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ORIGINAL PAPER

Characterization of an ATP-dependent DNA ligase from the acidophilic archaeon "*Ferroplasma acidarmanus*" Fer1

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Abstract Analysis of the genome of "*Ferroplasma acidarmanus*" Fer1, an archaeon that is an extreme acidophile, identified an open reading frame encoding a putative ATP-dependent DNA ligase, which we termed FaLig. The deduced amino acid sequence of FaLig contains 595 amino acids, with a predicted molecular mass of 67.8 kDa. "*F. acidarmanus*" Fer1 is classified as a Euryarchaeote, but phylogenetic analysis using amino acid sequences showed that FaLig is more similar to DNA ligases from Crenarchaeota, suggesting that lateral transfer of these genes has occurred among archaea. The gene sequence encoding FaLig was cloned into a bacterial expression vector harbouring an upstream His-tag to aid purification. Conditions for expression and purification from *Escherichia coli* were identified and recombinant FaLig was confirmed to be an ATP-dependent DNA ligase. Optimal conditions for nick-joining by the protein were pH 6–7, 0.5 mM ATP, in the presence of either Mg^{2+} or Mn^{2+}. Using a range of nicked, double-stranded nucleic acids, ligation was detected with the same substrates as previously determined for other DNA ligases. Although FaLig is the DNA ligase from one of the most extreme acidophilic organism yet studied, this characterization suggests that its biochemical mechanism is analogous to that of enzymes from other cellular systems.

Keywords DNA ligase · *Ferroplasma acidarmanus* · DNA nick-joining · Acidophilic · Archaea

Abbreviations

DTT	Dithiothreitol
FaLig	"*Ferroplasma acidarmanus*" Fer1 DNA ligase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani broth
T4Dnl	T4 DNA ligase

Introduction

"*Ferroplasma acidarmanus*" Fer1 (hereafter referred to as *F. acidarmanus* Fer1) is an acidophilic archaeon, which was first identified as a dominant prokaryote in the acid-drainage biofilm at Iron Mountain in Northern California (Dopson et al. 2004; Edwards et al. 2000; Golyshina and Timmis 2005; Tyson et al. 2004). *F. acidarmanus* Fer1 is mesophilic, with optimal growth at about 40°C, but it survives in extremely acidic conditions, with optimal growth at pH 1.2. This group of Archaea plays important roles in geochemical iron and sulphur cycles and has also been implicated as a major contributor in the process of acid mine drainage, which causes considerable environmental damage by the release of metal-rich acidic effluents into groundwater (Dopson et al. 2004; Edwards et al. 2000; Golyshina and Timmis 2005; Schleper et al. 2005). Coupling these points with its unusual microbiology, identification of

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factors involved in the metabolism of *F. acidarmanus* Fer1 holds considerable interest for both applied and basic sciences.

The two major phyla of the Archaea, the Crenarchaeota and the Euryarchaeota, both contain organisms that inhabit low pH environments (Makarova and Koonin 2003; Makarova and Koonin 2005; Schleper et al. 2005). The only family of cultured acidophiles among the Crenarchaeota are the *Sulfolobaceae*, which grow at pH 2–4 (Makarova and Koonin 2003; Makarova and Koonin 2005; She et al. 2001). The Euryarchaeota contains several members that grow at extremely low pH, including *F. acidarmanus* Fer1 and the other identified member of this family, *F. acidiphilum* (Edwards et al. 2000; Golyshina and Timmis 2005). The *Ferroplasmaceae* lie within the order of *Thermoplasmatales*, which also contains the other characterized acidophiles, the *Thermoplasmaceae* and *Picrophilaceae* (Darland et al. 1970; Futterer et al. 2004; Golyshina et al. 2006; Golyshina and Timmis 2005). The *Thermoplasmatales* are able to grow at very low pH, typically <pH 2, with *Picrophilus torridus* and *F. acidarmanus* Fer1 being the most extreme with growth observed at pH 0 (Dopson et al. 2004; Futterer et al. 2004). However, all of these organisms are believed to maintain an intracellular pH of around 5 (Golyshina et al. 2006; Golyshina and Timmis 2005; Macalady et al. 2004; Searcy 1976). Thus, the cytoplasmic conditions of these organisms are distinct from their environment, which is aided by the fact that their single cytoplasmic membrane has low permeability to protons (Golyshina et al. 2006; Golyshina and Timmis 2005). It would, therefore, be expected that the proteins of these organisms have optimal activity close to the mildly-acidic cytoplasmic pH, as supported by characterization of a novel DNA repair protein encoded by *F. acidarmanus* Fer1 (Kanugula et al. 2005). By contrast, a recent study identified that several intracellular or membrane-bound proteins of *F. acidiphilum* had optimal activity in the pH range 2–4 (Golyshina et al. 2006). It is not yet clear how this observation relates to the environment within the cell and its impact upon cell metabolism. However, if the cytoplasm of the archaeon experiences acidity levels of pH 2, even briefly, then it is likely that extensive DNA damage would be induced, with a concomitant effect on DNA metabolism.

DNA ligases are a class of proteins that are involved in many aspects of DNA metabolism. These enzymes act to join breaks in the backbone of DNA and are essential for all cellular organisms due to the requirement for completion of replication, but they also participate in the repair and recombination of DNA (Doherty and Suh 2000; Shuman and Lima 2004; Tomkinson et al. 2006; Wilkinson et al. 2001). All nucleotidyl transferases are believed to operate through similar biochemical mechanisms consisting of three separate steps (Doherty and Suh 2000; Lehman 1974; Shuman and Lima 2004; Timson et al. 2000; Wilkinson et al. 2001). For DNA ligases the first step involves the enzyme attacking the α-phosphate of the nucleotide co-substrate to form a covalent enzyme-adenylate (AMP) intermediate. For the Archaea, the AMP moiety is generally provided by ATP (EC 6.5.1.1), while the essential DNA ligases of bacteria obtain this from NAD^+ (EC 6.5.1.2) (Wilkinson et al. 2001). High resolution structures of several DNA ligases confirm that they exhibit a modular structure, with the common core required for adenylation linked to other domains that bind substrate (Doherty and Suh 2000; Shuman and Lima 2004; Tomkinson et al. 2006).

The first archaeal DNA ligase was identified in 1992 from a hyperthermophilic Crenarchaeon, *Delsulfurolobus ambivalens*, with the amino acid sequence of this protein being most similar to those of Eukaryotic viral and cellular ATP-dependent ligases (Kletzin 1992; Nakatani et al. 2000). This is not particularly surprising since Archaea have been shown to be similar to Eukarya in many aspects of DNA metabolism, despite their morphological and structural resemblance to Bacteria (Kelman and White 2005; White 2003). Generally, the putative DNA ligases of Archaea are of fairly uniform size and their primary structures are extensively conserved. Despite this overall similarity, DNA ligases from Crenarchaeota and Euryarchaeota differ in the sequence of motif V as well as in the spacing between some motifs (Lai et al. 2002) (also see Fig. 1b).

More recently, recombinant versions of DNA ligases have been characterized from a number of archaea that grow at high temperature or salinity . The observation of some unexpected characteristics provides increasing interest in this class of proteins. For example, ATP-dependent DNA ligases from *Thermococcus kodakaraensis* (Nakatani et al. 2000) and *T. fumicolans* (Rolland et al. 2004) are also able to use NAD^+ as the cofactor for ligation. The ability for DNA ligases to use both ATP or NAD^+ as a cofactor appears to be specific to DNA ligases from *Thermococcales*, an order of hyperthermophilic microorganisms that belongs to the Euryarchaeota (Rolland et al. 2004). The relationship between ATP- and NAD^+-dependent DNA ligases has taken a further twist upon analysis of *Haloferax volcanii*, since this organism encodes both types of protein that share the essential DNA-joining functions (Zhao et al. 2006).

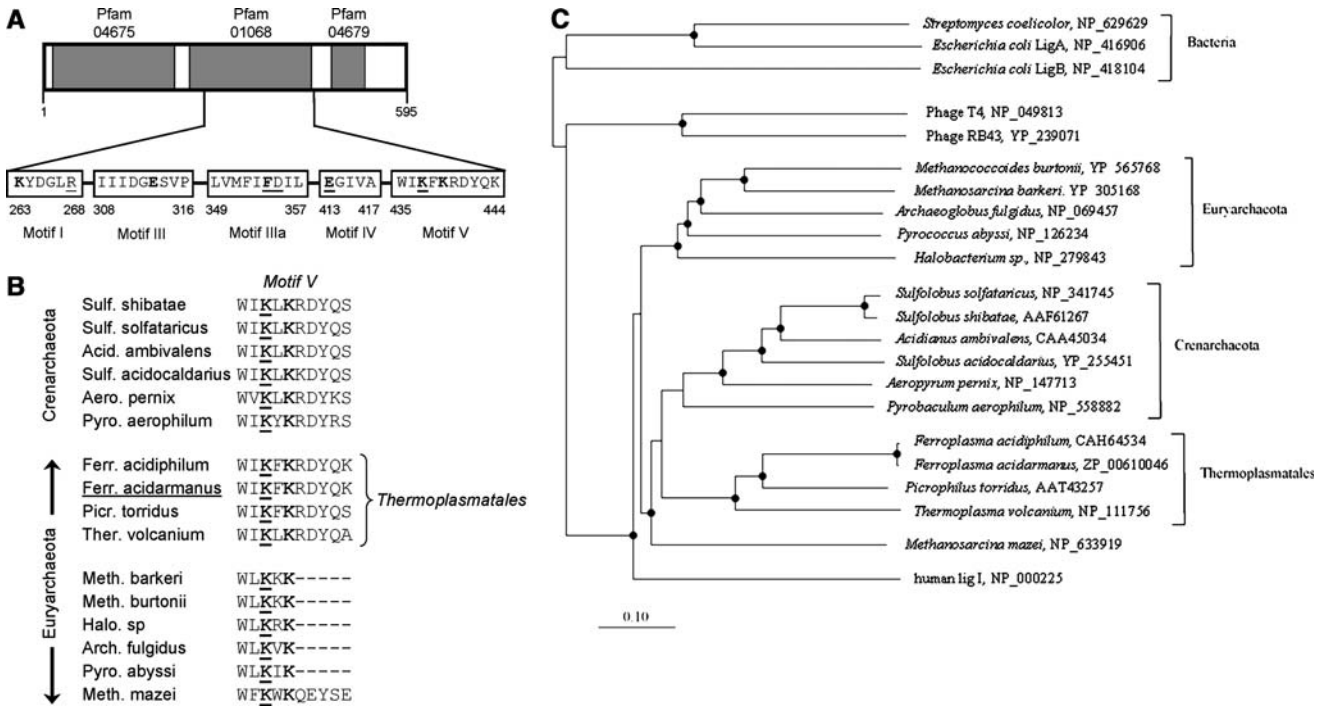

Fig. 1 *Ferroplasma acidarmanus* DNA ligase. *Standard single-letter abbreviations* indicate specific amino acids. *Residues in bold* are conserved components of the active site (Shuman and Lima 2004), whilst those *underlined* are identical to those conserved in bacterial ATP-dependent DNA ligases (Wilkinson et al. 2001). **a** Schematic diagram of conserved domains within the putative DNA ligase from *Ferroplasma acidarmanus* Fer1. All numbers refer to the position of the amino acid within the total protein of 595 amino acids. The position of five motifs conserved within ATP-dependent DNA ligases is indicated. Sections marked in *grey* and the Pfam names directly above them highlight significant similarity to domains within the conserved domain database (CDD) (Marchler-Bauer et al. 2005). Pfam04675 relates to a conserved N-terminal region among ATP-dependent DNA ligases; Pfam01068 relates to a conserved catalytic (adenylation) domain of DNA ligases; Pfam04679 relates to a conserved C-terminal region among ATP-dependent DNA ligases. **b** Alignment of motif V identified for DNA ligases from a variety of archaea. Fuller names of organisms are provided in (**c**). Complete sequences and organism names are available at the NCBI database (http://www.ncbi.nlm.nih.gov/). **c** Evolutionary distance dendogram of DNA ligase amino acid sequences estimated by neighbour-joining as described in the Materials and methods. The bacterial NAD^+-dependent ligase sequences, from *S. coelicolor* and *E. coli*, were used as the out-group. Branch points supported by the maximum-likelihood estimations are indicated by *filled circles*. Evolutionary distances are indicated by the sum of horizontal branch lengths and the *scale bar* represents changes per amino acid. Protein sequence accession numbers are included in the tree

To extend analysis of this essential class of enzymes even further, we have evaluated the DNA ligase from *F. acidarmanus* Fer1, the most extreme acidophile yet studied. We characterized a recombinant version of the DNA ligase by analysing the in vitro nick-joining activity of the enzyme using a variety of double-stranded nucleic acids. The biochemical properties of the recombinant protein were similar to previously described ATP-dependent DNA ligases, though optimal activity was obtained at pH 6–7, close to the measured intracellular pH of the organism.

Material and methods

Growth of bacterial cultures

Details of bacterial strains and host plasmids used for cloning and protein expression have been described previously (Lavesa-Curto et al. 2004; Wilkinson et al. 2003; Wilkinson et al. 2005). Growth of *E. coli* was performed at a variety of temperatures on plates and in liquid cultures. In all cases, Luria broth (LB) was the nutrient media. Antibiotics were added to media as required, with final concentrations of ampicillin at 100 μg ml^{-1} and kanamycin at 50 μg ml^{-1}. Stock cultures containing 25% glycerol were stored at –80°C and used to streak on to fresh LB-agar plates as required. Bacterial cells were made chemically competent for DNA transformation and stored in 200 μl aliquots at –80°C (Sambrook and Russell 2001).

Bioinformatic analyses

The genome sequence of *F. acidarmanus* Fer1 was obtained from http://www.genome.jgi-psf.org/draft_microbes/ferac/ferac.home.html. BLAST analysis was

performed upon sequences contained within the NCBI database (http://www.ncbi.nlm.nih.gov/). For phylogenetic analysis the DNA ligase amino acid sequences were managed using ARB, a software environment for sequence data (Ludwig et al. 2004). Multiple sequence alignments were performed using ClustalW. Evolutionary distance and tree topology was estimated using the neighbour-joining method and this was repeated by the maximum likelihood method using Dayhoff's model and star decomposition.

Cloning of *Ferroplasma acidarmanus* Fer 1 DNA ligase

Cloning of the predicted DNA ligase from *F. acidarmanus* Fer1 was performed following the strategies employed for bacterial DNA ligases described previously (Lavesa-Curto et al. 2004; Wilkinson et al. 2003; Wilkinson et al. 2005). The gene was amplified by PCR with a proof-reading DNA polymerase from genomic DNA using the following primers:

''Forward primer'': 5′-CAT ATG ACA AAA TCT TAT AAT ATA CTA TAT G-3′.

''Reverse primer'': 5′-GGA TCC TTA TTT TGT TTT TTT CTG CAT TTT ATA AAG-3′.

Proteins were over-expressed from pET-16b (Novagen) and contained a 10-His tag within 21 additional amino acids (2.5 kDa) at the N-terminus. To allow over-expression of proteins in *E. coli* GR501, the full-length gene plus the His-tag were excised from pET-16b vectors using the *Nco*I and *BamH*I sites and cloned into pTRC99A (Amersham Pharmacia), as described previously (Lavesa-Curto et al. 2004; Wilkinson et al. 2003; Wilkinson et al. 2005).

Protein purification

For protein expression, all *E. coli* cultures were grown in LB containing ampicillin and kanamycin. Initial experiments identified that over-expressed FaLig was highly insoluble in *E. coli*. The amount of soluble protein was not increased despite testing a wide range of growth conditions, including temperatures from 16 – 37 °C, slower rates of shaking, different concentrations of IPTG, cold- and heat-shock, and strains encoding extra copies of tRNA genes that are rare in *E. coli* (data not shown). Some soluble FaLig was obtained when the pET16b derivatives were transformed into *E. coli* BL21 (DE3) harbouring pOFX-bad-KJ1, which expresses the *E. coli* chaperone proteins DnaK and DnaJ under the control of the pBAD promoter (Castanie et al. 1997). After transformation, cells were plated on LB-agar containing antibiotics and grown overnight at 37°C. Single colonies were inoculated into 10 ml liquid media, grown overnight at 37°C and diluted 100-fold into fresh media (1 l). After growth at 25°C to mid log phase (OD_{600}=0.5), expression of chaperones from pOFX-bad-KJ1 was induced by addition of 0.2% L-arabinose and expression of FaLig was induced by addition of IPTG to 0.4 mM. After incubation at 25°C for 20 h, cells were harvested, sonicated and centrifuged to separate soluble and insoluble fractions. Proteins were purified from the soluble fraction using columns with affinity for the His-tag (Novagen His•Bind®). Fractions containing the purified protein were confirmed by SDS-PAGE, pooled together in volumes of 2.5 ml and the buffer was exchanged using disposable PD-10 desalting columns (Amersham Biosciences, UK), with the proteins being eluted in 20 mM Tris, pH 7.5, 200 mM NaCl. Protein concentrations within cell extracts were determined by the Bradford method (Bio-Rad Protein Assay). Since low amounts of FaLig were obtained, the amount of purified FaLig was estimated from comparison with known amounts of bovine serum albumin after electrophoresis and silver staining of SDS-PAGE (Sambrook and Russell 2001). In general, 40 μg of FaLig was obtained from each litre of induced culture. For long-term storage at –80°C, glycerol was added to a final concentration of 25% (v/v).

Analysis of ligation activity

In vitro assays of ligation activity were performed using the nicked, 40 bp DNA described previously (Lavesa-Curto et al. 2004; Wilkinson et al. 2003; Wilkinson et al. 2005). Additional experiments assessed the activity of FaLig with a variety of nicked, 20 bp RNA:DNA hybrids (Bullard and Bowater 2006). Oligonucleotides were purchased from MWG-Biotech, Germany. The nicked 40-bp substrates were used in end-point and time-course assays of the in vitro ligation activity. The buffer used for standard reactions was 100 mM Tris–acetate, pH 6.5, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP. End-point reactions were performed as follows: 0.66–1.28 pmoles ligase, 22.5 pmol oligonucleotide substrate in a total volume of 10 μl. The reaction mix was incubated at 30°C for 18 h then stopped using 10 μl formamide stop solution (Sambrook and Russell 2001). The effect of pH on the extent of ligation was examined by performing assays in reaction buffer as indicated above except that it contained Tris-acetate (pH 4–7) or Tris–HCl (pH 7–9); measurements of the pH of all solutions were performed at 20°C. Time-course reactions were performed as follows: 17.9 pmol ligase, 315 pmoles oligonucleotide substrate in a total

volume of 140 µl. The reaction mix was incubated at 30°C, with 10 µl aliquots being removed at various time points and added to 10 µl formamide stop solution. Reactions were also performed with a variety of nucleotides and divalent metals at the described concentrations in 100 mM Tris-acetate, pH 6.5, 20 mM DTT. In many reactions, the extent of ligation by T4 DNA ligase was compared using optimal amounts as identified previously (Bullard and Bowater 2006).

At the end of all ligation experiments, samples were heated in a 95°C heating block for 5 min and analysed on a 15% polyacrylamide-urea gel (8.3 × 6.2 cm) in 0.5 × TBE. Reaction products on the gel were visualized using a Bio-Rad Molecular Imager FX. Quantitation was performed using the public domain NIH Image/J program (available at http://www.rsb.info.nih.gov/nih-image/). For time-course experiments, the extent of ligation was expressed as mole of ligated product per mole of ligase per minute of reaction time. The initial rates of reactions were measured from the extent of ligation in the linear section of the plotted data.

To assay for ligation activity in vivo, we used *E. coli* GR501, which has a temperature-sensitive mutation in *ligA* (Dermody et al. 1979; Lavesa-Curto et al. 2004). Cells were transformed with p*Trc99A* containing *falig* downstream of an inducible promoter. To assist production of soluble FaLig, pOFX-bad-KJ1 was also transformed into the cells and the chaperone proteins DnaK and DnaJ were induced by addition of 0.2% L-arabinose (Castanie et al. 1997). Following strategies described previously (Lavesa-Curto et al. 2004), growth was assessed at 30 and > 43°C.

Results

Identification of an ATP-dependent DNA ligase within the genome of *Ferroplasma acidarmanus* Fer1

Ferroplasma acidarmanus Fer1 has a genome of 1.8 Mbp, which is 73% A+T base-pairs and encodes 1,713 candidate proteins (see http://www.genome.jgi-psf.org/draft_microbes/ferac/ferac.home.html). Annotation of the genome identified a homologue of an ATP-dependent DNA ligase (EC 6.5.1.1) encoded by gene 542 (NCBI accession number ZP_00610046). This is the only open reading frame in *F. acidarmanus* Fer1 predicted to be a functional DNA ligase so, for convenience, we refer to this gene as *falig* and its protein product as FaLig. Three regions of the predicted amino acid sequence have significant similarity to domains within the Conserved Domain Database (CDD) (Marchler-Bauer et al. 2005) (Fig. 1a). Each of these domains is found in other DNA ligases, as indicated by their Pfam nomenclature (Finn et al. 2006): Pfam04675 relates to a conserved N-terminal region present in many, but not all, ATP-dependent DNA ligases; Pfam01068 relates to the conserved catalytic (adenylation) domain of DNA ligases; Pfam04679 relates to a conserved C-terminal region present in many, but not all, ATP-dependent DNA ligases. Interestingly, BLAST analysis of FaLig at the NCBI database identified that eukaryotic sequences have relatively high similarity to FaLig. Among these, the closest homologs, at approximately 30% identity, were several types of mammalian DNA ligase I, which participate in DNA replication.

Along with RNA ligases and mRNA capping enzymes, DNA ligases form a group of enzymes known as covalent nucleotidyl transferases. These enzymes are defined by the essential lysine residue situated within motif I, the first of a series of conserved colinear motifs (Doherty and Suh 2000; Shuman and Lima 2004; Tomkinson et al. 2006). Closer examination of the sequences within the motifs of FaLig confirmed that it contains all amino acids that are essential for the function of nucleotidyl transferases (Shuman and Lima 2004) and all amino acids that are highly conserved in predicted bacterial ATP-dependent DNA ligases (Wilkinson et al. 2001) (Fig. 1a). Previous analysis suggests that enzymes from Crenarchaea are homologues of Eukaryotic DNA ligase I, whilst the versions from Euryarchaea are more similar to ATP-dependent enzymes from some Viruses (Lai et al. 2002). Interestingly, motif V of DNA ligases from the *Thermoplasmatales*, which includes FaLig, are more similar to DNA ligases from Crenarchaeota compared to those from Euryarchaeota (Fig. 1b). This relationship is supported by phylogenetic analysis of the complete DNA ligase sequences (Fig. 1c). ATP-dependent DNA ligases and the classical bacterial NAD^+-dependent enzymes are distant in evolutionary terms (Nakatani et al. 2000). Thus, the amino acid sequences of the NAD^+-dependent DNA ligases from *E. coli* and *Streptomyces coelicolor* (Wilkinson et al. 2003) were used as an out-group to improve confidence in the phylogenetic description. To allow comparison with ATP-dependent DNA ligases from other organisms, sequences from human DNA ligase I and bacteriophages T4 and RB43 were also included. In agreement with an earlier, more-detailed phylogenetic description (Nakatani et al. 2000), our analysis confirms that all archaeal ATP-dependent DNA ligases have a closer evolutionary relationship to each other than to other

ATP-dependent DNA ligases. In addition, our analysis shows that *Thermoplasmatales* are grouped in a clade that is separated from the other Euryarchaeota but branches with the Crenarchaeota (Fig. 1c). This is not congruent with the general evolutionary relationship of the *Thermoplasmatales* that places these organisms in the Euryarchaeota (Dopson et al. 2004; Ruepp et al. 2000). However, the tree topology was supported by both the neighbour-joining and the maximum likelihood analyses and suggests lateral gene transfer has occurred during the evolution of the DNA ligase genes of the Euryarchaeota.

In summary, bioinformatic and phylogenetic analysis of the predicted sequence of FaLig suggests that it is likely to be an ATP-dependent DNA ligase that has characteristics of the Eukaryotic class of enzymes that participate in DNA replication.

Purification of FaLig and confirmation of ATP-dependent DNA ligase activity

No detailed experimental analysis of a DNA ligase from such an extreme acidophile has yet been reported, so it is unclear if such a hostile environment affects the biochemical activity of these enzymes. To allow biochemical assessment of the activity of this class of enzymes, we wished to determine the ability of a recombinant form of FaLig to join nicks in double-stranded nucleic acids. The gene for FaLig was cloned into the bacterial expression vector pET-16b, over-expressed and purified by affinity chromatography to an in-frame N-terminal His-tag, as has been used in studies of a number of different nucleic acid ligases (Ho and Shuman 2002; Ho et al. 2004; Lavesa-Curto et al. 2004; Nandakumar et al. 2004; Nandakumar and Shuman 2004; Wilkinson et al. 2003; Wilkinson et al. 2005; Yin et al. 2003; Yin et al. 2004). Including the addition of the His-tag from pET-16b, the total molecular weight of FaLig is 70.3 kDa. Preliminary expression analysis of FaLig induced in several derivatives of *E. coli* BL21 identified that it was almost totally insoluble (data not shown). Since the gene has 61% A+T base pairs it is rather atypical for *E. coli*, and the use of some unusual codons may lead to problems with folding that could be a factor in producing insoluble protein. Solubility of proteins was improved by growth at 25°C and inclusion of plasmids expressing *E. coli* chaperonins (Castanie et al. 1997), but it was possible to obtain only relatively small amounts of FaLig; in the experiments reported here the concentration of His-tagged FaLig was approximately 12 μg/ml. Analysis on SDS-PAGE showed the final sample contained a number of polypeptides (Fig. 2), as is typical for samples prepared using the assistance of induced chaperonins (Castanie et al. 1997; Chen et al. 2003; Goenka and Rao 2001). Western blot analysis using an antibody to *E. coli* LigA (Lavesa-Curto et al. 2004) did not detect the presence of this NAD^+-dependent DNA ligase in the preparation of FaLig (data not shown).

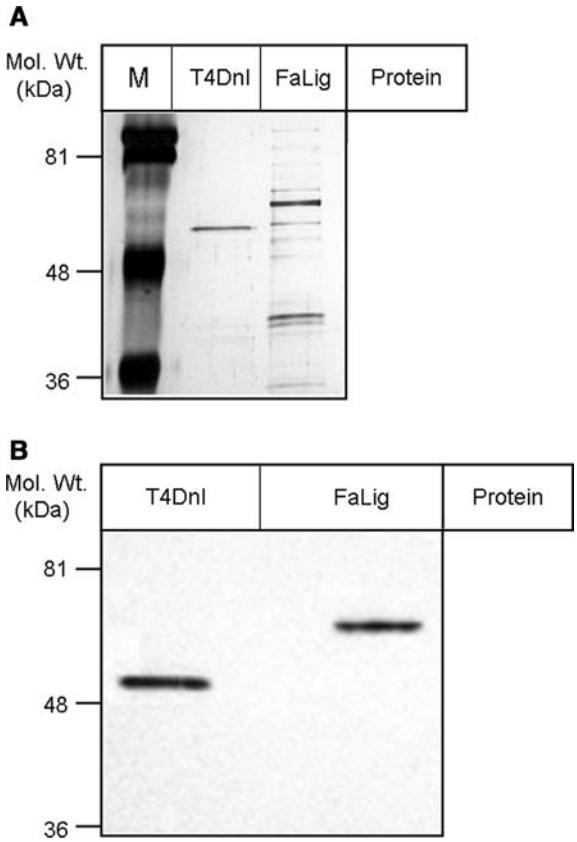

Fig. 2 Purified recombinant *Ferroplasma acidarmanus* DNA ligase. SDS-PAGE analysis of FaLig and T4DnI used during this study. Purified proteins were analyzed by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were identified by silver staining (**a**) or western blot using a monoclonal antibody to the His-tag (**b**). Molecular weights refer to proteins contained in a marker included on the same gel

The standard biochemical assay of nucleic acid ligation activity uses denaturing gel electrophoresis to detect increases in length of an oligonucleotide upon ligation. The nick-joining activity of FaLig was assessed using the substrate described previously (Lavesa-Curto et al. 2004; Wilkinson et al. 2003; Wilkinson et al. 2005), consisting of a 40-bp double-stranded oligonucleotide containing a gap between bases 18 and 19 of one strand. In all experiments we compared the activity of FaLig with a recombinant form of the ATP-dependent DNA ligase from bacteriophage T4 (T4DnI), which has been well characterized as being efficient in joining nicks in this substrate (Bullard and Bowater

2006). Preliminary experiments confirmed that FaLig harboured ATP-dependent DNA ligase activity under conditions that are used for T4 DNA ligase obtained from commercial sources, i.e. pH 7.5, 1 mM ATP (data not shown).

From this confirmation that FaLig is a DNA ligase, it is to be expected that it will join nicks that occur during replication—since it is the only DNA ligase encoded within its genome. To assess this function of the protein, we expressed the recombinant protein in *E. coli* GR501. This strain contains a temperature-sensitive mutation in chromosomal-encoded *E. coli* LigA (Dermody et al. 1979), which prevents growth on LB-agar plates at temperatures of 42 °C and above. Use of this strain in complementation experiments with a plasmid-expressed DNA ligase has proved useful for analysis of *in vivo* activity of a variety of DNA ligases, including human DNA ligase I, which is ATP-dependent (Kodama et al. 1991; Lavesa-Curto et al. 2004; Wilkinson et al. 2003, 2005). To assist production of soluble FaLig in *E. coli* GR501, the chaperone proteins DnaK and DnaJ were also induced from pOFX-bad-KJ1 (Castanie et al. 1997). Induction of the chaperone proteins allowed slight growth of *E. coli* GR501 on LB-agar plates, even up to 45°C, though the extent of growth was much reduced compared to strains expressing wild-type *E. coli* LigA. Over-expression of FaLig in *E. coli* GR501 allowed more vigorous growth of the host compared to over-expression of proteins without DNA ligase activity (data not shown). This observation is consistent with FaLig being able to seal nicks produced during replication of DNA in *E. coli*.

Biochemical characterization of nick-joining by FaLig

The most remarkable aspect of the physiology of *F. acidarmanus* Fer1 is its ability to grow at very low pH. Therefore, to assess whether pH might be an important influence on its ability to ligate DNA, we characterized the activity of FaLig and T4Dnl across a range of pH. Experiments were performed at 30°C from pH 4–9 using two different buffers (Ho and Shuman 2002; Ho et al. 2004; Yin et al. 2003): experiments at pH 4–7 were performed with Tris–acetate (Fig. 3a) and pH 7–9 were performed with Tris–HCl (Fig. 3b). All other components of the reaction were unchanged. Note that ligation was only detected in reactions containing buffer, cations and ATP and could not, therefore, be provided by residual NAD^+-dependent DNA ligase from the *E. coli* cell extract. Quantitation of the extent of ligation identified that T4Dnl had optimal nick-joining activity at pH 7–8, in broad agreement with that identified previously for T4Dnl (Bullard and Bowater, 2006). By contrast, for FaLig optimal nick-joining activity was observed at pH 6–7 (Fig. 3c).

The majority of archaeal DNA ligases that have been experimentally analysed use ATP as the co-factor for ligation, although those from some Euryarchaeota can also use other nucleotides (Jeon and Ishikawa 2003; Nakatani et al. 2000; Rolland et al. 2004; Zhao et al. 2006). To assess the situation with FaLig, we analysed nick-joining in the presence of a variety of co-factors (Fig. 4a). Ligation was only observed in the presence of ATP or, to a lesser extent, dATP. The lack of ligation in the presence of NAD^+ confirmed that the nick-joining activity was not due to the DNA ligase encoded on the *E. coli* chromosome. The concentration of the nucleotides used in the reaction was varied to provide a more detailed analysis of co-factor utilization (Fig. 4b). This experiment showed that optimal activity was obtained at concentrations equal and greater than 0.5 mM ATP; no inhibition of activity was observed up to 4 mM ATP. This analysis demonstrated that the maximal activity of FaLig in the presence of dATP was about 50 times less compared to that obtained with ATP. We also determined the nick-joining activity in the presence of a variety of cations (Fig. 5). Using the standard buffer conditions for FaLig, efficient nick-joining was observed in the presence of Mg^{2+} and Mn^{2+}, but no ligation was detected in the presence of Ca^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+} or Zn^{2+}.

To allow further comparison with previously studied DNA ligases, the rate of nick-joining by FaLig was determined. The extent of ligation at 30 °C was analysed at various times (Fig. 6a). The amount of ligation at each time-point was quantitated and the initial rates of nick-joining were determined for FaLig and T4Dnl under the same conditions (Fig. 6b). For FaLig, the extent of nick-joining increased linearly during a period of 2 h, giving a rate during this period of approximately 0.2 ligation events min^{-1}. The reaction with T4 Dnl occurred faster, with a maximal rate of approximately 2 ligation events min^{-1} during the initial 10 min. Thus, FaLig joins nicks in dsDNA approximately 10 times less efficiently than T4Dnl.

Recent studies identify that DNA ligases may join breaks in a variety of double-stranded nucleic acids (Martins and Shuman 2004; Pascal et al. 2004; Sekiguchi and Shuman 1997; Sriskanda and Shuman 1998; Tomkinson et al. 2006), with the range of active substrates varying compared to enzymes characterized as ''RNA'' ligases (Bullard and Bowater 2006). To assess how FaLig fitted into these categories, we analysed its nick-joining activity on a range of substrates. Each of

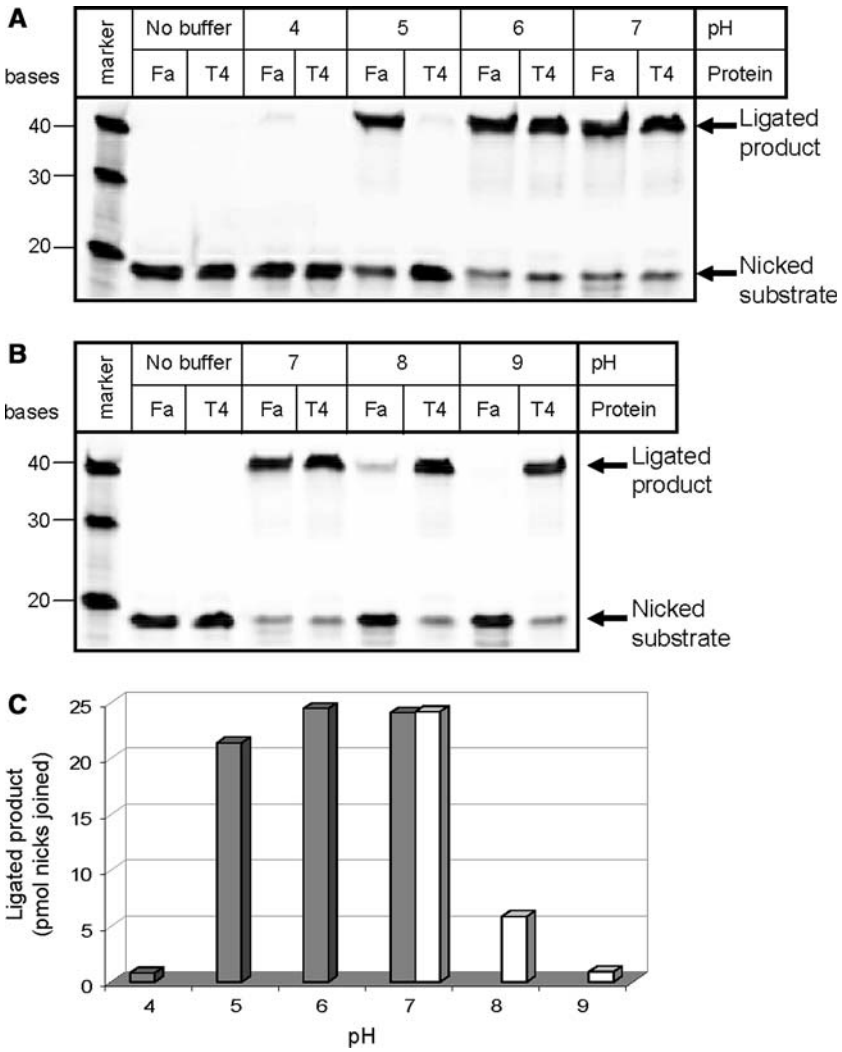

Fig. 3 Effect of pH on the nick-joining activity of *Ferroplasma acidarmanus* DNA ligase. Nicked 40-bp substrates were used in assays of the in vitro ligation activity of DNA ligase from *F. acidarmanus* Fer1 (FaLig) and bacteriophage T4 (T4Dnl) at various pH. The buffer for reactions was 100 mM Tris–acetate or Tris–HCl at the appropriate pH containing 20 mM $MgCl_2$, 20 mM DTT and 2 mM ATP. Reactions were performed in 10 µl for 18 h at 30°C with 1.28 pmol of FaLig or 1 pmol of T4Dnl. Samples were analysed on a denaturing polyacrylamide gel. The marker contained a mixture of fluorescein-labelled oligonucleotides of the specified size. The *lanes* referred to as ''No buffer'' are negative controls to assess the effect of buffer constituents and contained only protein, DNA and water. **a** Buffer used for all reactions contained Tris–acetate at pH 4–7. **b** Buffer used for all reactions contained Tris–HCl at pH 7–9. **c** Quantitation of extent of ligation by FaLig. Experiments performed with Tris–acetate and Tris–HCl are shown by the *grey* and *white bars*, respectively

the three individual strands used in substrate preparation was used as ribo- or deoxyribonucleotides. Appropriate mixing of each strand produced eight different combinations of double-stranded, nicked nucleic acids (Fig. 7a). These consisted of DNA only (substrate 1), RNA only (substrate 2) and a variety of DNA:RNA hybrids (substrates 3–8). End-point ligation analysis identified that FaLig was able to join the nicks in substrate 1 (dsDNA) most efficiently and that it also had significant activity with substrate 7, which has RNA as the donor of the 3'-OH at the nick (Fig. 7b). The same range of substrates was ligated by T4Dnl (Bullard and Bowater 2006), thus confirming that FaLig is effectively a functional ''DNA'' ligase.

Discussion

Due to their fundamental role during replication, DNA ligases are likely to have been required within the earliest cells. Although there are two types of enzyme that use different co-factors during ligation and there are distinct versions in the different kingdoms of life, it is clear that all DNA ligases harbour similar ''core''

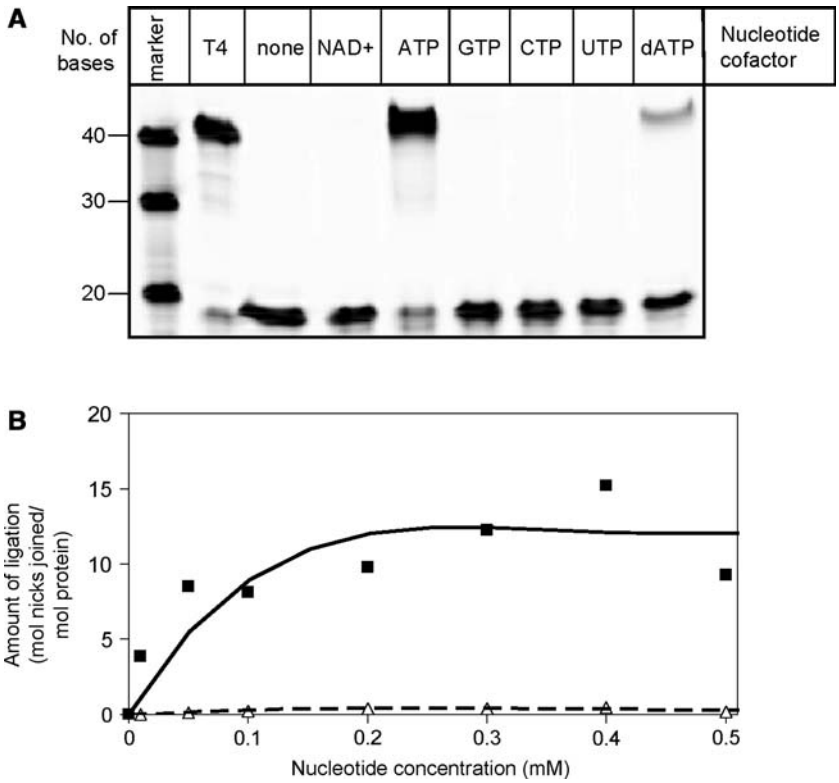

Fig. 4 Co-factor requirements for the nick-joining activity of *Ferroplasma acidarmanus* DNA ligase. Nicked 40-bp substrates were used to assess the effect of nucleotide on in vitro ligation activity of *F. acidarmanus* Fer1 DNA ligase (FaLig). **a** Reactions were performed in a total volume of 10 µl (100 mM Tris–acetate, pH 6.5, 20 mM $MgCl_2$, 20 mM DTT) for 18 h at 30°C with 1.28 pmol of FaLig using the specified co-factor at 2 mM, or 52 µM for NAD^+. All samples were analysed on a denaturing polyacrylamide gel. The marker contained a mixture of fluorescein-labelled oligonucleotides of the specified size. **b** Reactions were performed in a total volume of 10 µl for 18 h at 30°C with 1.28 pmol of FaLig using various concentrations of ATP (*filled squares*) or dATP (*open triangles*). The extent of ligation in each sample was quantitated from analysis of the samples on a denaturing polyacrylamide gel. The *lines* represent an approximate best fit through the data points

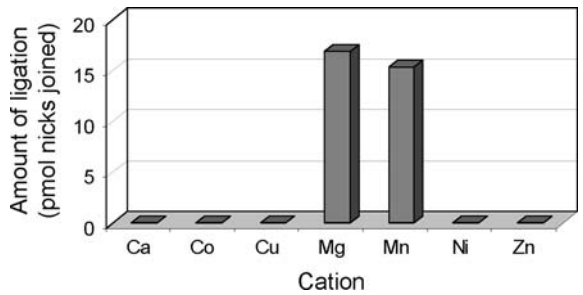

Fig. 5 Cation requirements for the nick-joining activity of *Ferroplasma acidarmanus* DNA ligase. Nicked 40-bp substrates were used to analyse the effect of cation on the in vitro ligation activity of *F. acidarmanus* Fer1 DNA ligase (FaLig). Reactions were performed in a total volume of 10 µl (100 mM Tris–acetate, pH 6.5, 20 mM DTT, 2 mM ATP) for 18 h at 30°C with 1.28 pmol of FaLig using the specified cation at 10 mM. After analysis on a denaturing polyacrylamide gel, the extent of nick-joining was quantitated

features (Shuman and Lima 2004; Tomkinson et al. 2006; Wilkinson et al. 2001). Recently, however, characterization of DNA ligases from some archaea have identified unexpected features, particularly in relation to the role of the co-factor in enzyme function (Jeon and Ishikawa 2003; Nakatani et al. 2000; Rolland et al. 2004; Zhao et al. 2006). We have cloned and characterized the single DNA ligase encoded by the genome of *F. acidarmanus* Fer1, an archaeon that is viable at extremely acidic conditions.

Bioinformatic analysis of the gene sequence suggested that gene 542 (*falig*) of the *F. acidarmanus* Fer1 genome would be an ATP-dependent DNA ligase and our *in vitro* biochemical studies using recombinant protein confirmed this to be the case. The bioinformatic analysis suggests that FaLig is likely to exhibit a modular three-dimensional structure, closely related to those obtained for other DNA ligases (Doherty and Suh 2000; Shuman and Lima 2004; Timson et al. 2000; Tomkinson et al. 2006). We observed that FaLig favoured Mg^{2+} over other divalent cations, though significant activity was retained with Mn^{2+}. FaLig was also able to join nicks between a ribonucleotide containing

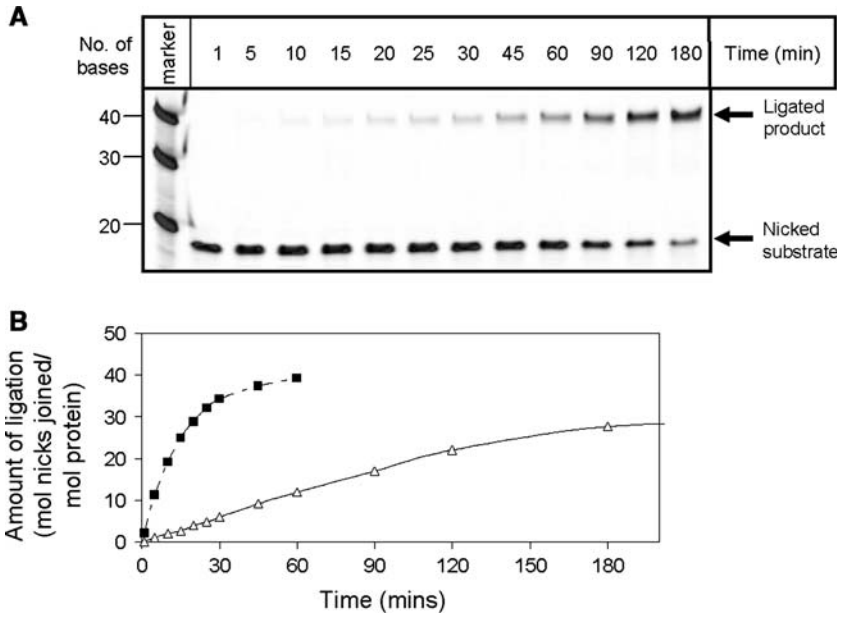

Fig. 6 Time-course analysis of the nick-joining activity of *Ferroplasma acidarmanus* DNA ligase. Nicked 40-bp substrates were used to determine the rates of in vitro ligation activity of DNA ligase from *F. acidarmanus* Fer1 (FaLig) and bacteriophage T4 (T4Dnl). **a** Representative reaction performed with FaLig for various times at 30°C. Reactions contained 315 pmol of substrate and 18 pmol of FaLig in a total volume of 140 µl. At each time-point, 10 µl of sample was removed and the reaction stopped. All samples were analysed on a denaturing polyacrylamide gel. The marker contained a mixture of fluorescein-labelled oligonucleotides of the specified size. **b** The extent of nick-joining was quantitated for T4Dnl (*filled square*, *dashed line*) and FaLig (*open triangle*, *solid line*)

the 3′-hydroxyl and deoxyribonucleotide containing the 5′-phosphate. These effects on biochemical activity have been observed for other nucleic acid ligases, including human DNA ligase I (Pascal et al. 2004), the bacterial cellular enzyme DraRnl from *Deinococcus radiodurans* (Martins and Shuman 2004) and viral enzymes *Chlorella* PBCV-1 DNA ligase (Sriskanda and Shuman 1998), *Vaccinia* DNA ligase (Sekiguchi and Shuman 1997) and T4 RNA ligase 2 (Nandakumar et al. 2004; Nandakumar and Shuman 2004; Nandakumar and Shuman 2005).

In most facets that we examined, FaLig was observed to be a rather typical ATP-dependent DNA ligase. This is not particularly surprising since it is the only DNA ligase encoded within the genome and it must, therefore, act in fundamental metabolic pathways, such as joining nicks at Okazaki fragments. As might be expected from such cellular requirements, FaLig has high levels of homology to eukaryotic DNA ligases that participate in DNA replication. Phylogenetic analysis of the sequences presented here is consistent with previous studies of DNA ligases (Nakatani et al. 2000). However, the addition of amino acid sequences for FaLig and related enzymes from other *Thermoplasmatales* identified that these are more similar to DNA ligases that have been characterized from Crenarchaeota (Jeon and Ishikawa, 2003; Kletzin 1992; Lai et al. 2002) compared to those from Euryarchaeota (Gunther et al. 2002; Keppetipola and Shuman 2005; Nakatani et al. 2000; Rolland et al. 2004; Sriskanda et al. 2000; Zhao et al. 2006). This phylogenetic description suggests that lateral gene transfer has occurred in the evolution of the DNA ligases of the Euryarchaeota. Detailed analysis of additional archaeal genome sequences will be required to resolve whether this observation is only associated with DNA ligases or whether additional genes have undergone such evolution.

The most significant result from this biochemical study is that optimal nick-joining by FaLig occurs at pH 6-7. Similar observations were made for an unusual type of DNA repair protein from *F. acidarmanus* Fer1 (Kanugula et al. 2005). These results suggest that at least some of the enzymes involved in DNA metabolism are optimized to function in the intracellular environment of this organism, which has been measured to be at pH 5.6 (Macalady et al. 2004). The situation may not be the same for all proteins, since a recent in vitro study suggests some intracellular or membrane-bound proteins of *F. acidiphilum*—a close relative of *F. acidarmanus* Fer1—have optimal activity in the pH range 2–4 (Golyshina et al. 2006). The precise explanation for the variations in observed pH optima in these different studies is unclear. It is possible that proteins experience different pH conditions

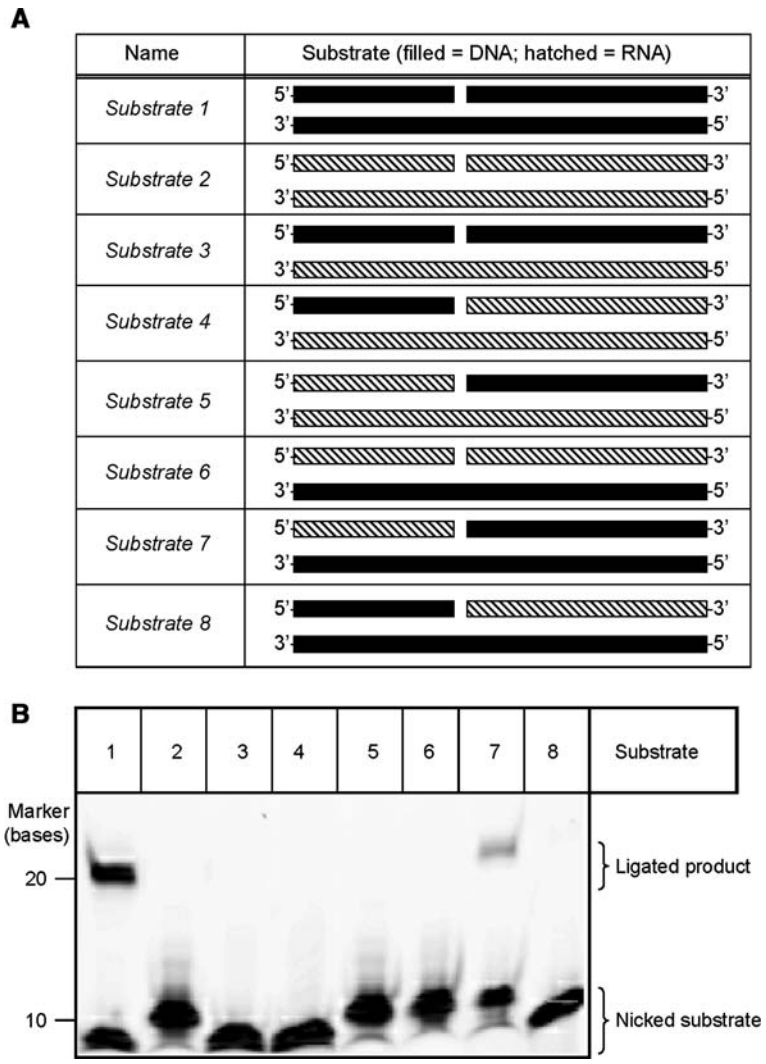

Fig. 7 Substrate specificity of *Ferroplasma acidarmanus* DNA ligase. The nick-joining activity of *F. acidarmanus* Fer1 DNA ligase (FaLig) was tested on 20 bp double-stranded substrates containing differing fragments of DNA and RNA. **a** Schematic diagram of the substrates used in the ligation assay, with DNA and RNA being represented by *filled and hatched boxes*, respectively. **b** In vitro ligation assays performed in a total volume of 5 µl (50 mM Tris–acetate, pH 6.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP) for 18 h at 30°C with 0.66 pmol of FaLig and the various substrates (45 pmol). Samples were analysed on a denaturing polyacrylamide gel

in *F. acidiphilum* and *F. acidarmanus* Fer1, but this seems unlikely given that all available evidence suggests that the organisms are extremely closely related. Perhaps more likely is that the differences are due to the studies of different types of enzymes and that this may indicate that different compartments within the organisms experience different pH (Golyshina et al. 2006). In this case, the pH optima of enzymes acting on DNA are closest to neutral (Kanugula et al. 2005), perhaps suggesting that the organisms have evolved mechanisms to protect their genome from harsh, acidic environments. A further explanation for the observations of different pH optima for proteins is that some of them may not be functioning at their optimal level within the cell. Further experimentation is required to identify whether these acidophilic organisms experience variation of pH within their cells or if some of the enzymes are not functioning at their optimal pH.

The pH profile of FaLig is similar to that observed for a variety of DNA ligases, but it is now clear that the optimal pH for activity varies for enzymes from different organisms. In terms of archaea that grow at neutral pH, the optimal activity of their DNA ligases has been detected to be pH 7–8.5 (Jeon and Ishikawa 2003; Keppetipola and Shuman 2005; Rolland et al. 2004; Sriskanda et al. 2000). For DNA ligase from *S. shibatae*, which is able to grow at pH 2–4, optimal nick-joining was detected at pH 6–7 (Lai et al. 2002). From these results it is tempting to speculate that those organisms that can grow in acidic environments have DNA ligases with pH optima that are more acidic. However, this is probably too simplistic, as indicated by the fact that there is a different pH optima for two nucleic acid ligases from bacteriophage T4, which will obviously experience the same cellular environment (Bullard and Bowater 2006).

Analysis of the activity of FaLig in the presence of different co-factors identified optimal ligation with ATP, with a low-level of activity with dATP. Similar observations were made with the DNA ligase from *Methanobacterium thermoautotrophicum* (Sriskanda et al. 2000). Extensive ligation by FaLig was detected in the presence of Mg^{2+} or Mn^{2+}, as observed with many other DNA ligases (Shuman and Lima 2004; Tomkinson et al. 2006). *F. acidarmanus* Fer1 can grow in the presence of high concentrations of a wide range of metals, but our in vitro experiments only detected nick-joining activity in the presence of Mg^{2+} or Mn^{2+}. Further studies will be required to assess whether FaLig may be active in the presence of other cations under cellular conditions present within *F. acidarmanus* Fer1.

In summary, this study describes the cloning and characterization of the single DNA ligase encoded by the genome of *F. acidarmanus* Fer1. In providing this characterization of a DNA ligase from an extreme acidophile, knowledge of these essential proteins is extended to another phylogenetic family.

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