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NOTE

Aldehyde dehydrogenase of the haloalkaliphilic archaeon *Natronomonas pharaonis* and its function in ethanol metabolism

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Abstract The genome of *Natronomonas pharaonis* encodes genes annotated as alcohol dehydrogenase (ADH; EC 1.1.1.1) and aldehyde dehydrogenase (ALDH; EC 1.2.1.3), enzymes involved in alcohol metabolism. These genes (*adh* and *aldH2*) occur in a single copy on the chromosome. We have studied the role of these genes in ethanol metabolism in *N. pharaonis*. Reverse transcription-PCR analysis showed that the *aldH2* gene was inducible by ethanol, but the *adh* gene was transcribed both in the presence and absence of ethanol. The gene encoding for ALDH of *N. pharaonis* (*Np*ALDH) was cloned into a pET41a vector containing a glutathione S-transferase tag, expressed in *Escherichia coli* and purified by glutathione sepharose affinity chromatography. The GST-*Np*ALDH fusion protein was cleaved by bovine enterokinase and the target enzyme showed a molecular mass of approximately 60 kDa by SDS-PAGE. The enzyme was thermophilic and alkaliphilic, the optimal temperature and pH being 60°C and 8.0, respectively. *Np*ALDH was salt independent, being most active at 0.25 M NaCl or KCl.

Keywords *Natronomonas pharaonis* · Haloalkaliphilic · Aldehyde dehydrogenase · Reverse transcription-PCR · Molecular cloning

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Introduction

Natronomonas pharaonis is an excellent model for physiological analysis of extremely haloalkaliphilic Archaea. *N. pharaonis* was first isolated from highly saline soda lakes in Egypt, where it is exposed simultaneously to two forms of stress: hypersaline conditions and high pH (Soliman and Trüper 1982). Phylogenetically it belongs to the family *Halobacteriaceae* (Euryarchaeota). It grows optimally in the presence of 3.5 M NaCl and at pH 8.5. Its genome has been completely sequenced, and the genome-wide proteomics of *N. pharaonis* has been established (Aivaliotis et al. 2007; Falb et al. 2005; Konstantinidis et al. 2007). The *N. pharaonis* genome contains a 2.6-Mb chromosome and two megaplasmids, PL131 and PL23, which are about 131 and 23 kb in size, respectively (Falb et al. 2005). Cytosolic and membrane proteomic data for *N. pharaonis* have enabled the identification of a total of 1,226 proteins, which cover 43% of the theoretical proteome inferred from the genome sequence (Konstantinidis et al. 2007).

The genome of *N. pharaonis* encodes genes annotated as alcohol dehydrogenase (ADH; EC 1.1.1.1) and aldehyde dehydrogenase (ALDH; EC 1.2.1.3), two key enzymes involved in degradation of ethanol. These genes

(*adh* and *aldH2*) occur in a single copy on the chromosome and are regulated by different promoters (Falb et al. 2005). Proteomic analysis shows that both the ADH and ALDH are likely to be cytoplasmic proteins (Konstantinidis et al. 2007). The *N. pharaonis* alcohol dehydrogenase and the gene encoding it have been the subject of an earlier study (Cao et al. 2008). Here we report the properties of the aldehyde dehydrogenase and the expression of both genes involved in alcohol transformation in response to exposure of *N. pharaonis* to ethanol.

Materials and methods

Strains and culture conditions

Natronomonas pharaonis CGMCC 1.1965^T (=DSM 2160^T) was obtained from the China General Microbiological Culture Collection Center. *N. pharaonis* was grown at 40°C in APB medium which contained 7.5 g casamino acids, 10 g yeast extract, 3 g Na_3-citrate, 2 g KCl, 0.1 g $MgSO_4{\cdot}7H_2O$, 10 g Na_2CO_3, and 200 g NaCl per liter at pH 8.5. When indicated, ethanol (0.5 or 1%, vol/vol) was added. Growth was followed by measurement of the OD at 600 nm. To test whether ethanol can be used as a growth substrate, we reduced the yeast extract concentration to 0.25 g/L, omitted casamino acids, and added 0, 0.25 of 0.5% ethanol.

Gas chromatography analysis

Ethanol concentrations in culture supernatants were determined using a gas chromatograph with a flame ionization detector (GC-FID; Tecbcomp 7890 series, China) equipped with a 30-m, 0.32-mm internal diameter, PGE-20M flexible glass capillary column. Operating conditions were as following: oven temperature 120°C; inlet temperature 140°C; and detector temperature 180°C. Nitrogen was used as a carrier gas at a flow rate of 17 mL min^{-1}.

RT-PCR analysis

Natronomonas pharaonis was grown in two 100-mL flasks with 30 mL APB medium with and without 0.5% ethanol (vol/vol), at 40°C with shaking at 200 rpm. After 48, 72, and 96 h (toward the end of the exponential growth phase), 1-mL samples were removed to pre-chilled 1.5-mL microcentrifuge tubes, and cells were pelleted by centrifugation (11,000×*g*, 1 min, 4°C).

Total RNA was isolated according to the protocol of Dyall-Smith (2006). The RNA concentration was determined by measuring OD at 260 nm. One microgram of each RNA sample was digested with 2 units DNase I (TaKaRa, Japan) at 37°C for 30 min. Then, the RNA was precipitated with ethanol and resuspended in water treated with diethyl pyrocarbonate (Sangon, China). A mixture of primers of *adh* reverse, *aldH2* reverse, and universal 16S 1492R (Table 1; 50 ng of each), DNase I treated RNA

Table 1 Bacterial strains, plasmids, and primers used

Strain, plasmid, or primer	Genotypes, comments, or sequences (5′ → 3′)
Strain	
N. pharaonis	CGMCC 1.1965^T (=DSM 2160^T)
E. coli DH5α	Cloning host, F^- *φ*80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA*1 *endA*1 *hsdR*17(r_k^- m_k^+) *phoA supE*44 λ^- *thi*-1 *gyrA*96 *relA*1
E. coli Rosetta (DE3)	Expression host, F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm lacY1* (DE3) pRARE^6 (Cm^R)
Plasmid	
pET41a	Cloning vector containing the GST·Tag, *Kan* gene
pET-*Np*ALDH	1.5-kb *aldH2* fragment containing *Eco*RI and *Xho*I sites cloned in pET41a
Primer	
adh forward	ATGCGCGCTGTCGTCTTCGAG
adh reverse	TCAGAACTCGTCGCAGACCG
aldH2 forward	ATGTCTGTATCTGAACAGTAC
aldH2 reverse	TCAGTCCTTCTGTGTCCGGAT
archaeal 16S 2F	TTCCGGTTGATCCTGCCGGA
universal 16S 1492R	GGTTACCTTGTTACGACTT
aldH2 cloning forward	GCCGAATTCATGTCTGTATCTGAACAGTAC, the *Eco*RI recognition site is underlined
aldH2 cloning reverse	TCTCTCGAGTCAGTCCTTCTGTGTCCGGATATC, the *Xho*I recognition site is underlined

(5 μg) and dNTP (1 mM) was heated at 65°C for 5 min, then immediately chilled on ice for 5 min. Reverse transcription-PCR (RT-PCR) was performed using PrimerScript Transcriptase (TaKaRa, Japan) at 50°C for 1 h with the above-described mixture and RNase inhibitor (TaKaRa, Japan). After heating at 75°C for 15 min, the cDNA from the RT reaction was PCR amplified using LA Taq polymerase (TaKaRa, Japan), dNTP, and gene specific primers (Table 1; *adh*, *aldH2*, and archaeal 16S rRNA gene, respectively), for 30 cycles (20 s at 98°C, 30 s at 65°C, 1 min 30 s at 72°C). RT-PCR products were analyzed by electrophoresis in a 1% agarose gel and ethidium bromide staining. The 16S rRNA gene RT-PCR was used as positive control.

Construction of *Np*ALDH expression plasmid

The *Np*ALDH (GenBank accession No. YP327159) gene was amplified by PCR using *Pfu* DNA polymerase (Shenergy, China). The genomic DNA template was prepared from *N. pharaonis* cells. *Eco*RI and *Xho*I recognition sites were introduced by synthetic *aldH2* cloning primers (Table 1). The plasmid pET41a (Novagen, Germany) was digested with *Eco*RI/*Xho*I (TaKaRa, Japan). The PCR product was then ligated into the gap introducing an N-terminal GST tag using T4 DNA ligase (TaKaRa, Japan), resulting in the construction of expression vector pET-*Np*ALDH.

Soluble expression and purification of *Np*ALDH in *Escherichia coli*

pET-*Np*ALDH was transformed into *E. coli* Rosetta (DE3). The transformed Rosetta cells were grown in 2 L of Luria–Bertani medium (containing 20 μg mL^{-1} kanamycin) at 37°C. After an OD_{600} of 0.6–0.8 was reached, 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) was added. Following an induction period of 5 h at 30°C, the cells were harvested by centrifugation (7,000×*g*, 5 min, 4°C).

To avoid insoluble inclusion body generation, we followed the method of Frangioni and Neel (1993). The pellet of bacteria expressing the fusion protein was resuspended in cold TGE buffer (50 mM Tris, pH 8.0; 1 mM EDTA; 150 mM NaCl; 5% glycerol, vol/vol), and treated with 100 μg mL^{-1} lysozyme for 15 min on ice, followed by the addition of DTT to 5 mM. Sarkosyl (Sangon, China) was then added to the lysozyme-treated bacteria at a concentration of 0.25% (vol/vol). The cell suspension was sonicated at a low power (20 W; 1 s sonifying vs. 2 s pause) in ice until the viscosity disappeared. After centrifugation (18,000×*g*, 20 min, 4°C), the supernatant was amended with Triton X-100 at a concentration of 0.5% (vol/vol), and was mixed gently for 30 min at room temperature. The fusion protein-containing buffer was loaded onto a chromatography column filled with glutathione sepharose 4B beads (GE Healthcare, USA) and was washed extensively with PBS buffer (140 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4, pH 7.3) to remove unspecifically bound proteins. Subsequently the *Np*ALDH-GST fusion protein was eluted in glutathione elution buffer (50 mM Tris, pH 8.0; 10 mM reduced glutathione). The fusion protein was cleaved with 1 unit bovine enterokinase (Sinobio, China) at 37°C for 8 h. The purified protein was ultrafiltrated against buffer containing 50 mM Tris–HCl (pH 8.0), and was stored at −80°C until used.

The *Np*ALDH-containing fraction was analyzed by SDS-PAGE, using a 12% polyacrylamide gel, which was stained with Coomassie brilliant blue R250 (Laemmli 1970). Protein concentration was determined by the method of Bradford (1976), using bovine-serum albumin as standard.

Enzymatic assays

Standard enzymatic assays were performed by monitoring the substrate-dependent absorbance change of NADH at 340 nm using a Beckman Coulter DU 800 nucleic acid/protein analyzer as previously described (Ziegenhorn et al. 1976). Aldehyde dehydrogenase was assayed in 1 mL reaction mixtures containing 50 mM Tris–HCl (pH 8.0), 250 mM NaCl, 2 mM DTT, 0.4 mM NAD^+, 1% acetaldehyde (vol/vol), and enzyme. One enzyme unit was defined as the amount of *Np*ALDH that catalyzed the reduction of 1 μmol NAD^+ per minute. Assay conditions were modified with respect to temperature, pH, and salt concentrations as indicated in the experiments.

Results and discussion

To determine the effect of ethanol on the growth of *N. pharaonis*, the organism was cultured in media which ethanol concentrations up to 1%. As shown in Fig. 1a, *N. pharaonis* tolerated 0.5% ethanol, but did not grow at 1%. In medium containing 0.5% ethanol, *N. pharaonis* reached the mid-exponential phase after 96 h, compared to 60 h for the control without ethanol. In the culture grown with 0.5% ethanol, the ethanol concentration decreased much faster than in a control experiment without inoculum (Fig. 1b), indicating that the cells mediated transformation of ethanol. The slow decrease in ethanol concentration in the uninoculated medium may be attributed to evaporation. To complement the data shown in Fig. 1, we grew the organism without casamino acids and at a reduced concentration of yeast extract (0.025%) with different

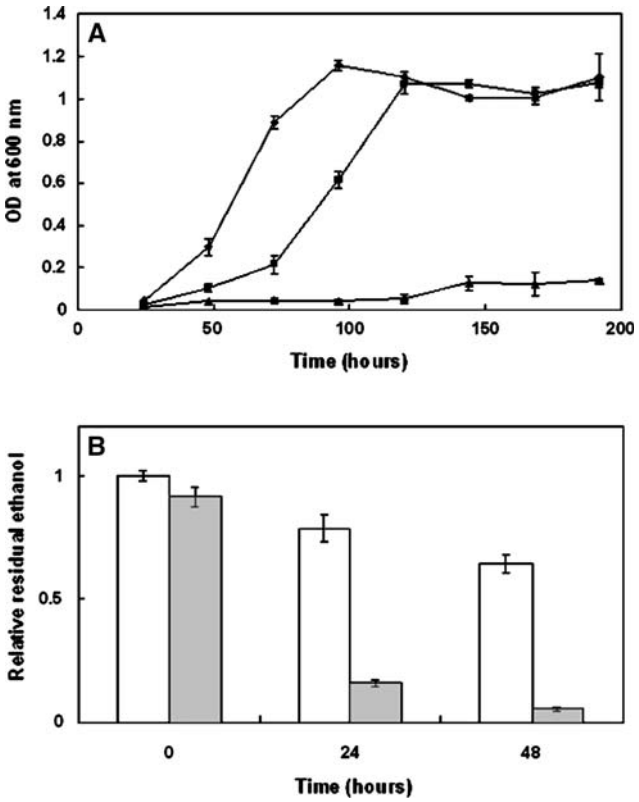

Fig. 1 Cultivation of *N. pharaonis* in media in the absence (*diamonds*) and in the presence of 0.5% (*squares*) and 1% ethanol (*triangles*) (**a**), and decrease of ethanol in the culture with 0.5% ethanol shown in **a** (*gray bars*) as compared to a control experiment in the absence of *N. pharaonis* cells (*white bars*) (**b**)

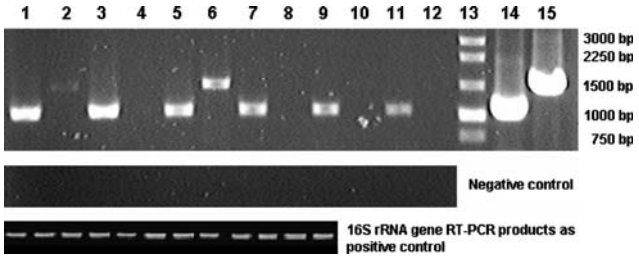

Fig. 2 RT-PCR analysis of *adh* and *aldH2* gene expression in *N. pharaonis*. a 1% agarose gel is shown after electrophoresis and staining with ethidium bromide. The RT-PCR products from RNAs (after DNase I treatment) were purified from *N. pharaonis* cultured in APB medium with 0.5% ethanol and APB medium without ethanol after different periods (48, 72, and 96 h) of growth, using *adh* and *aldH2* gene primers, respectively. *Lane 1* the *adh* RT-PCR products with ethanol at 48 h; *lane 2* the *aldH2* RT-PCR products with ethanol at 48 h; *lane 3* the *adh* RT-PCR products without ethanol at 48 h; *lane 4* the *aldH2* RT-PCR products without ethanol at 48 h; *lane 5* the *adh* RT-PCR products with ethanol at 72 h; *lane 6* the *aldH2* RT-PCR products with ethanol at 72 h; *lane 7* the *adh* RT-PCR products without ethanol at 72 h; *lane 8* the *aldH2* RT-PCR products without ethanol at 72 h; *lane 9* the *adh* RT-PCR products with ethanol at 96 h; *lane 10* the *aldH2* RT-PCR products with ethanol at 96 h; *lane 11* the *adh* RT-PCR products without ethanol at 96 h; *lane 12* the *aldH2* RT-PCR products without ethanol at 96 h; *lane 13* a DNA ladder marker; *lanes 14 and 15* contain the positive control PCR fragments using genomic DNA from *N. pharaonis* as template and *adh* and *aldH2* gene primers, respectively. The negative controls contain PCR products using the same RNAs as in *lanes 1–12*, respectively, after DNase I treatment but without reverse transcriptase. The 16S rRNA gene PR-PCR products were used as the positive controls

concentrations of ethanol (0, 0.25 or 0.5%). Addition of ethanol to the growth medium did not lead to increased growth yields, indicating that *N. pharaonis* could not efficiently utilize ethanol as carbon source.

To understand the regulation and expression patterns of the *adh* and *aldH2* genes in *N. pharaonis*, cells were grown in the presence and in the absence of ethanol, and transcripts were analyzed by RT-PCR. The two-step RT-PCR reactions were carried out on the purified RNA, using primers specific for the *adh* and *aldH2* genes. DNA bands of the expected size were obtained in each reaction (Fig. 2). The RT-PCR products with primers for the *adh* gene were of the expected size in both conditions. However, RT-PCR products of the *aldH2* gene were only detected in cells grown in the presence of ethanol in 48- and 72-h-old cultures, but disappeared when the cultures were 96-h old and hardly any ethanol was left in the medium. These data show that the *aldH2* gene encoding a putative aldehyde dehydrogenase is induced by ethanol.

Analysis of the genome sequence of *N. pharaonis* indicated that the putative *Np*ALDH gene includes a start codon (ATG) and a stop codon (TGA), is 1,518 bp in length, and encodes a protein of 505 amino acids. DNA sequencing showed that the *Np*ALDH gene cloned in the plasmid exhibited 100% identity to the coding region published in the database.

To generate large quantities of *Np*ALDH, *E. coli* Rosetta (DE3) carrying the plasmid pET-*Np*ALDH, which encodes a GST-*Np*ALDH fusion protein was grown in Luria–Bertani medium, and production of the recombinant enzyme was induced with IPTG. The GST-*Np*ALDH was purified by glutathione sepharose affinity chromatography methods and the target protein was cleaved with bovine enterokinase. A sharp protein (ALDH + GST) band located at 90 kDa was observed in the cell extract, and the expected 60 kDa fraction of *Np*ALDH was detected in 12% SDS-PAGE (Fig. 3). The yield of recombinant protein was 1.63 mg L^{-1} of culture.

The optimum temperature for *Np*ALDH activity was 60°C and the enzyme was inactive above 70°C (Fig. 4a). The recombinant enzyme displayed optimal pH at 8.0 and was inactive below pH 5.0 (Fig. 4b). Unlike most other enzymes isolated from halophilic organisms, *Np*ALDH was salt independent. The activity of *Np*ALDH was optimal at salt concentrations at 0.25 M NaCl or KCl, and only low activities were found at salt concentrations of 1 M and above (Fig. 4c). Salt was not required for long-term stability of the enzyme: following incubation for 24 h in the

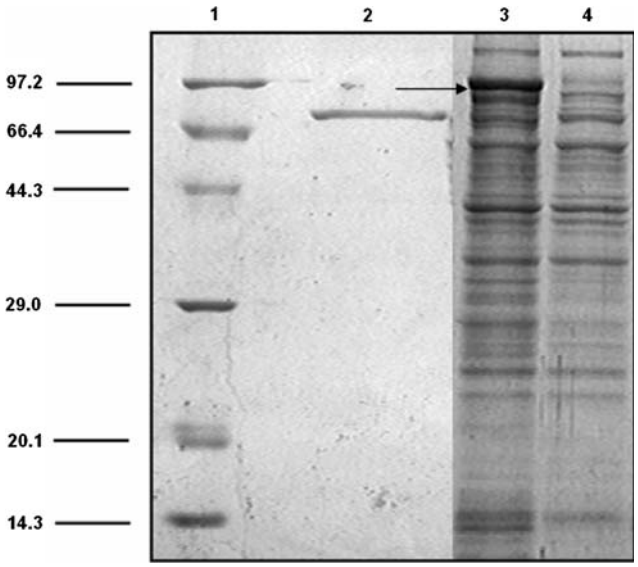

Fig. 3 SDS-PAGE of the recombinant protein. *Lane 1* protein molecular mass standards; *lane 2* purified recombinant *Np*ALDH; *Lane 3 E. coli* extract with fusion protein (ALDH + GST) induced by IPTG, indicated by a band at about 90 kDa (*arrow*); *Lane 4* extract of *E. coli* not induced by IPTG as negative control. Molecular mass standards are indicated in kDa

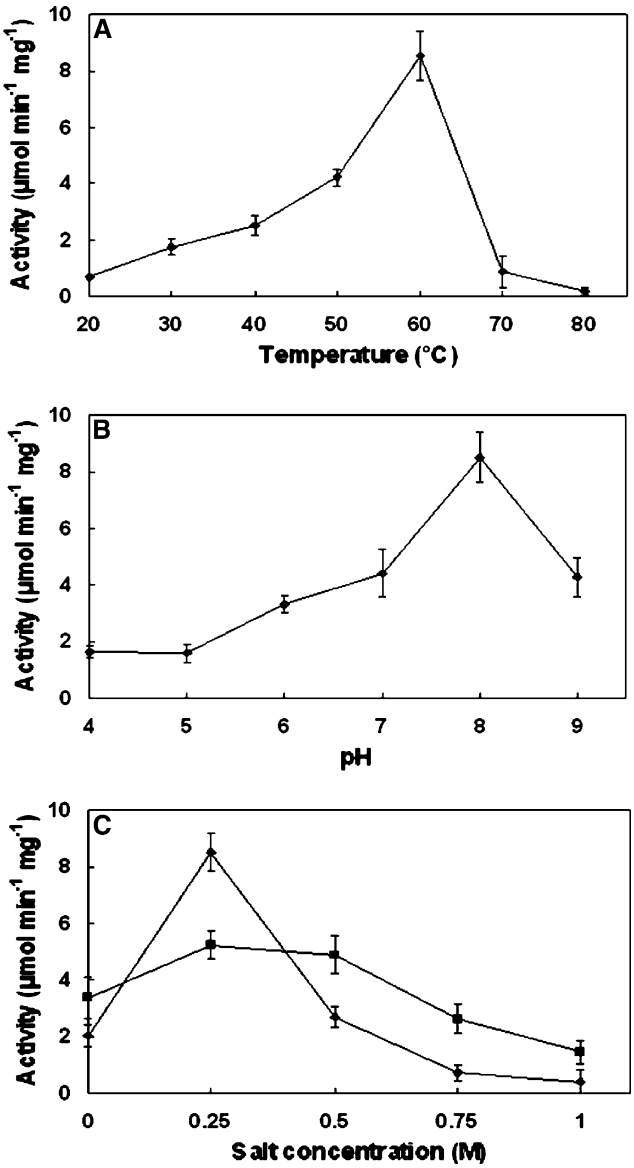

Fig. 4 Effect of temperature (**a**), pH (**b**), and salt concentration (**c**) on *Np*ALDH activity. **a** The effect of temperature on *Np*ALDH activity was measured for 10 min at temperatures from 20 to 80°C, with the reaction mixtures containing 0.25 M NaCl, pH 8.0. **b** The pH dependence of *Np*ALDH activity was determined between 4 and 9, with two buffer systems, 50 mM Na_2HPO_4-citric acid (pH 4.0–5.0), and 50 mM Tris–HCl (pH 6.0–9.0). Both buffers contained 0.25 M NaCl. Activity assays were performed at various pH values at 60°C for 10 min. **c** To test the effects of salts on *Np*ALDH activity, the NaCl (*diamond*) and KCl (*square*) concentrations in the assay were varied from 0 to 1 M. Activity was determined at 60°C, pH 8.0 for 10 min

presence of 0, 1, 2, 3, 4, and 5 M NaCl, the activity as tested in the presence of optimum salt concentrations was unchanged. The lack of salt requirement for activity and stability of the enzyme was unexpected in view of the typical 'halophilic' signature of the protein, with a large excess of acidic amino acids (48 Glu, 41 Asp, compared to 29 Arg and 14 Lys) and a predicted p*I* of 4.47.

Alcohol dehydrogenase and aldehyde dehydrogenase are the two key enzymes involved in ethanol metabolism, and RT-PCR analysis pointed out that the *aldH2* gene was induced by ethanol, possibly contributing to the alcohol resistance. However, *adh* gene transcription was not affected by ethanol, indicating that alcohol dehydrogenase of *N. pharaonis* may play other physiological roles in addition to ethanol degradation.

Natronomonas pharaonis alcohol dehydrogenase, one of the key enzymes of ethanol degradation, has been studied previously (Cao et al. 2008). The acetaldehyde produced can serve as substrate for aldehyde dehydrogenase and be further oxidized to acetic acid. The present study shows that ALDH from *N. pharaonis* is thermophilic and alkaliphilic. Like many other enzymes from halophiles that exhibit optimal activity at temperatures far above the maximum growth temperature of the organism (Bonete et al. 1986; Bonete et al. 1987; Cao et al. 2008; Oren 1983), *Np*ALDH has its maximum activity at 60°C, and can be classified as a thermoactive enzyme. *Np*ALDH is active at pH 8.0, and this agrees well with the intracellular alkaline pH generally observed in alkaliphiles (Padan et al. 1981; Padan et al. 2005). *Np*ALDH was salt independent, while the analogue in *Halobacterium salinarum* performed most pronounced activity at 1 M NaCl (Kim et al. 2006). To what extent the *Np*ALDH may be active intracellularly in the presence of the high KCl concentration found within the cells is not clear. On the other hand, the low salt

requirement of the *Np*ALDH made the expression of the active enzyme in recombinant *E. coli* relatively easy.

The question should be raised what function the alcohol dehydrogenase and aldehyde dehydrogenase genes may play in the physiology of the organism. No direct indication has yet been obtained for the importance of alcohols or aldehydes in the alkaline hypersaline lake environment, and the species description of *N. pharaonis* states that the organism cannot utilize ethanol as a carbon source. Acetate, the predicted product of the oxidation of ethanol via acetaldehyde is also not used as a growth substrate (Soliman and Trüper 1982).

Many halophilic Archaea oxidize glucose via modified Emden–Meyerhof and Entner–Doudoroff pathways (Siebers and Schönheit 2005), by which the intermediate pyruvate is produced. However, little is known about the metabolism of ethanol in the halophilic Archaea. Another possibility is that *N. pharaonis* may encounter ethanol in its environment where it may be produced by organisms such as *Halonatronum saccharophilum*, an organism isolated from the extremely hypersaline and alkaline Lake Magadi in Kenya, an environment that resembles that of the Wadi Natrun lakes to a large extent. *H. saccharophilum* produces major amounts of ethanol during sugar fermentation (Garnova and Krasil'nikova 2003; Zhilina et al. 2001). Such organisms may well be important inhabitants of hypersaline soda lakes which are rich in organic material and depleted in oxygen which is poorly soluble in salt-saturated brines. Ethanol oxidation by *N. pharaonis* may therefore be one of the mechanisms used by halophilic Archaea to degrade toxic compounds found in their habitat (Margesin and Schinner 2001). The fact that *adh* and *aldh* gene have been found in multiple copies in the genomes of other members of the *Halobacteriaceae* (*Halobacterium* sp. NRC-1, *Haloarcula marismortui*, *Haloquadratum walsbyi*) suggests that also the neutrophilic relatives of *Natronomonas* may occasionally need to cope with ethanol stress in their natural environment.

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