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Metallosphaera sedula TA-2, a calditoglycerocaldarchaeol deletion strain of a thermoacidophilic archaeon

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Abstract A spherical thermoacidophilic archaeon, strain TA-2, was obtained from acidic hot springs located in Ohwaku Valley, Hakone, Japan. This isolate is an obligate aerobic chemoorganoheterotroph that grows optimally at about 75°C, pH 2.8. The G + C content of DNA from TA-2 is 47 mol%. The 16S rRNA gene from TA-2 showed more than 99% similarity with those of *Metallosphaera sedula* and *Metallosphaera prunae* and less than 92% similarity with other members of the order *Sulfolobales*. DNA–DNA hybridization experiments showed more than 93% genomic DNA homology among TA-2, *M. sedula* DSM5348^T, and *M. prunae* DSM10039^T. However, TA-2 lacks calditoglycerocaldarchaeol derivatives, which are usually found in the membrane lipids of members of the order *Sulfolobales*. Therefore, calditoglycerocaldarchaeol may not be essential for survival in thermophilic and acidophilic environments. The isolate was deposited as *Metallosphaera sedula* TA-2 (JCM 9064, IFO 15160).

Key words Archaea · Thermophile · Acidophile · *Metallosphaera* · Calditoglycerocaldarchaeol

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Introduction

The membranes of archaeal species belonging to the order *Sulfolobales* are monolayers based on two types of cyclic tetraether core lipids, caldarchaeol (glycerol dialkyl glycerol tetraethers) and calditoglycerocaldarchaeol (glycerol dialkyl calditol tetraether, previously called glycerol dialkyl nonitol tetraether) (Gambacorta et al. 1995). The calditoglycerocaldarchaeol derivatives are typically found only in the members of the order *Sulfolobales* (De Rosa and Gambacorta 1988; Gambacorta et al. 1995; Sugai et al. 1995). In contrast, we have isolated several thermoacidophilic archaeal strains from hot springs in Japan and have analyzed their core lipid compositions. Both caldarchaeol and calditoglycerocaldarchaeol were detected in almost all these isolates, and calditoglycerocaldarchaeol was found to be a major core component that constituted 70%–80% of all core lipids. Surprisingly, one of the isolates, strain TA-2, lacked calditoglycerocaldarchaeol. Analysis of the 16S rRNA genes showed that this isolate was closely related phylogenetically to *Metallosphaera sedula* and *Metallosphaera prunae*. In this article, we describe the isolation and characterization of TA-2, a calditoglycerocaldarchaeol deletion strain of the genus *Metallosphaera*.

Materials and methods

Bacterial strains

The sample of muddy water was obtained from acidic hot springs (70°C, pH 3) of OhwakuValley, Hakone, Japan. The sample was transported at ambient temperature and cultured at 70°C in "TA (thermophilic acidophile) medium" ($g l^{-1}$): $(NH_4)_2SO_4$ (1.3), KH_2PO_4 (0.25), $MgSO_4 \cdot 7H_2O$ (0.25), $CaCl_2 \cdot 2H_2O$ (0.08), and yeast extract (1.0; Difco, Detroit, MI, USA). Before autoclaving, the pH was adjusted to 3.0 with 10% H_2SO_4 (w/v) at room temperature. The isolate was purified by single colony isolation on TA plates, composed of TA medium solidified by 0.6% gellan

gum (Merck, Darmstadt, Germany), at 70°C and pH3.0. *Sulfolobus acidocaldarius* ATCC33909^T was obtained from the American Type Culture Collection. *Metallosphaera sedula* DSM5348^T and *Metallosphaera prunae* DSM10039^T were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen.

Growth conditions

The isolate TA-2 was grown in modified Brock's basal salts mixture (Brock et al. 1972) (mg l^{-1}): $(NH_4)_2SO_4$ (1,300), KH_2PO_4 (280), $MgSO_4 \cdot 7H_2O$ (250), $CaCl_2 \cdot 2H_2O$ (70), $FeCl_3 \cdot 6H_2O$ (2), $MnCl_2 \cdot 4H_2O$ (1.8), $Na_2B_4O_7 \cdot 10H_2O$ (4.5), $ZnSO_4 \cdot 7H_2O$ (0.22), $CuCl_2 \cdot 2H_2O$ (0.05), $NaMoO_4 \cdot 2H_2O$ (0.03), $VOSO_4 \cdot 2H_2O$ (0.03), and $CoSO_4$ (0.01), supplemented with 1.0 g yeast extract (Difco) per liter, pH2.5, at 80°C. *S. acidocaldarius*, *M. sedula*, and *M. prunae* were grown in the same medium, at 80°C, 70°C, and 70°C, respectively.

For determination of the optimum temperature, TA-2 was cultured between 50° and 95°C in modified Brock's basal salts mixture supplemented with 1.0 g yeast extract per liter at pH2.5. For determination of the optimum pH, cells were cultured in the same medium at 75°C. pH of the media was adjusted by the addition of 10% H_2SO_4 (w/v) or 1 M NaOH and measured at room temperature.

All cells were grown aerobically in standing cultures in loosely capped glass tubes or long-necked conical flasks with stirring. Growth was determined either by measurement of the optical density at 600 nm (OD_{600}) or by direct cell counting using a Thoma counting chamber (Kayagaki, Tokyo, Japan). A good correlation was found between these two methods in both exponential- and stationary-phase cultures. An OD_{600} of 1.0 corresponded to a cell density of about 1.1×10^9 cells ml^{-1}. The doubling times were calculated from the slopes of the growth curves.

Anaerobic media were prepared using the technique of Balch et al. (1979). Modified Brock's basal salts mixture supplemented with S^0 (1 g l^{-1}) was reduced by the addition of sodium sulfide (0.5 g l^{-1}) and adjusted to pH2.5 with 10% H_2SO_4. Resazurin (1 mg l^{-1}) served as an O_2 indicator. Medium (5 ml) was distributed into pressure-culture tubes [18 mm (diameter) × 180 mm; Sanshin (Yokohama, Japan)] and pressurized with H_2–CO_2 (8:2, v/v, 200 kPa). A 1% inoculum of a dense culture of TA-2 (3–4 × 10^7 cells ml^{-1}) was added with a syringe. The culture was incubated at 75°C without shaking. Growth was determined by direct cell counting using a Thoma counting chamber.

Gram staining

A Gram-staining test of cells in log phase was carried out using the modified Hücker's method (Kruczak-Filipov and Shively 1992). *Escherichia coli* LE392 and *Bacillus subtilis* 162 were used for negative and positive controls, respectively, of staining.

Lipid analysis

Total cellular lipids were extracted from stationary-phase cells as described in a previous report (Sugai et al. 1995) and analyzed by thin-layer chromatography. Cyclic tetraether core lipids were prepared by acid methanolysis of total cellular lipids as described by Sugai et al. (1995). These core lipids were applied to thin-layer chromatography plates (Merck silica gel 60 HPTLC; Merck) and were separated by chromatography by two ascending runs with the following solvent systems: first step, chloroform : methanol, 4 : 1 (to 2 cm from the origin); second step, hexane : diethyl ether : acetic acid, 60 : 40 : 2.

DNA base composition and DNA–DNA hybridization

The G + C contents of DNA were determined by reversed-phase HPLC of the DNA digested with nuclease P1 (Tamaoka and Komagata 1984). Mean values were obtained from three independent experiments. Levels of genomic DNA relatedness were determined by DNA–DNA hybridization experiments according to the fluorescent method of Ezaki et al. (1989). *Sulfurisphaera ohwakuensis* TA-1^T (IFO 15161^T) was used as a reference strain.

Cloning and sequencing of the 16S rRNA gene

A 3.0-kbp *Xba*I fragment of TA-2 genomic DNA, which contained the 16S rRNA gene, was cloned from a genomic DNA library constructed on plasmid Bluescript SK– (Stratagene, La Jolla, CA, USA), using standard methods (Sambrook et al. 1989). Subclones of smaller fragments in plasmid Bluescript SK– or KS– (Stratagene) were used as sequencing templates. DNA sequence analysis was performed on both strands and carried out by the dideoxynucleotide chain termination method with Texas red-labeled primers using an automated DNA sequencer (SQ-5500; Hitachi, Tokyo, Japan). *Bca*-best DNA polymerase (Takara Shuzo, Kyoto, Japan) was used, and deoxyguanosine triphosphate (dGTP) was replaced with deoxyinosine triphosphate (dITP) in all four sequencing mixtures to avoid "peak compression."

Phylogenetic analysis

The 16S rRNA gene sequences from TA-2 and 11 type strains belonging to the order *Sulfolobales* were aligned with the sequence of *Aeropyrum pernix* (Sako et al. 1996) as outgroup species. All sites with gaps in any sequences and the regions of the polymerase chain reaction (PCR) primers were removed from the alignment. Pairwise distances between all sequences were estimated by Kimura's two-parameter method (Kimura 1980). The percentages of similarities within the 16S rRNA gene sequences were estimated using GENETYX-MAC software (Software Development, Tokyo, Japan). Phylogenetic trees were con-

structed using the neighbor-joining method (Saitou and Nei 1987). A total of 1,359 positions were used for construction of the phylogenetic trees. To test the stability of our phylogenetic trees, the sequence data were sampled 1,000 times for bootstrap analysis. These analyses were performed by the Clustal W program (Higgins 1998).

Nucleotide sequence accession numbers

The nucleotide sequence data of the 16S rRNA gene of TA-2 appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D85508. The previously published DNA database accession numbers of the strains are as follows: *Sulfolobus acidocaldarius* 98-3, D14876; *Sulfolobus solfataricus* P1, D26490; *Sulfolobus shibatae* B12, M32504; *Sulfolobus metallicus*, D85519; *Acidianus brierleyi* DSM1651, D26489; *Acidianus infernus*, D85505; *Acidianus ambivalens*, D85506; *Metallosphaera sedula* TH2, D26491; *Metallosphaera prunae* DSM10039, X90482; *Stygiolobus azoricus* DSM6296, D85520; *Sulfurisphaera ohwakuensis* TA-1, D85507; *Aeropyrum pernix* JCM9820, D83259.

Results

Isolation

Supernatants of muddy water samples (70°C, pH3) from the hot springs in Ohwaku Valley, Hakone, were inoculated into 10ml of modified Brock's basal salts, supplemented with 0.1% yeast extract, and incubated at 70°C. After 3days, 100µl turbid culture containing about 10^3–10^4 cells was spread onto each gellan gum plate. Hundreds of small colonies appeared after 5days of incubation and were grown continuously for 1day more. The colonies were replated to purify the organisms. We chose some stable cultures and analyzed their core lipid compositions. One of the isolates, named TA-2, had no calditoglycerocaldarchaeol, which is one of the major core lipids found in members of *Sulfolobales*. We investigated isolate TA-2 in detail for its characterization and taxonomic classification.

Morphology

Under a phase-contrast microscope, cells of the isolate TA-2 appeared as round to slightly irregular cocci about 0.5–1µm in diameter. Colonies of TA-2 on the gellan gum plates were smooth, roundly convex, and slightly yellow. The cells were not stained by Gram stain.

Growth

The temperature range for growth of TA-2 was from 55° to 83°C; the optimum temperature was 75°C. TA-2 grew between pH1.5 and pH3.5; the optimum pH was 2.8. The doubling time of TA-2 was 4.5h under optimal conditions. TA-2 was unable to grow under anaerobic conditions. TA-2 was able to grow on proteinaceous complex substrates such as yeast extract or tryptone but not able to grow autotrophically. From zinc sulfide, TA-2 extracted zinc ions at 270ppm after 20days of incubation. This value was almost the same level as that of *M. sedula* when grown in Brock's basal salts medium supplemented with 0.1% yeast extract and 1% ZnS instead of $FeCl_3$, $CuCl_2$, and $ZnSO_4$. Cultures frozen and stored at –80°C served as inocula for at least 2years.

Lipids

Cyclic tetraether core lipids were analyzed by thin-layer chromatography and compared with those of *M. sedula*, *M. prunae*, and *Sulfolobus acidocaldarius*. Both caldarchaeol and calditoglycerocaldarchaeol were detected in *M. sedula*, *M. prunae*, and *S. acidocaldarius*. Calditoglycerocaldarchaeol constituted about 80%, 50%, and 30% of all core lipids of *S. acidocaldarius, M. sedula*, and *M. prunae*, respectively; however, isolate TA-2 lacked calditoglycerocaldarchaeol (Fig. 1). The amounts of calditoglycerocaldarchaeol in strains of *Sulfolobales* other than TA-2 changed somewhat under different growth conditions. However, calditoglycerocaldarchaeol was never detected in isolate TA-2 grown under any conditions.

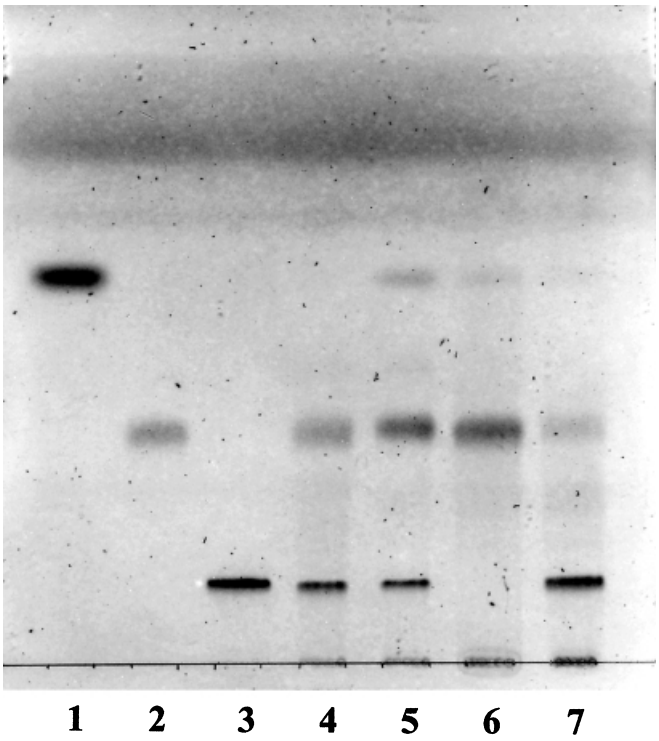

Fig. 1. Thin-layer chromatogram of core lipids. Purified archaeol, *lane 1*; purified caldarchaeol, *lane 2*; purified calditoglycerocaldarchaeol, *lane 3*; *Metallosphaera sedula*, *lane 4*; *Metallosphaera prunae*, *lane 5*; TA-2, *lane 6*; *Sulfolobus acidocaldarius*, *lane 7*

Table 1. Levels of DNA relatedness between TA-2, *Metallosphaera sedula, Metallosphaera prunae*, and *Sulfurisphaera ohwakuensis*

Strain	Relatedness (%) with biotinylated DNA from:				G + C
	TA-2	*M. sedula*	*M. prunae*	*S. ohwakuensis*	(%)
TA-2	100	97	95	2	47.1 ± 0.5
M. sedula	94	100	91	1	46.3 ± 0.2
M. prunae	93	98	100	0	46.9 ± 0.1
S. ohwakuensis	2	2	2	100	32.9 ± 0.8

DNA base composition and phylogenetic analysis

Genomic DNA of isolate TA-2, *M. sedula*, and *M. prunae* had almost the same G + C content, which was about 46.5 mol% as calculated by direct analysis of the nucleotides (Table 1). To analyze the phylogenetic position of TA-2, the 16S rRNA gene sequence from the isolate was determined and compared with the sequences of type strains belonging to the order *Sulfolobales*. The 16S rRNA gene of TA-2 showed more than 99% similarity with that of *M. sedula* and *M. prunae,* 91% similarity with that of *Acidianus* spp., and less than 90% similarity with *Sulfolobus* spp., *Sulfurisphaera ohwakuensis,* or *Stygiolobus azoricus*. We constructed phylogenetic trees by the neighbor-joining method (Fig. 2). TA-2, *M. sedula,* and *M. prunae* formed a cluster that is clearly distinguished from other clusters with 100% bootstrap probabilities. Values for DNA relatedness between TA-2, *M. sedula*, and *M. prunae* are shown in Table 1.

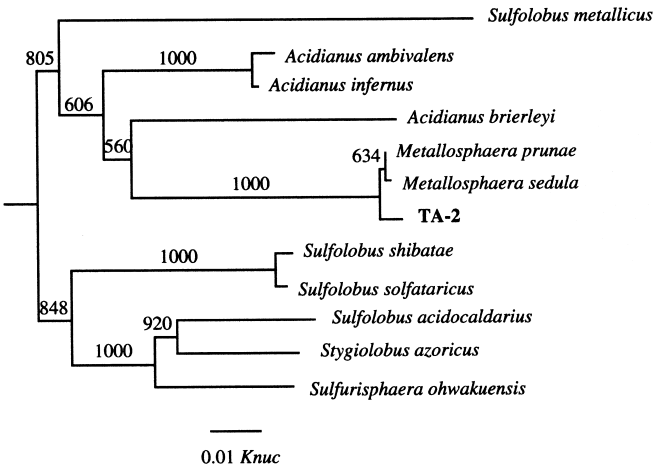

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequences constructed by the neighbor-joining method. Bootstrap scores of 1,000 trials are given *above* the internal branches. *Bar* represents 0.01 nucleotide substitution

Discussion

The isolate TA-2 is a cocci that does not stain by Gram stain and grows as an aerobe. The optimal temperature and pH for growth, doubling time, and G + C content of TA-2 are almost the same as those of *M. sedula* (Huber et al. 1989) and *M. prunae* (Fuchs et al. 1995). The sequence of the 16S rRNA gene of TA-2 is more than 99% identical to those of *M. sedula* and *M. prunae*. On the phylogenetic tree, these three strains form a cluster that is clearly distinguished from other members of *Sulfolobales* (see Fig. 2). To test the genomic DNA homology between TA-2, *M. sedula,* and *M. prunae*, DNA–DNA hybridization experiments were performed. The isolate TA-2 showed more than 93% genomic DNA homology to both *M. sedula* and *M. prunae*. This value clearly demonstrates that isolate TA-2 belongs to the same species as *M. sedula* or *M. prunae*.

In contrast with these characteristics, TA-2 is quite different in its lipid composition from *M. sedula*, *M. prunae*, and other members of *Sulfolobales*. TA-2 has only caldarchaeol as the tetraether-type core lipid whereas *M. sedula* and *M. prunae* have both calditoglycerocaldarchaeol and caldarchaeol (see Fig. 1). This characteristic is a very special feature of TA-2, because calditoglycerocaldarchaeol is one of the major core lipids, and its derivatives constitute 70%–80% or more of the total lipids of the members of the order *Sulfolobales* such as *Sulfolobus acidocaldarius, Acidianus brierleyi*, and *M. sedula* (Sugai et al. 1995). Therefore, calditoglycerocaldarchaeol may not be essential for survival in thermophilic and acidophilic environments.

The deletion of calditoglycerocaldarchaeol in strain TA-2 may be led by mutation of one or multiple gene(s) for calditoglycerocaldarchaeol synthesis, although those genes have not yet been identified. On the other hand, it is clear that the deletion of this core lipid in strain TA-2 did not occur by an adaptation to the environment because another strain isolated from the same sampling point, *Sulfurisphaera ohwakuensis* TA-1, does contain calditoglycerocaldarchaeol (Kurosawa et al. 1998). Thus, isolate TA-2 is worth using in further studies on the structure and the metabolism of the thermoacidophilic archaeal lipids.

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