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ORIGINAL PAPER

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Purification and characterization of an extremely halophilic acetoacetyl-CoA thiolase from a newly isolated *Halobacterium* strain ZP-6

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Abstract The extremely halophilic archaeon ZP-6 was isolated from Ai-Ding salt lake in Xinjiang Uighur Autonomous Region of the People's Republic of China. Based on its physiological properties, 16S rDNA sequence, and DNA–DNA homology with known haloarchaea, the isolate was tentatively identified as a *Halobacterium* sp. An acetoacetyl-CoA thiolase was purified and characterized from this organism. The native enzyme has a molecular mass of 80 ± 8 kDa and consists of two identical subunits of 43 ± 2 kDa each. The N-terminus 14 amino acid residues were sequenced and showed identity with the respective part of a putative thiolase (AcaB1) of *Halobacterium* sp. NRC-1. The purified enzyme has an optimal pH of 7.9 for acetoacetyl-CoA thiolysis. The thiolytic activity was inhibited by the presence of Mg^{2+} and was stimulated by KCl or NaCl. The thiolysis reaction of *Halobacterium* sp. ZP-6 thiolase can be inhibited by either substrate when present in excess. The distinct kinetic profile indicates that the thiolase from *Halobacterium* sp. ZP-6 may have a different catalytic mechanism from the so-called ping-pong mechanism employed by other thiolases. To our knowledge, this is the first report of the purification and characterization of a halophilic thiolase from an archaeal species.

Key words Archaeon · Acetoacetyl-CoA thiolase · *Halobacterium* sp. ZP-6 · Halophile · Purification and properties

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Introduction

The Archaea (Woese et al. 1990) constitute a separate domain of possibly ancient organisms that exist under extreme conditions such as high salinity and high temperature. Investigations on archaeal genomes and enzymology are of interest because they may provide information helpful in understanding the evolution of early life (Woese 2000).

Thiolases are ubiquitous enzymes that play important roles in many biochemical pathways (Kunau et al. 1995). Based on their substrate specificities and physiological functions, two classes of β-ketothiolases have been characterized in bacteria and eukaryotes. The first class, referred to as acetyl-CoA acetyltransferase or acetoacetyl-CoA thiolase (EC 2.3.1.9), is specific for acetoacetyl-CoA and is involved in biosynthetic pathways such as poly(hydroxybutyrate) (PHB) synthesis (Slater et al. 1998), isoprenoid chain initiation, and ketone body synthesis. The second class, referred to as acetyl-CoA acyltransferase or β-ketoacyl-CoA thiolase (EC 2.3.1.16), usually shows high specificity toward longer-chain substrates and is involved in fatty acid β-oxidation (Hiltunen and Qin 2000). Members of each class have been purified from bacteria and eukaryotes, and genes coding for them have been cloned (Peoples and Sinskey 1989; Petersen and Bennett 1991; Antonenkov et al. 2000). The three-dimensional structure of the biosynthetic thiolase from *Zoogloea ramigera* and the biodegradative thiolase from *Saccharomyces cerevisiae* were determined at high resolution (Mathieu et al. 1994; Modis and Wierenga 1999). In spite of considerable variations in the amino acid sequences of the two proteins, the three-dimensional structures show a high degree of homology. The thiolase reaction mechanism has been studied in some detail. Enzyme kinetics, active site labeling, and site-directed mutagenesis experiments with the biosynthetic thiolase from *Z. ramigera* allowed two conserved cysteine residues to be identified as important for catalysis (Thompson et al. 1989; Palmer et al. 1991). On the basis of those studies, a two-step "ping-pong" reaction mechanism was proposed (Masamune et al. 1989). More recently, the crystal structure of thiolase in a complex with

different reaction intermediates provided new insights into the detailed catalytic mechanism (Mathieu et al. 1997; Modis and Wierenga 2000).

Compared with what is known about thiolases in eubacteria and eukaryotes, the information on thiolases from the archaeal domain, especially from haloarchaea, is very sparse. The successful purification of a thiolase from haloarchaea has not yet been reported, and the catalytic properties of thiolases from haloarchaea are not known. To extend further our knowledge of thiolases, we describe the purification and characterization of an extremely halophilic acetoacetyl-CoA thiolase from the haloarchaeon *Halobacterium* sp. ZP-6.

Materials and methods

Isolation and characterization of *Halobacterium* sp. ZP-6

Halobacterium sp. ZP-6 was isolated in our laboratory from a sample recovered from the Ai-Ding salt lake (42°32′–42°43′ N, 89°10′–89°40′ E) located in Xinjiang Uighur Autonomous Region of the People's Republic of China. After enrichment of the sample in Sehgal and Gibbons medium (Sehgal and Gibbons 1960) at 40°C with shaking for 2 weeks, a pure culture was obtained by plating serial dilutions of enrichment and repeated transfers of separate colonies on agar plates of the same medium. The strain is deposited in the Type Culture Collection of the Chinese Academy of Sciences (Beijing, P.R. China), and is publicly available upon request (Deposition No: AS 1.2742).

Cultivation and harvesting of cells

Halobacterium sp. ZP-6 was cultivated on a shaker in a medium (Colwell et al. 1979) consisting of 0.5% (w/v) casamino acid, 0.5% yeast extract, 0.1% sodium glutamate, 0.3% sodium citrate, 2% $MgSO_4 \cdot 7H_2O$, 0.2% KCl, 20% NaCl, 0.0036% $FeCl_2 \cdot 4H_2O$, and 0.000036% $MnCl_2 \cdot 4H_2O$. The final pH value was adjusted to 7.2 with NaOH. Cells were harvested by centrifugation at 8,000 *g* for 15 min after 3- to 4-day cultivation, and washed three times with basic salt solution (25% NaCl, 2% $MgSO_4 \cdot 7H_2O$, 0.3% sodium citrate, 0.2% KCl).

Enzymatic activity assay and determination of protein concentration

Thiolase activity was assayed in the direction of the thiolysis of acetoacetyl-CoA by measuring the increase in absorption at 232 nm and at 25°C. The reaction mixture contained (1 ml total volume) 24 nmol acetoacetyl-CoA, 30 nmol CoA, 50 mM Tris-HCl, and 4 M KCl, pH 8.0. An extinction coefficient of 4.5 $mM^{-1}\,cm^{-1}$ was used to calculate the activity (Antonenkov et al. 1999). One unit was defined as the amount of enzyme required for the formation of 1 μmol of acetyl-CoA per minute.

Protein concentrations were determined according to Bradford (1976).

Purification of acetoacetyl-CoA thiolase

Step 1: Preparation of crude extract. Forty grams of wet cells of Halobacterium sp. ZP-6 was suspended in a 200-ml breakage buffer [50 mM Tris-HCl, pH 7.0, 2 M KCl, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF)], and disrupted in a sonicator for about 15 min at 4°–10°C. The cellular lysate was centrifuged at 12,000 g for 15 min, and the supernatant was used for enzyme purification.

Step 2: Polyethylene glycol 6,000 precipitation. Solid Polyethylene glycol (PEG) 6,000 (5,500–7,500 Da) was added at approximately 12% (w/v). After 30 min of stirring, the mixture was centrifuged at 12,000 *g* for 20 min. PEG 6,000 was then added to the supernatant to 30% saturation, followed by a repeat of the stirring (60 min) and centrifugation. The pellet was dissolved in buffer A (50 mM K_2HPO_4-KH_2PO_4, pH 7.0, 5 mM 2-mercaptoethanol, 1 mM PMSF, 0.2 M KCl) and dialyzed against the same buffer for 4 h.

Step 3: DEAE-cellulose chromatography. The dialyzed crude enzyme solution was loaded at a speed of 2 ml/min onto a DEAE-cellulose column (2.5 × 50 cm) previously equilibrated with buffer A. A linear gradient of KCl from 0.2 to 1 M in 500 ml buffer A was used to elute the enzyme at a speed of 1 ml/min. Fractions of 5 ml were collected in tubes with crystal KCl (about 1 g per tube). The thiolase activity was eluted with 1.5 M KCl.

Step 4: Benzyl-sepharose chromatography. The active fractions obtained from the previous step were combined and concentrated by dialysis against sucrose. Excess sucrose was removed by dialysis in buffer B (50 mM K_2HPO_4-KH_2PO_4, pH 6.6, 5 mM 2-mercaptoethanol, 1 mM PMSF, 2.5 M KCl). This sample was loaded onto the benzyl-sepharose column (2.5 × 4.0 cm) previously equilibrated with buffer B. After being washed with 400 ml buffer B, the column was eluted with 100 ml buffer C (50 mM K_2HPO_4-KH_2PO_4, pH 6.6, 5 mM 2-mercaptoethanol, 1 mM PMSF, 1.5 M KCl).

Step 5: Hydroxylapatite treatment. Fractions containing thiolase activity were collected and concentrated by dialysis against sucrose. Excess sucrose was removed by dialysis against buffer D (50 mM K_2HPO_4-KH_2PO_4, pH 6.6, 5 mM 2-mercaptoethanol, 1 mM PMSF, 2 M KCl). This sample was applied to a hydroxylapatite column (0.5 × 4.0 cm) equilibrated with buffer D. The column was eluted sequentially with 50 ml each of 50, 75, 100, and 150 mM potassium phosphate in buffer E (pH 6.6, 5 mM 2-mercaptoethanol, 1 mM PMSF, 2.0 M KCl). Acetoacetyl-CoA thiolase activity was recovered in fractions of 75 mM phosphate.

Determination of molecular mass

The molecular mass (M_r) of the native thiolase was determined by gel filtration on a Bio-Gel P-200 column (Bio-Rad, Hercules, CA, USA). The filtration was performed in 50 mM potassium phosphate buffer, pH 6.8, containing 2 M KCl and 5 mM 2-mercaptoethanol. Molecular weight standards (12–200 kDa) were obtained from Sigma (St. Louis, MO, USA). The molecular mass of the thiolase subunit was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by cetyltrimethylammonium bromide (CTAB)-PAGE (Eley et al. 1979).

N-terminal amino acid sequencing

After SDS-PAGE, the thiolase protein was blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) and stained with Coomassie Blue R-250. The stained proteins were excised and the N-terminal amino acid sequence was determined using an Applied Biosystems 491A liquid-phase sequencer (ABI, Foster City, CA, USA).

Determination of the 16S rRNA gene sequence and DNA–DNA hybridization

The total DNA of *Halobacterium* sp. ZP-6 was extracted by the method of Cline et al. (1989). The 16S rRNA gene was amplified by PCR and sequenced according to Xu et al. (1999), and is deposited in GenBank (Access No: AF 355101). The sequence obtained was aligned with the other reported halobacterial 16S rDNA sequences using the Clustal W (1.7) program (Thompson et al. 1994). DNA–DNA hybridization was performed using the membrane filter method, as described by Xu et al. (1999).

Results and discussion

Characterization of the isolate

Cells of *Halobacterium* sp. ZP-6 are nonmotile, gram-negative short rods. Cellular lysis occurred when exposed to media containing less than 1.0 M NaCl. Colonies on agar plates were pink, circular, and 0.5–1.0 mm in diameter. The strain grew between 1.5 M and saturation of NaCl, with optimum growth at 3.5–4.3 M. The optimal pH and temperature for growth are 7.2 and 40°C, respectively. Analysis of the 16S rDNA sequence of this strain indicated 98.8% and 99.6% similarity to *Halobacterium salinarum* and *Halobacterium* sp. NRC-1. The level of DNA–DNA hybridization of the isolate to *Halobacterium salinarum* is 65%. Based on these results, the isolate was identified as a member of the genus *Halobacterium* and tentatively named *Halobacterium* sp. ZP-6.

Table 1. Purification of acetoacetyl-CoA thiolase from *Halobacterium* sp. ZP-6

Step	Total protein (mg)	Specific activity (U/mg)	Enrichment (-fold)	Yield (%)
Crude extract	14,000	2.4	1	100
PEG 6,000	560	3.6	1.5	61
DEAE-cellulose 52	60	26.1	11	47
Benzyl-sepharose	1.15	404.3	170	14
Hydroxylapatite	0.36	833.3	347	9

Purification of thiolase from *Halobacterium* sp. ZP-6

The purification of thiolase is summarized in Table 1. At the end of purification, the enzyme was purified 347-fold. The final yield was 9%. The final enzyme preparation was found to be homogeneous as judged by electrophoresis on SDS-PAGE and by native electrophoresis on polyacrylamide gel. In all cases, there was only one protein band visualized after Coomassie Blue staining (Fig. 1).

During purification, we found that sucrose (>10%), 2-mercaptoethanol, or dithiothreitol (DTT) were necessary to maintain enzyme activity, while low temperature was detrimental to the activity. For example, enzyme activity is lost completely after 1-h incubation at 0°C. During PEG 6,000 precipitation and DEAE-cellulose chromatography, low-salt (0.2 M KCl) buffers were used to perform the chromatography smoothly.

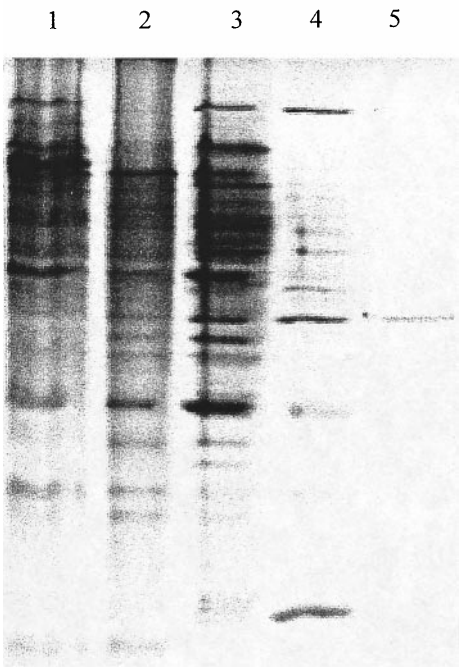

Fig. 1. Native polyacrylamide gel electrophoresis shows the homogeneity of the purified *Halobacterium* sp. ZP-6 thiolase. *Lane 1* crude extract, *lane 2* polyethylene glycol 6,000 precipitation, *lane 3* DEAE-cellulose chromatography, *lane 4* benzyl-sepharose chromatography, *lane 5* hydroxylapatite treatment

Molecular mass and subunits of the purified thiolase

Electrophoresis of the purified thiolase from *Halobacterium* sp. ZP-6 with anionic (SDS) and cationic (CTAB) detergents indicated that the thiolase corresponded to a subunit of 60 ± 4 kDa in SDS-PAGE and a subunit of 43 ± 2 kDa in CTAB-PAGE. As found previously, during purification of halophilic proteins such as *Halobacterium salinarum* arginine deaminase (Mondstadt and Holldorf 1990), *Haloferax mediterranei* glucose dehydrogenase (Bonete et al. 1996), *Haloarcula vallismortis* glyceraldehyde-3-phosphate dehydrogenase (Prüß et al. 1993), and halophilic serine proteinase from *Halobacterium halobium* (Izotova et al. 1983), the subunit M_r of *Halobacterium* sp. ZP-6 thiolase determined by SDS-PAGE was about 28% higher than that obtained by CTAB-PAGE. The value obtained from CTAB-PAGE is regarded as more reliable for highly acidic halophilic proteins. The native M_r of the thiolase was determined to be 80 ± 8 kDa by gel filtration. Based on these results, we conclude that this thiolase is a dimeric enzyme.

Effects of salt concentration on thiolase activity and stability

Thiolase activity was highly dependent on the concentration of KCl or NaCl in the buffer, being optimal at saturated KCl or NaCl (4.5 M). At lower concentrations, the stimulatory effect of KCl is higher than the effect of identical concentrations of NaCl (Fig. 2).

High salt concentrations increase the stability of the halophilic thiolase at either room or high temperatures. At room temperature, the purified thiolase was stable for 1 week in saturated KCl solution, while only 10% activity remained after 72 h in 0.2-M KCl solution. When the thiolase was incubated in 4 M KCl for 10 h at 70° and 85°C, 80% and 65% activity, respectively, remained.

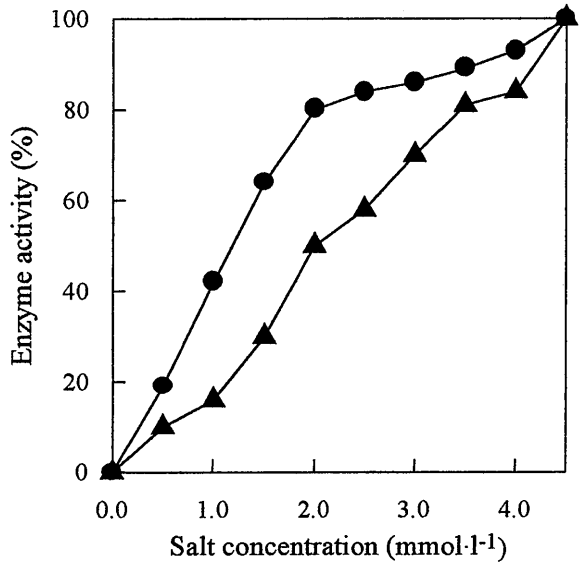

Fig. 2. Effect of salt concentration on thiolase activity; *circles* KCl, *triangles* NaCl. The reactions were carried out at 25°C in 50 mM Tris-HCl buffer (pH 7.9) containing 24 µmol/l of acetoacetyl-CoA and 30 µmol/l of CoA. The highest activity was taken as 100%

Influence of pH and Mg^{2+} on thiolase activity

Halobacterium sp. ZP-6 thiolase activity occurred over a very narrow pH range and its optimal pH was 7.9. There was no significant activity when the pH was higher than 8.6. The presence of Mg^{2+} adversely affected its activity, in common with other thiolases (Antonenkov et al. 1999). At 20 mM Mg^{2+}, only 40% of the activity remained.

Reaction kinetics of thiolysis

During the analysis of the kinetics of the thiolase, we found that the velocity versus substrate concentration profile did not coincide with simple Michaelis–Menten kinetics (Figs. 3, 4). As shown in Figs. 3 and 4, substrate inhibition was evident at high concentrations of acetoacetyl-CoA or CoA alone. However, this inhibitory effect could be partially neutralized by increasing the concentration of the second substrate. Substrate inhibition was also observed with thiolases from bacteria (Berndt and Schlegel 1975; Oeding and Schlegel 1973; Suzuki et al. 1987) and rat liver peroxisomes (Antonenkov et al. 1997, 2000). In such cases, one substrate, either CoA or acetoacetyl-CoA, mainly produced the inhibitory effect on the thiolysis reaction. However, the thiolysis reaction of *Halobacterium* sp. ZP-6 thiolase can be inhibited by any single substrate in excess. It appears that the ratio of one substrate to another is important for the optimal thiolysis reaction. Figures 3b and 4b also show the crossed-line Lineweaver–Burk plot rather than a family of parallel lines as shown in almost all well-studied thiolases. They indicate that the thiolase from *Halobacterium* sp. ZP-6 may have a catalytic mechanism different from the ping-pong mechanism (Cleland 1963) employed by other thiolases.

Analysis of N-terminal amino acid sequence

Examination of currently known fungal and bacterial thiolases revealed a conserved glycine (-G-) at the N-terminus (Fig. 5). *Halobacterium* sp. ZP-6 thiolase also contains this amino acid. The N-terminal 14 amino acids of *Halobacterium* sp. ZP-6 thiolase are identical to the respective part of one of the two putative thiolase sequences of *Halobacterium* sp. NRC-1 [acaB1 from the genome sequence (Ng et al. 2000)] (Fig. 5). This indicates that the two thiolases might have a common ancestor.

As far as we are aware, this is the first thiolase purified from an organism from the archaeal domain. This thiolase exhibits not only an extreme salt requirement but also has unique kinetic properties that differ significantly from examples in eubacteria and eukaryotes. It is possible that there is a special thiolytic mechanism in haloarchaea. To further elucidate the mechanism, work on the structure of this protein is now in progress.

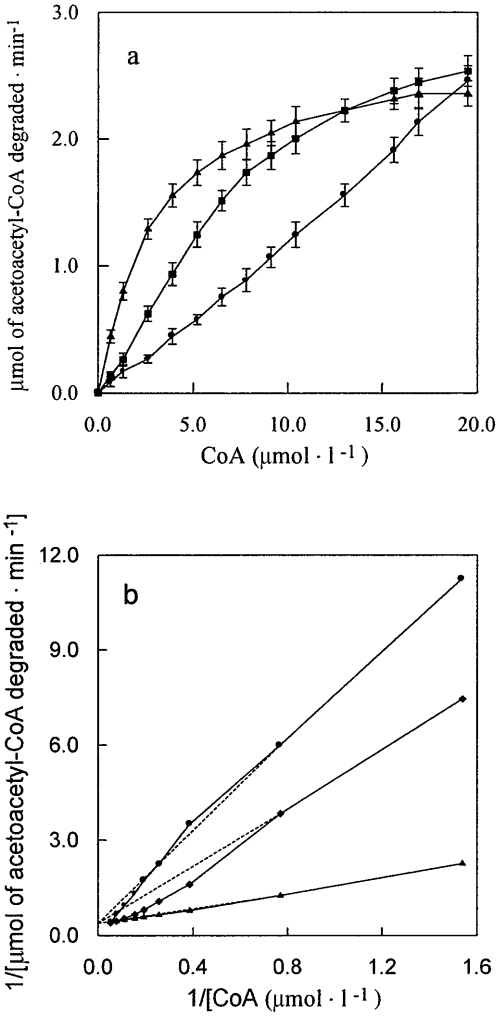

Fig. 3. a Substrate-saturation curves (CoA) of thiolase at three acetoacetyl-CoA concentrations. **b** Lineweaver–Burk plot. The concentrations of acetoacetyl-CoA (µmol/l) are 8 (*triangles*), 16 (*squares*), and 24 (*circles*)

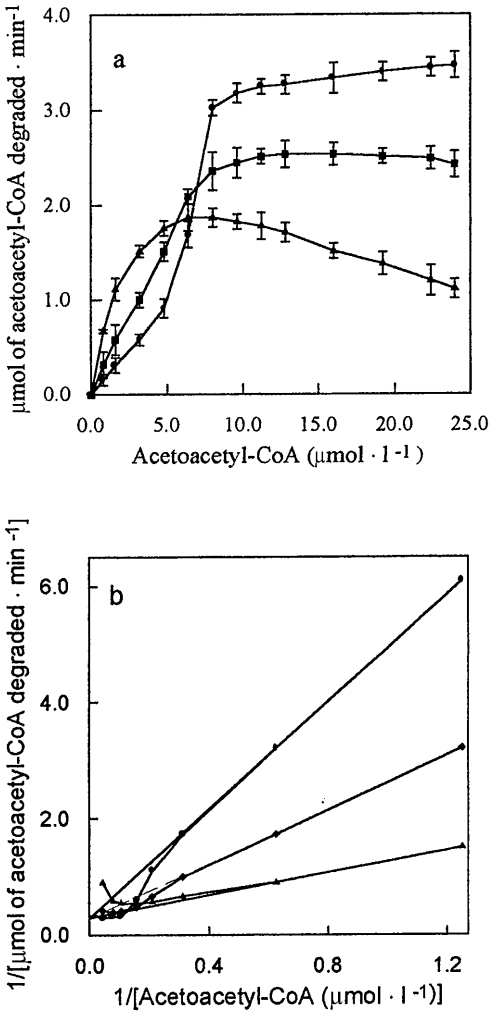

Fig. 4. a Substrate-saturation curves (acetoacetyl-CoA) of thiolase at three CoA concentration. **b** Lineweaver–Burk plot. The concentrations of CoA (µmol/l) are 6.5 (*triangles*), 19.5 (*squares*), and 32.5 (*circles*)

Fig. 5. Alignment of N-terminal sequences of thiolases with CLUSTAL W (1.7). All sequences, except for those of *Halobacterium* NRC-1 and *Halobacterium* sp. ZP-6, are from http://www.expasy.ch

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S.cerevisiae          ---MSQN-VYIVSTARTPIGSFQGSLSSKTAVELGAAALKGALAKV
C.tropicalis          ---TLPP-VYIVSTARTPIGSFQGSLSSLTYSDLGAHAVKAALAKV
Rhizobium sp.         ---MSNPSIVIASAARTAVGSFNGAFGNTLAHELGAAAIKAVLERA
Z.ramigera            ---MSTPSIVIAS-ARTAVGSFNGAFANTPAHELGATVISAVLERA
P.denitrificans       ---MTKA--VIVSAARTPVGSFLGSFANLPAHELGAIVLKAVVERA
R.eutropha            ---MTDV--VIVSAARTAVGKFGGSLAKIPAPELGAVVIKAALERA
C.vinosum             ---MSEN-IVIVDAGRSAIGTFGGSLSSLSATEIGTAVLKGLLART
T.violacea            ---MSDT-IVIVDAGRTAIGTFGGALSALQATDIGTTVLKALIERT
E.coli                ---MKD--VVIVGALRTPIGCFRGALAGHSAVELGSLVVKALIERT
M.tuberculosis        ---MTTS--VIVTGARTPIGKLMGSLKDFSASDLGAITIAAALKKA
Haemophilus sp.       ---MENV--VIVSAVRTPIGSFNGALSSVSAVDLGAIVIQEAIKRA
C.acetobutylium       MGVMNMREVVIASAARTAVGSFGGAFKSVSAVELGVTAAKEAIK
Halobacterium         ----MTDA-RVAGVGLTHFGVHPERTSRDLFAEAGLAALDDAGVA
sp.
ZP-6                  ----MTDA-RVAGVGLTHFG--
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