

Characterization of Bacteriophage T3 DNA Ligase

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DNA ligases of bacteriophage T4 and T7 have been widely used in molecular biology for decades, but little is known about bacteriophage T3 DNA ligase. Here is the first report on the cloning, expression and biochemical characterization of bacteriophage T3 DNA ligase. The polyhistidine-tagged recombinant T3 DNA ligase was shown to be an ATP-dependent enzyme. The enzymatic activity was not affected by high concentration of monovalent cations up to 1 M, whereas 2 mM ATP could inhibit its activity by 50%. Under optimal conditions (pH 8.0, 0.5 mM ATP, 5 mM DTT, 1 mM Mg²⁺ and 300 mM Na⁺), 1 fmol of T3 DNA ligase could achieve 90% ligation of 450 fmol of cohesive dsDNA fragments in 30 min. T3 DNA ligase was shown to be over 5-fold more efficient than T4 DNA ligase for ligation of cohesive DNA fragments, but less active for blunt-ended DNA fragments. Phylogenetic analysis showed that T3 DNA ligase is more closely related to T7 DNA ligase than to T4 DNA ligase.

Key words: ATP-dependent, bacteriophage, blunt-ended, cohesive, T3 DNA ligase.

DNA ligases catalyze the sealing of adjacent 5'-phosphate and 3'-hydroxyl termini in double-strand DNA. On the basis of cofactors required for ligation, DNA ligases are categorized into two groups: NAD⁺-dependent DNA ligases, which are mainly found in eubacterial organisms; and ATP-dependent DNA ligases, including bacteriophage T4 and T7 DNA ligases, which are widely found in bacteriophages, archaea and eukarya.

T4 DNA ligase is encoded by *Enterobacteria phage T4* and the enzyme has been purified and well characterized (1). It has a K_m^{ATP} of 14 μ M for ligation of cohesive DNA fragments (2). The joining of blunt-ended DNA can be achieved at 0.5 mM or lower ATP concentration in the presence of 1 mM Mg²⁺. However, the reaction can be inhibited almost completely by increasing ATP concentration to 5 mM or higher. T4 DNA ligase can also be inhibited by using 10 mM spermidine (3) or high concentration (200 mM) of monovalent cations (4). Mutation analysis of T4 DNA ligase showed that the inactive K159L substitution, although unable to self-adenylate, still possessed AMP-dependent DNA nicking activity. This finding was consistent with a three-step model proposed to explain the ligation reaction (5). In step I, in the presence of ATP, the adenylated enzyme scans DNA molecules searching for suitable 5'-phosphorylated ends; in step II, the adenylate group is transferred from the ligase to DNA and the deadenylated enzyme waits on DNA; and in step III, when a free 3'-OH end is available, ligation is completed and AMP is released. In the presence of ATP, the enzyme is readenylated to start a new cycle. In 2002, Cherepanov *et al.* proposed a dynamic mechanism of nick recognition and sealing to explain the specificity of ligation reaction (6).

T7 DNA ligase is produced by bacteriophage T7, a double-stranded DNA virus belonging to the Family *Pedoviridae*. Its crystallization and characterization were reported in 1996 (7), and its three dimensional structure with ATP was subsequently determined (8). The structure of ligase-ATP complex revealed that the nucleotide-binding pocket is situated in the cleft surrounded by N- and C-terminal domains of the enzyme. The K_m^{ATP} constants of ATP-dependent T7 DNA ligase for joining and exchange reactions are 6 μ M and 0.3 μ M, respectively (9, 11). The enzyme requires a divalent cation for activity, most commonly Mg²⁺ ion *in vivo* (9–11), and has an optimal pH range from 7.2 to 7.7 (9). Like T4 ligase, T7 DNA ligase can ligate DNA fragments with cohesive ends, but it has almost no activity for blunt-ended fragments. However, with 20 to 30% polyethylene glycol 6000 as macromolecular crowding agent, T7 ligase can join blunt-ended DNA with a comparably high ligation efficiency to that of T4 ligase (7).

Bacteriophage T3 is a T7-like virus. Although the whole bacteriophage T7 genome was reported early in 1983 (10), the complete sequence of T3 genome had not been readily available until 2002 (11). One open reading frame of 346 amino acids shows a high degree of identity to bacteriophage fYeO3-12 and T7 DNA ligases. This putative DNA ligase was referred to as T3 DNA ligase. So far, little has been reported on its characteristics. In this study we describe the cloning and overexpression of the T3 DNA ligase gene, a preliminary characterization of the enzyme and a phylogenetic analysis of ATP-dependent ligases.

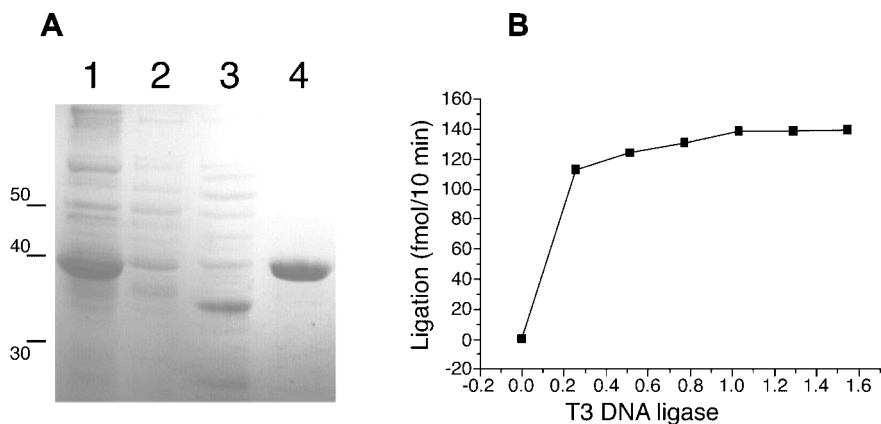
MATERIALS AND METHODS

Materials—T4 DNA ligase was purchased from *New England Bio-labs* (USA). Restriction endonucleases were purchased from *TaKaRa* (Japan). Chelating Sepharose™ was purchased from *Pharmacia* (USA). NADH was purchased from *Sigma* (USA). ATP and dNTPs were purchased from *Promega* (USA). BCA kit for protein quanti-

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Fig. 1. Purification and enzyme activity of T3 DNA ligase. (A) Coomassie Brilliant Blue staining of a 12% SDS-polyacrylamide gel showing the expression level of T3 ligase in BL21 cells and the purified fractions from the Ni-affinity chromatography. Lane 1, supernatant of cell lysate; lane 2, flow-through of buffer A; lane 3, flow-through of wash buffer containing 50 mM imidazole; lane 4, eluate fraction of elution buffer containing 250 mM imidazole. The positions and sizes (kDa) of marker proteins are indicated on the left. (B) Reaction mixture containing 450 fmol *Hind*III linearized pBluescript II SK (+) DNA, 66 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 5 mM DTT, 0.1 mM ATP, 300 mM NaCl and different amounts of T3 DNA ligase as indicated was incubated at 20°C for 30 min and the reaction was terminated by the addition of 60 mM EDTA and 0.1% SDS. The extent of ligation was plotted against the concentration of T3 DNA ligase.



tative assay was purchased from Pierce (USA). Plasmid pET-32a was purchased from Novagen (USA).

Cloning of T3 DNA Ligase—T3 DNA ligase gene was amplified from DNA samples isolated from *Escherichia coli* cells transfected with bacteriophage T3 (ATCC 11303) by polymerase chain reaction (PCR). The primers were designed according to the nucleotide sequence reported previously. The 5' primer, CGGGATCCATGACATCTTCAATACCAAC, contains a *Bam*HI site, and the 3' primer, GCGTCGACTGAGCCAGTGAGTTGGT-TAC, contains a *Sal*I site. PCR product was digested with *Bam*HI and *Sal*I and ligated directly into pET-32a. The resulting construct, pET-32a-T3, was verified by DNA sequencing.

Expression and Purification of T3 DNA Ligase—The expression construct pET-32a-T3 was transformed into *E. coli* strain BL21 (DE3). For large-scale expression, 100 ml of LB medium supplemented with 50 µg/ml ampicillin was inoculated with 2 ml of overnight culture and further incubated at 37°C with shaking. IPTG was added to a final concentration of 0.3 mM at mid-log phase ($A_{550} = 0.6$), and the incubation was continued with shaking at either 37 or 22°C for 4 h. The cells were harvested by centrifugation and lysed by sonication in phosphate-buffered saline. After removal of cell debris by centrifugation, the supernatant was loaded onto a Ni²⁺-charged Chelating Sepharose™ column that had been pre-equilibrated with buffer A (20 mM Tris-HCl, 500 mM NaCl, pH 7.9) at a flow rate of 1 ml/min. The column was then washed with 5 column volumes of buffer A followed by 5 column volumes of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 50 mM imidazole, pH 7.9), and eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 7.9). The eluted fractions containing T3 DNA ligase were pooled and subjected to dialysis, and the protein amount was determined.

Enzymatic Activity Assay—DNA fragments with either cohesive or blunt ends were prepared by digesting pBluescript II SK (+) plasmid with suitable restriction enzymes to completion. The digests were treated with phenol/chloroform extraction and recovered by ethanol precipitation. Ligation mixture in a total volume of 10 µl contained 450 fmol of linearized DNA, 66 mM Tris-HCl buffer, 5 mM DTT, and additional ingredients including different

amounts of T3 DNA ligase, ATP, MgCl₂ and NaCl as indicated. Then ligation reaction was usually carried out at 20°C for 30 min and terminated by addition of an equal volume of 60 mM EDTA and 0.1% SDS. Mixtures were then subjected to electrophoresis on 1.2% agarose gel, and the ligation efficiency was determined by gel scanning using an Eagle Imaging system.

Phylogenetic Analysis—Nucleotide sequences of ATP-dependent DNA ligases were downloaded from GenBank (NCBI, USA) and sequence alignment was performed using *ClustalX* 1.5b (12). Phylogenetic tree was constructed with *Phylip* 3.6 (Joseph Felsenstein, University of Washington).

RESULTS

Expression and Purification of T3 DNA Ligase—The gene encoding T3 DNA ligase was amplified by PCR with a pair of primers designed according to the nucleotide sequence registered in GenBank. His-tag was fused to the N-terminus of T3 DNA ligase in pET-32a vector. The resultant construct pET-32a-T3 was transformed into *E. coli* strain BL21 (DE3) for expression. Although cells cultured at 37°C and induced with 1 mM IPTG had higher productivity of T3 DNA ligase, the large body of the ligase formed inclusion bodies and no enzymatic activity was detected. On the contrary, cells grown at 22°C produced T3 DNA ligase in a soluble form, which accounted for more than 30% of the total soluble proteins (Fig. 1 A, lane 1). The His-tagged T3 DNA ligase was purified by chelating chromatography to near homogeneity (Fig. 1 A, lane 4).

Ligation Efficiency for Cohesive DNA—The enzymatic activity of His-tagged T3 DNA ligase purified by chelating chromatography from cell lysate was measured with *Hind*III-linearized pBluescript II SK (+) DNA as substrate. Different amounts of T3 DNA ligase, varying from 0 to 2.5 fmol were added to 10 µl of reaction mixture containing 450 fmol *Hind*III-linearized pBluescript II SK (+) DNA and incubated at 20°C for 30 min. Gel electrophoresis showed that 1 fmol of T3 DNA ligase could achieve 90% ligation of DNA with cohesive ends (Fig. 1B). To achieve 90% ligation, 6 fmol of T4 DNA ligase (New England Bio-labs, USA) was needed in parallel reactions

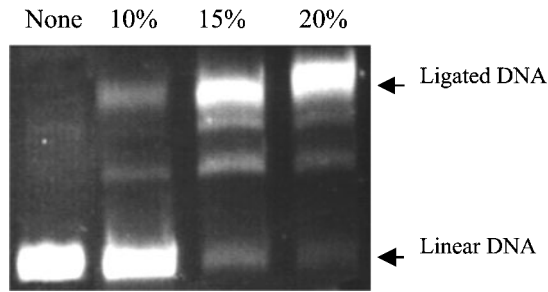


Fig. 2. **Effect of PEG on blunt-ended DNA ligation.** Reaction mixture contained 450 fmol pBluescript II SK DNA linearized with EcoR V, 66 mM Tris-HCl (pH 8.0), 5 mM DTT, 300 mM NaCl, 1 mM MgCl₂, 0.5 mM ATP, 1 fmol T3 DNA ligase and PEG 6000 as indicated. Reaction was performed at 20°C for 30 min and terminated by the addition of 60 mM EDTA and 0.1% SDS. The reaction products were analyzed by scanning the gels.

under the conditions recommended by the T4 ligase provider. Therefore, for cohesive end ligation, T3 DNA ligase is more efficient than T4 DNA ligase.

Ligation Efficiency for Blunt-Ended DNA—To investigate the ligation efficiency for blunt-ended DNA substrates, restriction endonucleases *Sma*I and *Eco*RV were used to digest pBluescript II SK (+) to generate G/C matched and A/T matched blunt ends, respectively. Ligation assay showed that T3 DNA ligase had little activity to join blunt-ended DNA, since no ligation was observed. However, in the presence of 15% PEG 6000, about 60–70% of the linear DNA could be ligated within 30 min (Fig. 2). This characteristic is quite similar to that of T7 DNA ligase. On the other hand, T4 DNA ligase has the capacity to ligate blunt-ended DNA without the aid of PEG 6000, although the addition of 10% PEG6000 can increase ligation efficiency greatly (4, 7). T3 DNA ligase was also found to have a higher efficiency in joining A/T matched blunt-ended DNA than G/C matched ends (data not shown).

Effect of Temperature on Enzymatic Activity—T3 DNA ligase was relatively insensitive to reaction temperature in the range of 16–37°C. In a 30-min reaction at 20°C, T3 DNA ligase produced the maximum amount of joined DNA (Fig. 3A). Extended incubation (over night) at lower

temperature (16°C) sealed almost all linear DNA fragments (data not shown), but enzymatic activity decreased drastically at temperatures higher than 45°C. The time course of ligase activity assay at 20°C (Fig. 3B) revealed that T3 DNA ligase could ligate up to 90% of cohesive DNA within 30 min.

Effect of Ion Concentration on Enzymatic Activity—With the supplement of 1 mM MgCl₂, the effect of different concentrations of NaCl on enzymatic activity of T3 DNA ligase was investigated. The results revealed that low concentrations of NaCl could promote the enzyme activity significantly, and the optimal concentration of NaCl for T3 DNA ligase was 300 mM. Surprisingly, the ligase still kept about 95% activity in the presence of 1 M NaCl (Fig. 4A). Potassium chloride had a similar effect on T3 DNA ligase (not shown). This finding is interesting, as most ligases from other sources are sensitive to high concentration of monovalent ions. The activity of the ligase from *Methanobacterium thermoautotophium* reduced dramatically when NaCl concentration rose above 10 mM (13), and *Saccharomyces cerevisiae* Cdc9p DNA ligase was even more sensitive to ions (14). T4 DNA ligase lost 70% activity in the presence of 300 mM NaCl (4).

The effect of several bivalent ions was also investigated in ligation reaction containing 300 mM NaCl (Fig. 4C). The presence of 5 mM Mg²⁺ or Ca²⁺ increased the activity 9-fold, while 5mM Mn²⁺ increased the activity 7-fold, compared to that without the ions. CaCl₂ concentration gradient assay (from 0 to 10 mM) showed that the enzyme reached the highest activity in the mixture containing 1 mM CaCl₂ (Fig. 4 B). MgCl₂ had similar effect on T3 ligase activity. T4 DNA ligase is also most active in the presence of 1 mM MgCl₂ (4).

Effect of pH on Enzymatic Activity—Although the isoelectric point of T3 DNA ligase calculated from its amino acid sequence is 5.14, ligation reactions showed that the T3 DNA ligase was most active at pH 8.0. This is quite close to the optimum pH of T4 DNA ligase (pH 7.2–7.8) and T7 DNA ligase (pH 7.2–7.7). The T3 DNA ligase retained about 80% of its activity at pH 6.5 and pH 8.5 (Fig. 4 D).

Specificity of Nucleotide Cofactors—T7 DNA ligase is ATP-dependent; most activity is achieved in the presence of 0.5 mM ATP (7). To define the specificity of T3 DNA

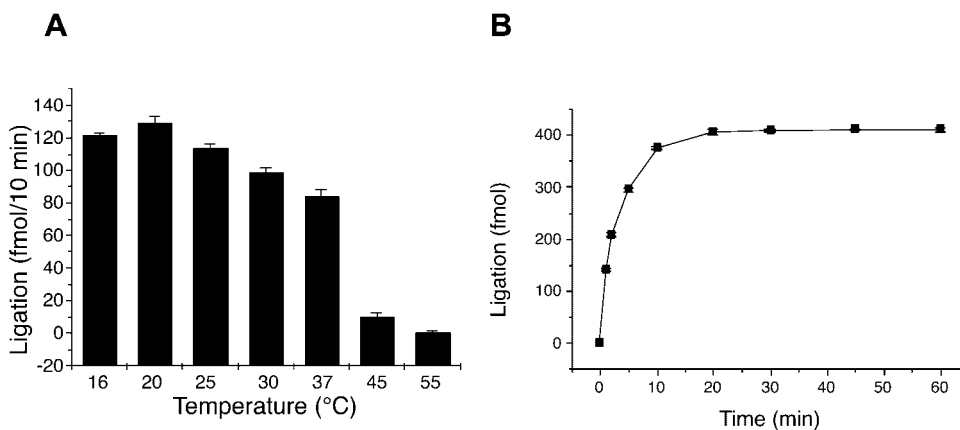


Fig. 3. **Temperature effect and time course.** (A) Effect of reaction temperature on ligation. Reaction mixture containing 450 fmol *Hin*dIII-linearized pBluescript II SK (+) DNA, 66 mM Tris-HCl (pH 8.0), 5 mM DTT, 1 fmol T3 DNA ligase, 300 mM NaCl, 1 mM MgCl₂ and 0.5 mM ATP was incubated for 30 min at different temperatures as indicated, and the reaction was terminated by the addition of 60 mM EDTA and 0.1% SDS. The ligation products were measured by gel scanning. (B) Ligation time course. A standard reaction mixture was incubated at 20°C for different times as indicated. The ligation was measured by gel scanning.

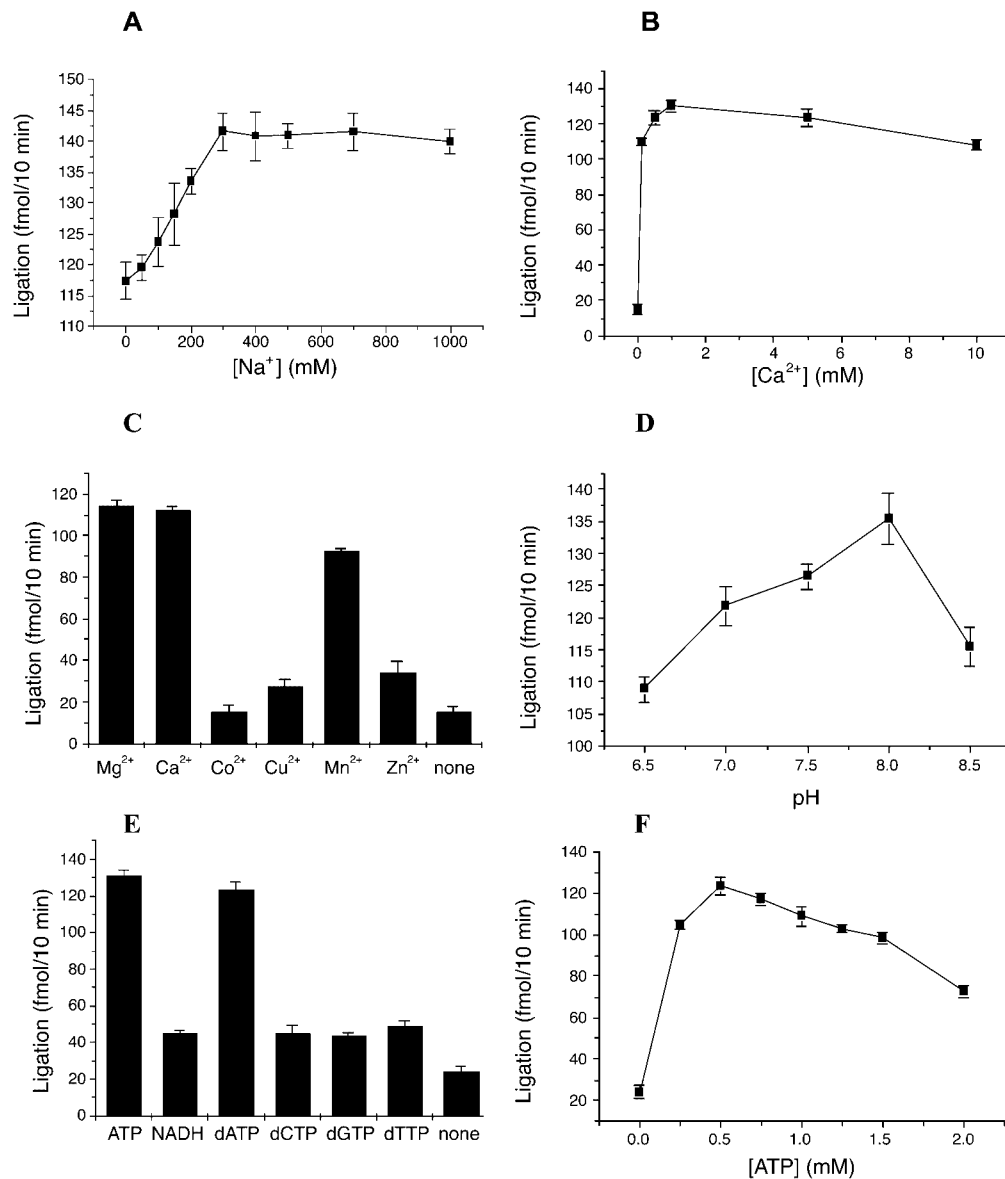


Fig. 4. Effect of metal ions, pH and nucleotide cofactors. Reaction mixture contained 450 fmol *Hind*III-linearized pBluescript II SK (+) DNA, 66 mM Tris-HCl (pH 8.0 unless otherwise indicated), 5 mM DTT, 1 fmol T3 DNA ligase and additional ingredients as indicated. The ligation reaction was performed at 20°C for 30 min. The extent of ligation was plotted as a function of sodium concentration in the presence of 0.5 mM ATP and 1 mM MgCl₂ (panel A) and calcium concentration in the presence of 0.5 mM ATP and 300 mM NaCl (panel B). Panels (C) and (E) show the ligase activity in the

mixture containing 0.5 mM ATP, 300 mM NaCl and 5 mM bivalent metal ions as indicated, and that in the mixture containing 300 mM NaCl, 1 mM MgCl₂ and 0.5 mM dNTPs or NADH as indicated, respectively. Ion and nucleotide were omitted from the corresponding control reaction (none). Panels (D) and (F) show the ligation extent plotted as a function of pH (mixture contained 0.5 mM ATP, 300 mM NaCl and 1 mM MgCl₂) and ATP concentration (reaction mixture contained 300 mM NaCl and 1 mM MgCl₂), respectively.

ligase to nucleotide cofactors, 0.5 mM ATP, dATP, dCTP, dGTP, dTTP or NADH was added to reaction mixtures, and ligation efficiencies were measured. As shown in Fig. 4E, T3 DNA ligase is an ATP-dependent enzyme, as the enzyme could be activated completely only in solution with ATP or dATP. The enzymatic activity of T3 DNA ligase increases with ATP concentration; it reaches the highest level at 0.5 mM, and then falls as the concentration goes higher (Fig. 4F). NADH, dCTP, dGTP and dTTP have slight stimulatory effect (close to 2-fold) compared to the non-ATP ligation.

Different concentrations of ATP were used as substrates in the activity assay in which all other components were included as needed, and the reaction was stopped at different time points by the addition of 60 mM EDTA and 0.1% SDS. Double reciprocal plotting revealed that K_m^{ATP} of T3 DNA ligase in the ligation of cohesive DNA is 14.4 μM (data not shown).

DISCUSSION

This is the first report about the characterization of bacteriophage T3 DNA ligase. T3 and T7 DNA ligases show

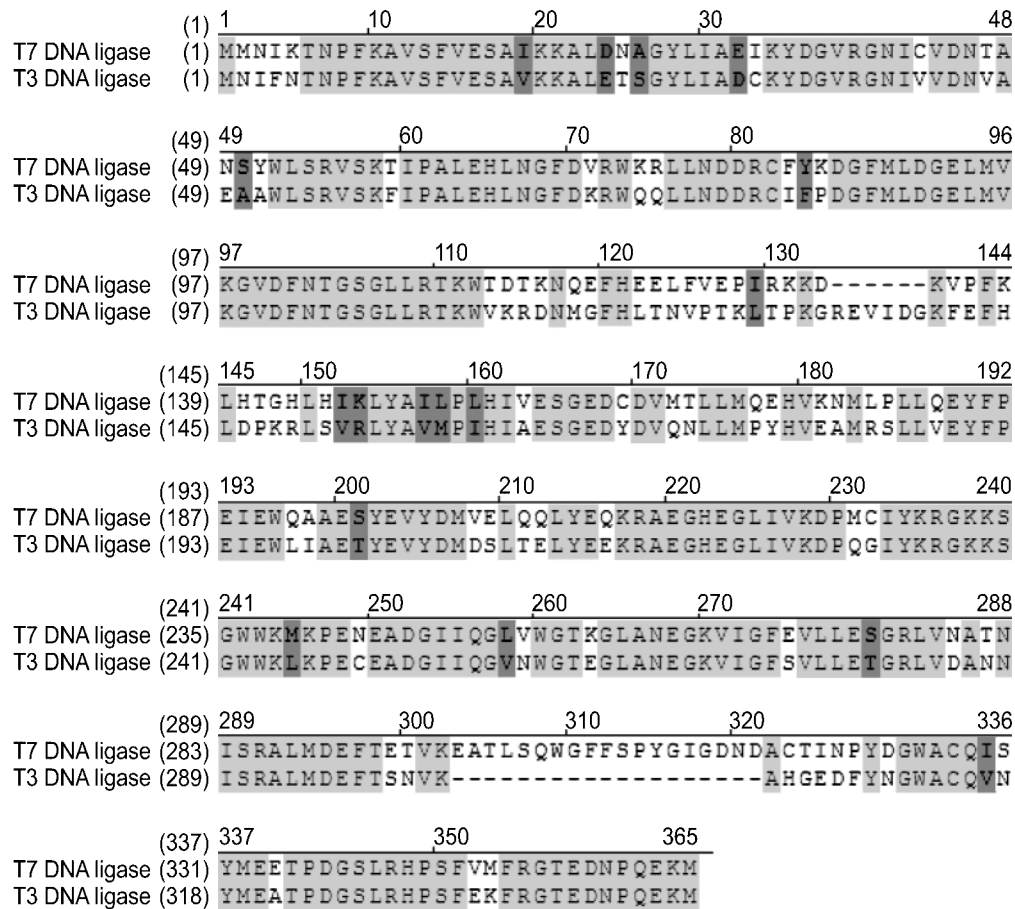


Fig. 5. Alignment of putative amino acid sequences of T3 and T7 DNA ligases. Amino acid sequences were obtained from GeneBank with accession number NP_523305.1(T3 DNA ligase) and

NP_041963.1 (T7 DNA ligase). Identical residues are shaded with yellow and similar residues with green. The alignment was performed with the *AlgnX* program in *Vector NTI* Suite 8 (InfoMax[®]).

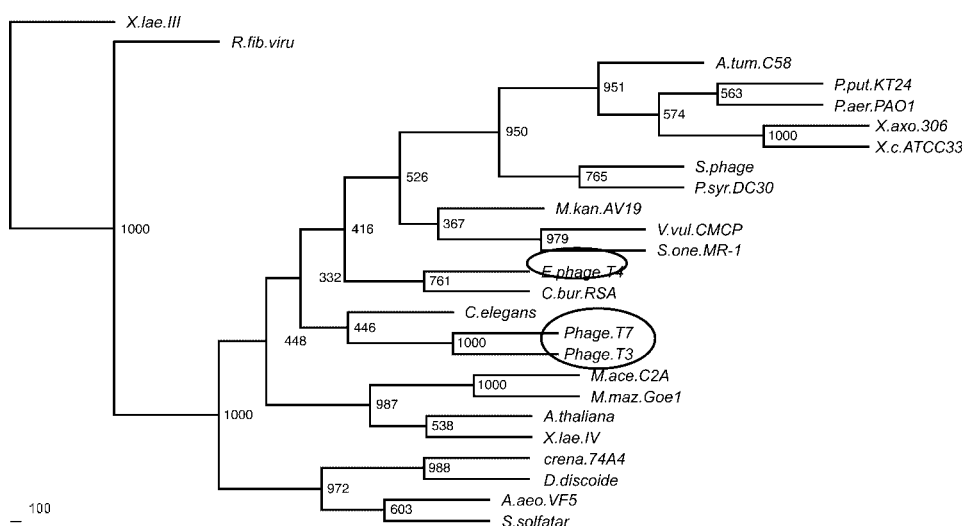
many similarities in biochemical properties. Both of them are monomeric enzymes (7); their optimal pHs are in the range 7.2–7.7 (7, 9); both require monocations (K^+ or Na^+) and bivalent cations (Mg^{2+} or Ca^{2+}) for in vitro ligation (7); and both can ligate cohesive DNA fragments at a similar rate to T4 DNA ligase. Neither can join blunt-ended fragments efficiently, except when polyethylene glycol exists in the reaction mixture (7). However, although 15% PEG 6000 enhances the ligation efficiency for blunt-ended DNA, neither T3 nor T7 DNA ligase would be a good alternative for in vitro DNA cloning, as the presence of a high concentration of PEG 6000 would greatly reduce the efficiency of the following transformation.

The alignment of amino acid sequences of T3 and T7 DNA ligases reveals 70% identity (Fig. 5). T3 ligase appears to have all the conserved motifs that are known to form the ATP-binding pocket and the groove running between the two domains in T7 DNA ligase (8). This finding suggests that T3 DNA ligase may be ATP-dependent, as is T7 DNA ligase. Our experiment demonstrated that ATP is indeed required for the ligase, as little ligation was found in the absence of ATP. However, it also has been observed that both T7 and T4 ligases can efficiently ligate DNA containing cohesive ends or nicked sites in the absence of ATP (7). One reason for this was proposed to be that a great proportion of the enzyme preparation

was in the adenylated form and the AMP adduct contributed to the following ligation reaction (7).

ATP may act a substrate in ligation reaction and follow similar mechanism as T7 DNA ligase. An interesting biochemical feature of T3 ligase, however, is that it displays the greatest activity at 0.5 mM of substrate ATP, and then the activity falls off, while T7 ligase retains a high level of activity at ATP concentration as high as 10 mM. The reason for this difference is not clear at present.

T3 DNA ligase can utilize ATP or dATP more efficiently than other nucleotides as its cofactors. It was suggested the ATP specificity could be explained by the interaction of 6-aminogroup of the adenine ring with the main-chain carbonyl of Ile-33 and the side chain of Glu-32 in T7 DNA ligase (8). In T3 DNA ligase, the amino acids at these two positions are Cys-33 and Asp-32, and the adjacent amino acid residues are highly conserved. At biological pH, aspartic acid and glutamic acid are negatively charged, and cysteine is a polar amino acid with higher hydrophilicity than the non-polar amino acid isoleucine. If T3 ligase has the same common fold as T7 ligase, a change in the amino acids that have been shown to cluster around the ATP-binding site in T7 ligase might cause it to have a differentiated structure, and this might help to explain why a high concentration of ATP has an inhibitory effect on T3 ligase but not on T7 ligase. How-



rcina acetivorans C2A); *M.maz.Goe1* (*Methanosarcina mazei* Goe1); *P.aer.PAO1* (*Pseudomonas aeruginosa* PAO1); *P.put.KT24* (*Pseudomonas putida* KT2440); *P.syr.DC30* (*Pseudomonas syringae* pv tomato str. DC3000); *R.fib.viru* (*Rabbit fibroma virus*); *S.one.MR-1* (*Shewanella oneidensis* MR-1); *S.phage* (*Sinorhizobium meliloti* phage PBC5); *S.solfatar* (*Sulfolobus solfataricus*); *V.vul.CMCP* (*Vibrio vulnificus* CMCP6); *X.axo.306* (*Xanthomonas axonopodis* pv. citri str. 306); *X.c.ATCC33* (*Xanthomonas campestris* pv. campestris str. ATCC33913); *X.lae.III* (*Xenopus laevis* III); *X.lae.IV* (*Xenopus laevis* IV); *crena.74A4* (*crenarchaeote* 74A4). The bacteriophage T3, T4 and T7 DNA ligases are circled.

ever, the exact mechanism involved awaits a structural study.

T3 DNA ligase also shows a considerable variation from T7 ligase in amino acids. Amino acid residues 122–142 and the carboxyl termini of dozens of amino acids are the two major sequences that are different between the T3 and T7 ligase.

DNA ligase plays an important role in DNA replication and repair system in cells. The unique property of T3 DNA ligase leads us to speculate that it may have followed a different evolution path from T4 and T7 DNA ligases. Here, we have constructed a NJ phylogenetic tree based on ATP-dependent DNA ligase DNA sequences available in *GenBank*. The phylogenetic tree containing bootstrap results could reveal some aspects of evolution.

Based on the phylogenetic tree of ATP-dependent DNA ligase sequences (Fig. 6), bacteriophage T3 and T7 are grouped together with 100% reliability (15). As they are classified into the same taxonomic family of *Pedoviridae*, it is thought that the evolution of the ligases has the same process as their organisms. Even if the ligase was admitted at a later stage from another organism, the invasion of ligase must have happened before the branching of *Pedoviridae*. Second, *Enterobacteria phage* T4 is positioned as an outgroup compared with bacteriophage T3 and T7, suggesting that *Enterobacteria phage* T4 has a different evolutionary process from bacteriophage T3. This might confer ligases with different characteristics, possibly those of ligating blunt-ended DNA. Various environmental selective pressures or different physiological contexts of hosts might lead to the attenuation of T3 DNA ligase blunt-ended DNA ligation activity. Third, *Rabbit fibroma* virus is positioned very close to the root of the tree, which reminds us that the ATP-dependent DNA ligase might be of viral origin. Fourth, DNA ligase III in *Xenopus laevis*, an amphibian model organism, is located at the root of the tree, while DNA ligase IV in *Xenopus*

laevis is a leaf of the tree. This discrepancy shows a picture in which DNA ligase IV in *Xenopus laevis* co-evolves with the organism, while DNA ligase III, only seen in mitochondria (16), is the result of virus ligase invading at a later stage of evolution. Furthermore, the “host” *Xenopus laevis* might give the DNA ligase III more protection than a virus against high frequency mutation, allowing it to retain its original appearance of an ATP-dependent DNA ligase.

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Fig. 6. Phylogenetic tree of ATP-dependent DNA ligases. The NJ phylogenetic tree was constructed with *Phylip* 3.6 (Joseph Felsenstein, University of Washington). Bootstrap values (1,000 replications) were indicated at nodes. All DNA sequences of ATP-dependent DNA ligases are obtained from *GenBank*. The full names of the hosts of DNA sequences are indicated in brackets. *A.tum.C58* (*Agrobacterium tumefaciens* str. C58); *A.aeo.VF5* (*Aquifex aeolicus* VF5); *A.thaliana* (*Arabidopsis thaliana*); Phage T3 (Bacteriophage T3); Phage T7 (Bacteriophage T7); *C.elegans* (*Caenorhabditis elegans*); *C.bur.RSA* (*Coxiella burnetii* RSA 493); *D.discoide* (*Dictyostelium discoideum*); *E.phage.T4* (*Enterobacteria phage* T4); *M.kan.AV19* (*Methanosarcina kandleri* AV19); *M.ace.C2A* (*Methanosarcina*

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