pEG50-WT were isolated from agarose gels and ligated. *E. coli* transformants were screened for the *Age*I site associated with the mutation.

Digestion with *Hind*III and *Bgl*II (expected fragments of 706 and 4586 base pairs) confirmed correct orientation as well as the presence of the *TMP1* promoter, which is missing in the donor plasmid pEGRBS.

Having thus linked the mutant *TMP1* genes to the *TMP1* promoter, the *Hind*III/*Sma*I fragment encompassing the entire *TMP1* gene and promoter was subcloned intoYCp50 cut with *Hind*III/*Nru*I (Figure 2.3). Since this disrupts the tetracycline resistance gene in YCp50, transformants that were tetracycline sensitive as well as Ura+ were picked for the screening of correct constructs. Presence of the *TMP1* gene and promoter was verified by *Hind*III/*Bgl*II restriction analysis which yielded the expected fragments of 706, 2565 and 6389 base pairs.

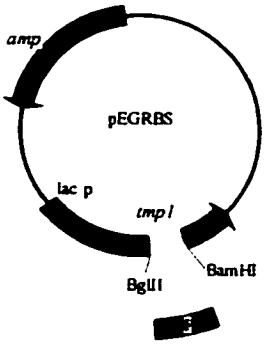
2.3. Subcloning *TMP1* Mutant Alleles into a Yeast Episomal Plasmid

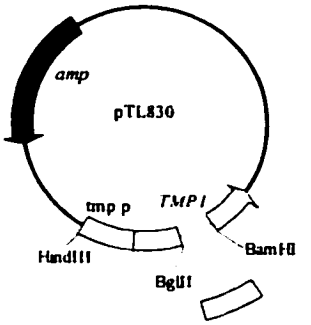
The *tmp1-F126* and *tmp1–F133* alleles were incorporated into a yeast episomal vector, pEM54, by the technique of "plasmid gap repair" in a yeast host (Raymond *et al.*, 1999; Ausubel *et al.*, 1989). First, pEM54 was cut with *Pvu*II in order to excise the 370 bp *Pvu*II fragment in *TMP1* which contains the Loop 2 region. The plasmid was recircularized with ligase, propagated in *E coli* and screened by *Hind*III and *Bam*HI digestion. The new plasmid, designated pEG54-Δ370, had a *Hind*III/*Bam*HI *TMP1*fragment of 590 bp instead of the original 960 bp fragment. It was then again linearized with *Pvu*II and transformed into a yeast *TMP1* null strain (RS5331) which harboured a mutant *TMP1* allele on centromere plasmid, either pEG50-FY (*tmp1-F126*) or pEG50-YF (*tmp1-F133*). The strategy was to let the yeast cell repair the missing

Figure 2.3. Construction of pEG50-FF.

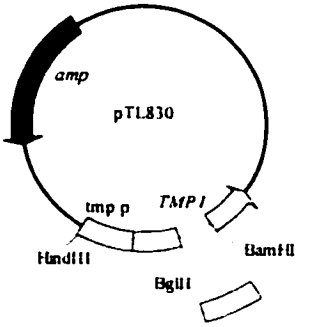
1. pEGRBS-FF and pTL830 were cut with *Bgl*II and *Hind*III.
2. The *Bgl*II/*Hind*III fragment containing the mutation was isolated on an agarose gel.
3. The fragment was ligated into the *Bgl*II/*Hind*III backbone of pTL830, (renamed pEG830-FF).
4. pEG830-FF was cut with *Hind*III and *Sma*I (blunt-end), yielding a 2.7 Kb fragment containing the mutant *TMP1* gene (the remaining backbone was digested with *Pvu* I).
5. The recipient vector, YCp50, was cut with *Hind*III and *Nru*I (blunt-end).
6. Digests (4) and (5) were mixed and ligated.
7. Transformants were selected for uracil prototrophy, and screened for tetracycline sensitivity by replica-plating.

1. Cut with *Bgl*II and *Bam*HI

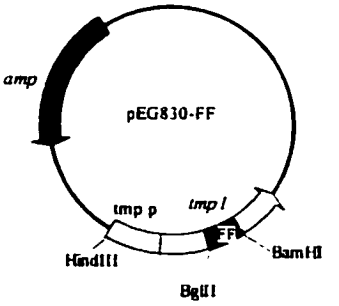




2. Gel isolate

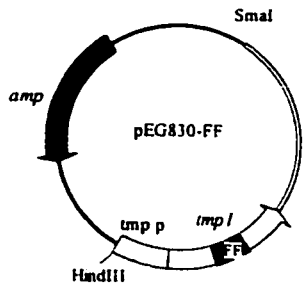


3. Ligate

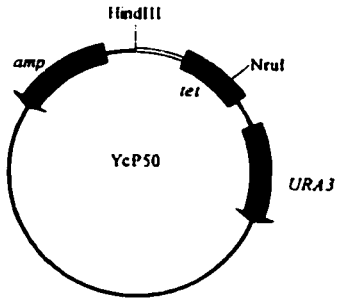


Continued.....

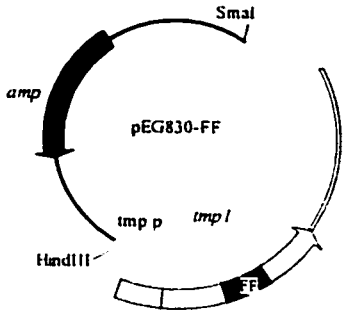
4. Cut with *Hind*III and *Sma*I

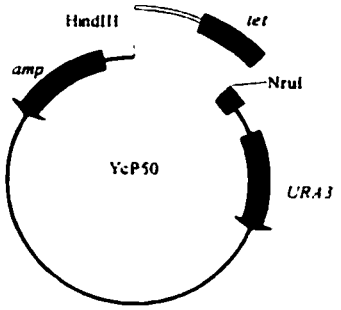


5. Cut with *Hind*III and *Nru*I

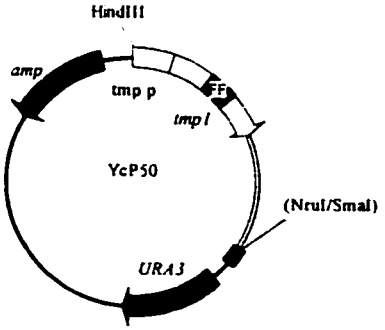


6. Mix, ligate





7. Select for Ura+, screen for tetracycline sensitivity



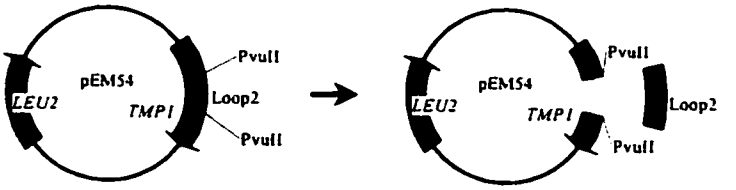
fragment of *TMP1* on pEG54-Δ370 by using the corresponding sequence on the centromere plasmid as template. Hence the gapped plasmid would be recircularized and would contain the mutation borne on the centromere plasmid (Figure 2.4). To screen for gap repair events, selection for leucine prototrophy was used since pEG54-Δ370 has a *LEU2* marker. Leucine prototrophs were pooled and cured of the pEG50-FY or pEG50-YF plasmid. Cured colonies were selected on FOA plates, since this selects against a functional *URA3* gene, the selective marker on the centromere plasmid. This also selected against leucine prototrophs that had uncut or religated pEG54-Δ370, since the loss of the centromere plasmid required that the *LEU2* plasmid must have a functional, hence repaired, *TMP1* gene. Plasmids were rescued from Leu+ /FOA^R yeast colonies into *E. coli*, propagated and analyzed by *Hind*III/*Bam*HI digestion. All plasmids retrieved had the correct *Hind*III/*Bam*HI fragment of 960 base pairs. Two isolates of each construct, designated pEG54-FY and pEG54–YF, were verified by DNA sequence analysis of both strands through the entire gap-repaired region.

Since we were unable to isolate spores of the *TMP1* null strain transformed with pEG50-FF, a different method was used to clone the double phenylalanine mutation into an episomal plasmid. The *Hind*III/*Bam*HI fragment of pEG50-FF was exchanged with the *Hind*III/*Bam*HI of pEG54-Δ370 by routine *in vitro* construction. The new construct, pEG54-FF, was screened in an *E. coli* strain (MC1066) that is auxotrophic for leucine. Selection for leucine prototrophs selected against transformants containing religated donor plasmid, pEG50-FF, since this plasmid has a *URA3* marker. Two isolates of this construct were analyzed by *Hind*III/*Bam*HI digestion followed by DNA sequencing.

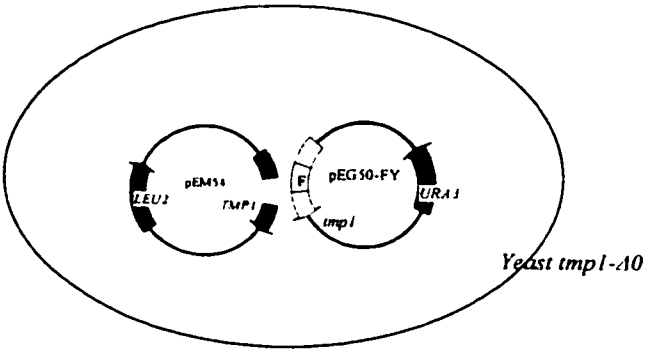
Figure 2.4. Strategy for the construction of pEG54-FY and pEG54-YF by gap repair.

1. Plasmid pEM54 was cut with *Pvu*II (to remove the Loop 2 region), religated, and propagated in *E. coli*. The new plasmid (pEG54-Δ370) was then linearized with *Pvu*II.
3. The linear plasmid was transformed into the *tmp1-Δ0* strain (haploid) already transformed with a centromere plasmid having either *tmp1-F126* or *tmp1-F133*.
4. The missing region is replaced by gap repair, using the centromere plasmid as template.
5. The mutation from the centromere plasmid has been copied into the gap repaired plasmid.

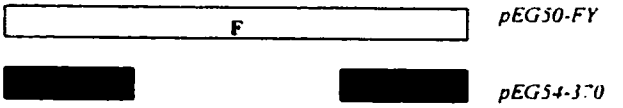
1.


pEM54
LEU2
TMP1
PvuII
Loop2
PvuII
pEM54
LEU2
TMP1
PvuII
Loop2
PvuII

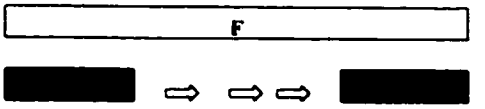
2.


pEM54
LEU2
TMP1
F
pEG50-FY
URA3
tmp1
Yeast tmp1-Δ0

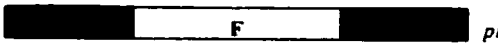
3.


F
pEG50-FY
pEG54-370

4.


F

5.


F
pEG54-FY

2.4. Subcloning the *thyA* Gene and *tmp1-S137* Allele into a Yeast Vector

The *thyA* gene was PCR amplified from plasmid pBTA (a pBR322 plasmid with a *Hind*III fragment containing *thy*A, obtained from M. Herrington) using primers with *Bam*HI floppy ends (5'AATTGGATCCATGAAACAGTATTTA3', 5'TTAAGGA TCCTTAGATAGCCACCGG3'). The PCR product (~800 bp) was cut with *Bam*HI and ligated into pYES2, also cut with *Bam*HI. The correct construct was verified by *Mlu*I digestion, which yielded the expected fragments of 6100 and 500 base pairs. The resulting plasmid, pEG-thyA, has *thyA* under the control of the yeast *GAL1* promoter, and has selectable markers for both yeast (*URA3*) and *E. coli* (Amp^R).

The *tmp1-S137* allele is a temperature sensitive allele of *TMP1* which has a serine substituted for a proline at position 137 (Taylor *et al.*, 1987). To subclone this allele into an episomal plasmid, it was PCR amplified from genomic DNA extracted from strain RS625-2B, a haploid strain which has the *tmp1-S137* allele. The primers used (5'-GAACAAGCTTCTTTCCCCTCTCG-3' and 5'-TACCGGATCCTTCTCCTTCTTTG-3') amplified the region from *Hind*III and *Bam*HI of *TMP1*, to yield a fragment of ~960 base pairs. This fragment was then exchanged with the *Hind*III/*Bam*HI fragment of pEG54-Δ370. The new construct (pEG54-S137) was confirmed by the presence of a *Hind*III/*Bam*HI fragment of 960 bp instead of the original 590 bp *Hind*III/*Bam*HI fragment of pEG54-Δ370.

2.5. Construction of a Yeast *TMP1* Knock-Out Strain

The *TMP1* locus was disrupted using a *GFP-HIS* cassette, which contains a green fluorescent protein-HIS fusion gene. The fusion gene was PCR amplified with primers that hybridize to *GFP-HIS* but have floppy ends homologous to the sequences that flank the *TMP1* ORF (primer TMP1-GFP, 5'ACAATAAAGTATCAAGGAGAGA GCTTCATAACAGAACGGTACAGGATGAGTAAAGGAGAAGAAC3'; primer HIS-TMP1, 5'GTGTATATACGTAAGTACATGATTTTGTTTCTCCTCGTGCTGTCAGC GCGCCTCGTTCAGAATG3'). The PCR product (~1.8 Kb) was transformed into a diploid strain (strain RS3695 obtained from H. Bussey), and histidine prototrophs were selected. To verify that the *GFP-HIS* fragment had recombined at the *TMP1* locus and disrupted it, genomic DNA was extracted and PCR amplified with a *GFP* primer and a *TMP1* promoter primer. The expected 1300 base pair fragment was obtained in one of two colonies tested, and not in the parental strain.

The disrupted strain (designated RS4664) was then verified by tetrad analysis. The diploid cells were incubated in 2 mL sporulation medium (1% potassium acetate, 0.005% zinc acetate) at room temperature and spores appeared after 4 or 5 days. Aliquots of 1 mL were washed 3 times with sterile water and the asci were digested for 5 minutes at 37° C with 30 µg/mL of zymolyase 20T. The tetrads were dissected on a Singer Micromanipulator and found to segregate in the expected 2:2 ratio of surviving to non-surviving spores. The disruption was then verified by transforming the diploid strain RS4664 with pEG50-WT or pEG-thyA (which both have a *URA3* marker), isolating spores that contained the plasmids (i.e. Ura+) and were also histidine prototrophs, and

showing that curing the cells of the plasmids by selecting for FOA resistance was not possible. Finally, the absence of *TMP1* in the cells was shown by PCR amplification of genomic DNA using primers specific for *TMP1* (Figure 3.11a, b, Results section).

2.6. Complementation Analysis

E. coli strain 264 transformed with plasmids containing wild type or mutant *TMP1* alleles was propagated in 2 x YT media with 100 μg/mL ampicillin and 50 μg/mL thymidine, either on agar plates or liquid medium. Complementation was tested by patching individual transformant colonies and by streaking for single colonies onto minimal medium plates without thymidine supplement. Also, *E. coli* cultures were grown to an optical density of 0.2 (600 nm) and 1 μL of cells was spotted onto minimal medium plates without thymidine. Complementation of TS deficiency was assessed by comparing the ability of the cells harbouring mutant *TMP1* alleles to grow without thymidine, using wild type *TMP1* transformants as controls.

Complementation was tested similarly in the yeast strain RS3236, except that yeasts were grown in YEPD medium supplemented with 200 μg/mL thymidylate and tested on YNBD plates supplemented with 50 μg/mL of the required amino acids, but without thymidylate.

Since the yeast strain RS5331 (*TMP1* knock-out, haploid) is unable to take up thymidylate, cell survival is dependent on the presence of a functional *TMP1* gene. The parent strain, a diploid with one *TMP1* gene deleted, was transformed with pEG-thyA using Ura+ selection and then sporulated. Spores were isolated and those that were

histidine and uracil prototrophs were presumed to have the *TMP1* deletion and pEG-thyA. This was confirmed by PCR analysis. The episomal plasmid pEG54-FF was then transformed into the haploid containing pEG-thyA, selecting for Leu+. Complementation was assessed by the ability to cure the cell of the pEG-thyA plasmid by FOA selection. The episomal plasmids pEG54-FY and pEG54-YF (generated directly in the *tmp1-Δ0* strain by gap repair) were shown to complement by curing the cells of the centromere plasmids by FOA selection (discussed in Results section).

To test complementation by the centromere plasmids pEG50-FY, pEG50-YF, and pEG50-FF, these were transformed directly into the diploid strain RS4664 and tetrads were dissected. A plasmid was considered to complement if the following 3 requirements were met: 1) more than 2 of 4 spores in most tetrads survived, 2) all spores that were His+ were also Ura+, and 3) His+ segregants could not be cured of the plasmid by FOA selection.

2.7. Generation of Chromosomal *tmp1-F126* and *tmp1-F133* Alleles

These strains were made in order to test the activity of *tmp1-F126* and *tmp1-F133* in a different genetic background and also for future use for a synthetic lethal screen (Tong *et al.*, 2001) The first step was to link the mutant allele to a *URA3* marker. *URA3* was amplified by PCR from plasmid p4348 (obtained from C. Boone) with primers 5'-CTAT CGCGAATTCGAGCTCGTTTAAACTGGA-3' and 5'-GCATCGCGAGATCTGTTTAG CTTGCCTCGT-3', using Pfu polymerase which generates blunt ends. The *URA3*

Table 2

Genotypes of yeast and *E. coli* strains used

Strain Designation	Genotype	Reference or Source
E. coli strains:		
MC1066	*hsdR*⁻ *leuB6 trpC*9830 *pyrF*674::Tn5 (*cam*ʳ) *lacX*74 *galK galU strA*ʳ	Casadaban *et al.*, 1983
RZ1032	*dut*⁻ *ung*⁻ *thi*-1 *relA spoT*1/F' *lysA*	Quantum Biotechnologies
DH5α	*supE*44 *lacU*169 (Φ80 *lacZ* ΔM15) *hsdR*1 *recA*1 *endA*1 *gyrA*96 *thi*-1	Hanahan *et al.*, 1983
264	*HfrH thi lac thy* Δ64	Belfort and Pedersen-Lane, 1984
Yeast strains:		
RS3236	*MATa tmp1-6 leu2-3,112 ura3-52 trp1::hisG tup*⁻	Munro *et al.*, 1995
RS3695	*leu2-3 ura3-52 hisΔ200 lys2-801 trpΔ902 suc2-Δ9/ leu2-3 ura3-52 hisΔ200 lys2-801 trpΔ902 suc2-Δ9*	H. Bussey
RS4664	*leu2-3 ura3-52 hisΔ200 lys2-801 trpΔ902 suc2-Δ9/ leu2-3 ura3-52 hisΔ200 lys2-801 trpΔ902 suc2-Δ9 tmp1::GFP/HIS*	This work
RS5331	*leu2-3 ura3-2 hisΔ200 lys2-801 trpΔ902 suc2-Δ9 tmp1::GFP/HIS*	This work
RS5487	can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0	C. Boone
RS5510	can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 tmp1-F126::URA3	This work
RS5511	can1Δ::MFA1pr-HIS3-MFα1pr-LEU2 his3Δ leu2Δ0 ura3Δ0 met15Δ0 lys2Δ0 tmp1-F133::URA3	This work
RS625-2B	MATa leu2-3,112 his4 cdc21	R. Storms

Table 3

Relevant features of plasmids used

Plasmid	Relevant Features	Parent plasmid	Mutant Derivatives[g]
pEGRBS-WT[a]	amp^R, pBR322 ori, *TMP1*, *lacZ* promoter	pTL830	pEGRBS-FY, pEGRBS-YF, pEGRBS-FF, pEGRBS-SF, pEGRBS-FS, pEGRBS-FYF, pEGRBS-Lma, pEGRBS-Δloop2
pTL830[b]	amp^R, pBR322 ori, *TMP1*	pUC9 (Vieira and Messing, 1982)	pEG830-FY, pEG830-FF
pEG50-WT[c]	amp^R, pBR322 ori, *TMP1*, *URA3*, *CEN4*, *ARS1*	YCp50 (Rose *et al.*, 1987)	pEG50-FY, pEG50-YF, pEG50-FF
pEM54[d]	amp^R, pBR322 ori, *TMP1*, *LEU2*, *REP3*, *ARS-2μm*	YEp13 (Broach *et al.*, 1979; Lagosky *et al.*, 1987)	pEG54-FY, pEG54-YF, pEG54-FF, pEG54-S137 pEG54-Δ370
pEG-thyA[e]	amp^R, pBR322 ori, *URA3*, *ARS-2μm*, *GAL1* promoter	pYES2	
pEM89[f]	amp^R, pBR322 ori, *TMP1*, *TRP1*, *ARS1*, fl *ori*	pEMBLYr25 (Baldari and Cesareni, 1985; Dente *et al.*, 1983)	pEG89-ΔE2a-K117

[a] PCR fragment of *TMP1* (from the start codon to the *Bgl*II site), exchanged for the *Hind*III to *Bgl*II fragment of pTL830

[b] genomic *TMP1* fragment (*Hind*III to second *Bgl*II site) ligated into *Hind*III to *Bam*HI backbone of pUC9

[c] *Hind*III to *Sma*I fragment of pTL830 ligated into *Hind*III to *Nru*I backbone of YCp50

[d] *Hind*III to *Sma*I fragment of pTL830 ligated into *Hind*III to *Pvu*II backbone of YEp13

[e] PCR fragment of *thyA* amplified from pTBA (Belfort *et al.*, 1983) and ligated into *Bam*HI backbone of pYES2

[f] *Hind*III to *Sma*I fragment of pTL830 ligated into *Hind*III and *Sma*I sites of the *lacZα* segment of pEMBLYr25

[g] Constructed in this work

Table 4

Nomenclature of mutants of yeast TS

Name by convention	Abbreviation	Allele designation
TSΔ124-136,F117K[a]	TSΔEUK2a-K117	*tmp1-ΔE2a-K117*
TSΔ121-138::EL[b]	TSΔloop2	*tmp1-Δloop2*
TS-Y126F	TS-FY	*tmp1-F126*
TS-Y133F	TS-YF	*tmp1-F133*
TS-Y126F,Y133F	TS-FF	*tmp1-FF*
TS-Y126F,Y133S	TS-FS	*tmp1-FS*
TS-Y126S,Y133F	TS-SF	*tmp1-SF*
TS-Y126F,Y133F,::Y130	TS-FYF	*tmp1-FYF*
TS-K125D,T128G,C129F,D130E, D131A,D132N,T134D,Q136E	TS-Lma	*tmp1-Lma*
TS-G137S	TS-S137	*tmp1-S137*

[a] Amino acids 124 to 136 are deleted, F117 is replaced by K
[b] Amino acids 121 to 138 are deleted, E,L are inserted

fragment was then ligated into pEG50-FY or pEG50-YF, at the unique, blunt-end *Sna*BI site about 50 base pairs downstream of the *TMP1* stop site. The construct was verified by restriction analysis and then PCR amplified using primers that hybridize to the sequences that flank the *TMP1* coding region. The PCR fragment, ca. 2.8 Kb encompassing the *TMP1* coding region and *URA3*, was used for gene replacement by transforming into yeast strain RS5487 (obtained from C. Boone), with selection for Ura+ colonies. Ura+ transformants were then screened for the mutation by amplifying the *TMP1* gene and digesting the fragment with *Apo*I or *Age*I, the restriction sites closely linked to the F126 and F133 mutations respectively.

2.8. Gel Filtration Chromatography

This method was used to determine the molecular weights of the deletion mutants TSΔEUK2a and TSΔEUK2b. A 1x100 cm column was packed with Sephacryl S-200 (Pharmacia) to a bed volume of 74.5 mL and equilibrated with buffer (20 mM KPO_4, pH 7, 0.2 mM EDTA) at a flow rate of 20 mL/hour. The void volume (V_o) was measured as the elution volume of a 1 mL sample of Dextran blue (2 mg/mL) from the start of loading to the middle of the elution peak. A standard curve was obtained from 1 mL samples of yeast alcohol dehydrogenase (5 mg/mL), bovine serum albumin (2 mg/mL), horse cytochrome *c* (2mg/mL) and TSΔEUK1 (4.5 mg/mL). Elution volumes (V_e) were measured and known molecular weights (M_r) were plotted as a function of corresponding V_e/V_o. After establishing the standard curve, TSΔEUK2a and TSΔEUK2b (4.5 mg/mL) were run separately but with internal standards of cytochrome *c*. M_r for the mutant

proteins was extrapolated from the standard curve generated with EXCEL (Microsoft) and fitted to single exponential decay.

2.9. Tritium Release Assays for Thymidylate Synthase

2.9.1. Assays on Crude Protein Extracts of *E. coli*

To prepare *E. coli* crude protein extract, 50 mL cultures were grown overnight in 2 xYT media with ampicillin (100 μg/mL) and thymidine (50 μg/mL). Cells were harvested by centrifuging at 6000 rpm (4969 x *g*) for 10 minutes in a Beckman JA-17 rotor, and resuspended in 5 mL of 20 mM Tris-HCl pH 7.4, 0.2 mM EDTA, 1 mM PMSF. Cells were lysed by sonicating with a Fisher Scientific 550 Sonic Dismembrator, using a 0.5 inch probe, setting of 3, 10 bursts of 10 seconds on, 10 seconds off, on ice. Cellular debris were removed by twice centrifuging at 10500 rpm (15188 x g), at 4°C for 20 minutes in a JA-17 rotor. Cell extracts were aliquoted and stored at –80°C.

For the assays, 100 μL of crude protein (concentrations ranging from 1 to 4 μg/μL) was mixed with 70 μL of assay buffer (30% glycerol, 15 mM β-mercaptoethanol, 40 mM $MgCl_2$, 3 mM EDTA, Tris-HCl) and 10 μL of THF stock (2 mM THF from Sigma, in 30 mM $NaHCO_3$, 15 mM HCHO, 100 mM β-mercaptoethanol). Reactions were started by adding 20 μL of 1 mM dUMP (prepared by mixing 200 μL of 1 mM dUMP with 5 μL of [5-^{3}H]-dUMP (purchased from ICN). The assays were always carried out at 25°C. Reactions were quenched by adding 500 μL of 12% activated charcoal powder in 0.1N HCl and placing on ice for at least 30 minutes. The charcoal was

pelleted by centrifuging at 13000 x g for 15 minutes. Supernatants were clarified by loading into Eppendorf tubes that were pierced on the bottom and packed with a GF/A filter. Filtrates were collected in a second Eppendorf tube by centrifuging 30 seconds and DPMs were counted with a Rackbeta Model 1215 scintillation counter.

2.9.2. Assays on Permeabilized Cells

Cultures of yeasts and *E. coli* were grown in rich media (YEPD for yeast, 2xYT with 100 μg/mL ampicillin for *E. coli*), to log phase (O.D. = 0 .5). Cells were harvested in 20 mL batches, by centrifuging at 6000 rpm (4960 x *g*) in a Beckman JA-17 rotor, 7 minutes at 4°C. Pellets were resuspended in 1 mL ice-cold water, transferred to Eppendorf tubes, centrifuged at 13000 x *g* (30 seconds for yeast, 1 minute for *E. coli*), frozen in liquid nitrogen after removing the water, and stored at -80°C.

The assays were done as described above, except that 2 to 3 cell pellets were resuspended in 100 μL of Buffer A (20% glycerol, 10 mM β–mercaptoethanol, 10 mM $MgCl_2$, 1 mM EDTA, 20 mM Tris-HCl pH 7.4, 3% Brij), followed by the addition of 80 μL of Buffer B (20 mM $MgCl_2$, 2 mM EDTA, 250 mM Tris-HCl pH 7.4) and 10 μl of THF stock (2 mM THF in 30 mM $NaHCO_3$, 15 mM formaldehyde, 100 mM β–mercaptoethanol). For yeast, an alternate method used for permeabilizing the cells was to thaw the cells, and subject them to 2 more rounds of freezing in liquid N_2 and thawing at room temperature for 15 minutes each. When this method was used, Brij was omitted from Buffer A.

2.10. Immunoblot Analysis

Protein extracts were prepared by harvesting 50 mL yeast or *E. coli* cultures grown to log phase, resuspending the cells in 1 mL SDS-PAGE loading buffer with 1 mM PMSF, boiling for 5 minutes and centrifuging at 16000 x *g* for 15 minutes to remove insoluble material. The proteins were resolved by electrophoresis on 12.5% polyacrylamide gels, which were then equilibrated in transfer buffer (20% methanol, 192 mM glycine, 25 mM Tris, pH 8 .3) for 30 minutes. The proteins were transferred from gels to nitrocellulose membranes in a Bio-Rad Electroblotter apparatus, overnight at 4°C at a setting of 350 mA. The membranes were blocked in TBST (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 5% milk, for 1 hour and then treated for 3 hours with affinity-purified anti-TS antibody (prepared by Dr. Pak Poon) at a dilution of 1/400. After three rinses in TBST-5% milk, the membranes were treated with alkaline phosphatase conjugated goat anti-rabbit immunoglobulin (Bio-Rad) diluted 1/3000 in TBST-5% milk. Membranes were washed twice with TBST-5% milk, once with TBS, once with color development buffer (100 mM NaCl, 5 mM $MgCl_2$, 100 mM Tris, pH 9.5) and then incubated with 0.03% nitro blue tetrazolium (3% stock solution prepared in 70% dimethylsulfoxide) and 0.015 % 5-bromo-4-chloro-3-indolyl phosphate (2.5% stock solution prepared in 100% dimethylsulfoxide) in color development buffer. When the color was sufficiently developed (typically 10-30 minutes) reactions were stopped by thoroughly rinsing the nitrocellulose membranes with water.

2.11. Other Procedures

Transformation of *E. coli* was routinely done by the calcium chloride method (Maniatis *et al.*, 1989). For rescuing plasmids from yeast, electroporation (Maniatis *et al.*, 1989) was used since a high transformation efficiency was required.

Yeast transformations were done according to the method of Schiestl and Gietz (1989) except that lithium chloride was used instead of lithium acetate, and carrier DNA was not sonicated.

For screening and analysis, plasmid DNA was extracted from *E. coli* with a Wizard Miniprep Kit (Promega). DNA used for constructs was prepared by the alkali lysis method described in Maniatis *et al.* (1989). Restriction enzymes and modifying enzymes (ligases, polymerases, phosphorylases) were purchased from MBI and reactions were performed according to the included protocols.

Plasmid rescue from yeast and extraction of yeast genomic DNA was done as described by Ausubel *et al.* (1989).

PCR amplifications were done with Taq polymerase (MBI) when the DNA was used for screening purposes and with high fidelity PfuTurbo polymerase (MBI) when the DNA was used for construction or gene replacement. The reactions were set up according to the protocols included with the enzymes and done in a Techgene Thermal Cycler. Typically, 300 ng of genomic DNA or 10 ng of plasmid DNA were used as template, primers were diluted to 0.5 μM, and the annealing temperature was set at 5° C below the primer T_m.

Protein concentrations were estimated following the protocol included with the Bio-Rad Protein Assay Kit (Bradford, 1976) and BSA was used for the standard curve.

DNA sequencing was done at the Concordia Center for Structural and Functional Genomics with a Beckman CEQ 2000 XL DNA Analysis System.

2.12. Culture Media

E. coli cultures were grown in 2 x YT media consisting of 1.6 % Bacto-tryptone, 1.0% yeast extract and 0.5% NaCl, adjusted to pH 7, with 2% agar added for plates. Plasmid selection was maintained with 100 μg/mL ampicillin, and medium for thymidine auxotrophs was supplemented with 50 μg/mL thymidine. Thymidine prototrophs were tested on minimal medium consisting of 10.5 g/L dibasic potassium phosphate, 4.5 g/L monobasic potassium phosphate, 1 g/L ammonium sulfate, 0.5 g/L sodium citrate, 0.2% glucose and 1mM magnesium sulfate, supplemented with 2 μg/mL thiamine and 100 μg/mL ampicillin for plasmid selection.

Yeast cultures were grown in YEPD medium consisting of 1% yeast extract, 2% peptone, and 2% dextrose, supplemented with 200 μg/mL deoxythymidine monophosphate (Sigma) if the strain was Tmp- and permeable to dTMP (*tup*- strains). Selection media (YNBD) consisted of 1.75 g/L yeast nitrogen base (Difco) without amino acids, 5 g/L ammonium sulfate, and 2% dextrose, supplemented with 50 μg/mL of the required amino acids and 20 μg/mL uracil when necessary.

FOA plates were prepared by filter-sterilizing a stock solution containing 2 mg/mL FOA (Sigma) and adding to an equal volume of 2% yeast extract, 4% peptone,

4% dextrose, and 4% agar. Hydroxyurea plates were prepared similarly, except that the hydroxyurea stock solution was diluted with sterile water to give final concentrations ranging from 5 mM to 200 mM.

3. RESULTS

3.1. Deletion Mutations in Loop 2

In a previous study we altered Loop 2 of yeast thymidylate synthase by removing the eukaryotic inserts such that the modified enzyme mimicked TS from prokaryotes (Munro *et al.*, 1999). Mutant *tmp1-ΔEUK2a* was engineered to mimic Loop 2 from phage T4 while *tmp1-ΔEUK2b* was made to resemble that of *E. coli*. These modifications of yeast TS completely abolished activity. The mutant enzymes no longer complemented TS deficiency either in yeast or in *E. coli*, and showed no activity when assayed *in situ* or *in vitro*.

3.1.1. Quaternary Structure of TSΔEUK2a and TSΔEUK2b

Since Loop 2 is located at the dimer interface of the two TS subunits, we hypothesized that disturbing its structure might impede or prevent the formation of stable dimers. To test this, the purified mutant enzymes were passed through a gel filtration column to compare their relative masses to those of known standards. Figure 3.1 shows that TSΔEUK2a and TSΔEUK2b have a relative mass (M_r) of ca. 35 kDa, while TSΔEUK1, a mutant enzyme that retains some activity, has a M_r of around 73 kDa, the expected mass of a TS dimer. These results suggest that indeed the altered structure of Loop 2 in the mutants shifts the equilibrium toward the monomeric form.

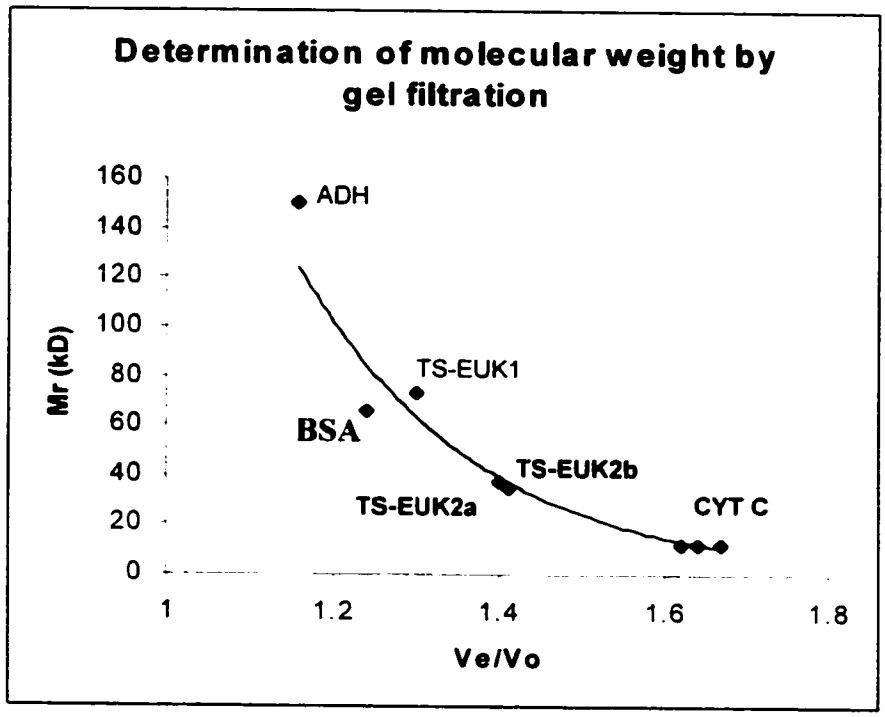


Figure 3.1: Determination of molecular weight of mutant TS by gel filtration chromatography. Left to right: yeast alcohol dehydrogenase (M_r = 150000), bovine serum albumin (M_r = 66000), TSΔEUK1 (M_r = 73000), TSΔEUK2a (M_r = 37000); TSΔEUK2b (M_r = 35000); horse cytochrome c (M_r =12400) (3 samples). Molecular weight for TSΔEUK1 was determined in a previous experiment. M_r for TSΔEUK2a and TSΔEUK2b were calculated by fitting to the standard curve shown above.

3.2. Generation of New Loop 2 Mutants

The aim of this study is to assess the role of the eukaryotic insert of Loop 2 in yeast TS. We attempt to identify what may be key residues in this insert and determine their importance in enzyme function.

To this end, a set of mutants was generated using as vector a pUC derivative which had been previously engineered to optimize expression in *E. coli* (E. Munro, Master's Thesis). This plasmid, designated pEGRBS, insures high gene copy number, high expression because *TMP1* is under the control of the *lac Z* promoter, and efficient translation resulting from an *E. coli* ribosomal binding site which was inserted near the start codon. The reason for choosing this vector was twofold: high expression of the mutant proteins in *E. coli* would provide a quick and sensitive complementation assay that would serve as a screening system for inactivated enzymes, and this vector could eventually be used for protein expression for purification purposes if necessary.

Mutagenesis was carried out as described in Materials and Methods. For point mutations, each primer contained the desired mutation as well as a second, silent mutation that would either introduce a novel restriction enzyme site or eliminate an existing one. This provided a convenient screening and identification strategy for the various mutant alleles (Table 5, Figure 3.2). For the large deletion mutations, the mutagenic primer was composed of the sequences flanking the region to be deleted so as to force it out and exclude it from the newly synthesized strand.

Table 5

Restriction enzyme analysis of plasmids harbouring *TMP1* mutant alleles

Plasmid	Template [a]	Screen Site	Fragments (base pairs) [b]
pEG89-E2a-K117	pEG89-EUK2a	-*Eco*RI	~4000, 1500, 1400, 1000 (3500, 1500, 1400, 1000, 500)
pEGRBS-Δloop2	pEGRBS-WT	+*Sac*I (with *Hind*III [c])	4508, 379 (4932)
pEGRBS-FY	pEGRBS-WT	+*Apo*I	2720, 909, 511, 365, 306, 121 (2720, 909, 817, 365, 121)
pEGRBS-YF	pEGRBS-WT	+*Age*I	3148, 1784 (4932)
pEGRBS-FF	pEGRBS-FY	+*Age*I	3148, 1784 (4932)
pEGRBS-FS	pEGRBS-FY	+*Age*I	3148, 1784 (4932)
pEGRBS-SF	pEGRBS-FF	-*Apo*I	2720, 909, 817, 365, 121 (2720, 909, 511, 365, 306, 121)
pEGRBS-FYF	pEGRBS-FF	+*Dra*I	2717, 1507, 692 (4221, 692)
pEGRBS-Lma	pEGRBS-WT	+*Bst*BI (with *Hind*III [c])	4525, 407 (4932)

[a] Plasmid used for site-directed mutagenesis
[b] Fragment sizes of the template are shown in parentheses
[c] *Hind*III was used in conjunction with the screening enzyme to facilitate the comparison between the new construct and the template plasmid

Figure 3.2. Restriction enzyme analysis for screening mutants generated on pEGRBS plasmids.

Lanes 1 and 2 represent 2 isolates of each mutant, lane 3 represents the template DNA from which the mutant was made.

A. *Apo*I digest of pEGRBS-FY (1,2) and pEGRBS-WT (3)

B. *Age*I digest of pEGRBS-YF (1,2) and pEGRBS-WT (3)

C. *Age*I digest of pEGRBS-FF (1,2) and pEGRBS-FY (3)

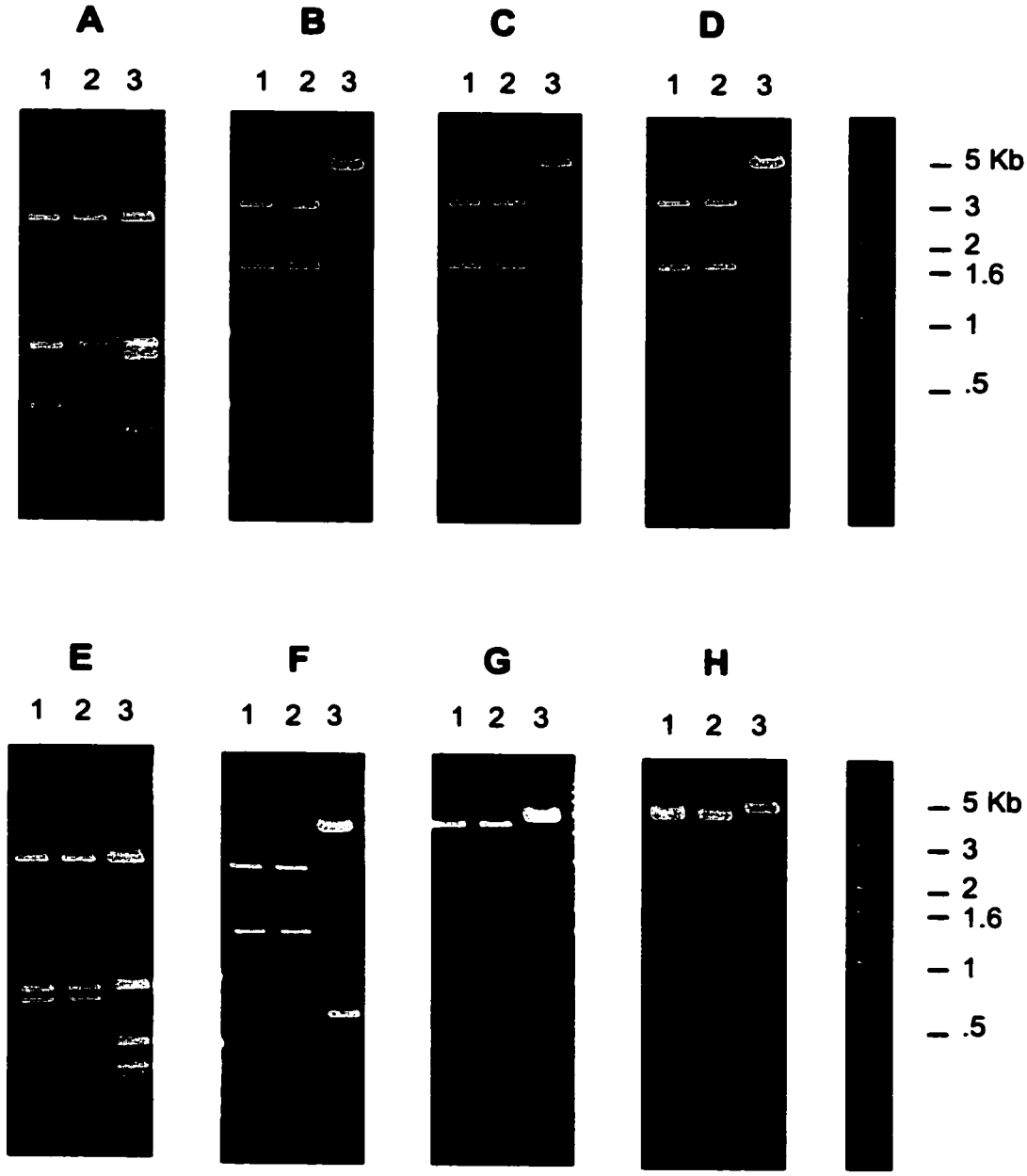
D. *Age*I digest of pEGRBS-FS (1,2) and pEGRBS-FY (3)

E. *Apo*I digest of pEGRBS-SF (1,2) and pEGRBS-FF (3)

F. *Dra*I digest of pEGRBS-FYF (1,2) and pEGRBS-FF (3)

G. *Sac*I/*Hind*III digest of pEGRBS-Δloop2 (1,2) and pEGRBS-WT

H. *Bst*BI/*Hind*III digest of pEGRBS-Lma (1,2) and pEGRBS-WT


A
B
C
D
1 2 3
1 2 3
1 2 3
1 2 3
5 Kb
3
2
1.6
1
.5
E
F
G
H
1 2 3
1 2 3
1 2 3
1 2 3
5 Kb
3
2
1.6
1
.5

Two isolates of all mutants were sequenced through the region of Loop 2 and adjacent sequences, top and bottom strand, to confirm the presence of the desired mutations (Figure 3.3).

3.2.1. Deletion of the Entire Loop 2 Region

When Loop 2 was engineered in mutants *tmp1-ΔEUK2a* and *tmp1-ΔEUK2b*, the effect was to decrease its size but enough residues were retained to form a small loop between the flanking aligned helices. We considered the possibility that this truncated loop remnant might create steric hindrance and prevent the subunits from interacting properly at their ß-sheet interface. We reasoned that we might produce a functional mutant by simply removing the entire loop and replacing it with a small turn between the helices. To achieve this, mutant *tmp1-Δloop2* was generated, in which residues 121 to 138 are removed and replaced by a glutamate and a leucine. This deletion mutant was made from the plasmid pEGRBS-WT serving as template, and was screened by its novel *Sac*I site.

As mentioned previously, TSΔEUK2a and TSΔEUK2b mimic prokaryotic enzymes, in which Loop 2 projects toward the dimer interface. By contrast, in the eukaryotes, Loop 2 is folded back on its subunit and "caps" a region of the protein which is exposed in the prokaryotic protein. One of the residues of this region, phenylalanine 117, is conserved in the eukaryotes but is replaced by a polar residue in the prokaryotes.

We attempted to restore activity to TSΔEUK2a by replacing phe117 by lysine, a residue which appears at this position in several prokaryotes. This mutation was made

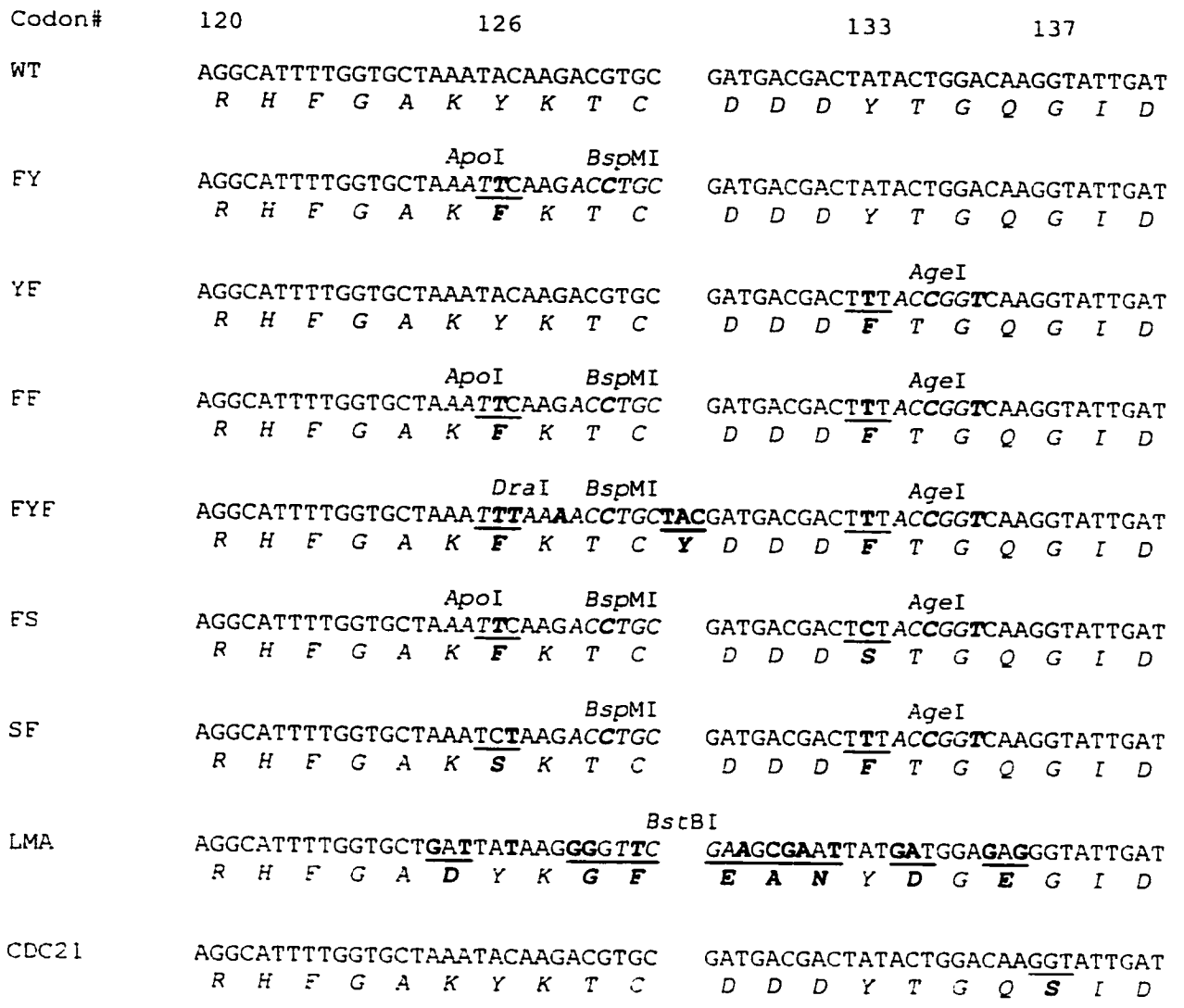


Figure 3.3. Sequences of mutants as determined by automated sequence analysis.

All sequences were determined by a Beckman CEQ 2000 XL DNA Analysis System. Bases that differ from the wild type sequence are shown in bold print. New restriction enzyme sites are shown in italics, and the restriction enzymes are indicated above the sites. Altered codons resulting in amino acid substitutions are underlined. Amino acid sequences are shown beneath the DNA sequences.

from pEG89-ΔEUK2a (a yeast episomal plasmid) and was screened by the loss of an *Eco*RI site. The new plasmid was designated pEG89-ΔE2a-K117.

3.2.2. Point Mutations in Loop 2

Inspection of an amino acid sequence alignment of TS from 29 organisms (Figure 3.4) revealed that tyrosine 126 is conserved in all eukaryotes, while tyrosine 133 is conserved in all but one, *T. brucei*, where it is replaced by a serine residue. Because these tyrosines are conserved and since they contain a hydroxyl in their side chain that can participate in a number of intra- or inter-molecular interactions, we chose these residues as the target of our study.

The first two point mutants which were generated, designated as *tmp1-F*126 (TS-FY) and *tmp1-F133* (TS-YF), had phenylalanine substitutions at positions 126 and 133 respectively. The rationale was to eliminate the potentially important hydroxyl group with minimal alteration to the structure and size of the tyrosine residue. Mutants containing either phenylalanine 126 or 133 were generated using as template wild type *TMP1* on the plasmid pEGRBS-WT, and were identified by their novel restriction enzyme sites, *Apo*I and *Age*I respectively.

A third mutant, designated *tmp1-FF* (TS-FF), was made in which both tyrosine 126 and 133 were substituted with phenylalanine, to test whether the enzyme would tolerate the removal of both tyrosine hydroxyls present in the eukaryotic insert. The *tmp1-F*126 mutant served as template since it already had phenylalanine at position 126, and the screening site was a new *Age*I site.

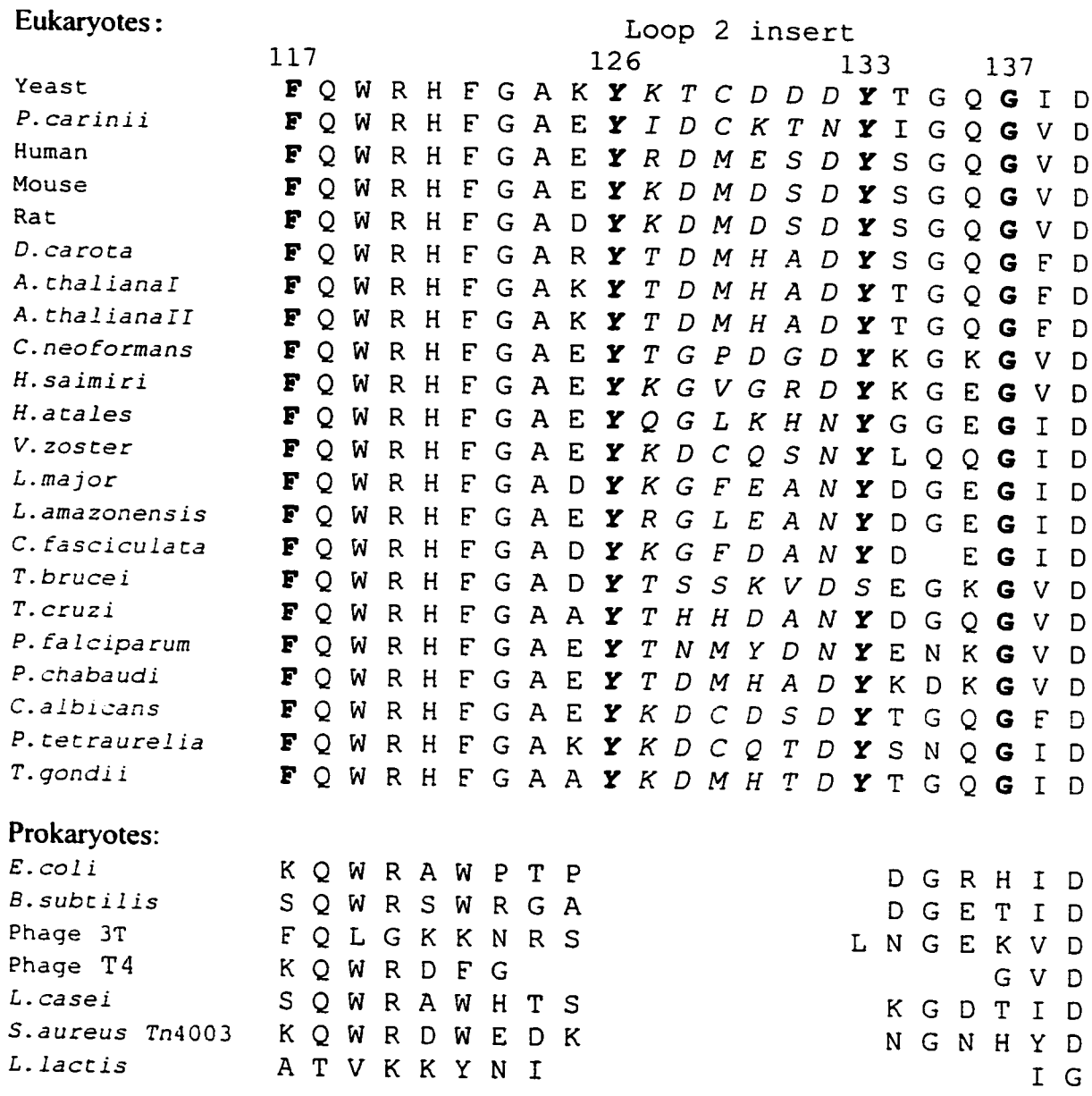


Figure 3.4: Amino acid sequence alignment of Loop 2 in eukaryotes and prokaryotes. Loop 2 spans residues 120 to 139. The 8 residue insert of the eukaryotes is in italics. The conserved residues examined in this study, F117, Y126, Y133 and G137, are in bold print.

To test if the conserved hydroxyls at positions 126 and 133 could be provided by a residue other than tyrosine, we constructed a pair of mutants having both tyrosine 126 and 133 removed, but substituted with serine at one position and phenylalanine at the other. The mutant with phe126 and ser133 (*tmp1-FS*, TS-FS) was made from *tmp1-F126* template and was screened with a new *Age*I site. For the ser126/phe133 mutant (*tmp1-SF*, TS-SF) the template was *tmp1-FF* and the screen was the removal of an *Apo*I site.

To determine whether the precise positioning of tyrosine was important, another mutant was made in which both tyrosine126 and 133 were replaced by phenylalanines, but a single tyrosine was inserted at position 130. This mutant, designated *tmp1-FYF* (TS-FYF), was generated from *tmp1-FF* template and was screened with a novel *Dra*I site.

3.2.3. Replacement of the Insert Sequence by the *L. major* Sequence

To test the importance and specificity of the tyrosine environment to enzyme function, one last mutant was constructed in which the eukaryotic insert specific to the yeast TS sequence was removed and replaced by that of *Leishmania major*. That is, the sequence **KYKTCDDDYTGQ** was replaced by **DYKGFEANYDGE**, thus mimicking Loop 2 of *L. major*. This mutant was made from wild type template, with a primer homologous to both sequences flanking the region to be removed, and containing the corresponding *L. major* sequence. The correct construct, designated *tmp1-Lma* (TS-Lma), was screened with a novel *Bst*BI site.

3.3. Analysis of Mutant Enzymes in *E. coli*

The behaviour of the yeast mutants in *E. coli* was assessed in a strain (strain 264) that has a large deletion in its *thyA* gene, which codes for thymidylate synthase. The use of a deletion mutant ensured that there would be no reversion to wild type in the host chromosomally expressed TS, and that the results would not be affected by a mutant TS other than our intended mutants. Two isolates of each mutant gene were transformed into this strain and tested for complementation, enzyme activity and TS protein levels.

3.3.1. Complementation

Complementation was assessed by the cells' ability to grow without thymidine. Thymidine enables *E. coli* to survive without thymidylate synthase activity because *E. coli* cells have kinases that convert the nucleoside to thymidylate. Cells transformed with the mutant genes were always grown with thymidine supplement and then patched or spotted onto plates without thymidine. This was to minimize selective pressure for wild type revertants in our point mutants. Complementation was tested both by patching transformant colonies and spotting log-phase cultures onto minimal media plates that did not contain thymidine (Figures 3.5 and 3.6).

The deletion mutants *tmp1-ΔE2a-K117* and *tmp1-Δloop2* failed to complement as assessed by failure to grow in the absence of thymidine (Figure 3.5, C-3 and F-3). No difference in patch or spot size or rate of growth was observed between cells containing *tmp1-F126*, *tmp1-F133*, *tmp1-Lma* and the wild type *TMP1*. In contrast, none of the

Figure 3.5. Complementation results in *E. coli*.

Strain 264 (*thyA* deletion strain) was transformed with various plasmids. Transformants were selected on rich media plates (2 x YT) supplemented with thymidine and colonies were then patched onto minimal media without thymidine.

A. pEGRBS plasmids (pUC): 1) WT, 2) FS, 3) SF, 4) FYF

B. pEGRBS plasmids: 1) WT, 2) FY, 3) YF, 4) FF

C. pEGRBS plasmids: 1) WT, 2) Lma, 3) Δloop2

D. pEG50 plasmids (centromere): 1) WT, 2) FY, 3) YF, 4) FF

E. pEG54 plasmids (episomal): 1) WT, 2) FY, 3)YF, 4) FF

F. Episomal plasmids: 1) pEM54 (WT), 2) pEG54-S137, 3) pEG89-E2a-F117, 4) pEG-thyA

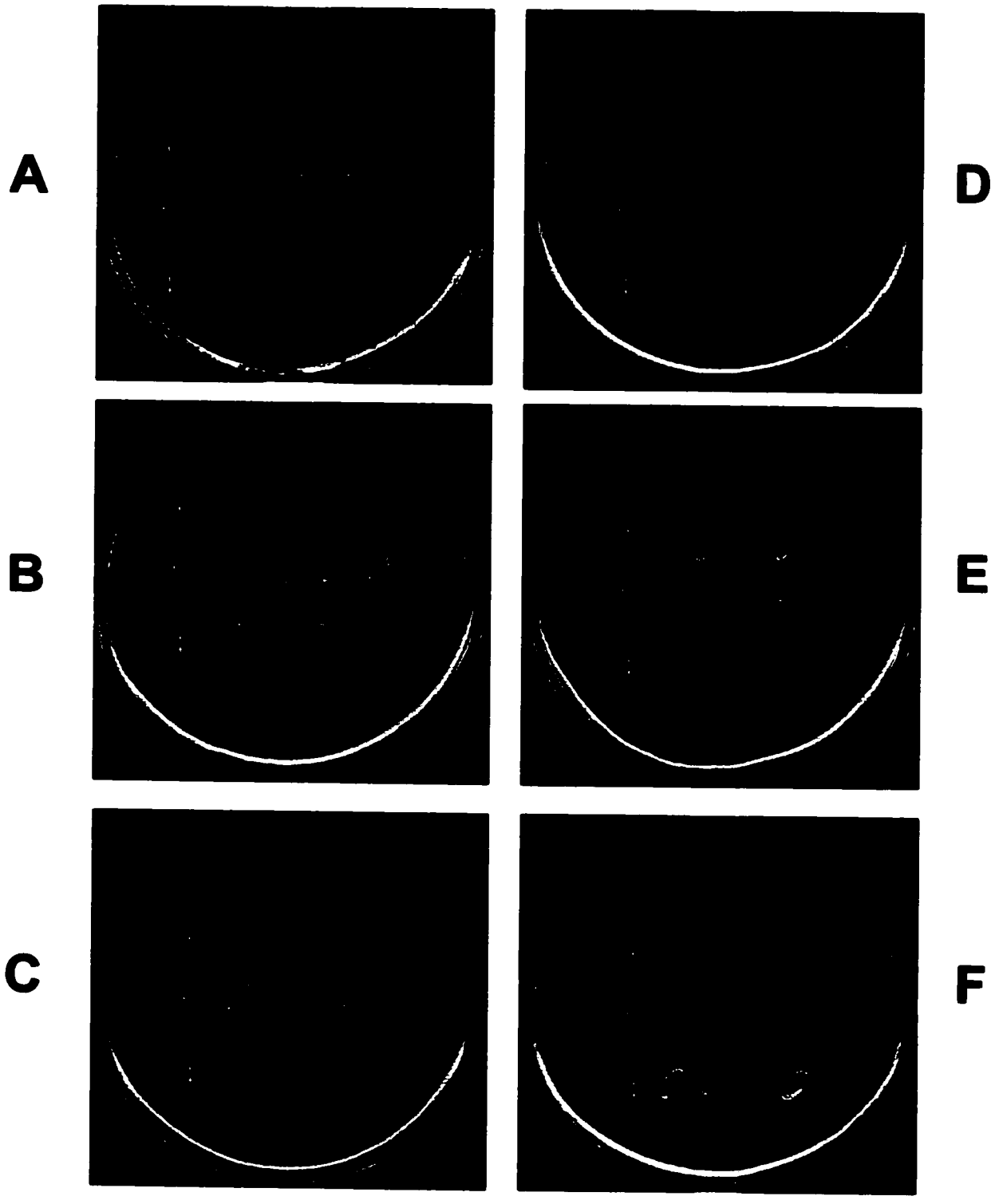
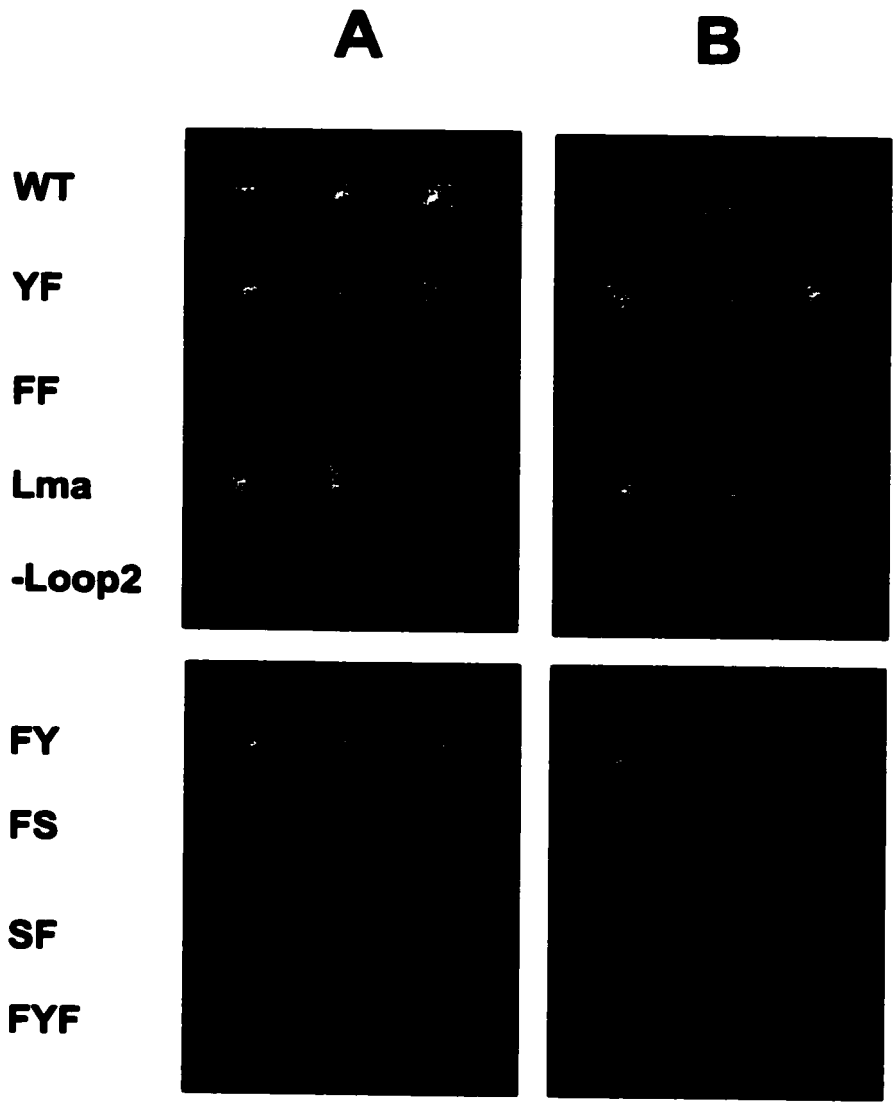

A
D
B
E
C
F

Figure 3.6. Complementation in *E. coli* with pEGRBS plasmids (pUC).

Strain 264 (*thyA* deletion) was transformed with *TMP1* wild type or mutant alleles expressed from a high copy plasmid, pEGRBS. Cells were grown in 2 x YT liquid media with thymidine until they reached an optical density (at 600 nm) of 0.2. Spots of 1 μl of culture were then deposited onto minimal media plates without thymidine and incubated at 37°C. Panels A and B represent two separate isolates of each mutant.


A
B
WT
YF
FF
Lma
-Loop2
FY
FS
SF
FYF

mutants with both tyrosines substituted (*tmp1-FF*, *tmp1-FS*, *tmp1-SF*, *tmp1-FYF*) were able to complement the *thyA* deletion.

3.3.2. Thymidylate Synthase Activity Assays

Although the active mutants (TS-FY, TS-YF, TS-Lma) showed no visible difference with wild type *TMP1* in their ability to complement TS deficiency, we wanted to determine whether enzyme activity was the same in all three mutants and how it compared to that of the wild type. As well, we were interested in testing whether the inactive mutants had any detectable level of activity.

TS activity of the mutant enzymes was determined using a tritium release assay in permeabilized *E. coli* cells transformed with the mutant alleles. This assay uses a substrate (dUMP) which is labeled with a tritium atom at position C5. The first step of the TS enzymatic conversion of dUMP to dTMP is the removal of the C5 hydrogen, which is released into the bulk solvent. This radiochemical assay is thus a measurement of the first step of the TS-mediated reaction.

The assays were performed on the same TS deletion *E. coli* strain transformed with the pEGRBS plasmids used for the complementation analysis. The cells were grown in media supplemented with thymidine in order to support growth of the non-complementing mutants. The first set of assays was done using 100 μg of crude protein extract incubated with excess substrate (dUMP) and cofactor (MTHF) for one hour. Results in Figure 3.7 show that surprisingly, only the wild type yeast TS had detectable activity. None of the mutants had any activity significantly above background levels of

the assay, regardless of their ability to complement TS deficiency in the host cell. To avoid possible damage by sonication or by proteases to the mutant proteins during the extraction procedure, assays were repeated with whole cells permeabilized with Brij detergent. As was the case with the protein extract assays, mutant enzyme activity was not detectable when the assays were done *in situ* (Figure 3.7).

3.3.3. Expression Levels of Mutant TS

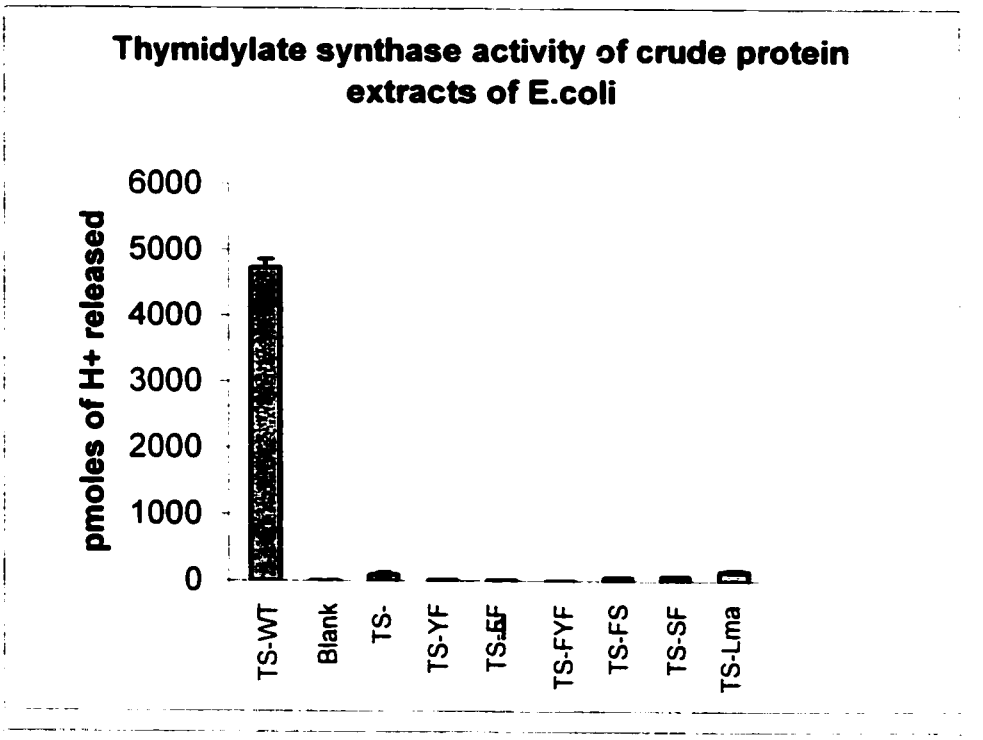
To test whether the dramatic loss of activity was a result of unstable protein products, the amount of mutant protein present in the cells was determined by immunoblot analysis. Crude protein extracts were prepared from aliquots of the *E. coli* cultures used for the enzymatic assays and immunoblotted with antibodies specific for yeast thymidylate synthase. Figure 3.8 panel A, a comparison of TS levels between wild type TS and mutants with point mutations, shows that all mutant enzymes were expressed and that levels were about the same as wild type TS. Expression of the deletion mutants TS-Δloop2 and TS-ΔE2a-Kll7 is shown in panel B, in which the gel was more finely resolved to verify the difference in size between the wild type and mutant TS. Although the mutant protein bands are lighter than the wild type band, this was due to the fact that less total protein was loaded on the gel for these samples, as verified by a Ponceau stain of the nitrocellulose membrane (not shown). In this gel, the TS bands are resolved into two species, one migrating slightly more slowly than the other. This is characteristic of yeast TS (Storms lab, unpublished observation), though yet unexplained, and is frequently observed when the proteins are resolved by longer migration and by loading smaller

Figure 3.7. Tritium release assays in *E. coli*.

Strain 264 was transformed with wild type *TMP1* or mutant alleles expressed from pEGRBS plasmids.

Top panel: Tritium release assays on crude extracts of *E. coli* for each reaction. 100 μg of protein extract was incubated with100 mM dUMP and 100 mM THF for 1 hour at 25°C, except for the pEGRBS-WT assay which was quenched after15 minutes (~25% substrate depletion).

Bottom panel: Tritium release assays on *E. coli* whole cells permeabilized with 3% Brij 35. Mutant TS activity is compared to that of chromosomally expressed wild type TS of the *E. coli* strain DH5α, and *E. coli* strain 264, which has a deletion in *thyA*, is the negative control. 10^9 cells were used for each reaction. Reactions were quenched after1 hour, except for the wild type DH5α which was quenched after15 minutes (~8% substrate depletion). Reported results are averages of 2 sets of assays each done in duplicate with error bars indicating one standard deviation.


Thymidylate synthase activity of crude protein extracts of E.coli
pmoles of H+ released
6000
5000
4000
3000
2000
1000
0
TS-WT
Blank
TS-
TS-YF
TS-FF
TS-FYF
TS-FS
TS-SF
TS-Lma

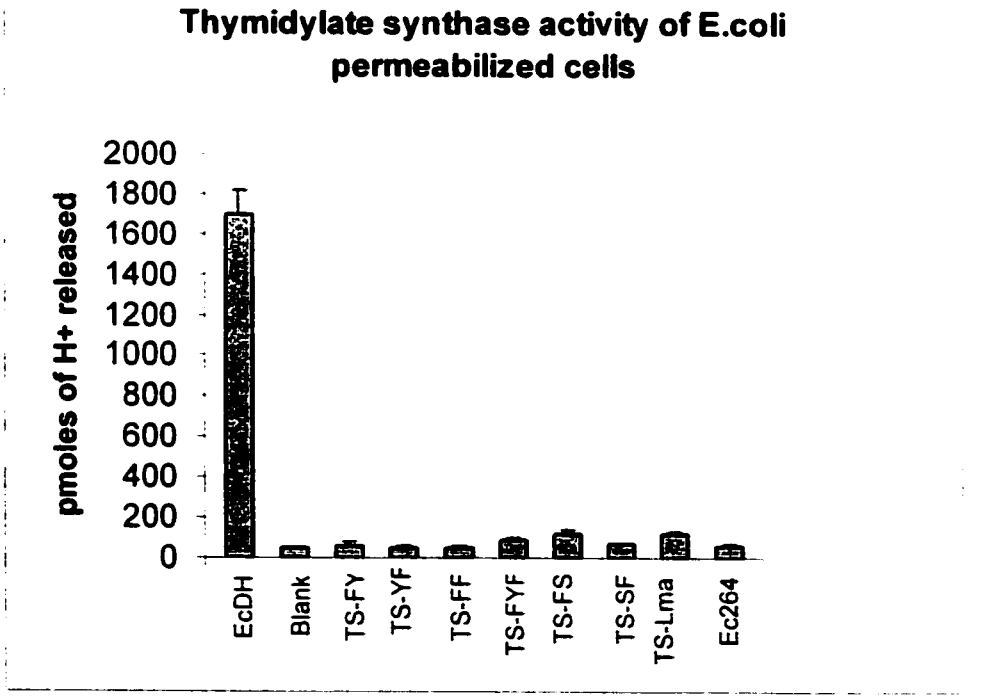

Thymidylate synthase activity of E.coli permeabilized cells
pmoles of H+ released
2000
1800
1600
1400
1200
1000
800
600
400
200
0
EcDH
Blank
TS-FY
TS-YF
TS-FF
TS-FYF
TS-FS
TS-SF
TS-Lma
Ec264

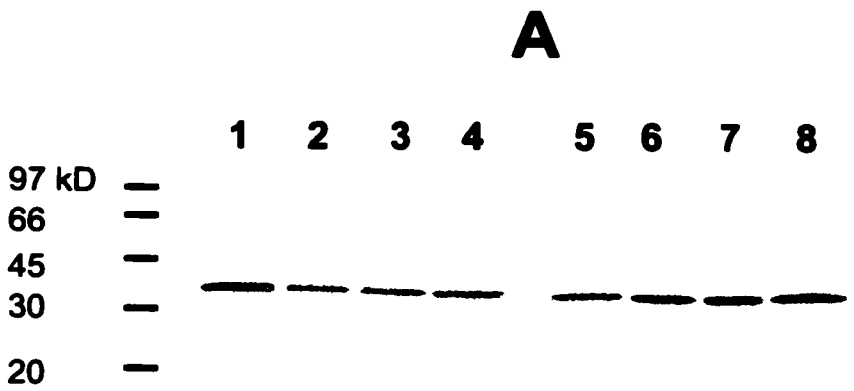
Figure 3.8 Immunoblots of yeast thymidylate synthase wild type and mutant proteins expressed in *E. coli*.

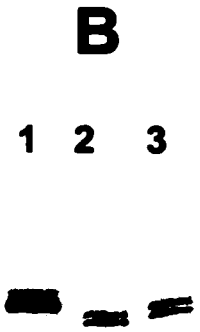
A. Extracts from *E. coli* strain 264 transformed with pEGRBS plasmids, 10 μg of total protein per lane, incubated with purified TS antibodies.

   1) WT, 2) FY, 3)YF, 4) FF, 5) FYF, 6) FS, 7) SF, 8) Lma

B. Extracts from *E. coli* strain 264, incubated with purified TS antibodies, 10 μg of total protein in lane 1, 3 μg in lanes 2 and 3.

   1) pEGRBS-WT, 2) pEGRBS-Δloop2, 3) pEM89-E2a-K117


A
1 2 3 4 5 6 7 8
97 kD
66
45
30
20


B
1 2 3

amounts of protein on the gel. Interestingly, when the deletion mutants are expressed in *E. coli*, the relative amounts of the larger species increases (panel B, lanes 2 and 3), while the smaller species of the wild type TS doublet (lane 1) is far more intense than the larger one.

3.4. Analysis of Loop 2 Function in Yeast

To further assess the requirement of yeast TS for tyrosine residues in Loop 2, we evaluated three of the mutants in their natural environment, the yeast cell. We chose to further investigate the behaviour of mutants *tmp1-F126*, *tmp1-F133* and *tmp1-FF*, since our preliminary results, complementation and enzyme assays in *E. coli* (Figures 3.5, 3.6 and 3.7), indicated partial loss of activity if one tyrosine was substituted and even more severe loss of activity when both tyrosines were removed.

3.4.1. Complementation by Mutant Alleles Expressed from a Centromere Plasmid

In order to compare the thymidylate synthase activity of these mutants to normal levels found in wild type yeast cells, we moved the mutant alleles into a centromere plasmid, which is typically present in one or two copies per cell, hence would simulate chromosomally expressed enzyme levels. Because the pEGRBS plasmids did not contain the *TMP1* promoter region, the mutant *tmp1* alleles first had to be linked to the yeast promoter and then inserted into a centromere plasmid, as described in Materials and Methods.

Three centromere plasmids were made, designated pEG50-FY, pEG50-YF, and pEG50-FF, containing *tmp1-F126*, *tmp1-F133* and *tmp1-FF* respectively, under the control of the *TMP1* promoter. These were transformed into a yeast strain (RS3236) which has a point mutation in its *TMP1* gene that inactivates thymidylate synthase. This strain also has a mutation at another locus that allows it to take up thymidylate so that supplementation with thymidylate enables it to survive without a functional thymidylate synthase (Wickner, 1975).

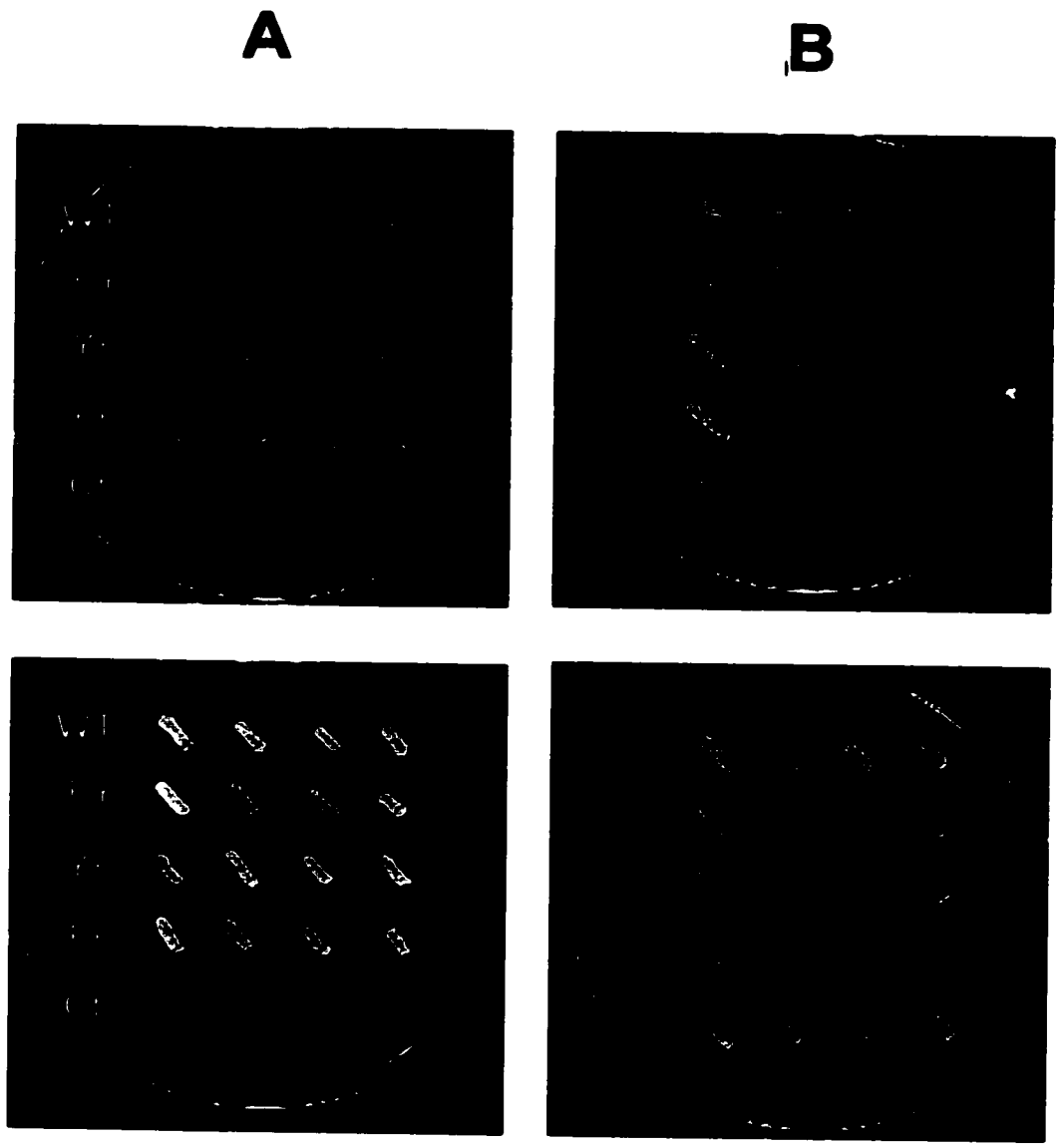
Figure 3.9 shows that surprisingly, all three mutants complemented TS deficiency in this genetic background, and grew as well as the wild type control (pEG50-WT) in the absence of thymidylate. These new constructs were also tested in *E. coli* since they function as shuttle vectors suitable for both yeast and *E. coli*. As seen earlier, the *tmp1-FF* mutant did not complement in *E. coli*, but in contrast to the results with the pEGRBS plasmids, *tmp1-F126* and *tmp1-F133* no longer complemented (Figure 3.5, plate D).

To verify that these results were reproducible, the three centromere plasmids were rescued from yeast and retransformed into *E. coli*. Again, all three mutants failed to complement in *E. coli* whereas they had complemented in yeast in a way that was comparable to the wild type gene. At this point, the mutant sequences were verified by restriction enzyme analysis and again confirmed by DNA sequence analysis.

3.4.2. Construction of a Yeast Strain with a Null Mutation in *TMP1*

Since our test strain for complementation of TS deficiency in yeast merely had a *TMP1* point mutation, we had to consider the possibility that formation of heterodimers

Figure 3.9. Complementation results in yeast strain RS3236 (*tmp1-6* point mutant). Strain RS3236 (*tup-*, hence capable of thymidylate uptake) was transformed with mutant or wild type *TMP1* expressed from centromere plasmids (pEG50). Transformants were selected on minimal media plates containing thymilydate, then colonies were patched onto minimal media without thymidylate (A and C), and with thymidylate (B and D). FY denotes the *tmp1-F126* allele; YF, the *tmp1-F133* allele; FF, the *tmp1-FF* allele. The control (bottom row on each plate) is the same strain transformed with a centromere plasmid (Ycp50) that has no *TMP1* gene. Plates A and C represent four independent transformants obtained with two independent constructs of each mutant.


A
B
C
D

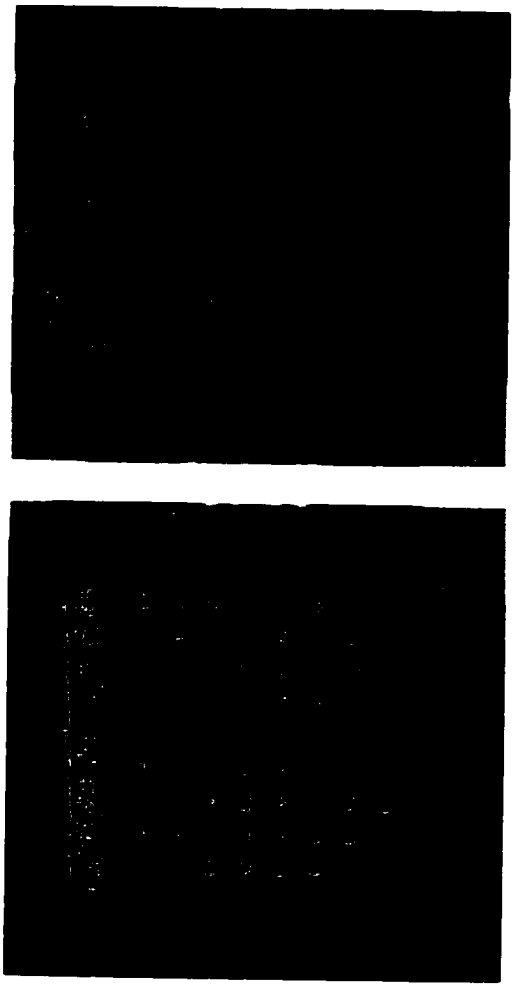
of two different TS mutants was restoring activity to otherwise inactive homodimers. To circumvent this problem, we constructed a new strain in which the *TMP1* locus was deleted. The entire *TMP1* ORF was knocked out by gene replacement using a *GFP-HIS* cassette (described in Materials and Methods). Since the strain of choice was incapable of thymidylate uptake, it was necessary to use a diploid form of the strain so that one functional *TMP1* gene would ensure viability of the cells. Once the null mutation was made, the strain was sporulated and verified by tetrad analysis (Figure 3.10, plate A). All tetrads dissected segregated in a 2:2 ratio of surviving versus non-surviving spores. The survivors were all histidine auxotrophs as expected. A transformant containing plasmid pEG50-WT, a centromere plasmid that has *TMP1* as well as *URA3* for selection, was also analyzed by tetrad dissection. In this case, the number of surviving spores per tetrad was 2 to 4, depending on whether the centromere plasmid segregated with the *TMP1* or *tmp1-Δ0* allele. All survivors that were histidine prototrophs (*TMP1* disruptants) were also uracil prototrophs, indicating a requirement for the plasmid and presumably, the *TMP1* gene.

To confirm this dependence for the plasmid-encoded *TMP1* gene, we attempted to cure several haploid colonies of the centromere plasmid. Spores were picked and patched onto minimal media plates containing the appropriate supplements to determine their genotypes. Several spore-derived colonies that were prototrophic for histidine (which selects for the *TMP1* disruption) and uracil (which selects for the centromere plasmid, pEG50-WT) were chosen for further analysis. First we verified that all colonies were none derived from haploid cells by growing them in sporulation media and ensuring that

Figure 3.10. Tetrad analysis of yeast strain RS4664 (*TMP1* knock-out).

Plate A is the untransformed strain, plate B is the knock-out strain transformed with a multicopy plasmid that contains the *thyA* gene of *E. coli*. The 4 spores of each tetrad were deposited in vertical arrays. Over 30 tetrads were dissected each for the transformed and untransformed strain. The untransformed strain consistently had 2 surviving spores per tetrad, while the transformed strain frequently had greater than 2 surviving spores per tetrad.

A



B

formed spores. These were then grown without selection for uracil prototrophy to see whether it would be possible to cure the cells of the plasmid containing the *TMP1* gene. As a control, we used cells that had the pEG50-WT plasmid but were auxotrophic for histidine, indicating that the chromosomal *TMP1* gene is intact. After subculturing 3 times into 2 mL of fresh media, cells were plated onto media containing fluoro-orotic acid (FOA). This inhibitor of thymidylate synthase is a powerful tool for selecting uracil auxotrophs since a cell lacking a functional *URA3* gene cannot synthesize the inhibitory substance, F-dUMP, that binds covalently and irreversibly to thymidylate synthase. For the histidine prototrophs (presumably auxotrophic for thymidylate), no colonies appeared on plates that contained FOA at a concentration of 240 μg/mL or greater (Table 6). For the histidine auxotrophs (His-, Tmp1+), thousands of colonies appeared at all FOA concentrations. This demonstrates a dependence of the *tmp1-Δ0* haploid strain for the *URA3* gene-containing plasmid, which confirms the disruption of the *TMP1* gene.

We also used PCR to confirm the disruption of *TMP1*. Genomic DNA was extracted from 3 strains: RS3695 (diploid with two wild type *TMP1* genes), RS4664 (diploid derivative of RS3695 with one disrupted *TMP1* gene), and RS5331 (haploid obtained by sporulating RS4664, transformed with a plasmid containing the *E. coli* gene for thymidylate synthase, *thyA* - discussed in the next section). When PCR amplified with a *TMP1* promoter primer and a *GFP*-specific primer, the expected 1100 base pair fragment appeared in the disrupted strain (RS4664) but not in the parent strain (RS3695) (Figure 3.11a, b). When amplified with primers specific for the sequences flanking the *TMP1* coding region, the expected fragment of 1400 base pairs appeared in the parent

Table 6

Testing for loss of *URA3* plasmids by selecting for FOA resistance

Strain	Plasmid	µg/mL of FOA						
		100	200	240	280	320	380	1000
RS4664[a]		+++	+++	+++	+++	+++	+++	+++
RS4664[b]	pEG50-WT	+++	++	-	-	-	-	-
RS5331-A[c]	pEG-thyA	+++	+++	+++	+++	+++	+++	+++
RS5331-B[d]	pEG-thyA	+++	++	-	-	-	-	-
RS5332[e]	pEM54, pEG-thyA							+++
RS5500[f]	pEG50-FYa							-
RS5501[f]	pEG50-FYb							-
RS5502[g]	pEG50-YFa							-
RS5503[g]	pEG50-YFb							-
RS5504[h]	pEG50-WT							-
RS4664	pEG50-FY							+++
RS4664	pEG50-YF							+++

[a] Diploid strain with one *TMP1* gene disrupted, Ura-, used as control for FOA resistance
[b] Same as above, but transformed with a *URA3* plasmid, grown with selective media to determine strain sensitivity to FOA
[c] Haploid, derived by sporulation of RS4664 transformed with pEG-thyA, His- colony chosen as indication of presence of wild type *TMP1* gene
[d] Haploid, derived by sporulation of RS4664 transformed with pEG-thyA, His+ colony indicating disruption of *TMP1* gene
[e] Haploid, derived by sporulation of RS4664 transformed with pEG-thyA and pEM54
[f] Haploid, derived by sporulation of RS4664 transformed with pEG50-FY
[g] Haploid, derived by sporulation of RS4664 transformed with pEG50-YF
[h] Haploid, derived by sporulation of RS4664 transformed with pEG50-WT

All strains (except RS4664[a,b]) were grown without selection for *URA3* plasmids and subcultured 4 times after growing to saturation. 20 µl of a 1:100 dilution of the last subculture was spread on YEDP plates containing 1000 µg/mL FOA.
(+++) denotes hundreds of colonies on a plate, (-) denotes absence of a single colony.

Figure 3.11a. Strategy for generating a *TMP1* knock-out strain.

A cassette of the *GFP-HIS* fusion gene was amplified with primers whose 5' ends were comprised of 45 base pairs of sequence immediately flanking the *TMP1* coding region (1). The PCR fragment (~1800 bp) was transformed into a yeast diploid strain (strain RS3695 from H. Bussey) to recombine at one of the two *TMP1* loci (2). Gene replacement resulted in a larger fragment inserted between the *Hind*III site of the promoter and the end of the *TMP1* coding region. (2200 bp versus 1400 bp) (3).

Figure 3.11b. PCR verification of the *TMP1* knock-out strain.

DNA from the *TMP1* knock-out diploid strain (RS4664) was PCR-amplified using primer 3 (see diagram) and an internal *GFP* primer, yielding the expected 1100 base pair product (lane 2). When the same primers were used to amplify the parent strain RS3695, no PCR product was obtained, as expected (lane 1). The haploid form of the knock-out strain, transformed with the plasmid-borne *thyA* gene of *E. coli*, was amplified using primers 3 and 4 (specific for upstream and downstream sequences of the *TMP1* coding region), yielding the expected fragment of 2200 base pairs that contains *GFP-HIS* (lane 4). The diploid parent strain, RS3695, was PCR amplified with primers 3 and 4, yielding the expected 1400 base pair fragment that contains the *TMP1* coding region (lane 3).

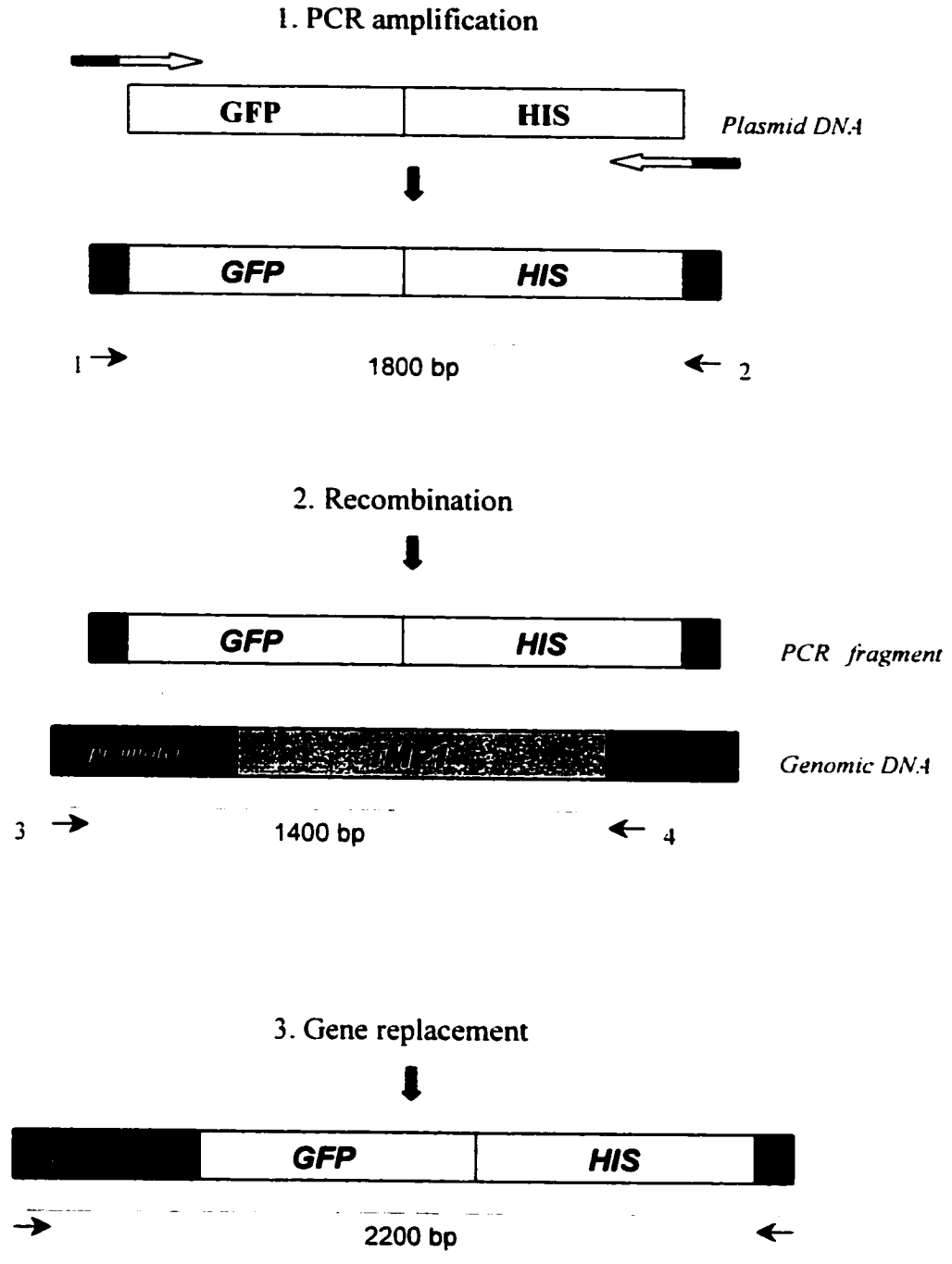


Figure 3.11a. Strategy for generating a *TMP1* knock-out strain.

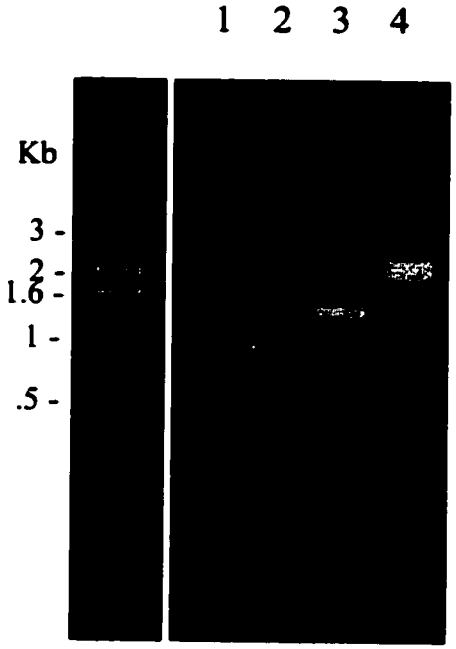


Figure 3.11b. PCR verification of the *TMP1* knock-out strain.

strain (RS3695), but a larger fragment of 2200 base pairs appeared in the disrupted haploid (RS5331) transformed with *thyA*.

3.4.3. Complementation in Yeast by the *thyA* Gene of *E. coli*

In *E. coli*, thymidylate synthase is encoded by the *thyA* gene, whose protein product is significantly smaller than in yeast and other eukaryotes, having 264 amino acid residues per subunit instead of 300 or more. One of the regions missing in the *E. coli* enzyme is the Loop 2 insert which as discussed earlier is specific to the eukaryotes. To investigate how the absence of the insert and/or conserved tyrosine residues would affect function in the context of the yeast cell, we chose the alternate approach of testing complementation of yeast TS deficiency by the *thyA* gene.

The *thyA* gene was amplified by PCR from plasmid pBTA and subcloned into a pYES2 yeast vector as described in Materials and Methods. The new construct (pEG-thyA) was then transformed into the *TMP1*-disrupted strain described above and cells were sporulated. The tetrad analysis results (Figure 3.10, plate B), show a number of surviving spores greater than 2 per tetrad in several tetrads dissected. This suggests that the *thyA* gene does complement in yeast since the yeast intact *TMP1* gene should be present in only 2 of the 4 meiotic products. Since pEG-thyA has a *URA3* selectable marker, we were able to show that His+ haploid cells could not be cured of the pEG-thyA by our inability to obtain any FOA resistant colonies.

Complementation by *thyA* was also tested and confirmed in the yeast strain containing the *tmp1-6* point mutation (RS3236), by patching transformants onto minimal

media plates not supplemented with thymidylate. Cells transformed with pEG-thyA grew as well as cells transformed with pEM54, a multi-copy plasmid that contains wild type *TMP1*. Cells transformed with pYES2, the parent plasmid of pEG-thyA but not containing a gene for thymidylate synthase, did not grow (data not shown).

Finally, an immunoblot of the *tmp1-Δ0* haploid transformed with pEG-thyA failed to show any band bound to TS antibody, demonstrating the absence of yeast TS in this strain, hence complementation by *thyA*. That the *thyA*-encoded TS was not detected was not unexpected since the TS antibody was raised specifically against yeast TS.

3.4.4. Complementation Using Centromere Plasmids in the Yeast *TMP1* Null Strain

Plasmids pEG50-FY, pEG50-YF, pEG50-FF and the wild type control, pEG50-WT, were transformed into strain RS4664, the diploid containing the *TMP1* disruption. As was done for pEG-thyA, complementation was tested by tetrad analysis. For pEG50-WT, pEG50-FY and pEG50-YF transformants, 2-4 surviving spores were obtained per tetrad and all His+ spores were also Ura+. Three spores were picked from each transformation, grown and subcultured several times without Ura+ selection, and plated on FOA. No FOA resistant colonies were obtained, indicating that the plasmids could not be cured from the cells. The control plates however, pEG50-FYand pEG50-YF transformed into the diploid parent, RS4664, had thousands of FOA resistant colonies. The plasmids were rescued from the yeast cells into *E. coli* and verified by DNA sequencing to confirm that the mutations were still present.

Figure 3.12. Tetrad analysis of yeast strain RS4664 (*TMP1* knock-out), transformed with centromere plasmids.

(A) pEG50-WT, (B) pEG50-FY, (C) pEG50-YF, (D)YCp50, (E,F) pEG50-FF. The 4 spores of each tetrad were deposited in a vertical array. Plates A, B and C show 2 to 4 surviving spores per tetrad, indicating that the plasmid complements the *tmp1-Δ0* mutation. Plates D, E and F show 2 surviving spores per tetrad, indicating inability to complement. YCp50 (plate D) is a centromere plasmid without a *TMP1* gene and served as a negative control. Over 30 spores were dissected for samples A, B, C and D, over 60 spores were dissected for sample E, F.

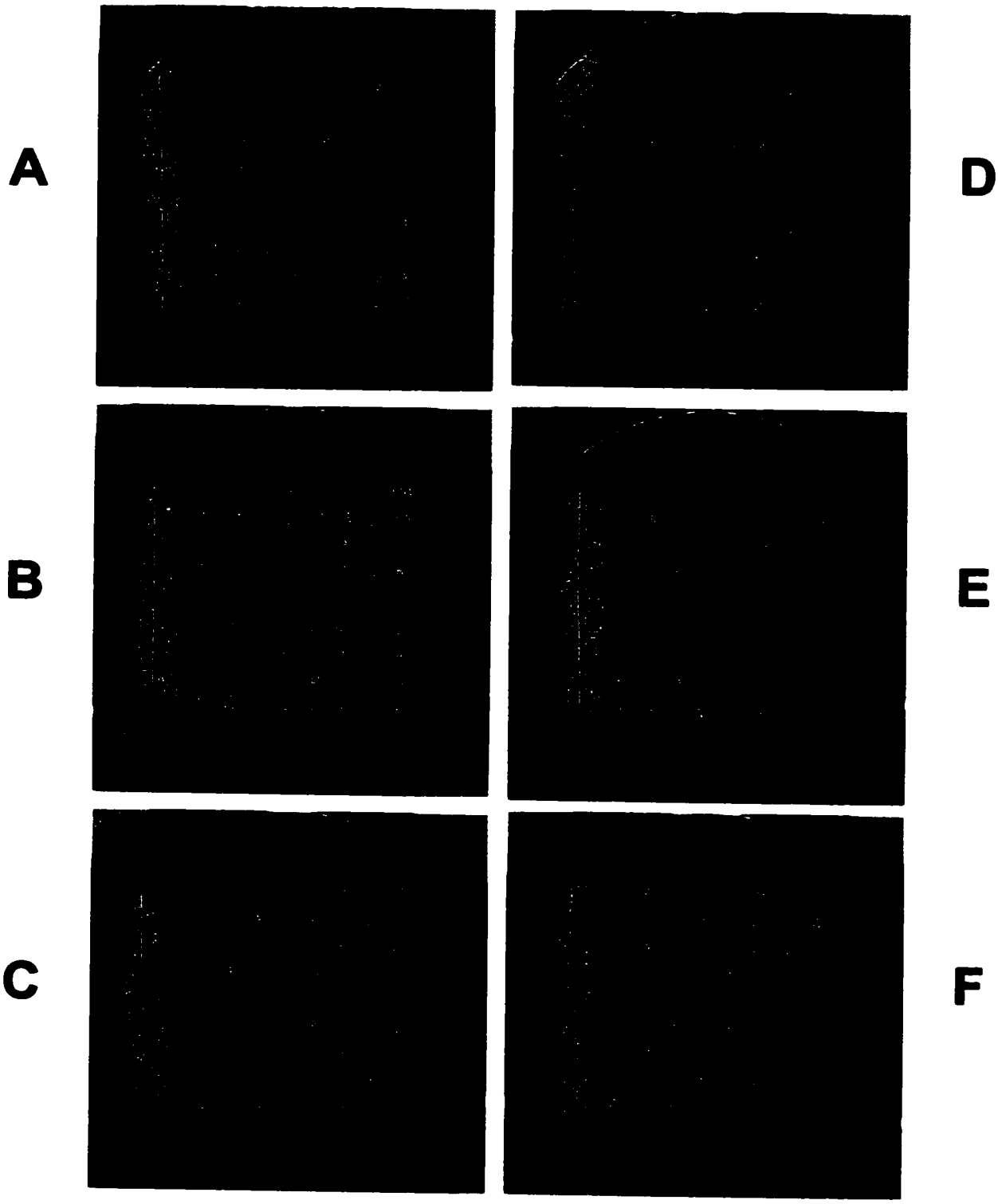

A
D
B
E
C
F

Table 7

Complementation results in *E. coli* and yeast by mutant *TMP1* alleles expressed from *E. coli* plasmids, yeast centromere plasmids and yeast episomal plasmids

TMP1 Allele	**E. coli** (264)[a]	Yeast (RS3236)[b]	Yeast (RS5331)[c]
E. coli Plasmids (pEGRBS)			
tmp1-Δloop2	-	n/a[d]	n/a
tmp1-F126	+	n/a	n/a
tmp1-F133	+	n/a	n/a
tmp1-FF	-	n/a	n/a
tmp1-FS	-	n/a	n/a
tmp1-SF	-	n/a	n/a
tmp1-FYF	-	n/a	n/a
tmp1-Lma	+	n/a	n/a
Yeast Centromere Plasmids (pEG50)			
tmp1-F126	-	+	+
tmp1-F133	-	+	+
tmp1-FF	-	+	-
Yeast Episomal Plasmids (pEG54)			
tmp1-F126	-	+	+
tmp1-F133	-	+	+
tmp1-FF	-	+	+
tmp1-ΔE2a/K117	-	n/d[e]	n/d

[a] *E. coli* strain with a large deletion in *thyA*
[b] Yeast haploid strain with a point mutation in *TMP1* (*tmp1-6*)
[c] Yeast haploid strain with *TMP1* deleted (*tmp1-Δ0*)
[d] Not applicable
[e] Not done

For pEG50-FF, the number of survivors was consistently 2 per tetrad, and these were all His- (Figure 3.12, E, F). In over 60 tetrads dissected, only 3 had greater than 2 surviving spores but these were all His- and probably resulted from mixing tetrads during the dissection, or by gene conversion. This plasmid was also transformed into strain RS5331 that contained pEM54 (wild type *TMP1*, *LEU2*) to attempt to cure the cells of pEM54. After several rounds of subculturing without selection for leucine (ca. 40 generations), no leucine auxotrophs were obtained in over 200 colonies screened by patching onto plates with or without leucine. These results suggest that the single tyrosine mutants complement in this genetic background while the double mutant does not.

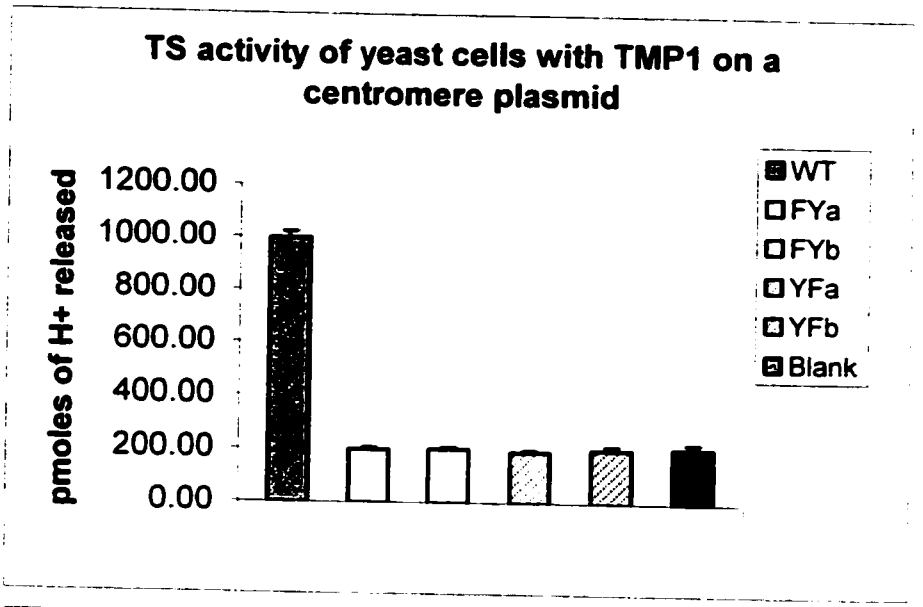
3.4.5. Activity Assays of Mutant TS Expressed from a Centromere Plasmid

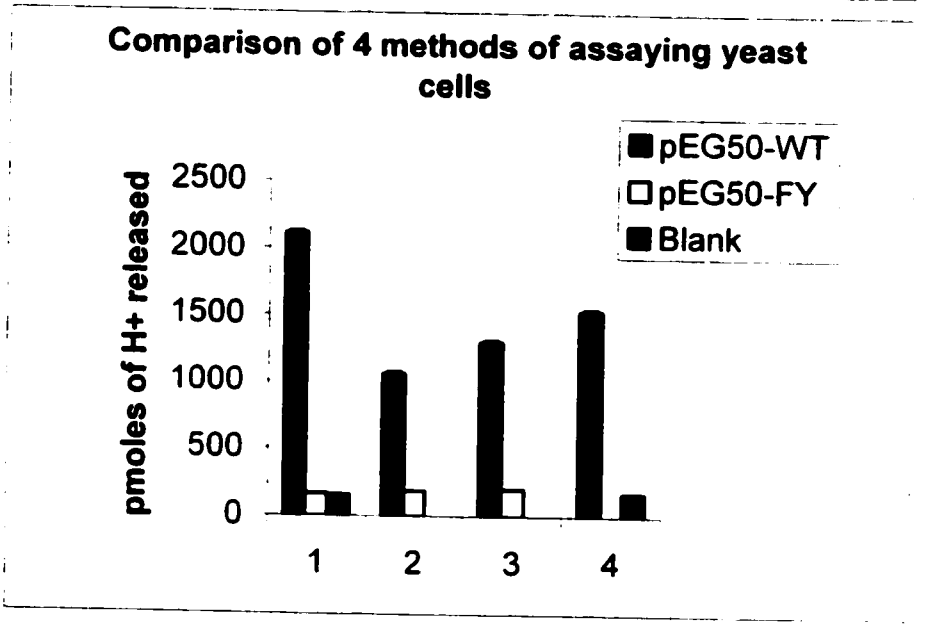
Having established that *tmp1-F*126 and *tmp1-F133* complement in yeast, we wanted to measure the enzyme activity of these mutants. Tritium release assays were done on permeabilized cells. Contrary to our expectations, the mutants did not exhibit any measurable activity using this sensitive assay (Figure 3.13, top panel). Since the permeabilization buffer used for the assays contained a small amount of Brij detergent, we speculated that the mutants might be sensitive to this buffer. Using cells containing wild type TS, we tested alternate methods of permeabilizing the cells (Figure 3.13, bottom panel). We found that the highest TS activity was obtained from cells permeabilized simply by freezing and thawing three times, without Brij detergent, hence we chose this method for subsequent assays.

Figure 3.13. Tritium release assays on strain RS4664 transformed with centromere plasmids.

Top panel: Tritium release assays on yeast cells permeabilized with 3% Brij 35. The yeast strain was RS4664 (*TMP1* knock-out) transformed with pEG50-WT, pEG50-FY (*tmp1-F126*) or pEG50-YF (*tmp1-F133*). The cells were sporulated and spores that had the deleted *TMP1* gene and the centromere plasmid were propagated and assayed. Assays were incubated for 3 hours, and 2.5 x 10^8 cells were used per assay. Reported values are averages of 6 reactions and error bars indicate one standard deviation. For each mutant TS, 2 separate spores were isolated and tested, each arising from a different original mutagenesis event, designated "a" or " b".

Bottom panel: Comparison of 4 methods of assaying yeast cells. 1) cells were frozen and thawed 3 times in liquid nitrogen; 2) cells were treated with SDS and chloroform (Ausubel *et al.*, 1989); 3) crude protein was extracted by vortexing with glass beads (Ausubel *et al.*, 1989); 4) cells were treated with 3% Brij 35.


TS activity of yeast cells with TMP1 on a centromere plasmid
pmoles of H+ released
1200.00
1000.00
800.00
600.00
400.00
200.00
0.00
WT
FYa
FYb
YFa
YFb
Blank


Comparison of 4 methods of assaying yeast cells
pmoles of H+ released
2500
2000
1500
1000
500
0
1
2
3
4
pEG50-WT
pEG50-FY
Blank

One set of assays was repeated on the mutant TS-FY, using the freeze-thaw method without Brij buffer to permeabilize the cells. The results however were the same as in the previous assay, that is, we could not measure any tritium release significantly above background levels (Figure 3.13, bottom panel).

Finally, we performed one last set of assays in which we added trehalose to the buffer. Trehalose is a sugar synthesized in yeast cells, which has been shown to stabilize proteins or protect them from degradation when added in large quantities (Sampedro *et al.*, 2001). The use of this compound however did not increase the activity of our mutant enzymes (data not shown).

We then determined expression levels of the mutant enzymes by immunoblot analysis. Protein levels were low and difficult to detect, but this is due to the low gene copy number of the centromere plasmid. Although mutant protein levels were detectable, they were slightly lower than wild type levels. Interestingly, as was observed earlier in *E. coli* the faster migrating band of the TS doublet was much more prominent in the wild type than in the mutant samples (Figure 3.14, panels A and B).

3.4.6. Expressing the Mutant Alleles from a Yeast Episomal Plasmid

Since TS activity could not be measured when the mutant enzymes were expressed from a centromere plasmid, the mutant alleles were moved into a yeast episomal plasmid, which is maintained in multi-copy number in the yeast cell. We reasoned that increasing the amount of TS in the cell might boost activity to measurable levels.

Figure 3.14. Immunoblots of yeast thymidylate synthase wild type and mutant proteins expressed in yeast.

A. Extracts from yeast *tmp1* knock-out strain, haploid, transformed with centromere plasmids (pEG50), incubated with crude TS antibodies, 20 μg of protein per lane.

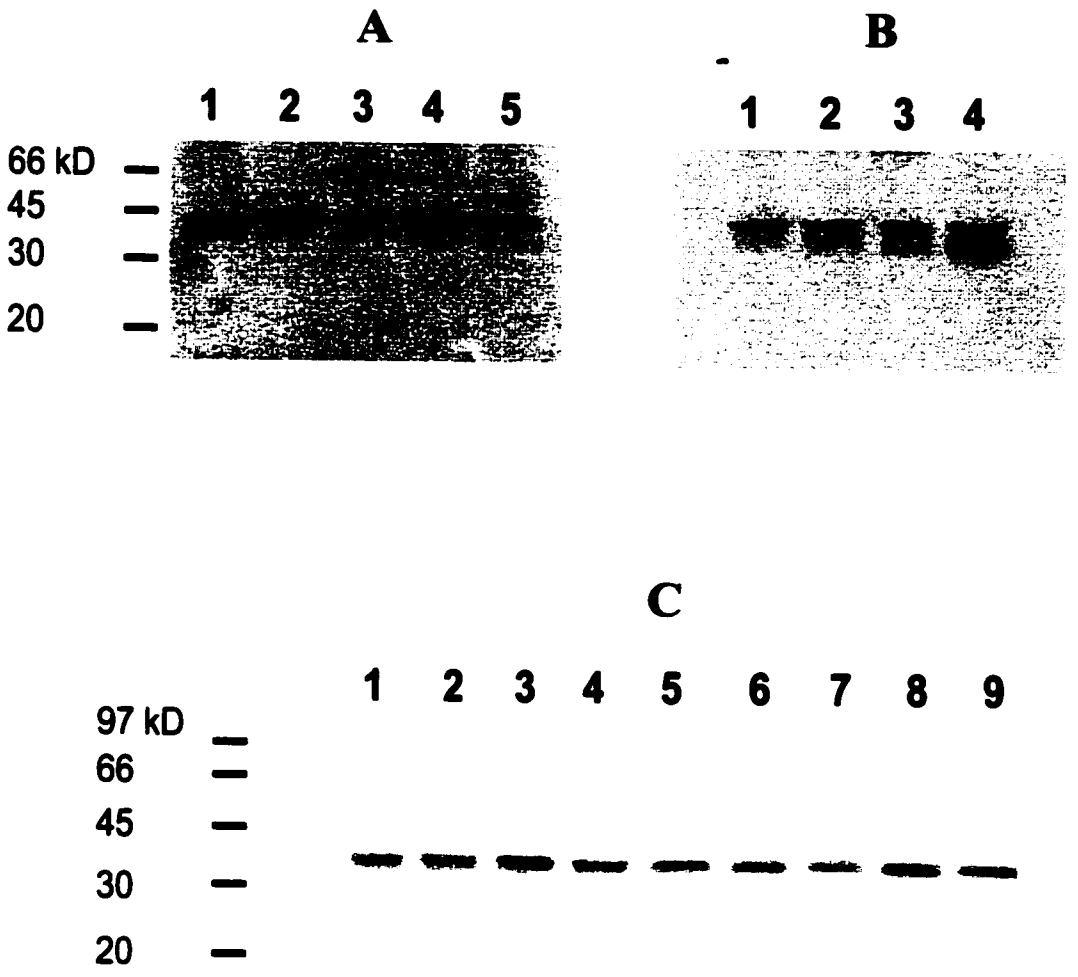
   1) WT, 2) FYa, 3) FYb, 4) YFa, 5) YFb

B. Same as A, but incubated with purified TS antibodies.

   1) FYa, 2) FYb, 3) YFa, 4) WT

C. Extracts from yeast *tmp1* knock-out strain, haploid, transformed with episomal plasmids (gap-repaired).

   1) to 4) pEG54-FY (4 isolates), 5) to 8) pEG54-YF (4 isolates), 9) pEM54 (WT)


A
B
1 2 3 4 5
1 2 3 4
66 kD
45
30
20
C
1 2 3 4 5 6 7 8 9
97 kD
66
45
30
20

For *tmp1-F126* and *tmp1-F133*, we made the new constructs by "gap repair" in yeast (Raymond *et al.*, 1999; Ausubel *et al.*, 1989). In this technique, the yeast is transformed with a gene that has a gap in the region to be replaced. The cell then repairs the gap using its own DNA as template for the missing region. To move the mutations into an episomal plasmid, we used a derivative of pEM54, in which we made a gap by cutting out a *Pvu*II fragment within *TMP1* that encompasses the Loop 2 region. We transformed the gapped plasmid into the haploid *tmp1-Δ0* strain already harbouring the *tmp1-F126* or *tmp1-F133* alleles on the centromere plasmid. The strategy was to repair the gap in the episomal plasmid by using the mutant allele of the centromere plasmid as template. Since the episomal plasmid has a *LEU2* gene for selection while the centromere plasmid has a *URA3* marker, we selected for repaired episomal plasmids by plating the transformants on minimal media lacking leucine. The leucine prototrophs were then scraped from the plates, grown without Ura+ selection and replated on FOA plates. This ensured that the surviving transformants had lost the *URA3*-containing centromere plasmid, hence the *TMP1* mutant allele also present on that plasmid. Fifty transformants of each mutant were phenotyped. All were prototrophic for leucine and histidine but auxotrophic for uracil. This indicates loss of the centromere plasmid, presence of the episomal plasmid, and presence of the *TMP1* gene replacement cassette (*GFP-HIS*). Several transformants of each mutant were picked and the plasmids were rescued in *E. coli*. They were then verified by restriction enzyme analysis and DNA sequencing of both strands through the entire gap-repaired region.

Since we were unable to obtain a *tmp1-Δ0* haploid transformed with pEG50-FF, the *tmp1-FF* allele was moved into the episomal plasmid by conventional *in vitro*

construction described in Materials and Methods. We also wanted to compare our mutants to another mutant allele of *TMP1*, containing a point mutation (G137S) in Loop 2 designated as *tmp1-S137* (Hartwell, 1973; Game, 1976, Bisson and Thorner, 1977; Taylor *et al.*,1986), which renders the cells temperature sensitive. We PCR-amplified this mutation from genomic DNA of the temperature sensitive *tmp1-S137* strain (RS625-2B) and ligated the PCR fragment into pEM54 (designated pEG54-S137, described in Materials and Methods).

3.4.7. Complementation by Mutant Alleles Expressed from an Episomal Plasmid

The new plasmids constructed by gap repair, designated pEG54-FY and pEG54-YF, were already known to complement in the *tmp1-Δ0* strain since this strain had been used for the gap repair construction of the plasmids and since the strain had been successfully cured of the centromere plasmid. The episomal plasmid harbouring *tmp1-FF*, designated pEG54-FF, was transformed into the *tmp1-Δ0* yeast strain which also contained a pEG-thyA plasmid. The strain was then cured of the pEG-thyA plasmid, demonstrating complementation by the double phenylalanine mutant which in contrast had been unable to complement in the null strain when expressed from a centromere plasmid (Figure 3.15, B-3).

Figure 3.15. Complementation in yeast by various *TMP1* alleles, incubation at 30°C (3.15a) or at 36°C (3.15b)

pEG50 plasmids are centromere plasmids (single copy). All others are multi-copy plasmids. Strain RS4664 is a diploid yeast with one *TMP1* gene deleted (*tmp1-Δ0*). Strain RS5331 is a haploid derivative of RS4664, containing the *TMP1* deletion.

A. Sector 2 is the untransformed diploid strain RS4664.

   All others are the haploid RS5331 transformed as follows:

   1) pEG50-FY, 3) pEG54-S137, 4) pEG50-YF

B. RS5331 transformed with the following:

   1) pEM54, 2) pEG-thyA, 3) pEG54-FF, 4) pEG54-YF, 5) pEG54-FY

C. Strain RS3236 (*tmp1-6* point mutant) transformed with:

   1) pEG50-FY, 2) pEG50-WT, 3) pEG50-FF, 4) pEG50-YF

D. 1) RS4664, 2) RS625-2B, (S137 mutation), 3) pEG50-FF in RS3236

   4) pEG50-FY in RS5331, 5) pEG50-YF in RS5331

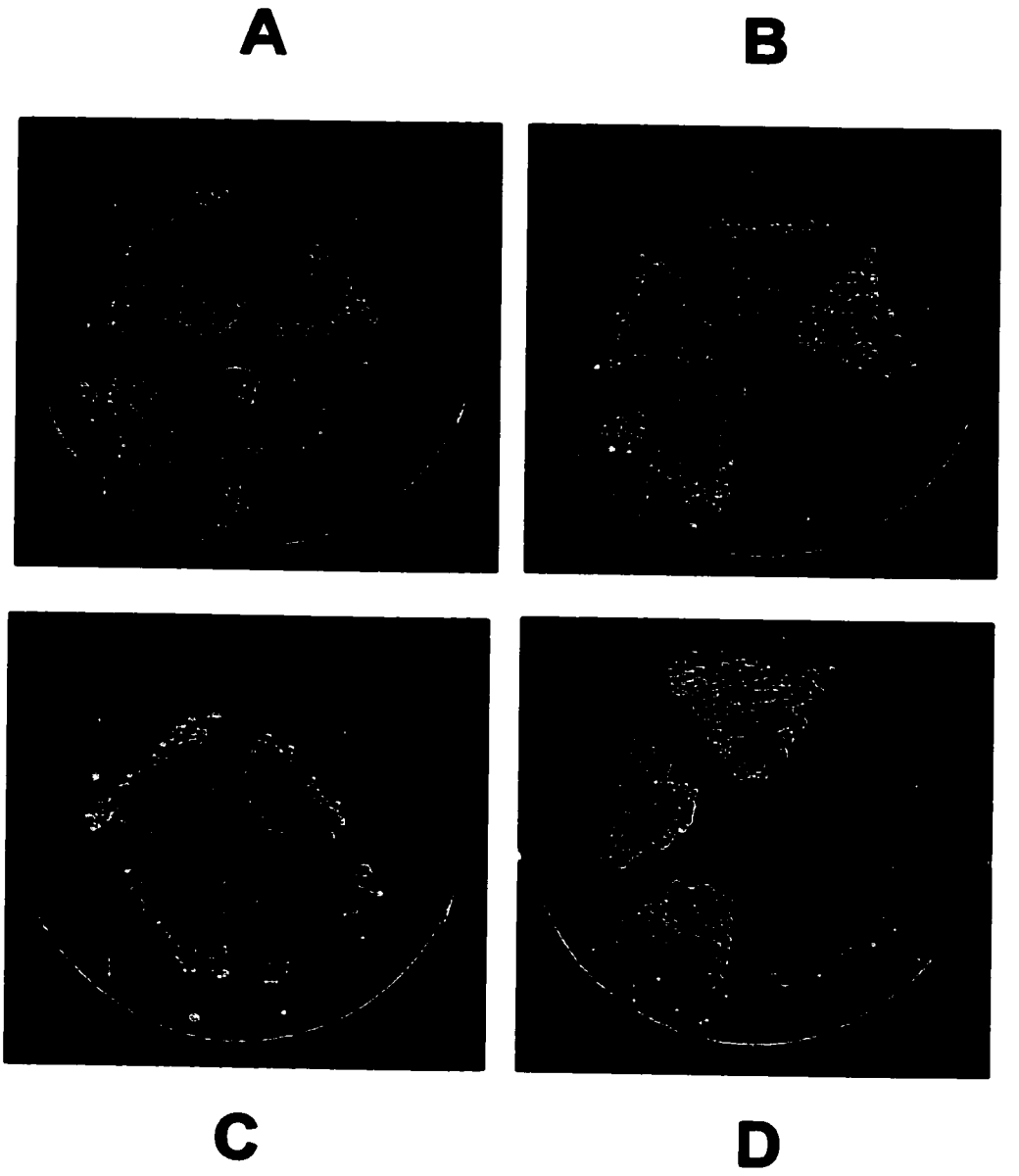


Figure 3.15a. Complementation in yeast, incubation at 30 degrees Celsius.

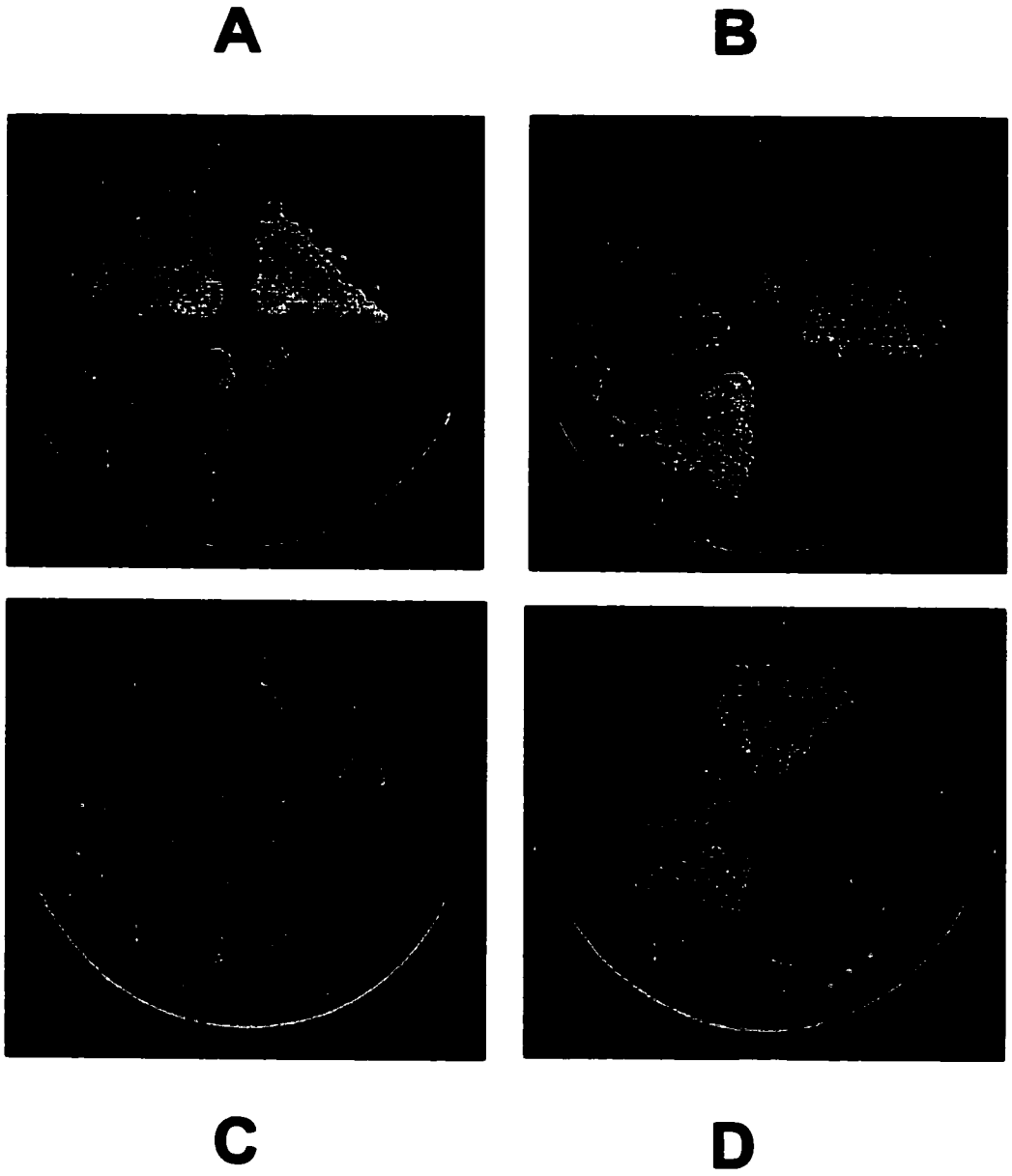


Figure 3.15b. Complementation in yeast, incubation at 36 degrees Celsius.

3.4.8. Activity Assays of Mutant TS Expressed from an Episomal Plasmid

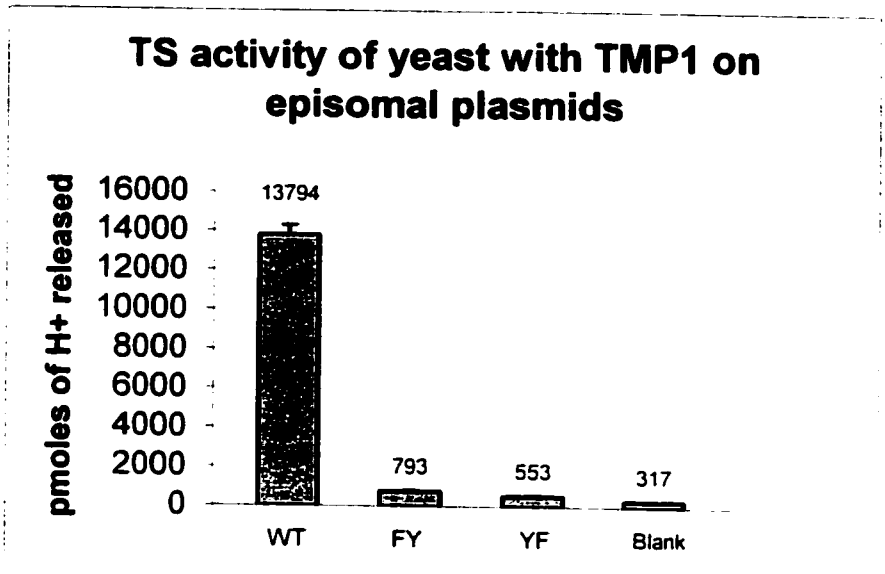
Since no enzyme activity was detected from mutant enzymes expressed from centromere plasmids, assays were performed on the *tmp1-Δ0* strain which contained the gap-repaired episomal plasmids, pEG54-FY and pEG54-YF. Figure 3.16, top panel, shows that although activity was significantly above background, it was nonetheless too low to enable an accurate comparison to that obtained from the wild type *TMP1* expressed from the same plasmid. In order to obtain measurable levels of tritium release, the assays were allowed to proceed for 2 hours and 40 minutes, at which point the wild type assay was no longer linear (60% substrate depletion). In other experiments, the pEM54 plasmid typically gives an activity of 830 to 850 picomoles of tritium release per minute per 10^9 cells. The mutant enzyme activity was calculated to be 15 pmol/min/10^9 cells for pEM-FY transformants and 10 pmol/min/10^9 cells for pEM-YF.

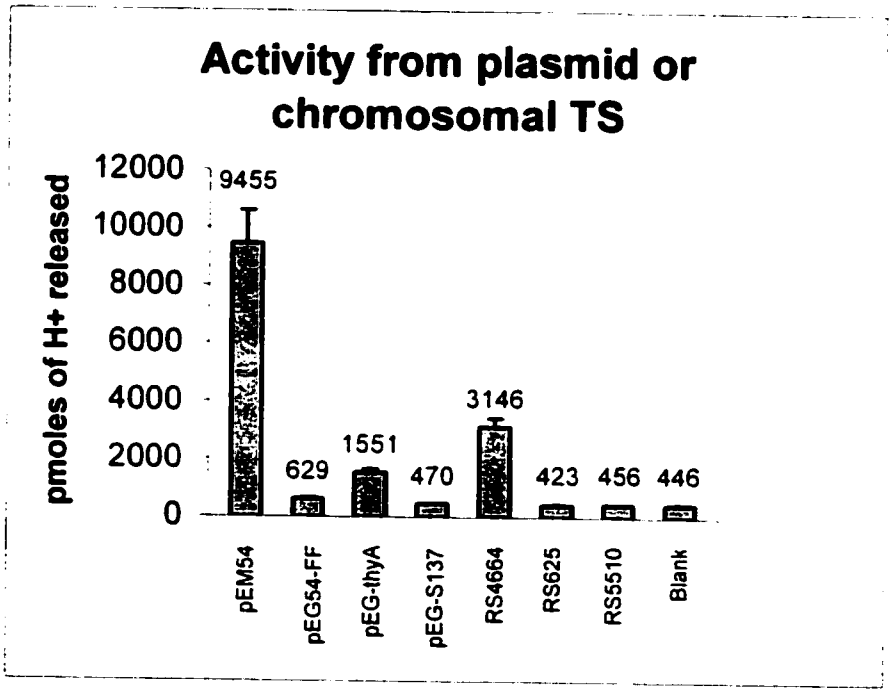
Figure 3.16, bottom panel, shows the activity of pEM-FF transformed in the *tmp1-Δ0* strain as well as several other strains. The calculated activity for TS-FF expressed from this episomal plasmid was 4 pmol/min/ 10^9 cells. The *E. coli* TS expressed from an episomal plasmid (pEG-thyA) had an activity of 24 pmol/min/10^9 cells, while the diploid form of the *tmp1-Δ0* strain had an activity of 57 pmol/min/10^9, which is comparable to other wild type strains assayed in other experiments. The activity for the temperature sensitive mutant (TS-S137) was not significantly above background, when expressed either chromosomally (RS625-2B) or from an episomal plasmid (pEG54-S137). Likewise, a haploid strain in which we introduced the *tmp1-F126* allele by gene replacement had no detectable activity (RS5110).

Figure 3.16. Tritium release assays on various yeast strains and transformants.

Top panel: Assays on yeast cells permeabilized by freezing and thawing. The strain is the *tmp1-Δ0* strain, harbouring "gap repaired" episomal plasmids. The reported values are averages of 4 isolates for each mutant, with assays done in triplicate and error bars indicating one standard deviation. The 4 isolates represent two distinct original mutagenesis events and two separate gap repair events. 2 x 10^8 cells were used per reaction, and reactions were quenched after 2 hours 40 minutes. For the wild type control (pEM54), 60% of the substrate was depleted at the time of termination.

Bottom panel: Comparison of TS activity of various strains, with *TMP1* expressed from a multi-copy plasmid or chromosomally encoded. All assays contained 3 x 10^8 cells, except for the wild type control (pEM54 transformed in the *tmp1-Δ0* strain) which contained 6 x 10^7 cells. Reported values are averages of 2 separate assays done in duplicate, error bars indicating one standard deviation. pEG-thyA, pEG54-FF, pEG54-S137 are harboured in the *tmp1-Δ0* haploid strain. RS4664 is a diploid with one *tmp1-Δ0* and one wild type *TMP1* gene. Strain RS5110 is a haploid strain with a *tmp1-F126* allele generated by gene replacement. Strain RS625-2B is a haploid strain with a temperature sensitive *TMP1* allele (*tmp1-S137*).


TS activity of yeast with TMP1 on episomal plasmids
pmoles of H+ released
16000
14000
12000
10000
8000
6000
4000
2000
0
13794
793
553
317
WT
FY
YF
Blank


Activity from plasmid or chromosomal TS
pmoles of H+ released
12000
10000
8000
6000
4000
2000
0
9455
629
1551
470
3146
423
456
446
pEM54
pEG54-FF
pEG-thyA
pEG-S137
RS4664
RS625
RS5510
Blank

Since activity levels were surprisingly low for the mutant enzymes even when expressed from a multi-copy plasmid, protein expression levels from pEG54-FY and pEG54-YF were verified by immunoblot analysis. Figure 3.14 (panel C) shows that all mutant proteins are expressed in amounts comparable to the wild type, hence lower expression does not account for the reduced activity.

3.49. Growth Rate, Temperature and Hydroxyurea Sensitivity of Cells Harbouring Mutant *TMP1* Alleles

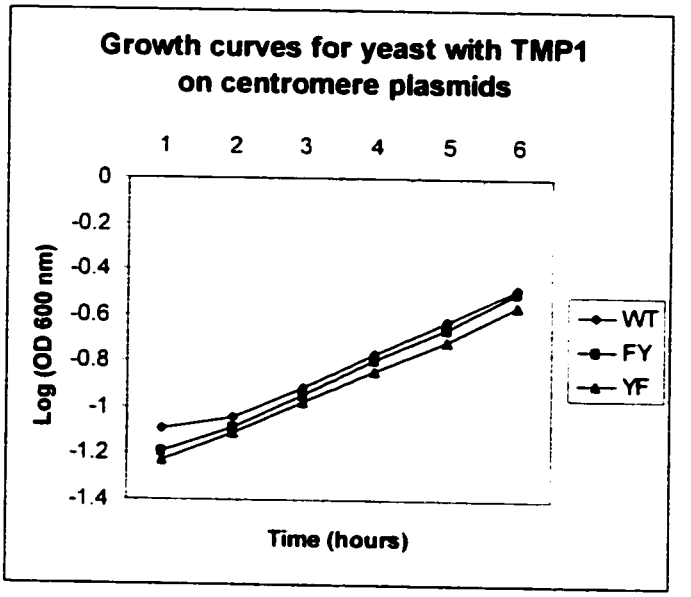
Since the mutant genes do complement in yeast yet fail to show any significant activity in the tritium release assay for thymidylate synthase, we speculated that there might be subtle differences between the wild type and mutants not detectable by the complementation assay. Growth rates of the *TMP1* null strain transformed with the centromere plasmids containing *tmp1-F126* and *tmp1-F133* were measured during log phase and found to be the same as those of wild type *TMP1* transformants with doubling times of 2 hours 10 minutes in rich media (Figure 3.17, top panel). Similarly, cells transformed with pEM54 (wild type *TMP1*), pEG54-FY and pEG54-YF grew at the same rate (2 hours 10 minutes doubling time). Transformants harbouring pEG54-FF however, grew more slowly with a doubling time of over 4 hours (Figure 3.17, bottom panel).

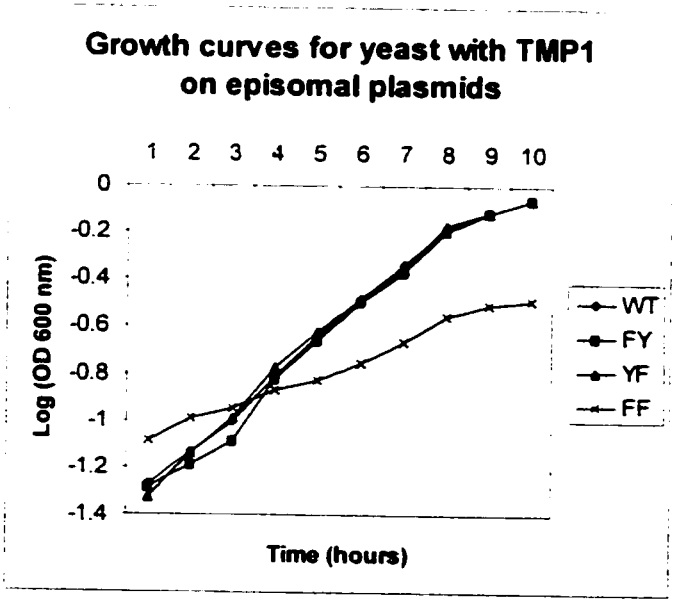
We also tested the ability of the mutants to grow at various temperatures to see if we could detect any temperature sensitivity. Figure 3.15b shows various strains and transformants grown at 36°C for 72 hours. The temperature sensitive mutant (*tmp1-S137*) did not grow when expressed chromosomally (D-2) or from an episomal plasmid (A-3).

Figure 3.17. Growth curves for yeast *TMP1* knock-out strain transformed with *TMP1* alleles expressed from centromere or episomal plasmids

Top panel: Strain RS4664 was transformed with centromere plasmids containing *TMP1* wild type or mutant alleles and sporulated. Spores containing the *tmp1-Δ0* allele and the centromere plasmid were propagated in rich media (YEPD) and their growth rates were measured during log phase. The doubling time for all three strains is 2 hours and 10 minutes.

Bottom panel: Growth curves for the haploid form of the *tmp1-Δ0* strain, transformed with episomal plasmids containing wild type or mutant *TMP1*. The strains harbouring the wild type (pEM54), *tmp1-F126* (pEG54-FY) or *tmp1-F133* (pEG54-YF) alleles all grew at the same rate, with a doubling time of 2 hours and 10 minutes. The strain with the *tmp1-FF* allele (pEG54-FF) grew more slowly with a doubling time of over 4 hours.


Growth curves for yeast with TMP1
on centromere plasmids
1 2 3 4 5 6
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Log (OD 600 nm)
Time (hours)
WT
FY
YF

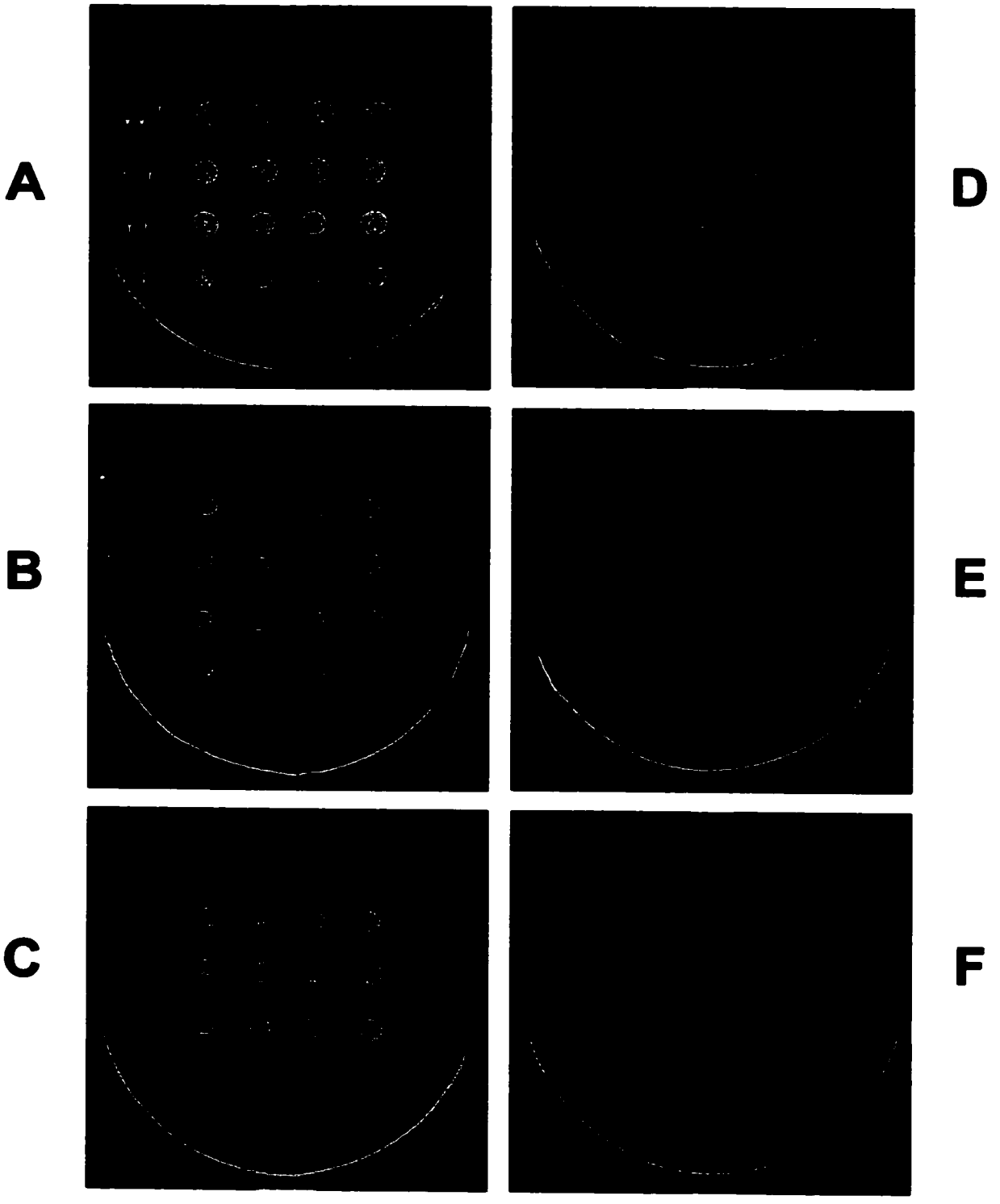

Growth curves for yeast with TMP1
on episomal plasmids
1 2 3 4 5 6 7 8 9 10
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Log (OD 600 nm)
Time (hours)
WT
FY
YF
FF

The double phenylalanine mutant was temperature sensitive when expressed from an episomal plasmid in the *tmp1-Δ0* strain (B-3), but surprisingly, did not exhibit this phenotype when expressed from a centromere plasmid in the *tmp1-6* point mutant (C-3 and D-3). TS-FY and TS-YF were not temperature sensitive and grew like the wild type when expressed from an episomal plasmid in the *tmp1-Δ0* strain (B-1, 4, 5). When TS-YF was expressed from a centromere plasmid in the *tmp1-Δ0* strain, cells grew more slowly (A-4, D-5) than those with TS-FY. When TS was expressed from a centromere plasmid in the *tmp1-6* point mutant, all four transformants (WT, FY,YF, FF) grew at comparable rates (plate C). The *tmp1-Δ0* strain transformed with the *thyA* gene (B-2) grew as well as the untransformed *TMP1*-disrupted diploid, RS4664 (A-2, D-1).

One final comparison which we made between the wild type and mutant proteins was their tolerance to a range of hydroxyurea concentrations. This compound is an inhibitor of ribonucleotide reductase, the enzyme that provides the nucleotide substrate (dUMP) used by thymidylate synthase. We reasoned that decreasing the dUMP pools of the cells might affect the mutant enzymes more drastically than the wild type, since the mutant enzymes display very low activity when tested by the tritium release assay. For this test, we used *tmp1-F126*, *tmp1-F133* and *tmp1-FF* expressed from the episomal plasmids transformed in the *tmp1-Δ0* strain. Surprisingly, there was no difference between TS-WT, TS-FY and TS-YF at any hydroxyurea concentration over a range from 5 to 200 mM (Figure 3.18). The TS-FF mutant however was significantly more sensitive at the lowest concentration used, 5 mM, and was unable to grow at 50 mM or higher. For TS-WT, TS-FY and TS-YF, decrease in growth rate began at 75 mM and cessation of growth occurred at some point between 120 mM and 200 mM hydroxyurea.

Figure 3.18. Sensitivity of mutant TS to hydroxyurea.

Strain RS5331 (*TMP1* knock-out, haploid), transformed with episomal plasmids pEM54 (WT), pEG54-FY, pEG54-YF, or pEG54-FF. Cells were grown to an optical density of 0.1 (at 600 nm) and 1 µl spots were deposited on YEPD plates containing hydroxyurea at the following concentrations: 5 mM (A), 25 mM (B), 50 mM (C), 75 mM (D), 120 mM (E), and 200 mM (F). All mutants grew as well as the wild type on control plates without hydroxyurea (not shown).


A
D
B
E
C
F

4. DISCUSSION

4.1. Deletion Mutations in Loop 2

Several three-dimensional structures of thymidylate synthase have been solved and published. The Loop 2 structure examined in this study has two distinct conformations in the TS structures reported so far. In the prokaryotes *E. coli* (Matthews *et al.*, 1990a) and *L. casei* (Finer-Moore *et al.*, 1993), Loop 2 projects toward the dimer interface and interacts with its counterpart on the opposite subunit. In the three eukaryotes reported, TS from rat (Sotelo-Mundo *et al.*, 1999), *L. major* (Knighton *et al.*, 1994), and *P. carinii* (Anderson *et al.*, 2000) this larger version of Loop 2 is folded back over its own subunit, away from the axis of symmetry of the dimer (Figures 1.4 and 1.5, pages 13 and 16). In both eukaryotes and prokaryotes, Loop 2 is constricted at the base by an almost invariant arginine (R120 in yeast) and aspartate (A139 in yeast) that form a salt linkage through their side chains. This salt bridge circularizes the loop and perhaps more importantly may help align the adjacent helices along a common longitudinal axis (Matthews *et al.*, 1990a; Hardy *et al.*, 1987).

Parts of the amino acid sequence of Loop 2 are well conserved among eukaryotes (Figure 3.4, page 69). A five-residue motif, RHFGA, is strictly conserved in the eukaryotes and initiates the amino end of the loop, beginning with the arginine that forms the salt bridge at its base. The other contributor to this salt linkage, the aspartate that terminates the loop region, is also fully conserved. Two more residues are absolutely conserved, tyrosine 126 and glycine 137, while tyrosine 133 is conserved with one

exception (*T. brucei*) where it is replaced with another hydroxylated residue, serine. The RHFGA motif lies in a region of TS which exhibits very low variability. This region spans parts of the two surface loops (Loop 1 and Loop 2) with eukaryote-specific inserts, and the small helix that connects them. The high conservation extends over the carboxy end of Loop 1, the connecting helix and the amino end of Loop 2, encompassing a sequence of 15 residues.

The other striking feature of the eukaryotic Loop 2 is the presence of the 8-residue insert that the prokaryotes lack and which includes tyrosines 126 and 133. In a previous study (E. Munro, Master's Thesis: Munro *et al.*, 1999) we investigated the significance of the Loop1 and Loop 2 inserts in enzyme structure and function by generating mutants with deleted insert sequences (shown in Figure 1.7, page 30). The Loop1 deletion affected enzyme kinetics by decreasing the apparent V_{max} and increasing the K_m for the nucleotide substrate. For Loop 2, we generated two deletion mutants. In TSΔEUK2a, the loop was reduced from its largest naturally occurring form (20 amino acids) to its smallest (7 amino acids) by the removal of residues 124-136, thus mimicking the prokaryotic Loop 2 of Phage T4 and *L. lactis*. In TSΔEUK2b the loop was reduced from 20 residues to12 by removing residues124-136 and introducing a sequence from *E. coli*, TDGPR. These deletion mutants had no complementation activity in either *E. coli* or yeast, and no enzyme activity was detected using the tritium release assay. Gel filtration chromatography (Figure 3.1, page 61) of purified proteins suggests that these mutant enzymes do not form stable dimers. One reason could be that the truncated versions of Loop 2 are not tethered to the subunit like in the wild type protein. This could create two possible problems: 1) steric hindrance between the two sister loops preventing normal

interaction of the subunits at the β-sheet interface and/or 2) exposure of the hydrophobic phenylalanine 117, normally buried by the "capping"of the wild type Loop 2, to bulk solvent thus causing local destabilization of the structure. Consistent with this notion is the fact that position 117 is occupied by a polar residue in the prokaryotes. Precedence of this has been reported; Schellenberger *et al.* (1994) inactivated *L. casei* TS by removing an *L. casei*-specific insert of 50 residues, and then partially restored activity by replacing an exposed phenylalanine by an aspartate. We attempted to restore activity to one of our deletion mutants (TSΔEUK2a) by substituting F117 with lysine, the residue found at this position in two prokaryotic sequences. However, the modified deletion mutant TSΔEUK2a-K117 continued to lack complementation activity when tested in *E. coli*.

We also attempted to create an active deletion mutant by completely deleting Loop 2 to prevent any possibility of steric hindrance between sister loops of opposite subunits. In order to bridge the two flanking helices we introduced a small glutamate-leucine turn. This mutant however was also unable to complement TS deficiency and had no detectable enzyme activity. Although deleting the entire loop removed the possibility of interference between unanchored sister loops, this may have introduced other problems leading to misfolding or instability. For example, glutamates do not occur frequently at the carboxy end of a helix since the negative charge of a glutamate side chain and the negative polarity of the carboxy end of a helix have a repelling effect on each other. Furthermore, the use of only two residues in the connecting turn would likely introduce a rotation and re-orientation of the downstream helix. The side chains of this clearly amphipathic helix that normally face the interior of the enzyme would then be exposed to the surface, preventing normal interactions with underlying residues of the enzyme core

and exposing hydrophobic side chains to solvent. Hence it is still inconclusive whether or not the earlier deletion mutants failed to dimerize because of steric hindrance at the dimer interface.

4.2. Effect of Point Mutations in *E. coli*

We next focused on the conserved residues of the Loop 2 insert, tyrosines 126 and 133. The substitution of tyrosine by phenylalanine at either position did not affect the ability to complement TS deficiency in *E. coli* when the genes were expressed from a high copy plasmid (pUC). When expressed from a yeast vector however, either centromeric or episomal, TS-FY (F126) and TS-YF (F133) no longer complemented. Another effect of these single tyrosine mutations observed in *E. coli* was the absence of tritium release activity.

When both tyrosines were substituted, either by two phenylalanines or by a phenylalanine/serine combination, the mutant genes failed to complement even though they were expressed from the pUC-derived plasmid. The serine hydroxyls (in TS-FS and TS-SF) did not suffice to restore activity levels to those of TS-FY and TS-YF, perhaps because of incorrect orientation or insufficient binding energy for hydrogen bonding (discussed later). In wild type TS from *T. brucei*, a serine hydroxyl is substituted for a tyrosine hydroxyl at position 133; our results however suggest that one serine hydroxyl cannot by itself provide the stability and/or conformation required for wild type activity.

When a tyrosine was provided at a position other than 126 or 133, in the TS-FYF mutant, complementation still failed. It appears then that at least one tyrosyl-hydroxyl is

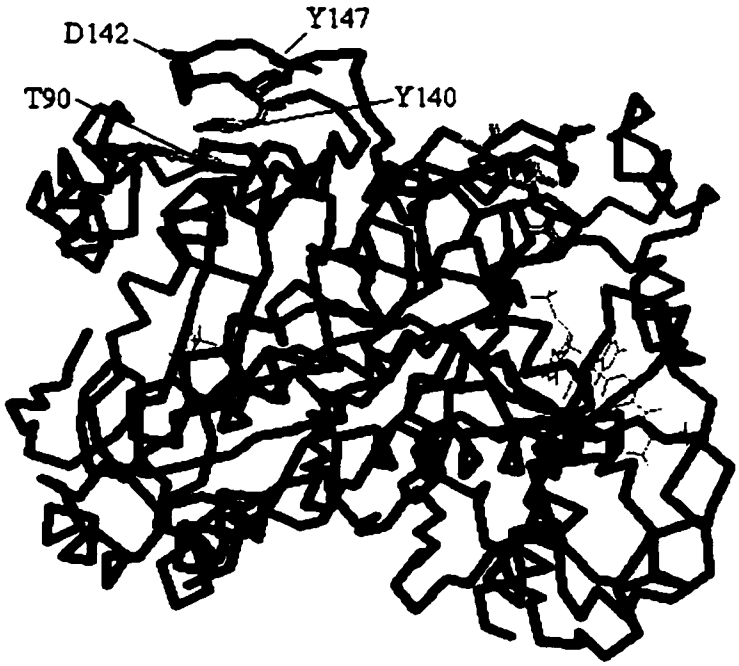
required at either position 126 or 133 in order to achieve some level of complementation. Further support of this came from the TS-Lma mutant, in which the tyrosines are present at their normal positions and which complements despite the substitution of six other residues within the loop. However, although TS-FY, TS-YF and TS-Lma complement when expressed from the pUC plasmid, none of these mutants show tritium release activity when assayed.

In 1999 Sotelo-Mundo *et al.* published the TS structure from rat, showing the Loop 2 conformation in detail (Figure 4.1). They reported that both tyrosines participate in hydrogen bonding. Tyr140 (126 in yeast) hydrogen bonds with Thr90 (76 in yeast), which is conserved in all sequences except one, where it is substituted with another hydroxylated side chain, serine. It seems surprising that all TS's, including those from the prokaryotes even though they lack the Loop 2 insert, possess a hydroxyl side chain at this position.

The other tyrosine, Tyr147 (133 in yeast), hydrogen bonds with Asp142; in yeast this aspartate is replaced by a threonine (Thr128) which could potentially hydrogen bond with the tyrosine as well. Thus one tyrosine stabilizes the loop structure by anchoring it to the enzyme core, while the other tyrosine stabilizes it by pinching in within the loop and forming what appears to be a short β-strand pair. Our results in *E. coli* suggest that these hydrogen bonds are important for preserving enzyme function, and that at least one of these interactions is required in order to retain some level of activity.

Figure 4.1. Interactions of conserved tyrosines in Loop 2.

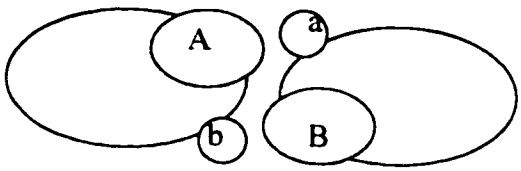
Eukaryotic Loop 2 (rat TS) showing hydrogen bonding involving conserved tyrosines 140 and 147 (Tyr126 and 133 in yeast). Tyrosine 147 hydrogen bonds within the loop with aspartate142 (Thr128 in yeast). Tyrosine 140 interacts with a core residue outside the loop, threonine 90 (Thr76 in yeast)


D142
Y147
T90
Y140

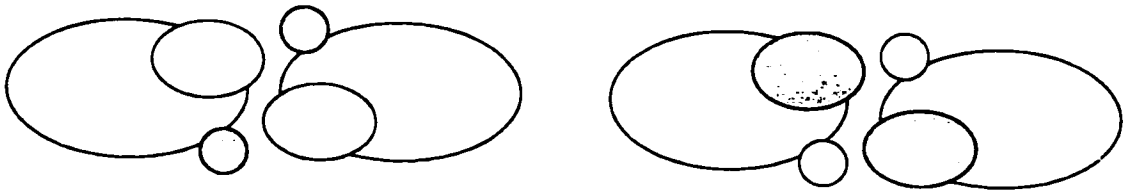
4.3. Complementation in the Yeast *tmp1-6* Point Mutant Strain

The *tmp1-F126*, *tmp1-F133* and *tmp1-FF* mutations, expressed from centromere plasmids, were first tested for complementation activity in the yeast haploid strain having a point mutation (*tmp1-6*) in its chromosomally encoded *TMP1* gene. All three mutants complemented in this strain. Ability to complement from this low copy plasmid was surprising since no tritium release activity had been observed in *E. coli*. It was all the more unexpected for TS-FF, since this mutant had not complemented in *E. coli*. For TS-FF, this result was verified by rescuing the plasmids from the yeast cell and re-transforming *E. coli* and yeast, as well as sequencing the rescued plasmids.

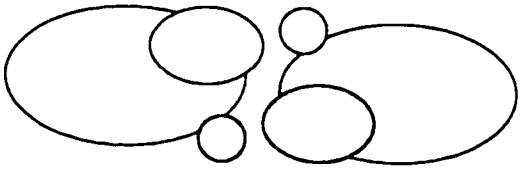
One possible explanation for the fact that the TS-FF mutant complements in a *TMP1* point mutant strain could be that heterodimers form between the *tmp1-6* and the *tmp1-FF* encoded proteins. Such cases of "subunit complementation" in thymidylate synthase have been reported previously (Pookanjanatavip *et al.*, 1992, Greene *et al.*, 1993). Since TS is a dimer enzyme with two active sites, and each active site is contributed to by both subunits, it is possible to restore activity to one active site by the formation of heterodimers between two inactive but distinct mutations, if these mutations are in a different region of the protein (Figure 4.2). The point mutant strain used in our study has an active site mutation at the conserved glycine 211 which is substituted with asparagine. Our *tmp1-FF* mutation lies in a surface loop that is unlikely to affect the active site directly but which clearly affects enzyme activity in some way. Loop 2 is proximal to the region of TS where the two arginine residues that contribute to the active site of the opposite subunit are located; i.e. these residues (Arg155, 156) are in a coil adjacent to the



TS dimer with 2 functional active sites, Aa and Bb



TS dimers with two non-functional active sites

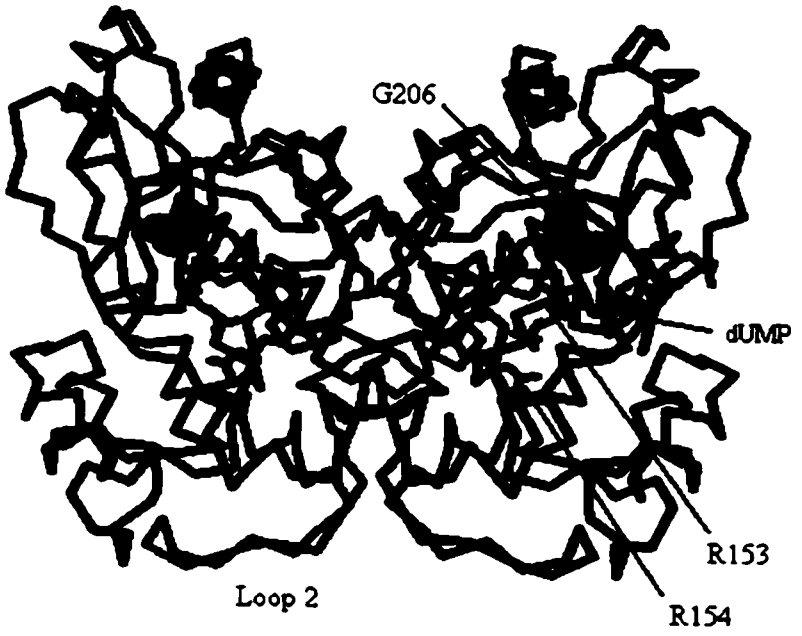


TS heterodimer with one functional and one non-functional active site

Figure 4.2: Subunit complementation in the TS heterodimer. The TS active site is comprised of a major (A,B) cavity contributed by one subunit, and a minor (a,b) component contributed by the other. The above diagram depicts how two inactive mutants can form a heterodimer with one functional and one non-functional active site.

Figure 4.3. Active site contribution of arginine 153 and 154 in *P. carinii*.

Conserved arginine 153 and 154 (Arg155, 156 in yeast) reach across the dimer interface to stabilize the phosphate moiety of dUMP, bound in the active site of the opposite subunit. The arginines thus form a "minor" component of the active site. These residues are adjacent to the helix that is downstream of Loop 2. Arg153 and 154 from subunit A (shown in light blue) contribute to the active site of subunit B (shown in red). Glycine 206 (Gly211 in yeast) from subunit B also participates in the formation of active site B. Subunit complementation could thus occur between a mutant of Gly211 (*tmp1-6*) and a mutant that affects the orientation of Arg155 and Arg156 in yeast.


G206
dUMP
R153
R154
Loop 2

helix downstream of Loop 2. Destabilizing the loop by removal of the tyrosine contacts might affect the orientation of the adjacent helix and coil region and interfere with the "minor" component of the active site (Figure 4.3). It is therefore possible that formation of heterodimers is the mechanism by which *tmp1-FF* complements in the point mutant strain. In support of this hypothesis, it has been previously reported that *tmp1-6* and *cdc21-1* complement each other in heteroallelic diploids (Game, 1976). Moreover, the *cdc-21-1* mutation also occurs in Loop 2 (discussed later).

4.4. Complementation in the Yeast Knock-Out Strain

To eliminate possible interaction between our mutants and the *tmp1-6* encoded TS, a *TMP1* knock-out strain was constructed by gene replacement with a *GFP-HIS* cassette. Evidence of the *TMP1* deletion was demonstrated by the following: 1) tetrad dissection showed a 2:2 ratio of surviving versus non-surviving spores, 2) dissection of the knock-out diploid transformed with *TMP1* or *thyA* resulted in 2-4 surviving spores, suggesting that a gene coding for TS could rescue otherwise inviable haploids 3) all haploid colonies that contained the *GFP-HIS* marker also possessed the *TMP1* or *thyA* plasmid; the cells could not be cured of the plasmid, indicating a dependence for a plasmid encoded gene for TS, 4) PCR amplification of *TMP1*-disrupted haploids transformed with *thyA* did not produce a fragment corresponding to *TMP1* when using appropriate primers, 5) immunoblots done with yeast TS antibodies on the *TMP1*-disrupted haploids transformed with *thyA* did not produce a TS band, 6) *TMP1*-disrupted haploids transformed with

mutant *TMP1* alleles had drastically reduced tritium release activity. Although the haploid colonies that grew from sporulation of the diploid parent strain exhibited a disparity in size ranging from quite small to fairly large, this variation in colony morphology did not correlate with the presence or absence of the *TMP1* deletion, but rather, appeared to be associated with the segregation of an unlinked allele of unknown function. Furthermore, colonies of different sizes were tested for complementation and growth rates and no variation in behaviour was observed from one to another. Since four mutants of one type from four different spores were tested and found to behave similarly, we assume that the initial size of the colony had no bearing on the behaviour of the mutants.

TS-FF did not appear to complement in the knock-out strain, since we could not isolate transformants that had both the chromosomal deletion and the *tmp1-FF* allele encoded on the centromere plasmid (pEG50-FF), either by sporulation or by plasmid shuffling. Tetrad dissection of the knock-out strain transformed with pEG50-FF consistently led to 2 surviving spores per tetrad. Double transformants of the knock-out strain containing pEG50-FF and pEM54 (wild type *TMP1* on a episomal plasmid) could not be cured of the episomal plasmid.

Of the three mutations tested in yeast, *tmp1-FF* exhibits the highest deviation from wild type behaviour. This mutant failed to complement in *E. coli* when expressed from any plasmid. As mentioned above, in yeast it does not appear to complement in a knock-out strain when expressed from a centromere plasmid, although it does complement in this strain when copy number is increased by using an episomal plasmid. However, even though increasing the gene copy number enables the mutant allele to complement, the

cells nonetheless exhibit a variety of phenotypes that differ from the wild type, TS-FY, and TS-YF transformants. The growth rate of the TS-FF transformants was markedly slower than wild type transformants, with a generation time twice as long as that of wild type TS. Sensitivity to hydroxyurea occurred at very low hydroxyurea concentrations (5 mM) with complete growth inhibition at 50 mM; the wild type and other mutants still grew fairly well at 120 mM. Since hydroxyurea is a specific inhibitor of ribonucleotide reductase and this enzyme provides the nucleotide substrate dUMP, the use of hydroxyurea presumably lowers the substrate pools available to TS. It appears then that TS-FF is more susceptible to decreased cellular levels of dUMP than the single tyrosine mutants or wild type enzyme.

Temperature sensitivity was observed at 36°C; at this temperature, TS-FF expressed from the episomal plasmid in the knock-out strain showed the same growth impairment as the temperature sensitive TS (*tmp1-S137*, also designated *cdc21-1*, Hartwell, 1973; Bisson and Thorner, 1981; Taylor *et al.*, 1987) either chromosomally or plasmid encoded. Interestingly this temperature sensitive mutation, a glycine substituted by a serine, is also located in Loop 2, four residues downstream of Tyr133 and two residues away from the carboxy end where the loop is constricted. Perhaps packing the larger serine side chain in a narrow region of the loop places a constraint that overcomes the salt linkage at the higher temperature.

When expressed from a centromere plasmid in the *tmp1-6* point mutant strain, TS-FF was not temperature sensitive, which is consistent with the observation that this genetic background is more favourable to this mutant, perhaps by enabling the formation of heterodimers.

The TS-FY and TS-YF mutants, by contrast, did not exhibit any of these phenotypes. They grew at the same rate as the wild type, showed the same degree of hydroxyurea sensitivity as the wild type, and were not temperature sensitive at 36°C. In either yeast strain (point mutant or knock-out) and expressed from either plasmid (centromere or episomal) these mutants were indistinguishable from the wild type in their complementation activity and phenotypes listed above. There were however three distinct deviations from wild type behaviour: 1) failure to complement in *E. coli* when expressed from yeast shuttle vectors, 2) shift toward the slower TS band in immunoblot analyses and 3) dramatically reduced level of tritium release in enzymatic assays.

The larger band in the TS doublet has been observed in our lab at higher protein resolutions but is yet unexplained. It could arise from modifications to the protein, such as tagging for degradation (Hiller *et al.*, 1996, Hong *et al.*, 1996) or phosphorylation since one recent study suggests that TS is a phosphoprotein (Samsonoff *et al.*, 1997). A slower TS band is also characteristic of enzyme that has been incubated with F-dUMP, a potent competitive inhibitor of TS which forms a covalent ternary complex with the enzyme and the cofactor MTHF (Santi and McHenry, 1972). Perhaps the shift toward the slower band observed with the mutants is an indication that the mutant protein is more readily targeted for degradation than the wild type protein, or that it is more susceptible to the formation of covalent complexes with inhibitors that may be present in the cell. An even more intriguing notion is that perhaps the putative phosphorylation of TS somehow enhances its activity, either by directly activating the protein or by preventing its binding to its own mRNA, an event that has been shown to take place and decrease TS expression by repressing translation (studies of human TS, Chu *et al.*, 1991, Chu and Allegra, 1996).

The fact that TS-FY and TS-YF ceased to complement in *E. coli* when switched to lower copy vectors (from pUC to yeast vectors) was not unexpected since the tritium release assays in *E. coli* extracts and whole cells had indicated that the activity of these mutants was severely impaired compared to wild type TS activity. More astonishing however, was the fact that in yeast this assay continued to give very low activity for these mutants, even though they showed no other abnormalities. The low activity of TS-FF was expected since we had observed other phenotypic consequences of this mutation. Consistent with all the Loop 2 mutations, the temperature sensitive mutant TS-S137 had no tritium release activity, either when the protein was chromosomally or plasmid encoded.

The low tritium release activity of mutants able to complement remains the single most perplexing result of this study and is yet unexplained, although this phenomenon has been reported in other studies with mutant TS from *E. coli*. Belfort *et al.* reported in 1984 that *thyA* mutants with greater than 5% wild type tritium release activity could complement, while those with less than 2% were unable to. They did not however indicate the level of expression of the plasmids used in their study. In 1996, Kupiec *et al.* also reported that some *thyA* mutants that had lower than 1% tritium release activity relative to wild type could complement in *E. coli*; these mutant genes however were expressed from very high copy plasmids (500 copies per cell). This is consistent with our observations that TS-FY and TS-YF which have no detectable tritium release activity in *E. coli*, complement when expressed from the high copy pUC plasmid, but do not complement when expressed from the lower copy yeast shuttle plasmids. In contrast,

Schellenberger *et al.* (1994) reported that an *L. casei* TS mutant could not complement in *E. coli* even though tritium release activity was about 4% of the wild type activity.

The peculiarity of our mutants TS-FY and TS-YF, is that while they fail to complement in *E. coli* when plasmid copy number is reduced, they show no anomalies when expressed from a one-copy plasmid in yeast, other than reduced tritium release activity. We speculate on several possible explanations for this phenomenon. The most interesting hypothesis would be that the yeast cell environment provides an unknown factor which enhances the mutant protein's ability to synthesize dTMP, and that the assay conditions disrupt this interaction. For example, another protein might conceivably associate with the mutant TS and help to stabilize an otherwise unstable tertiary or quaternary conformation, or simply channel substrates efficiently and facilitate their binding to the mutant enzyme. There are several possible candidates for these types of favourable intervention: 1) proteins that might associate to form a DNA replication complex (Reddy and Fager, 1993, and references therein), 2) proteins that comprise the dTMP synthesis pathway and might benefit from a substrate channelling system, (Knighton *et al.*, 1994), 3) proteins that may anchor or direct TS to the nuclear membrane (Poon and Storms, 1993; Samsonoff *et al.*, 1997). Work is underway to test this attractive hypothesis, which will make use of a novel technique recently developed in C. Boone's lab (Tong *et al.*, 2001) to carry out a synthetic lethal screen on the ca. 5100 non-essential yeast genes. In this procedure, a haploid yeast strain containing a chromosomal query mutation (tagged with *URA3*) is mated to haploid cells representing the entire deletion set of non-essential genes. The resulting diploids are sporulated, and an ingenious triple selection strategy selects for cells that are haploid, that contain the query mutation, and

that contain the knock-out allele. This system could be useful in identifying genes that are essential for the function of our TS-FY or TS-YF mutants.

Another hypothesis that could explain why the mutants complement without conspicuous phenotypic aberrations yet have no tritium release activity, is that the commercial folate cofactor supplied for the assay differs from the cofactor produced in the cells. The synthetic THF used for the assays was mono-glutamylated, whereas the cells produce poly-glutamylated forms of folate; as discussed in Section 1, TS generally has a higher affinity for the poly-glutamylated folate. If the use of mono-glutamylated folate does affect enzyme activity adversely, it clearly has a more severe effect on the mutants than on the wild type enzyme; this could reflect that the mutation affects the cofactor binding site. This possibility could be addressed in future work by testing the mutants in a strain with a deficient glutamyl transferase, the enzyme that attaches glutamate residues to folates; alternately the cofactor-independent debromination assay of BrdUMP could be used to compare the rate of dehalogenation in the mutants versus wild type TS (Garret *et al.*, 1979).

Finally, a third hypothesis that would explain these singular results is that dTMP levels synthesized by wild type TS might far exceed the cellular requirements for the nucleotide. This scenario however, is unlikely. Our results indicate that when expressed from episomal plasmids, TS-FY and TS-YF have approximately 1% of the wild type TS activity. If we extrapolate this to centromere-plasmid-expressed TS, for which we could not measure activity, 1% of wild type activity would be around 0.6 pmoles of protons per minute per 10^9 cells. A Loop 1 deletion mutant which we constructed and assayed in a previous study (Munro *et al.*, 1999), had 4% of wild type TS activity (~2.5 pmol

H+/min/10^9 cells); yet when this allele (*tmp1-ΔEUK1*) was expressed from a centromere plasmid in the *tmp1-6* strain, the cells did not grow as well as the wild type transformants. TS-FY and TS-YF have less tritium release activity than TS-ΔEUK1, yet they complement better. This favours the hypothesis that the intact yeast cell enhances the function of TS-FY and TS-YF, or that these mutants are unable to use mono-glutamylated folate as efficiently as the poly-glutamylated form.

4.5. Complementation by the *thyA* gene

When we initiated the study of the eukaryotic inserts in Loops 1 and 2, we hypothesized that these inserts might have a cellular function in the eukaryotic host. We constructed mutants with deleted inserts in the hope that this would not drastically affect enzyme activity, and that the mutants would complement in a prokaryote but cease to complement in a eukaryote. However, for all mutants that we have made so far, whether deletion mutations in either Loop1 or 2, or point mutations or substitutions in Loop 2, the effect is to impair tritium release activity or abolish it completely. This suggests that these loops, despite being located on the surface of the protein and not integrated into the core structure, have an important effect on enzyme structure. Since these loops cannot be easily modified without affecting enzyme activity, it is difficult to assess whether they have an *in vivo* role in the cell, mediated via their inserts. Hence we chose to test the *thyA* gene, in yeast cells, to see if the presence of these inserts was essential for complementation.

Our results demonstrate that the *E. coli thyA* gene, which has no insert in Loop 1 or Loop 2, complements TS deficiency as well as wild type TS in the two yeast strains tested (*tmp1-6* and *tmp1-Δ0*). In our construct, the *thyA* gene is driven by the GAL promoter, but complementation was tested using glucose as a carbon source, therefore we expect expression to be at most, uninduced levels of the enzyme. However we could not determine TS protein levels since our yeast TS antibodies did not recognize the *E. coli* enzyme. The tritium release activity of TS from *thyA* was lower than chromosomally expressed yeast TS but higher than any mutant TS from multi-copy plasmids. These results suggest that a prokaryotic TS, lacking eukaryotic inserts, functions nearly as well in the yeast cell as a eukaryotic TS with the inserts. Hence, if the inserts do have a cellular function in addition to their structural contribution, this role does not appear to be essential.

It is interesting to note at this point that Loop 2, which in yeast is highly sensitive to even as slight an alteration as the removal of a single hydroxyl group, exhibits in *E. coli* a high adaptability to mutations that would seem potentially more radical. Michaels *et al.* (1990) have reported that the Loop 2 salt bridge of *E. coli* TS is sufficiently tolerant to substitution by serine or tyrosine to allow complementation. Even more startling is the report of Kupiec *et al.* (1996) that Loop 2 is the most permissive region of *E. coli* TS for incorporating exogenous sequences up to 50 residues in length, without loss of complementation activity when expressed from a very high copy plasmid (500 copies per cells). This would suggest that the *E. coli* enzyme may have greater plasticity of structure than does yeast TS. Perhaps the yeast and other eukaryotes have evolved such that their

core structures have acquired mutations that accommodate and depend on a differently ordered Loop 2 than that of the prokaryotes.

4.6. Conclusions

This study has demonstrated unequivocally that the Loop 2 region of yeast TS is highly sensitive to modification, in spite of its location on the protein surface, away from the enzyme core and active site. Large deletions in this loop that mimic prokaryotic TS impede dimerization. The importance of the hydrogen bonds (shown in rat TS) involving the conserved tyrosines 126 and 133 is clearly demonstrated by the fact that these tyrosines cannot both be substituted by the structurally similar phenylalanine without incurring several deleterious consequences. These consequences however can be partially offset by increased gene dosage or heteroallelic complementation by *tmp1-6*. Single tyrosine/phenylalanine substitutions greatly diminish enzyme activity; this is demonstrated by low tritium release activity in both yeast and *E. coli* and loss of complementation in *E. coli* when gene copy number is reduced. For reasons yet unexplained, this decreased activity does not affect yeast cells in any way that is manifest, even when gene copy number is minimal. Lastly, the eukaryotic inserts of Loops 1 and 2 are not critical for TS function in yeast since *thyA* complements in a yeast *TMP1* knock-out strain.

4.7. Future Work

The generation of TS mutants that have reduced activity and exhibit several other deviations from wild type properties should be useful for investigating other cellular roles of TS or its possible interaction with other proteins. We have already begun the work for a synthetic phenotype screen (discussed earlier) by producing two strains that have the *tmp1-FY* and *tmp1-YF* alleles chromosomally encoded. The screen might reveal that combining these mutations with various deletion strains will produce a synthetic phenotype and lead to the identification of genetic interactions. If such proteins are detected, immunoprecipitation and/or two-hybrid studies should ensue to determine whether dependence on the putative "helper" protein is accompanied by physical association.

Also underway is the testing of these mutants in a genetic background that is deficient for producing the poly-glutamylated form of THF. This may shed light as to whether the mutants can use mono-glutamylated forms of THF and whether this is the reason why their activity is so drastically reduced. Another approach would be to use the debromination assay, since this reaction is cofactor-independent. In contrast, covalent F-dUMP binding absolutely requires concomitant binding of the cofactor and this assay could be used as a complementary approach.

Another interesting area of study would be to test if complementation of the *tmp1-6* and *tmp1-FF* alleles occurs via the formation of heterodimers. A starting point for this could be to cotransform the two alleles into *E. coli* and the yeast *TMP1* knock-out strain and test for complementation activity in these two different genetic backgrounds. In

addition, the mutant proteins could be overexpressed, purified, and subjected to dissociation followed by re-association conditions to determine whether partial enzyme activity could be restored by this process.

One particularly intriguing area of study would be to elucidate the phosphorylation state of TS protein in yeast and to address several related issues. Perhaps yeast TS becomes phosphorylated to prevent translational repression of its own mRNA, and this phosphorylation state could be regulated by the dTMP levels in the cell. This would be an interesting hypothesis to test in view of the fact that our low activity but complementing mutants reveal a shift toward a slower TS band in immunoblots.

Lastly, in order to delineate the structural role of Loop 2 in maintaining the integrity of the conformation of TS, purification of the enzyme for physico-biochemical studies and perhaps even crystallization of wild type yeast TS as well as mutant TS could enhance and enrich the already vast and growing body of knowledge published thus far on this central and significant enzyme.

5. LITERATURE CITED

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