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Enzymatic Characterization of a Mutant of *Escherichia coli* with an Altered DNA Ligase

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ABSTRACT **A temperature-sensitive, radiation-sensitive mutant of *Escherichia coli* has been assayed for DNA ligase activity *in vitro*. The strain contains a markedly reduced amount of DNA-joining activity, which is thermolabile. The formation of the ligase-adenylate intermediate is also temperature-sensitive *in vitro*. Two temperature-resistant revertants of the mutant contain normal amounts of a thermostable ligase. The mutant is killed by growth at 42°C, a temperature at which it displays aberrant DNA synthesis. These results suggest that the ligase is necessary for normal DNA metabolism and viability in this strain.**

Polynucleotide-joining enzymes (ligases) have been implicated in DNA replication (1) and repair (2), and in genetic recombination (2). The function of these enzymes *in vivo* has generally been studied in T4 phages with amber and temperature-sensitive mutations in the structural gene for the phage-induced ligase (3, 4). An inherent difficulty in interpreting the results obtained with such mutants has been uncertainty in regard to the extent to which the *Escherichia coli* ligase can substitute for the phage-induced enzyme. We have, in fact, recently found that the specific activity of the phage-induced ligase in extracts of T4-infected cells is only two- to three-fold higher than that of the host enzyme when the two are assayed under optimal conditions (5).

Gellert and Bullock (6) have recently characterized several *E. coli* strains defective in ligase that they isolated by a novel selection technique. The three mutants studied had 40–150% of normal joining activity when analyzed at 30°C, but only 4–14% of normal activity when assayed at 42°C. Adenylylation of the enzyme was reduced in two of the mutants, but was not temperature sensitive. Except for the case of their *lop*8 *lig*4 mutant (150% of normal joining at 30°C, 4% of normal at 42°C), which became UV sensitive at 42°C, the mutants grew normally on minimal or rich media and showed no obvious defect in their DNA metabolism. These results do not permit unambiguous conclusions concerning the physiological role of the ligase.

In 1968, Pauling and Hamm described a conditional-lethal mutant of *E. coli* that was temperature sensitive in a late step of dark repair (7). They also observed that at restrictive temperatures, the newly replicated DNA accumulated in the form of small (10S) fragments (8). These properties led them to suggest that the mutant had a defective DNA ligase. Using as an assay the joining of oligo(dT) annealed to poly(dA) (9), they compared partially purified fractions derived from the mutant ts-7 and the parent TAU-bar; they found that the ligase activity in the mutant was significantly reduced compared to that of the parent. However, no difference in the thermolability of the two enzyme fractions could be detected (8). Measurement of ligase activity by the (dA)·(dT) method may have been complicated by the presence of exonucleases in these relatively crude enzyme preparations. We have recently developed a new assay for DNA ligase that measures the conversion of linear molecules of poly(dA-dT) copolymer to closed-circular structures and have found it to be relatively unaffected by the nuclease activities present in most cell extracts (5). Using this method, we have reexamined the ligase activity in extracts and in partially purified enzyme fractions of TAU-bar, ts-7, and several revertants of ts-7.

Abbreviation: NMN, nicotinamide mononucleotide.

MATERIALS AND METHODS

E. coli strains and media

TAU-bar, ts-7, and two revertants of ts-7 (ts-7 rev 1, ts-7 rev 2) were generously provided by Dr. Crellin Pauling (University of California, Riverside). Cultnres were grown with aeration in H broth (10) supplemented with thymidine (10 μg/ml) at 25°C, unless specified otherwise. Viable-cell titers were determined by plating immediately on tryptone agar plates supplemented with thymidine (10 μg/ml). The plates were incubated at 25°C overnight.

Enzyme fractionation and assays

Cultures were grown to an A_{595} of 0.8, harvested by centrifugation, and crude extracts were prepared as was described (5). Where indicated, the extracts were purified through the ammonium sulfate step described by Olivera and Lehman (9). Additional purification of the ammonium sulfate fraction was obtained by adsorption to and elution from alumina C_γ gel as follows: The ammonium sulfate pellet was dissolved in 0.02 M potassium phosphate buffer (pH 6.5)–2 mM EDTA–1 mM β-mercaptoethanol to 40 mg/ml of protein. This was diluted to a protein concentration of 7 mg/ml with 0.01 M potassium phosphate (pH 7.5)–0.01 M $(NH_4)_2SO_4$, and treated with 0.3 volume of alumina C_γ gel (25 mg/ml in H_2O, BioRad). After 20 min at 0°C, the gel was collected by centrifugation and washed successively with 1 volume (volume relative to 7 mg/ml protein solution) of 0.03 M potassium phosphate buffer (pH 7.5)–0.01 M $(NH_4)_2SO_4$ and 1 volume of 0.4 M potassium phosphate (pH 7.5)–0.01 M $(NH_4)_2SO_4$–2 mM EDTA–1 mM dithiothreitol. The latter fraction was adjusted to 70% saturation with solid $(NH_4)_2SO_4$ and the precipitate, which was collected by centrifugation, was dissolved in 0.02 M potassium phosphate (pH 6.5)–2 mM EDTA–1 mM β-mercaptoethanol.

The DNA-joining assay measured the rate of conversion of linear poly(dA-dT) (number average chain length: 700–1000

TABLE 1. *Assay of ligase activity by measurement of (dA-dT) circle formation*

Strain	Enzyme fraction	Specific activity (units/mg protein) 25°C	40°C
TAU-bar	Extract	2.9	2.9
TAU-bar	Ammonium sulfate	8.9	8.9
ts-7	Extract	0.1	≤0.01
ts-7	Ammonium sulfate	0.05	≤0.02
ts-7 rev 1	Extract	3.1	3.5
ts-7 rev 2	Extract	3.4	2.8

nucleotides) to the circular form (5) the reaction mixture was made 0.01 M in $(NH_4)_2SO_4$ and incubation was at the temperature indicated. One unit is defined as the amount of activity converting 100 nmol of nucleotide to the circular form in 30 min under the assay conditions.

The formation of the enzyme-adenylate intermediate, the first step of the ligase reaction (11, 12), was assayed by a method devised by Dr. Richard Gumport. To discharge any ligase already in the adenylylated form, the enzyme fractions were made 4 mM in $MgCl_2$ and 0.16 mM in nicotinamide mononucleotide (NMN), and incubated at 25°C for 5 min. After addition of EDTA, to a final concentration of 0.01 M, the fractions were dialyzed against 1000 volumes of 0.05 M Tris·HCl (pH 7.4)–1 mM EDTA–0.1 M NaCl–2 mM β-mercaptoethanol for 4 hr; then against two changes (500 volumes each) of 0.05 M Tris·HCl (pH 7.4)–1 mM EDTA–2 mM β-mercaptoethanol for a total of 3–4 hr. The reaction mixtures (0.05 ml) contained 0.01 M Tris·HCl (pH 8.0), 5 mM $MgCl_2$, 1 mM EDTA, 0.01 M β-mercaptoethanol, 0.81 μM [^{32}P]DPN, bovine plasma albumin (50 μg/ml), and 0.1–0.5 mg of protein from the NMN-treated, dialyzed preparations (the enzyme fractions were incubated at the temperature of the assay for 5 min before the addition of the other components to initiate the reaction). The [^{32}P]DPN was labeled in the AMP moiety: specific radioactivity, 1.6×10^4 cpm/pmol; labeled AMP was prepared by the method of Symons (13), and was converted to DPN by the method of Shuster *et al.* (14). After incubation at the indicated temperature for 10 min, at which time adenylylation is essentially complete, the reactions were stopped by chilling to 0°C and adding 0.45 ml of 2 mM EDTA–bovine plasma albumin (1 mg/ml), followed by 1 ml of cold 10% trichloroacetic acid. After 5 min at 0°C, the precipitates were collected by centrifugation for 5 min at 20,000 × *g* and dissolved in 1 ml of 0.02 M NaOH–0.02 M sodium pyrophosphate. They were then chilled and 0.1 ml of cold 50% trichloracetic acid was added. After 5 min at 0°C, the precipitates were collected on glass filters (Whatman GF/C, 2.4-cm circles) and then washed with five 10-ml portions of cold 1 N HCl and three 10-ml portions of cold absolute ethanol. The filters were dried and radioactivity was determined in a Nuclear-Chicago Unilux Spectrometer. Blank values were obtained by making the reaction mixture 2–4 mM in NMN and assaying in the standard way.

For both the joining and the adenylylation assays, the values shown represent the average of several determinations. The ratio of the extent of enzyme-adenylate formation to the rate of (dA-dT) circle formation varied only slightly with the purity of the enzyme [0.14 pmol of AMP bound per (dA-dT) unit in crude extracts, compared to 0.22 pmol of AMP bound per unit for purified enzyme].

TABLE 2. *Formation of enzyme-adenylate intermediate*

Strain	Fraction	Specific activity (pmol of E-AMP/mg protein) 25°C	40°C
Expt. 1			
TAU-bar	Extract	0.34	0.39
ts-7	Extract	0.27	≤0.01
ts-7 rev 1	Extract	0.36	0.39
ts-7 rev 2	Extract	0.42	0.36
Expt. 2			
TAU-bar	Extract	0.24	0.21
TAU-bar	Ammonium sulfate	0.75	0.65
ts-7	Extract	0.10	≤0.005
ts-7	Ammonium sulfate	0.39	0.07

Rates of thymidine incorporation

0.5-ml aliquots were removed from exponentially growing cultures and added to tubes, prewarmed to the culture temperature, that contained 20 μCi of [^{3}H]thymidine (6 Ci/mmol). After 3 min of incubation with shaking at the appropriate temperature, incorporation was stopped by the addition of 0.2 ml of cold 17% trichloroacetic acid that contained thymidine (2.5 mg/ml), and the tubes were chilled to 0°C. After 30 min at 0°C, the precipitates were collected on glass filters (Whatman GF/C, 2.4-cm circles) and washed with 15 10-ml portions of cold 1 N HCl and three 10-ml portions of cold absolute ethanol. The filters were dried and radioactivity was counted in a Nuclear-Chicago Mark I spectrometer.

RESULTS

Thermolability of DNA joining in ts-7

As shown in Table 1, extracts of ts-7 had only 3% of the joining activity found in the parent strain, TAU-bar, even when the (dA-dT)-joining assays were performed at 25°C, the permissive temperature for ts-7. The low activity found in extracts of ts-7 was also extremely thermolabile, decreasing by at least 10-fold when assayed at 40°C, the nonpermissive temperature for the mutant. This result contrasts with that found for extracts of TAU-bar, where there was no change in the specific activity of the enzyme when the assay temperature was increased from 25°C to 40°C. Experiments (not shown) in which extracts of TAU-bar and ts-7 were mixed and then assayed demonstrated that the reduced activity found in ts-7 was not due to a freely-diffusable inhibitor of the joining reaction present in extracts of the mutant strain.

The thermolability of the ligase from ts-7 persisted through the ammonium sulfate stage of purification (9). Moreover, although the specific activity of the enzyme from TAU-bar increased about 3-fold upon fractionation, that from ts-7 actually decreased. We attribute this effect to the instability of the ts-7 enzyme; the ammonium sulfate fraction had a half-life of about 1 day when stored at 0°C, whereas the TAU-bar enzyme was stable under these conditions.

Since ts-7 was isolated directly from a nitrosoguanidine-mutagenized stock of TAU-bar, it was important to establish that the physiological properties of the strain, and the apparent ligase defect, are attributable to a single mutational event. Several temperature-resistant revertants of ts-7 have been isolated by Dr. C. Pauling and were found to occur with a frequency compatible with a single base change. These revertants have lost the abnormal physiological properties that char-

acterize ts-7 and have a phenotype very similar to that of TAU-bar (C. Pauling, personal communication). As shown in Table 1, the two revertant strains have normal amounts of joining activity with thermal stabilities similar to the TAU-bar enzyme. This result suggests that the abnormal properties of ts-7 *in vivo* and the DNA ligase defect as measured *in vitro* are due to the same mutation.

Thermolability of enzyme-adenylate formation in ts-7

The ligase reaction proceeds via a covalent enzyme-adenylate intermediate (11, 12). Since the formation of this intermediate can be measured independently of the overall joining reaction, we determined whether the ligase defect in ts-7 is also expressed at this step in the catalysis. As shown in Table 2, extracts of TAU-bar, ts-7, and the two revertants of ts-7 all have a similar capacity to form enzyme-adenylate at 25°C. At 40°C, however, the marked temperature sensitivity of ts-7 became apparent; the yield of enzyme AMP from extracts of ts-7 decreased as much as 30-fold when the assay temperature was increased from 25°C to 40°C, whereas the yield from extracts of TAU-bar and the revertants was almost independent of temperature in this range. The thermolability of enzyme-AMP formation by the ts-7 enzyme was evident after purification through the ammonium sulfate step, which resulted in a 4-fold increase in specific activity as measured by this assay and removal of most of the nucleic acid (A_{280}/A_{260} = 1.4). This increase in capacity to form enzyme-AMP contrasts with the decrease in joining activity observed upon fractionation (Table 1), and supports the idea that the decrease in overall joining activity after ammonium sulfate fractionation is due to the instability of the ts-7 enzyme.

A decrease in the thermolability of enzyme-adenylate formation by the ts-7 enzyme was generally observed upon ammonium sulfate fractionation; at present this decrease is not understood. However, a further 3-fold purification of the ammonium sulfate fraction by adsorption to and elution from alumina C_γ gel (a step in which the mutant and wild-type enzymes behaved similarly) gave a preparation whose thermolability was comparable to that found in crude extracts.

Enzyme-adenylate formation, assayed as described in the legend to Table 2, is essentially complete at the end of the 10-min incubation period. To determine whether there is a

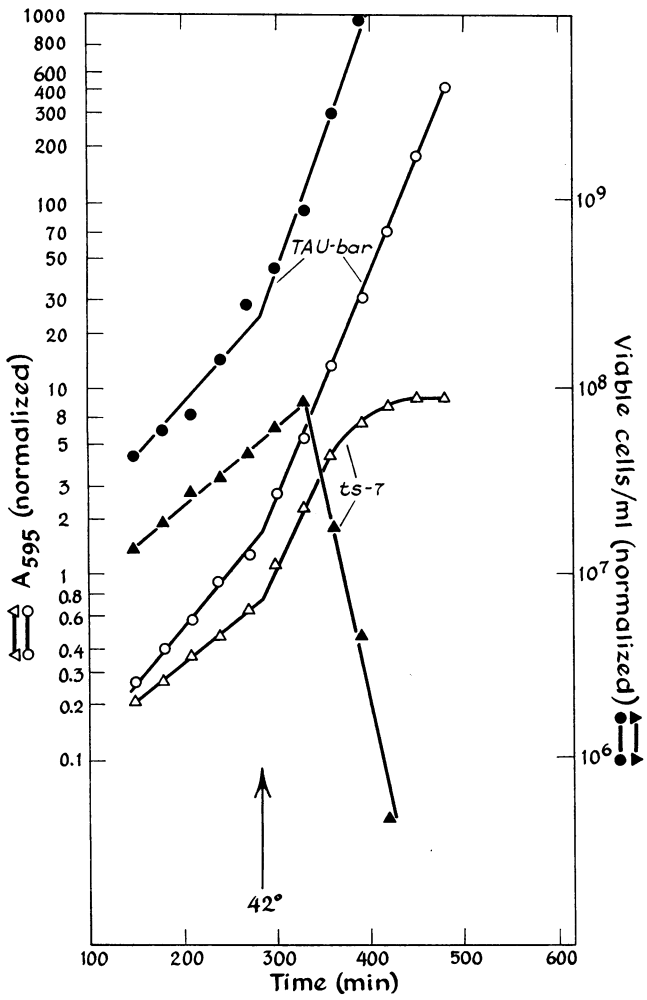

FIG. 1. Growth rate and viability of TAU-bar and ts-7. H broth (10), supplemented with thymidine (5 μg/ml), was innoculated from fresh overnight cultures of TAU-bar or ts-7 and incubated at 25°C with shaking. After 285 min at 25°C, the flasks were transferred to 42°C. At the times indicated, aliquots were removed to determine the A_{595} and the viable-cell titer. The cultures were maintained in exponential growth by dilution into fresh, prewarmed media as necessary to keep the $A_{595} < 0.7$. Viable counts and A_{595} were corrected for dilution by normalizing all readings to the volume at time 0.

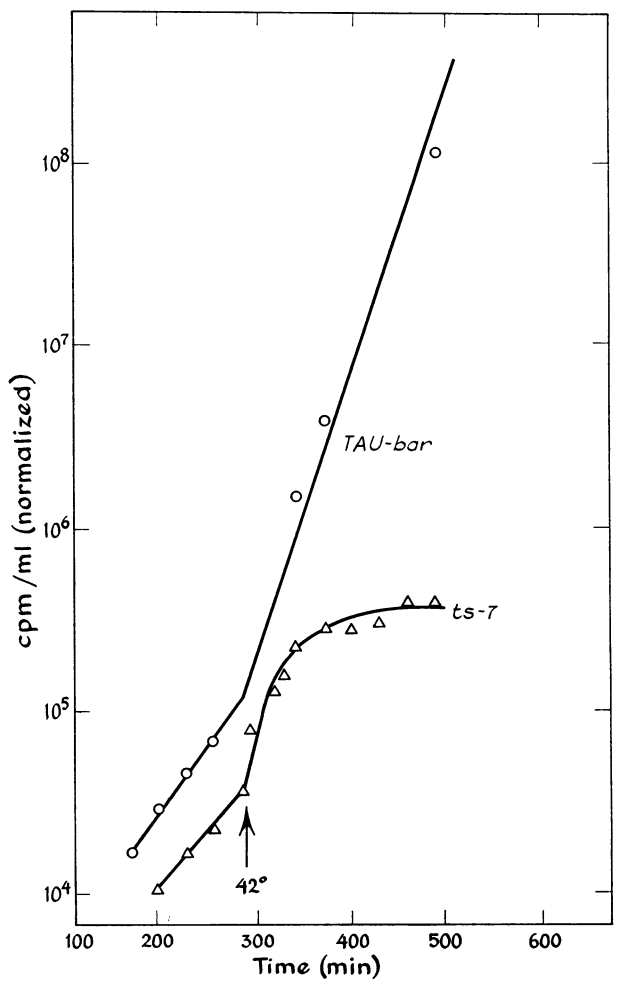

FIG. 2. Rate of [^{3}H]thymidine incorporation by TAU-bar and ts-7. Aliquots were removed from the cultures as described in the legend to Fig. 1 and pulsed with [^{3}H]thymidine (at 25°C for $t < 285$ min, at 42°C for $t > 285$ min). All values are corrected for culture dilutions by normalizing to the volume at time 0.

difference in the rate of enzyme-AMP formation at 25°C between the TAU-bar and ts-7 enzymes, measurements were made at 15- to 30-sec intervals for 10 min; however, no significant difference was found in the initial rates of enzyme-AMP formation in the two extracts (0.05 pmol/min per mg for TAU-bar, compared with 0.07 pmol/min per mg for ts-7).

DNA synthesis in ts-7

We have examined the effect of the ligase mutation in ts-7 on DNA synthesis *in vivo*. Figs. 1 and 2 represent the results of an experiment in which viability, A_{595}, and the rate of DNA synthesis (as determined by 3-min pulses of [^{3}H]thymidine at the indicated times) were followed in parallel cultures of TAU-bar and ts-7 before and after a temperature shift from 25°C to 42°C. At 25°C, several differences between the two strains were apparent: ts-7 grew at a slightly lower rate than TAU-bar, and the rate of thymidine incorporation per viable cell in ts-7 was almost twice that in TAU-bar. When the cultures were shifted to 42°C, the differences between the two strains became even more pronounced. In the case of TAU-bar, the viable cell titer, the A_{595}, and the rate of thymidine incorporation all continued to increase exponentially after the shift-up in temperature. However, in the case of ts-7, the A_{595} increased about 10-fold, then leveled off after 2 hr at 42°C. The rate of thymidine incorporation followed a similar pattern, increasing progressively for about 2 hr at 42°C, then leveling off at a value 10 times that found just before the temperature shift. On the other hand, the viable-cell count increased by only 60%, after which the cells began to die exponentially, with a half-time of 12 min, a result similar to that already reported (7).

One explanation for the increased rate of thymidine incorporation despite the rapid fall in viability after a shift-up of temperature is that extensive repair replication occurs after transfer to the higher temperature. In the absence of a functional ligase, single-strand breaks might accumulate that could act as initiation sites for repair replication; alternatively, if the ligase is required to terminate repair synthesis, then in its absence such incorporation might be expected to continue unchecked. This hypothesis is compatible with our observation that, although ts-7 grows more slowly than TAU-bar at 25°C, thymidine incorporation proceeds at a higher rate per viable cell in the mutant than in TAU-bar. It is also consistent with the previous observation that after ultraviolet irradiation, more extensive repair replication occurs in ts-7 than in TAU-bar (at 25°C or 40°C), and the finding that a large fraction of the DNA made at 40°C by ts-7 is not the product of semiconservative replication (7).

DISCUSSION

It is clear that ts-7 contains an altered DNA ligase. The low amount of joining activity observed in extracts of ts-7, even when the assay is performed at 25°C, is presumably a manifestation of its thermolability. The defect in the enzyme is apparent in the overall joining reaction and in the formation of the enzyme–AMP intermediate. The fact that two revertants to the parental phenotype display normal ligase activity strongly suggests that the aberrations observed in ts-7 *in vivo* are due to the ligase mutation.

In an attempt to relate the results reported here to those of Gellert and Bullock (6), it should be pointed out that ts-7 grows well at 25°C, a temperature at which it has only 3% of the normal joining activity; this value is comparable to that found for the *lop*8 *lig*4 strain at 42°C. Furthermore, the ts-7 mutation seems to belong to a different class than that of the *lop*8 *lig*-defective strains, since the thermolability of the ts-7 ligase is apparent not only in the joining reaction, but also in the formation of enzyme–AMP. Thus, ligase may be necessary for viability and Gellert's strains may not be defective enough to allow this requirement to be observed. Alternatively, ligase may be required for the growth of TAU-bar and related strains, but not for the strains of Gellert and Bullock. With regard to this latter possibility, it could be imagined that inactivation of the ligase could cause induction of a defective virus carried by TAU-bar.

Unfortunately, the ts-7 strain presents a number of problems in attempting a more definitive analysis of the effect of the ligase mutation on DNA metabolism *in vivo*. Like TAU-bar, ts-7 probably contains at least three kinds of plasmids (15, 16) that could interfere with studies of the function of the ligase in replication of the *E. coli* chromosome. Furthermore, ts-7, like its parent, cannot be infected with many of the *E. coli* bacteriophages [ϕX-174 is a notable exception (17)], thus making a study of the role of the host ligase in phage infection impossible. Additional work on the effects of the mutation we have studied clearly require that it be transferred to another, more easily manipulated, strain.

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