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ORIGINAL PAPER

Abundance and diversity of archaeal *accA* gene in hot springs in Yunnan Province, China

Zhao-Qi Song · Li Wang · Feng-Ping Wang · Hong-Chen Jiang · Jin-Quan Chen · En-Min Zhou · Feng Liang · Xiang Xiao · Wen-Jun Li

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Abstract It has been suggested that archaea carrying the *accA* gene, encoding the alpha subunit of the acetyl CoA carboxylase, autotrophically fix CO_2 using the 3-hydroxypropionate/4-hydroxybutyrate pathway in low-temperature environments (e.g., soils, oceans). However, little new information has come to light regarding the occurrence of archaeal *accA* genes in high-temperature ecosystems. In this study, we investigated the abundance and diversity of archaeal *accA* gene in hot springs in Yunnan Province, China, using DNA- and RNA-based phylogenetic analyses and quantitative polymerase chain reaction. The results showed that archaeal *accA* genes were present and expressed in the investigated Yunnan hot springs with a wide range of temperatures (66–96 °C) and pH (4.3–9.0). The majority of the amplified archaeal *accA* gene sequences were affiliated with the ThAOA/HWCG III [thermophilic ammonia-oxidizing archaea (AOA)/hot water crenarchaeotic group III]. The archaeal *accA* gene abundance was very close to that of AOA *amoA* gene, encoding the alpha subunit of ammonia monooxygenase. These data suggest that AOA in terrestrial hot springs might acquire energy from ammonia oxidation coupled with CO_2 fixation using the 3-hydroxypropionate/4-hydroxybutyrate pathway.

Keywords *accA* gene · *amoA* gene · Archaea · Hot springs · Yunnan Province

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Z.-Q. Song · J.-Q. Chen · E.-M. Zhou · W.-J. Li (✉)
Key Laboratory of Microbial Diversity in Southwest China, Ministry of Education, and Laboratory for Conservation and Utilization of Bio-resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, China
e-mail: wjli@ynu.edu.cn

Z.-Q. Song · L. Wang · F. Liang
Engineering Technology Research Center of Biomass Degradation and Gasification, University of Henan Province, Shangqiu Normal University, Shangqiu 476000, China

F.-P. Wang · J.-Q. Chen · X. Xiao
Key Laboratory of MOE for Microbial Metabolism and School of Life Sciences and Biotechnology, State Key Laboratory of Ocean Engineering, Shanghai Jiao Tong University, Shanghai 200240, China

H.-C. Jiang (✉)
State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan 430074, China
e-mail: hongchen.jiang@gmail.com

W.-J. Li
Key Laboratory of Biogeography and Bioresource in Arid Land, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, Ürűmqi 830011, China

Introduction

The *accA* gene encodes the alpha subunit of the Acetyl CoA carboxylase (ACCase), a biotin-dependent enzyme that catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA (Brownsey et al. 1997). This gene is present in a wide range of organisms and is known to be involved in fatty acid biosynthesis (Moss Lane 1971). However, ACCase would recently be expected to be specific to carbon fixation in Archaea due to their unique lipids and lack of fatty acids (Berg et al. 2007, 2010a). Specifically, ACCase represents one of the key enzymes responsible for CO_2 fixation through the 3-hydroxypropionate/4-hydroxybutyrate cycle (Berg et al. 2007). This pathway was first discovered in a

thermophilic chemolithoautotroph *Metallosphaera sedula* (belonging to the archaeal order *Sulfolobales*) isolated from a terrestrial hot spring (Berg et al. 2007) and has also been suggested to function in other autotrophic members of *Sulfolobales* (Berg et al. 2007, 2010b) and mesophilic *Crenarchaeota* (*Thaumarchaeota*, which includes all known ammonia-oxidizing archaea, i.e. AOA) as well (Hallam et al. 2006; Berg et al. 2007; Hatzenpichler et al. 2008; Tourna et al. 2011).

Recently, several studies investigated the diversity and abundance of archaeal *accA* and *amoA* genes in agricultural soils and ocean waters and showed that the 3-hydroxypropionate/4-hydroxybutyrate pathway has significant correlation with archaeal ammonia oxidation process (Yakimov et al. 2009, 2011; Hu et al. 2011a, b; Pratscher et al. 2011). AOA are widespread in terrestrial geothermal sites in Iceland (Reigstad et al. 2008), Kamchatka (Russia) (Zhang et al. 2008; Zhao et al. 2011), Great Basin and Yellowstone National Park (United States) (de la Torre et al. 2008; Zhang et al. 2008), and Yunnan geothermal zone (China) (Zhang et al. 2008; Jiang et al. 2010). The Yunnan geothermal zone is one of the most active geothermal areas in the world. It possesses thousands of hot springs characteristic of a variety of hydrothermal features, such as hydrothermal explosion craters, geysers, fumaroles, and boiling springs (Song et al. 2013). Previously, two cultivation-independent studies showed that AOA *amoA* genes (encoding the subunit A of ammonia monooxygenase) were abundant and diverse in Yunnnan hot springs with temperatures higher than 74 °C and up to 94 °C (Zhang et al. 2008; Jiang et al. 2010). However, little is known about whether the AOA found in hot springs also autotrophically fix CO_2 through the 3-hydroxypropionate/4-hydroxybutyrate pathway.

In order to fill the above knowledge gap, the abundance and diversity of archaeal *accA* gene were investigated in the samples collected from the Yunnan hot springs with a range of temperature 66–96 °C and pH 4.3–9.0. An integrated approach was employed, including DNA- and RNA-based phylogenetic analyses and quantitative polymerase chain reaction (qPCR).

Materials and methods

Field measurements and sampling

Eight hot springs were selected in this study and they were located in three thermal areas (Tengchong, Longling, and Eryuan) in the Yunnan geothermal zone (Table 1). At each hot spring, water temperature and pH were determined using a portable meter (PT-10, SARTORIUS, Germany). Concentrations of nitrite, nitrate, ammonium, phosphate, and ferrous iron were measured using HACH colorimeter (model CEL 850/product #: 2687900, Hach Chemical Co., Iowa, USA) according to the manufacturer's instructions in the field. The measurements of major and trace elements were performed with inductively coupled plasma mass

Table 1 Water chemistry and temperature of the eight hot springs in Yunnan Province, China, and description of samples collected from these hot springs

Sample code	Sample description	GPS location (N/E)	Temp (°C)	pH	PO_4^{3-}	NH_4^+	NO_2^-	NO_3^-	Fe^{2+}	SiO_4^{2-}	Al	Ca	K	Mg	Mn	Na
Zzq	Brown sandy sediment	24°57′03″/ 98°26′09.5″	96	4.3	1.96	164.27	2.21	5.49	0.201	645.68	0.5373	3.901	25.32	0.5995	0.521	59.64
Dgg	Ashen geyserite	24°57′12.7″/ 98°26′17.4″	94	8.1	1.74	BD	0.36	3.35	BD	677.39	0.134	1.75	116.4	0.1197	BD	387.4
Gmd	Gray Mat	24°57′12.6″/ 98°26′15.7″	84	9.0	0.87	36.36	0.24	3.26	BD	679.76	0.342	1.39	96.38	0.11	0.00	364.7
Hmz2	Black sediment mix mat	24°57′12.6″/ 98°26′17.5″	82	7.8	4.99	0.56	0.67	3.9	BD	679.22	0.0537	1.975	69.32	0.1429	BD	299.3
Eynj2	Black mat	26°15′01.2″/ 99°59′22.2″	73	7.3	1.06	BD	0.48	1.88	BD	643.7	0.0253	37.06	38.51	14.04	0.034	206.3
Wm3	Gray Mat	24°57′12.6″/ 98°26′15.6″	70	7.2	1.24	96.49	3.29	39.84	BD	173.37	0.074	1.42	83.84	0.086	0.00	360.4
Enp	Gray sandy sediment	26°15′01.1″/ 99°59′22.3″	68	7.2	3.17	167.87	ND	4.18	BD	656.45	0.624	56.23	43.02	19.12	0.062	213.4
Sx4	Black sandy sediment	24°39′23.3″/ 98°40′03.4″	66	8.0	8.2	382.08	0.02	2.34	BD	629.74	0.3423	28.09	8.213	1.371	0.0524	73.94

Values are reported as milligrams per liter

The collected samples were labeled as: *Zzq* Zhenzhuquan, *Dgg* Dagunguo, *Gmd* Gumingquan downstream, *Hmz2* Hamazui #2, *Wm3* Wumingquan #3, *Eynj2* Eryuanniujie #2, *Enp* Eryuanniujie park, *Sx4* Shangxiao #4, *BD* below the detection limit (0.001 mg/L), *ND* not determined

spectroscopy (ICP-MS, Thermo, USA) in laboratory. After chemical measurements, mat-containing sinter or sediments were collected into 50-mL Falcon tubes and immediately stored in liquid nitrogen. The samples were kept in liquid nitrogen in the field and during transportation, and then were stored at −80 °C in the laboratory until further analyses.

Nucleic acid isolation

Both DNA- and RNA-based molecular approaches were employed. Total DNA and RNA were extracted from 1 to 2 g of sediment using E.Z.N.A. ® Soil DNA Kit (Omega Bio-Tek Inc., USA) and E.Z.N.A. ® Soil RNA Kit (Omega Bio-Tek Inc., USA), respectively, according to the manufacturer's instructions. To remove the residual DNA, the soluble crude RNA was digested with RNase-free DNase I (Fermentas, USA). The DNase-digested RNA samples were checked for potential genomic DNA contamination by PCR amplification with specific primer sets of Arch21F/Arch958R, Bac27F/Univ1492R, and Crena_529F/Crena_981R (Table 2) for archaeal and bacterial 16S rRNA genes and *accA* gene, respectively.

cDNA synthesis by reverse transcription

The checked RNA samples were reverse-transcribed into cDNA using the Fermentas AMV Reverse Transcriptase (Fermentas, USA) and random hexamer primer according to the manufacturer's protocol. Absence of contamination from DNA and chemical reagents was verified by conducting the same reactions without the AMV reverse transcriptase and template, respectively.

Quantitative PCR (qPCR)

The archaeal *accA* and *amoA* genes and archaeal and bacterial 16S rRNA genes were quantified by qPCR with the primer sets listed in Table 2. Amplification conditions were 95 °C for 10 min, followed by 40 cycles of 15 s at 94 °C, 45 s for annealing at temperatures specified in Table 2, 1 min at 72 °C for extension, and data collection. The reaction volume is 20 μL, containing 20–50 ng DNA, 10 μL of SYBR®PREMIX TaqTM (2×) (TaKaRa, China), and 10 pmol of each primer. Plasmids of an *accA* gene clone (obtained in this study), one *amoA* gene clone (obtained in this study), and 16S rRNA genes of *Shewanella piezotolerans* WP3 and *Natronomonas* sp. were used as standard templates for the qPCR of archaeal *accA* and *amoA* genes and bacterial and archaeal 16S rRNA genes, respectively. Standard templates were made into serial dilutions of 10^2–10^8 gene copies per micro-liters. R^2 values of the standard curves were 0.996–0.967 for the targeted genes. The qPCR amplification efficiencies were in the range of 85–95 %. All qPCR reactions were performed in triplicate with an ABI7500 real-time thermal cycler (ABI, USA). Melting curve analysis was performed using the default conditions set in ABI7500 (95 °C). The melting curve had only one peak, indicating that the SYBR green signals were not from primer-dimer artifacts or non-specific PCR amplification.

Table 2 Primers used in this study

Primers	Primer sequence (5′→3′)	Target group	Annealing temp. (°C)	Usage	References
Crena_529F	GCW ATG ACW GAY TTT GTY RTA ATG	Archaeal *accA* gene	50/52	qPCR/Clone library	Yakimov et al. (2009)
Crena_981R	TGG WTK RYT TGC AAY TAT WCC				
Arch-*amoA*F	STA ATG GTC TGG CTT AGA CG	Archaeal *amoA* gene	56	qPCR	Francis et al. (2005)
Arch-*amoA*R	GCG GCC ATC CAT CTG TAT GT				
Bac331F	TCC TAC GGG AGG CAG CAG T	Bacterial 16S rRNA gene	60	qPCR	Nadkarni et al. (2002)
Bac797R	GGA CTA CCA GGG TCT AAT CCT GTT				
Bac27F	AGA GTT TGG ATC MTG GCT CAG		56	PCR	Lane (1991)
Univ1492R	CGG TTA CCT TGT TAC GAC TT				
Arch349F	GYG CAS CAG KCG MGA AW	Archaeal 16S rRNA gene	60	qPCR	Takai and Horikoshi (2000)
Arch806R	GGA CTA CVS GGG TAT CTA AT				
Arch21F	TTC YGG TTG ATC CYG CCR GA		55	PCR	Lane (1991)
Arch958R	CGG TTA CCT TGT TAC GAC TT				

PCR amplification of archaeal *accA* gene

Archaeal *accA* genes from DNA and cDNA extracts were PCR amplified using archaeal *accA* gene-specific primers Crena_528F and Crena_981R (Yakimov et al. 2009; see Table 2 for detail). PCR reactions were carried out in a total volume of 50 μL containing 1× PCR buffer with 1.5 mM Mg^{2+}, dNTPs (100 μM each), 0.25 μM each primer, 2.5 U of DNA polymerase (Ex-Taq) (TaKaRa, Dalian, China), and 1–10 ng of total DNA/cDNA. Amplification consisted of an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 1 min and extension at 72 °C, and a final extension at 72 °C for 20 min. PCR products were purified using an E.Z.N.A. ® Gel Extraction Kit (Omega Bio-Tek Inc., USA) according to the manufacturer's instructions.

Clone library construction and phylogenetic analysis

The archaeal *accA* gene clone libraries were constructed according to the procedures previously established (Jiang et al. 2010). Briefly, the purified PCR products were ligated into pMD18-T Vector system (TaKaRa, Dalian, China) and transformed into competent *Escherichia coli* JM109 cells. The transformed cells were plated on Luria–Bertani (LB) plates containing 100 μg/mL of ampicillin, 80 μg/mL of X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) and 0.5 mM IPTG (isopropyl-b-D-thiogalactopyranoside), and incubated overnight at 37 °C. A total of 20–40 randomly chosen colonies per sample were analyzed for the insert *accA* gene sequences by PCR amplification with the *accA* gene specific primer set (Crena_528F/Crena_981R). Archaeal *accA* gene clone were sequenced using primer M13+ with the BigDye Terminator version 3.1 chemistry (Applied Biosystems, Foster City, CA, USA). The *accA* gene sequences were determined with an ABI 3730 automated sequencer. The obtained raw archaeal *accA* clone sequences were edited using the DNASTAR program v.5.0 and their validity was checked by using TBLASTX (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Operational taxonomic units (OTUs) of the obtained archaeal *accA* gene clone sequences were identified using the DOTUR 1.53 program (Schloss and Handelsman 2005) at 98 % nt identity cutoff. Coverage (C) was used to assess the adequacy of clone sampling. Coverage of the clone libraries was calculated as follows: $C = 1 - (n1/N)$, where $n1$ is the number of OTUs that occurred only once in the clone library and N is the total number of clones analyzed (Jiang et al. 2010). One representative sequence was selected from each OTU for further analysis. The selected representative OTU sequences were translated into amino acid sequences using TBLASTX. The representative sequences and their closest references were aligned using CLUSTALX1.83. Phylogeny was constructed using neighbor-joining and maximum likelihood methods with the MEGA 5.0 program (Tamura et al. 2011). Bootstrap analysis was performed using 1000 replications. The archaeal *accA* gene sequences obtained in this study were deposited in the GenBank database under accession numbers KC433711–KC433733.

Statistical analysis

The diversity indices of Chao 1, Shannon, and Simpson were calculated using the SPADE software (Chao 2010). Pearson correlation analysis and Mantel test (http://bioinformatics.psb.ugent.be/webtools/zt/) were performed to assess the correlation between the environmental variables and the archaeal *accA* gene abundance and diversity according to the procedures described previously (Jiang et al. 2009).

Results

Environmental characteristics

Water temperature, pH, and major ions were determined at all the investigated hot springs (Table 1). Temperature ranged from 66 to 96 °C, and pH from 4.3 to 9.0. Most of the examined ions were variable in the investigated sites. For example, the highest concentrations of ammonia and nitrate were 382 and 40 mg/L, respectively, but the lowest concentrations of these two variables were below detection limits.

Abundances of archaeal *accA* and *amoA* and archaeal and bacterial 16S rRNA genes

The archaeal *accA* gene abundance ranged from 2.9×10^4 to 6.8×10^5 copies per gram of dry solids, a little higher than (but was very close to) the archaeal *amoA* gene abundance, in the investigated hot springs. The abundance of *accA* gene was markedly lower than that in soils and oceans (Yakimov et al. 2009; Pratscher et al. 2011; Hu et al. 2011b). The abundances of archaeal *accA* and *amoA* genes were three to six orders of magnitude lower than that of archaeal 16S rRNA gene copies (Fig. 1).

DNA-based diversity and phylogeny of archaeal *accA* gene

Eight DNA-based clone libraries were constructed (one for each sample). A total of 224 archaeal *accA* gene clone sequences were obtained and they could be grouped into 23

OTUs at the cutoff of 98 % identity (Table 3). The coverage ranged from 89 to 100 %, indicating that the sampled clones represent well the AOA *accA* gene diversity in the studied hot springs. Chao 1, Simpson's inverse index (*D*), and Shannon index were 4.5–14.0, 2.1–6.3, and 1.1–2.3, respectively.

Neighbor-joining and maximum likelihood phylogenetic trees consistently showed that all the obtained archaeal *accA* gene clone sequences could be divided into three clusters: Clusters A, B, and C (Fig. 2). Clusters A and B were affiliated with AOA lineages. Cluster A was affiliated with Group 1.1a (Dawson et al. 2006) and it was composed of two OTUs (OTU1 and OTU2), representing 6.3 % of the obtained archaeal *accA* gene clone sequences from hot springs Gmd, Wm3 and Sx4 (temperature 66–84 °C, pH 7.2–9.0) (Fig. 3a). Cluster B consisted of fourteen OTUs (OTU3 to OTU16) and was the dominant group representing of 86 % of the obtained archaeal *accA* gene clone sequences (Fig. 2). Cluster B has a relatively deep-branching association with all the known lineages of AOA and forms a distinct, well supported sister lineage to Group 1.1a and 1.1b. BLAST analysis showed that clone sequences in Cluster B had 68–71 % amino acid identity with all the closely related sequences known up to date in the NCBI database. Seven OTUs (OTU17 to OTU23) tightly formed Cluster C, which was affiliated with the *Desulfurococcales* (Fig. 3a). The clone sequences within Cluster C represented a total of 8.5 % of the obtained archaeal *accA* gene clone sequences.

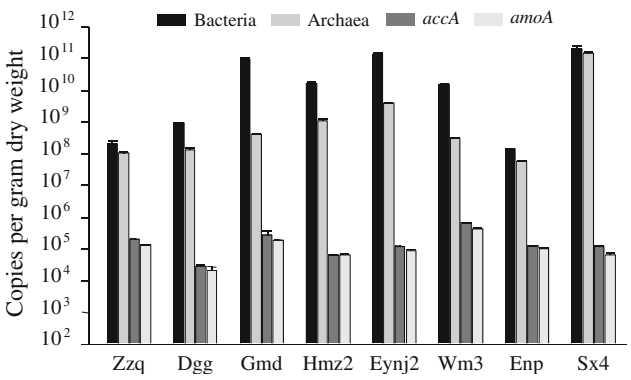


Fig. 1 Abundances of bacterial and archaeal 16S rRNA genes and archaeal *accA* and *amoA* genes in the investigated hot spring samples

cDNA-based diversity and phylogeny of AOA *accA* gene

cDNA-based clone libraries were successfully constructed on four hot spring samples (Gmd, Wm3, Eynj2, and Sx4). *accA* gene transcripts were not successfully amplified from the other four samples. The failure was possibly due to the following aspects: (1) the four hot spring samples may contain inhibiting substances affecting RNA harvest; and/or (2) RNA expression of *accA* gene may be low in failed samples. So the four failed samples were not included in downstream cDNA-based analyses.

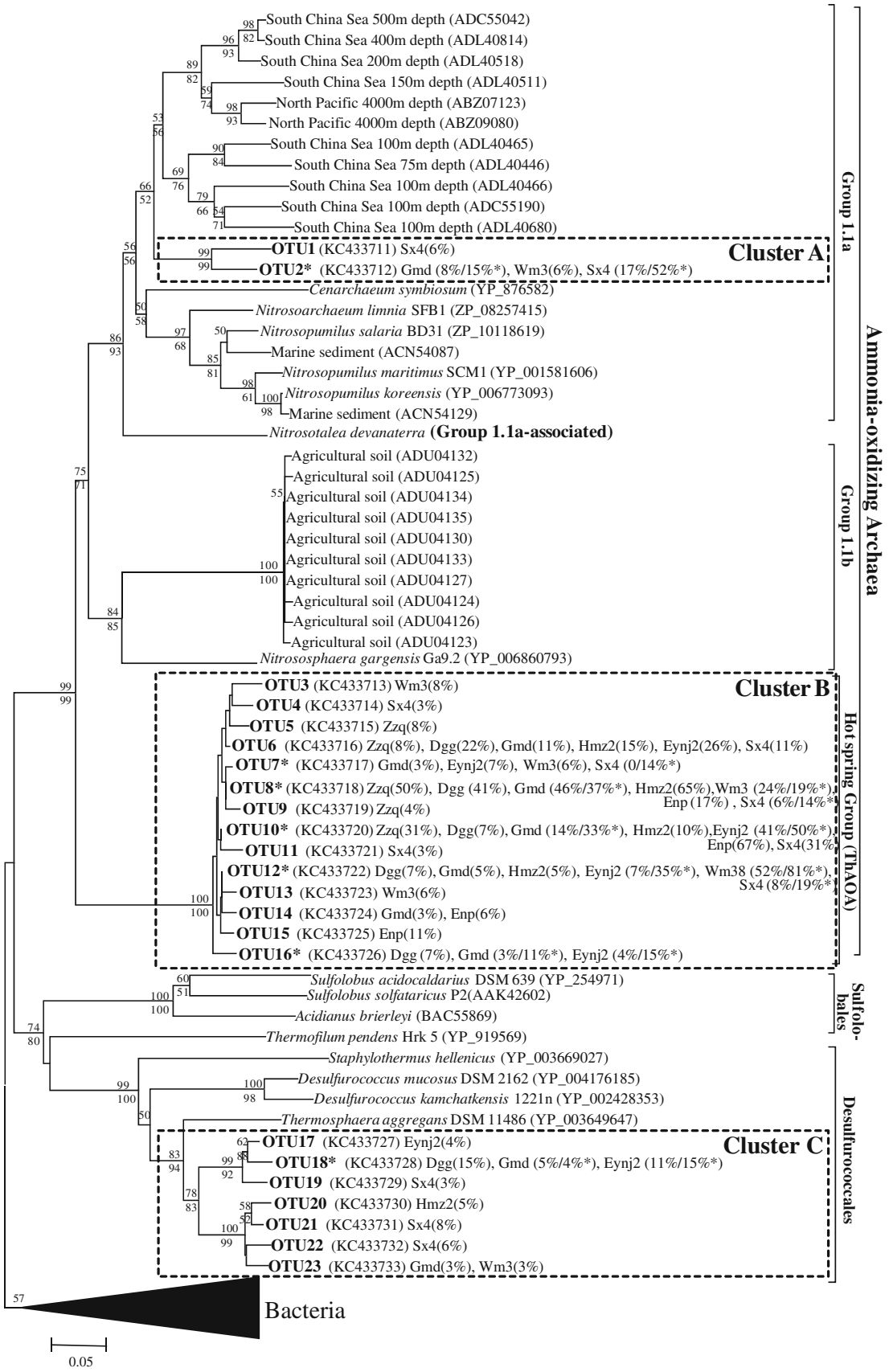
A total of 84 cDNA-based archaeal *accA* gene clone sequences were obtained and they were grouped into seven OTUs at the cutoff of 98 % amino acid similarity. Phylogenetic analyses showed that the cDNA-based OTUs fell into AOA-related Clusters A and B and *Desulfurococcales*-related Cluster C, and they were affiliated with their DNA-based counterparts (Fig. 2). Clusters A and B include 80 cDNA-based archaeal *accA* gene clone sequences. The Cluster A-related *accA* genes were expressed in Gmd and Sx4 hot springs (temperature 66 and 84 °C, respectively), and cluster B-related *accA* genes were expressed in all the four hot springs (temperature 66 to 84 °C). *Desulfurococcales*-related Cluster C contains four cDNA-based *accA* gene clone sequences, indicating that *Desulfurococcales*-related *accA* genes were expressed in Gmd and Eynj2 hot springs (temperature 73 and 84 °C, respectively) (Fig. 3b).

Statistical analysis

The simple Mantel tests and Pearson correlation showed that the abundances of the archaeal *accA* and *amoA* genes were significantly correlated ($r > 0.99$, $P < 0.005$) with each other, and they were significantly positively correlated with concentrations of nitrate ($r > 0.8$, $P < 0.005$) and nitrite ($r > 0.7$, $P < 0.005$), and were negatively correlated ($r < -0.8$, $P < 0.005$) with silicate concentration.

Table 3 Diversity indices of archaeal *accA* gene clone libraries retrieved from the eight hot springs in Yunnan Province, China

Community	Zzq	Dgg	Gmd	Hmz2	Eynj2	Wm3	Enp	Sx4
Library size (number of clones)	26	27	37	20	27	33	18	36
Number of OTUs	5	6	10	5	7	7	4	11
Coverage (%)	97	100	89	90	93	94	94	92
Chao 1	5.3	6.0	14.0	7.0	8.0	7.7	4.5	12.5
Shannon index	1.4	1.6	2	1.3	1.8	1.6	1.1	2.3
Simpson's inverse index (*D*)	2.8	3.9	3.9	2.2	3.9	3.0	2.1	6.3


South China Sea 500m depth (ADC55042)
South China Sea 400m depth (ADL40814)
South China Sea 200m depth (ADL40518)
South China Sea 150m depth (ADL40511)
North Pacific 4000m depth (ABZ07123)
North Pacific 4000m depth (ABZ09080)
South China Sea 100m depth (ADL40465)
South China Sea 75m depth (ADL40446)
South China Sea 100m depth (ADL40466)
South China Sea 100m depth (ADC55190)
South China Sea 100m depth (ADL40680)
OTU1 (KC433711) Sx4(6%)
OTU2* (KC433712) Gmd (8%/15%*), Wm3(6%), Sx4 (17%/52%*)
Cluster A
Cenarchaeum symbiosum (YP_876582)
Nitrosoarchaeum limnia SFB1 (ZP_08257415)
Nitrosopumilus salaria BD31 (ZP_10118619)
Marine sediment (ACN54087)
Nitrosopumilus maritimus SCM1 (YP_001581606)
Nitrosopumilus koreensis (YP_006773093)
Marine sediment (ACN54129)
Nitrosotalea devanaterra (Group 1.1a-associated)
Group 1.1a
Agricultural soil (ADU04132)
Agricultural soil (ADU04125)
Agricultural soil (ADU04134)
Agricultural soil (ADU04135)
Agricultural soil (ADU04130)
Agricultural soil (ADU04133)
Agricultural soil (ADU04127)
Agricultural soil (ADU04124)
Agricultural soil (ADU04126)
Agricultural soil (ADU04123)
Nitrososphaera gargensis Ga9.2 (YP_006860793)
Group 1.1b
OTU3 (KC433713) Wm3(8%)
OTU4 (KC433714) Sx4(3%)
OTU5 (KC433715) Zzq(8%)
OTU6 (KC433716) Zzq(8%), Dgg(22%), Gmd(11%), Hmz2(15%), Eynj2(26%), Sx4(11%)
OTU7* (KC433717) Gmd(3%), Eynj2(7%), Wm3(6%), Sx4 (0/14%*)
OTU8* (KC433718) Zzq(50%), Dgg (41%), Gmd (46%/37%*), Hmz2(65%),Wm3 (24%/19%*), Enp (17%) , Sx4 (6%/14%*)
OTU9 (KC433719) Zzq(4%)
OTU10* (KC433720) Zzq(31%), Dgg(7%), Gmd (14%/33%*), Hmz2(10%),Eynj2 (41%/50%*), Enp(67%), Sx4(31%)
OTU11 (KC433721) Sx4(3%)
OTU12* (KC433722) Dgg(7%), Gmd(5%), Hmz2(5%), Eynj2 (7%/35%*), Wm38 (52%/81%*), Sx4 (8%/19%*)
OTU13 (KC433723) Wm3(6%)
OTU14 (KC433724) Gmd(3%), Enp(6%)
OTU15 (KC433725) Enp(11%)
OTU16* (KC433726) Dgg (7%), Gmd (3%/11%*), Eynj2 (4%/15%*)
Cluster B
Hot spring Group (ThAOA)
Sulfolobus acidocaldarius DSM 639 (YP_254971)
Sulfolobus solfataricus P2(AAK42602)
Acidianus brierleyi (BAC55869)
Sulfolo-bales
Thermofilum pendens Hrk 5 (YP_919569)
Staphylothermus hellenicus (YP_003669027)
Desulfurococcus mucosus DSM 2162 (YP_004176185)
Desulfurococcus kamchatkensis 1221n (YP_002428353)
Thermosphaera aggregans DSM 11486 (YP_003649647)
OTU17 (KC433727) Eynj2(4%)
OTU18* (KC433728) Dgg(15%), Gmd (5%/4%*), Eynj2 (11%/15%*)
OTU19 (KC433729) Sx4(3%)
OTU20 (KC433730) Hmz2(5%)
OTU21 (KC433731) Sx4(8%)
OTU22 (KC433732) Sx4(6%)
OTU23 (KC433733) Gmd(3%), Wm3(3%)
Cluster C
Desulfurococcales
Ammonia-oxidizing Archaea
Bacteria
0.05

◀**Fig. 2** Neighbor-joining tree showing the phylogenetic relationships of archaeal AccA protein sequences (151 amino acid residues) obtained from the studied samples to closely related sequences from the GenBank database. The sequence of *Nitrosotalea devanaterra* was provided by Dr Laura Lehtovirta (University of Aberdeen). One clone type within each OTU is shown, and the accession number is shown after the OTU number. The relative abundance of clones represented by each OTU in each of the studied samples is shown after the OTU GenBank accession number. The percentages in the parentheses indicate the abundances of DNA/RNA (two percentages separated by a solidus)-based clones represented by the OTU type, respectively. *Asterisk* represented that the archaeal *accA* gene expression was detected. Neighbor-joining- and maximum likelihood-based bootstrap values of >50 % (for 1000 iterations) are indicated above and below the nodes, respectively. Scale bars indicate the Jukes–Cantor distances

Discussion

Occurrence of archaeal *accA* genes in Yunnan hot springs

To our knowledge, this study is the first to characterize archaeal *accA* gene diversity and abundance in terrestrial hot springs. The data presented here proved archaeal *accA* genes were present in Yunnan hot springs with a wide range of temperature and pH and could be expressed at least in four of the investigated sites with temperatures ranging from 66 to 84 °C (Fig. 3), which was consistent with the temperature range for the distribution and expression of archaeal *amoA* genes in these hot springs (Zhang et al. 2008; Jiang et al. 2010). Furthermore, the abundances of the archeal *accA* and *amoA* genes were almost equal with each other at each investigated site (Fig. 1), which was in agreement with other previous studies in marine and soil environments (Yakimov et al. 2009; Hu et al. 2011b). Taken together, our results suggested that the retrieved archaeal *accA* genes were mainly derived from AOA. The AOA in the Yunnan hot springs may acquire energy from ammonia oxidation coupled with CO_2 fixation using the 3-hydroxypropionate/4-hydroxybutyrate pathway, as has been suggested for their relatives in mesophilic environments (Auguet et al. 2008; Yakimov et al. 2009, 2011; Zhang et al. 2010; Pratscher et al. 2011).

Phylogeny of archaeal *accA* gene in Yunnan hot spring

The archaeal *accA* gene-based phylogenetic analysis in this study substantiated the wide distribution of ThAOA in terrestrial geothermal habitats. The cluster B has a relatively deep-branching association with all the known AOA lineages and forms a well-supported lineage distinct from groups 1.1a and 1.1b (Fig. 2). The phylogenitic position of this cluster strongly corresponds to the ThAOA/HWCG III group (Thermophilic Ammonia-Oxidizing Archaea/Hot Water Crenarchaeotic Group III), which represented a high-temperature archaeal *amoA* gene lineage (Nunoura et al. 2005; de la Torre et al. 2008; Stahl and de la Torre 2012; Hatzenpichler 2012). Cluster B might be related to ThAOA although the *accA* gene sequence of the representative organism Candidatus Nitrosocaldus yellowstonii is lacking, as the genome sequence is not yet available.

Environmental factors affecting AOA *accA* genes

Erguder et al. (2009) have reviewed the environmental factors affecting AOA abundance and/or diversity, and such factors include organic carbon, temperature, salinity, DO levels, pH, sulfide levels, and phosphate. Gubry-Rangin et al. (2011) proposed that pH was the major factor governing AOA community structure in global soil ecosystems. However, in this study, the diversity and

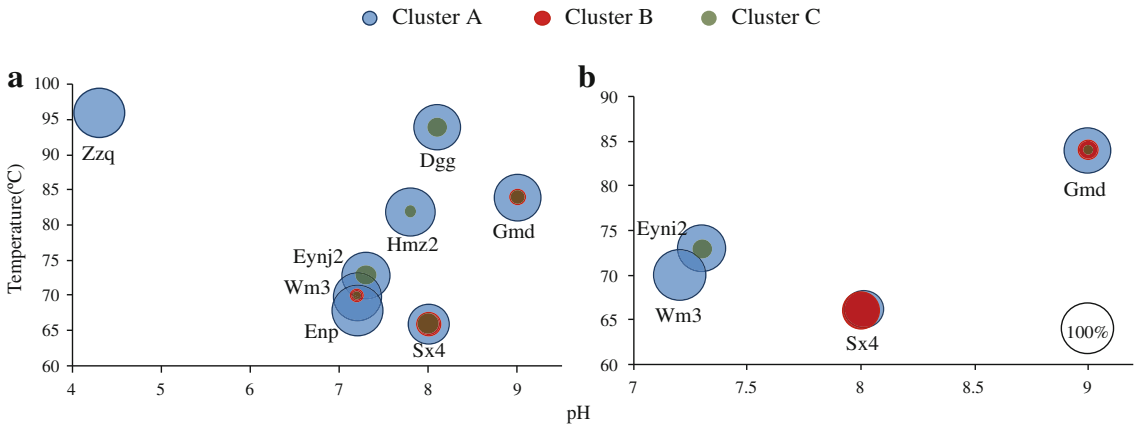


Fig. 3 Percentages of different phylogenetic groups as a function of temperature and pH in the investigated hot springs. **a**, **b** Indicate results of DNA- and RNA-based clone libraries, respectively. The *hollow circle* in the plots is scaled to 100 %

abundance of archaeal *accA* genes were not correlated with pH in the investigated hot springs. Our previous study also showed the community structures of *Crenarchaeota* and *Thaumarchaeota* in Yunnan hot springs were not affected by pH (Song et al. 2010). In addition, recent studies showed that the "Group 1.1a-associated" AOA preferred acidic or acido-neutral niches in soil ecosystems (Lehtovirta-Morley et al. 2011; Gubry-Rangin et al. 2011); however, AOA *accA* genes belonging to this group were not detected in acid or acido-neutral soil environments (Lehtovirta-Morley et al. 2011). In contrast, previous archaeal *amoA* gene-based studies showed that "Group 1.1a-associated" AOA were distributed in Yunnan and Yellowstone hot springs with a wide range of pH (pH 3.4–8.0) (de la Torre et al. 2008; Zhang et al. 2008). Taken together, the AOA populations in hot springs may not be limited by pH only.

In this study, a significant correlation was found between the concentrations of nitrate and nitrite and the abundances of archaeal *accA* and *amoA* genes in the studied hot springs. This correlation can be explained by the fact that nitrite and nitrate are the products of ammonia and nitrite oxidation, respectively. Such correlation was also indicative of nitrification activity. However, the concentration of ammonia (serving as the substrate for AOA) was not correlated with the abundances of archaeal *accA* and *amoA* genes. This can be explained by the fact that AOA subsisted on a very low level of ammonia (Hatzenpichler 2012), so the concentration of ammonia likely was not limited to AOA present in the investigated hot springs.

Desulfurococcales-related *accA* genes in Yunnan hot springs

It is notable that a small amount of archaeal *accA* gene clone sequences obtained in this study were affiliated with *Desulfurococcales*. Up to date, no research has ever shown that *Desulfurococcales*-related species autotrophically fix CO_2 using the 3-hydroxypropionate/4-hydroxybutyrate pathway; instead, they use another pathway of the dicarboxylate/4-hydroxybutyrate cycle (Huber et al. 2008; Berg et al. 2010a, b; Berg 2011). This inconsistency raises questions about of the function of the *accA* gene in *Desulfurococcales*-related organisms in geothermal ecosystems, and awaits further investigations. In summary, our study demonstrates the presence and expression of archaeal *accA* genes in Yunnan hot springs with a wide range of temperatures and pH. The obtained archaeal *accA* genes were mainly derived from AOA. The AOA in Yunnan hot springs might acquire energy from ammonia oxidation coupled with CO_2 fixation using the 3-hydroxypropionate/4-hydroxybutyrate pathway.

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