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Expression of thymidylate synthase from a

temperature sensitive *thyA* mutant of

Escherichia coli

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A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements
for the Degree of Master in Science at
Concordia University
Montreal, Quebec, Canada

March 1989

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ABSTRACT

Expression of thymidylate synthase

from a temperature sensitive *thyA* mutant of

Escherichia coli

H. Ricky Kurzman

This study attempted to examine the proteins expressed from the region of the *thyA* gene in a temperature sensitive strain of *Escherichia coli* K12. The temperature sensitive *thyA* allele of strain N4316 was cloned and compared to a wild type *thyA* allele carried on the plasmid pBTA. Thymidylate synthase specific activity was compared between strains carrying the wild type and mutant *thyA* alleles on plasmid. The strain carrying the wild type plasmid had a thymidylate synthase specific activity higher than that of the strain carrying the mutant allele. As the growth temperature was increased, the specific activity of thymidylate synthase expressed from the mutant allele decreased more than thymidylate synthase activity expressed from the wild type allele. The two plasmids were transferred into a maxicell strain. Maxicell analysis showed three peptides being synthesized from the plasmid. As the growth temperature was increased, the amount of one of these proteins decreased. This peptide was identified to be thymidylate synthase. It appears that the mutant allele of *thyA* seems to affect the synthesis of thymidylate synthase, resulting in relatively less protein being made as compared to the wild type However, the possibility that the mutant allele increases the rate of degradation cannot be completely ruled out.

ACKNOWLEDGEMENTS

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Introduction:

Thymidylate synthase (EC 2.1.1.45) catalyzes the conversion of dTMP from dUMP. This reaction is the only *de novo* source of dTMP for DNA synthesis in *Escherichia coli*, suggesting that this enzyme is critical for cell growth. *E. coli* cells can also synthesize dTMP through the salvage pathway. *E. coli* cells which have non-functional thymidylate synthase (Thy⁻) undergo thymineless death when starved for thymine or thymidine, and surviving cells exhibit an increased mutation rate. (Barclay *et al*, 1982).

The lack of dTMP for growing *E. coli* cells results in the incorporation of uracil instead of thymine within the DNA (Kunz & Glickman, 1985). As a result of low or no thymidylate synthase activity, these stressed *E. coli* cells exhibit a number of additional phenomena.

E. coli cells which have low or no thymidylate synthase activit, have been found to have increased levels of ribonucleotide reductase (Lammers & Follman, 1983; Mollgaard & Neuhard, 1983; Biswas, Hardy & Beck, 1965).

In addition, thy⁻ strains of *E. coli* suppress nonsense and frameshift mutants of phage T4 (Herrington, Lapchak & Kohli, 1983, Herrington Kohli & Lapchak, 1984). It was proposed that suppression occurs during translation, suggesting that thymidylate synthase levels are critical for translational fidelity (Herrington, Kohli & Lapchak, 1984).

These phenomena suggest that thymidylate synthase synthesis and activity levels are critical for cell growth. Studies in yeast and higher eucaryotes show a very rigid regulation of both synthesis and degradation of thymidylate synthase during the cell cycle (Storms *et al*, 1984). Studies at the level of the protein reveal that the enzyme is found at very low levels within the cell, and thymidylate synthase activity levels do not vary much under different growth conditions.

Thymidylate synthase has been purified from *E. coli* by a series of chromatographic steps (Haertle, Wohlrab and Guschlbauer, 1979), and is composed of two identical monomers 264 amino acids in length (Belfort & Pedersen-Lane, 1984). Each monomer has a molecular weight of approximately 33000, as determined by labelling the enzyme with tritiated-FdUMP

(5 fluorodeoxyuridine monophosphate) and estimating the molecular weight from an autoradiogram of the proteins' migration on SDS-polyacrylamide gels (Belfort, Maley & Maley, 1983; R. Clarizio, unpublished results).

The *E. coli ThyA* gene has been cloned and characterized. The 1163 base pair sequence includes all of the regular transcriptional and translational control elements, including a promoter, a Shine-Dalgarno sequence, as well as transcriptional a d translational termination sequences. The gene was further subcloned into an expression vector which allowed for the isolation of large quantities of the enzyme for amino acid sequencing. The molecular weight of the enzyme subunit is 30441 molecular weight, as determined from the amino acid sequence (Belfort *et al*, 1983). The enzyme's active site includes cysteine-146 as shown by F-dUMP binding (Belfort, Maley & Maley, 1983). Further localization was accomplished by binding a THF (5,6,7,8- tetrahydrofolate) analogue to the enzyme, in order to find the THF binding site (Maley & Maley, 1987, 1988).

Sequencing of the *thyA* gene reveals an open reading frame extending into the 5' portion of the *thyA* gene. This open reading frame ends just before the transcriptional initiation region, and is followed by a functional transcription terminator at about 285 base pairs from the start codon of *thyA*. Observations which raise the possibility of another gene whose 3' end overlaps the 5' end of thymidylate synthase include this functional transcriptional terminator within the *thyA* sequence, and the inability to obtain viable 5' deletion mutants of *thyA* (Chu *et al*, 1984). Williams *et al*, upon examining the 5' region of *thyA*, have recently found evidence that there is an essential gene which extends into *thyA* (Williams *et al*, 1989).

Overlapping sequences have been observed in other systems. For example, the T4 gene encoding for dihydrofolate reductase shows a four-base overlap with the downstream gene, which specifies the functionally related enzyme thymidylate synthase (Purohit & Mathews, 1984). Within the reductase sequence, there are three open reading frames. One open reading frame would code for a small peptide 80 amino acids in length. The second contains 517 bases, with the characteristic ribosome binding site and control elements. This codes for the dihydrofolate

reductase (dhfr) protein. The third open reading frame starts at the end of the dhfr sequence (at base pair 914) and extends through the 3' end into the amino portion of thymidylate synthase. This four base overlap, ATGA, may be analogous to the occurence of the overlapping genes in bacteriophage lambda. In that case, there are a series of nine interlinked genes in the *ninR* region, with overlapping reading frames. At the boundaries between two consecutive open reading frames an ATGA sequence is repeatedly observed in a position that would allow it to act as a terminator (TGA) or an initiator (ATG). Each open reading frame is preceeded by a ribosomal recognition site which overlaps the terminal codons of the proximal reading frame (Kroger & Hobom, 1982). Translation of one of the *nin* genes blocks the initiation of transcription of the downstream gene. The conformation of the mRNA may affect the initiation of translation of the overlapping gene, so that the ribosomal binding site for the second gene is buried within the mRNA structure. Conformational change of the mRNA allows the ribosome binding site to be available for a ribosome. Thus, expression of the downstream gene is regulated by the amount of synthesis of the upstream gene. In such a case, two mutually exclusive mRNA secondary structures are possible. The formation of one over the other would be determined by interaction with the translation system, and indirectly by the levels of controlling molecules. One structure allows continuation of transcription into the operon, while the other causes termination of the transcript prior to the coding region of the downstream gene. If such is the case in thymidylate synthase, a structural mutation within the *thyA* locus may affect translation by altering the conformation of the mRNA in such a way as to either cause the ribosome to "hang up" on the mRNA to cause premature termination, or to alter the levels of synthesis of the enzyme.

Roodman and Greenberg (1971a, 1971b) studied the thymidylate synthase of a temperature sensitive mutant strain of *E. coli B*, and found that the rate of synthesis of the enzyme was affected by a mutation in the structural portion of the gene. Two experiments led to this hypothesis. On shifting a growing culture of this temperature sensitive strain from $37^\circ C$ to the permissive temperature of $25^\circ C$, synthesis of active thymidylate synthase ocurs immediately

at a rate which is greater than that of a control enzyme. This suggests that thymidylate synthase may have been present in an incomplete form, and just had to be completed. Following the initial burst of completed enzyme, a slower, *de novo* phase of synthesis occurs.

The second experiment involves density labelling of the thymidylate synthase protein. The strain is grown in heavy water (D_2O) at the restrictive temperature of 37°C and then shifted to fresh medium (H_2O) at the permissive temperature. Upon separating the proteins on cesium chloride gradients, it was found that the newly synthesized thymidylate synthase protein consisted of two species. One component, the heavy peak, must have been synthesized from deuterium-labelled precursors at 37°C even though active enzyme was not being completed. They claim that the mutation prevents the growing polypeptide chain from assuming the proper conformation. This alteration in structure prevents the movement of the ribosome, and results in partially synthesized protein. When the temperature is shifted down to 25°C, the protein changes its conformation to allow the ribosome to continue its passage, which results in the completion of the protein.

E. coli strain N4316 suppresses nonsense and frameshift mutations in phage T4 (Herrington, Kohli & Lapchak, 1984). This suppression is caused by the temperature sensitive *thyA* mutation found in this strain (Herrington, Kohli & Lapchak, 1984). The *thyA* mutation has been mapped and is a structural one, located somewhere between nucleotides 268 and 361 (Belfort & Pedersen-Lane, 1984). R. Clarizio and M.B. Herrington (unpublished results) have compared the thymidylate synthase from strain N4316 to that of a closely related Thy+ strain D10. The specific activity of thymidylate synthase of strain D10 decreased slightly as the growth temperature increased. Strain N4316 contained about 19% of the specific activity of D10 when grown at 31°C, and the activity decreased substantially as the growth temperature increased to about 2% of wild type levels. Since N4316 grows well without thymidine at 31°C, it was concluded that less than 19% of wild type thymidylate synthase activity levels is sufficient for cell growth. This is consistent with the results of Belfort and Pedersen-Lane (1984)

who found that 5% of wild type levels is the minimum for cell growth.

Heat inactivation studies have shown that, when crude extracts are heated at 45°C for varying lengths of time before assay, the activity of thymidylate synthase from strain N4316 decreases to a greater extent than does the thymidylate synthase activity of strain D10 (R. Clarizio, unpublished results). This observation is consistant with a structural mutation, as was shown by genetic mapping (Belfort & Pedersen-Lane, 1984). This seems to indicate that the structural mutation affects the lability of the enzyme.

There are a number of ways in which a structural mutation can alter the apparent levels of an enzyme relative to wild type levels. The amount of enzyme may remain the same but the activity per molecule may be changed. This would arise if the structural mutation causes an altered conformation of enzyme, creating a population of molecules which cannot bind dUMP as effectively, thus lowering thymidylate synthase activity. Another possibility would be that the amount of enzyme may be decreased, either by a reduction in the synthesis or by an increase in degradation. A change in the amount of synthesis may result if the structural mutation causes a conformational change of the mRNA thereby masking the ribosomal binding site, or causing an obstacle or block for the ribosome to continue its passage along the mRNA.

J. Basso (M.Sc. thesis, Concordia University, Montreal, 1987) investigated mRNA levels of the two strains, N4316 and D10, in order to see if there is any change in amount or types of mRNA specific to *thyA*. If translational interference occurs in strain N4316, the mRNA transcript might be affected. It may be possible that the frequency of premature termination of transcription might be increased, thereby resulting in less thymidylate synthase protein being synthesized. No change in the size or level of the *thyA* specific transcripts was found; therefore, the rate of transcription is probably not affected. In this study, we wished to examine the relationship between the activity levels and enzyme levels in strain D10 and the temperature sensitive mutant strain N4316. We cloned the mutant *thyA* gene from strain N4316 in order to compare it to a wild type thymidylate synthase contained on the plasmid pBTA (Belfort *et al*, 1983).

We compared restriction enzyme patterns of the two plasmids, and thymidylate synthase activity of the plasmid carrying strains to that of strains N4316 and D10. As expected, thymidylate synthase from the cells carrying the thy^{ts} allele on the plasmid showed a similar activity and heat lability to that of N4316.

Sancar, Hack & Rupp (1979) developed a technique used to identify plasmid coded proteins. This technique uses maxicells, which are ultraviolet-irradiated E. coli cells carrying recA and uvrA mutations. In this system, because of extensive unrepaired damage to DNA, extensive expression of chromosomal genes is prevented, whereas some copies of genes carried on multicopy plasmids are not damaged and these can direct the synthesis of proteins which can be detected by labelling with ^{35}S-methionine. The advantage of using this technique is two-fold. By using plasmids, it is easy to manipulate the DNA and to amplify the gene products. Additionally, by using maxicells, the number of proteins being labelled are very few, thus making it much simpler to study the proteins of interest. It is possible to use the maxicell system to determine the order of a series of genes located on a large plasmid (Dykstra, Prasher & Kushner, 1984; Lerner, Stephenson & Switzer, 1987), or to identify polypeptides being synthesized from various genes located on a plasmid (Berg, Squires & Squires, 1987; Maruyama, Yamamoto & Hirota, 1988). However, labelling proteins with ^{35}S may also result in the detection of polypeptides of unknown origin, or sizes different from those expected (Lerner, Stephenson & Switzer,1987). This may be due to the opening or closing of a reading frame in the plasmid such that either a larger or smaller peptide is synthesized. The preparation of maxicells has also been used to study the electrophoretic mobility of mutant proteins (Betz and Fall, 1988).

We used the maxicell system to study the expression of thymidylate synthase from the plasmids to determine if the amount of thymidylate synthase changes as the growth temperature increases. We wanted to examine peptides being coded for by the region surrounding the thyA gene, and then examine the amount of thymidylate synthase synthesized as the growth temperature is altered. This would allow us to see if the lower thymidylate synthase specific activity

found in strain N4316 may be due to lower synthesis of the enzyme when compared to the wild type strain D10.

Materials and Methods

Bacterial and Plasmid Strains:

The genotypes and sources of the *Escherichia coli K12* bacterial strains used in this study are listed in Table 1. Plasmid carrying derivatives of strains are described by the strain name and plasmid name separated by a slash.

The plasmid pBR322 (Maniatis, Fritsch & Sambrook, 1982) was obtained from B. Glick.

The plasmid pBTA, which was obtained from M. Belfort, is a derivative of pBR322 with the thyA gene on a 7.2kb *HindIII* insert derived from a l*thyA* transducing phage (Belfort *et al*, 1983).

The plasmid pRK11 was constructed in this study, as was the plasmid pRK12, and plasmid pJB6.

Storage and Maintenance of Bacterial Cells:

The bacterial strains were restreaked on agar plates once a month. For long term storage, 2ml cultures of newly constructed strains were grown overnight in either AB or appropriately supplemented minimal medium. An equal volume of 80% glycerol was added to the cultures and this was transferred to sterile vials which were stored at -70°C.

Media:

AB medium (Apirion, 1966) contained 10g Difco Nutrient broth, 10g Difco Vitamin Free casamino acids in one litre of distilled water. AB agar plates were solidified with 15g of Bacto-agar per litre of liquid media.

Minimal medium A (Miller, 1972) consists of 10.5g K_2HPO_4, 4.5g KH_2PO_4, 1.0g $(NH_4)_2SO_4$, and 0.5g sodium citrate.$2H_2O$ per litre medium. One ml of 20% $MgSO_4$ and 10ml of 20% glucose was added to each litre of minimal medium prior to use. Required supplements (amino acids, thymidine) were added at concentrations of 50ug/ml of thymidine and 40ug/ml of amino acids. Minimal medium A plates were solidified with 15g of Bacto-agar per litre media.

K medium (Rupp *et al*, 1971), which consisted of M9 medium containing casamino acids and thiamine, was used for growth of cells for maxicell analysis.

Table 1: *Escherichia coli K12* strains

Strain	Genotype	Source and reference
CSR603	uvrA6 recA1 phr-1 thr leu pro his thi arg lac gal ara xyl mtl rpsL	A. Sancar (Sancar *et al*, 1979)
D10	metB1 rna-10	M.C. Ganoza, (Gesteland, 1966)
JF1754	hsdr lac gal metB leuB hisB436	Storms *et al*, 1981.
MH293	hsdr lac gal metB leuB hisB436 thyA729	M.B.Herrington (Herringtor: *et al*, 1988)
MH295	hsdr lac gal metB leuB hisB436 thyA729 recA:Tn10	M.B.Herrington (Herrington *et al*, 1988)
MH461	metB1 rna-10 thyAD64	M.B. Herrington (Herrington *et al*,1988)
N4316	metB1 rna-10 thyA(Ts) sts	M.C. Ganoza (Phillips *et al*, 1969)

The media was made up of 11.3g $Na_2HPO_4 \cdot 7H_2O$, 3.0g KH_2PO_4, 1.0g NH_4Cl, and 10.0g casamino acids per litre media. For 100ml, the following was added prior to use: 0.1ml 20%$MgSO_4$, 0.2ml 50mM $CaCl_2$, 1.0ml 20% glucose, and 0.1ml 1% thiamine.

Modified Hershey medium (Worcel & Burgi, 1974) was used for maxicell analysis. Sulphate free Hershey salts, per litre, contained 5.4g NaCl, 3.0g KCl, 1.1g NH_4Cl, 15mg $CaCl_2 \cdot 2H_2O$, 0.2g $MgCl_2 \cdot 6H_2O$, 0.2mg $FeCl_3 \cdot 6H_2O$, 87mg KH_2PO_4, 12.1g Tris, pH7.4. Sulphate free Hershey medium for strain CSR603, per 100ml Hershey salts, contains 2ml of 20% glucose, 2.5ml threonine (4mg/ml), 2ml leucine (4mg/ml), 5ml proline (4mg/ml), 5ml arginine (4mg/ml) and 0.5ml thiamine (2mg/ml).

Antibiotics:

Ten ml of filter-sterilized ampicillin solution (10mg/ml) was added per litre media used for propagation of plasmid-carrying cells prior to use. Ampicillin was added to all growing cultures containing plasmid to maintain selection for the plasmid, in order to ensure that none of the cells would lose the plasmid.

Scintillation counting:

Bray's scintillation fluid (Bray, 1960) contained 60g scintanalyzed naphthalene, 4g scintanalyzed PPO (2,5-Diphenyloxazole), 0.2g dimethyl POPOP (2,2'-p-phenyl-enebis (4-methyl-5-phenyl)-oxozole), 100ml methanol, 20ml ethylene glycol and p-dioxane made up to a volume of one litre in a foil covered volumetric flask. The fluid is dissolved overnight in a fumehood, and then transferred and stored in a brown bottle.

Radioactive samples to be counted were placed in 7.0ml of scintillation fluid and counted in the LKB liquid scintillation counter.

Agarose Gels:

Agarose gels (1%) were made by dissolving 1g ultrapure agarose (IBI) in 100ml 1X TAE buffer. A concentrated stock solution of 50x TAE consists of 242g Tris, 57,1 ml of glacial acetic acid, and 100 ml of 0,5M EDTA (pH 8,0) (Maniatis et al, 1982). Gels were run at 100V, stained for twenty minutes with ethidium bromide (approximately one mg/100ml TAE buffer), destained in TAE

buffer for approximately ten minutes, and DNA was observed by looking at the stained gel on a Transilluminator (Ultraviolet Products Inc).

Isolation of *Escherichia coli* Chromosomal DNA:

DNA was extracted by the freeze-thaw method as described in Schlief & Wensink (1981).

Five ml of cells were grown overnight and collected by centrifugation at 5000 rpm for five minutes. They were resuspended in one ml media and transferred to Eppendorf tubes. The cells were centrifuged for one minute and resuspended in 15ul lysozyme mix (2mg/ml lysozyme in 0.15M NaCl, 0.1M EDTA). The mix was incubated at 37°C for 20 minutes, just until the cells began to lyse, and were then quickly frozen in liquid nitrogen. 125ml of SDS mix (1% SDS in 0.1M NaCL, 0.1M Tris pH9) was added and the mixture was stirred as the cells thawed. 150ul phenol saturated with the SDS mix was added and the mixture was vortexed. After five minutes centrifugation, the upper aqueous phase which contains the DNA was removed and precipitated with 300ml 95% ethanol for 60 minutes at -20°C. After a further ten minute centrifugation, the DNA was dissolved in 0.1x SSC. 20x SSC was made by dissolving 175.3g of NaCl and 88.2g of sodium citrate in one litre of water, with the pH adjusted to 7.0 (Maniatis *et al*, 1982). Five microlitres of 20xSSC was added, and the sample was then treated with RNAase, 4ml of RNAaseT1 (0.8mg.ml) and 3ml RNAaseA (2mg/ml), and incubated for 30 minutes at 30°C. 100ml of phenol saturated with SSC was added, and after five minutes of centrifugation, the upper aqueous DNA phase was removed. The phenol was removed with ether, and the DNA was precipitated with two volumes of 95% ethanol at -20°C for at least one hour followed by a 15 minute centrifugation at 4°C. The DNA was washed twice with 75% ethanol, dessicated, and resuspended in 100ml TE (0.01M Tris, 0.001M EDTA, pH7.6). The concentration of the DNA was determined by measuring the absorbance at 260nm, and the DNA was stored at 4°C until needed.

Isolation of Plasmid DNA:

Small amounts of plasmid DNA for constructions or rapid identification of correct inserts was isolated by the alkali-SDS method of Birnboim and Doly (1979), as described by Maniatis *et al* (1982).

A one ml overnight cell culture was centrifuged and resuspended in

100ml lysis solution (2mg/ml lysozyme in 25mM Tris, 10mM EDTA, 50mM glucose, pH8) and kept on ice for 30 minutes. 200ml alkaline SDS (0.2N NaOH, 1% SDS) was added to the sample and kept on ice for five minutes, after which 150ml of 3M sodium acetate was added. This was kept on ice for at least one hour, and then the proteins were precipitated by centrifugation for ten minutes. The supernatant carrying the DNA was transferred to a new tube and precipitated with 0.2ml 95% ethanol and 0.1ml 0.1M sodium acetate for at least ten minutes at -20°C. The DNA was centrifuged for 5 minutes, washed with 70% ethanol, dried and resuspended in 50ml TE.

Large scale isolation of Plasmid DNA:

For large amounts of plasmid DNA, the alkali-lysis method described in Maniatis et al (1982) was followed.

Cells were grown overnight in 250ml AB medium with 2.5ml ampicillin (10mg/ml) and harvested by centrifugation at 9000g for 15 minutes. The cells were resuspended in a minimal amount of 25mM Tris, 10mM EDTA pH8, separated into 4 Oakridge tubes, and spun down. The cells were then resuspended in 5ml of lysozyme solution (5mg/ml lysozyme in 50mM glucose, 10mM EDTA 25mM Tris pH8.0) and allowed to stand at room temperature for ten minutes. To this mix, 10 ml of SDS was added (1% SDS in 0.2N NaOH), and the tubes were mixed by gently inverting them several times. The tubes were kept on ice for about twenty minutes, at which time 7.5ml acetate was added. The acetate should be balanced between potassium and acetate. Therefore, the stock solution was 60ml of 5M potassium acetate, 11.5ml of glacial acetic acid, and 28.5ml of water. The resultant solution was 3M potassium, and 5M acetate. The mix was inverted a number of times, and left to stand on ice for about 20 minutes. The proteins and cell debris were pelleted by centrifugation at 35000g for one hour at 4°C. Equal quantities of supernatant was transferred into two Oakridge tubes, and was mixed with 0.6 volumes isopropanol, and was kept at room temperature for 15 minutes to allow the DNA to precipitate. The DNA was collected by centrifugation at 14000g for 30 minutes at room temperature. The pellet was washed with 70% ethanol at room temperature, and dried with a stream of nitrogen gas. The DNA was resuspended in 6.85ml of TE, and 7.4g cesium chloride and 0.9ml ethidium bromide (10mg/ml) were added. The suspension of DNA in Cesium chloride was

spun in the Beckman L8-70 ultracentrifuge at 37000rpm at 4^oC for at least 60 hours. Under these conditions the cesium chloride forms a gradient which separates the DNA species by density.

After centrifugation, the plasmid DNA was isolated by observing the ethidium bromide-stained DNA under UV light. The lower band containing plasmid DNA was removed by inserting a needle into the side of the tube. The plasmid band was transferred to a tube containing about 1.5ml 70% isopropanol saturated in NaCl, in order to remove the ethidium bromide. This first wash was done in the dark. The top phase, which was pinkish in colour, was discarded, and about two volumes of isopropanol mix was added to the bottom phase containing the DNA. This washing was repeated until the top phase was clear in colour. In order to remove all traces of cesium chloride, the lower phase was dialyzed twice for at least four hours each against TE. The DNA was precipitated with 95% ethanol for several hours at -20^oC, spun, washed in 70% ethanol, and resuspended in 200ml TE. The concentration of the DNA was determined by measuring the absorbance at 260nm, and stored at 4^oC until needed (Davis, Botstein and Roth, 1980. p. 126).

Southern transfer:

After electrophoresis, DNA was transferred to Pall Biodyne A nylon membrane according the the manufacturer's guidelines. After electrophoresis, the agarose gel was placed in 150ml of denaturing solution (2.5M NaCl, 0.5M NaOH) for 30 minutes with gentle agitation. The buffer was then replaced with neutralizing solution (3M sodium acetate pH5.5), and shaken gently for another 30 minutes. The excess buffer was removed from the gel surface, and the gel was then placed on a sheet of filter paper resting on a glass plate, with the ends soaking in a reservoir of 20X SSC (3M NaCl, 0.3M NaCitrate pH7.0). The gel was flanked with strips of Saran Wrap^{tm} to prevent short circuiting, such that the blotting buffer would go through the gel and not around it. The Biodyne A membrane was placed on top of and completely covering the gel surface. All trapped air bubbles were removed. The membrane was covered with filter paper, a stack of paper towels, a glass plate, and a weight. The transfer continued for about 18 hours, and the paper towels were changed as they absorbed moisture. The membrane was then removed from the gel surface and blotted dry. It was then baked at 80^oC for one hour.

Hybridization:

The membrane was placed in a plastic bag with 4ml of hybridization solution per 100cm^2 membrane. Hybridization solution contained (final concentration): 5x Denhardt's buffer (100x Denhardt's: 2% ficoll, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 5x SSPE (20X SSPE: 3.6M NaCl, 0.2M NaH$_2$PO$_4$, 20mM EDTA, pH7.7), 0.2% SDS. Non-homologous, denatured DNA was added to a final concentration of 100mg/ml. This was usually salmon sperm DNA. The bag was heat sealed and immersed in a 65°C bath for one hour. The hybridization solution was replaced with fresh solution, and the radioactive probe was added. The bags were then resealed and immersed in the 65°C bath for the duration of the hybridization, between 18 to 24 hours. After hybridization, the membrane was sealed in a plastic bag with wash buffer in a ratio of 250ml per 100 cm^2. Wash buffer consisted of 5mM NaH$_2$PO$_4$, 1mM EDTA, 0.2% SDS pH7.0. The membranes were washed three times, for 30 minutes each at room temperature. The membranes were air dried, and were then ready for autoradiography.

Preparation of radioactive probes:

Nick translation was carried out as described by the manufacturers of the Nick translation kit (NEN or Amersham).

A typical reaction sample, with a final volume adjusted to 25ml contained 5ml dCTP (a-^{32}P, specific activity 3000 Ci/mmol), 5ml Nick translation buffer, 4ml cold dNTP mixture, 2ml DNA polymeraseI, 2ml DNAaseI to start the reaction, and 1.0mg plasmid DNA. The reaction was allowed to proceed for two hours at a temperature of 15°C.

The reaction was stopped by adding 20ml of 0.25M EDTA, and precipitated with 4ml 3M sodium acetate, 1ml tRNA (1mg/ml) and 2-3 volumes of 95% ethanol. The DNA was precipitated at -70°C for 5-6 hours, or overnight at -20°C. The DNA was centrifuged for twenty minutes, and then precipitated a second time in 95% ethanol for a few hours at -20°C. It was then centrifuged, dessicated and resuspended in a small volume of sterile water (approximately 20ml). The probe was then ready to be hybridized to the membrane.

To check for incorporation of label, 2ml of the probe sample was added to

100ml of carrier DNA in a disposable tube. a 10ml portion was spotted onto a nitrocellulose filter. Two ml of cold 10% TCA/0.1M sodium pyrophosphate was added to the remaining 90ml, left on ice for 30 minutes, and then filtered through a nitrocellulose filter. The filter was washed with cold TCA and ethanol and dried. The two nitrocellulose filters were placed in Bray's scintillation fluid and then counted in the liquid scintillation counter. The first count measured total radioactivity, whereas the second, the TCA precipitated one. counted only the ^{32}P incorporated DNA. A typical Nick translation gave incorporation of approximately 80%.

Transformation of _Escherichia coli_ with plasmid DNA:

Cells were made competent by the calcium chloride method as described by Davis, Botstein and Roth (1980). Cells from an overnight culture were subcultured into 40ml of fresh AB medium. The cells were collected in early log phase, at an OD_{600} of about 0.3. They were sedimented at 3500g for 15 minutes at 4°C. The cells were resuspended in 20ml of 50mM $CaCl_2$ and kept on ice for 10-60 minutes (usually 20 minutes). The cells were centrifuged at 3500g for 15 minutes at 4°C, and resuspended in 2ml of $CaCl_2$ and left on ice for 10-60 minutes (usually 20 minutes). 0.1ml cells were added to about 5ng of plasmid DNA (in 10ml sterile water). The mixture was left on ice for ten minutes, heat treated for two minutes at 45°C, and then put back on ice. 900ml of sterile water was added to the competent cells and DNA. 100ml were plated onto each plate, and incubated. Efficiency of transformation using pBTA was approximately 250 colonies per plate.

Preparation of crude extracts for thymidylate synthase assay:

Cells were grown in 250 ml minimal media to an OD_{600} of 0.5. The cells were collected by centrifugation at 3500g for 15 minutes at 4°C, and resuspended in about 20ml 0.1M Tris-$MgCl_2$ pH7.4. The cells were centrifuged a second time, and resuspended in the same buffer at a concentration of 1ml per 0.2g cells. The ice cold cell suspension was then sonicated using a Sonifier Cell Disruptor. Three to four twenty second sonic bursts were used with a 30 second cooling period between each. Cell debris was removed by centrifugation at 35000g for 30 minutes at 4°C. The protein concentration of the supernatant was determined by doing a Lowry protein assay using bovine serum albumin as a standard (Lowry _et_

al, 1951). The crude extracts can be stored at -20°C for at least six months, without any loss in thymidylate synthase activity.

Thymidylate synthase assay:

The assay for thymidylate synthase is a tritium release assay (Roodman & Greenberg, 1971a).

A mix stock solution was prepared containing 1.1ml 1M Tris pH7.4, 0.28ml 14M B-Mercaptoethanol, 0.5ml 0.01M EDTA, 0.12ml 37% formaldehyde, and 7.0ml water, and was stored at 4°C. A tetrahydrofolic acid (THF) stock solution was prepared by adding 3ml of THF (0.5g in 3ml 1M B-mercaptoethanol, Calbiochem) to 3ml of B-mercaptoethanol, 16.7ml Tris pH7.4, and 10.6ml water. This was distributed into 0.1ml aliquots, and quickly frozen in liquid nitrogen. The THF stock samples were then stored at -20°C until needed. At the time of the assay, 900ml of mix stock was combined with 100ml of THF stock and was dispensed in 50ml aliquots in Eppendorf micro test tubes, 10ml ^{3}H-dUMP (6.02x10^5 dpm/mmole) was added, and 0.01M Tris-0.01M MgCl$_2$ buffer was added so that the final volume after adding crude extract would be 200ml. The tubes were kept on ice. To begin the assay, reaction tubes were incubated at 31°C for one minute, then crude extract was added. The reaction tubes were incubated for ten minutes and the reaction was then stopped by adding 500ml of Norite A (6g activated Norite A suspended in 50ml 0.01M HCl). The tubes were centrifuged for ten minutes in an Eppendorf Microcentrifuge, and 200ml of the supernatant was placed in scintillation fluid and counted in the liquid scintillation counter. The specific activity of the enzyme was determined by the following calculation,

SA=U/VC,   where U=D/FST

SA= specific activity

V= volume of protein added (ml)

C= concentration of protein extract (mg/ml)

D= DPM measured

F= fraction of reaction counted (usually 0.7)

S= specific activity of ^{3}H-dUMP (dpm/mmole)

T= time of reaction (min)

A unit of activity was defined as the amount of enzyme required to convert

1mmole of dUMP to dTMP per minute. Under the conditions used, this assay was sensitive enough to detect approximately 0.005 units.

For heat inactivation studies, crude extract containing 340mg of protein in 140ml Tris-MgCl$_2$ buffer was incubated at 45°C for varying lengths of time before continuing the assay at 31°C.

Assays on crude extracts were generally done several times and data from two or three extracts were pooled. The variability on replicate assays was less than 15%.

Maxicell Analysis:

Maxicell analysis requires the maxicell strain CSR603 (Sancar, Hack & Rupp, 1979). This strain was transformed with the plasmids used in this study.

An overnight culture of CSR603 cells carrying the plasmids were subcultured into 10ml of fresh K medium and grown at 37°C with vigorous aeration to early log phase (OD$_{600}$ of about 0.3).

The cells were irradiated using a UV dose of 3-5J/m^2 in a glass petri dish, with shaking. Using a Gelman-Camag universal UV lamp, with a wavelength of 254nm, irradiating the cells for five minutes was found to be sufficient. The cells were transferred to a sterile flask and incubated at 37°C for one hour with rapid shaking. Cycloserine was added to the cultures to a final concentration of 100mg/ml. Incubation was continued for 8-10 hours at 37°C, in the dark. The cells were collected by centrifugation and washed twice with Hershey salts. The cells were resuspended in 6ml of Hershey medium, separated into 2ml aliquots, and pre-incubated at 31°C, 37°C, or 43°C for one hour. ^{35}S-methionine (1000Ci/mmole) was added to a final concentration of 5mCi/ml. Incubation was continued another hour, and the cells were collected by centrifugation. They were washed once with 100ml of 0.01M Tris-0.01M MgCl$_2$ buffer, lysed by boiling, and frozen and stored at -20°C until needed.

Densitometric analysis of autoradiographs:

Autoradiographs of polyacrylamide gels were analyzed on a densitometer. Specifications were set according to manufacturer's instructions. The gels were passed through the densitometer, and peaks were cut and weighed on a Sartorius

analytical balance in order to determine an estimated amount of labelled protein. The tracings obtained determine the relative intensities of the bands, and the mass of the peaks can be used to estimate of the amount of labelled protein.

Construction of pRK11:

The plasmid pRK11 contains a 7kb insert carrying the *thyA* gene from strain N4316. For constructing the plasmid, the shotgun cloning approach was used. DNA from strain N4316 was extracted and digested with *HindIII*. It was extracted with phenol, phenol:chloroform, and chloroform. Traces of chloroform were removed with ether, and the DNA was precipitated with ethanol. pBR322 DNA was also digested with *HindIII* and treated in the same way. For ligation, chromosoal DNA was mixed with 20x less plasmid DNA in an Eppendorf tube. T4 DNA ligase was added, and the ligation was allowed to proceed for at least two hours at room temperature. Competent MH295 cells (a strain which has a non-temperature sensitive thymine auxotrophy) were transformed with the ligation mix and plated on AB+thy+ampicillin plates and incubated for two days at 31°C. Ampicillin resistant colonies were picked and tested for thymidine requirements at 31°C, 37°C and 43°C on minimal A media containing ampicillin, methionine, histidine, and leucine. Colonies showing growth without thymidine at 31°C, but no growth at 43°C were picked for further study.

Construction of pRK12:

To remove some of the DNA flanking *thyA* on pBTA, pBTA was digested with *SmaI* restriction endonuclease and ligated to pUC13. The ligation mix was used to transform strain JM101, white colonies were selected, and these were then tested for Thy requirements. Plasmid DNA was isolated from those white colonies which were found to be ampicillin resistant and Thy+ at 43°C, and this plasmid DNA was used to transform strain CSR603 so that maxicells would be prepared.

Construction of pJB6:

The plasmid pJB6, which has a deletion of approximately 400bp in the *thyA* gene, was constructed by John Basso. *E. coli* strain MH461, which has an approximate 400bp deletion in *thyA* was transformed with pBTA (which carries

wild type *thyA*), and ampicillin resistant transformants were selected on AB plates. One hundred single colonies were picked, and pooled. The plates were washed with a minimal amount of AB liquid and the cells were collected. These pooled cells were allowed to grow overnight in about two ml of fresh media, and plasmid DNA was then isolated. Strain MH461 was transformed with this total plasmid DNA and plated onto minimal media plates containing methionine, thymidine, ampicillin, and trimethoprim. Cells which grew were trimethoprim resistant as well as ampicillin resistant. Under these conditions, trimethoprim resistant cells are usually Thy⁻(Herrington *et al*, 1984). Mini-preps were performed on individual colonies, to screen for plasmids smaller than pBTA. Restriction mapping allowed us to identify the clones carrying the *thyA* allele with the deletion (J. Basso, unpublished results).

Chemicals:

Common chemicals (reagent grade) were generally obtained from Fisher Scientific Co. The chemicals used for Bray's scintillation fluid were Fisher scintillation grade. Biochemicals were from Sigma Chemical Co. Restriction enzymes were obtained from Boeringer-Mannheim or Pharmacia. Tetrahydrofolate was obtained from Calbiochem. ^{3}H-dUMP was obtained from Calbiochem, ^{32}P-dATP from Amersham, and ^{35}S-met from Amersham.

Results
Construction and Characterization of pRK11:

Studying an enzyme which is found in very small amounts in the cell is made simpler if the gene which codes for that enzyme is transferred into a plasmid. This allows for easier manipulation of the gene and facilitates transferring the gene from one strain to another. Belfort *et al* (1983) cloned and mapped the *thyA* gene. The plasmid pBTA, which carries the wild type allele of *thyA* is pBR322 with a *HindIII* insert 7.2kb in length. This insert has been derived from a specialized thyA+ transducing phage. The 7.2kb *thyA HindIII* fragment was excised from *thyAI* and ligated into the *HindIII* site of pBR322 to yield pBTA (Belfort *et al*, 1983). To determine if the *thyA* gene in the temperature sensitive strain N4316 is located on a similar *HindIII* fragment of chromosomal DNA, the plasmid pBTA was used to probe Southern blots of chromosomal DNA from strain N4316 which had been digested with *HindIII*. Figure 1 shows that the DNA from both strain N4316 and strain D10 contain a unique *HindIII* fragment which hybridizes with pBTA DNA and is similar in size to the *thyA HindIII* insert of pBTA. Therefore, a plasmid similar to pBTA but carrying the mutant *thyA* allele can be constructed.

Cloning of the temperature sensitive *thyA* gene was done by digesting total DNA from strain N4316 with *HindIII* and allowing it to ligate with similarly digested pBR322. The Thy⁻ strain MH295 was transformed with the total ligation mix and plated on AB plates containing thymidine and ampicillin. The ampicillin selection is to select for transformants which incorporated a plasmid, since it is not certain if the plasmid would definitely complement *thyA*.

There are a number of ways to determine if the gene of interest has been cloned. The first way is by phenotypic analysis. If the gene product is necessary for growth, and there exists a mutant strain lacking this gene product, one can test whether the cloned gene complements the mutant gene. In this case we want to identify the plasmid which codes for a mutant thymidylate synthase. Strain MH295 and transformed derivatives which lack the correct plasmid will not grow at any temperature unless thymine or thymidine is added to the medium. Derivatives of strain MH295 containing the plasmid should be ThyA+ at the permissive temperature of 31°C. The cells would therefore be able to grow at the

Figure 1
Hybridization analysis of the *thyA* allele in chromosomal
DNA digests

Approximately 0,01μg of DNA was digested with *HindIII*, separated on a 1% agarose gel at 100V, and transferred to Pall Biodyne A membrane. The gel was probed with approximately 1μg of cesium-chloride purified plasmid DNA which was nick translated as described by the manufacturers of the Nick translation kit. The membrane was exposed to X-ray film for up to one week and the autoradiograph was then developed. (kb= Kilobase)
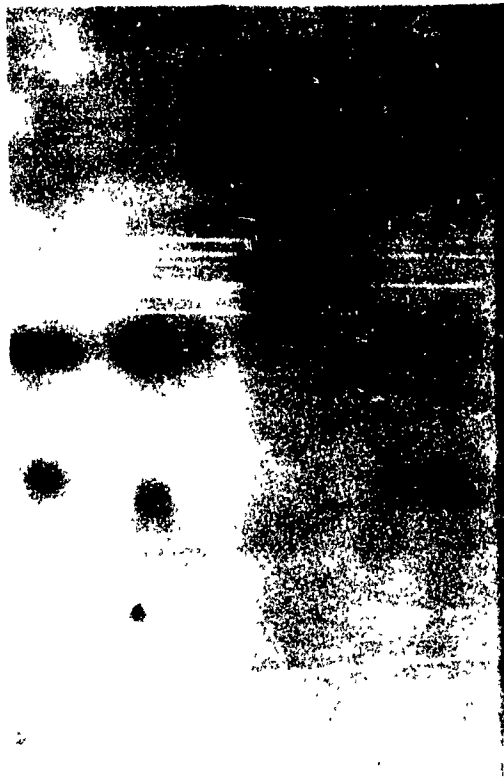
Lane 1: D10 chromosomal DNA digested with *HindIII*

Lane 2: N4316 chromosomal DNA digested with *HindIII*

Lane 3: pBTA undigested

Lane 4: pBTA cut with *HindIII*

1   2   3   4

<--7,2kb

<--4,3kb

permissive temperature of 31°C without any added thymine or thymidine in the medium. However, at 43°C, these cells should not be able to grow unless exogenous thymine or thymidine is added. Transformants which grew on AB plates containing thymidine and ampicillin (ca1200) were screened on minimal medium plates containing ampicillin and lacking thymidine at 31°C and those that grew were further tested for thymidine requirements at 37°C and 43°C. Four colonies which grew at 31°C but did not grow without thymidine at 43°C were selected.

Restriction analysis should show whether the plasmids from these strains are the same size and have the same restriction pattern as that of pBTA. Preliminary results suggested that the four newly constructed plasmids all had the same HindIII fragment inserted as in pBTA, but that it was in an orientation opposite to that of pBTA.

We used only one of the four strains for further investigation. To obtain a plasmid with the insert in the same orientation as that of pBTA, we digested the plasmid with HindIII, religated it, and transformed strain MH295 with the ligation mix. We picked ten transformants, and isolated plasmid DNA. One of the isolates, plasmid pRK11, showed similar fragment numbers and sizes as pBTA when comparing the various restriction endonuclease patterns produced. Figure 2 compares these patterns. The discrepancy in the DNA digested with PvuII is due to incomplete digestion.

Growth patterns in the presence and absence of thymidine in the media was tested. In the presence of thymidine, all strains grew at all three growth temperatures. In the absence of thymidine, the Thy⁻ strain MH295 did not grow, as expected. When that strain was transformed with pRK11, however, it grew well at 31°C, and not at all at 43°C, similar to the pattern shown in the thy^{ts} strain N4316 (Table 2).

Thymidylate synthase activity in Thy- cells transformed with pRK11:

Thymidylate synthase activity levels of cells grown at various temperatures were compared. R. Clarizio (unpublished data) has shown that in the wild type strain D10, thymidylate synthase specific activity decreases slightly as the growth temperature of the cells is increased.

Figure 2
Restriction enzyme patterns of plasmid DNA

A 1ml overnight culture was grown in AB media containing Ampicillin. Plasmid DNA was isolated as described, and 3µl was digested with 1unit of enzyme for 2 hours at 37°C. The DNA was separated on a 1% agarose gel (2% gel for NciI and TaqI digestion), stained with ethidium bromide, and DNA was viewed under a Transilluminator.

Lane 1: pRK11 digested with NciI

Lane 2: pBTA digested with NciI

Lane 3: pRK11 digested with Taq1

Lane 4: pBTA digested with Taq1

Lane 5: pRK11 undigested

Lane 6: pBTA undigested

Lane 7: pRK11 digested with HindIII

Lane 8: pBTA digested with HindIII

Lane 9: pRK11 digested with PstI

Lane 10: pBTA digested with PstI

Lane 11: pRK11 digested with PvuII

Lane 12: pBTA digested with PvuII
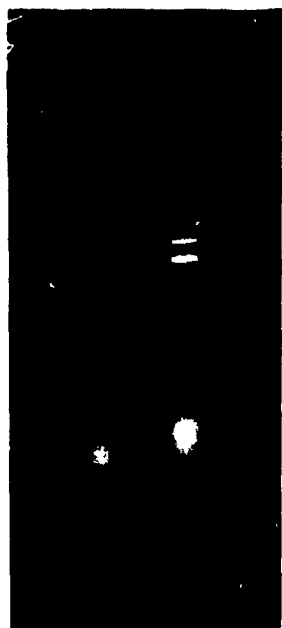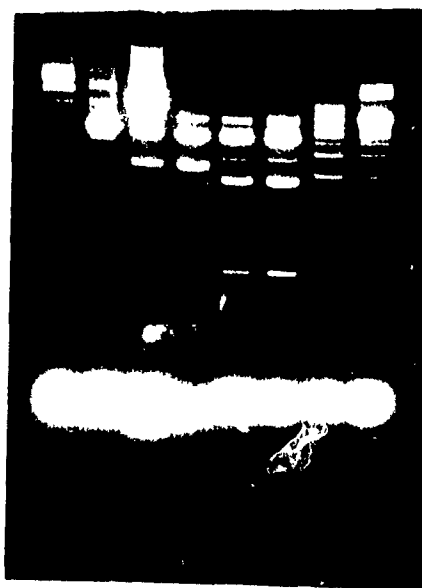
Table 2: Growth of *E. coli* cells on minimal medium plates
lacking exogenous thymine or thymidine

	Growth temperature		
Strain	31°C	37°C	43°C
D10	+ + +	+ + +	+ + +
N4316	+ + +	+	-
MH295	-	-	-
MH295/pBTA	+ + +	+ + +	+ + +
MH295/pRK11	+ + +	+ +	-

However, in strain N4316, as the growth temperature is raised from 31°C to 43°C, the specific activity of thymidylate synthase drops dramatically. If pRK11 does indeed carry the *thyA* allele from strain N4316, one would expect that the activity of the plasmid encoded thymidylate synthase from strain MH295/pRK11 should drop to a greater extent that does the thymidylate synthase activity of strain MH295/pBTA. Strain MH295 is Thy⁻, and extracts of this strain did not contain a detectable amount of thymidylate synthase activity.

Crude extracts were made from cells grown in the presence of exogenous thymidine at 31°C, 37°C, or 43°C and the activity of thymidylate synthase in these extracts was measured using the tritium release assay (Roodman and Greenberg, 1971a). All assays were performed at 31°C. The specific activity of thymidylate synthase in strain MH295/pRK11 is lower than that of strain MH295/pBTA at all three growth temperatures (Table 3A). The specific activity of thymidylate synthase in both strains decreases as the growth temperature increases, but when the specific activity of thymidylate synthase is expressed relative to the activity shown of cells grown at 31°C, the specific activity of strain MH295/pRK11 drops much more than does the thymidylate synthase activity of strain MH295/pBTA (Table 3B).

Belfort observed that cells that have less than 5% of the wild type thymidylate synthase activity appear to be Thy⁻(Belfort *et al*, 1984). Strain N4316, when grown at 37°C, has only about 2% of the thymidylate synthase activity. This strain does grow, occasionally, without exogenous thymine or thymidine in the media (Cheung & Herrington, 1982; Herrington *et al*, 1986; R. Clarizio, unpublished results) (Table 3B). In contrast, strain MH295/pRK11, which has 19% of thymidylate synthase specific activity (Table 3B), can grow. The higher levels of thymidylate synthase observed in plasmid carrying strains may be due to the multicopy plasmid, such that sufficient quantities of enzyme could be synthesized to allow growth.

Table 3A

Thymidylate synthase activity in crude extracts

Growth (°C) temperature	Tritium release Activity (munits/mg protein)			
	MH295/pBTA	MH295/pRK11	D10[1]	N4316[1]
31	0.70±0.05	0.31±0.02	0.5	0.094
37	0.55±0.05	0.13±0.02	0.4	0.021
43	0.44±0.05	<0.025	0.39	0.01

(1) R. Clarizio, unpublished results

Table 3B
Comparison of the relative levels of thymidylate synthase activity

Growth (°C) Temperature	Relative thymidylate synthase activity (%)			
	MH295/pBTA[1]	MH295/pRK11[1]	D10[2]	N4316[2]
31	100	44	100	19
37	79	19	80	4.2
43	63	<3.6	78	2

(1) the activity is the specific activity of thymidylate synthase in extracts of strains MH295/pBTA and MH295/pRK11 normalized to the value obtained for strain MH295/pBTA grown at 31°C.

(2) the activity is the specific activity of thymidylate synthase in extracts of strains D10 and N4316 normalized to the value obtained for strain D10 grown at 31°C. (R. Clarizio, unpublished results).

Heat inactivation of thymidylate synthase activity:

Clarizio (unpublished results) found that the thymidylate synthase from strain N4316 is affected by heat to a greater extent than is the thymidylate synthase of the wild type strain D10. Crude protein extracts were incubated at 45°C for varying lengths of time prior to assaying thymidylate synthase activity. The activity of thymidylate synthase from strain MH295/pRK11 decreases to a greater extent than the activity of the strain carrying the wild type *thyA* allele in a wild type strain JF1754 (Figure 3). This heat inactivation profile is similar in magnitude to that seen with the thymidylate synthase from strain N4316 (R. Clarizio, unpublished results).

Results that indicate that pRK11 does carry the *thyA*^{ts} allele from the temperature sensitive E. *coliK12* strain N4316 include: (a) Strain MH295 transformed with the *thyA* allele from N4316 has a growth pattern similar to the temperature sensitive strain N4316 on minimal media containing no exogenous thymidine, (b) the restriction enzyme digests of the two plasmids pBTA and pRK11 suggest that the plasmids are similar in size and contain the same restriction enzyme cut sites, (c) the thymidylate synthase specific activity of strain MH295/pRK11 at various growth temperatures has a pattern similar to the activity of strain N4316, and finally (d) the heat inactivation profile of strain MH295 transformed with pRK11 is similar in magnitude to that of N4316.

Proteins expressed from pRK11 in maxicells:

To compare levels of synthesis of thymidylate synthase in the strains carrying the wild type and temperature sensitive *thyA* alleles on plasmids, maxicells were prepared. The maxicell strain CSR603 has mutations within the *uvrA* and *recA* loci, which makes the strain very sensitive to UV damage. When the growing cells carrying the plasmids are irradiated with UV light, the chromosomal DNA is damaged beyond repair. However, with multicopy plasmids, some plasmid molecules in the cell are not damaged. These cells will continue to synthesize proteins which are coded by the plasmid DNA (Sancar, Hack and Rupp, 1979). ^{35}S-methionine is added to the media, such that only newly synthesized proteins will be radioactively labelled. The proteins are then extracted and separated on SDS-PAGE and the labelled proteins are viewed by preparing an autoradiograph of the protein separation.

Figure 3:
Heat Inactivation of thymidylate synthase

Crude extracts of cells grown at 31°C were incubated at 45°C. Samples were taken at various times and assayed at 31°C for thymidylate synthase activity. The extracts were in 0.01M Tris-HCl pH7.4, 0.01M MgCl$_2$. % activity remaining is expressed relative to the activity seen at 0 minutes.

+   JF1754

O   MH295/pRK11
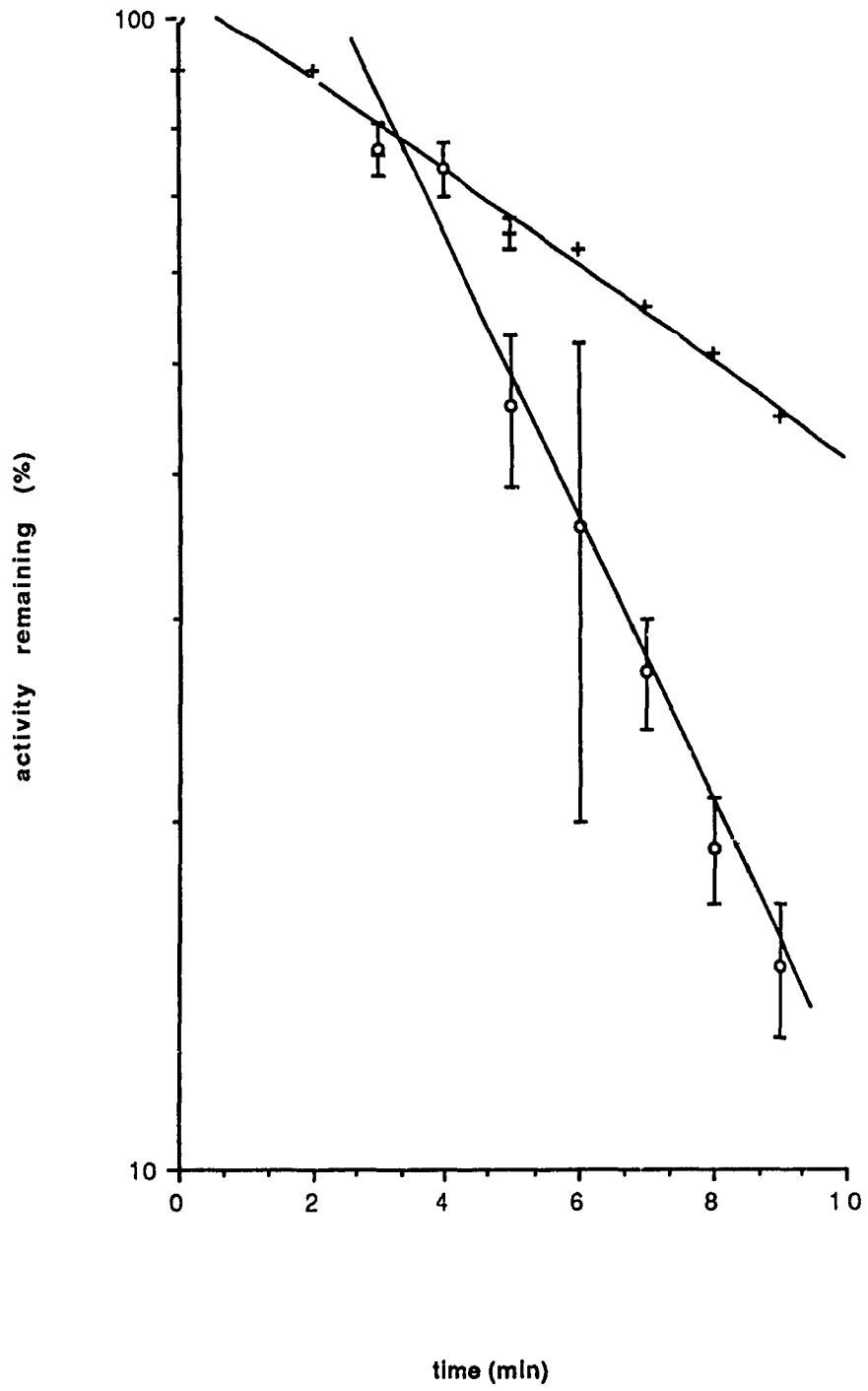
The maxicell strain, CSR603 was transformed with pBR322, pBTA, and pRK11, and maxicells were prepared as described in Materials and Methods. The cells were grown at 37°C, irradiated with UV light and treated with cycloserine. The cells were washed, divided into equal aliquots and then incubated at the experimental test temperatures for one hour prior to adding the radioactive label. The cells were then incubated for an additional hour and extracts were prepared. SDs-polyacrylamide gel electrophoresis of the crude protein extracts was performed, and autoradiographs of the dried gels were prepared.

Fig. 4 shows the bands obtained with strain CSR603 carrying the plasmids pBTA and pRK11, and the single band obtained from strain CSR603/pBR322. The single band found in this strain has a molecular weight of 28000 and is presumably B-lactamase (Sancar, Hack and Rupp, 1979). This band is also found in the strains carrying the other plasmids, pBTA and pRK11, at all three temperatures. In addition, strains CSR603/pBTA and CSR603/pRK11 synthesize three other peptides. These are approximately 33000 molecular weight, 31000 molecular weight, and 25000 molecular weight. Similar patterns of bands and intensities were observed in 3 separate experiments. The faint bands seen near the top of the autoradiographs are thought to be artifacts, faint peptides that appear possibly due to incomplete inactivation of chromosomal DNA, since these bands do not consistantly appear from one experiment to another. The smaller bands also are probably artifacts, or due to incomplete degradation of chromosomal DNA, since these bands are seen in the plasmid-carrying strains as well as in the host strain CSR603, which does not have any plasmid. These bands, therefore, are not from the *thyA* insert.

Identification of the product of *thyA* on gels:

The molecular weight of thymidylate synthase is 30441 (Belfort *et al*, 1983). Since the mobility of SDS gels are only estimates of molecular weight, either the 31000 molecular weight peptide or the 33000 molecular weight peptide could be thymidylate synthase.

To try to eliminate peptides which are not coded by the *thyA* locus, a smaller fragment of DNA carrying the *thyA* gene was subcloned by digesting pBTA with *SmaI* and ligating it into the *SmaI* site of pUC13. Since pUC13 is a much smaller plasmid than pBR322, and found in higher copy numbers (Ausubel *et al*,

Figure 4
Expression of *thyA* and *thyA*^{ts} in maxicells

Maxicells were prepared as described in methods. Plasmid coded proteins were labelled with ^{35}S-methionine (final concentration 5µCi/ml) for one hour at the respective temperature. Cells were washed, resuspended in 100µl Tris-MgCl$_2$ buffer and lysed by boiling in 200µl of gel loading buffer. 10µl of sample was loaded on a 15% SDS-polyacrylamide gel, and run until the dye would run off the gel at 18milliamps.(Bla: ß-lactamase)

> Lanes 1-3 CSR603/pRK11
>> Lane 1: 31ºC
>>
>> Lane 2: 37ºC
>>
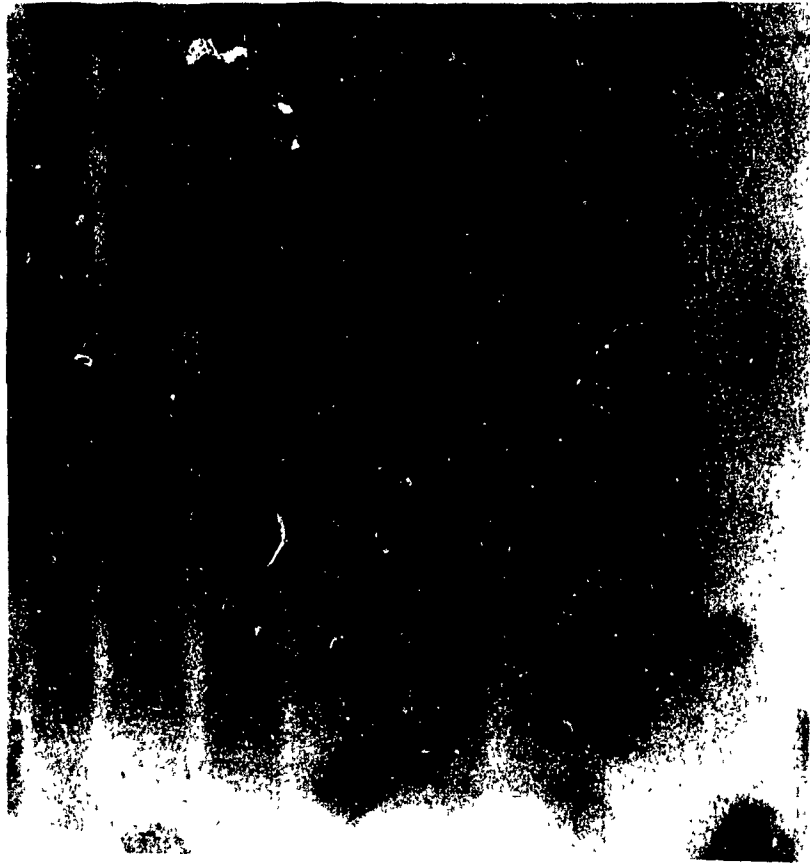>> Lane 3: 43ºC
>
> Lane 4: CSR603/pBR322
>
> Lane 5-7: CSR603/pBTA
>> Lane 5: 31ºC
>>
>> Lane 6: 37ºC
>>
>> Lane 7: 43ºC

1989), we would expect to obtain higher quantities of peptides synthesized.

Maxicell analysis of the resulting strain CSR603/pRK12 showed the same four peptide bands as observed with strain CSR603/pBTA (figure 5). In this case, maxicells of strain CSR603/pBR322 were prepared using tetracycline as selection, in order to allow the protein responsible for tetracycline resistance to be induced and expressed. The band due to tetracycline is seen in these autoradiographs, as having a molecular weight of approximately 40000. Sancar, Hack and Rupp (1979) found the molecular weight of tetracycline to be approximately 37000, and the protein synthesized from the *tet* gene must be induced. This is why the *tet* band is not seen in any of the previous maxicell experiments, since the protein was not induced. B-lactamase is readily identified with the molecular weight of 28000. Thus, this experiment did not permit identification of thymidylate synthase.

A second approach to identify thymidylate synthase was to isolate a plasmid with a deletion in *thyA*. Strain MH461 is a *thy⁻* strain which has an approximate 400 base pair deletion in *thyA* (Belfort & Pedersen-Lane, 1984) This deletion was transferred into pBTA by recombination (see methods) and the resulting plasmid pJB6 was identified because it did not complement the *thyA* mutation. Restriction analysis was used to show that it was similar to pBTA but had an approximate 400 base pair deletion. Strain CSR603 was transformed with pJB6 and maxicells were prepared. The deletion of a large segment of the coding region of thymidylate synthase should either alter the mobility of the product on gels or eliminate it. Therefore, by comparing the peptides synthesized from maxicells carrying pBTA with those carrying pJB6, any loss or shift of peptide would identify which was thymidylate synthase. Figure 6, lane 2 clearly illustrates that the top band as well as the lowest band disappear in the strain carrying pJB6. Therefore, the top band must be thymidylate synthase, since it is found in strain CSR603/pBTA and not found in strain CSR603/pJB6 which contains the plasmid with the deletion in *thyA*. The low band or molecular weight 25000 may be a degradation product of thymidylate synthase, or a product of a gene which overlaps *thyA*, or the product of another gene which is regulated by thymidylate synthase.

Figure 5

Identification of thymidylate synthase in maxicells

In order to attempt to identify thymidylate synthase, maxicells of strains carrying pBTA, pRK11, and pRK12 were prepared as described. All incubations were carried out at 37°C. (Bla: ß-lactamase, units are x10³ molecular weight).

Lane 1: CSR603/pRK11

Lane 2: CSR603/pRK12

Lane 3: CSR603/pBTA

Lane 4: CSR603/pBR322

Figure 6
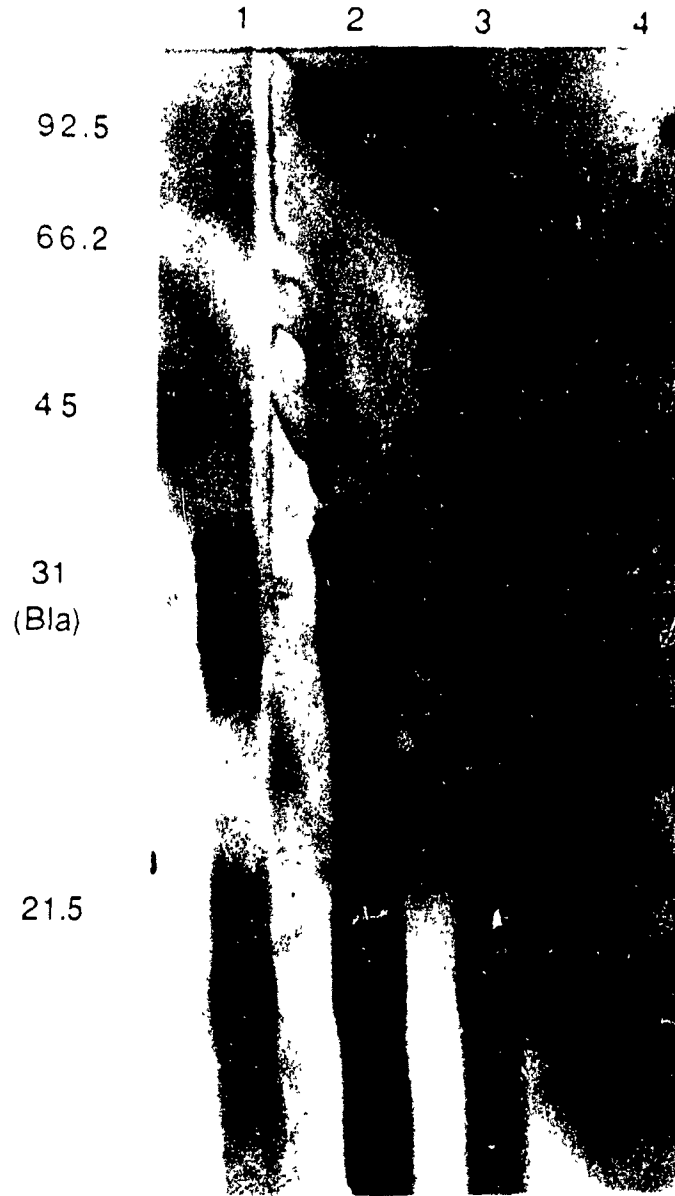## Identification of Thymidylate synthase in maxicells

Thymidylate synthase was identified by the disappearance of a peptide in maxicells of strain CSR603/pJB6, due to an approximate 400 base pair deletion within *thyA*. Maxicells were prepared as described, and proteins were separated on a 15% SDS-polyacrylamide gel.(Bla: ß-lactamase)

     Lane 1: CSR603/pBR322

     Lane 2: CSR603/pJB6

     Lane 3: CSR603/pBTA

Synthesis of Peptides from the *thyA* insert:

We wished to compare the amounts of protein synthesized under the varying temperatures. By passing the autoradiograph shown in Figure 4 through a densitometer, the tracings obtained allows one to determine the relative intensities of the bands on the autoradiogram (data not shown). The area under the curves is then an estimate of the amount of labelled protein. Four peaks were identified in the autoradiographs as being derived from the plasmid. These were cut out of the densitometer tracings and were weighed. These measurements provide an estimate of the relative amounts of peptides synthesized.

Figure 7 illustrates the amount of each peptide synthesized from the plasmid in the two strains, CSR603/pBTA and CSR603/pRK11. The amount of B-lactamase (peak 3) is found to be fairly constant in both strains at all temperatures.

At all three incubation temperatures, the amounts of peptides 2,3, and 4 synthesized from strain CSR603/pBTA were similar. In contrast, the amount of peptide 1, which was identified as being thymidylate synthase, decreased slightly as the incubation temperature was increased.

As incubation temperature was increased, the amounts of peptides 2,3, and 4 synthesized from strain CSR603/pRK11 did not change significantly. However, peptide 1, thymidylate synthase, decreased markedly as the incubation temperature was increased to 43°C.

The intensities of the measured bands were done with identical settings of the densitometer for each group of cells. Therefore, the differences seen between the two strains are probably due to sample differences in the pre-splitting phase of maxicell preparation; there were more growing cells of strain CSR/pBTA than strain CSR/pRK11 before they were separated to the three test temperatures. Since similar amounts of peptides 2,3, and 4 were seen at each temperature for either strain, it is clear that an equivalent amount of cells was incubated, proteins were equally labelled and an equivalent amount of protein extract was loaded onto the gel for each incubation temperature.

Figure 7
Mass of peaks from densitometer tracings of peptides
synthesized from maxicells

The autoradiograph from Figure 4 was passed through a densitometer, and tracings of the peaks were cut out and weighed, allowing determination of the relative intensities of the bands. The area under the curves is an estimate of the amount of labelled protein.

A: mass of peaks from strain CSR603/pBTA

B: mass of peaks from strain CSR603/pRK11

■ peak 1: thymidylate synthase

▨ peak 2

▩ peak 3: ß-lactamase

☐ peak 4

A
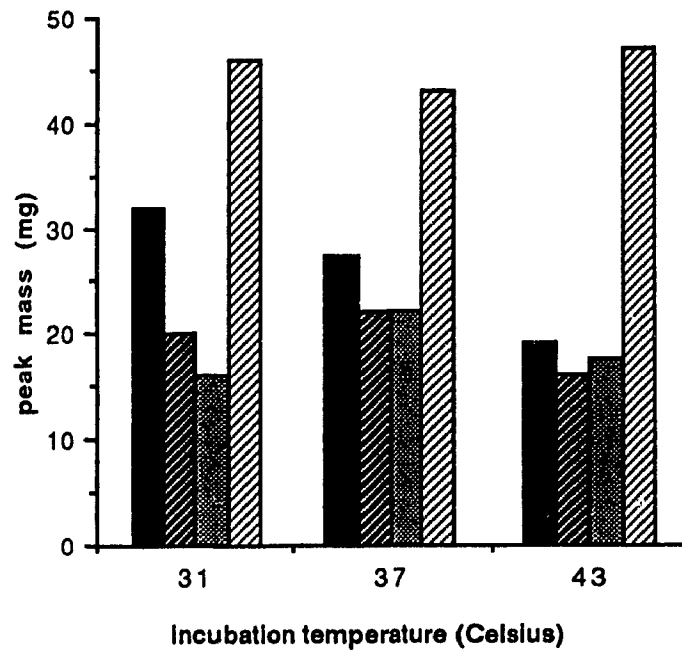
B
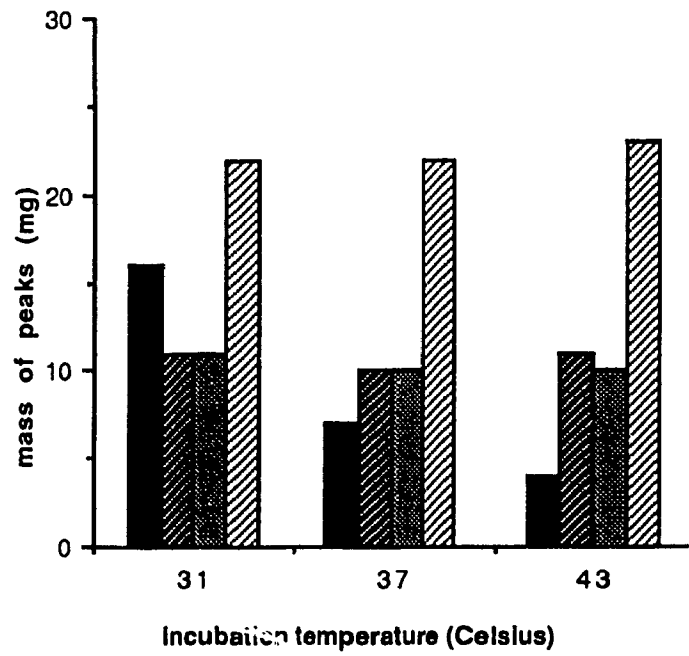
Both groups of cells were treated and handled in the same way. They were treated with UV light for equivalent amounts of time. They are only shifted to their specific growth temperatures when labelling with ^{35}S-methionine during the last two hours of incubation which consists of a one hour incubation before labelling to become acclimatized to the new temperature, and a second one hour incubation with label in the media.

To correct for possible differences between samples resulting from differences in plasmid copy number or in preparation of maxicells and extracts, abundancies of thymidylate synthase (peak 1) were expressed as the ratio of thymidylate synthase to B-lactamase (peak 3).

When we examined the relationship between thymidylate synthase specific activity and abundancies of thymidylate synthase corrected to B-lactamase, we saw that the amount of thymidylate synthase decreases as the growth temperature is increased. Strain CSR603/pBTA has about 54% of the protein at 43°C than at 31°C. Strain CSR/pRK11 on the other hand, loses most of its thymidylate synthase as the temperature is increased, dropping from 64% to about 10% of the wild type levels.

Figure 8 compares the relationship between activity and amount of thymidylate synthase. To facilitate the comparison of activity and relative amounts of protein, the scales were normalized in a way such that the relative amount was chosen so that the height of the bar of pBTA at 31°C was equivalent to the activity bar. The scales were chosen so that at 31°C the activity was made to be equivalent to the wild type activity. As the temperature is increased, the amount of protein is decreased relative to the activity. In strain CSR603/pBTA the activity is always slightly higher than the amount of protein. Both the amounts of protein, as well as the thymidylate synthase specific activity decreases as the growth temperature is increased. In strain CSR603/pRK11, the activity is always somewhat lower than the amount of protein. However the relative amount of thymidylate synthase protein is always lower than that seen with the wild type plasmid.

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Figure 8

Relationship between thymidylate synthase specific activity and abundancies of thymidylate synthase protein on maxicells

■ thymidylate synthase specific activity pBTA

▨ relative amount thymidylate synthase pBTA (peptide1/peptide 3)

▨ thymidylate synthase specific activity pRK11

▨ relative amount thymidylate synthase pRK11 (peptide1/peptide 3)
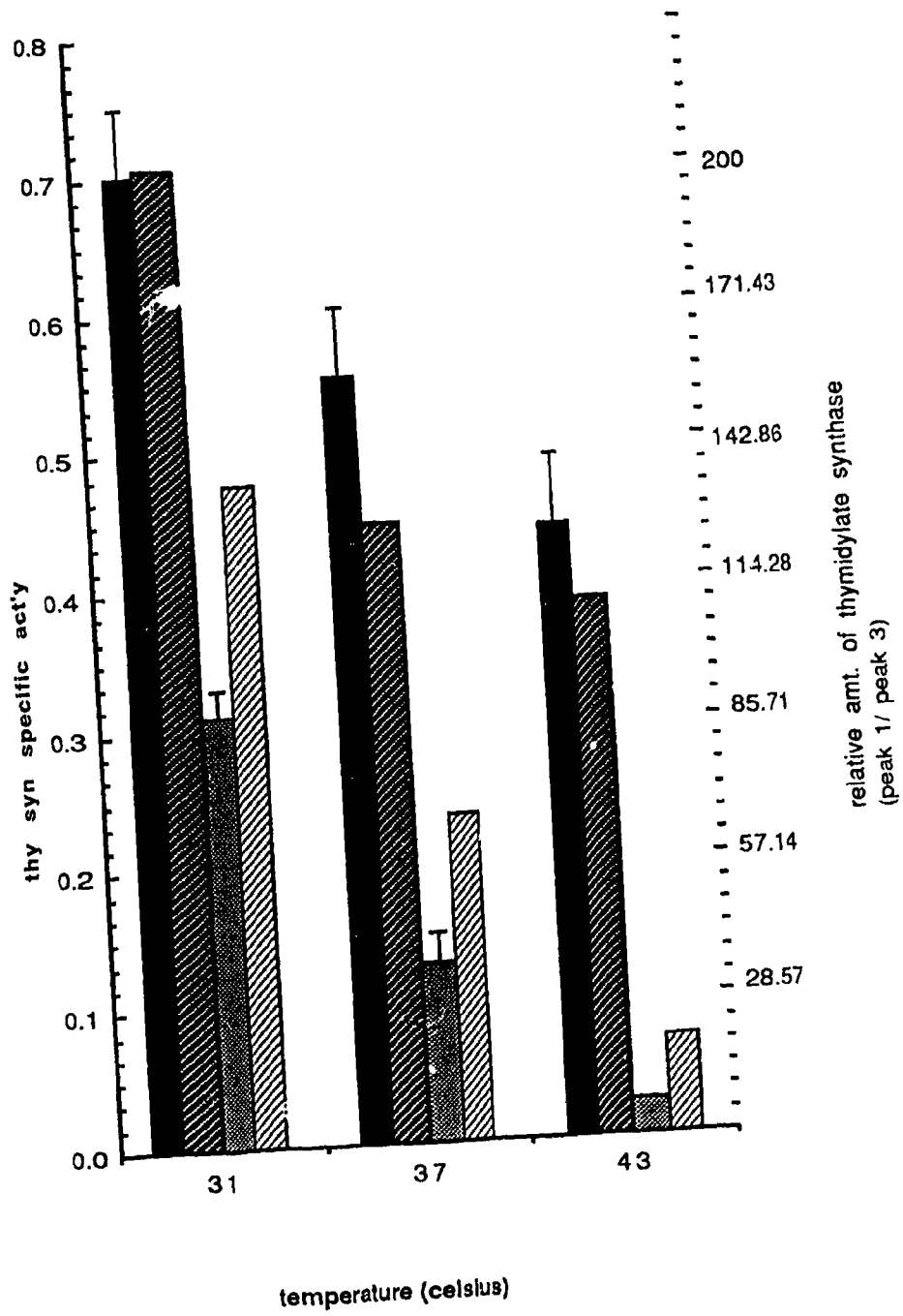
DISCUSSION

Construction and Characterization of pRK11:

The aim of this research was to isolate the *thyA* allele of the temperature sensitive *E. coli* strain *N4316* and to compare its thymidylate synthase with that of pBTA, a plasmid carrying a wild type allele of ... 4..

A plasmid similar to pBTA, but carrying the temperature sensitive *thyA* allele from strain N4316 was constructed. Plasmid pRK11, constructed by digesting total genomic DNA from strain N4316 with *HindIII* and ligating it with similarly digested pBR322 was used to transform competent thy⁻ cells of strain MH295.

Plasmid DNA from transformants was isolated, and restriction analysis showed that this plasmid and pBTA both have similar number of fragments and patterns when digested with a variety of restriction endonucleases. Any discrepancies shown in the gel figures is due to incomplete digestion with the enzyme, or contamination of the plasmid DNA with chromosomal DNA.

Strain MH295 is thy⁻, and cannot grow in medium lacking exogenous thymine or thymidine. Strain MH295, transformed with pRK11 was tested for thymidine requirements at 3 growth temperatures. At 43°C, this strain was unable to grow. At 37°C, this strain grew, albeit slowly. At 31°C, the strain grew well. This growth pattern is similar to that seen with strain N4316. Strain MH295 transformed with pBTA grew well at all three temperatures. Since strain MH295 is thy⁻, it does not grow at all without any exogenous thymine or thymidine.

Thymidylate synthase specific activity of the two *thyA* alleles in similar thy⁻ host backgrounds was compared. Activity profiles from the two strains follow trends similar to those of strain D10 and strain N4316. Previous studies (R. Clarizio, unpublished results) have shown that the thymidylate synthase specific activity of the mutant strain is five times less than the activity of the wild type strain, and that there is a greater decrease in activity in the mutant strain than in the wild type as the growth temperature is increased.

The specific activity of the strains carrying the plasmids shows a similar pattern. The thymidylate synthase specific activity of strain MH295/pRK11 decreases to a much greater extent than does the activity of strain MH295/pBTA

as the growth temperature is increased. The thymidylate synthase activity in the mutant strain drops ten-fold, whereas the activity of the wild type strain drops only two-fold as the growth temperature is raised from 31°C to 43°C. One would expect that the thymidylate synthase specific activity in the strains carrying the plasmid to be higher than in the strains without the plasmids, since there is a higher copy number of the gene; however, the regulation of the synthesis seems to keep the thymidylate synthase specific activity only somewhat higher than in the strains without the plasmid, suggesting that the regulation and expression is not solely dose dependant.

Further evidence that suggests that the mutant allele from strain N4316 has been cloned includes examining the heat lability of the enzyme. R. Clarizio (unpublished results) found that upon preincubating crude protein extracts prior to assaying for thymidylate synthase activity, the enzymatic activity from strain N4316 drops substantially more, and at a faster rate, than does the activity from strain D10. When preincubating crude protein extracts, thymidylate synthase specific activity of strain MH295/pRK11 drops to 20% of the initial activity, whereas that of a wild type strain drops only about 50% of its initial activity.

Proteins coded in Maxicells:

Maxicell analysis of the plasmid pBTA shows evidence that three peptides are consistently being synthesized from the 7,2kb *thyA* insert. Two of these, of 31000 molecular weight, and 33000 molecular weight, as determined by the proteins' migration on SDS-polyacrylamide gels may be thymidylate synthase. Both Hickson, Atkinson and Emmerson (1982) and Dykstra, Prasher and Kushner (1984) identify thymidylate synthase on autoradiographs of maxicell preparations as being larger than B-lactamase, as having a molecular weight of approximately 33000. The third peptide synthesized from pBTA, of 25000 molecular weight, cannot be thymidylate synthase.

Dykstra *et al* (1984) investigated the regions around *thyA* and the downstream genes, *recC* and *recB*. Their maxicell preparation identified a number of peptides synthesized upstream of the *HindIII* site which interrupts the recC gene in the plasmid pBTA. These peptides were identified as thymidylate synthase (33000 molecular weight), and a peptide of unknown function of 22000 molecular weight (p22). Hickson *et al* (1982) prepared maxicells on a similar

region around *thyA*, and they also found a number of peptides being synthesized. They identified thymidylate synthase by looking at maxicells of preparations where the transposon Tn1000 was inserted within the *thyA* locus. They did not try to identify any of the other polypeptides being synthesized. Dykstra *et al* (1984) suggests that there may be an essential gene coding for a protein of 22000 molecular weight upstream, which extends into *thyA*. This p22 peptide was later identified as being the *umpA* gene of 25000 molecular weight (Williams *et al*, 1989). Williams *et al* (1989) suggest that transcription of the *umpA* gene may serve to regulate the expression of *thyA*. The discrepancy between the p22 polypeptide and the protein of 25000 molecular weight may be due to the fact that Dykstra's p22 peptide did not consist of the entire protein, but extended further upstream of *thyA*.

However, neither of these investigators identified a second peptide of approximately 31000 molecular weight. The preparations of Dykstra *et al* (1984) were 7,5% polyacrylamide gels, whereas the polyacrylamide gels of Hickson *et al* (1982) were 13%. Close examination of Dykstra's gels suggest that the thymidylate synthase peptide may in fact be composed of two unique bands close together. Due to the better separation of these 15% polyacrylamide gels, there may be better separation and resolution between the two bands. When they inactivated *thyA* by a Tn1000 insertion, the peptide of 33000 molecular weight (thymidylate synthase) disappeared, but another peptide of 31000 molecular weight was seen. This in fact may be the second band seen in these gels.

Since the *thyA* gene in pBTA and pRK11 is bounded by flanking DNA, it seemed possible that there may be other genes coding for proteins in that region. Not much is known about the upstream region. Finch *et al* (1986) investigated the region downstream of *thyA*, the intergenic region between *thyA* and *recC*. They identified three open reading frames that could code for polypeptides, two of which are preceded by possible ribosome binding sites. These polypeptides would have molecular weights of 30000 and 12500. Both these peptides could possibly be synthesized by pBTA. In fact, the peptide of approximately 30000 molecular weight may be this p30.

In an attempt to simplify identification of thymidylate synthase, a substantial part of the flanking DNA was removed, so that only those proteins

coded by the DNA in a smaller region around *thyA* would be synthesized.

Approximately 4,8kb of DNA was removed by digesting pBTA with *SmaI* and subcloning the *SmaI* fragment into pUC13 According to results suggested by Finch *et al* (1986), construction of plasmid pRK12 would prevent the p12 peptide from being synthesized. With respect to the region upstream of *thyA*, not much was known. Our results showed that the same peptides were synthesized, suggesting that these three bands are the result of peptides being synthesized in the immediate vicinity surrounding *thyA*. The *umpA* gene maps between the *SmaI* restriction site and 5' end of *thyA*, so the gene product would still be synthesized in pRK12. Therefore, the 25000 molecular weight band shown in these autoradiographs may indeed by the *umpA* gene product. Since both pBTA and pRK12 gave similar patterns, we can conclude that none of these synthesized polypeptides come from a junction between the insert and vector; and that these peptides are unlikely to be truncated proteins.

An alternative way to identify thymidylate synthase was to construct a deletion plasmid; a plasmid in which the thymidylate synthase has a large deletion such that a complete active protein cannot be synthesized. The plasmid pJB6 has an approximate 400bp deletion in the middle of *thyA*. With this plasmid, either the peptide of thymidylate synthase would disappear if very small, or if no protein is synthesized; or the mobility of the peptide would be shifted. By comparing the maxicell analysis of pJB6 to that of pBTA, it is possible to identify the upper band of 33000 molecular weight as being thymidylate synthase, since this band was not seen in maxicells of pJB6.

The peptide of 25000 molecular weight also disappears in maxicells of pJB6. This suggests that the 3' end of the overlapping gene which extends into *thyA* extends into the deleted region, preventing the protein from being completely synthesizer Dykstra *et al* were able to localize the protein only to the 5' end of *thyA*. Williams *et al* only suggested that the *clm* locus extends somewhat into *thyA*, and the 400bp deletion is not accurately localized either. Therefore, it is possible that the coding region of this essential gene may extend into the deleted region of *thyA*.

Thymidylate synthase coded by pRK11:

We wished to examine whether the amounts of thymidylate synthase

synthesized changes as the incubation temperature is altered. With respect to most *E. coli* proteins, as the incubation temperature is increased, there will usually be a limited increase in the rate of protein synthesis. The maxicell experiment was designed such that all cells were grown at 37°C to a similar optical density, and irradiated with an equivalent amount of ultraviolet light, so that there should be an equivalent amount of chromosomal DNA degradation. The cells were only separated to the different test temperatures prior to labelling with ^{35}S-methionine. This is, to the best of our knowledge, the first description of maxicells used to monitor a synthesis of plasmid encoded proteins at different temperatures.

Ultimately, we wanted to examine the mutant thymidylate synthase of the temperature sensitive strain N4316, and compare the amounts of thymidylate synthase being synthesized with that of a closely related wild type Thy$^+$ strain. Previous studies (R. Clarizio, unpublished results) have shown that the thymidylate synthase of the mutant strain has a specific activity lower than that of the wild type, and that it decreases substantially more as the growth temperature is increased. A number of possible explanations for this observation may be raised:

1. The amount of thymidylate synthase protein being synthesized is the same in both the wild type and mutant strains, but the average activity per molecule is different, either because there is less activity per molecule, or because there is a mixed population of active and inactive proteins; i.e. those that can and cannot bind dUMP.

2. There is a similar amount of thymidylate synthase protein being synthesized in the mutant and wild type strains, but, because the mutation alters the amino acid sequence of the protein, the mutant protein is more likely to be degraded than the wild type protein.

3. Because of the structural mutation in strain N4316, there is less thymidylate synthase synthesized in the mutant strain than in the wild type strain. The structural mutation may cause a hang-up while the ribosomes travel along the mRNA. This would ultimately result in the failure to complete the synthesis of the complete enzyme, resulting in less protein being synthesized. This possibility is similar to that proposed by Roodman and Greenberg (1971).

The apparent drop in the amounts of thymidylate synthase in the mutant corresponds to the drop shown in the thymidylate synthase specific activity; whereas the amount of thymidylate synthase, as well as its specific activity stays relatively stable in the wild type. This seems to suggest that the structural mutation in the thymidylate synthase of strain N4316 affects the synthesis of the protein, such that there is less being made, thereby reducing the specific activity of the enzyme.

On the other hand, the mutant protein may be synthesized at the same rate and amounts as the wild type protein, but may just be degraded more rapidly. The data cannot distinguish this possibility. However, when Betz and Fall (1988) examined maxicells of a series of missense mutations, they observed distinct degradation products. This would suggest that if degradation was occuring in the case of thymidylate synthase, similar degradation products should be detected on the autoradiographs of the maxicells. Thus, the structural mutation of thymidylate synthase of the temperature sensitive *E. coli* strain N4316 may affect the synthesis of the enzyme.

The amount of activity is less than what the protein levels would predict, as seen in Figure 8. This may be explained by the first hypothesis, spec ically, that there is a certain amount of thymidylate synthase protein being synthesized, but only a fraction of it is active, only a part of it may bind dUMP and catalyze the reaction. These results would show a combination of the two hypotheses, that there is less total thymidylate synthase enzyme being synthesized, and within that group, there is less enzyme which can bind dUMP and carry out the reaction.

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