Extremophiles (2007) 11:675–684
DOI 10.1007/s00792-007-0083-0

ORIGINAL PAPER

A single amino acid substitution in the DNA-binding domain of *Aeropyrum pernix* DNA ligase impairs its interaction with proliferating cell nuclear antigen

Shinichi Kiyonari · Toru Kamigochi · Yoshizumi Ishino

Received: 22 January 2007 / Accepted: 9 April 2007 / Published online: 9 May 2007

Abstract Proliferating cell nuclear antigen (PCNA) is known as a DNA sliding clamp that acts as a platform for the assembly of enzymes involved in DNA replication and repair. Previously, it was reported that a crenarchaeal PCNA formed a heterotrimeric structure, and that each PCNA subunit has distinct binding specificity to PCNA-binding proteins. Here we describe the PCNA-binding properties of a DNA ligase from the hyperthermophilic crenarchaeon *Aeropyrum pernix* K1. Based on our findings on the *Pyrococcus furiosus* DNA ligase–PCNA interaction, we predicted that the aromatic residue, Phe132, in the DNA-binding domain of *A. pernix* DNA ligase (ApeLig) would play a critical role in binding to *A. pernix* PCNA (ApePCNA). Surface plasmon resonance analyses revealed that the ApeLig F132A mutant does not interact with an immobilized subunit of ApePCNA. Furthermore, we could not detect any stimulation of the ligation activity of the ApeLig F132A protein by ApePCNA in vitro. These results indicated that the phenylalanine, which is located in our predicted PCNA-binding region in ApeLig, has a critical role for the physical and functional interaction with ApePCNA.

Communicated by K. Horikoshi.

S. Kiyonari · T. Kamigochi · Y. Ishino (✉)
Department of Genetic Resources Technology,
Faculty of Agriculture, Kyushu University,
6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi,
Fukuoka 812-8581, Japan
e-mail: ishino@agr.kyushu-u.ac.jp

S. Kiyonari · T. Kamigochi · Y. Ishino
BIRD-Japan Science and Technology Agency,
6-10-1 Hakozaki, Higashi-ku, Fukuoka-shi,
Fukuoka 812-8581, Japan

Keywords Archaea · *Aeropyrum pernix* K1 · DNA ligase · Proliferating cell nuclear antigen · PIP box · DNA replication

Abbreviations

DBD	DNA-binding domain
DTT	Dithiothreitol
FEN-1	Flap endonuclease 1
IDCL	Interdomain connecting loop
K-Glu	Potassium glutamate
PCNA	Proliferating cell nuclear antigen
PIP box	PCNA interaction protein box
SPR	Surface plasmon resonance

Introduction

DNA ligases catalyze nick-sealing reactions via three nucleotidyl transfer steps, as described in recent review articles (Wilkinson et al. 2001; Tomkinson et al. 2006). In the first step, DNA ligases form a covalent enzyme-AMP intermediate by reacting with ATP or NAD^+ as a cofactor (step 1). In the second step, DNA ligases recognize the substrate DNA, and the AMP is subsequently transferred from the ligases to the 5′-phosphate terminus of the DNA, to form a DNA-adenylate intermediate (step 2). Then, in the final step, the 5′-DNA-adenylate intermediate is attacked by the adjacent 3′-hydroxy group of the DNA to form a phosphodiester (step 3).

Generally, DNA ligases are grouped into two families, according to their requirement for ATP or NAD^+ as a nucleotide cofactor in the step 1 reaction. ATP-dependent DNA ligases are widely found in all three domains of life

(eukarya, bacteria, and archaea), whereas NAD^+-dependent DNA ligases exist in bacteria and some eukaryotic viruses (Benarroch and Shuman 2006). A number of archaeal ATP-dependent DNA ligases, which are homologous to eukaryotic DNA ligase I, have been biochemically characterized (Sriskanda et al. 2000; Lai et al. 2002; Keppetipola and Shuman 2005; Jackson et al. 2006). It is noteworthy that the ATP-dependent DNA ligases of hyperthermophilic euryarchaea, *Thermococcus kodakaraensis*, *Thermococcus fumicolans*, and *Thermococcus* sp. NA1, can utilize NAD^+ as a cofactor (Nakatani et al. 2000; Rolland et al. 2004; Kim et al. 2006). Interestingly, the genetic analysis of a DNA ligase from the halophilic euryarchaeon *Haloferax volcanii* revealed the existence of both ATP- and NAD^+-dependent DNA ligases in one organism (Zhao et al. 2006; Poidevin and MacNeil 2006). Based on a phylogenetic analysis, it was proposed that the NAD^+-dependent DNA ligase in *H. volcanii* was acquired by lateral gene transfer from bacteria. In addition, some archaeal ligases efficiently utilize ADP as well as ATP as a cofactor, as described below.

In Eukarya and Archaea, three enzymes, DNA polymerase, flap endonuclease 1 (FEN-1), and DNA ligase, are required for lagging strand processing. These enzymes interact with proliferating cell nuclear antigen (PCNA) to accomplish the effective processing of lagging strand synthesis (Maga and Hubscher 2003). It is well known that PCNA interacting proteins from Eukarya and Archaea interact with PCNA via a consensus sequence, termed the PCNA interacting protein box (PIP box) (Vivona and Kelman 2003). The PIP box consists of the sequence Qxxhxxaa, where x represents any amino acid, h represents hydrophobic residues (e.g., Leu, Ile, or Met), and a represents aromatic residues (e.g. Phe, Tyr, or Trp). Human DNA ligase I was reported to form a stable complex with a PCNA homotrimer that is topologically linked to duplex DNA via an N-terminal PIP box motif (Levin et al. 1997; Montecucco et al. 1998). In general, PCNA is a homotrimer in eukaryotes and euryarchaea. However, the authors of a biochemical study of *Sulfolobus solfataricus* PCNA (SsoPCNA) proposed that the crenarchaeal PCNA forms a heterotrimeric structure, which consists of three distinct subunits, PCNA1, 2, and 3. Furthermore, it was found that *S. solfataricus* DNA ligase (SsoLig) specifically binds to the PCNA3 subunit to form a functional complex (Dionne et al. 2003). We have been studying DNA ligase (PfuLig) and PCNA (PfuPCNA) from *Pyrococcus furiosus*. We found the pentapeptide motif QKSFF, which seems to be a shorter version of a typical PIP box, in the middle loop region of the DNA-binding domain of PfuLig, and demonstrated that the motif actually plays an important role in binding to the PfuPCNA homotrimer (Kiyonari et al. 2006).

The biochemical properties of *Aeropyrum pernix* DNA ligase (ApeLig) have already been characterized (Jeon and Ishikawa 2003). The ApeLig is particularly interesting because of its utilization of both ADP and ATP as cofactors. A recent study revealed that a DNA ligase from *Staphylothermus marinus* also utilizes both ATP and ADP (Seo et al. 2006). A phylogenetic analysis of archaeal DNA ligases revealed that the *S. marinus* DNA ligase is most closely related to ApeLig. Thus, some types of crenarchaeal DNA ligases may be able to utilize ADP during the step 1 reaction; however, the underlying molecular mechanisms and their biological meaning are not fully understood. Accordingly, it is interesting to investigate the cofactor specificity of DNA ligases from their structural and functional aspects.

The three PCNA proteins were also identified in *A. pernix*, and their stimulation effects on the DNA polymerase activity were reported (Daimon et al. 2002). An extensive study of *A. pernix* PCNA (ApePCNA) interacting proteins revealed that ApeLig preferentially binds to PCNA3, among the three subunits (Imamura et al., submitted). In this study, we have characterized the physical and functional interactions between ApeLig and ApePCNA. We also predicted the ApePCNA interacting site in ApeLig, based on the amino acid sequence alignment of PfuLig and ApeLig, and examined it by site-specific mutation analyses.

Materials and methods

Cloning the genes for ApeLig and its mutant proteins

The DNA ligase gene (*lig*) was amplified by PCR directly from *A. pernix* genomic DNA using the oligonucleotides 5′-GCGAACCATATGGGGTGTCTGGTTTTGGCT-3′ and 5′-GCTGGATCCTACTACACCTGCTCCGCCGGC-3′ as the forward and reverse primers, respectively. The amplified gene was cloned into the pGEM-T Easy vector (Promega), and its nucleotide sequence was confirmed. The cloned gene was digested by *Nde*I–*Bam*HI and inserted into the corresponding sites of pET-21a (Novagen). The resultant plasmid was designated as pET-ApeLig. To prepare the expression plasmid for the amino acid substitution (F132A), A PCR-mediated mutagenesis was performed (QuickChange site-directed mutagenesis kit, Stratagene, CA, USA) using F132A-F 5′-GTCACCCTGGAGGCGgccATGGCGGGAGGG-3′ and F132A-R 5′-CCCTCCCG CCATggcCGCCTCCAGGGTGAC-3′ as the forward and reverse primers, respectively (lowercase letters show the desired nucleotide changes). The gene for the N-terminal truncated ApeLig was amplified by PCR using Lig(18-619)-F 5′-GCGCCATATGCCTTTCAAGCCCGTGGCTG

A-3′ and Lig(18-619)-R 5′-GCTGGATCCTACTACACC TGCTCCGCCGGC-3′ as the forward and reverse primers, respectively, and pET–ApeLig as the template DNA. The amplified DNA was inserted into pGEM-T Easy, and the nucleotide sequence was confirmed. The cloned gene was digested by *Nde*I–*Bam*HI and inserted into the corresponding sites of pET-21a. The resultant plasmid was designated as pET–ApeLigΔN.

Overproduction and purification of ApeLig proteins

To obtain the recombinant ApeLig, *E. coli* BL21 codonPlus^{TM}(DE3)-RIL cells (Stratagene) carrying pET–ApeLig were grown in 1 l of LB medium, containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C. The cells were cultured to an A_{600}=0.50, and expression of the *lig* gene was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and continuing the culture for 3 h at 37°C. After cultivation, the cells were harvested and disrupted by sonication in buffer A (50 mM Tris–HCl, pH 8.0, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol). The soluble cell extract, obtained by centrifugation at 12,000×*g* for 20 min was heated at 80°C for 30 min. The heat-resistant fraction obtained by centrifugation was treated with 0.15% polyethylenimine to remove the nucleic acids. The soluble proteins were precipitated by 80% saturated ammonium sulfate. The precipitate was resuspended in buffer B (50 mM Tris–HCl, pH 8.0, 1 M $(NH_4)_2SO_4$, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol) and was subjected to chromatography on a Hitrap Phenyl column (GE Healthcare Biosciences). The proteins were eluted at 0 M ammonium sulfate, and the eluted proteins were dialyzed against buffer C (50 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol). The dialysate was loaded onto a Hitrap Q column (GE Healthcare Biosciences), and the proteins were eluted in the flow-through fraction. The eluted proteins were dialyzed against buffer D (50 mM Tris–HCl, pH 8.0, 0.05 M NaCl, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol), and the dialysate was subjected to chromatography on a Hitrap SP column (GE Healthcare Biosciences). The proteins were eluted at 0.1–0.15 M sodium chloride, pooled, and stored at 4°C. The mutant ApeLig proteins were purified by the same procedures. The purity of each protein used in this study was evaluated by SDS-PAGE. No extra band was detected by Coomassie Brilliant Blue staining of the gel containing 2 μg of each purified protein. The protein concentrations were calculated by measuring the absorbance at 280 nm. The theoretical molecular absorption coefficient of each protein was calculated, based on its tryptophan and tyrosine content.

Overproduction and purification of ApePCNA proteins

Preparation of *A. pernix* PCNA was performed as described previously (Imamura et al. 2007). Briefly, *E. coli* Rosetta^{TM}(DE3) cells (Novagen), carrying pET-PCNA-1, -2, or -3, were grown in 1 l of LB medium, containing 50-μg/ml ampicillin and 34-μg/ml chloramphenicol, at 37°C. The cells were cultured to an A_{600} of 0.5, and then the expression of the *pcna* genes was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the cultivation was continued for a further 5-h at 37°C. The cells were harvested and disrupted by sonication in buffer A, containing 50 mM Tris–HCl, pH 8.0, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol. The soluble cell extracts, obtained by centrifugation (12,000×*g*, 20 min), were heated at 75°C for 15 min. The heat-resistant fractions obtained by centrifugation were treated with 0.15% polyethylenimine to remove the nucleic acids. The soluble proteins were precipitated by 80% saturation with ammonium sulfate. The precipitate was resuspended in buffer B, containing 50 mM Tris–HCl, pH 8.0, 1 M $(NH_4)_2SO_4$, 0.5 mM DTT, 0.1 mM EDTA, and 10% glycerol, and was subjected to chromatography on a Hitrap Phenyl column (GE Healthcare Biosciences). The PCNA1, PCNA2, and PCNA3 proteins were eluted with ammonium sulfate at 0.15–0.45, 0.35–0.55, and 0.3–0.5 M, respectively. The eluted proteins were dialyzed against buffer A. The dialysates were loaded onto a Hitrap Heparin column (GE Healthcare Biosciences), and the proteins were eluted at 0.3–0.35 M sodium chloride. The eluted proteins were dialyzed against buffer A, and the dialysate was subjected to chromatography on a Hitrap Q column (GE Healthcare). The proteins were eluted with 0.1–0.15 M sodium chloride, pooled, and stored at 4°C.

Based on our biochemical analyses, *A. pernix* PCNAs can form several different oligomeric states in solution, and the heterotrimeric form of PCNA1-PCNA2-PCNA3 seems to be preferential when all three subunits exist in the solution (Imamura et al. 2007). To isolate the heterotrimer of PCNA1-PCNA2-PCNA3, purified PCNA subunits were mixed with equal molar ratio and the mixture was subjected to a gel filtration chromatography as described previously (Imamura et al. 2007).

Western blot analysis

Aeropyrum pernix cells were disrupted by sonication in buffer A, and the extract was obtained by centrifugation (25,000×*g* for 15 min). The *A. pernix* cell extract (35 μg of protein) and the purified ApeLig proteins (3 ng) were separated by 10% SDS-PAGE, blotted onto polyvinylidene difluoride membranes, and reacted with

anti-ApeLig antiserum. The bands were detected by using an enhanced chemiluminescence system (ECL Plus, GE Healthcare Biosciences) according to the supplier's recommendations.

DNA ligation assay

The substrate DNA used in the ligation assay was a 49-bp DNA duplex containing a single nick at the center. The 22-mer deoxynucleotide (5′-AATTCGTGCAGG CATGGTAGCT-3′), which was labeled with ^{32}P at the 5′-terminus, and the 27 mer deoxynucleotide (5′-AG CTATGACCATGATTACGAATTGCTT-3′) were annealed to the 49-mer deoxyoligonucleotide with a complementary sequence, in 40 mM Tris–acetate (pH 7.8) and 0.5 mM magnesium acetate. The purified ApeLig proteins (at different concentrations for each experiment, as described in the figure legends) were incubated with the nicked DNA substrate (5 nM), prepared as described above, in 20 µl of ligation buffer, containing 50 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 1 mM DTT, 0.1 mM ATP, 0.1% Tween 20, and 0.1 mg/ml BSA, at 60°C for 15 min. Reactions were initiated by the addition of enzyme and were terminated with 5 µl of stop solution, containing 98% formamide, 10 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. Samples were heated at 100°C for 5 min and chilled rapidly on ice prior to loading onto a 10% polyacrylamide gel containing 8 M urea. After electrophoresis, the gels were dried and were scanned with an FLA5000 imaging analyzer (Fuji Film, Japan) to detect the ^{32}P-labeled DNA. Three independent experiments were carried out in succession for each ligation condition in this study, and the standard errors are shown as vertical lines on the plots in each graph.

Surface plasmon resonance analysis

A BIAcore system was used to study the physical interaction between ApeLig and ApePCNA3. Highly purified recombinant ApePCNA3 was fixed on a CM5 sensor chip (research grade; Biacore) according to the manufacturer's recommendations. To measure the kinetic parameters, various concentrations of ApeLig (0.50, 0.75, 1.0, 1.5, 2.0 µM) were applied to the immobilized ApePCNA3. All measurements were performed at a continuous flow rate of 30 µl/min, in a buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, and 0.005% Tween 20. At the end of each cycle, the bound protein was removed by washing with 2 M NaCl. The kinetic constants for ApeLig binding to ApePCNA3 were determined from the association and dissociation curves of the sensorgrams, using the BIA-evaluation program (Biacore).

Results

Expression of the ApeLig gene in *A. pernix*

The complete genome sequence of *A. pernix* was determined (Kawarabayasi et al. 1999), and the putative DNA ligase gene (APE1094) was identified. The predicted ApeLig gene encodes a protein of 619 amino acids, with a calculated mass of 69196.2 Da. The amino acid sequence alignment shows that ApeLig has an N-terminal extended region, which is absent from all known archaeal DNA ligases (Fig. 1a). Recently, the genes in the *A. pernix* genome were completely re-annotated, based on the results of proteome analyses (Yamazaki et al. 2006), which can be accessed at http://www.bio.nite.go.jp/dogan/GeneralFeature?GENOME_ID=ape_G1. One interesting finding from that work was that *A. pernix* preferentially uses TTG, rather than ATG and GTG, as the translation initiation codon. Therefore, some ORFs were modified in the revised database. The re-annotated ApeLig gene encodes a protein of 602 amino acids (67747.6 Da), in which 17 amino acids were truncated from the N-terminus of the originally annotated protein (Fig. 1b). However, no experimental evidence for this 602 aa protein has been obtained yet. To determine the actual initiation codon of the ApeLig gene in the *A. pernix* cells, we analyzed the size of the ApeLig protein produced in vivo, by a western blot analysis with anti-ApeLig antiserum. As shown in Fig. 1c, the protein band detected in the *A. pernix* cell extract migrated to exactly the same position as that of the recombinant ApeLig(1-619) produced in *E. coli*. On the other hand, the ApeLig(18-619) protein migrated was slightly faster than the ApeLig(1-619) protein and the protein detected in *A. pernix* cell extracts. In addition, we examined the specific activities of the two recombinant ApeLig proteins. As a result, the specific activities for the nick sealing reaction of the two ApeLigs, (1-691) and (18-619), were almost the same in vitro (Fig. 2), and therefore, the N-terminal region with the extra 17 amino acids seems to be dispensable for, at least, the DNA ligation activity. Based on the western analysis, we decided to use the ApeLig (1-619) protein, which hereafter we refer to as wild-type ApeLig in this report. Elucidation of the functional role of the N-terminal 17 amino acids remains as an interesting pursuit.

Physical interaction between the ApeLig and PCNA3 proteins

We previously reported that PfuLig interacts with PCNA via a novel PCNA-binding motif. Interestingly, this novel PCNA binding motif is widely conserved in the same region of other archaeal DNA ligases (Kiyonari et al. 2006). Therefore, we examined whether the corresponding region

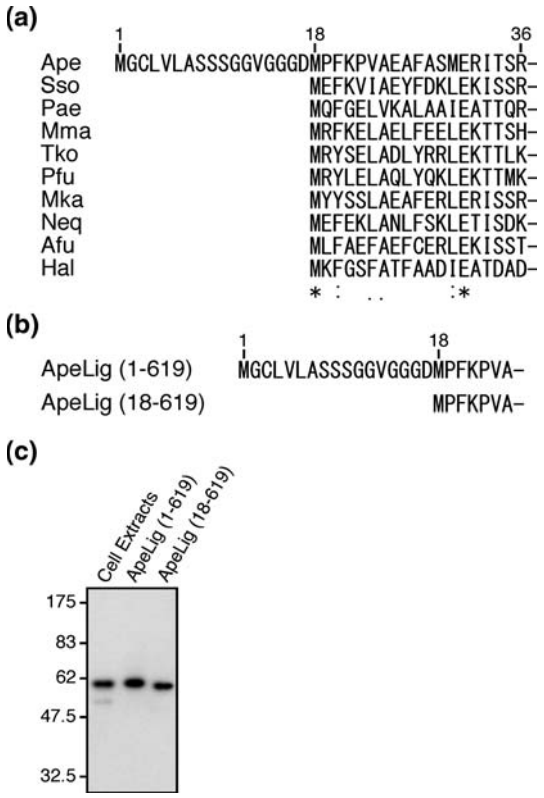

Fig. 1 ApeLig has an N-terminal extended region (**a**) Amino acid sequence alignment of the N-terminal regions of archaeal DNA ligases. Identical residues among the ligases are marked as ''*'', and residues with conserved and semi-conserved substitutions are marked as '':'' and ''.'', respectively. Ape, *Aeropyrum pernix*; Sso, *Sulfolobus solfataricus*; Pae, *Pyrobaculum aerophilum*; Mma, *Methanosarcia mazei*; Tko, *Thermococcus kodakaraensis*; Pfu, *Pyrococcus furiosus*; Mka, *Methanopyrus kandleri*; Neq, *Nanoarchaeum equitans*; Afu, *Archaeoglobus fulgidus*; Hal, *Halobacterium* sp. NRC-1. **b** N-terminal amino acid sequences of the two ApeLig proteins. ApeLig (1-619) is the originally annotated protein and ApeLig (18-619) is the re-annotated protein. **c** *A. pernix* cell extracts (35 μg) and recombinant ApeLig proteins (3 ng) were separated by SDS-10% PAGE and then were analyzed by western blotting with anti-ApeLig antiserum. The positions and sizes (in kilodaltons) of pre-stained marker polypeptides are indicated on the left. The ApeLig protein usually migrates faster than the position based on the calculated molecular weight (69196.2 Da) in comparison to the protein size markers we used

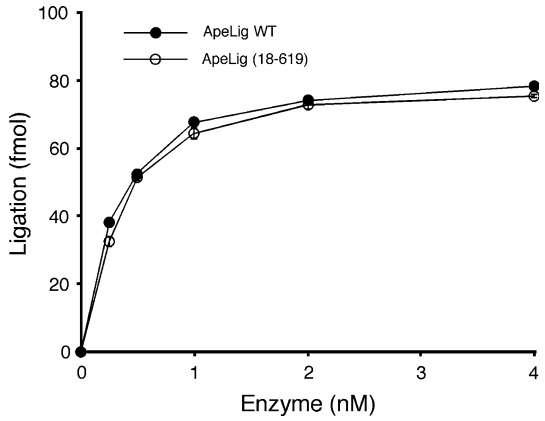

Fig. 2 The enzymatic activity of the N-terminally truncated mutant protein. The ligation activities of wild-type (*WT*) and ApeLig (18-619) were compared. Various amounts of ApeLig proteins, as indicated, were incubated with 100 fmol of nicked DNA in the reaction mixture, as described in the Materials and methods. The ligation efficiency is plotted as a function of the enzyme concentration

of ApeLig actually plays an important role in binding to ApePCNA. Based on amino acid sequence alignments with PfuLig, we predicted that one phenylalanine residue at position 132 (Phe132) in the putative PCNA-binding region is important for the interaction with ApeLig–PCNA3 (Fig. 3). We constructed the expression plasmid and purified the produced alanine-substitution mutant, ApeLig F132A, and then performed surface plasmon resonance (SPR) experiments to analyze the physical interaction between ApeLig and ApePCNA. *A. pernix* has three PCNA proteins, PCNA1, 2, and 3, and these proteins preferentially form functional heterotrimers consisting of PCNA1-2-3. We already determined that ApeLig has specific affinity to the PCNA3 subunit among them (Imamura et al., submitted). Therefore, the purified PCNA3 protein was immobilized on the Biacore CM5 sensor chip, and subsequently, the wild type and F132A proteins were injected at different concentrations. Physical interactions between immobilized ApePCNA3 and ApeLig wild type were detected, but ApeLig F132A showed no binding affinity, according to the SPR sensorgrams (Fig. 4). The calculated equilibrium constant (K_D) for ApeLig wild type was 7.0×10^{-7} M, which is comparable to that of the PfuLig–PCNA interaction determined by the SPR analysis (K_D: 1.1×10^{-7} M) (Kiyonari et al. 2006). These results show that the PCNA-binding modes of archaeal DNA ligases are remarkably conserved, in terms of the interaction motifs and equilibrium constant.

The ligation activity of ApeLig is enhanced by the PCNA heterotrimer at high salt concentrations

It is well known that most archaea, which exist in high salt as well as high temperature environments, contain high intracellular potassium ion concentrations (Roberts 2004). The exact salt concentration in the *A. pernix* cells is not known yet; however, the salt concentration required for the optimal growth condition of *A. pernix* is 3.5% (Sako et al. 1996). We initially examined the effect of increasing the salt concentration on the nick-joining activity of ApeLig by supplementing the reaction with KCl and potassium glutamate (K-Glu) salts. The enzyme activity was reduced by each of these monovalent salts in a concentration-dependent manner (data not shown), as

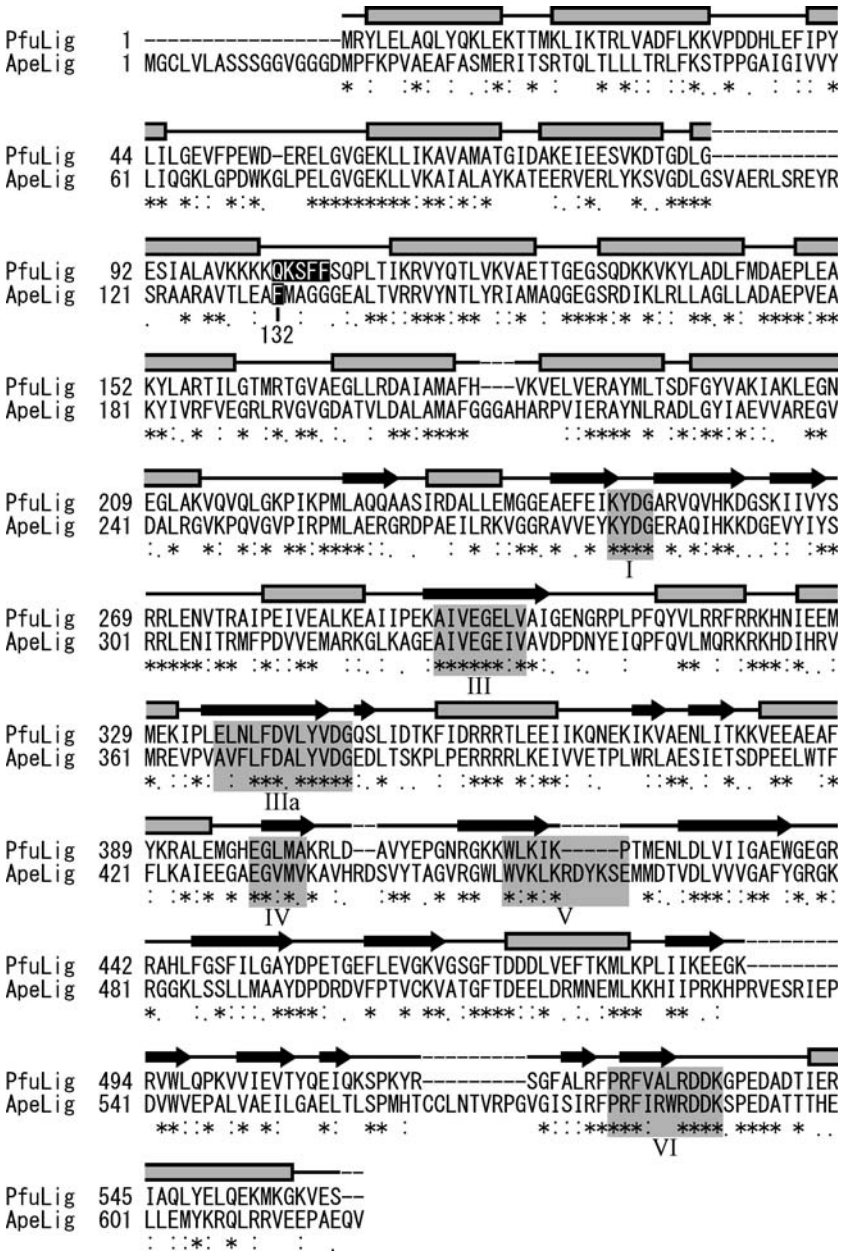

Fig. 3 Amino acid sequence comparison of PfuLig and ApeLig. The dashes indicate gaps, and identical residues between the two ligases are marked as ''*'', and residues with conserved and semi-conserved substitutions are marked as '':'' and ''.'', respectively. The secondary structure of PfuLig is shown above the corresponding residues, based on the crystal structure (Nishida et al. 2006). *Shaded boxes* I to VI present the six motifs commonly found in ATP-dependent DNA ligases. The PCNA binding sequence QKSFF, found in PfuLig, is enclosed in *black box*, and the aromatic residue Phe132, found in the corresponding region of ApeLig, is indicated

reported previously (Jeon and Ishikawa 2003). We reported that a functional interaction between PfuLig and PCNA was observed not at low ionic strength, but at high K-Glu concentrations, at which a DNA ligase alone cannot bind to a nicked DNA substrate, and discussed the possibility of the inhibitory effect of a high concentration of chloride ion on the enzyme activity (Kiyonari et al. 2006). Therefore, we examined whether the ApePCNA heterotrimer can stimulate the ligation activity of ApeLig wild type and ApeLig F132A at high K-Glu concentrations. Before we compared the effects of ApePCNA on these enzyme activities, ligation reactions were performed to determine the specific activities of each enzyme. As shown in Fig. 5a, the wild type and F132A enzymes showed almost the same ligation activity in the reaction conditions without the addition of salts. The stimulation effect of the ApePCNA heterotrimer (1-2-3) on the wild-type enzyme was observed at 0.3 M K-Glu, but the effect was decreased at >3 nM PCNA heterotrimer (Fig. 5b). The optimum K-Glu concentrations for the ApePCNA-dependent ligation activity of ApeLig are 0.2–0.3 M (data not shown). These salt concentrations are slightly lower than the case of the reaction with PfuLig and PfuPCNA interaction (0.3–0.4 M K-Glu, Kiyonari et al. 2006). Consistent with our SPR analysis observations, the ApeLig F132A was not stimulated by the PCNA heterotrimer, probably due to the loss of the binding ability to the PCNA3 subunit. Taken together, we concluded that the

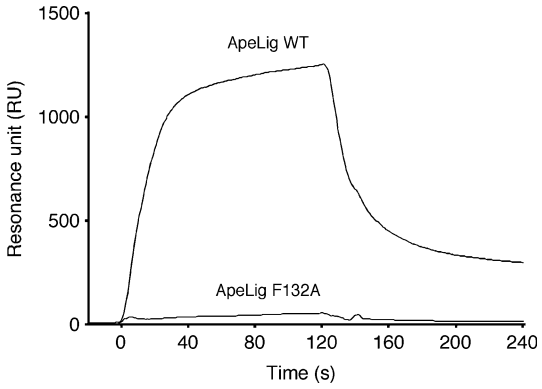

Fig. 4 Physical interaction between ApeLig and ApePCNA3 protein. An SPR analysis was performed using a Biacore system to detect the physical interaction between the ApeLig and ApePCNA3 proteins. Purified ApePCNA3 was immobilized on a Biacore sensor chip, and the wild-type (*WT*) and F132A mutant protein (1 μM) were each injected for 120 s, at a constant flow rate of 30 μl/min

aromatic residue Phe132 has a critical role for the functional interaction with ApePCNA.

Discussion

We prepared two ApeLig proteins (1-691) and (18-619), based on the open reading frames started by GTG and ATG, respectively, in this study. A comparison of the two recombinant proteins with the native ApeLig in the *A. pernix* cells by a western blot analysis suggested that the (1-691) protein is probably produced in *A. pernix*. However, the specific activities for the in vitro DNA ligation reaction are the same between the two enzymes. Therefore, the N-terminal 17 amino acids seem to be dispensable for the ligation activity. Our preliminary study showed that ApeLig (1-691) is more heat-stable than ApeLig (18-691), when these enzymes were incubated at 110°C for 20 min (data not shown). The N-terminal region probably contributes, at least, to the stability of the ApeLig protein in the hyperthermophilic archaeal cells. This N-terminal region might be involved in interactions with other proteins that regulate the ApeLig activity to adjust it to different processes in the DNA transactions.

It is known that human DNA ligase I forms a stable complex with PCNA. However, the functional role of the complex formation is unclear, because several groups have reported contradictory observations, about the stimulatory effect of PCNA on DNA ligase activity (Levin et al. 1997; Jonsson et al. 1998; Tom et al. 2001). We demonstrated that the ligation activity of PfuLig is obviously stimulated by PfuPCNA at a physiological salt concentration, although a large amount of PCNA is required for the stimulation of PfuLig activity in vitro (The PfuPCNA

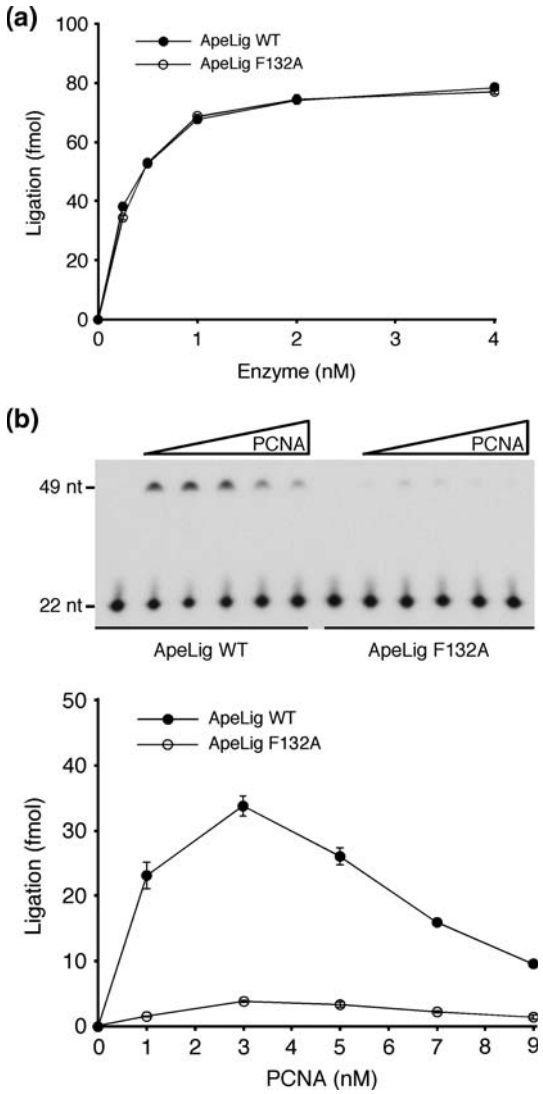

Fig. 5 Functional interaction between ApeLig and the PCNA heterotrimer. **a** The ligation activities of the wild-type (*WT*) and F132A mutant proteins were compared. Various amounts of ApeLig proteins, as indicated, were incubated with 100 fmol of nicked DNA in the reaction mixture, as described in the Materials and methods. The ligation efficiency is plotted as a function of the enzyme concentration. **b** The stimulatory effect of the ApePCNA heterotrimer on the ligation activity of the ApeLig proteins. The ApeLig proteins (4 fmol) were incubated with 100 fmol of nicked DNA and 0–9 nM PCNA heterotrimer in a reaction mixture containing 0.3 M K-Glu, as described in the Materials and methods. The ligation efficiency is plotted as a function of the enzyme concentration

concentration-dependent stimulation of PfuLig activity reaches to the maximum at 50 nM for the 5 nM DNA substrate), probably due to the difficulty of loading of the PCNA ring onto the double-stranded DNA substrate in the assay mixture (Kiyonari et al. 2006). The PCNA ring was probably loaded by diffusion onto the ends of the DNA fragment, and therefore, this step may be a rate-limiting process in the in vitro reaction using oligodeoxynucleotide

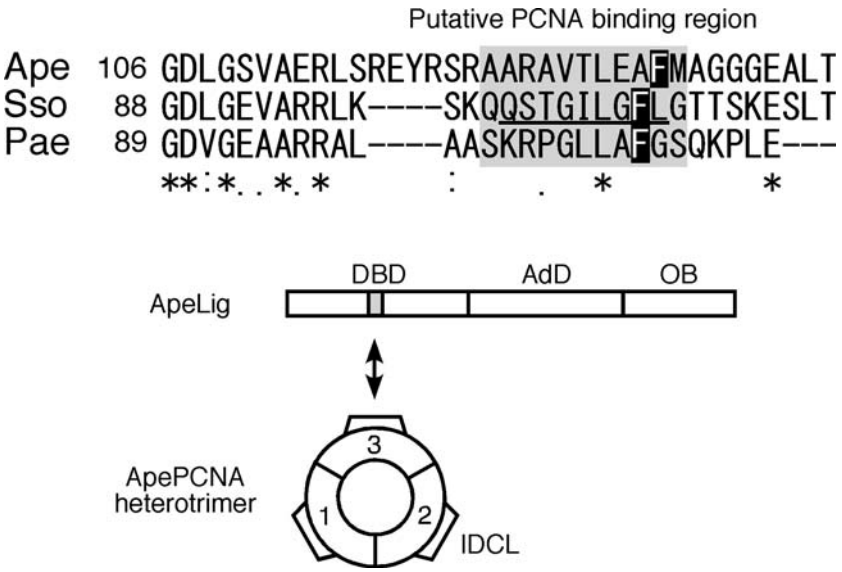

Fig. 6 Amino acid sequence alignment of the PCNA binding regions in crenarchaeal DNA ligases. Identical residues among the ligases are marked as ''*'', and residues with conserved and semi-conserved substitutions are marked as '':'' and ''.'', respectively. Phenylalanine residues are enclosed in a *black box* and the PCNA binding motif of SsoLig is underlined (Pascal et al. 2006). The interaction between ApeLig and the PCNA heterotrimer is schematically drawn. Eukaryotic DNA ligase comprises three domains, N-terminal DNA-binding domain (*DBD*), the middle adenylation domain (*AdD*), and the C-terminal OB (oligonucleotide/oligosaccharide binding)-fold domain (*OB*). ApeLig interacts with the PCNA3 subunit via the binding motif located in the middle of the DBD. Ape, *Aeropyrum pernix*; Sso, *Sulfolobus solfataricus*; Pae, *Pyrobaculum aerophilum*

substrates, as discussed previously (Tom et al. 2001). As shown in Fig. 5b, the ApeLig activity was enhanced by ApePCNA in a concentration-dependent manner, and the stimulatory effect was decreased at >3 nM PCNA. The difference of the concentration dependency between PfuPCNA and ApePCNA suggested that the ApePCNA heterotrimer can be loaded onto the DNA substrate more efficiently than the PfuPCNA homotrimer, and once it is formed, the PCNA heterotrimer–DNA complex may be quite stable. In the reaction mixtures with high concentrations of ApePCNA, accumulation of an excess amount of PCNA heterotrimer might prevent the binding of DNA ligase to the nicked site on the double-stranded DNA substrate. The binding affinities of these PCNAs to DNA should be compared to address this issue. Since the calculated equilibrium constant for the ApeLig–PCNA3 interaction is similar to that of the PfuLig–PCNA interaction, the inhibitory effect of ApePCNA on ligation by ApeLig with a lower concentration as compared with PfuPCNA is not due to the formation of the ApeLig–ApePCNA complex without the DNA substrate.

Human DNA ligase I interacts with PCNA via a typical PIP box motif, 2-QRSIMSFF-9, which is located at the N-terminus of the DBD (Montecucco et al. 1998). The crystal structure of human DNA ligase in complex with a nicked DNA substrate and biochemical analyses of the enzyme revealed that eukaryotic DNA ligase comprises the N-terminal DNA-binding domain (DBD), the middle adenylation domain, and the C-terminal OB (oligonucleotide/oligosaccharide binding)-fold domain (Pascal et al. 2004). The authors proposed a model structure of Lig I–PCNA. However, their crystal analysis used an N-terminally truncated protein lacking the PIP box for Lig I, and therefore, it is difficult to discuss the interaction at the N-terminal PIP box from this work. Archaeal DNA ligases do not seem to have a clear PIP box motif (Warbrick 2000; Grabowski and Kelman 2003). Based on our crystal structure (Nishida et al. 2006) and mutational analyses of PfuLig (Kiyonari et al. 2006), we identified a novel PCNA-binding motif, 103-QKSFF-107. This novel motif is located in a loop structure in the middle of the DBD, but not at the N-terminus of PfuLig. During the preparation of this manuscript, the crystal structure of SsoLig was reported (Pascal et al. 2006), in which the PCNA-binding motif was identified in the loop region corresponding to that we identified in PfuLig. In the crystal structure of SsoLig, the loop region is disordered, and therefore, the PCNA-binding region is predicted to be flexible. A structural comparison of the novel motif found in the PfuLig with the PIP box motif of the *P. furiosus* RFC (replication factor C) large subunit, complexed with PCNA (Matsumiya et al. 2002), revealed that locations of the amino acid residues responsible for the hydrophobic and ionic interactions clearly correspond between the two PCNA-binding motifs. Notably, the three-dimensional positions of Q103 in PfuLig and Q470 in RFCL (for ionic interaction) and F106/F107 in

PfuLig and F476/L473 in RFCL (for hydrophobic interaction) are remarkably conserved between the two PCNA interacting motifs. An amino acid sequence alignment of the putative PCNA-binding regions of the crenarchaeal DNA ligases showed that the glutamine residue is not conserved in the *A. pernix* and *Pyrobaculum aerophilum* DNA ligases (Fig. 6). Our present observations of the ApeLig–ApePCNA interaction suggest that the glutamine residue may not be required for PCNA-binding. Consistent with this idea, we found that although the glutamine residue is conserved in the PCNA-binding motif 103-QKSFF-107 of PfuLig, the single substitution of the aromatic amino acid residue of PfuLig, F106A, abolished the binding ability to PfuPCNA (Kiyonari et al. 2006). Further crystallographic and biochemical studies are required to understand the detailed interaction mode (role of each amino acid) in the PCNA-binding motifs of the archaeal DNA ligases. A recent crystallographic study of the SsoPCNA1–PCNA2–FEN1 complex shed light on the mechanism of the binding specificity for each PCNA-binding protein (Dore et al. 2006). *S. solfataricus* FEN-1 binds specifically to the PCNA1 subunit via the typical PIP box sequence, 339-QTGLDRWF-346, at its C-terminus. The PIP box binds to the interdomain-connecting loop (IDCL) of PCNA1. A structural comparison between the two SsoPCNA proteins revealed that the IDCL of PCNA1 adopts a unique conformation, which is suitable for FEN1, but not for DNA polymerase and ligase. This structural study supports the idea that the PCNA-binding specificity of the crenarchaeal replicative enzymes is regulated by the sterical matching and hindrance between IDCL and PIP box. X-ray crystallographic studies of the ApeLig–PCNA3 complex will be required to provide a framework to understand the interaction mode and the binding specificity of ApeLig to ApePCNA. Continuous research on the archaeal PCNA and PCNA-binding proteins will greatly contribute toward elucidating the basic molecular mechanisms of the DNA transaction processes not only in the archaeal cells, but also in the more complicated eukaryotic cells.

Acknowledgments We are grateful to the Human Frontier Science Program (HFSP) for their support in the form of a research grant to Y. I. This work was also supported in part by Grants-in Aid from the Ministry of Education, Culture, Sports, Science, and Technology, Japan, to Y. I. We thank the Hou-ansha Foundation for the support of our work.

References

Benarroch D, Shuman S (2006) Characterization of mimivirus NAD+-dependent DNA ligase. Virology 353:133–143

Daimon K, Kawarabayasi Y, Kikuchi H, Sako Y, Ishino Y (2002) Three proliferating cell nuclear antigen-like proteins found in the hyperthermophilic archaeon *Aeropyrum pernix*: interactions with the two DNA polymerases. J Bacteriol 184:687–694

Dionne I, Nookala RK, Jackson SP, Doherty AJ, Bell SD (2003) A heterotrimeric PCNA in the hyperthermophilic archaeon *Sulfolobus solfataricus*. Mol Cell 11:275–282

Dore AS, Kilkenny ML, Jones SA, Oliver AW, Roe SM, Bell SD, Pearl LH (2006) Structure of an archaeal PCNA1-PCNA2-FEN1 complex: elucidating PCNA subunit and client enzyme specificity. Nucleic Acids Res 34:4515–4526

Grabowski B, Kelman Z (2003) Archeal DNA replication: eukaryal proteins in a bacterial context. Annu Rev Microbiol 57:487–516

Imamura K, Fukunaga K, Kawarabayasi Y, Ishino Y (2007) Specific interactions of three proliferating cell nuclear antigens with replication-related proteins in *Aeropyrum pernix*. Mol Microbiol 64:308–318

Jackson BR, Noble C, Lavesa-Curto M, Bond PL, Bowater RP (2006) Characterization of an ATP-dependent DNA ligase from the acidophilic archaeon ''Ferroplasma acidarmanus'' Fer1. Extremophiles (in press)

Jeon SJ, Ishikawa K (2003) A novel ADP-dependent DNA ligase from *Aeropyrum pernix* K1. FEBS Lett 550:69–73

Jonsson ZO, Hindges R, Hubscher U (1998) Regulation of DNA replication and repair proteins through interaction with the front side of proliferating cell nuclear antigen. EMBO J 17:2412–2425

Kawarabayasi Y, Hino Y, Horikawa H, Yamazaki S, Haikawa Y, Jinno K, Takahashi M, Sekine M, Baba S, Ankai A, Kosugi H, Hosoyama A, Fukui S, Nagai Y, Nishijima K, Nakazawa H, Takamiya M, Masuda S, Funahashi T, Tanaka T, Kudoh Y, Yamazaki J, Kushida N, Oguchi A, Kikuchi H (1999) Complete genome sequence of an aerobic hyper-thermophilic crenarchaeon, Aeropyrum pernix K1. DNA Res 6:83–101, 145–152

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

Kim YJ, Lee HS, Bae SS, Jeon JH, Yang SH, Lim JK, Kang SG, Kwon ST, Lee JH (2006) Cloning, expression, and characterization of a DNA ligase from a hyperthermophilic archaeon *Thermococcus* sp. Biotechnol Lett 28:401–407

Kiyonari S, Takayama K, Nishida H, Ishino Y (2006) Identification of a novel binding motif in *Pyrococcus furiosus* DNA ligase for the functional interaction with proliferating cell nuclear antigen. J Biol Chem 281:28023–28032

Lai X, Shao H, Hao F, Huang L (2002) Biochemical characterization of an ATP-dependent DNA ligase from the hyperthermophilic crenarchaeon *Sulfolobus shibatae*. Extremophiles 6:469–477

Levin DS, Bai W, Yao N, O'Donnell M, Tomkinson AE (1997) An interaction between DNA ligase I and proliferating cell nuclear antigen: implications for Okazaki fragment synthesis and joining. Proc Natl Acad Sci USA 94:12863–12868

Maga G, Hubscher U (2003) Proliferating cell nuclear antigen (PCNA): a dancer with many partners. J Cell Sci 116:3051–3060

Matsumiya S, Ishino S, Ishino Y, Morikawa K (2002) Physical interaction between proliferating cell nuclear antigen and replication factor C from *Pyrococcus furiosus*. Genes Cells 7:911–922

Montecucco A, Rossi R, Levin DS, Gary R, Park MS, Motycka TA, Ciarrocchi G, Villa A, Biamonti G, Tomkinson AE (1998) DNA ligase I is recruited to sites of DNA replication by an interaction with proliferating cell nuclear antigen: identification of a common targeting mechanism for the assembly of replication factories. EMBO J 17:3786–3795

Nakatani M, Ezaki S, Atomi H, Imanaka T (2000) A DNA ligase from a hyperthermophilic archaeon with unique cofactor specificity. J Bacteriol 182:6424–6433

Nishida H, Kiyonari S, Ishino Y, Morikawa K (2006) The closed structure of an archaeal DNA ligase from Pyrococcus furiosus. J Mol Biol 360:956–967

Pascal JM, O'Brien PJ, Tomkinson AE, Ellenberger T (2004) Human DNA ligase I completely encircles and partially unwinds nicked DNA. Nature 432:473–478

Pascal JM, Tsodikov OV, Hura GL, Song W, Cotner EA, Classen S, Tomkinson AE, Tainer JA, Ellenberger T (2006) A flexible interface between DNA ligase and PCNA supports conformational switching and efficient ligation of DNA. Mol Cell 24:279–291

Poidevin L, MacNeill SA (2006) Biochemical characterisation of LigN, an NAD+-dependent DNA ligase from the halophilic euryarchaeon Haloferax volcanii that displays maximal in vitro activity at high salt concentrations. BMC Mol Biol 7:44

Roberts MF (2004) Osmoadaptation and osmoregulation in archaea: update 2004. Front Biosci 9:1999–2019

Rolland JL, Gueguen Y, Persillon C, Masson JM, Dietrich J (2004) Characterization of a thermophilic DNA ligase from the archaeon *Thermococcus fumicolans*. FEMS Microbiol Lett 236:267–273

Sako Y, Nomura N, Uchida A, Ishida Y, Morii H, Koga Y, Hoaki T, Maruyama T (1996) *Aeropyrum pernix* gen. nov., sp. nov., a novel aerobic hyperthermophilic archaeon growing at temperatures up to 100 degrees C. Int J Syst Bacteriol 46:1070–1077

Seo MS, Kim YJ, Choi JJ, Lee MS, Kim JH, Lee JH, Kwon ST (2006) Cloning and expression of a DNA ligase from the hyperthermophilic archaeon *Staphylothermus marinus* and properties of the enzyme. J Biotechnol (in press)

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

Tom S, Henricksen LA, Park MS, Bambara RA (2001) DNA ligase I and proliferating cell nuclear antigen form a functional complex. J Biol Chem 276:24817–24825

Tomkinson AE, Vijayakumar S, Pascal JM, Ellenberger T (2006) DNA ligases: structure, reaction mechanism, and function. Chem Rev 106:687–699

Vivona JB, Kelman Z (2003) The diverse spectrum of sliding clamp interacting proteins. FEBS Lett 546:167–172

Warbrick E (2000) The puzzle of PCNA's many partners. Bioessays 22:997–1006

Wilkinson A, Day J, Bowater R (2001) Bacterial DNA ligases. Mol Microbiol 40:1241–1248

Yamazaki S, Yamazaki J, Nishijima K, Otsuka R, Mise M, Ishikawa H, Sasaki K, Tago S, Isono K (2006) Proteome analysis of an aerobic hyperthermophilic crenarchaeon, *Aeropyrum pernix* K1. Mol Cell Proteomics 5:811–823

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