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Acidianus manzaensis sp. nov., a Novel Thermoacidophilic *Archaeon* Growing Autotrophically by the Oxidation of H_2 with the Reduction of Fe^{3+}

Naoki Yoshida,[1] Masanori Nakasato,[2] Naoya Ohmura,[3] Akikazu Ando,[2] Hiroshi Saiki,[4] Masaharu Ishii,[1] Yasuo Igarashi[1]

[1]Department of Biotechnology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657, Japan
[2]Department of Bioresources Science, Graduate School of Science and Technology, Chiba University, 648 Matsudo, Matsudo City, 271-0092, Chiba, Japan
[3]Central Research Institute of Electric Power Industry, 1646 Abiko, Abiko City, Chiba, 270-1194, Japan
[4]School of Bionics, Tokyo University of Technology, 1404-1 Kataoka, Hachiouji, 192-0982, Tokyo,

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Abstract. A novel thermoacidophilic iron-reducing *Archaeon*, strain NA−1, was isolated from a hot fumarole in Manza, Japan. Strain NA-1 could grow autotrophically using H_2 or S^0 as an electron donor and Fe^{3+} as an electron acceptor, and also could grow heterotrophically using some organic compounds. Fe^{3+} and O_2 served as electron acceptors for growth. However, S^0, NO_3^-, NO_2^-, SO_4^{2-}, Mn^{4+}, fumarate, and Fe_2O_3 did not serve as electron acceptors. The ranges of growth temperature and pH were 60–90°C (optimum: 80°C) and pH 1.0–5.0 (optimum: pH 1.2–1.5), respectively. Cells were nearly regular cocci with an envelope comprised of the cytoplasmic membrane and a single outer S-layer. The crenarchaeal-specific quinone (cardariellaquinone) was detected, and the genomic DNA G + C content was 29.9 mol%. From 16S rDNA analysis, it was determined that strain NA-1 is closely related to *Acidianus ambivalens* (93.1%) and *Acidianus infernus* (93.0%). However, differences revealed by phylogenetic and phenotypic analyses clearly show that strain NA-1 represents a new species, *Acidianus manzaensis*, sp. nov., making it the first identified thermoacidophilic iron-reducing microorganism (strain NA-1^T = NBRC 100595 = ATCC BAA 1057).

Dissimilatory microbial iron reduction is thought to play an important role in mineral formation in a variety of environments like subsurface soils, lake sediments, ground water, hot springs, and deep sea hydrothermal vents [10]. Until now, a number of iron-reducing microorganisms have been isolated, and found to be widely distributed in phylogenetic positions among *Bacteria* and *Archaea* [14]. On the basis of optimum pH and temperature for growth, they can be classified into the following three groups; (i) iron-reducing bacteria mainly belonging to the Proteobacteria such as *Geobacter sulfurreducens* [2] and *Shewanella putrefaciens* [16], which grow by the reduction of insoluble iron compounds under neutral pH conditions at around 30°C; (ii) thermophilic iron-reducing microorganisms such as *Geothermobacterium ferrireducens* [8], *Geoglobus ahangari* [11] and *Pyrobaculum islandicum* [9], which grow by iron reduction under neutral pH conditions in a thermal environment over 70°C; and (iii) acidophilic iron-reducing bacteria such as *Acidithiobacillus ferrooxidans*, growing by anaerobic iron respiration under acidic conditions at around 30°C through the reduction of soluble iron (Fe^{3+}) by the oxidation of H_2 or S^0 [17].

Naoki Yoshida's present address is Central research institute of Electric Power Industry, 1646 Abiko, Abiko city, Chiba 270-1194, Japan
Strain NA−1 has been deposited in the culture collections of the National Institute of Technology and Evolution (NBRC 100595) and American Type Culture Collection (ATCC BAA 1057). The 16S rDNA sequence has been deposited at GenBank under accession number AB182498.
Correspondence to: Masaharu Ishii; *email:* amishii@mail.ecc.u-tokyo.ac.jp

However, there have been no reports on iron-reducing microorganisms growing at low pH and high temperature. Here we describe the first isolation and characterization of an acidothermophilic iron-reducing *Archaeon*, *Acidianus manzaensis* strain NA-1, which can chemolithoautotrophically grow through the reduction of Fe^{3+} by the oxidation of H_2.

Materials and Methods

Isolation and culture conditions. The soil was sampled from a fumarole (85–94°C, pH 1.0–2.0) in Manza, Gunma, Japan. The iron-reducing (IR) basal medium contained (L^{-1}): 0.132 g:$(NH_4)_2SO_4$, 0.052 g:KCl, 0.041 g:K_2HPO_4, 0.490 g:$MgSO_4 \cdot 7H_2O$, 0.009 g: $CaCl_2 \cdot 2H_2O$, 0.001 g:$ZnSO_4 \cdot 7H_2O$, 0.002 g:$CuSO_4 \cdot 5H_2O$, 0.001 g: $MnSO_4 \cdot 5H_2O$, 0.0005 g:$NaMoO_4 \cdot 2H_2O$, 0.0005 g:$CoCl_2 \cdot 6H_2O$, 0.001 g:$NiCl_2 \cdot 6H_2O$, and 0.005 g:$FeSO_4 \cdot 7H_2O$. In addition, 14.7 g of $Fe_2(SO_4)_3 \cdot nH_2O$ was added as an electron acceptor. The pH of the medium was adjusted to 1.5 with 10 N H_2SO_4. Four hundred milliliters of the medium was added to 1-L anaerobic bottles, which were then sealed with a silicon cap. The head space gas was packed with H_2/CO_2 (4:1 (v/v)) to 150 kPa. After suspending a small amount of the soil in IR medium, it was injected into an anaerobic culture bottle prepared as described above and cultivated at 70°C while shaking (80 rpm). The concentrations of Fe^{2+} were measured using *o*-phenanthroline as described previously by Ohmura [17], and cells were counted under a light microscope.

Electron donors and acceptors. The growth potential of stain NA-1 was tested with a variety of combinations of potential electron donors and acceptors. All experiments were performed using 30 ml of IR basal medium in 120-ml anaerobic bottles. The 22 electron donors including (L^{-1}) 1.6 g:S^0, 3.0 g:potassium tetrathionate, 5.0 g:$FeSO_4 \cdot 7H_2O$, 1.0 g: yeast extract, 5.5 g:sodium pyruvate, 8.2 g:sodium acetate, 8.9 g: sodium L-malate, 16.2 g:disodium succinate, 1.0 g:peptone, 6.8 g: sodium formate. 8.0 g:sodium fumarate, 1.0 g:glycerol, 1.0 g:sodium lactate, 29.4 g:sodium citrate, 3.0 g:glycine, 1.0 g:tryptone, 1.0 g:casamino acids, 1.0 g:beef extract, 1.0 g:sucrose, 1.0 g:mannose, 1.0 g:lactose monohydrate, and 1.0 g:glucose were tested. The concentration of each donor was adjusted by following the report by Kashefi *et al.* [8]. In the same manner, the nine electron acceptors for anaerobic respiration were individually tested at 14.7 g/ L:$Fe_2(SO_4)_3 \cdot nH_2O$, 12.4 g/L:Fe(III)-citrate, 8.0g/L:Fe_2O_3 (crystalline iron oxide), 5.3 g/L:$Fe(OH)_3$, 14.7 g/L:SO_4^{2-} as Na_2SO_4, 1.6 g/L:S^0, 50 mM:NO_3^- as $NaNO_3$, 50 mM:NO_2^- as $NaNO_2$, 50 mM:Mn^{4+} as MnO_2 and 50 mM:fumarate.

Optimal temperature, pH, salt, and concentration of O_2. The potential for growth was tested using H_2 as an electron donor, Fe^{3+} as an electron acceptor, and CO_2 as a carbon source at various temperatures and various pH values while shaking (80 rpm). The temperature was varied within a range of 50–95°C in a hot-air incubator. The pH was varied within a range of pH 0.8–6.0, and growth occurring in the range of pH 2.0–6.0 was also investigated under aerobic conditions in which 10% O_2 was added and Fe^{3+} was omitted. The potential for growth at various concentrations of Fe^{3+} or O_2 was also investigated. The concentration of Fe^{3+} was varied from 0 to 150 mM, while the concentration of O_2 was set by varying its proportion in the $H_2/CO_2/O_2$ (4:1:X (v/v/v), where X defines the proportion of O_2 in the total gas; 150 kPa,).

Electron microscopy. Cells in the late-exponential phase fixated with 4.0% glutaraldehyde for 12 h and dehydrated by ethanol, were observed through scanning electron microscopy (S-4500, HITACHI, Japan). Thin sections of the cells were prepared by rapid freezing and substitution fixation as described by Dempsey [4], and examined under a transmission electron microscope (H-7500; HITACHI, Japan).

Quinone analysis. Quinones were extracted using a mixture of chloroform and methanol (2:1 (v/v)) and identified by HPLC (LC-10; Shimadzu, Japan) using a reverse-phase column (ZORBAX-ODS; Shimadzu, Japan) as described by Collins [3].

Determination of the DNA G + C content. The G + C content was determined by reverse-HPLC (LC-10; Shimadzu, Japan) using a Develosil RPAQUEOUS column (4.6 mm × 250 mm; Nomura Chemical, Japan) as described by Katayama-Fujimura et al. [12].

16S rDNA sequence analysis. The 16S rDNA from strain NA-1 was determined using the archaeal-specific primers ARC20F (5′-TTCCGGTTGATCCYGCCRG-3′) and Uni1500R (5′-GGTTACCT TGTTACGACTT-3′) [5]. The sequence obtained from strain NA-1 was then subjected to a BLAST homology search (DDBJ gene bank, Japan), and the 16S rDNA sequences of close relatives and some typical archaea (listed in Fig. 5). These were aligned for phylogenetic analysis using Clustal X version 1.83 [19]. A phylogenic tree was then constructed with MEGA version 2.1 [13].

Results

Isolation of strain NA-1. Microorganisms in different soil samples were enriched by culture in an IR medium containing Fe^{3+} under an anaerobic atmosphere of H_2/CO_2 (4:1(v/v), 150 kPa) at 70°C and pH 1.5. The cultures showing Fe^{3+} reduction after 7–8 days were subcultured under the same conditions more than ten times. Samples of the microorganisms were then cultured on IR medium plates containing 3.0% gellan gum and Fe^{3+} under H_2/CO_2 (4:1(v/v), 150 kPa) at 70°C, and after 5–7 days colonies appeared. After repeating the colony isolation, strain NA-1 was isolated in a pure state.

Growth on iron respiration. Cell numbers increased from 1.0×10^6 to 1.0×10^8 cells/ml over a period of 150 h (Fig. 1A), and the growth correlated with the reduction of Fe^{3+} (Fig. 1B). No growth was observed in the absence of Fe^{3+}, H_2 or CO_2, and there was no reduction of Fe^{3+} without cells. Thus, strain NA-1 is clearly capable of chemolithoautotrophic growth on the oxidation of H_2 by the reduction of Fe^{3+}. Growth on anaerobic iron respiration was confirmed by its dependence on the concentration of the electron acceptor in the medium. When strain NA-1 was inoculated into a medium containing 10-60 mM Fe^{3+}, the cell density after 1 week of incubation was approximately proportional to the initial Fe^{3+} concentration (Fig. 2). This confirms that strain NA-1 is able to grow autotrophically using H_2 as an electron donor and Fe^{3+} as an electron acceptor.

Utilization of electron donors and acceptors. S^0, yeast extract, pyruvate, peptone, tryptone, casamino acid,

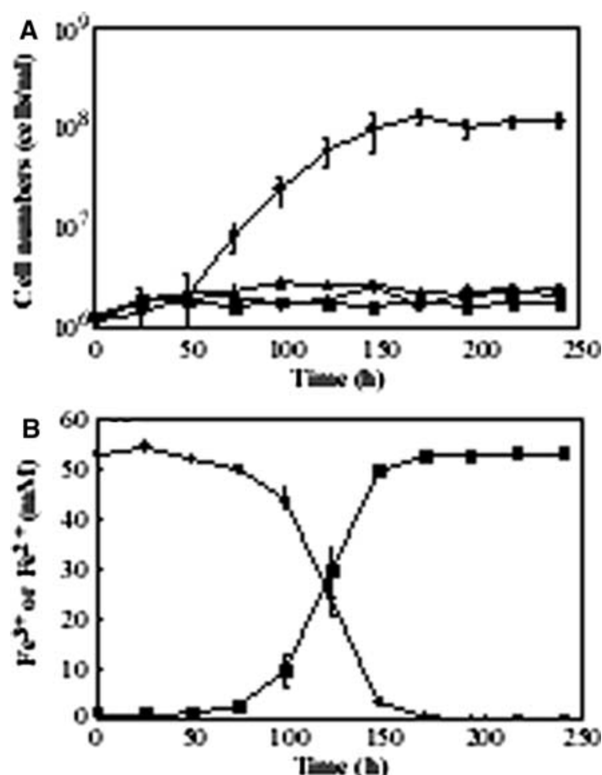

Fig. 1. Chemolithoautotrophic growth of strain NA-1 on the anaerobic reduction of Fe^{3+} by the oxidation of H_2. Strain NA-1 was cultivated at 70°C and pH 1.5. A: Cell numbers in the presence of $H_2 + CO_2 + Fe^{3+}$ (●), $H_2 + CO_2$(▲), $H_2 + Fe^{3+}$ (■), and $CO_2 + Fe^{3+}$ (♦). B: The concentrations of Fe^{3+} (●) and Fe^{2+}(■) in the presence of H_2, CO_2, and Fe^{3+}.

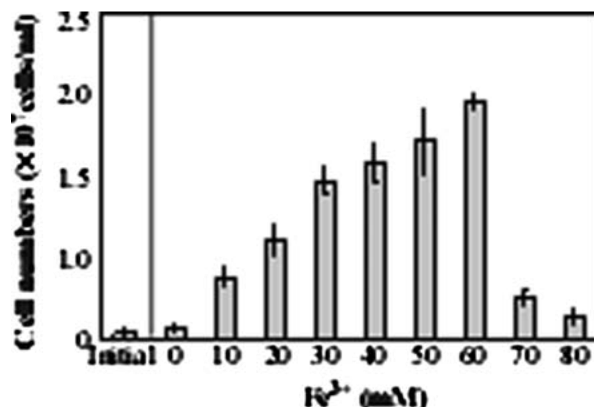

Fig. 2. Relative proportion between cell growth and Fe^{3+} concentration. Cell numbers at each concentration were obtained after 7 days cultivation.

sucrose, mannose, lactose, and glucose were all capable of supporting anaerobic growth as electron donors with the reduction of Fe^{3+}. Fe^{3+} and O_2 served as electron acceptors for growth. However, S^0, NO_3^-, NO_2^-, SO_4^{2-}, Mn^{4+}, and fumarate did not serve as electron acceptors. Furthermore, strain NA-1 could utilize $Fe_2(SO_4)_3$, Fe^{3+}-citrate, or $Fe(OH)_3$, but not Fe_2O_3 (crystalline iron oxide) in the anaerobic iron reduction.

Electron microscopy. The cells appeared as regular cocci (Fig. 3A), about 0.6–1.0 μm in diameter under transmission electron microscopy of thin sections (Fig. 3B). Figure 3C indicated that the cell envelope was composed of a cytoplasmic membrane, a periplasmic space, and a single outer S-layer, which is typical of Crenarchaea. In addition, the cells contained caldariellaquinone, which is a quinone found specifically in Crenarchaea.

Optimum temperature, pH, salt concentration, and O_2 partial pressure. Growth was observed at temperatures between 60 and 90°C, with the optimum at 80°C (Fig. 4A). No growth and no reduction of Fe^{3+} were observed below 55°C or at 94–95°C. The pH range for anaerobic iron-based growth was 1.0–2.0, with the optimum at 1.2–1.5 (Fig. 4B). In addition, the pH range for aerobic growth was 1.0–5.0, with the optimum at 1.2–2.0. No growth was detected at pH 5.5 or above. The growth was observed within a range of 1.0–20.0% O_2 partial pressure, with the optimum at 5.0% (Fig. 4C).

DNA base composition and evolutional analysis. The genomic DNA G + C content of strain NA-1 was 29.9 mol%. Phylogenetic analysis based on 16S rDNA indicated that the closest relatives of strain NA-1 are *Acidianus ambivalens* (93.1% similarity), *A. infernus* (93.0% similarity), *A. brierleyi* (89.9% similarity), and *A. tengchongensis* (89.9% similarity). which all belong to the archaeal genus *Acidianus* within the order *Sulfolobales*. The phylogenetic tree, determined by comparing 1358 bases from strain NA-1 with those of other archaeal species, showed that strain NA-1, *Acidianus infernus* and the other *Acidianus* strains clustered, suggesting strain NA-1 is a new species in the genus *Acidianus* (Fig. 5).

Discussion

Proposal of strain NA-1 as a new species of the genus *Acidianus*. Some phenotypic characteristics of strain NA-1 were the same as those of the four species in the genus *Acidianus* in its acidophilic and thermophilic growth: aerobic oxidation of H_2 or S^0 as an electron donor; the morphologic shape of cocci; the morphology of the cell envelope consisting of an S-layer attached to the cytoplasmic membrane; the presence of caldariellaquinone, which is a quinone found specifically in Crenarchaea; and the G + C content of

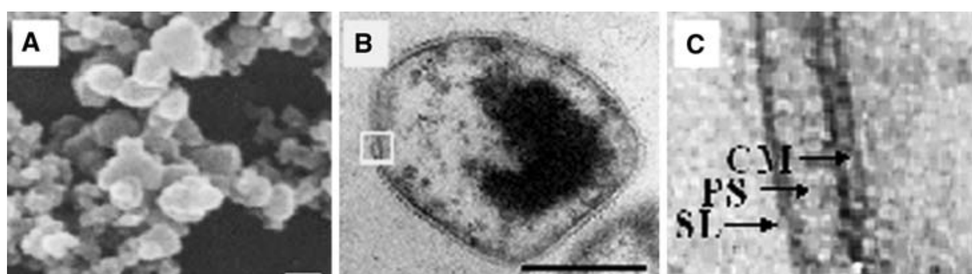

Fig. 3. Cells of strain NA-1 grown chemolithoautotrophically on anaerobic iron respiration. (A)Scanning electron micrograph; (B) Representative electron micrograph of a thin section of cells. (C) Higher magnification of membrane region of the cell boxed in B. CM, cytoplasmic membrane; PS, periplasmic space; SL, S-layer. Bars = 0.5 μm.

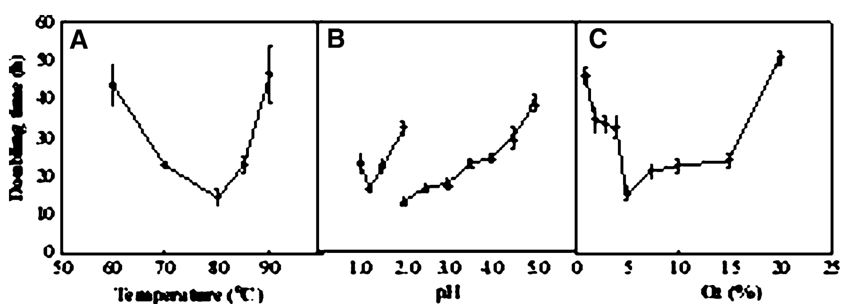

Fig. 4. Optical temperature, pH, and O_2 on growth of strain NA-1. (A) Optical growth temperature of strain NA-1 under an anaerobic condition of H_2, CO_2, and Fe^{3+}. (B) Influence of pH on the growth of strain NA-1. Fe^{3+} (•) or O_2 (▼) was supplied as an electron acceptor (•). (C) Effect of O_2 on aerobic growth on H_2.

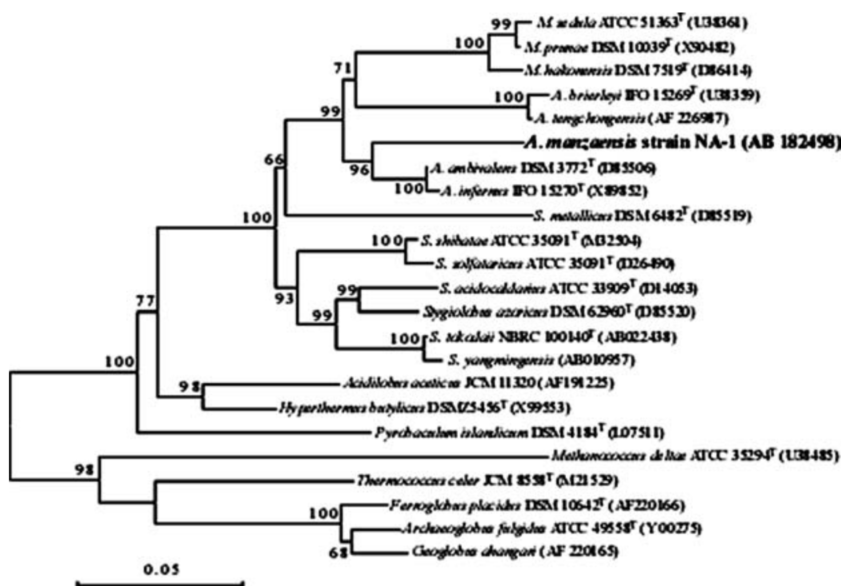

Fig. 5. Phylogenetic tree based on the analysis of 16S rDNA showing the relationship between strain NA-1 and other archaea. Significant bootstrap values are indicated as percentages at each branching points. The bar indicates 5 nucleotides per 100 nucleotides.

the DNA as 29.9 mol% (Table 1) [1, 6, 7, 18]. These common characteristics between strain NA-1 and the other *Acidianus* species suggested that strain NA-1 belongs to genus *Acidianus* in the cluster II of the order *Sulfolobales*. Furthermore, low similarities of strain NA-1 and *Acidianus* species based on the 16S rDNA gene sequences suggested that strain NA-1 differs from the closest relatives, *A. ambivalens*, and *A. infernus*. The phylogenetic tree shows that strain NA-1 forms a branch together with *Acidianus* and *Metallosphaera* species. However, these two genera are physiologically different in terms of their aerobic or anaerobic growth. *Acidianus* species can grow aerobically or anaerobically, while *Metallosphaera* species can grow obligately aerobically, like *Sulfolobus* species. Furthermore, the mol% G + C contents of *Acidianus* or *Metallosphaera* species as

Table 1. Physiological and phylogenetical comparisons between strain NA-1 and *Acidianus* species

	Strain NA-1	*A. inferms*	*A. ambivalens*	*A. brierleyi*	*A. tengchongensis*
Growth temparature (optimal temperature)	60–90°C (80°C)	65–96°C (90°C)	70–87°C (80°C)	45–75°C (70°C)	60–75°C (70°C)
Growth pH (optimal pH)	1.0–5.0 (1.2–1.5)	1.0–5.5 (2.0)	1.0–3.5 (2.5)	1.0–6 (1.5–2.0)	1.0–5.5 (1.5-2.0)
Electron donor utility	H_2, S^o and organic compounds (Yeast extract, peptone, tryptone, cassamino acid, glucose)	H_2, S^o	H_2, S^o	H_2, S^o and organic compounds (Yeast extract, peptone, tryptone, cassamino acid, beef extract)	H_2, S^o
Electron acceptor utility	Fe^{3+} and O_2	S^o and O_2	S^o and O_2	S^o and O_2	S^o and O_2
autotrophy	Facultative autotroph	Obligate autotroph	Obligate autotroph	Facultative autotroph	Obligate autotroph
G+C contents (%)	29.9	31.0	32.7	31.0	31.0
16S rDNA similarity	1.00	0.93	0.93	0.90	0.91

around 31 or 45% suggests that these two genera were completely separated. Based on these classifications, faculative anaerobic growth and the 29.9 mol% of G + C contents in strain NA-1 clearly show that this strain belongs to genus *Acidianus*. Therefore, strain NA-1 should be classified to genus *Acidianus*, not to *Metallosphaera*, though strain NA-1 forms a cluster branching to these two genera. Additionally, the genus *Acidianus* is split into two monophyletic groups based on 16S rDNA analysis (Fig. 5). This split has no correlation with physiological classification by physiological characters of electron donor utility and autotrophy, which suggests the genus could be split into two groups as one group composed of *A. infernus*, *A. ambivalens*, and *A. tengchongensis*, the other of strain NA-1 and *A. brierleyi*. Therefore, *Acidianus* species should be reclassified in the near future.

Moreover, differences in metabolism were seen as strain NA-1 and *A. brierleyi* were both able to use the tested organic compounds as electron donors or carbon sources, while the other three *Acidianus* species were not able to do so. It is also particularly noteworthy that *A. infernus*, a close relative to strain NA-1, is an obligate autotroph; in contrast, strain NA-1 represented facultatively autotrophic growth under aerobic or anaerobic respiration. The most notable differences between strain NA-1 and the other *Acidianus* species are that strain NA-1 can grow autotrophically on the reduction of Fe^{3+} by the oxidation of H_2, while the other *Acidianus* can not grow by Fe^{3+} reduction utilizing H_2 or S^0, which was experimentally confirmed by anaerobic growth and Fe^{3+} reduction in *A. infernus*, *A. ambivalens*, and *A. brierleyi*, or by the report of *A. tengchongensis* [7]. Furthermore, all of the four *Acidianus* species are capable of anaerobic utilization of S^0 as an electron acceptor with the oxidation of H_2, but strain NA-1 is not. These differences suggest that strain NA-1 should be classified as a novel species. We, therefore, propose that strain NA-1 is a new species of the genus *Acidianus*, which we have named *Acidianus manzaensis* (= NBRC 100595 = ATCC BAA 1057).

Anaerobic Metabolisms in Strain NA-1. One of the most distinguishing characteristics of strain NA-1 is its chemolithoautotrophic growth on the reduction of Fe^{3+} by the oxidation of H_2. This metabolism in strain NA-1 is consistent with Lovley's proposal that the ability for iron reduction coupled with H_2 oxidation is much more universal among thermophilic microorganisms [14], and similar to the ancient metabolism in pre-biotic Earth proposed by Martin and Russell [15]. However, the utilization of soluble iron, not insoluble iron oxides in strain NA-1, is different from the hypothesized reaction. At this point, comparison between the biological aspects of strain NA-1 and those of other thermophilic iron-reducing microorganisms should be performed. Another distinguishing characteristic of strain NA-1 is the oxidization of S^0 as an electron donor with the reduction of Fe^{3+}, which is also the first case among *Archaea*. There is only one report on sulfur-oxidizing and iron-reducing microorganisms, acidophilic iron-reducing bacterium, *Acidothiobacillus ferrooxidans*. This bacterium grows autotrophically on the reduction of soluble iron by the oxidation of S^0 or H_2 [17]. In summary, we successfully isolated and characterized a novel thermoacidophilic iron-reducing archaeron, strain NA-1 growing autotrophically by the oxidation of H_2 with the reduction of Fe^{3+}. This is the first case in which iron reduction plays an important role even in extreme acidic and thermal conditions. More details about an iron-reducing mechanism in strain NA-1 are necessary to better understand dissimilatory iron reduction.

Description of *Acidianus manzaensis* sp. nov. *Acidianus manzaensis* (manzaen'sis. M.L. masc. adj. *manzaensis*, pertaining to Manza, Japan) is isolated from a hot fumarole in Manza, Japan. It is a facultative autotroph capable of both anaerobic and aerobic growth. H_2, S^0, yeast extract, pyruvate, peptone, tryptone, cassamino acids, sucrose, mannose, lactose, and glucose can serve as electron donors using Fe^{3+} or O_2 as an electron acceptor for anaerobic and aerobic respiration, respectively. S^0, NO_3^-, NO_2^-, SO_4^{2-}, Mn^{4+}, fumarate, or Fe_2O_3 cannot serve as electron acceptors for anaerobic respiration. Growth occurs at 60–90°C and pH 1.0–5.0. The cells are nearly regular cocci, approximately 0.5–0.8 μm in diameter. The cell envelope consists of an S-layer attached to the cytoplasmic membrane and contains caldariellaquinone. The G + C content of the DNA is 29.9 mol%. The type strain is *Acidianus manzaensis* strain NA-1^T, deposited in the culture collections of the National Institute of Technology and Evolution (NBRC 100595).

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