Isolation and characterization of a thermophilic sulfur- and iron-reducing thaumarchaeote from a terrestrial acidic hot spring

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Received: 10 September 2018 / Revised: 18 April 2019 / Accepted: 3 May 2019 / Published online: 6 June 2019
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Abstract

A deep-branching clade of *Thaumarchaeota*, conventionally called Terrestrial hot spring creanarchaeotic group (THSCG), is a missing link between thaumarchaeotic ammonia oxidizers and the deeper-branching non-ammonia oxidizers, such as *Crenarchaeota* and *Candidatus Korarchaeota*. Here, we report isolation of the first cultivated representative from the THSCG, named as NAS-02. Physiological characterization demonstrated that the isolate was a thermoacidophilic, sulfur- and iron-reducing organoheterotroph, which was supported by gene contents encoded in its complete genome. There was no evidence for ammonia oxidation by the isolate. Members in THSCG are likely thermophiles, and may play roles in degrading cell debris as a scavenger and in biogeochemical cycling of sulfur and iron in the hot environments, as suggested by the physiological characteristics of the isolate and the geographical distribution of the 16S rRNA gene sequences of THSCG in terrestrial hot springs and marine hydrothermal fields. Phylogenetic analysis suggests that the THSCG lineage represented by NAS-02 has gained the ability of sulfur reduction via horizontal gene transfer. Based on the phylogeny and physiology, we propose the name *Conexivisphaera calidus* gen. nov., sp. nov. to accommodate the isolate.

Introduction

Archaea are ubiquitous and play a significant role in biogeochemical cycling in the environment. Recent culture-independent metagenomics and single-cell genomics have allowed to determine genome sequences of individuals of

novel archaeal clades without isolated representatives [1–6]. These findings have strongly pushed forward our knowledge of the phylogenetic diversity and metabolic potentials in the *Archaea*, and moreover, evolution of life. Remarkably, all archaeal isolates reported so far belong to only several clades in *Crenarchaeota*, *Euryarchaeota*, and *Thaumarchaeota*. The physiology of the remaining uncultured archaeal clades has not been demonstrated yet [7, 8].

Terrestrial hot springs harbor diverse as-yet-uncultured archaea in higher-taxonomic-level clades [9–11]. One of the uncultured clades is the Terrestrial hot spring creanarchaeotic group (THSCG) [12], also referred to as Hot Thaumarchaeota-related Clade (HTC) [13]. THSCG is phylogenetically located between ammonia-oxidizing thaumarchaeota (AOT) and deeper lineages, such as *Crenarchaeota*, *Ca. Aigarchaeota*, and *Ca. Korarchaeota* [14]. Thus, THSCG is a key group to understand the evolution of *Archaea* from non-ammonia-oxidizers to AOT that play a significant role in biogeochemical cycling of nitrogen and carbon in the present Earth [15–17]. Metagenome-assembled genomes (named Beowulf BS1 and Dragon DS1) reported in hot springs in Yellowstone National Park, USA, have suggested that THSCG contains sulfur-oxidizing nitrate/oxygen-reducers and heterotrophic sulfur-reducers

Supplementary information The online version of this article (<https://doi.org/10.1038/s41396-019-0447-3>) contains supplementary material, which is available to authorized users.

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[18]. However, the ecophysiology of THSCG is still poorly understood because of the absence of any isolated representative. Here, we report the isolation and characterization of a sulfur- and iron-reducing thermoacidophilic archaeon, strain NAS-02, belonging to THSCG. We performed physiological, morphological and genomic analyses of this first isolate of THSCG.

Materials and methods

Sampling and site description

We collected fluid samples at a thermoacidic spring at Oku-Shiobara, Tochigi, Japan (36°57'17" N, 139°46'47" E), as previously described [19]. The fluid chemistry of the upper, middle and lower points of a stream (Fig. S1; Table S1) were measured in April 2012. Temperature, pH and dissolved O₂ concentration were measured using a portable meter (D-55; Horiba, Kyoto, Japan). Salinity was measured using a hand-held refractometer (MASTER-S/Millα; Atago, Tokyo, Japan). Concentration of total sulfide ion was measured using a Gastec detector tube (Type 211; Kanagawa, Japan). Concentration of Fe²⁺ was measured by ferrozine assay [20]. For PCR clone analysis of 16S rRNA genes, we collected the fluid samples (1 L for each sampling point). The collected samples were stored with cold storage packs in a cooler box, brought back to our laboratory, and filtered using 0.2-μm-pore-sized membrane filters within a day. The filters were stored in −20 °C until DNA extraction. For cultivation, fluid samples were collected into sterile glass bottles at the middle point of the stream in August 2012. The collected samples were brought back to our laboratory without cooling, inoculated into an enrichment medium, and incubated within a day.

Enrichment and isolation

Using the collected sample from the middle stream as an inoculum, we performed enrichment cultivations with the culture medium contained (per liter); 0.2 g (NH₄)₂SO₄, 3.0 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 0.25 g CaCl₂·2H₂O, 1.8 mg MnCl₂·4H₂O, 4.5 mg Na₂B₄O₇·10H₂O, 0.22 mg ZnSO₄·7H₂O, 0.05 mg CuCl₂·2H₂O, 0.03 mg Na₂MoO₄·2H₂O, 0.03 mg VOSO₄·nH₂O, 0.01 mg CoSO₄·7H₂O, 0.5 g yeast extract (BD-Difco), 10 mM Fe(III)-citrate and 10 ml vitamin mixture [21]; pH adjusted to 5.0. The medium was placed in culture bottles under a gas phase of N₂/CO₂ (4:1, v/v), and reduced with 0.25 mM L-cysteine.HCl and 1.3 mM FeCl₂·4H₂O. The cultivation was performed at 70 °C for 1 month, and microbial growth was observed in the enrichment culture by phase-contrast microscopy. An archaeal strain was isolated by a repeated dilution-to-

extinction method, designated as NAS-02. Purity of the strain was confirmed by morphological observation and the 16S rRNA gene clone analysis as previously reported [19] using bacterial and archaeal universal primers: Arch9F [10] and Uni1406R [22] for archaea, and EB-20F [23] and Uni1406R for bacteria. The purity was also proved by the whole-genome sequencing, showing that only the sequences of the NAS-02 genome were detected.

Physiological characterization

During growth characterization, NAS-02 was found to grow better in a modified enrichment medium supplemented with 2.5 g L^{−1} Na₂S₂O₃·5H₂O, instead of Fe(III)-citrate. Unless otherwise indicated, it was routinely cultivated with S₂O₃^{2−} at 65 °C at pH 5.0 under N₂-CO₂ (4:1, v/v). NAS-02 was tested for growth on organic and inorganic substrates. As electron acceptors, S₂O₃^{2−}, SO₄^{2−}, NO₃[−], S⁰, and Fe(III) citrate were added (final 20 mM). For the gas phase composition, N₂, N₂-CO₂ (80:20, v/v), H₂-CO₂ (80:20, v/v), N₂-CO₂-O₂ (79:20:1, v/v/v), and N₂-CO₂-O₂ (78:20:2, v/v/v) were tested. Growth at various pHs was examined with the following buffers (10 mM each): pH 4.0–5.5, HOMO-PIPES; pH 5.0–7.0, MES; 6.5–8.0, HEPES. The pH of the culture medium was adjusted from 3.5 to 6.5 at 0.5 intervals. The cultivations were performed at 55 °C to 80 °C at 5 °C intervals. As electron donors and carbon sources, a variety of organic carbon compounds, such as sugars, organic acids, alcohols and proteinaceous substances, were added (Table S2). Growth was determined using a fluorescence microscopy (BX51, Olympus, Japan) by direct counts of cells stained with SYBR green I.

Morphology

Morphology of cells was observed by phase-contrast microscopy and transmission electron microscopy (TEM). For preparation of ultra-thin sections for TEM, we employed a method using freeze-substitution after glutaraldehyde fixation [24] with minor modifications to preserve ultrastructure of the cells. The cells grown at 70 °C for 3 weeks were fixed with formaldehyde (final 4%) in the culture at 70 °C for 30 min, and then these were fixed with glutaraldehyde (final 2.5%) in the culture at room temperature. The fixed specimens were frozen in a high-pressure freezing apparatus (EM-PACT2; Leica, Wetzlar, Germany). The frozen samples were substituted with OsO₄ (final 2%) in acetone for 4 days at −80 °C. The samples were warmed gradually to room temperature, rinsed with acetone, stained with uranyl acetate (final 2%) in methanol, and then embedded in epoxy resin (TAAB Laboratories Equipment Ltd, Aldermaston, UK). Ultra-thin sections (70 nm in thickness) were prepared using an ultra-

microtome (EM-UC7; Leica), and were stained with uranyl acetate (final 2%) and lead-stained solution (lead nitrate and lead acetate, final 0.3% each). The sections were observed by a TEM (Tecnai G2 20; FEI, Hillsboro, OR, USA) at an acceleration voltage of 120 kV. We performed elemental analysis using energy dispersive X-ray spectrometry (EDS) (EDAX EDS System; AMETEK, Inc., Minneapolis, MN, USA) equipped with the TEM.

Genome analysis

Genomic DNA was extracted from cell suspension of NAS-02 after 40 days cultivation as previously described [19]. The DNA library was constructed using a Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA), and genome sequencing was performed on the Illumina MiSeq platform with MiSeq Reagent Kit v3 (2x300). The extracted DNA was also applied to whole-genome sequencing using an Ion Torrent PGM system (Life Technologies) and 454 pyrosequencing (Roche) at the Center for Omics and Bioinformatics of the University of Tokyo, Japan. We performed removal of adapter sequences from reads, removal of the reads shorter than 100 bps, and trimming of low-quality ends of the reads using CLC Genomics Workbench version 9.5.3 (QIAGEN Aarhus A/S) with the default setting. All the treated reads were assembled using SPAdes [25] version 3.9.0 with the following parameter (-k 33, 55, 77, 99), resulting in six contigs. Gaps were closed by sanger sequencing of PCR-amplified gap regions. The genome sequences were annotated using Prokka [26] version 1.11 that includes Prodigal [27], Infernal [28] and RNAmmer [29] for detection of protein-coding DNA sequences (CDSs), non-coding RNA and rRNA, respectively. The tRNAs in the genome were predicted using tRNAscan-SE v.2.0 [30]. We also used RAST [31] version 2.0 for gene prediction and annotation. The results of gene prediction and annotation were manually checked and curated. The position of OriC was predicted based on the previous report [32]. Homology searching of all of the CDSs against a non-redundant (nr) database in NCBI (downloaded at 12 April 2018) were performed using Blastp [33] version 2.7.1 with the default parameters. Using the searching result with top 100 hits, the taxonomic classification of the CDSs were determined using MEGAN community edition [34] version 6.11.2 with the default setting by the lowest common ancestor algorithm [35]. All of the CDSs were classified into Orthologous Groups (OGs) using eggNOG version 4.5.1 [36]. The prediction of metabolic pathways was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway tool [37]. Functional properties of proteins coded by CDSs were predicted using InterProScan [38] version 5.24–63.0. Extracellular

transport signals of CDSs were identified using PSORTb 3.0 [39]. The peptidase database MEROPS was used to classify peptidase-like CDSs [40]. Optimal growth temperatures were estimated from genome sequences using Tome version 1.1 [41].

Phylogenetic analysis

To construct a phylogenetic tree for the 16S rRNA gene of NAS-02, its relatives were collected from public databases, such as Genbank/DDBJ/EMBL and Silva database [42]. The sequences of NAS-02 and the relatives were aligned by SINA [43] using an aligned sequences curated by Silva [42] as a reference. The alignment was trimmed using TrimAl [44] version 1.2re59 with the “-automated1” option. The trimmed alignment was used for Maximum-Likelihood tree construction using RAxML [45] version 8.2.10 with the GTRGAMMA model. To construct a genome tree, i.e., a phylogenetic tree of concatenated amino acid sequences from the 43 single copy marker proteins (Table S3) as determined previously [46], the dataset of the sequences were collected from the databases and aligned using MUSCLE [47] version 3.8.31. Based on the alignment trimmed using TrimAl, the genome tree was constructed using RAxML with the PROTOGAMMALG model. We also constructed phylogenetic trees of amino acid sequences encoded by each CDS as described above. For all trees, bootstrap support values were computed with 1000 resamples.

16S rRNA gene survey

The 16S rRNA genes related to THSCG deposited into Sequence Read Archive (SRA) in National Center for Biotechnology Information (NCBI), which were produced by next-generation sequencing methods, such as pyrosequencing and Illumina technology, were surveyed using an IMG-NGS (Integrated Microbial Next-Generation Sequencing) platform [48] with a 95% similarity threshold. In this survey, we used 10 representative sequences of the reported THSCG 16S rRNA genes (AB007307, DQ834113, AB007308, EF156533, HE574567, AB019732, DQ243777, and FJ797319 for NCBI accession number, 2263083025 (DS1) for JGI-IMG accession number, and that of NAS02) as queries. The 16S rRNA genes affiliated in THSCG in the Silva database release 132 were also surveyed.

16S rRNA gene PCR clone analysis

Genomic DNA was extracted from the membrane filters as described above. Archaeal 16S rRNA genes were amplified by PCR using Arc9F and Uni1406R, and the PCR products were cloned and sequenced as previously described [10].

Table 1 Summary of habitat, physiology, and genomic feature of NAS-02 and close relatives

Strain, MAG/SAG ID	NAS-02	Dragon (DS1)	Beowulf (BS1, BS4)	AB-179-E04	HRbin06	Fn1
Habitat	Terrestrial hot spring (57 °C, pH 2.2)	Terrestrial hot spring (70–72 °C, pH 3.1)	Terrestrial hot spring (65–68 °C, pH 3.0)	Terrestrial hot spring (75–85 °C, no pH data)	Terrestrial hot spring (69–72 °C, pH 6.1–6.4)	Fen (no temp. data, pH 4.5–4.8)
Physiology						
Growth pH	4.5–5.5 (5.0) ^a	N.A. ^b	N.A.	N.A.	6.8–8.0	N.A.
Growth Temp.	60–70 °C (65 °C) ^a	N.A.	N.A.	N.A.	70 °C	N.A.
Estimated optimal growth temp. ^c	66 °C	71 °C	58 °C	77 °C	71 °C	42 °C
Electron donor ^d	Org. C ^e	Org. C	Org. C, elemental sulfur	Org. C	Org. C	
Electron acceptor ^d	thiosulfate, elemental sulfur, ferric iron	elemental sulfur	oxygen, nitrate	N.A.	oxygen, nitrate	fumarate
Carbon source ^d	Org. C	Org. C	Org. C	N.A.	Org. C	Org. C
Genomic feature						
DNA source	Isolate	Environment	Environment	Environment	Enrichment culture	Environment
Size (bp)	1,593,902	1,485,980	1,532,059	1,202,119	291,490	1,716,974
Number of contigs	1	38	76	110	20	90
GC content (%)	62.1	40.2	45.6	41.9	45.9	56.3
Number of CDSs	1608	1685	1112	1411	327	1891
Number of rRNA operons	1	1	1	1	1	1
Number of tRNAs	46	48	20	22	4	34
Completeness (%) ^f	98.1	88.2	38.3	84.0	33.5	97.3
Contamination (%) ^f	0	0.97	0	0.97	0	2.91
Accession number ^g	AP018732	2263082000	2502957026	2519899514	ASPK000000000	2558309099
Reference	This study	Beam et al. (2014)		Rinke et al. (2013)	Kato et al. (2018)	Lin et al. (2015)

^aOptimal values in parentheses^bNot applicable^cDetermined by Tome^dGene-based prediction in italics^eOrganic carbon^fCalculated by CheckM in this study^gJGI or NCBI number

QIIME 1.9.1 [49] was used for chimera removal, OTU clustering, and taxonomic affiliation of the obtained sequences. The detected clones and close relatives were collected from public databases, the sequences were aligned using SINA, gap positions were removed, and a ML tree of the 16S rRNA sequences was constructed using RAxML, as described above.

Accession number

The complete genome sequence of the NAS-02 was deposited into the DDBJ under the following accession number, AP018732. The archaeal 16S rRNA genes used in the clone library analysis were deposited into the DDBJ under the following accession numbers; AB931042–AB931105, LC158371–LC158446, and AB930951–AB931041, for the upper, middle, and lower stream samples, respectively.

Results and discussion

We isolated NAS-02 from a thermoacidic water sample (57 °C and pH 2.2) using an anaerobic medium amended with Fe(III)-citrate as the sole electron acceptor. We determined its complete genome sequence (Table 1, Fig. S2, Table S4), and constructed phylogenetic trees with a concatenated alignment of conserved single copy marker proteins (called genome tree) and with 16S rRNA genes (Fig. 1a, b, respectively) extracted from the genome. The results indicated that NAS-02 belonged to THSCG, and supported that THSCG branched out between the AOT clade and other archaeal clades, such as *Crenarchaeota* and *Ca. Aigarchaeota*, as reported previously [14, 18]. NAS-02 clustered with the metagenome-assembled genomes of Beowulf BS1 and Dragon DS1 [18]. The closest 16S rRNA gene sequence deposited in public databases to that of NAS-02 was the fosmid clone 1m19 recovered from a Kamchatka thermoacidic spring, Russia [13] with a 99.3% similarity. Based on the 16S rRNA gene phylogeny (Fig. 1b), NAS-02 was affiliated with Group I.1f/HTC2, a sub-clade of the THSCG [13, 18]. The closest 16S rRNA gene sequence of cultured species to that of NAS-02 was *Hyperthermus butylicus* (85.1%), followed by *Pyrolobus fumarii* (84.8%) and *Ca. Nitrosocaldus yellowstonii* (83.3%).

Considering the phylogenetic positions of THSCG on the basis of the single copy marker proteins or the 16S rRNA gene, THSCG should represent a major clade different from AOT or class *Nitrososphaera* among the phylum *Thaumarchaeota* [50]. At present, NAS-02 is the sole cultivated member of THSCG, and we propose tentatively the name *Conexivisphaera calidus* to accommodate NAS-02 (=JCM 31663). The genus will also represent the higher taxa (i.e.,

Fig. 1 Phylogenetic trees for NAS-02. The maximum-likelihood trees were constructed using RAxML for **a** concatenated single copy marker proteins (a total of 43 proteins) and for **b** 16S rRNA genes. Bootstrap values are indicated as filled circles ($\geq 70\%$) and open circles (≥ 50 and $< 70\%$) at nodes. The scale bar represents 0.1 amino acid or nucleotide substitutions per sequence position. AOT, ammonia-oxidizing thaumarchaeota; THSCG, Terrestrial Hot Spring Crenarchaeotic Group

Fig. 2 Morphology of NAS-02 cells. **a** An image of phase-contrast microscopy. **b** An image of ultrathin-section transmission electron microscopy. S-layer and cell membrane were observed (enlarged image in the left bottom box). Particles showing high electron density were observed both inside and outside a cell

family, order and class) of the THSCG lineage within the phylum *Thaumarchaeota*.

Physiological characterization demonstrated that NAS-02 was a moderate thermophilic, weak acidophilic, iron- and sulfur-reducing organoheterotrophic archaeon. Its physiology is summarized in Table 1. A growth curve of NAS-02 cultivated with $S_2O_3^{2-}$ as the electron acceptor at the optimal conditions (65 °C and pH 5.0) is shown in Fig. S3. Its doubling time was 40 h at the exponential phase. The maximal cell density was $\sim 1.0 \times 10^7$ cells per mL. NAS-02 grew with sulfur species and Fe^{3+} as sole electron

acceptors, but neither NO_3^- nor O_2 . Neither fermentative nor autotrophic growth was observed. It used proteinaceous substances (such as yeast extract and peptone) as the electron donors and carbon sources, but not the other organic carbon compounds tested (Table S2). Cells of NAS-02 were in irregular-coccoid shape, $\sim 0.5\text{--}0.8\ \mu\text{m}$ in diameter (Fig. 2). No motility was observed, and neither flagella-like nor pili-like structures was found. There were intracellular particles containing S, Fe, As, Se, and P in the cells growing with thiosulfate (Fig. S4). It is possible that sulfide is produced by thiosulfate reduction, and then precipitated as metal sulfide (or phosphate) within the cells. Such particles were also observed outside the cells (Fig. 2b, Fig. S4).

The genomic characteristics reflect the thermophily of NAS-02. The genome encodes a gene for reverse gyrase (NAS2_0035) that is a hallmark of (hyper)thermophiles [51]. This gene was also found in DS1, but not in other relatives (such as BS1, HRbin06, and AB-179-E04) from hot environments possibly due to incompleteness of these genomes. We estimated optimal growth temperature ($66\ ^\circ\text{C}$) of NAS-02 based on its proteome-wide 2-mer amino acid composition [41], which was consistent with its actual growth temperature. In addition, the estimated optimal growth temperatures of its relatives were also consistent with the in situ temperatures of their habitats (Table 1). Thus, these results strongly support that the deep-branching lineages of *Thaumarchaeota* include thermophiles, as suggested previously [18].

Anabolic and catabolic metabolisms deduced from the annotatable CDSs (Fig. 3; Table S4) were consistent with the above physiological characterization. The NAS-02 genome encodes genes involved in peptide and fatty acid degradation and elemental sulfur/thiosulfate reduction. For the peptide degradation, genes encoding extracellular and intracellular peptidases, aminotransferases of branched amino acids and aspartate, glutamate dehydrogenase are likely involved. NAS-02 is also likely able to degrade fatty acids via beta oxidation pathway. The acetyl-CoA, oxaloacetate, and 2-oxoglutarate produced by degradation of peptides and fatty acids could be used for production of biomass. ATP and reduced cofactors, such as NADPH, reduced ferredoxin (Fd_{red}), and quinol (QH), could be produced via the Embden–Meyerhof–Parnas (EMP) pathway, pentose phosphate (PP) pathway, and tricarboxylic acid (TCA) cycle, of which complete gene sets were found in the genome. Acetate might be incorporated into acetyl-CoA via AMP-forming acetyl-CoA synthase (ACS) using ATP.

The NAS-02 genome encodes genes for NAD(P)H:sulfur oxidoreductase (Nsr), thiosulfate reductase / polysulfide reductase (Phs/Psr), and sulfur reductase (Sre), which are likely involved in the reduction of S^0 and $\text{S}_2\text{O}_3^{2-}$. The genome also encodes a gene for thiosulfate:quinone oxidoreductase (Tqo), which may be used for quinol oxidation

and $\text{S}_2\text{O}_3^{2-}$ reduction. In addition, we found genes for ferredoxin:NADP⁺ oxidoreductase (FNR) and electron transfer flavoprotein (ETF):quinone oxidoreductase (Fix), which are likely involved in electron bifurcation. We found genes for NAD(P)H:quinone oxidoreductase (Nuo)-like complex. The Nuo-like complex contained homologs of subunits NuoA–D and H–N, but not NuoE, F, and G that are necessary to obtain electron from NAD(P)H. Such Nuo-like complex without NuoE–G is thought to be a progenitor of Complex I as Fd_{red} :quinone oxidoreductase [52]. Indeed, a Nuo-like complex without NuoE–G in a methanogenic euryarchaeote *Methanosaeta thermophila* oxidizes Fd_{red} [53]. Oxidation of the reduced cofactors on the cell membrane via the Sre, Phs/Psr, and/or Nuo-like complex could create proton motive force (PMF), and eventually, ATP could be generated by the ATPase using the PMF. The H^+/Na^+ antiporter could be used for maintaining homeostasis of pH and salinity.

The NAS-02 genome encodes a total of 18 genes related to molybdoenzymes containing a molybdenum cofactor (MoCo) binding site (Fig. S5). Molybdoenzymes include a variety of oxidoreductases, which has been classified into three families, i.e., the xanthine oxidase (XO) family, dimethyl sulfoxide (DMSO) reductase family, and sulfite oxidase family [54]. The XO family includes carbon monoxide dehydrogenase (Cox) and aldehyde oxidoreductase (AOR), in addition to XO. The DMSO family includes formate dehydrogenase (Fdh), and Sre and Phs/Psr. The NAS-02 genome encodes 5 and 13 genes for the XO (Fig. S6) and DMSO families (Fig. S7A–F), respectively. Although genes of the XO family were also found in the genomes of the NAS-02 relatives (e.g., DS1 and BS1), those of DMSO families were rarely found in them possibly due to their incompleteness. Although these molybdoenzymes (except Sre and Phs/Psr) appeared to be involved in oxidation/reduction of carbon monoxide, organic carbon such as formate and aldehydes, and redox cofactors, their exact functions are still unclear because of no supporting physiological data. Further physiological and biochemical analyses are needed to reveal their functions.

The genome encodes cytochrome bd ubiquinol oxidase, Bcp-type thiol peroxidase, and superoxide dismutase, which might be involved in resistance to oxidative stress. Indeed, NAS-02 was tolerant to traces O_2 (i.e., growth without a reducing reagent, such as L-cysteine or sodium sulfide, into the media), but could not grow at 1% or higher O_2 levels in the headspace.

Although NAS-02 grew on Fe^{3+} , there were no homologs for known ferric iron reductase and multi-heme c-type cytochromes, which are involved in iron reduction in archaea and bacteria [55–57]. NAS-02 may have an unknown mechanism for Fe reduction, as reported for the other Fe-reducing archaea, such as *Pyrobaculum*

Fig. 3 Metabolisms deduced from annotatable CDSs. The list of the CDSs is shown in Table S4

spp., *Pyrococcus* spp. [58], and members in *Ca. Marsarchaeota* [6].

As reported in the nearly complete genomes of deeper lineages of *Thaumarchaeota* [18, 59, 60], the NAS-02 complete genome also lacked *amo* for ammonia monooxygenase, a key enzyme for ammonia oxidation [15]. This strongly supports that the deeper lineages of *Thaumarchaeota* do not represent ammonia oxidizers.

We focused on the evolutionary history of genes encoding Nsr, Sre, and Phs/Psr that are involved in reduction of sulfur compounds. The phylogenetic analysis (Fig. 4a) showed that Nsr of NAS-02 was located within a crenarchaeal clade of the class *Thermoproteaceae*. Although a *nsr* was also found in DS1, it was phylogenetically distant from that of NAS-02 (Fig. 4a). The

phylogenetic analysis of the catalytic subunit of Sre (Fig. 4b) showed that Sre of NAS-02 was located within a crenarchaeal clade of the class *Sulfolobaceae*. Neither *phs/psr* nor *sre* has been found in the genomes of the deep-branching relatives (e.g., DS1 and BS1) of *Thaumarchaeota* so far. Based on the phylogenies (Fig. 4a, b), it seems that the lineage of NAS-02 has gained the genes of Nsr and Sre from members of *Crenarchaeota* via horizontal gene transfer (HGT). Indeed, the Nsr and Sre of NAS-02 were most closely related to those of *Vulcanisaeta* spp. of *Thermoproteaceae* and *Acidianus* spp. of *Sulfolobaceae*, of which 16S rRNA genes were detected in the sampling point by PCR clone library analysis (Fig. S8). In contrast, Phs/Psr of NAS-02, as well as those of a bacteria clade containing members (e.g., *Sutterella* spp. and *Dakarella* spp.) of the

Fig. 4 Phylogenetic trees for CDSs involved in sulfur reduction. The maximum-likelihood trees were constructed using RAXML for **a** Nsr and **b** Sre and Phs/Psr. Taxonomic names at order and class levels for *Crenarchaeota* are shown. Stars indicate the presence of the same species in each tree. Bootstrap values are indicated as filled circles ($\geq 70\%$) and open circles ($\geq 50\%$ and $< 70\%$) at nodes

family *Sutterellaceae* of *Betaproteobacteria*, was located between crenarchaeal clades of *Sulfolobales* and *Thermoproteales*. If NAS-02 had vertically gained the genes of Nsr,

Sre, and Phs/Psr from the common ancestor of THSCG and *Crenarchaeota*, the NAS-02 genes would have been located outside a large clade including *Sulfolobales* and

Thermoproteales at least. It should be noted that the genes of Nsr, Sre, and Phs/Psr have likely experienced multiple events of gene duplication, gene loss, and HGT, as suggested by the phylogenetic trees (Fig. 4). Thus, the evolutionary history of the genes involved in sulfur reduction are complicated. Further determination and analysis of genome sequences of members in THSCG and relative clades are needed to reveal the detailed evolutionary history of the genes.

To assess geographical distribution of THSCG, we surveyed 16S rRNA gene sequences of THSCG in public databases. Although members of THSCG were hardly detected in the hot spring water obtained for this study, the 16S rRNA gene sequences of THSCG have been frequently detected in terrestrial acidic hot springs of several countries, such as Russia, China, Argentina and Japan, and also in marine hydrothermal fields, such as Okinawa Trough, Juan de Fuca Ridge, Mid Atlantic Ridge, and Mohn Ridge (Fig. S9). As consistent with the thermophily of NAS-02, the geographical distribution of THSCG appears to be restricted to thermal environments. Based on the NAS-02 physiology shown in the present study and the previous metagenomics insights [18], members in THSCG may play a role in degrading organic detritus as a “scavenger” and in geochemical cycling of sulfur and iron in such environments.

Acknowledgements We would like to thank the owner of the spa for permitting us to collect hot spring samples. We also would thank Dr. Kenshiro Oshima and Dr. Masahira Hattori (The University of Tokyo) for their help on genome sequencing. We would also like to thank anonymous reviewers for their helpful and insightful suggestions. This work was supported by RIKEN Special Postdoctoral Researchers Program, RIKEN Incentive Research Grant and Institute of Fermentation (IFO), Osaka, and partially by the Cabinet Office, Government of Japan, through the Next-generation Technology for Ocean Resources Exploration (called as Zipangu-in-the-ocean project) in the Cross-ministerial Strategic Innovation Promotion Program (SIP).

Author contributions S.K., T.I., and Mo.O. conceived this study. S.K., T.I., T.T., K.S., and Mo.O. supervised experiments and analyses. S.K., T.I., M.N., Ma.O., M.Y., and K.U. performed analyses. S.K., T.I., and Mo.O. wrote the paper with contributions from all authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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