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Biochemical and Enzymatic Characterization of a Thermostable DNA Ligase Encoded by Thermophilic Acidophilic Archaebacterium Strain JP2*

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Abstract A thermostable DNA ligase gene was identified, and the biochemical and enzymatic properties of the ligase were characterized from JP2 strain which was enriched from geothermally active sites in Papua New Guinea. The nucleotide and amino acid sequences showed much high identities compared with that of archeabacterium species *Sulfolobus solfataricus* and *Sulfolobus shibatae*, especially in the six conserved motif sequences, which are known to be closely related to the key function of ligase. Recombinant JP2 ligase showed high activity in nick-joining reaction. It was the most active when Mn^{2+} present as divalent metal cofactor rather than Mg^{2+} and Ca^{2+} *etc*.. Assay of thermostability over a range of temperatures showed that at 50～80℃ the enzyme displayed relative high activity. Further thermostability experiment indicated that the activity of JP2 ligase could last for a long time at 80℃ and 85℃, however, at 90℃ and 95℃, it became unstable quickly. An investigation on the acquired thermotolerance of recombinant JP2 ligase was done by applying a chaperonin known as TF55 in thermophile on JP2 ligase reaction. Result showed that TF55 could not help in improving thermostability of ligase at 85℃. The possible reason might be that at 85℃ *in vitro*, the chaperonin itself was denatured.

Key words DNA ligase, *Sulfolobus spp*., thermophilic acidophilic archaebacterium, thermotolerance, molecular chaperone TF55

DNA ligase is an essential component in DNA replication, recombination and repair systems, it catalyses the formation of phosphodiester bond between adjacent 3′-hydroxyl and 5′-phosphoryl groups at a single-stranded break in double-stranded DNA[1]. There are three sequential reactions involved in this process: first, ligase is activated through the covalent addition of AMP to the conserved active site lysine from ATP or NAD^+; then AMP is transfer to the 5′-phosphoryl group of the nick on the DNA strand, finally the nick is sealed upon the release of AMP from the adenylated DNA intermediate[2].

DNA ligases can usually be classified into two groups: ATP-dependent and NAD^+-dependent ligases, based on cofactor requirement. ATP-dependent ligases are found predominantly in the eukarya and archaea whereas NAD^+-dependent DNA ligases are found only in the eubacteria and in entomopoxviruses[3]. However, some simple eubacteria and archaeon seem to encode both group of ligases, while some higher eukaryotes may employ several ATP-dependent ligases to carry out diverse biological functions[3～5]. Although they share very limited homology with sequence, they have good structural similarity over the six conserved motifs (Ⅰ, Ⅲ, Ⅲa, Ⅳ, Ⅴ and Ⅵ), which play key roles in nucleotide binding, nick recognition and nucleotidyl transfer[6,7], this suggests both ATP-dependent and NAD^+-dependent DNA ligases may catalyze their reactions through a common mechanism.

Archaea belong to the third kingdom of life. From the biochemical and genetic analyses of various DNA ligases, archaea have been shown to be similar to eukarya in many aspects of DNA metabolism despite their morphological and structural resemblance to

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bacteria[8,9]. There are many of the archaeal ligase genes being identified in recent years, which have been proved to be closely related to DNA ligase Ⅰ from eukarya[10]. Up to now, there have at least five archaeal DNA ligases being biochemically characterized. They are from *Methanobacterium thermoautotrophicum* [11], *Thermococcus kodakaraensi*[10], *Sulfolobus shibatae*[12], *Pyrococcus horikoshii* [13], and the latest report species——*Haloferax volcanii* [3], they are all ATP-dependent ligase, and have some similarities in basic enzymatic and biochemical properties. Interestingly, in *H. volcanii*, two classes of DNA ligases- ATP-dependent DNA ligase and NAD^+-dependent DNA ligase which was firstly identified in archaea were found to be co-opted in functioning.

In present study, we identified a ligase gene from a hyperthermoacidophilic archaeal strain——JP2 which was enriched from samples collected from geothermally active sites in Papua New Guinea. To understand the biochemical and enzymatic properties, JP2 ligase gene was cloned, overexpressed, and the enzymatic characteristics have been studied. For investigation of the acquired thermotolerance of JP2 ligase, an experiment with molecular chaperone——TF55 protein applying on JP2 ligase reaction at high temperature was also presented.

1 Materials and methods

1.1 Genomic DNA Extraction of JP2 strain

Thermophilic archaeal strain JP2 was grown in a basal nutrient medium as described in Plumb *et al.* (2002) [14] at 75℃ for 3 days, and harvested by centrifugation at 4 000 *g* for 15 min. Genomic DNA was extracted according to Ramakrishnan *et al.* (1995)[15].

1.2 Isolation and sequence analysis of JP2 ligase gene

Based on the genomic and ligase gene sequence of *Sulfolobus solfataricus* (P2), two primers were designed from immediate outside of the ORF sequence of the P2 ligase gene: 5′ TAATTGCCGAGTATTC-ATCATCC 3′ / 5′ TTTACAAGATTCTGCACATT-AATACC 3′. PCR was performed with JP2 genomic DNA as template. A 2.0 kb fragment was purified from PCR products and sequenced. Based on both ends of putative ORF sequence of JP2 ligase gene, another two new primers were designed to complete the whole sequence of JP2 ligase gene from genomic DNA: *Mlu* Ⅰ site was introduced at 5′ end primer (in bold and underline), 5′ CGC**ACGCGT**ATGGAG-TTTAAAGTT ATTGCCGAG, and *Xho* Ⅰ at 3′ end primer (in bold and underline), 5′ GCG**CTCGAG**-GCTCCG ATATATTTTAAACG CTCT. PCR product was cleaved and inserted into cloning vector for sequencing.

1.3 Expression of recombinant JP2 ligase gene

A 1.8 kb DNA fragment was cleaved with *Mlu* Ⅰ and *Xho* Ⅰ from the clone vector, then inserted into the *Mlu* Ⅰ -*Xho* Ⅰ site of pETBlue-2 vector (Novagen, Madison, WI, USA), which was a T7 RNA polymerase-based prokaryotic expression vector, and had an optional C-terminal His6-Tag sequence, it can be induced to express in host strain Tuner(DE3) pLac Ⅰ (Novagen, Madison, WI, USA) with isopropyl-D-thiogalacto-pyranoside (IPTG). The pETBlue-*lig* recombinant plasmid was transferred into Tuner (DE3) pLacI, the transformant was grown at 37℃ in LB medium containing 50 mg/L ampicillin and 34 mg/L chloramphenical till A_{550}=0.5. IPTG was added to a final concentration of 1 mmol/L, and continued incubation for 3 h to induce ligase expression.

1.4 Purification of recombinant JP2 ligase

Well-induced cells harbouring with the recombinant JP2 ligase gene were harvested by centrifugation (5 000 *g*, 10 min, 4℃), washed one time and then resuspended with lysis buffer (pH 8.0) containing 50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 10 mmol/L imidazole. Cells were lysed by sonication till the slurry became clear, and cell debris was removed by centrifugation (13 000 *g*, 30 min, 4℃). The supernatant was heat-treated at 80℃ for 20 min, and precipitation was removed at 13 000 *g* 4℃ for 45 min. Added 1/4 sample volume of 50% Ni-NTA resin slurry (Qiagen, Clifton Hill, Victoria, Australia) to the clarified lysate and mixed gently by shaking (200 r/min on a rotary shaker) at 4℃ for 60 min, then loaded the lysate-Ni-NTA mixture into a column, washed the column twice with 1 sample volume of wash buffer pH 8.0 (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 20 mmol/L imidazole), then eluted the protein 4 times with a total of 1/2 sample volume of elution buffer (50 mmol/L NaH_2PO_4, 300 mmol/L NaCl, 250 mmol/L imidazole, pH 8.0), pooled the eluate and dialyzed at 4℃ against buffer containing 50 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol for overnight. Concentrated the dialyzed enzyme solution with Amicon Centricon Concentrator (cut-off *M* 30 ku) (Millipore, Danvers, MA, USA) to a

final volume of 100 μl, determined the enzyme concentration by comparison with concentration-known bovine serum albumin (BSA), then added 40% of glycerol and 100 mg BSA/ml to the enzyme, stored at −20℃.

1.5 Preparation of crude recombinant JP2 ligase

Appropriate amount of well-induced cells of the recombinant JP2 ligase overproducer were harvested by centrifugation (5 000 *g*, 10 min, 4℃), washed with buffer containing 50 mmol/L Tris-HCl, pH 8.0, 0.1 mmol/L EDTA, 1 mmol/L DTT, then resuspended completely. The cells were sonicated till the slurry become clear, and debris was removed by centrifugation (13 000 *g*, 30 min, 4℃). The supernatant was heat-treated at 80℃ for 20 min, and removed precipitation at 13 000 *g* 4℃ for 60 min, saved the supernatant on ice for ligase activity assay.

1.6 Ligase substrates

Three short oligonucleotides were synthesized for ligase activity assay. Donor strand was 42 bp (5′ TCCGCGGATCCTGAGGTGAAATGTAAATGAAA AAGCCTGAAC 3′), acceptor strand was 38 bp (5′ CGTCGAGCAGCGAACCTACTGCGTGGCTTCCG GAGCTA 3′), and the complement ary strand was 80 bp (5′ GTTCAGGCTTTTTCATTTACATTTCA-CCTCAGGATCCGCGGATAGCTCCGGAAGCCAC GCAGTAGGTTCGCTGCTCGACG 3′). The donor strand was labelled with [γ-^{32}P] ATP (Amersham Biosciences Pty Ltd, Castle Hill, NSW, Australia) by phosphorylation at its 5′ terminus, purified by ethanol precipitation [16]. Preparation single-nicked DNA by using plasmid pETBlue-2, the details described as previously[17].

1.7 Assay of ligase activity

(1) With nicked plasmid DNA: incubation JP2 ligase (crude enzyme, 10～20 ng) with single-nicked plasmid pETBlue-2 (300～500 ng) for 15min at 65℃ or specified temperatures in buffer (50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ATP, 10 mmol/L DTT, 25 μg BSA/ml, 10 mmol/L $MnCl_2$) (standard reaction) or specified divalent cations in a total volume of 20 μl. The ligation mixtures were subjected to electrophoresis through a 0.8% agarose gel containing 0.5 μg ethidium bromide/ml in 1×Tris-acetate-EDTA buffer, and the gel was photographed under UV light. (2) With [γ-^{32}P]ATP labelled DNA substrates: incubation JP2 ligase (crude enzyme, 10～20 ng) with 1 pmol ^{32}P-labelled 42-mer donor strand, 5 pmol 38-mer acceptor strand and 5 pmol 80-mer complementary strand in buffer (50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L ATP, 10 mmol/L DTT, 25 μg BSA/ml, 10 mmol/L $MnCl_2$) in a total volume of 20 μl. The reaction was performed for 15 min at 65℃, then stopped by addition of 20 μl loading buffer (94% formamide, 20 mmol/L EDTA, 0.01% bromophenol blue & xylene cyanol FF) and cooling on ice. Samples were heated at 99℃ for 3 min, then subjected to electrophoresis through a denatured 10% polyacrylamide-7 mol/L urea gel in 1 × Tris-borate-EDTA buffer. After unloaded, the gel was wrapped in a fresh film, pressed in a Phosphoimager (Molecular Dynamics, Sunnyvale, CA. USA) and stored for 2～3 h, then analysed.

1.8 Purification of JP2 chaperonin TF55 and assay the effect on JP2 ligase

To induce high expression of heat-shock protein TF55, JP2 cells were grown at 75℃ to A_{550}=0.5 as described previously[18,19], then shift the culture to 85℃ and keep growing for 2 h, finally at 90℃ for another 4 h. Cells were harvested by centrifugation at 5 000 *g*, 4℃ for 10 min. About 1.5 g wet cells from 1.0 L culture were resuspended in 5 ml buffer containing 50 mmol/L Tris-HCl, pH 8.0, 15 mmol/L $MgCl_2$, 1 mmol/L DTT. Cells were lysed at 0℃ by addition of Triton X-100 to a final concentration of 0.5%, and clarified by centrifugation (20 600 *g*, 4℃, 60 min), then concentrated the supernatant with Amicon Concentrator (cut-off *M* 30 ku) (Millipore, Danvers, MA, USA) to a final volume of 500 μl. The crude extract was loaded onto a Superose 6 column (Pharmacia Biotech; 1.5 cm × 30 cm) and eluted with buffer containing 10 mmol/L Tris-HCl, pH 8.4, 100 mmol/L NaCl. The fractions with chaperonin were pooled and concentrated again, then stored at 0℃. For testing the acquired thermotolerance of JP2 ligase when crude chaperonin was applied at high temperature, 500 ng crude ligase and 500 ng chaperonin crude fraction were incubated in buffer containing 35 mmol/L Tris-HCl, pH 8.0, 50 mmol/L ATP, 1 mmol/L KCl, 50 mmol/L $MgCl_2$ at specified temperatures and desired time periods, then took 10 μl of this mixture to perform ligase assay with labelled DNA substrate (as described in **Materials and methods 1.7**).

2 Results

2.1 Cloning and sequencing of JP2 ligase gene

In previous study, JP2 strain was identified as

being most closely related to *S. solfataricus* (known as P2) [14,20]. Based on the published whole genomic sequence of P2 and the open reading frame (ORF) sequence of DNA ligase, a ~1.8kb PCR product of JP2 was obtained. After sequencing, the 1.8 kb DNA fragment was inserted into a prokaryotic expression vector, T7 RNA polymerase-based pETBlue-2, in which his6-tag sequence is attached for protein purification.

After comparing the sequence of 1.8 kb PCR fragment with published DNA ligase gene in GenBank we found, putative JP2 ligase gene ORF had 1 806 base pair of nucleotides, and it corresponds to a polypeptide of 601 amino acids with calculated molecular mass of 67.573 ku and an isoelectric point of 5.86. Its amino acid sequence showed the highest identities with crenarchaeota species of *S. shibatae* and *S. solfataricus* as 98% and 96%, respectively. It displayed relatively lower homology with Euryarchqeota species of *Thermococcus kodakaraensis* KOD1 and *Methanobacterium thermoautotrophicum*, being 38% and 34%, respectively. When comparing with eukarya, eubacteria and viruses, it was more similar to human ligase Ⅰ (33%) than human ligase Ⅲ (24%), Vaccinia virus (24%) and phage T7 (13%). It has poor homology with *E. coli* (NAD^+-dependent DNA ligase) as 2% only[10].

The amino acid sequence analysis showed that JP2 ligase had six conserved motifs as all other ATP-dependent ligase, and they were completely identical with that of *S. shibatae* and *S. solfataricus* (Figure 1). These motifs were located at the C-terminal domain, which were the core parts responsible for the nick-joining activity. Although the complete protein sequence of Euryarchaeota have higher homology with Crenarchaeota, while on the motifs conservation, the Crenarchaeota is more similar to Eucarya, especially with motif Ⅴ and Ⅵ.

	Motif Ⅰ		Motif Ⅲ		Motif Ⅲa		Motif Ⅳ		Motif Ⅴ		Motif Ⅵ	
JP2	259-	KYDG	-41-	FIIEGEIV	-33-	NVFLFDLMYYED	-51-	EGVMV	-17-	WIKLKRDYQSE	-118-	PRFIRWRDDK	-32
Ssh	259-	KYDG	-41-	FIIEGEIV	-33-	NVFLFDLMYYED	-51-	EGVMV	-17-	WIKLKRDYQSE	-118-	PRFIRWRDDK	-32
P2	259-	KYDG	-41-	FIIEGEIV	-33-	NVFLFDLMYYED	-51-	EGVMV	-17-	WIKLKRDYQSE	-118-	PRFIRWRDDK	-32
Mth	250-	KYDG	-41-	YIVEGEII	-32-	SLFLFDVLYHR-	-54-	EGIMI	-15-	MLKFK-----A	-101-	PVVKRIRDDL	-24
Tko	251-	KYDG	-41-	AIVEGELV	-33-	ELNLFDVMFVDG	-51-	EGLMA	-15-	WLKIK-----P	-101-	PRYVALREDK	-25
Hu1	567-	KYDG	-44-	FILDTEAV	-31-	CLYAFDLIYLNG	-52-	EGLMV	-17-	WLKLKKDYLDG	-120-	PRFIRVREDK	-37
Hu3	420-	KYDG	-43-	MILDSEVL	-27-	CLFVFDCIYFND	-52-	EGLVL	-15-	KVKKDYLNEGA	-119-	PRCTRIRDDK	-51
Vvi	230-	KYDG	-43-	IVLDSEIV	-26-	CLFVFDCLYFDG	-52-	EGLVL	-15-	KIKRDYLNEGS	-118-	PRFTRIREDK	-17
T7	75-	LLND	-38-	QEFHEELF	-25-	ILPLHIVESGED	-54-	EGLIV	-15-	WKMKP------	-101-	PSFVMFRGTE	- 7
Eco	322-	FVRD	-42-	LRIGDKVV	-32-	PVCGSDVERVEG	-65-	ERMGP	-17-	FARFLYALGIR	-117-	TDLVIAGEAA	-26

Fig. 1 Multiple peptide sequence alignment of six conserved motifs of DNA ligases from Crenarchaeota (JP2, Ssh, P2), Euryarchaeota (Mth, Tko), Eucarya (Hu1, Hu3), Virus (Vvi), Phage (T7) and *E. coli* (Eco)
This analysis was conducted using Clustal W[21]. Ssh: *Sulfolobus shibatae* (GenBank accession number: AAF61267); P2: *Sulfolobus solfataricus* (AAK40535); Mth: *Methanobacterium thermoautotrophicum* (U51624); Tko: *Thermococcus kodakaraensis* KOD1 (AB042527); Hu1: human DNA ligase Ⅰ (NP_000225); Hu3: Human ligase Ⅲ (P49916); Vvi: *Vaccinia virus* (P16272); T7: Phage T7 (P00969); Eco: *Escherichia coli* (P15042). Motif Ⅰ, Ⅲ, Ⅲa, Ⅳ, Ⅴ, Ⅵ show six conserved sequences which play important roles in ligase. The numbers separating the motifs indicate amino acid residues.

2.2 Overexpression of the recombinant JP2 ligase gene and protein purification

To understand the enzymatic properties of JP2 ligase, the putative JP2 ligase gene was expressed in prokaryotic expression vector-pETBlue-2. Cells harbouring pETBlue-2-*lig* plasmid were induced with IPTG to express recombinant ligase to a high level. After sonication, the expression proteins were purified successively as steps described in **Materials and methods**.

The protein expression profile showed that the target protein was expressed as expected, with the size of about 67 ku (Figure 2a), but the expression level was relatively low. After purification and concentration, two major bands were observed (Figure 2b), the size of band Ⅰ was about 67 ku, band Ⅱ was about 55 ku. It was not clear why two major bands were presented. According to previous report that Archael proteins showed aberrant migration rates on SDS-PAGE gels[22], sometime the protein was modified (such as cleavage) during denaturation with SDS and reducing agents [10], the 55 ku band might be the modified fragment of ligase, but the final conclusion should be made on the Western blot result.

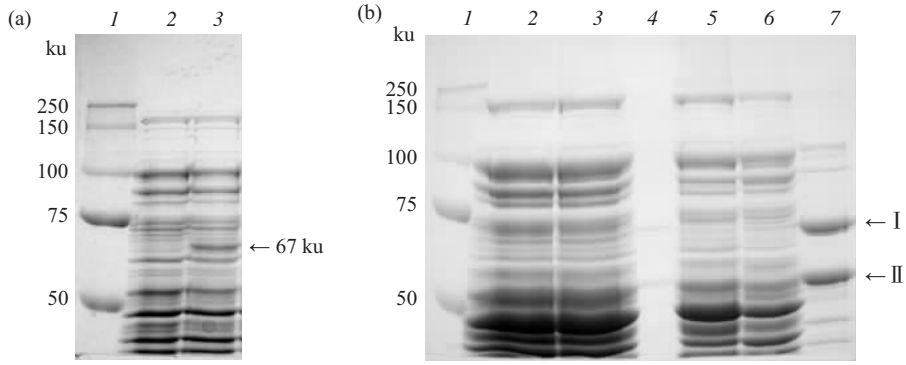

Fig. 2　JP2 ligase gene expression and protein purification

(a) Ligase gene expressed in pETBlue-2 vector. (b) Protein purification with heat treatment and Ni-NTA resin. *1*: Protein molecular mass standard; *2*: Extract of uninduced cells harbouring with recombinant plasmid; *3*: Extract of induced cells harbouring with recombinant plasmid; *4*: Supernatant of extract of induced cells after heat treatment at 80℃ for 20 min; *5*: Flowthrough of Ni-NTA resin after applying heat-treated sample; *6*: Washthrough of Ni-NTA resin after applying heat-treated sample; *7*: Concentrated eluate of Ni-NTA resin. Arrowheads in (a) show the expressed protein bands; Arrowheads in (b) show the two major protein bands after purification.

2.3　Catalytic properties of recombinant JP2 ligase

We employed single-nicked pETBlue-2 plasmid as substrate to examine the catalytic properties of JP2 recombinant ligase. Because the sealed double stranded plasmid would form supercoiled DNA and migrate faster than the nicked DNA in electrophoresis of agarose gel in the presence of the ethidium bromide, so when ligase functions properly, there will have a smaller band present. In this experiment, 10～20 ng of purified ligase was added into the mixture of single-nicked pETBlue-2 plasmid (500 ng) plus 1 mmol/L ATP and 10 mmol/L $MnCl_2$, after incubation at 65℃ for 30 min, we found nearly half amount of substrate converted to supercoiled DNA (Figure 3). This experiment indicated that recombinant JP2 ligase was properly expressed and showed ligase activity.

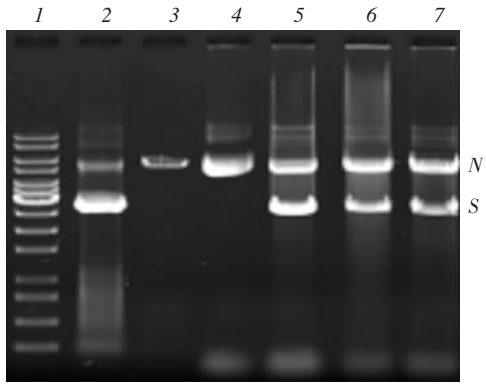

Fig. 3　Catalytic activity of JP2 recombinant ligase on the single-nicked plasmid DNA

Ligation was performed under different ligase concentrations. Reaction mixtures were subjected to agarose gel electrophoresis. *1*: DNA molecular mass standard (gene-ruler 1 kb ladder); *2*: pETBlue-2 plasmid DNA(500 ng); *3*: Linear pETBlue-2 DNA; *4*: Single-nicked pETBlue-2 plasmid; *5*: Single-nicked DNA (500 ng) incubated with T4 ligase (400 U) at ambient for 30 min; *6*, *7*: Single-nicked DNA (500 ng) incubated with JP2 ligase at 20 ng and 10 ng concentration, respectively. *N*: Nicked DNA; *S*: Supercoiled DNA.

2.4　Specificity of divalent cation cofactor

The ligation reaction was performed in the presence of various divalent cations to investigate the activity of recombinant JP2 ligase on the metal cofactor requirement. Results of nick-joining activity

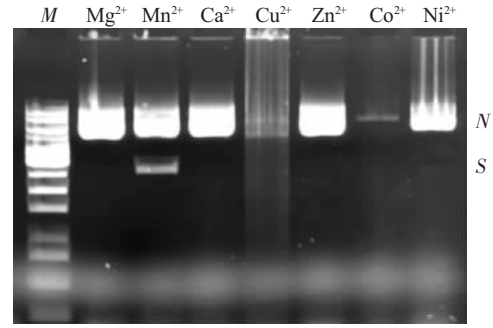

Fig. 4　Divalent cation cofactor specificity of recombinant JP2 liase

Ligation reaction was performed with recombinant JP2 ligase for 10 min at 65℃ with 10 mmol/L of $MgCl_2$, $MnCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, $ZnCl_2$ and $NiSO_4$. Reaction mixtures were resolved by agarose gel electrophoresis. The gel was photographed under UV light. Covalent cations were indicated above each lane. *M*: DNA molecular mass standard (Gene-ruler 1 kb ladder); *N*: Single-nicked plasmid; *S*: Supercoiled plasmid.

of electrophoresis in agarose gel indicated that JP2 ligase was active only when Mn^{2+} was present, with other cations, there had no supercoiled plasmid observed (Figure 4). However, comparing with assay of the radioactive isotope, the agarose gel had lower sensitivity, just from this experiment we could not tell if other cations such as Mg^{2+}, Ca^{2+} or Co^{2+} would in some extent satisfy recombinant JP2 ligase.

2.5 Effect of temperature on DNA ligation of recombinant JP2 ligase

When ligation reactions were performed at various temperatures, recombinant JP2 ligase showed distinctive activity over a range of temperature from 40℃ to 95℃, the optimal temperature was 60～70℃ (Figure 5). At 90℃ and 95℃, although the nicked substrate had already partially degraded, a band of supercoiled plasmid still could be observed.

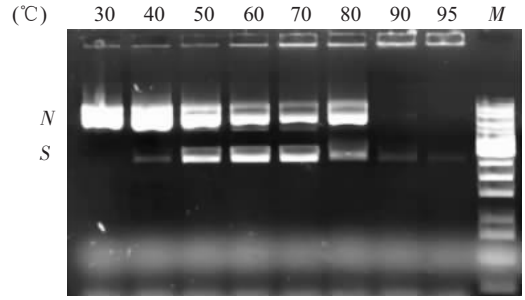

Fig. 5 Effect of temperature on activity of JP2 ligase

Ligation reaction using single-nicked plasmid with recombinant JP2 ligase for 10 min at various temperatures. Reaction mixtures were subjected to agarose gel electrophoresis. Temperatures were indicated above each lane; *M*: DNA molecular mass standard (Gene-ruler 1 kb ladder); *N*: Single-nicked plasmid; *S*: Supercoiled plasmid.

Based on these results, the thermostability of the recombinant JP2 ligase was investigated. The lysate of cells with the recombinant JP2 ligase overproducer was incubated at 80℃, 85℃, 90℃ and 95℃ respectively for a certain period of time, then applied to the ligation reaction with [γ-^{32}P] ATP labelled DNA substrates. The principle of this assay is: the labelled 42-mer donor strand and the non-labelled 38-mer acceptor strand are complementary with the 80-mer non-labelled strand, if the ligase functions properly, the 42 bp DNA fragment and 38 bp fragment which combined on the 80 bp DNA fragment during annealing should be joined together and form an 80 bp labelled fragment, after denaturalized, an 80 bp band will be observed in addition to the 42 bp band on an X-ray film. Ligase activity (%) can be calculated as: (exposure intensity of 80 bp fragment divide by exposure intensity of 42 bp fragment of initial loading) multiply 100. Data showed that recombinant JP2 ligase activity decreased when incubation time increased (Figure 6), but it is fairly different at various temperatures. When enzyme was incubated at 80℃ and 85℃, 5 h later ligase still remained rather high activity. But when exceeded 90℃, especially at 95℃, the activity of ligase decreased very quick.

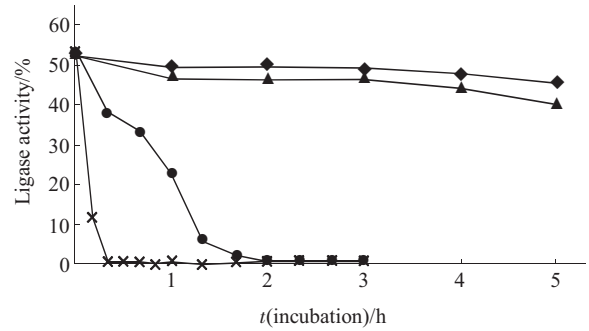

Fig. 6 Thermostability of recombinant JP2 ligase

The lysate of the cells harbouring with recombinant JP2 ligase gene was incubated at a specific temperature for a certain length of time, then the ligation activity of treated samples were assayed with labelled substrate as described in **Materials and methods**. ◆—◆: 80℃; ▲—▲: 85℃; ●—●: 90℃; ×—×: 95℃.

2.6 Effect of chaperonin (TF55) on the acquired thermotolerance of recombinant JP2 ligase

In order to investigate the thermotolerance of JP2 ligase at high temperature (more than 85℃) *in vitro*, we purified a heat shock protein [molecular chaperone, also called temperature factor 55 (TF55) in thermophile] [18,23] from JP2 and applied to crude

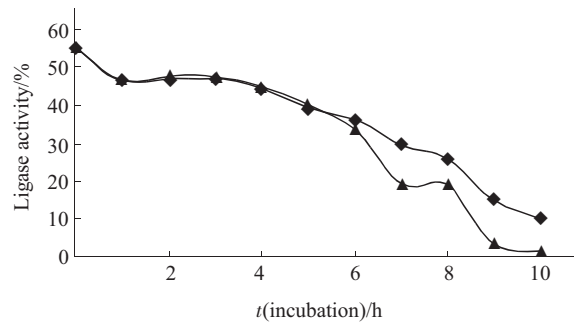

Fig. 7 Thermostability of recombinant JP2 ligase at 85℃ when applying JP2 chaperonin in the test

The lysate of the cells harbouring with recombinant JP2 ligase gene and the JP2 chaperonin gel filtration fraction were incubated at 85℃ for a certain length of time, then the ligation activity of treated samples were assayed with labelled substrate as described in **Materials and methods.** ◆—◆: Ligase; ▲—▲: Ligase+chaperonin.

preparation of recombinant JP2 ligase. Data suggested that JP2 chaperonin-TF55 seemed to have no effect on enhancement of the thermotolerance of ligase *in vitro* at 85℃ (Figure 7). Moreover, 6h later, ligation activity of the ligase without chaperonin seemed to be higher than the chaperonin treated ligase. Further experiments are necessary to demonstrate the details.

3 Discussion

We presented here the preliminary biochemical and enzymatic characterization of a thermostable ATP-dependent DNA ligase in a newly isolated archaebacterium strain from geothermally active sites in Papua New Guinea. It belongs to a kind of thermophilic acidophile which is able to degrade refractory mineral sulphides.

Based on previous study of the preliminary identification of the JP2 strain[14] and reported sequence of *S. solfataricus* [20], we got the ORF nucleotide and putative amino acid sequences of JP2, data showed very high identities among JP2, *S. solfataricus* and *S. shibatae*[12], especially on the conserved sequences of six motifs, which are known to be closely related to the key function of ligase.

The purified recombinant JP2 ligase showed high nick-sealing activity under optimal reaction conditions. But it is not clear yet that whether the ligase isolated in this study is the only ligase in JP2 or not, because a recent discovery from halophile *Haloferax volcanii* suggested that there are two classes of ligases (ATP-dependent DNA ligase-LigA and an NAD^+-dependent DNA ligase-LigN) co-existing in this archaeon, they are non-essential for cell viability separately, but simultaneous inactivation of both proteins is lethal indicating that they share an essential function. Perhaps the LigN protein may provide additional ligase activity under conditions of high genotoxic stress for LigA function[3].

Concerning on the divalent cation cofactor requirements, many ATP-dependent DNA ligases show highest activity with Mg^{2+}, some would prefer both Mg^{2+} and Mn^{2+}[11,24]. In our study, recombinant JP2 ligase showed much high activity when Mn^{2+} present rather than Mg^{2+} or other divalent cations, this property is similar with that of *S. shibatae*[12]. On the aspect of nucleotide cofactor, such as NAD^+, UTP, GTP, CTP, dATP, dTTP, dGTP and dCTP, when ATP was presented, it displayed high activity in nick-joining reaction, but the ligase did not show ligation activity when ATP was absent (data not shown). To our knowledge, so far one class of ligase usually prefer one kind of nucleotide as cofactor, except for *T. kodakaraensis* DNA ligase, but comparing with ATP, this ligase displayed very low activity when employed NAD^+ as the cofactor[10]. So JP2 ligase most likely belongs to the ATP-dependent DNA ligase.

JP2 strain was isolated from geothermally active sites, the optimal growth temperature is between 72～75℃, but it can function in a more extensive range of temperature. Based on the ligation assay of single-nicked plasmid, recombinant JP2 ligase displayed rather high activity at 50～80℃ *in vitro*, even at 95℃, it still formed a small amount of sealed supercoiled plasmid, although at the moment the single-nicked plasmid substrate had almost totally degraded. Further thermostability assay of recombinant JP2 ligase indicated, at 80℃ and 85℃, activity of JP2 ligase reduced not much within 5 h, but at 90℃ and 95℃, the activity decreased rapidly (Figure 6 and Figure 7).

With regard to the acquired thermotolerance of ligase under high temperature, it has been suggested that heat shock proteins in thermophile (temperature factor 55, TF55) [22,25] form a double-ring structure playing an essential role in helping damaged proteins refolding and recovering from heat-stress-related damages [26,27]. Generally, high thermal stability is a common feature of chaperonins. In present study, however, data showed that JP2 ligase with chaperonin applying at 85℃ did not improve the thermotolerance, by contraries, it made the ligase activity reduced. The probable reason might be, at 85℃ *in vitro*, the chaperonin itself was unstable[28].

References

1 Lehman I R. DNA ligase: structure, mechanism, and function. Science, 1974, **186** (4166): 790～797

2 Engler M J, Richardson C C. DNA ligases. In: Boyer PD ed. The Enzymes. **15**. New York: Academic, 1982. 3～29

3 Zhao A, Gray F C, MacNeill S A. ATP- and NAD^+-dependent DNA ligases share an essential function in the halophilic archaeon Haloferax volcanii. Mol Microbiol, 2006, **59** (3): 743～752

4 Deckert G, Warren P V, Gaasterland T, *et al*. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. Nature, 1998, **392** (6674): 353～358

5 Fleischmann R D, Adams M D, White O, *et al*. Whole-genome random sequencing and assembly of *Haemophilus* influenzae Rd. Science, 1995, **269** (5223): 496～512

6 Singleton M R, Hakansson K, Timson D J, *et al*. Structure of the

adenylation domain of an NAD+-dependent DNA lidase. Structure, 1999, **7** (1): 35～42

7 Timson D J, Singleton M R, Wigley D B. DNA ligases in the repair and replication of DNA. Mutat Res, 2000, **460** (3～4): 301～318

8 Edgell D R, Doolittle W F. Archaea and the origins of DNA replication proteins. Cell, 1997, **89** (7): 995～998

9 Olsen G J, Woese C R. Archaeal genomics: an overview. Cell, 1997, **89** (7): 991～994

10 Nakatani M, Ezaki S, Atomi H, *et al*. A DNA ligase from a hyperthermophilic archaeon with unique cofactor specificity. J Bacteriol, 2000, **182** (22): 6424～6433

11 Sriskanda V, Kelman Z, Hurwitz J, *et al*. Characterization of an ATP-dependent DNA ligase from the thermophilic archaeon Methanobacterium thermoautotrophicum. Nucleic Acids Res, 2000, **28** (11): 2221～2228

12 Lai X Q, Shao H B, Hao F Y, *et al*. Biochemical characterization of an ATP-dependent DNA ligase from the hyperthermophilic crenarchaeon *Sulfolobus shibatae*. Extremophiles, 2002, **6**(6): 469～477

13 Keppetipola N, Shuman S. Characterization of a thermophilic ATP-dependent DNA ligase from the euryarchaeon *Pyrococcus horikoshii*. J Bacteriol, 2005, **187** (20): 6902～6908

14 Plumb J J, Gibbs B, Stott M B, *et al*. Enrichment and characterisation of thermophilic acidophiles for the bioleaching of mineral sulphides. Minerals Engineering, 2002, **15** (11): 787～794

15 Ramakrishnan V, Adams M W W. Preparation of genomic DNA from sulfur-dependent hyperthermophilic archaea. In: Robb FT ed. Thermophiles (Archaea: A Laboratory Manual 3). New York: Cold Spring Harbour Laboratory Press, 1995. 95～96

16 Sambrook J, Fritsch E F, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd. New York: Cold Spring Harbor Laboratory Press, 1990. 284～286

17 Clark D J, Felsenfeld G. Formation of nucleosomes on positively supercoiled DNA. EMBO J, 1991, **10** (2): 387～395

18 Cerchia L, Rossi M, Guagliardi A. An archaeal chaperonin-based reactor for renaturation of denatured proteins. Extremophiles, 2000, **4** (1):1～7

19 Quaite-Randall E, Joachimiak A. Purification of chaperonins. J Chromatog Sect B, 1999, **722** (1～2): 153～177

20 She Q, Singh R K, Confalonieri F, *et al*. The complete genome of the crenarchaeon *Sulfolobus solfataricus* P2. Proc Natl Acad Sci USA, 2001, **98** (14): 7835～7840

21 Thompson J D, Higgins D G, Gibson T J. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res, 1994, **22** (22): 4673～4680

22 Ezaki S, Miyaoku K, Nishi K, *et al*. Gene analysis and enzymatic properties of thermostable β-glycosidase from *Pyrococcus kodakaraensis* KOD1. J Biosci Bioeng, 1999, **88** (2): 130～135

23 Trent J D. A review of acquired thermotolerance, heat shock proteins, and molecular chaperones in archaea. FEMS Microbiol Rev, 1996, **18** (2～3): 249～258

24 Tong J, Barany F, Cao W. Ligation reaction specificities of an NAD (+)-dependent DNA ligase from the hyperthermophile *Aquifex aeolicus*. Nucleic Acids Res, 2000, **28** (6): 1447～1454

25 Hartl F U, Hayer-Hartl M. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science, 2002, **295**(5561): 1852～1858

26 Peeples T L, Kelly R M. Bioenergetic response of the extreme thermoacidophile *Metallosphaera sedula* to thermal and nutritional stresses. Appl Environ Microbiol, 1995, **61** (6): 2314～2321

27 Trent J D, Kagawa H K, Yaoi T. The role of chaperonins *in vivo*: the next frontier. Ann NY Acad Sci, 1998, **841**: 42～54

28 Large A T, Kovacs E, Lund PA. Properties of the chaperonin complex from the halophilic archaeon *Haloferax volcanii*. FEBS Letters, 2002, **532** (3): 309～312

一种嗜热耐酸古细菌 JP2 菌株编码的热稳定 DNA 连接酶的生物化学及酶学特性研究 *

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摘要　对分离自巴布亚新几内亚地热活跃区的一种嗜热耐酸古细菌——JP2 菌株中的 DNA 连接酶基因进行了克隆、表达、纯化，并对其生物化学及酶学特性进行了研究. 对其核酸及氨基酸序列的分析表明：JP2 菌株的 DNA 连接酶与古细菌种 *Sulfolobus solfataricus* 和 *Sulfolobus shibatae* 的 DNA 连接酶具有很高的同源性，尤其在与功能紧密相关的 6 个保守结构基序的一致性更高. JP2 连接酶表现出高的 DNA 缺口连接活性，在二价金属辅因子的选择方面，JP2 连接酶更倾向于 Mn^{2+} 离子而不是 Mg^{2+}、Ca^{2+} 及其他离子. 不同温度时的热稳定性测试显示：JP2 连接酶在 50～80℃时为较适连接温度，当温度不超过 85℃时，连接酶的活性在 5 h 内保持相对稳定，但在 90℃以上活性则很快降低. 还分离纯化了 JP2 的分子伴侣——TF55，并将其应用于增加 JP2 连接酶的热稳定性研究. 结果表明：在体外 85℃时，分子伴侣未增加连接酶的热稳定性，可能的原因是在 85℃体外状态下 TF55 本身就表现出不稳定性.

关键词　DNA 连接酶，*Sulfolobus spp.*，嗜热耐酸古细菌，耐热性，分子伴侣 TF55
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