

Isolation and Characterization of an *Escherichia coli* Mutant Deficient in dTMP Kinase Activity

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Escherichia coli LD0181 is sensitive to 15 µg of 2',3'-dideoxythymidine per ml. A derivative that was resistant to 40 µg of the same chemical per ml at 30°C and that had lost the ability to grow on enriched medium at 42°C was isolated after nitroso-guanidine mutagenesis. This mutant, TD105, produced a dTMP kinase with 25-fold lower specific activity and a 5-fold higher K_m for dTMP than the parental strain. The dTMP pool in TD105 was 4.4-fold higher than in the parent. In addition to temperature sensitivity and resistance to 2',3'-dideoxythymidine, the mutant exhibited a hypersensitivity to 5-bromo-2'-deoxyuridine. All three of these phenotypes are cotransducible. The *tmk* gene was mapped by cotransduction to approximately 30 min on the *E. coli* map.

The metabolic pathways used by *Escherichia coli* for synthesizing the deoxynucleotide precursors of DNA have been well characterized genetically and biochemically (21). dTMP kinase, the enzyme responsible for specifically catalyzing the conversion of dTMP to dTDP, remains an exception. Since phosphorylation of dTDP is catalyzed by a nonspecific nucleotide diphosphate kinase, dTMP kinase is the last unique enzyme in the pathway leading to dTTP biosynthesis. To date, no mutations have been reported in the gene coding for dTMP kinase.

It has previously been shown that 2',3'-dideoxythymidine triphosphate (ddTTP) can serve as a substrate of DNA polymerase I (2, 13). The rate of attachment to the 3' terminus of a growing DNA strand is 0.1% that of dTTP. However, once ddTTP is incorporated, an absolute inhibition of chain elongation results. Furthermore, a polynucleotide with a 3' ddTTP is over 1,000-fold less susceptible to 3' → 5' exonucleolytic cleavage than is a polynucleotide with a 3' dTMP. The same inhibitory effects have not been investigated for DNA polymerase III. However, since DNA polymerases employ a mechanism of chain elongation requiring a 3' hydroxyl group (13, 27), an analogous effect seems likely. A similar analog, 2',3'-dideoxyadenosine, causes irreversible inhibition of *E. coli* DNA synthesis (24). This suggests that dideoxynucleosides can serve as substrates for nucleoside kinases. In contrast, most *E. coli* K-12 derivatives are resistant to high levels of 2',3'-dideoxythymidine (TddR) (2). Possibly, TddR and its phosphorylated derivatives are either poor substrates for the appropriate nucleotide kinases or they are metabolically unstable.

E. coli LD0181, which contains mutations in *dcd*, *cdd*, and *tpp*, was found to be sensitive to TddR. All three mutations are associated with functions concerning dTTP biosynthesis. The primary source of deoxyuridine nucleotides used for the de novo synthesis of dTTP is dCTP, which is deaminated by the *dcd* gene product (20). Deoxyuridine can also be formed by cytidine deaminase, coded for by *cdd*. Double mutations in *dcd* and *cdd* do not result in an absolute requirement for exogenous thymine since UDP can also serve as source of uridine deoxynucleotides (11, 15). However, these mutations result in a lower thymidine (TdR) nucleotide pool (20). Thus, for a given amount of exogenous TddR added, the

relative intracellular concentration may be higher in strains carrying these two mutations. The additional mutation in *tpp* prevents added TddR from being degraded by TdR phosphorylase into thymine and dideoxy-ribose-1-phosphate (22).

A mutant defective in *tmk*, the gene coding for dTMP kinase, was isolated from strain LD0181 on the basis of resistance to TddR and sensitivity to growth at 42°C on rich medium. Strains carrying this mutation have an elevated dTMP pool, are sensitive to bromodeoxyuridine (BUdR), and were shown by enzyme assay to be defective in dTMP kinase activity. The *tmk* gene was mapped at approximately 30 min on the *E. coli* chromosome.

MATERIALS AND METHODS

Materials. [³H]TdR and [³H]TMP were both obtained from Amersham Corp. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Aldrich Chemical Co. TddR was obtained from P-L Biochemicals, Inc. All other chemicals were obtained from Sigma Chemical Co.

Bacterial strains. The strains used in this study are all derivatives of *E. coli* K-12 and are listed in Table 1.

Growth of bacteria. Cells were grown in a New Brunswick model G76 Gyrotory water-bath shaker in L-broth (18), Davis minimal medium (8), or MOPS (morpholino-propane sulfonate) minimal medium (19). Minimal media were supplemented with nutrients as required, per milliliter: glucose, 2 mg; thymine, 20 µg; uracil, 10 µg; thiamine, 4 µg; histidine, 30 µg; leucine, 80 µg; methionine, 30 µg; arginine, 100 µg; tryptophan, 25 µg; proline, 50 µg; threonine, 100 µg; phenylalanine, 60 µg; tyrosine, 40 µg; cysteine, 30 µg. In some experiments, glucose was supplemented with 0.05% Cas-amino Acids or replaced by 1.0 mg of maltose per ml.

Growth was monitored with a Beckman DB spectrophotometer at 660 nm. Unless otherwise stated, cells were harvested while growing exponentially at a density of approximately 2×10^8 cells per ml.

Mutagenesis. A culture of strain LD0181 growing exponentially in Davis minimal medium at 37°C was harvested by centrifugation and suspended in an equal volume of 0.05 M Tris-maleate buffer (pH 6.5). This culture was treated for 15 min with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine at a concentration of 0.2 mg/ml, centrifuged, washed, and suspended in Davis medium. Samples containing approximately 10^7 cells

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TABLE 1. Bacterial strains used

Strain	Partial genotype ^a	Source or reference
HD1038	F ⁻ <i>metB leu hisA argG lacY mala xyl mtl rpsL dcd-1</i>	This laboratory (20)
LD0178	HD1038 <i>tpp-75</i>	This laboratory
LD0181	LD0178 <i>cdd-50 argG⁺</i> (P1)	This laboratory
TD105	LD0181 <i>tmk-1</i>	This study
TD205	LD0181 <i>zcyj-297::Tn10 tmk-1</i>	This study
TD1005	LD0181 <i>thyA</i>	This study
TD120	TD105 <i>thyA</i>	This study
TD135	TD105 <i>tyrR</i>	This study
TD401	LD0178 <i>tmk-1</i>	This study
TD402	TD401 <i>zcyj-297::Tn10 tmk⁺</i>	This study
TD405	TD401 <i>zcyj-297::Tn10 tmk⁺</i>	This study
KMBL54	F ⁻ <i>thi-1 thyA6 pyrF101 lacY1 codA1 supE44</i>	K. Berends (1)
JRG861	F ⁻ <i>fnr-1 trpA gal-25 strA</i>	CGSC ^b (14)
TD1106	JRG861 <i>tyrR</i>	This study
JP2144	F ⁻ <i>tyrR trpA his-85 Ilv-632 tsx-89</i>	CGSC (6)
DG17	F ⁻ <i>argG metB his-1 leu-6 mtl-2 xyl-7 mala gal-6 lacY thyA rpsL tonA4 supE44</i>	D. Glaser (23)
SG915	DG17 <i>dnaL708</i>	D. Glaser (23)
KL99	Hfr <i>thi-1 relA1 lac-42</i>	CGSC (16)
KL14	Hfr <i>thi-1 relA1</i>	CGSC (16)
HfrP3	Hfr <i>metB1 relA1</i>	CGSC
KA197	Hfr <i>thi-1 pheA97 relA1</i>	CGSC (10)
PC0254	F ⁻ <i>thi tyrA his-68 trp-45 purB lacY mala mtl-2 xyl-7 gal-6 rpsL tonA2 tsx-70 supE44</i>	CGSC
BH20	F ⁻ <i>thi his cysB lac gal mal mtl xyl rpsL tonA supE44 recA</i>	This laboratory

^a All strains used in this study were λ⁻.

^b CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine.

were plated on Davis minimal agar containing 20 μg of TddR per ml.

Ether-permeabilized cells. Exponentially growing cultures were harvested by centrifugation, suspended in 1/100 volume basic medium, and treated with an equal volume of ether (25). This suspension was gently inverted 30 times over a period of 60 s. The ether was then pipetted off, and the residual ether was allowed to evaporate for 5 min at 0°C before storing at -20°C. Protein content was determined by the Hartree modification (9) of the Lowry assay (17).

Assay for dTMP kinase activity. Five-microliter samples of ether-treated cultures were assayed for 10 min in 0.17 M Tris-hydrochloride buffer (pH 7.7) containing 0.013 M MgCl₂, 0.13 M KCl, 0.0067 M ATP, and 0.00017 M [³H]TMP (21 μCi/μmol) made to a total volume of 30 μl. The reaction was stopped by adding EDTA to a final concentration of 0.04 M and placing the samples on ice. Samples of 10 μl were spotted on polyethyleneimine-cellulose thin-layer plates, and the spots were allowed to dry. The plates were washed twice with absolute methanol and were dried before the separation of TMP from TDP and TTP with 1.0 N sodium formate buffer (pH 3.4). The origin of the chromatogram containing TDP and TTP was cut out, and the nucleotides were eluted by shaking for 15 min with 1.0 ml of 0.02 M Tris-hydrochloride (pH 7.0) made 0.7 M in MgCl₂. After the addition of 10 ml of Aquasol II-xylene (2.5:1), the radioactivity was measured with a Beckman LS-235 liquid scintillation counter.

TdR phosphate pools. Cultures growing in exponential phase at 37°C in MOPS medium containing 0.04 mM TdR

were harvested by centrifugation, washed, and suspended in fresh medium containing 0.04 mM [³H]TdR (1.25 mCi/μmol). After 40 min of labeling, cells were harvested from 100-μl samples by centrifugation, suspended in 40 μl of 0.4 M formic acid, and kept on ice for 2 h. Cellular debris was removed by centrifugation, and the supernatant was spotted on polyethyleneimine-cellulose thin-layer plates with TdR nucleotide markers (50 nM each, TMP, TDP, and TTP). The plates were washed three times in absolute methanol and were dried. TdR nucleotides were separated by using a step gradient (0.45 M LiCl in saturated boric acid, followed by 1.0 M LiCl in 1.0 N formate buffer, pH 3.4). Elution of nucleotides and counting were performed as described above.

RESULTS

Rationale for selection. The triphosphate derivative of the TdR analog TddR inhibits DNA synthesis. Competition of TddR with TdR, the naturally occurring nucleoside, is not efficient, and high levels are required to effect toxicity in most *E. coli* strains. The strain used in this study, LD0181, is sensitive to low levels of TddR (10 to 15 μg/ml). Three mutations carried by this strain are required for sensitivity: *dcd-1*, *cdd-50*, and *tpp-75* (alleles of genes coding for cytidine deaminase, deoxycytidine deaminase, and thymine phosphorylase, respectively). The *dcd* mutation results in a low TdR nucleotide pool (20) and thus would be expected to increase the relative concentration of the pools of an exogenously added TdR analog. Another effect of this mutation is to increase the dCTP pool. Breakdown of dCTP may increase the deoxycytidine levels so that deoxycytidine deaminase contributes significantly to the formation of TdR nucleotides. A mutation in *cdd* prevents the deamination of deoxycytidine. Finally, the defect in thymine phosphorylase prevents catabolism of TdR and TdR analogs (22). Based on this scheme, it was hypothesized that a mutation resulting in the production of a dTMP kinase with only partial activity would increase the steady-state levels of dTMP and TdR. As a consequence, this type of mutation could render a strain resistant to TddR. Mutations in *tmk* would have to produce a partially active enzyme since dTMP kinase is essential for the synthesis of dTTP.

Based on the above rationale, a culture of strain LD0181 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and was plated onto Davis minimal agar containing 20 μg of TddR per ml. These plates were incubated at either 30°C or 42°C. Resistant clones were then replicated onto L-agar at both 30 and 42°C to detect thermolabile or cold-sensitive conditional lethal mutants. This selection process resulted in the isolation of 15 cold-sensitive and 29 thermolabile mutants.

These mutants were assayed for dTMP kinase activity to detect those with mutations in *tmk*. Resistance to TddR could be conferred by a variety of mutations: reversion of one of the three mutations responsible for the original sensitive phenotype; regulatory mutations leading to constitutive synthesis of thymine nucleotides; mutations altering the substrate specificity of nucleoside phosphate kinases; or mutations altering the ability of TddR to be either incorporated into or efficiently excised from DNA. To identify those strains which have mutations specifically in *tmk*, the conditional lethal mutants obtained were assayed for dTMP kinase activity. Three cold-sensitive and three thermolabile mutants were found to have decreased dTMP kinase activity relative to the parent when assayed at nonpermissive temperatures. Under the conditions used in this study, we

obtained *tmk* mutants at a frequency of approximately 10^{-5} to 10^{-6} per cell.

Derivatives of one temperature-sensitive mutant, TD105, were used for further studies. Table 2 shows that the specific activity of dTMP kinase from strain TD120, a *thyA* derivative of TD105, is 4% of the parental activity regardless of the temperature at which it is assayed. Figure 1 indicates that the K_m of the defective dTMP kinase is fivefold higher than that of the TD1005 enzyme, suggesting that the defect is due to a mutation in the structural gene coding for the enzyme. Despite the observation that the kinase activity of TD105 was lower at all temperatures examined, mutants were able to grow on L-agar at 30 or 37°C, but not at 42°C. This can be explained if one assumes that sensitivity to temperature is growth rate dependent rather than due to thermolability of the protein itself. In support of this, it was observed that TD105 was able to grow at 42°C when plated on minimal medium plates rather than on rich agar. Lethality at 42°C was not observed in liquid minimal medium unless it was supplemented with a casein hydrolysate.

Strain TD105 was also found to be unable to grow on medium containing 20 μg of BUdR per ml. The basis for sensitivity to levels of BUdR at which the parent was unaffected was not investigated. However, selection for BUdR resistance was used to derive *tmk*⁺ recombinants in mapping studies.

The rationale used to isolate a mutation in *tmk* predicts that the steady-state level of dTMP would be higher in a *tmk* strain than in an isogenic *tmk*⁺ strain. This was confirmed by measuring the dTMP pools in derivatives of the parent and mutant strains. These derivatives carried mutations in *thyA* which allowed the specific activity of intracellular [³H]TdR to be controlled. Table 3 shows that TD120 was found to have a 4.4-fold higher dTMP pool than the *tmk*⁺ parental control. There was no significant difference between these strains in the intracellular amounts of dTDP and dTTP.

Mapping studies. Mapping of *tmk* was complicated by the fact that the phenotype of a *tmk* strain is dependent on the presence of other mutations. For this reason, transposon Tn10 was introduced into the chromosome near the *tmk* gene, and tetracycline resistance conferred by the transposable element was mapped. The strain used for these experiments was a derivative of LD0178, the *cdd*⁺ parental strain of LD0181. LD0181 is lysogenic for P1 and presented difficulties in mapping. LD0178 is not as sensitive to TddR as LD0181 is; it showed a significant amount of background growth when plated on 40 μg of TddR per ml. However, we were able to construct a *tmk* derivative of this strain by mating with TD105/F'101 (used as an Hfr). The resulting strain, TD401, retained the phenotype of BUdR sensitivity exhibited by the analogous derivative of LD0181, but it was able to grow at 42°C, even in rich medium.

The techniques of Kleckner et al. (12) were used to generate a derivative of strain TD120 containing a Tn10

TABLE 2. dTMP kinase activity at three temperatures in exponentially growing cultures

Temp (°C)	Activity ^a		TD120/TD1005 ratio
	TD1005	TD120	
30	0.53	0.023	0.043
37	0.78	0.029	0.037
42	0.93	0.035	0.038

^a Expressed as picomoles of TDP and TTP formed per minute per milligram of total cellular protein.

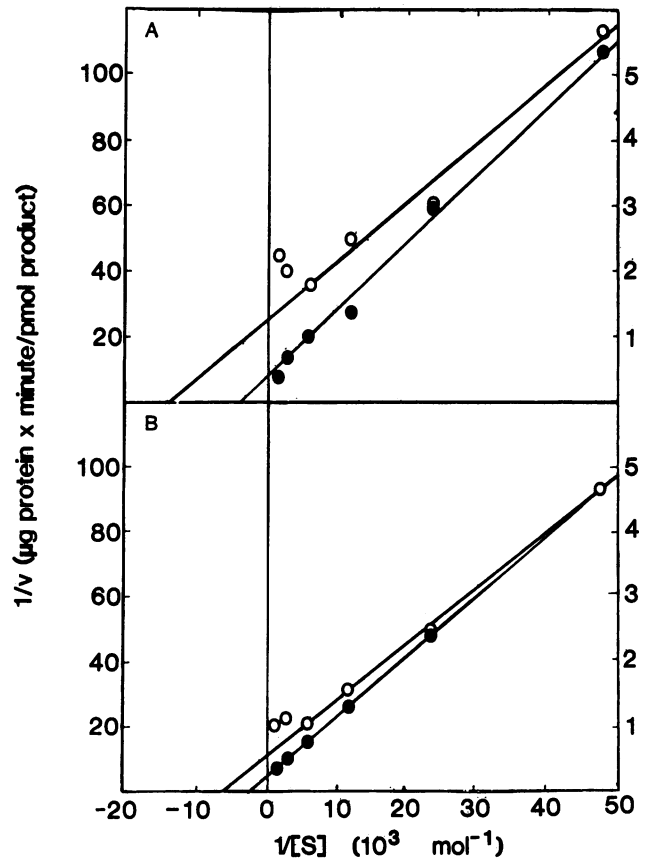


FIG. 1. Double-reciprocal plots of dTMP kinase activity at 30°C (A) and 42°C (B) in strains TD1005 (*tmk*⁺) (○) and TD120 (*tmk*) (●). The values for 1/v were determined by enzyme assay, as described in the text, using a range of dTMP concentrations from 0.0416 to 0.67 mM. The Y-axis on the left pertains to TD120; that on the right pertains to TD1005.

element that was cotransducible with *tmk*. A pool of Tn10 insertions was made in a *malA*⁺ derivative of LD0178 by using λ 561::Tn10 as a vector, selecting for tetracycline resistance. Bacteriophage P1 grown on this pool was then used to transduce TD401 to tetracycline resistance. These transductants were tested for cotransduction of *tmk*⁺ on the basis of BUdR resistance. Individual BUdR-resistant, tetracycline-resistant derivatives were used to transduce TD401 to tetracycline resistance, and the frequency of cotransduction of *tmk*⁺ was determined. One of these derivatives, TD402, showed 56% cotransduction of the two markers. This strain was used to construct TD405, a *tmk zcj-297*::Tn10 derivative of TD401, which was used to map *tmk*.

The localization of *tmk* to the 27- to 30-min region of the *E. coli* map was determined by long-term Hfr matings as well as

TABLE 3. TdR phosphate pools in cultures growing exponentially at 37°C

Strain	Nucleotide (pm/mg of total protein)			Ratio	
	TMP	TDP	TTP	TMP/TTP	TDP/TTP
TD1005	2.0	1.3	0.94	2.2	1.4
TD120	9.3	1.2	0.96	9.7	1.3

TABLE 4. Cotransduction of genetic markers with *tmk*

Donor	Recipient	Selected donor marker ^a	No. of transductants	Unselected markers ^b	
				Type	%
JP2144	TD401	<i>tmk</i> ⁺	168	<i>tyrR</i>	2
TD1106	TD205	<i>tyrR</i>	156	Tn10 <i>tmk</i> ⁺	4 1
TD405	TD135	Tn10	61	<i>tmk</i> ⁺ <i>tyrR</i> <i>tmk</i> ⁺ <i>tyrR</i>	51 23 5

^a Selection for resistance to 30 µg of BUdR per ml was used to isolate *tmk*⁺ transductants. Isolation of *tyrR* transductants was achieved by selecting for resistance to 80 µg of *m*-fluoro-DL-tyrosine per ml. Transduction of Tn10 was determined by isolating clones resistant to 15 µg of tetracycline per ml.

^b Unselected markers were examined by testing for sensitivity to BUdR (*tmk*), tetracycline (Tn10), or *m*-fluoro-DL-tyrosine (*tyrR*).

by interrupted mating experiments with the Hfr strains listed in Table 1 (data not shown). These results were confirmed by introducing F'123 into a *tmk-1* strain (3). The resulting merodiploid is *Tmk*⁺, indicating that the mutation is recessive. More detailed localization was accomplished by using P1 transduction techniques with derivatives of strain TD105 (Table 4). Both BUdR resistance conferred by *tmk*⁺ and tetracycline resistance conferred by *zcy-297::Tn10* were utilized for selection. All phenotypes associated with the original *tmk-1* allele were 100% cotransducible in the proper genetic background. Although temperature sensitivity was not evident in TD401, BUdR-sensitive transductants of LD0181 derived with TD401 as the donor were also temperature sensitive. A gene located at 29 min on the *E. coli* map, *tyrR* (26), was found to cotransduce with the Tn10 insertion at a frequency of 28%. Selection for TyrR (fluoro-tyrosine resistance) (7) gave five- to sixfold lower cotransduction frequencies with *tmk*⁺ and the *zcy::Tn10* insertion than the reciprocal crosses did. Aberrant recombination frequencies may be an artifact arising as a consequence of the transposable element inserted into this region. Lower cotransduction frequencies of *tyrR* with *tmk*⁺ were also observed when the latter was used as the selected marker. Transduction to *tmk*⁺ was accomplished by selecting for BUdR resistance. Some of these BUdR-resistant clones may actually be *dcd*⁺ or *tpp*⁺ transductants. Since these were not characterized, cotransduction frequencies would be expected to underestimate the linkage between *tyrR* and *tmk*. The most precise estimation of this linkage is 5% cotransduction by P1, as indicated by transduction of strain TD135 to tetracycline resistance. This translates as a separation of 1.3 min by the formula of Wu for converting transduction frequencies into map distance (28). Transduction experiments with *cysB*, *trpA*, *purB*, and *pyrF*, located counterclockwise to *tyrR*, showed no cotransduction of either *tmk* or the linked transposable element. The results suggest that the gene order in this region is *pyrF*, *tyrR*, Tn10, *tmk* and that *tmk* is located at around 30 min on the *E. coli* genetic linkage map.

DISCUSSION

The metabolic pathways used by *E. coli* for the synthesis of deoxynucleotide precursors of DNA are fairly well understood, both genetically and biochemically. Phosphorylation

of dTMP is the last step in dTTP biosynthesis catalyzed by an enzyme that is specific to that pathway. Phosphorylation of dTDP is catalyzed by a nonspecific nucleotide diphosphate kinase. Given its key position in the synthesis of dTTP and the fact that thymine is unique to DNA, the control of the synthesis of this enzyme could be important in the overall control of DNA synthesis. This report describes the isolation and characterization of a mutation leading to a deficiency in dTMP kinase.

The mutation, *tmk-1*, was isolated by selecting for mutants that rendered a *dcd cdd tpp* strain resistant to TddR, a TdR analog. The mutant was found to produce a dTMP kinase with 4% of wild-type activity. This mutant kinase also has a fivefold higher *K_m* for the substrate, dTMP. Resistance is attributed to a buildup of the dTMP pool as a result of this defective kinase. The mutant has a conditional lethal phenotype, being unable to grow at 42°C on a nutritionally rich medium. However, the protein does not appear to be thermolabile since the specific activity was lowered by the same degree at all temperatures at which it was assayed. Instead, conditional lethality seems to be dependent on the growth rate. Cultures were able to grow at 42°C when plated onto minimal agar plates. This phenomenon can be explained if it is assumed that the defect in dTMP kinase does not allow DNA synthesis to proceed at a rate sufficient to balance cell growth when conditions for growth are optimal.

An additional phenotype of strains carrying this mutation is an increased sensitivity to BUdR, another TdR analog. Transductional analysis indicates that TddR resistance, BUdR sensitivity, and temperature sensitivity are 100% linked in *dcd cdd tpp* strains. We were surprised to discover two seemingly contradictory phenotypes, resistance to TddR and sensitivity to BUdR. This phenomenon may be due to an alteration in the substrate specificity of the mutant enzyme in addition to the general reduction in specific activity.

Genetic mapping studies have located *tmk* at around 30 min on the *E. coli* chromosome. The *tmk-1* allele is recessive and is covered by F'123, which has its point of origin near the *rac* locus at 30 min on the linkage map. There was 5% cotransduction of *tmk* and *tyrR* when bacteriophage P1 was used. Transposon Tn10 inserted between these two genes was 28% cotransducible with *tyrR* and 56% cotransducible with *tmk*. Transductional analysis of this region of the chromosome is difficult due to a paucity of genetic markers. Recently, a number of Tn10 insertions in the *rac* locus have been obtained (4, 5). These could be useful in locating *tmk* more precisely.

A large number of temperature-sensitive mutants have been isolated in *E. coli* as defective for DNA synthesis (23). One of these mutations, *dnaL708*, maps in the same general region as *tmk*. Since a mutant dTMP kinase would be expected to result in defective DNA synthesis and since no function has been associated with the *dnaL* gene product, we thought that these two genes might be identical. However, assays for dTMP kinase activity in the *dnaL* mutant, SG915, and in its parental strain, DG17, indicated no differences in activity between the two. In addition, *dnaL* and *tmk* did not cotransduce in experiments with bacteriophage P1 (data not shown).

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