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Isolation and physiological characterization of two novel, piezophilic, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent chimney

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Abstract:

Two novel, thermophilic piezophiles, capable of chemolithoautotrophic growth, are successfully cultivated and isolated from a black smoker chimney at the TAG field (Mid Atlantic Ridge: MAR) by using a piezophilic cultivation technique. Both strains (strains 106 and 108) represent dominant cultivated populations of the microbial communities in the chimney surface habitat. Strain 106 represents typically thin, long spiral cells under the piezophilic growth condition but short bent cells under the non-piezophilic condition. It is a strictly chemolithoautotrophic gammaproteobacterium using reduced sulfur compounds as the electron donors, and nitrate and O_2 as the electron acceptors. Based on the 16S rRNA gene sequence, strain 106 would represent a novel genus of the previously uncultivated group (Symbiont Group I; a potentially novel family) within the Gammaproteobacteria, and 'Thioprofundum lithotrophica' gen. nov., sp. nov. is proposed. Strain 108 is a short, oval rod at any of the growth pressures. It is a facultative chemoautotroph, capable of both chemolithoautotrophic growth with H_2 and S oxidations and organotrophic growth with complex organics or organic acids using nitrate and O_2 as the electron acceptors. The chemolithoautotrophic growth is strictly piezophilic and under the organotrophic growth condition, it grows at conventional pressures (0.1 MPa). Strain 108 is phylogenetically distinctive from any of the previously described genera of the family Rhodobacteraceae within the Alphaproteobacteria, and 'Piezobacter thermophilus' gen. nov., sp. nov. is proposed. The piezophilic cultivation technique can be a powerful tool to isolate and characterize the previously uncultivated phylotypes in the deep-sea hydrothermal vent environments.

INTRODUCTION

Research on deep-sea piezophiles has half century of history since the pioneering work by Zobell & Morita (1957). For 50 years, most of the targeted microorganisms have been heterotrophs, while very few piezophilic chemolithoautotrophs were described (Bernhardt *et al*., 1988; Kato, 2006; Miller *et al*., 1988; Park *et al.*, 2006). Thermophilic hydrogenotrophic methanogens *Methanocaldococcus jannaschii* and *Methanothermococcus thermolithotrophicus* were isolated from deep-sea and deep subsurface environments originally by conventional, non-piezophilic cultivation techniques but were later found to be facultative piezophilic based on the hyperbaric pressure cultivation experiments (Bernhardt *et al*., 1988; Miller *et al.*, 1988;). Recently, a new hydrostatic pressure cultivation technique has been developed, which has enabled cultivation of various types of deep-sea chemolithoautotrophs such as hydrogenotrophic methanogen, sulfate-reducer and H_2- and S-oxidizing nitrate- and O_2-reducers previously isolated by non-piezophilic techniques (Takai *et al*., 2008a). This technique was successfully applicable even for the most hyperthermophilic microorganism, *Methanopyrus kandleri*, which had been also known to be quite hard to cultivate (Takai *et al*., 2008a). Nevertheless, all the deep-sea piezophilic chemolithoautotrophs studied so far were initially isolated by the cultures under the conventional gas pressures (up to 0.4 MPa), and none of the chemolithoautotrophs is obtained by a high-pressure culture directly from a deep-sea habitat.

Direct piezophilic enrichment from deep-sea and deep subsurface microbial communities would be advantageous to obtain the numerically predominant or the functionally significant microbial populations in the *in situ* habitats. This is not only

because the *in situ* hydrostatic pressure is a potential key physical parameter directly affecting their growth (Abe *et al.*, 1999; Kato, 2006) but also because the pressure and temperature serve as the primary physicochemical factors preparing the thermodynamic states of energy and carbon metabolisms for their growth. That is to say, using conventional gas pressures of media, available gaseous substrates dissolved in the liquid phase of microbial medium often become much less abundant as compared to the concentrations in the *in situ* environments. For instances, the highest dissolved concentrations of H_2, N_2, CH_4 and CO_2 in the deep-sea hydrothermal fluids are recorded to be up to 16, 36, 54 and 2700 mM, respectively (Charlou *et al.*, 2002; Lupton *et al.*, 2008; Person *et al.*, 2005) but in the test tubes or bottles at 60 ˚C under a 0.2 MPa of gas pressure, theoretically the soluble concentrations are less than 1.6, 1.2, 2.0 and 23.5 mM, respectively. The different abundance of the gaseous energy and carbon sources may be a great metabolic bias for enrichment of certain populations that are less predominant in the *in situ* habitats but are energetically favorable in laboratory experiments. In contrast, if the piezophilic cultivation provides quite dissimilar thermodynamic states compared to those of the *in situ* habitats, it may lead to enrichment of previously uncultivated and unidentified microbial components of the similar environments.

In this study, we tried to cultivate H_2- and/or sulfur-oxidizing chemolithoautotrophs using the piezophilic cultivation technique from a black smoker chimney obtained from the TAG field in the Mid Atlantic Ridge (MAR). The TAG field is located at water depth of between 3620 and 3660 m and is among the world deepest hot hydrothermal systems. The H_2- and/or sulfur-oxidizing chemolithoautotrophs are one of the most predominant microbial populations in the global deep-sea hydrothermal environments and a diversity of *Aquificae* and *Epsilonproteobacteria* members have

been isolated as the primary components (Takai *et al*., 2006a; Nakagawa and Takai 2006; 2008). Although only one pressure-temperature (PT) condition of 50 ˚C and 36 MPa and only one medium was used in this study, two novel chemolithoautotrophic bacteria were enriched and isolated by purification under different pressure conditions. These piezophilic chemolithoautotrophs represented the previously uncultivated phylotypes of *Proteobacteria*. The physiological properties of the new deep-sea piezophiles were characterized under the piezophilic cultivation condition.

MATERIALS AND METHODS

Sample collection and fluid chemistry: A sample from a black smoker chimney was obtained from the TAG hydrothermal field (26˚08.23N, 44˚49.57W) in the Mid Atlantic Ridge (MAR) at a depth of 3626 m by means of the remotely operative vehicle VICTOR6000 (Ifremer) in Dive#263 during the EXOMAR cruise performed in August 2005. This chimney was one of the numerous chimneys comprising so-called “the black smoker complex (BSC)” in the TAG field (e.g., Charlou *et al*., 1996). The temperature of the black smoker fluid hosted by this chimney (named as Matomo chimney) was measured to be maximally 345 ˚C. The chimney portions were broken by a manipulator of the VICTOR6000 and directly dropped into a sample box that had been in advance decontaminated and filled with the sterilized distilled water (Postec *et al*., 2005). The lid of the sample box was closed immediately after the sampling at the seafloor although the box did not preserve the hydrostatic pressure. However, due to the tightly sealed lid, no additional seawater mixing was expected during returning to sea surface.

Immediately after the recovery of the chimney sample onboard, a relatively large piece of structure, which preserved the intact structure as much as possible, was subsampled into the representative microbial habitats as previously described (Takai *et al*., 2001; 2008b). The chimney outer surface (thickness 1 to 2 mm) and chimney inside part (thickness 1 to 2 cm) were collected for the nucleic acid extraction, the microscopic observation and the cultivation. For the subsamples of the cultivation, each of the outer surface (2 g wet weight) and the inside part (40 g wet weight) was suspended in 20 ml of seawater filtered with a 0.22 μm pore size filter in the presence and absence of 0.05% (w/v) neutralized sodium sulfide in a 100 ml glass bottle (Schott Glaswerke, Mainz,

Germany), and then tightly sealed with a butyl rubber cap under a gas phase of 100% N_2 (0.2 MPa). For the subsamples of the microscopic observation, approx. 1 g (wet weight) of the subsample was fixed with 3 ml of filter-sterilized seawater containing 3.7% (v/v) formalin for 24 h and then stored at -80 ˚C. All the samples for cultivation were transferred to our laboratory under refrigeration and were preserved at 4 ˚C prior to experiments.

The hydrothermal fluid chemistry in the TAG field is already characterized in detail (Campbell *et al*., 1988; Charlou *et al.*, 1996; Chiba *et al.*, 2001). It is known that the hydrothermal fluids emitting from the BSC of the TAG field have one endmember fluid source (Chiba *et al*., 2001). The chemical composition of the endmember fluid is determined and is found to be temporally stable (Campbell *et al.*, 1988; Charlou *et al.*, 1996; Chiba *et al.*, 2001).

Total direct cell counts: Microbial community densities in the chimney subsamples were determined by 4',6-diamidino-2-phenylindole (DAPI)-staining direct count. The frozen formalin-fixed chimney subsample was thawed, and then vigorously suspended with a vortex mixer. After 5 min of static state, 0.5 ml of formalin-fixed supernatant was added to 0.5 ml of filter-sterilized phosphate-buffered saline (PBS, pH 7.2) containing DAPI (10 μg/ml), and incubated at room temperature for 30 min. After the mixture was filtered, each filter was rinsed twice with 2 ml of filter-sterilized PBS. The filters were examined under epifluorescence using a phase-contrast Olympus BX51 microscope with the Olympus DP71 digital camera system. An average total cell count was obtained from more than 100 microscopic fields from three separate filters.

Liquid serial dilution cultures: To estimate the abundance of culturable microorganisms (viable counts) represented by a variety of physiological and metabolic

146 characteristics, a series of serial dilution cultures were done from the chimney
147 subsamples under the various cultivation conditions, which were in a same manner as
148 previously described (e.g., Takai *et al*., 2008b). Since the detail methods and results will
149 be described elsewhere, only the media and culture conditions that gave positive
150 cultivation results were described (Table 1). For anaerobic, heterotrophic
151 sulfur-reducing thermophiles such as members of *Thermococcales* and *Thermotogales*,
152 MJYPS medium (Takai *et al*., 2000) was used (Table 1). For hydrogen- and/or
153 sulfur-oxidizing chemolithoautotrophs, such as members of *Aquificales*,
154 *Gammaproteobacteria* and *Epsilonproteobacteria*, MMJHS medium (Takai *et al*. 2003)
155 was used (Table 1). For anaerobic dissimilatory Fe(III)- and/or sulfate-reducers, such as
156 members of *Archaeoglobales*, *Deferribacteres*, *Thermodesulfobacteria* and
157 *Deltaproteobacteria*, a MMJHFe medium (Takai *et al*. 2008b) was used (Table 1).
158 A serial dilution culture using a piezophilic cultivation technique (Takai *et al*.,
159 2008a) was also performed with the chimney surface sample at 50 ˚C and at 36 MPa
160 (Table 1). The detail procedure is described below.
161 The microbial growth was identified by turbidity and/or microscopic observation
162 for maximally 2 months. The microorganism present in the most diluted series of the
163 medium at each temperature was isolated by the subsequent extinction-dilution method
164 (Takai and Horikoshi, 2000). The partial sequences of the 16S rRNA genes (approx.
165 700-1000 bp) of the isolates were determined as described elsewhere (Takai *et al*..
166 2004).
167 **Enrichment and purification with piezophilic cultivation techniques:** The
168 chimney surface slurry preserved in the absence of sodium sulfide was inoculated into
169 the test tubes containing MMJHS medium (Takai *et al*., 2003) under a gas phase of 80%

H_2 + 20% CO_2 (0.2 MPa) with every 1/5 dilution. A 1 ml portion of the inoculated culture was taken from each of the serial dilution of test tubes into a piezophilic cultivation syringe and then, a 250 μl of 100% H_2 was added to the syringe (corresponding to approx. 10 mM of soluble H_2 and 30 mM of ΣCO_2 under the piezophilic condition) (Takai *et al*., 2008a). The syringe was sealed by a needle sticking into a butyl rubber stopper (Takai *et al*., 2008a). Finally, the pressure vessels containing the cultivation syringes were compressed by a hydraulic pomp at 36 MPa and incubated at 50 ˚C for two weeks. The serial dilution culture under the piezophilic condition was conducted twice.

After two weeks of incubation, the possible enrichment was examined by a microscopic observation. Up to a certain dilution of culture, two morphotypes of microorganisms were identified; one was a motile, thin, long spiral cell and the other was a non-motile, short, oval rod. The short, oval rods were also observed in two more dilution steps of culture than the one containing two morphotypes. Thus, the highest dilution of culture of oval rods was further conducted to the extinction-dilution culture for the purification under the same condition. For the purification of long spiral cells, the highest dilution of culture including the long spiral and short oval cells were inoculated into a serial dilution of MMJHS medium under a gas phase of 80% H_2 + 20% CO_2 (0.2 MPa) and incubated under the non-piezophilic condition at 50 ˚C. Under the non-piezophilic culture condition, only the long spiral cells were grown. The highest dilution obtained from the non-piezophilic cultivation was again applied to the serial dilution cultivation under the piezophilic cultivation condition (36 MPa). This procedure provided only the growth of long spiral cells under the high pressure. Thus, the extinction-dilution purification of this morphotype was conducted by the repeated

194 cultivation under every piezophilic and non-piezophilic condition.

195 Finally, two morphotypes were isolated and were designated as strain 106 (long
196 spiral cell) and strain 108 (short oval rod), respectively. The purity was confirmed
197 routinely by microscopic examination and by repeated partial sequencing of the 16S
198 rRNA gene using several PCR primers.

199 **Morphology:** Cells were routinely observed under a phase-contrast Olympus
200 BX51 microscope with the Olympus DP71 digital camera system. Transmission
201 electron microscopy of negatively stained cells was carried out as described by Zillig *et*
202 *al.* (1990). Cells grown in MMJHS medium under the optimal hydrostatic pressures
203 (strains 106 and 108) or the conventional gas pressure (0.2 MPa) (strain 106) were
204 negatively stained with 2% (w/v) uranyl acetate and observed under a JEOL JEM-1210
205 electron microscope at an accelerating voltage of 80 kV.

206 **Growth characteristics:** Both strains were routinely cultivated at 16 MPa (for
207 strain 106) or 36 MPa (for strain 108) in Piezo-MMJHS medium, which containing 10
208 mM of dissolved H_2 and 30 mM of ΣCO_2 in 1 ml of modified MMJHS medium (Takai
209 *et al*., 2003) in a 5 ml of piezophilic cultivation syringe. The original MMJHS medium
210 contained 10 mM of sodium nitrate but the modified MMJHS medium including 20 mM
211 of sodium nitrate was used for the subsequent experiments. The pH of the medium was
212 routinely checked before, during and after the growth at a room temperature. The initial
213 pH of Piezo-MMJHS medium was adjusted to 6.5.

214 Growth was measured by direct cell counting after staining with DAPI using a
215 phase-contrast Olympus BX51 microscope. All experiments described below were
216 conducted in duplicate. To test the effects of hydrostatic pressure and temperature on
217 growth, cultures with Piezo-MMJHS medium were incubated at a varying hydrostatic

pressure of 0.2 (non-piezophilic condition), 5, 16, 36, 50 or 65 MPa at 50 ˚C and at every 5 ˚C between 25 and 60 ˚C at the optimal pressures. For testing the effect of pH on growth, the pH of Piezo-MMJHS medium was adjusted to lower pH than pH 6.5 with decreasing concentration of sodium bicarbonate and by adding HCl in MMJHS medium, while was adjusted to higher pH than 6.5 with increasing concentration of sodium bicarbonate and by adding NaOH in MMJHS medium. The growth was conducted at 50 ˚C and the optimal pressures. The effect of NaCl on growth was test in Piezo-MMJHS medium by varying a NaCl concentration in MMJHS medium.

The potential nutrients required for growth such as selenite, tungstate and vitamins were examined with Piezo-MMJHS medium under an optimal pressure with and without the specified nutrients. The nitrogen source (NH_4Cl, $NaNO_2$, N_2, $NaNO_3$ and yeast extract) for growth was also examined with Piezo-MMJHS medium including none of the nitrogen sources under an optimum pressure. Antibiotics susceptibility was tested with Piezo-MMJHS medium at an optimal pressure by using a 50 μg/ml of ampicilin, kanamycin, chloramphenicol, streptomycin or vancomycin.

Energy and carbon sources: In an attempt to determine potential electron donors for autotrophic growth of both strains, each of the H_2 (10 mM), elemental sulfur (3%, w/v), thiosulfate (10 mM), sulfide (1.25 mM), cystein-HCl (2.5 mM), tetrathionate (5 mM), and sulfite (2.5 mM) was tested instead of a combination of H_2, elemental sulfur and thiosulfate as a sole electron donor in Piezo-MMJHS medium (nitrate as a sole electron acceptor) at 50 ˚C and an optimal pressure. In contrast, to examine potential electron acceptors for autotrophic growth of both strains, each of nitrate (20 mM) and O_2 (0.04, 0.12, 0.4, 1.2, 2 or 4 mM) was tested instead of a combination of nitrate, elemental sulfur and thiosulfate as a sole electron acceptor in MMJHS medium

(thiosulfate as a sole electron donor) at 50 ˚C and an optimal pressure. If H_2 (10 mM) was used as a sole electron donor, each of elemental sulfur (3% w/v), thiosulfate (10 mM), sulfite (2.5 mM) and ferrihydrite (5 mM) was also tested.

Heterotrophic growth was tested for both strains using Piezo-MMJHS medium including yeast extract (0.1%, w/v), tryptone (0.1%, w/v), Casamino acid (0.1 %, w/v), formate (5 mM), acetate (5 mM) or pyruvate (5 mM) instead of 30 mM of ΣCO_2 at 50 ˚C and under an optimum pressure. The pH of the media was adjusted at 6.5 in advance. In addition, organotrophic growth was tested using Piezo-MMJHS medium including yeast extract (0.1%, w/v), tryptone (0.1%, w/v), Casamino acid (0.1 %, w/v), formate (5 mM), acetate (5 mM) or pyruvate (5 mM) at pH 6.5 instead of H_2, thiosulfate, elemental sulfur and 30 mM of ΣCO_2 at 50 ˚C and under an optimal pressure.

Time course of oxidations of H_2 (strain 108) and thiosulfate (strains 106 and 108) by nitrate-reduction and concomitant growth of both strains were examined with Piezo-MMJHS medium as H_2 or thiosulfate as a sole electron donor, of which all the sulfate salts were replaced by the chloride salts, at 50 ˚C and under an optimal pressure. The concentrations of nitrate, thiosulfate and sulfate were analyzed by ion chromatography using a Shim-pack IC column (Shimadzu, Kyoto, Japan) and the concentration of H_2, N_2O and N_2 was measured by a gas chromatography Micro GC CP2002 (GL Sciences, Tokyo, Japan) with a thermal conductivity detector. Nessler's reagent was employed to monitor the production of ammonium ion.

Cellular fatty acid composition: Cellular fatty acid composition was analyzed using cells autotrophically grown at 50 ˚C under the optimal pressure and the non-piezophilic condition for strain 106, and using cells harvested from the piezophilic autotrophic condition (at 50 ˚C and 36 MPa) and the non-piezophilic organotrophic

condition (at 50 ˚C and 0.1 MPa) for strain 108. Lyophilized cells (30 mg) were placed in a Teflon-lined, screw-capped tube containing 1 ml of anhydrous methanolic HCl and heated at 100 ˚C for 3 h. The resulting fatty acid methyl esters (FAMEs) were extracted twice with n-hexane and concentrated under a stream of nitrogen gas. The FAMEs were analyzed using a gas chromatography-mass spectrometer (Xcalibur for Trace DSQ, Thermoelectron).

Nucleic acid analyses: DNA was prepared as described by Marmur & Doty (1962). The G+C content of DNA was determined by direct analysis of deoxyribonucleotides on HPLC (Tamaoka and Komagata, 1984). The 16S rRNA gene was amplified by PCR using Bac 27F and 1492R primers (DeLong, 1992; Lane, 1985). The nearly complete sequences of the16S rRNA genes from both strains were directly sequenced by both strands using the dideoxynucleotide chain termination method with a DNA sequencer Model 3100 (Perkin Elmer/Applied Biosystems Co., Foster City, CA, USA). The nearly complete sequences were manually aligned to the representative sequences according to the secondary structures using ARB (Ludwig *et al.*, 2004). Phylogenetic analyses were restricted to unambiguously aligned nucleotide positions. Evolutionary distance matrix analysis (using the Jukes & Cantor correlation method) and neighbor-joining analysis were performed using PHYLIP package (http://evolution.genetics.washington.edu/phylip.html). Bootstrap analysis was performed to provide confidence estimates for phylogenetic tree topologies.

The 16S rRNA gene sequences of strains 106 and 108 were deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB468957 and AB468958, respectively.

Whole cell fluorescence in situ hybridization (FISH) analysis: The whole cell

FISH analysis was conducted to estimate the cellular abundance of strains 106 and 108 and their relatives in the *in situ* chimney habitat by using the 16S rRNA gene sequences. The targeted microbial populations were the whole microbial cells (DAPI-stained cell fraction and both bacterial- and archaeal-probes-binding cell fraction), the strain 106 cells (a specific probe-binding cell fraction) and the strain 108 cells (a specific probe-binding fraction), respectively. The EUB338 (Stahl and Amann, 1991) and the ARC915 (Amann *et al*., 1990) probes were used for the detection of both bacterial and archaeal populations. The ribosomal RNA-targeted oligonucleotide probes specifically binding to the 16S rRNAs of strains 106 and 108, respectively, were designed using ARB (Ludwig *et al*., 2004) based on the multiply aligned sequences including those of strains 106 and 108. Among several potential sites for the probes, the probes designated as TF1110 (5'-CTCCATCTCTGGAGCCTTCC-3') and PB1001 (5'-TGGTAACTGAGGGCGTGGGT-3') were finally chosen through the sequence specificity analysis using the BLAST program to all the nucleic acid sequences in the DDBJ/EMBL/GenBank nucleotide sequence databases. In the *in silico* analysis, the TF1110 probe was found to have at least three bases of mismatch with any other 16S rRNA sequences and the PB1001 probe also had more than three bases of mismatch with any other 16S rRNA sequences except for the 16S rRNAs of *Methanobrevibacter* spp. (one mismatch).

The whole cell FISH experiments were performed as previously described (Sekiguchi *et al*., 1999). The frozen formalin-fixed chimney subsample was thawed, and then vigorously suspended with a vortex mixer. After 5 min of static state, 0.5 ml of formalin-fixed supernatant was centrifuged at a 15000 rpm at 4 ˚C for 30 min. After washing with a 0.5 ml of PBS (pH 7.2) twice, the microbial cells were immobilized on a

positive charged glass slide. Hybridization was performed with both the Alexa488-labeled EUB338 and the ARC915 probes and with each of the Cy3-labeled specific probes TF1110 and PB1001 at 46 ˚C for 3 h. The hybridization stringency was adjusted with varying concentrations of formamide in the hybridization buffer (30% for the ARC915, TF1110 and PB1001 probes, and 10% for the EUB338 probe). After the hybridization and the washing, the cells were stained with PBS (pH 7.2) containing DAPI (10 μg/ml) for 30 min. The slide was examined under an Olympus BX51 epifluorescence microscopy with the Olympus DP71 digital camera system. The cells of *E. coli* strain K12 and the other strain cells of strains 106 and 108 were used as the negative controls. An average of the ratio of probe-hybridized cells to the DAPI-stained cells was determined from more than 100 microscopic fields.

RESULTS AND DISCUSSION

Chimney habitats and total, viable and FISH cell counts: At the present, it is still very difficult to determine directly the physical and chemical conditions of the microbial habitats in the chimney structure by using some of the *in situ* hardware such as temperature-, pH- and other chemical-sensors at the deep seafloor. Thus, the potential chemical conditions for a given temperature range of chimney habitat have been often extrapolated by the mixing between the hot endmember hydrothermal fluid and the cold ambient seawater (McCollom and Shock, 1997; Shock and Holland, 2004; Tivey, 2004).

According to the chemical composition of the endmember hydrothermal fluid in the BSC of the TAG field already reported (Campbell *et al*., 1988; Charlou *et al*., 1996; Chiba *et al*., 2001), the potential concentration ranges of gaseous energy and carbon substrates in the chimney habitats were estimated. If the microbially habitable temperature range was assumed to be up to ~130 ˚C that could be brought by a simple mixing of approx. 1:2 ratio between the hydrothermal fluid and the seawater in the case of the TAG field, the available concentrations of dissolved H_2, N_2, CO_2, CH_4 and H_2S are estimated to be up to 0.12, 0.3, 3.4, 0.05 and 2.3 mM, respectively, based on the highest values reported by Campbell *et al*. (1988) and Charlou *et al*. (1996). The total dissolved concentrations of H_2, ΣCO_2 and reduced sulfur compounds were about 10, 30 and 10 mM in Piezo-MMJHS medium and <1.3, 30 and 10 mM in non-piezophilic MMJHS medium at 50 ˚C, respectively. Thus, the enrichment by using Piezo-MMJHS medium might provide a larger chemical dissimilarity than that under the non-piezophilic condition, particularly in dissolved H_2 concentration, between the *in*

situ habitats and the laboratory experiments. The dissolved H_2 concentration and hydrostatic pressure could be significantly different under piezophilic and non-piezophilic enrichment culture conditions.

The total cell counts in the chimney inside and surface habitats were $3.2 \times 10^5 \pm 7.2 \times 10^4$ and $9.6 \times 10^6 \pm 4.0 \times 10^5$ cell/g (wet weight), respectively. These total cell counts were very comparable to those in other black smoker chimneys studied so far in different deep-sea hydrothermal systems (e.g., Takai *et al*., 2008b). In addition to the total cell counts in the chimney habitats, the viable counts of the representative cultivated populations in the habitats were also determined under the conventional cultivation conditions. From the chimney surface habitat, members of the genera *Thermococcus*, *Deferribacter*, *Persephonella* and *Sulfurimonas* were cultivated as the predominant populations. The viable cell counts of these members were 10^6-10^7 cells/g for *Thermococcus*, 10^3-10^4 cells/g for *Deferribacter*, 10^5-10^6 cells/g for *Persephonella*, and 10^5-10^6 cells/g for *Sulfurimonas*, respectively. Based on these viable cell counts by the serial dilution cultivation experiments under the non-piezophilic condition, the predominant H_2 and/or S-oxidizing chemolithoautotrophic members in the TAG black smoker chimney surface were represented by *Persephonella* spp. within the *Aquificae* and *Sulfurimonas* spp. within the *Epsilonproteobacteria*, which has been commonly observed in other chimney habitats of the different hydrothermal systems (e.g., Takai *et al*., 2008b).

On the other hand, based on the estimation of the viable cell counts under the piezophilic cultivation condition, strains 106 and 108 represented $0.6\text{-}3.0 \times 10^3$ cells/g and $1.5\text{-}7.5 \times 10^4$ cells/g, respectively. These population sizes were lower than that of the *Persephonella* members growing at the same temperature under the non-piezophilic

cultivation condition, but represented 0.01-0.3% (for strain 106) and 0.2-7.5% (for strain 108) of the sum of viable cell counts in the chimney surface habitat. In the highest dilution culture of a serial dilution experiment using the non-piezophilic MMJHS medium at 50 ˚C, a minor but certain proportion of thin, long spiral cells were observed together with abundant, short rod cells of the *Persephonella* members. This morphotype of microorganism was successfully cultivated and isolated from the highest dilution of culture using Piezo-MMJHS medium. The partial 16S rRNA gene sequence of the isolate was 99.8% identical to that of strain 106. Thus, the viable cell count of strain 106 under the non-piezophilic cultivation condition was considered to be 10^5 - 10^6 cells/g, which was equivalent to that of the *Persephonella* members and 1-50% of the sum of viable cell counts. These results suggested that both strains 106 and 108 were novel microbial components previously uncultivated from other deep-sea hydrothermal systems but potentially represented the functionally significant H_2- and/or S-oxidizing chemolithoautotophic components in the chimney structure of the TAG field.

The estimation of the cellular abundance of strains 106 and 108 by the FISH analysis indicated that both strains can be not only the functionally and metabolically significant members in the viable microbial populations but also the numerically considerable components in the total cell count of the microbial community. The proportion of strains 106 and 108 cells in the total cell counts determined by the DAPI-staining and the hybridization with either the bacterial or the archaeal probe (EUB338 or ARC915) was 3.4 and 3.6 % for strain 106 and 1.1 and 1.2 % for strain 108, respectively. These results were not so inconsistent with the proportion of viable populations of strains 106 and 108 in the sum of viable cell counts determined by the cultivation experiments. It seems likely, therefore, that the piezophilic cultivation

technique is a powerful experimental scheme to explore the previously uncultivated but ecophysiologically significant chemolithoautotrophs in the deep-sea hydrothermal environments.

Morphological, physiological and metabolic properties of strain 106: Cells of strain 106 were motile (not all the cells but some), long, thin spiral and approximately 6–20 μm long and 0.4–0.6 μm wide, with a polar flagellum under the piezophilic cultivation condition (Fig. 1a). Under the conventional gas pressure condition (0.2 MPa), however, most of the cells became shorter (up to 4 μm long) with motility (Fig. 1b). As compared to other deep-sea chemolithoautotrophs having similar growth temperature ranges with the *Aquificae* and *Epsilonproteobacteria*, strain 106 was morphologically novel.

Strain 106 autotrophically grew at a temperature range between 30 and 55 ˚C (optimally 50 ˚C) (Fig. 2a), at a pH range between 5.8 and 7.6 (optimally pH 7.0) (Fig. 2b) and at a NaCl range from 1.2 to 4.2 (%; w/v) (optimally 3%) (Fig. 2c) under the piezophilic cultivation condition. In addition, the growth of strain 106 was observed at a hydrostatic pressure range between 0.2 and 36 MPa, and the highest growth rate was identified at 16 MPa (Fig. 3). Thus, strain 106 was piezophilic but not a strict piezophile.

The ability of strain 106 to utilize various electron donors, electron acceptors, and carbon sources was investigated using a range of substrates under the piezophilic and non-piezophilic conditions (Table 2). Strain 106 grew chemolithoautotrophically with elemental sulfur, thiosulfate, tetrathionate or sulfite as a sole electron donor and with nitrate or O_2 as a sole electron donor (Table 2). Organic compounds served as neither energy nor carbon sources. Strain 106 was found to be a strict chemolithoautotroph. In

addition, strain 106 was able to utilize O_2 as the electron acceptor but its O_2 utilization represented the microaerophilic mode (up to 0.4 mM of dissolved O_2 concentration or 5% of O_2 partial pressure) (Table 2). Strain 106 utilized nitrate, ammonium and yeast extract as a nitrogen source. Molecular nitrogen (N_2) could also support the growth as a sole nitrogen source, suggesting the possible N_2 fixation, although the existence of genetic components of N_2 fixation enzymes (*nif* genes) and the $^{15}N_2$ incorporation into the cellular nitrogens were not experimentally examined. Selenium, tungsten and vitamins were not required for the growth.

A time course of oxidation of thiosulfate by nitrate-reduction and concomitant growth under the piezophilic condition were characterized (Fig. 4a). During the growth, thiosulfate and nitrate were consumed, and sulfate, N_2 and N_2O were accumulated (Fig. 4a). None of the other potentially intermediate products such as sulfite and nitrite from the thiosulfate-oxidation and the nitrate-reduction was detected (Fig. 4a). The consumption of thiosulfate and nitrate was stoichiometrically equivalent to the accumulation of sulfate and gaseous nitrogen compounds (Fig. 4a). These results clearly demonstrated that strain 106 was a strictly chemolithoautotrophic, facultatively anaerobic piezophile oxidizing the reduced sulfur compounds to sulfate by O_2- or nitrate-reduction.

Strain 106 was found to be sensitive to a variety of antibiotics, including chloramphenicol (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), ampicillin (50 μg ml^{-1}) and vancomycin (50 μg ml^{-1}).

Morphological, physiological and metabolic properties of strain 108: Cells of strain 108 were non-motile, short, oval and approximately 1–1.5 μm long and 0.6–0.7 μm wide under any of the cultivation conditions (Fig. 1c). No flagellum was observed

445 (Fig. 1c).

446 Strain 108 autotrophically grew at a temperature range between 30 and 55 ˚C
447 (optimally 50 ˚C) (Fig. 2a), at a pH range between 5.5 and 7.5 (optimally pH 6.5-7.0)
448 (Fig. 2b) and at a NaCl range from 1.2 to 5.4 (%; w/v) (optimally 2%) (Fig. 2c) under
449 the piezophilic cultivation condition. The autotrophic growth of strain 108 was strictly
450 piezophilic and was observed at a hydrostatic pressure range between 16 and 65 MPa,
451 and the highest growth rate was obtained at 36 MPa (Fig. 3). Although the piezophilic
452 response in the organotrophic growth of strain 108 was not fully determined, strain 108
453 was able to grow with the organic substrates under the conventional non-piezophilic
454 condition (0.1 MPa of Air). The optimal hydrostatic pressure for the autotrophic growth
455 was equivalent to the in situ pressure of the seafloor at which the TAG hydrothermal
456 field is located. It has been reported that many deep-sea hydrothermal vent
457 hyperthermophilic heterotrophs demonstrate the greater hydrostatic pressure optima for
458 growth than the pressure corresponding to their seafloor habitats (Deming and Baross,
459 1993). The difference in hydrostatic pressure at which the hyperthermophiles grow
460 favorably in the laboratory experiments and naturally in the in situ seafloor may point to
461 the possible existence of deep, hot subseafloor biosphere beneath the hydrothermal
462 active seafloor (Deming and Baross, 1993). Unlike many hyperthermophilic
463 heterotrophs such as *Thermococcales* members, the growth pressure optima of strains
464 106 and 108 matched the water depth range of many seafloor hydrothermal systems in
465 the present Earth (>1000 – <4000 m). Thus, these strains may represent the indigenous,
466 functionally active microbial components thriving at the hydrothermally associated
467 habitats at around the seafloor.

468 The energy and carbon metabolisms of strain 108 were quite different from those

of strain 106 (Table 2). It was capable of both chemolithoautotrophic and organotrophic growth (Table 2). The possible inorganic electron donor to support the growth was H_2, elemental sulfur, thiosulfate, cystein-HCl, tetrathionate or sulfite, and various organic compounds such as yeast extract, tryptone, acetate and pyruvate also served as the energy sources (Table 2). All these organic compounds and Casamino acid were utilized as the carbon source instead of the inorganic carbons. Both the chemolithoautotrophic and organotrophic growth were based on the electron acceptors of nitrate and O_2 (Table 2), and the O_2-dependent growth was fully aerobic (Table 2). These results demonstrate that strain 108 is a piezophilic, facultatively anaerobic and chemolithoautotrophic, thermophile. Strain 108 utilized nitrate and ammonium as a nitrogen source. Similarly as strain 106, molecular nitrogen (N_2) could be utilized as a sole nitrogen source although the existence of genetic components of N_2 fixation enzymes (*nif* genes) and the $^{15}N_2$ incorporation into the cellular nitrogens were not experimentally checked. Selenium, tungsten and vitamins were not required for the growth.

A time course of oxidation of either H_2 or thiosulfate by nitrate-reduction and concomitant growth under the piezophilic condition were characterized (Figs. 4b and 4c). During the growth, either H_2 or thiosulfate and nitrate were consumed, and either H_2O (not identified) or sulfate and N_2O were accumulated (Figs. 4b and 4c). The consumptions of thiosulfate and nitrate were stoichiometrically equivalent to the accumulation of sulfate and N_2O during the thiosulfate-dependent growth (Fig. 4c), while H_2 consumption was too much as expected by the extent of nitrate-reduction (Fig. 4b). As mentioned in the previous study for development of this piezophilic cultivation technique (Takai *et al*., 2008a), it is inevitable that a certain proportion of H_2 gas leaks from the piezophilic cultivation syringe during the experiment. Probably, the excess

amount of H_2 consumption observed was due to the excess amount of H_2 leak as compared to the control experiment without the microbial inoculum. These results demonstrated that strain 108 was a piezophilic, N_2O-producing H_2- and S-oxidizer under the chemolithoautotrophic growth condition.

Strain 108 was also sensitive to a variety of antibiotics, including chloramphenicol (50 μg ml^{-1}), streptomycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), ampicillin (50 μg ml^{-1}) and vancomycin (50 μg ml^{-1}).

Cellular fatty acid composition: Cellular fatty acid compositions of strains 106 and 108 grown at the optimal pressures and at the conventional pressures are shown in Table 3. Each of the fatty acid components was typical as the components observed in the *Proteobacteria* except for small amounts of odds number of carbon chains (C_{15} and C_{17}) found in strain 106 (Table 3). The different patterns in the compositions of both strains were notable between the cells grown under the piezophilic and non-piezophilic conditions (Table 3). Since the first discovery (DeLong and Yayanos, 1985; 1986), it has been well known that the fatty acid compositions of many deep-sea piezophilic bacteria vary as a function of pressure. At the present, it has become evident that the pressure-controlled variability in the fatty acid compositions of the deep-sea piezophilic bacteria is not simply generalized (e.g., Mangelsdorf *et al*., 2005). Nevertheless, an increasing ratio of total unsaturated fatty acids to saturated fatty acids with an increasing hydrostatic pressure initially suggested by DeLong and Yayanos (1985; 1986) still works as a very practical principle in most cases. Indeed, the pressure-induced enrichment of the unsaturated fatty acids was identified in our new piezophilic, chemolithoautotrophic bacteria (Table 3). This is the first evidence showing that the pressure-induced enrichment of the cellular unsaturated fatty acids was applicable not

517 only to the deep-sea heterotrophic bacteria but also to the deep-sea piezophilic,
518 chemolithoautotrophic bacteria.

519 **Genetic characteristics:** The G+C contents of the genomic DNA were found to
520 be 65.7 mol% for strain 106 and 66.2 mol% for strain 108, respectively.

521 The nearly complete 16S rRNA gene sequences of both strains (1487 bp for strain
522 106 and 1302 bp for strain 108) were determined and applied to the sequence similarity
523 analysis. The 16S rRNA gene sequence of strain 106 was the most closely related to the
524 sequences of a deep-sea hydrothermal vent gastropod *Alviniconcha hessleri* gill
525 endosymbiont (94.0%) obtained from the Mariana Trough deep-sea hydrothermal
526 system (Suzuki *et al*., 2005), while it was distantly related with any of the previously
527 described gamma-proteobacterial species of which the most similar sequence was that
528 of *Sulfurivirga caldicuralii* (90.5%) (Takai *et al*., 2006b). The 16S rRNA gene sequence
529 of strain 108 was distantly related to any of the sequences phylogenetically affiliated
530 with the family *Rhodobacteraceae* within the *Alphaproteobacteria*. The most similar
531 sequence of the previously described species was that of *Paracoccus koreensis* (93.9%)
532 (La *et al*., 2005). These results strongly suggested that the new thermophilic piezophiles
533 were previously uncultivated phylotypes within the *Proteobacteria.*

534 To characterize the phylogenetic relationship between the new isolates and the
535 previously described species within the *Alphaproteobacteria* and *Gammaproteobacteria*,
536 the phylogenetic tree was reconstructed together with the representative environmental
537 clones and species reported so far (Fig. 5). The tree indicated that strain 106 was
538 phylogenetically associated with many of the thioautotrophic gamma-proteobacterial
539 endosymbionts in the deep-sea chemosynthetic animals, which are tentatively classified
540 into the Symbiont Group I including the endosymbionts in tubeworms and snails and

541 the Symbiont Group II consisting of the thioautotrophic endosymbionts of mussels and
542 clams (Fig. 5). On the other hand, strain 108 was firmly affiliated within the family
543 *Rhodobacteraceae* of the *Alphaproteobacteria* (Fig. 5). However, strain 108 was
544 phylogenetically distinctive from any of the previously described genera of the family
545 (Fig. 5). Strain 108 was the first member of the *Rhodobacteraceae* and the first
546 thermophilic and chemolithoautotrophic alpha-proteobacterium isolated from the
547 deep-sea hydrothermal vent environments (e.g., Nakagawa and Takai, 2006; 2008), and
548 even represented a novel alpha-proteobacterial phylotype identified in the global
549 deep-sea hydrothermal vent environments (Takai *et al*., 2006a)..

550 **Taxonomic and ecological implications:** The exploration of thermophilic H_2-
551 and/or S-oxidizing chemolithoautotrophs under the piezophilic condition corresponding
552 to an *in situ* hydrostatic pressure of the TAG hydrothermal field has lead to the
553 successful isolation of previously uncultured, two novel, piezophilic,
554 chemolithoautotrophic *Proteobacteria*. These new piezophilic chemolithoautotrophs
555 represented the functionally and metabolically significant members in the viable
556 microbial populations and even the numerically considerable components in the
557 microbial community of the black smoker chimney habitat.

558 In this study, the piezophilic cultivation condition was designed to reproduce the
559 in situ hydrostatic pressure but not to reproduce the chemical condition, particularly
560 dissolved concentrations of gaseous energy and carbon sources, of the *in situ* habitats.
561 The dissimilarity in the chemical conditions may result in the successful enrichment and
562 isolation of novel piezophilic chemolithoautotrophs in this study. This is one aspect of
563 application of the piezophilic cultivation technique to the microbial exploration in the
564 deep-sea and deep subsurface. In contrast, using our piezophilic cultivation technique or

similar techniques, the nearly complete reproduction of physical and chemical conditions of the *in situ* habitats in the laboratory could promote to excavate the numerically and functionally dominating but previously uncultivated microorganisms.

The isolation of two novel chemolithoautotrophic *Proteobacteria* from the TAG hydrothermal field is also of great interest from the aspect of systematic and evolutionary bacteriology. Based on the phylogenetic characterization, both strains might represent novel genera of the *Proteobacteria*. Strain 106 is the first fully characterized S-oxidizing chemolithoautotroph of the phylogenetic group within the *Gammaproteobacteria* consisting only of the thioautotrophic endosymbionts of the deep-sea animals (Fig. 5). It is a free-living relative evolutionarily related with the endosymbionts although the growth temperature range is a little different between them. Thus, strain 106 will be an excellent comparative research target for elucidating the evolutionary transition between the free-living and symbiotic life forms among the related deep-sea *Gammaproteobacteria*. Strain 108 is also the first member of the family *Rhodobacteraceae*, which consists of many chemolithotrophic and phototrophic members (Garrity *et al.*, 2005), from the deep-sea hydrothermal vent environments. Thus, the further investigation of strain 108 may provide an important insight into the metabolic and habitational interaction and evolution associated with interrelationship between the chemolithotrophy, phototrophy and piezophily. Finally, based on physiological and molecular properties of both strains, we propose here new species of new genera named "*Thioprofundum lithotrophica*" and "*Piezobacter thermophilus*" for strains 106 and 108, respectively.

Description of *Thioprofundum* gen. nov.: *Thioprofundum* (Thi.o.pro'fu.n.dum. Gr. n. *thios* sulfur; L. n. *profundus* deep; N.L. neut. n. *Thioprofundum* sulfur-oxidizer

589 from deep-sea). Bent to spiral rod. Facultatively anaerobic and piezophilic.
590 Thermophilic and neutrophilic. Chemolithoautotrophic. Able to utilize reduced sulfur
591 compounds as electron donors and nitrate and molecular oxygen as electron acceptors.
592 NaCl absolutely required for growth. G + C content of genomic DNA is about 66%.
593 Major cellular fatty acids are C_{16}, anteiso-C_{17}, C_{18} and $C_{18:1}$. Based on 16S rRNA gene
594 analysis, the genus *Thioprofundum* is related to the endosymbionts of the deep-sea
595 animals within the *Gammaproteobacteria*. The type species is *Thioprofundum*
596 *lithotrophica*.

597 **Description of *Thioprofundum lithotrophica* sp. nov.:** *Thioprofundum*
598 *lithotrophica* (li.tho.tro'phi.ca. Gr. n. *lithos* stone; Gr. adj. *trophikos* feeding; N.L. neut.
599 adj. *lithotrophica* referring to its lithotrophic metabolism). Motile, short bent to long
600 spiral rods with a polar flagellum, with a mean length of 1–20 μm and a mean width of
601 0.4–0.6 μm. Anaerobic to microaerobic. The temperature range for growth is 30 ˚C to
602 55 ˚C (optimum 50 ˚C). The pH range for growth is 5.8–7.6 (optimum growth at pH
603 7.0). NaCl in the concentration range of 1.2–4.2% is an absolute growth requirement;
604 optimum growth occurs at 3%. Strictly chemolithoautotrophic with the reduced sulfur
605 compounds such as elemental sulfur, thiosulfate, tetrathionate or sulfite as an electron
606 donor, and nitrate or molecular oxygen as an electron acceptor. Thiosulfate is oxidized
607 to sulfate and nitrate is reduced to N_2 and N_2O during growth. Nitrate, ammonium,
608 molecular nitrogen or yeast extract are utilized as a sole nitrogen source. The major
609 cellular fatty acids under the conventional pressure condition are C_{12} (1.2%), C_{14} (7.2%),
610 C_{15} (2.1%), C_{16} (41.3%), $C_{16:1}$ (14.3%), anteiso-C_{17} (14.3%), C_{17} (1.2%), C_{18} (4.2%) and
611 $C_{18:1}$ (14.1%). The G + C content of DNA is about 66 mol% (by HPLC). The organism
612 was isolated from a black smoker chimney of the TAG field in the Mid Atlantic Ridge.

The type strain is *Thioprofundum lithotrophica* strain 106, JCM 14596, Japan Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN), Japan, and DSM 19353, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

Description of *Piezobacter* gen. nov.: *Piezobacter* (Pie.zo.bac.ter. Gr. v. *piezo* to press; N.L. masc. n. *bacter* rod; N.L. masc. n. *Piezobacter* piezophilic rod). Oval rod. Facultatively anaerobic. Piezophilic. Thermophilic and neutrophilic. Facultatively chemolithoautotrophic. Able to utilize H_2 or reduced sulfur compounds as an electron donor and nitrate or molecular oxygen as an electron acceptor. NaCl absolutely required for growth. G + C content of genomic DNA is about 66%. Major cellular fatty acids are C_{16}, C_{18} and $C_{18:1}$. Based on 16S rRNA gene analysis, the genus *Piezobacter* is related to members of the family *Rhodobacteraceae* within the *Alphaproteobacteria*. The type species is *Piezobacter thermophila*.

Description of *Piezobacter thermophilus* sp. nov.: *Piezobacter thermophilus* (ther.mo.phi'lus. Gr. adj. *thermus* hot; Gr. adj. *philos* loving; N.L. masc. adj. *thermophilus* heat-loving). Non-motile, short oval rod, with a mean length of 1–1.5 μm and a mean width of 0.6–0.7 μm. Anaerobic to fully aerobic. The temperature range for growth is 30 ˚C to 55 ˚C (optimum 50 ˚C). The pH range for growth is 5.5–7.5 (optimum growth at pH 6.5-7.0). NaCl in the concentration range of 1.2–5.4% is an absolute growth requirement; optimum growth occurs at 2%. Facultatively chemolithoautotrophic with molecular hydrogen and reduced sulfur compounds such as elemental sulfur, thiosulfate, cystein-HCl, tetrathionate and sulfite as electron donors, and nitrate and molecular oxygen as electron acceptors. Able to grow organotrophically with yeast extract, tryptone, acetate and pyruvate. Chemolithoautotrophic growth is

637 strictly piezophilic. Thiosulfate is oxidized to sulfate and nitrate is reduced to N_2O
638 during growth. Nitrate, ammonium or molecular nitrogen are utilized as a sole nitrogen
639 source. The major cellular fatty acids under the conventional pressure condition are C_{14}
640 (3.4%), C_{16} (21.4%), C_{18} (37.5%) and $C_{18:1}$ (37.7%). The G + C content of DNA is
641 about 66 mol% (by HPLC). The organism was isolated from a black smoker chimney of
642 the TAG field in the Mid Atlantic Ridge.

643 The type strain is *Piezobacter thermophilus* strain 108, JCM 14636, Japan
644 Collection of Microorganisms, Institute of Physical and Chemical Research (RIKEN),
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646

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797 *Bacteriol* **73:** 563-568.

798 Table 1. Medium and condition of liquid serial dilution cultures.

Medium	Gas phase (gas pressure or hydrostatic pressure)	Cultivation temperature (˚C)	pH of medium	Possible electron donor	Possible electron acceptor	Subsamples inoculated	Reference
MJYPS	100% N_2 (0.2 MPa)	55, 70, 85, 95	7	YE*, TT†	NO_3, SO_4, S^0, fermentation	With sodium sulfide	Takai *et al*., 2000
MMJHS	80% H_2 + 20% CO_2 (0.2 MPa)	30, 50, 55, 70, 85, 95	6.5	H_2, S^0, S_2O_3	S^0, S_2O_3, NO_3, CO_2	Without sodium sulfide	Takai *et al*., 2003
	80% H_2 + 19% CO_2 + 1% O_2 (0.2 MPa)	30, 50, 55, 70, 85, 95	6.5	H_2, S^0, S_2O_3	O_2, S^0, S_2O_3, NO_3, CO_2	Without sodium sulfide	Takai *et al*., 2003
Piezo-MMJHS	10 mM H_2, 30 mM ΣCO_2 (36 MPa)	50	6.5	H_2, S^0, S_2O_3	S^0, S_2O_3, NO_3, CO_2	Without sodium sulfide	In this study
MMJHFe	80% H_2 + 20% CO_2 (0.2 MPa)	30, 55, 70, 85, 95	6.5	H_2, YE, Formate, Acetate, Pyruvate, Lactate, Citrate	soluble & insoluble Fe (III), SO_4, CO_2, fermentation	With sodium sulfide	Takai *et al*., 2008b

799 *YE means yeast extract.

800 †TT means tryptone.

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803 Table 2. Utilization of electron donor/acceptor of strains 106 and 108 under piezophilic and non-piezophilic
804 cultivation conditions.

Electron donor/acceptor:	**Strain 106** Piezophilic condition at 16 MPa	**Strain 106** Non-piezophilic condition at 0.2 MPa gas pressure	**Strain 108** Piezophilic condition at 36 MPa	**Strain 108** Non-piezophilic condition at 0.2 MPa gas pressure
H_2 (10 mM)/NO_3 (20 mM)	N.G.*	N.A.†	+§	N.A.
H_2 (80%; v/v)/NO_3 (20 mM)	N.A.	N.G.	N.A.	N.G.
H_2 (80%; v/v)/O_2 (1%; v/v)	N.A	N.G.	N.A	N.G.
H_2 (10 mM)/S^0 (3%; w/v)	N.G.	N.A	N.G.	N.A.
H_2 (10 mM)/S_2O_3 (10 mM)	N.G.	N.A	N.G.	N.A.
S^0 (3%; w/v)/NO_3 (20 mM)	++	++	++	N.G.
S_2O_3 (10 mM)/NO_3 (20 mM)	++	++	++	N.G.
S_2O_3 (10 mM)/NO_2 (2.5 mM)	N.G.	N.G.	N.G.	N.G.
S_2O_3 (10 mM)/O_2 (0.04 mM)	++	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (0.12 mM)	+	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (0.4 mM)	+	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (1.2 mM)	N.G.	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (2 mM)	N.G.	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (4 mM)	N.G.	N.A	++	N.A
S_2O_3 (10 mM)/O_2 (0.1%; v/v)	N.A	++	N.A	N.G.
S_2O_3 (10 mM)/O_2 (0.3%; v/v)	N.A	++	N.A	N.G.
S_2O_3 (10 mM)/O_2 (1%; v/v)	N.A	+++	N.A	N.G.
S_2O_3 (10 mM)/O_2 (3%; v/v)	N.A	+++	N.A	N.G.
S_2O_3 (10 mM)/O_2 (5 %; v/v)	N.A	+++	N.A	N.G.
S_2O_3 (10 mM)/O_2 (10 %; v/v)	N.A	N.G.	N.A	N.G.
H_2S+HS (1.25 mM)/NO_3 (20 mM)	++	++	++	N.G.
Cys-HCl (2.5 mM)/NO_3 (20 mM)	N.G.	N.G.	++	N.G.
S_4O_6 (5 mM)/NO_3 (20 mM)	+	+	++	N.G.
SO_3 (2.5 mM)/NO_3 (20 mM)	+	+	++	N.G.
Yeast Extract (0.1%; w/v)/NO_3 (20 mM)	N.G.	N.G.	+++	++
Tryptone (0.1%, w/v)/ NO_3 (20 mM)	N.G.	N.G.	+++	++
Casamino acid (0.1%, w/v)/NO_3 (20 mM)	N.G.	N.G.	N.G.	N.G.
Formate (5 mM)/NO_3 (20 mM)	N.G.	N.G.	N.G.	N.G.
Acetate (5 mM)/NO_3 (20 mM)	N.G.	N.G.	+++	++
Pyruvate (5 mM)/NO_3 (20 mM)	N.G.	N.G.	+++	++
Yeast Extract (0.1%; w/v)/O_2 (0.3%; v/v)	N.A	N.G.	N.A	++
Yeast Extract (0.1%; w/v)/O_2 (1%; v/v)	N.A	N.G.	N.A	+++
Yeast Extract (0.1%; w/v)/O_2 (3%; v/v)	N.A	N.G.	N.A	+++
Yeast Extract (0.1%; w/v)/O_2 (5 %; v/v)	N.A	N.G.	N.A	+++
Yeast Extract (0.1%; w/v)/O_2 (10 %; v/v)	N.A	N.G.	N.A	+++
Yeast Extract (0.1%; w/v)/O_2 (20 %; v/v)	N.A	N.G.	N.A	+++

805 *N.G.; not grown.

806 †N.A.; not attempted.

807 §+; final cell yield is <1.0 x 10^8 cells/ml, ++; between 1.0 x 10^8 and 5.0 x 10^8, +++; >1.0 x 10^9.

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811 Table 3. Cellular fatty acid compositions of strains 106 and 108 under the piezophilic
812 and non-piezophilic cultivation conditions.

Fatty acids	**Strain 106** **Autotrophically grown at 16 MPa**	**Strain 106** **Autotrophically grown at 0.2 MPa (gas pressure)**	**Strain 108** **Autotrophically grown at 36 MPa**	**Strain 108** **Organotrophically grown at 0.1 MPa (air)**
C_{12}	1.2%	1.8%	n.d.*	n.d.
C_{14}	7.2%	4.7%	3.4%	3.2%
C_{15}	2.1%	1.9%	n.d.	n.d.
C_{16}	41.3%	41.3%	21.4%	26.8%
$C_{16:1\ \omega7}$	14.3%	5.3%	n.d.	n.d.
Anteiso C_{17}	14.3%	17.1%	n.d.	n.d.
C_{17}	1.2%	1.1	n.d.	n.d.
C_{18}	4.2%	10.9%	37.5%	42.0%
$C_{18:1\ \omega2}$	n.d.	n.d.	16.5%	12.2%
$C_{18:1\ \omega7}$	14.1%	15.9%	21.2%	15.8%
Total	99.9%	100%	100%	100%
UF/SF†	0.397	0.269	0.605	0.389
$C_{average}$§	16.29	16.52	17.44	17.34

813 *n.d.; not detected.

814 †UF/SF; unsaturated fatty acids/saturated fatty acids.

815 §$C_{average}$; An average carbon chain number of fatty acids.

816

FIGURE LEGENDS

Figure 1. Electron micrographs of negatively stained cell of strain 106 grown under the piezophilic cultivation condition at 50 ˚C and 16 MPa (a) and under the non-piezophilic cultivation condition at 50 ˚C and 0.2 MPa of gas pressure (80% H_2 + 20% CO_2) (b), and of strain 108 grown under the piezophilic cultivation condition at 50 ˚C and 16 MPa (c). Bar indicates 2.0 μm (a) and 1.0 μm (b and c), respectively.

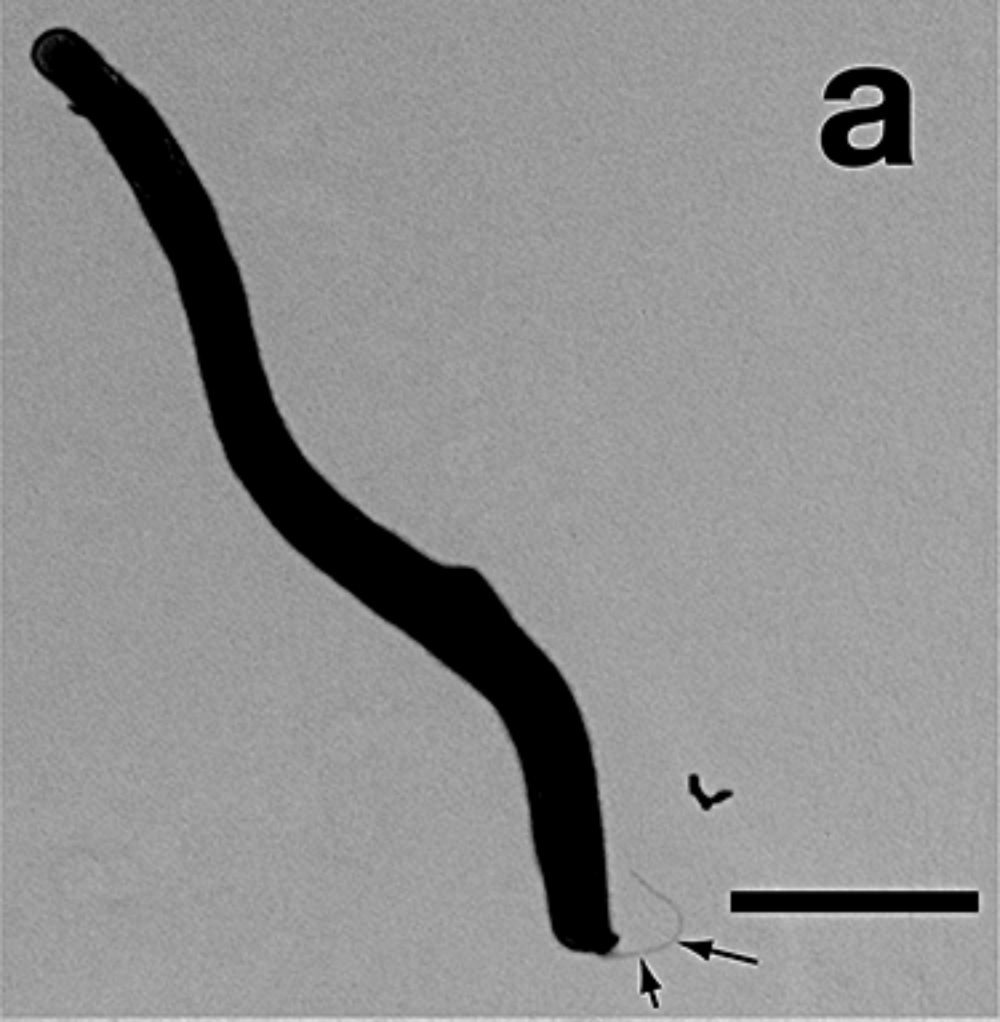
Figure 2. Effects of temperature (a), pH (b) and NaCl concentration (c) on growth of strain 106 (●) or strain 108 (□). (a) Growth curves at different temperatures were determined in Piezo-MMJHS medium at pH 6.5 at the optimal pressures. (b) Effect of pH on growth was determined in Piezo-MMJHS medium having varying pH at 50 ˚C and the optimal pressures. The pH value was monitored before (● and □) and after (○ and ■) the growth. (c) Effect of NaCl concentration on growth was determined in Piezo-MMJHS medium containing a varying concentration of NaCl at 50 ˚C and pH 6.0 under the piezophilic condition.

Figure 3. Effect of hydrostatic pressure on growth of strains 106 (●) or 108 (□). Growth curves were determined at 50 ˚C and pH 6.5 under the autotrophic growth condition.

Figure 4. Time course of oxidation of electron donor, reduction of electron acceptor and concomitant growth of strain 106 grown with thiosulfate and nitrate (a), strain 108 grown with H_2 and nitrate (b) or strain 108 grown with thiosulfate and nitrate (c). (a) Consumption of thiosulfate and nitrate, production of sulfate and nitrogenous gases and cellular proliferation of strain 106 were determined at 50 ˚C, pH 6.5 and 16 MPa. (b) Consumption of H_2 and nitrate, production of nitrogenous gases and cellular proliferation of strain 108 were determined at 50 ˚C, pH 6.5 and 36 MPa. The concentration N_2O is indicated as 5 times higher value for easy perception. (c) Consumption of thiosulfate and nitrate, production of nitrogenous gases and cellular proliferation of strain 108 were determined at 50 ˚C, pH 6.5 and 36 MPa.

Figure 5. Phylogenetic tree of representative species and endosymbionts within the *Alphaproteobacteria* and *Gammaproteobacteria* including strain 106 and 108. The tree was inferred from 16S rRNA gene sequences using the neighbor-joining method on the 1022 homologous sequence positions for each sequence. The number at each node represents the bootstrap value (in percent) determined by 1000 replicates. The scale bar

852 indicates 5 substitutions per 100 nucleotides.
853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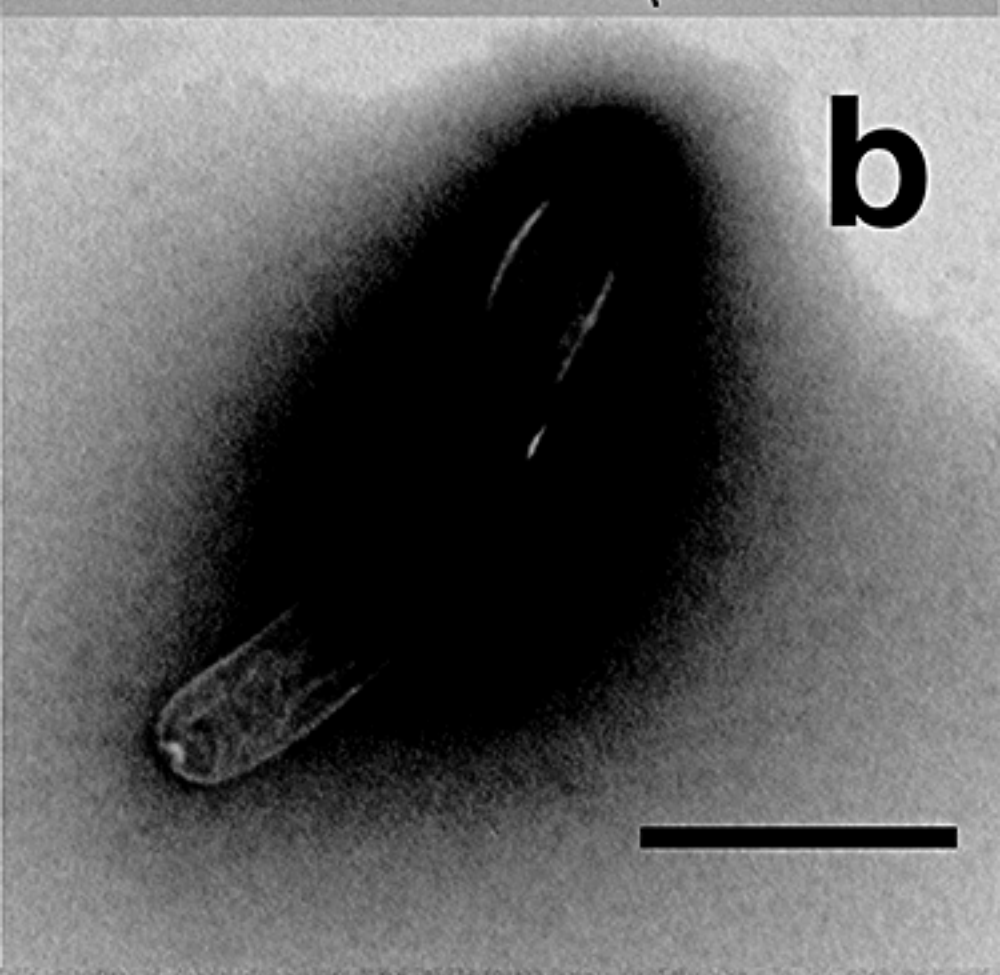
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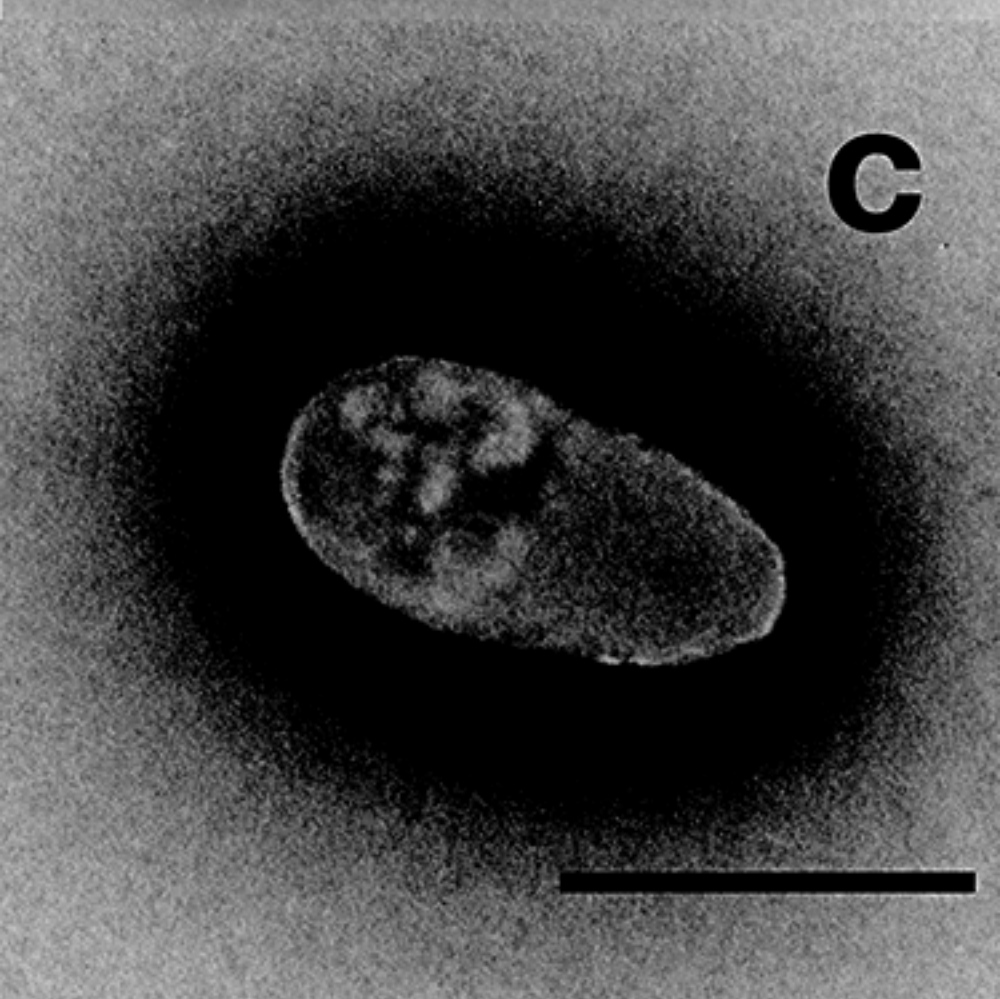
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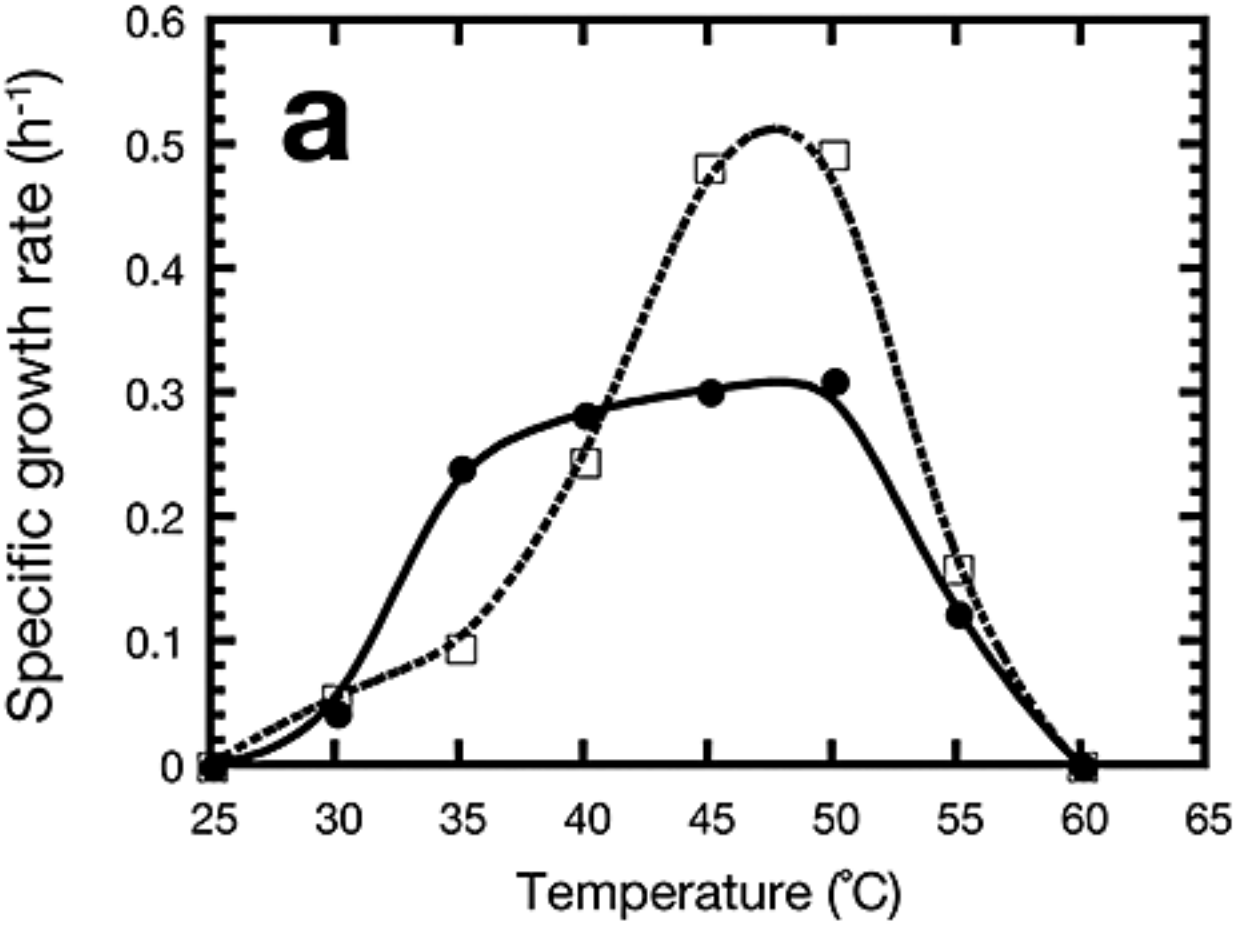
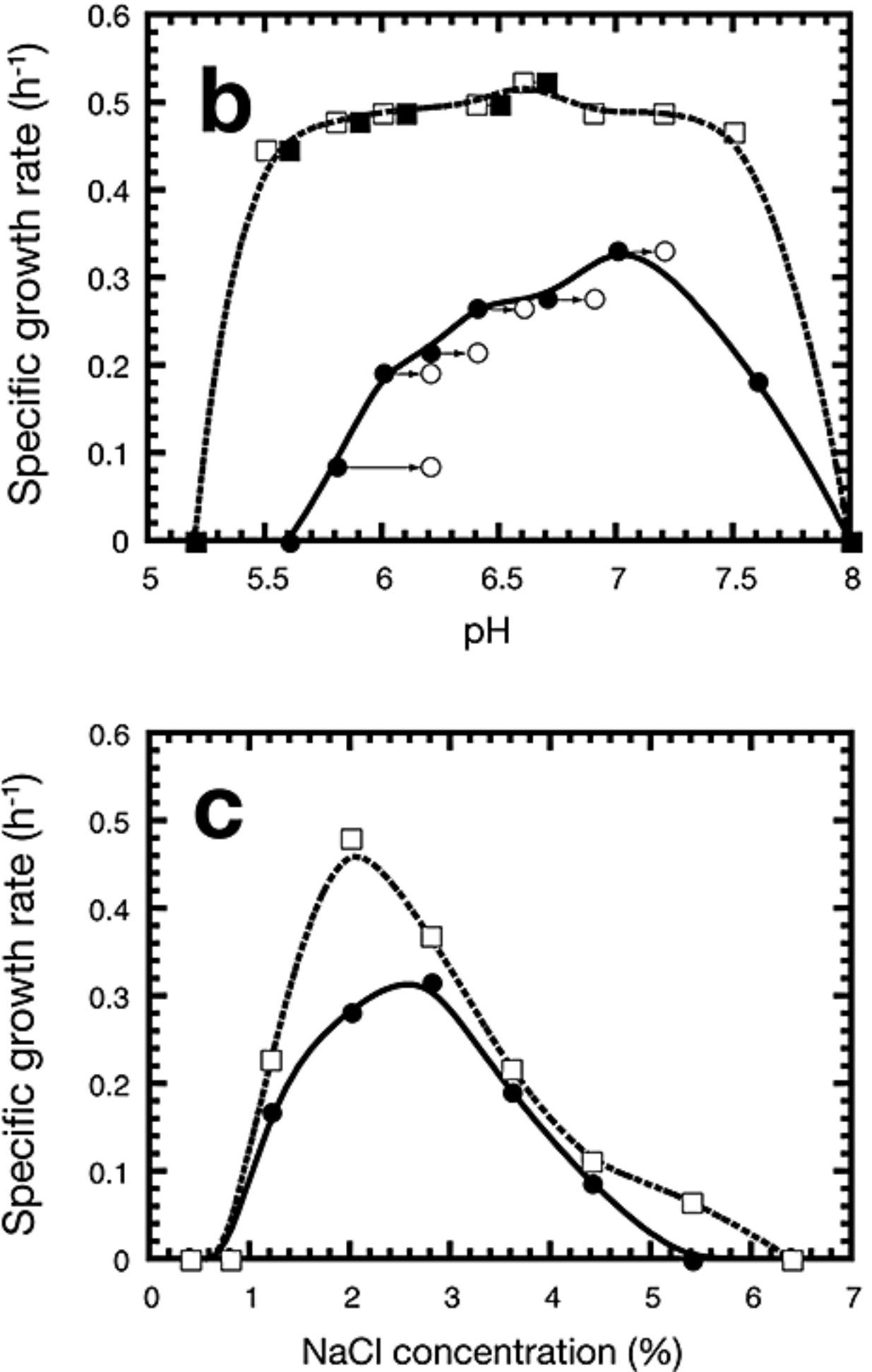
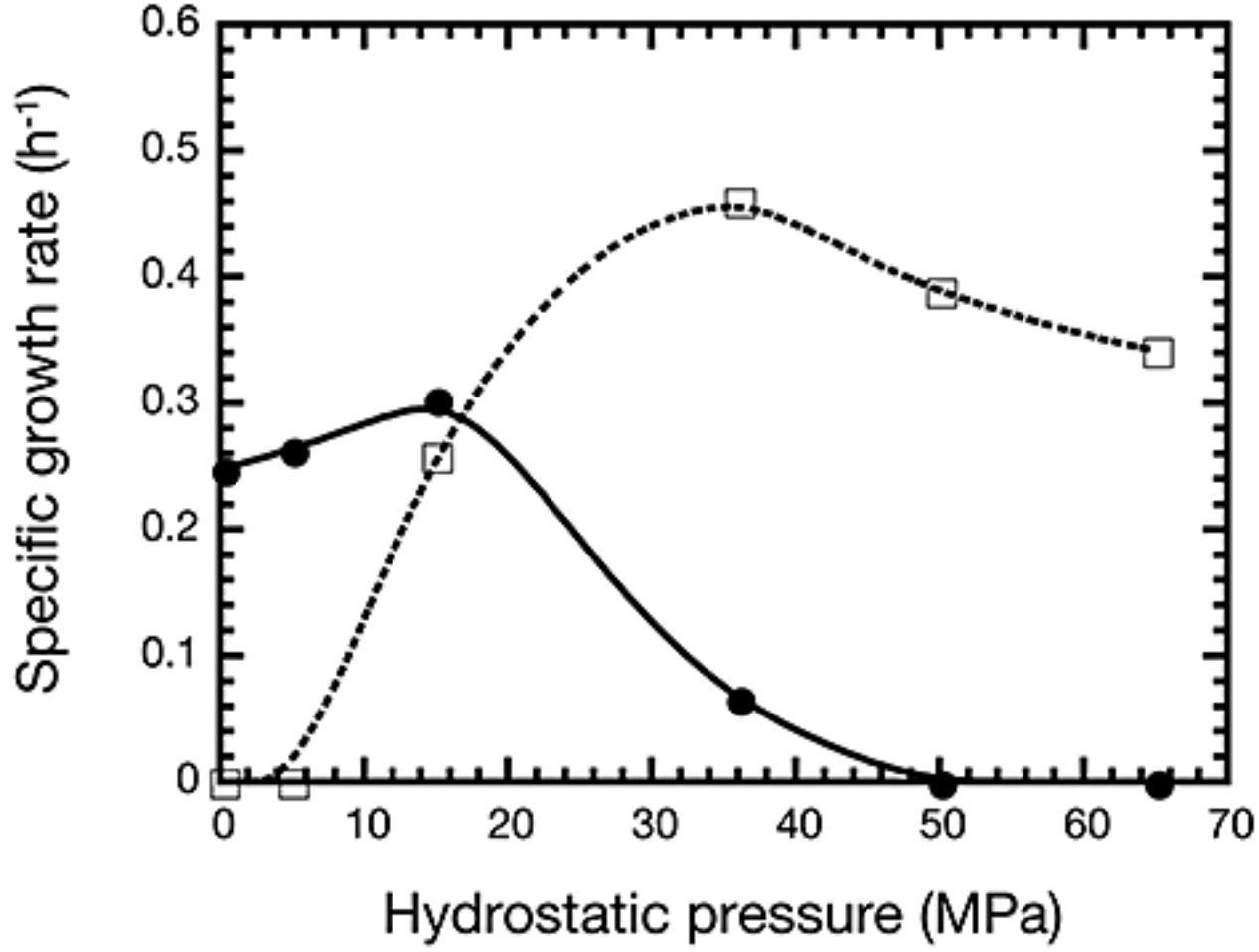
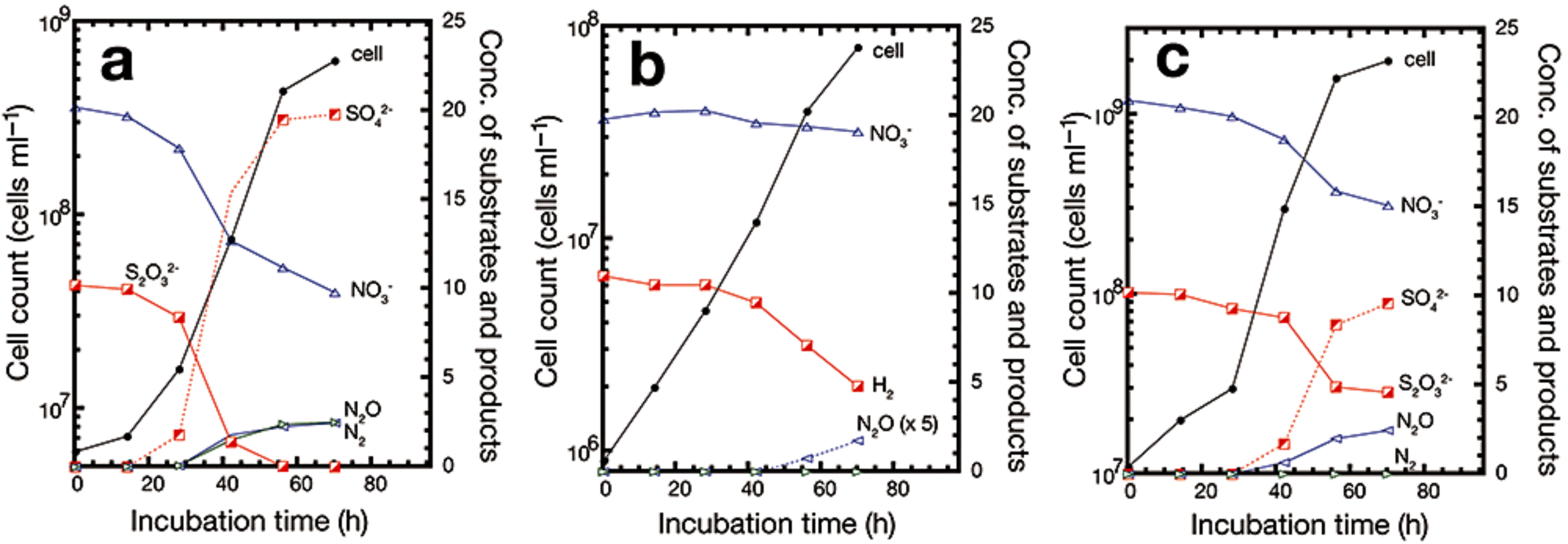
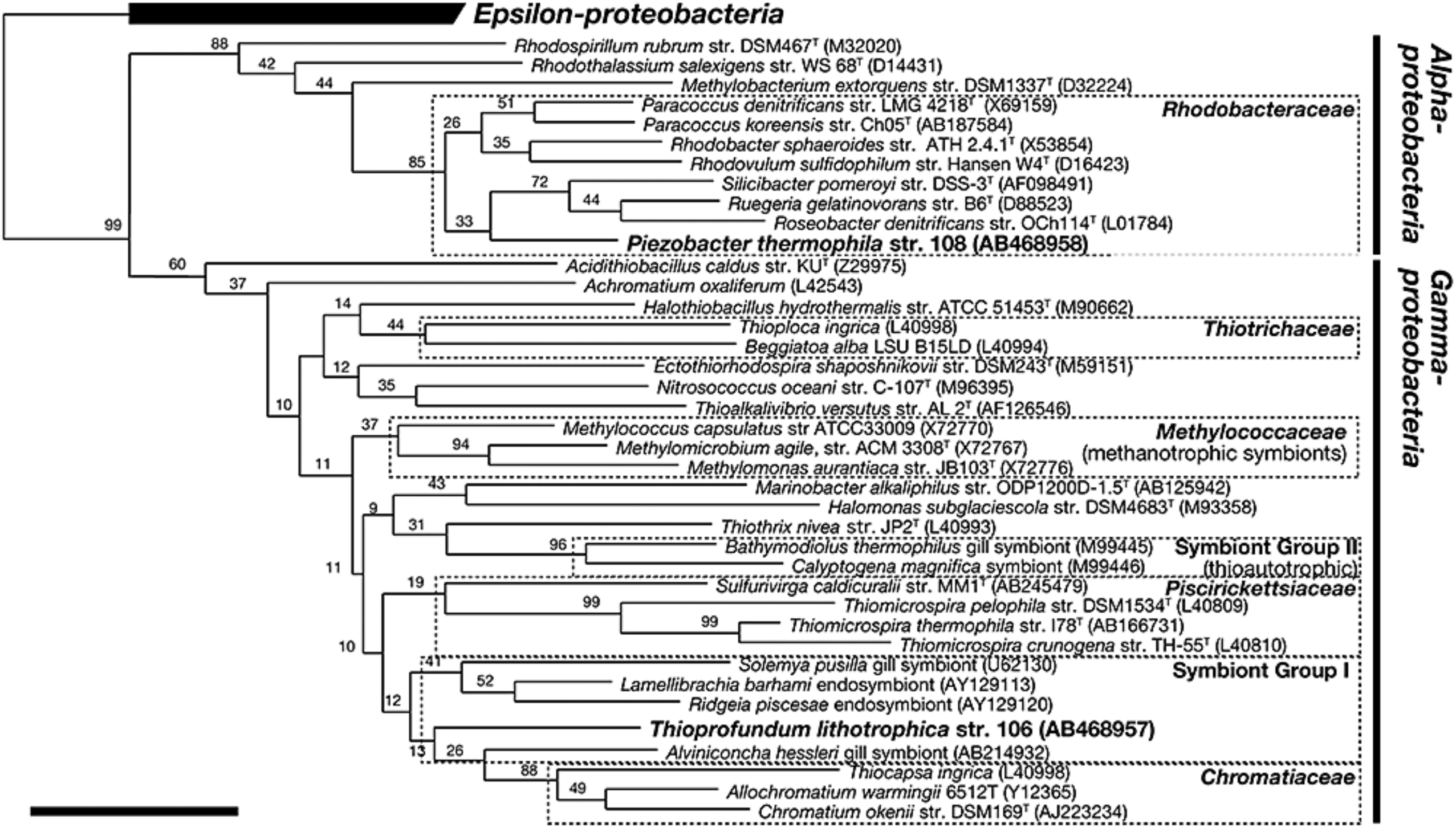

a
Specific growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
25
30
35
40
45
50
55
60
65
Temperature (°C)


b
Specific growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
5
5.5
6
6.5
7
7.5
8
pH

c
Specific growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
NaCl concentration (%)


Specific growth rate (h-1)
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
60
70
Hydrostatic pressure (MPa)


a
b
c
cell
SO_4^{2-}
$S_2O_3^{2-}$
NO_3^-
N_2O
N_2
H_2
N_2O (x 5)
Cell count (cells ml^{-1})
Conc. of substrates and products
Incubation time (h)


Epsilon-proteobacteria
Alpha-proteobacteria
Gamma-proteobacteria
Rhodospirillum rubrum str. DSM467T (M32020)
Rhodothalassium salexigens str. WS 68T (D14431)
Methylobacterium extorquens str. DSM1337T (D32224)
Paracoccus denitrificans str. LMG 4218T (X69159)
Paracoccus koreensis str. Ch05T (AB187584)
Rhodobacter sphaeroides str. ATH 2.4.1T (X53854)
Rhodovulum sulfidophilum str. Hansen W4T (D16423)
Silicibacter pomeroyi str. DSS-3T (AF098491)
Ruegeria gelatinovorans str. B6T (D88523)
Roseobacter denitrificans str. OCh114T (L01784)
Rhodobacteraceae
Piezobacter thermophila str. 108 (AB468958)
Acidithiobacillus caldus str. KUT (Z29975)
Achromatium oxaliferum (L42543)
Halothiobacillus hydrothermalis str. ATCC 51453T (M90662)
Thioploca ingrica (L40998)
Beggiatoa alba LSU B15LD (L40994)
Thiotrichaceae
Ectothiorhodospira shaposhnikovii str. DSM243T (M59151)
Nitrosococcus oceani str. C-107T (M96395)
Thioalkalivibrio versutus str. AL 2T (AF126546)
Methylococcus capsulatus str ATCC33009 (X72770)
Methylomicrobium agile, str. ACM 3308T (X72767)
Methylomonas aurantiaca str. JB103T (X72776)
Methylococcaceae
(methanotrophic symbionts)
Marinobacter alkaliphilus str. ODP1200D-1.5T (AB125942)
Halomonas subglaciescola str. DSM4683T (M93358)
Thiothrix nivea str. JP2T (L40993)
Bathymodiolus thermophilus gill symbiont (M99445)
Calyptogena magnifica symbiont (M99446)
Symbiont Group II
(thioautotrophic)
Sulfurivirga caldicuralii str. MM1T (AB245479)
Thiomicrospira pelophila str. DSM1534T (L40809)
Thiomicrospira thermophila str. I78T (AB166731)
Thiomicrospira crunogena str. TH-55T (L40810)
Piscirickettsiaceae
Solemya pusilla gill symbiont (U62130)
Lamellibrachia barhami endosymbiont (AY129113)
Ridgeia piscesae endosymbiont (AY129120)
Symbiont Group I
Thioprofundum lithotrophica str. 106 (AB468957)
Alviniconcha hessleri gill symbiont (AB214932)
Thiocapsa ingrica (L40998)
Allochromatium warmingii 6512T (Y12365)
Chromatium okenii str. DSM169T (AJ223234)
Chromatiaceae