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Isolation and physiological characterization of two novel, piezophilic, thermophilic chemolithoautotrophs from a deep-sea hydrothermal vent chimneyKen Takai^{1,*}, Masayuki Miyazaki¹, Hisako Hirayama¹, Satoshi Nakagawa¹, Joël Querellou² and Anne Godfroy²¹ Sunground Animalcule Retrieval (SUGAR) Program, Japan Agency for Marine-Earth Science & Technology, 2-15 Natsushima-cho, Yokosuka 237-0061, Japan² Ifremer, Ctr Brest, Laboratoire de Microbiologie des Environnements Extrêmes, UMR 6197, F-29280 Plouzané, France.

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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 O₂ 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 'Thiopfundum 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 H₂ and S oxidations and organotrophic growth with complex organics or organic acids using nitrate and O₂ 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.

43 **INTRODUCTION**

44

45 Research on deep-sea piezophiles has half century of history since the pioneering
46 work by Zobell & Morita (1957). For 50 years, most of the targeted microorganisms
47 have been heterotrophs, while very few piezophilic chemolithoautotrophs were
48 described (Bernhardt *et al.*, 1988; Kato, 2006; Miller *et al.*, 1988; Park *et al.*, 2006).
49 Thermophilic hydrogenotrophic methanogens *Methanocaldococcus jannaschii* and
50 *Methanothermococcus thermolithotrophicus* were isolated from deep-sea and deep
51 subsurface environments originally by conventional, non-piezophilic cultivation
52 techniques but were later found to be facultative piezophilic based on the hyperbaric
53 pressure cultivation experiments (Bernhardt *et al.*, 1988; Miller *et al.*, 1988;). Recently,
54 a new hydrostatic pressure cultivation technique has been developed, which has enabled
55 cultivation of various types of deep-sea chemolithoautotrophs such as hydrogenotrophic
56 methanogen, sulfate-reducer and H₂- and S-oxidizing nitrate- and O₂-reducers
57 previously isolated by non-piezophilic techniques (Takai *et al.*, 2008a). This technique
58 was successfully applicable even for the most hyperthermophilic microorganism,
59 *Methanopyrus kandleri*, which had been also known to be quite hard to cultivate (Takai
60 *et al.*, 2008a). Nevertheless, all the deep-sea piezophilic chemolithoautotrophs studied
61 so far were initially isolated by the cultures under the conventional gas pressures (up to
62 0.4 MPa), and none of the chemolithoautotrophs is obtained by a high-pressure culture
63 directly from a deep-sea habitat.

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

67 because the *in situ* hydrostatic pressure is a potential key physical parameter directly
68 affecting their growth (Abe *et al.*, 1999; Kato, 2006) but also because the pressure and
69 temperature serve as the primary physicochemical factors preparing the thermodynamic
70 states of energy and carbon metabolisms for their growth. That is to say, using
71 conventional gas pressures of media, available gaseous substrates dissolved in the liquid
72 phase of microbial medium often become much less abundant as compared to the
73 concentrations in the *in situ* environments. For instances, the highest dissolved
74 concentrations of H₂, N₂, CH₄ and CO₂ in the deep-sea hydrothermal fluids are recorded
75 to be up to 16, 36, 54 and 2700 mM, respectively (Charlou *et al.*, 2002; Lupton *et al.*,
76 2008; Person *et al.*, 2005) but in the test tubes or bottles at 60 °C under a 0.2 MPa of gas
77 pressure, theoretically the soluble concentrations are less than 1.6, 1.2, 2.0 and 23.5 mM,
78 respectively. The different abundance of the gaseous energy and carbon sources may be
79 a great metabolic bias for enrichment of certain populations that are less predominant in
80 the *in situ* habitats but are energetically favorable in laboratory experiments. In contrast,
81 if the piezophilic cultivation provides quite dissimilar thermodynamic states compared
82 to those of the *in situ* habitats, it may lead to enrichment of previously uncultivated and
83 unidentified microbial components of the similar environments.

84 In this study, we tried to cultivate H₂- and/or sulfur-oxidizing
85 chemolithoautotrophs using the piezophilic cultivation technique from a black smoker
86 chimney obtained from the TAG field in the Mid Atlantic Ridge (MAR). The TAG field
87 is located at water depth of between 3620 and 3660 m and is among the world deepest
88 hot hydrothermal systems. The H₂- and/or sulfur-oxidizing chemolithoautotrophs are
89 one of the most predominant microbial populations in the global deep-sea hydrothermal
90 environments and a diversity of *Aquificae* and *Epsilonproteobacteria* members have

91 been isolated as the primary components (Takai *et al.*, 2006a; Nakagawa and Takai
92 2006; 2008). Although only one pressure-temperature (PT) condition of 50 °C and 36
93 MPa and only one medium was used in this study, two novel chemolithoautotrophic
94 bacteria were enriched and isolated by purification under different pressure conditions.
95 These piezophilic chemolithoautotrophs represented the previously uncultivated
96 phylotypes of *Proteobacteria*. The physiological properties of the new deep-sea
97 piezophiles were characterized under the piezophilic cultivation condition.

98

98 MATERIALS AND METHODS

99

100 **Sample collection and fluid chemistry:** A sample from a black smoker chimney
101 was obtained from the TAG hydrothermal field (26°08.23N, 44°49.57W) in the Mid
102 Atlantic Ridge (MAR) at a depth of 3626 m by means of the remotely operative vehicle
103 VICTOR6000 (Ifremer) in Dive#263 during the EXOMAR cruise performed in August
104 2005. This chimney was one of the numerous chimneys comprising so-called “the black
105 smoker complex (BSC)” in the TAG field (e.g., Charlou *et al.*, 1996). The temperature
106 of the black smoker fluid hosted by this chimney (named as Matomo chimney) was
107 measured to be maximally 345 °C. The chimney portions were broken by a manipulator
108 of the VICTOR6000 and directly dropped into a sample box that had been in advance
109 decontaminated and filled with the sterilized distilled water (Postec *et al.*, 2005). The lid
110 of the sample box was closed immediately after the sampling at the seafloor although
111 the box did not preserve the hydrostatic pressure. However, due to the tightly sealed lid,
112 no additional seawater mixing was expected during returning to sea surface.

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

122 Germany), and then tightly sealed with a butyl rubber cap under a gas phase of 100% N₂
123 (0.2 MPa). For the subsamples of the microscopic observation, approx. 1 g (wet weight)
124 of the subsample was fixed with 3 ml of filter-sterilized seawater containing 3.7% (v/v)
125 formalin for 24 h and then stored at -80 °C. All the samples for cultivation were
126 transferred to our laboratory under refrigeration and were preserved at 4 °C prior to
127 experiments.

128 The hydrothermal fluid chemistry in the TAG field is already characterized in
129 detail (Campbell *et al.*, 1988; Charlou *et al.*, 1996; Chiba *et al.*, 2001). It is known that
130 the hydrothermal fluids emitting from the BSC of the TAG field have one endmember
131 fluid source (Chiba *et al.*, 2001). The chemical composition of the endmember fluid is
132 determined and is found to be temporally stable (Campbell *et al.*, 1988; Charlou *et al.*,
133 1996; Chiba *et al.*, 2001).

134 **Total direct cell counts:** Microbial community densities in the chimney
135 subsamples were determined by 4',6-diamidino-2-phenylindole (DAPI)-staining direct
136 count. The frozen formalin-fixed chimney subsample was thawed, and then vigorously
137 suspended with a vortex mixer. After 5 min of static state, 0.5 ml of formalin-fixed
138 supernatant was added to 0.5 ml of filter-sterilized phosphate-buffered saline (PBS, pH
139 7.2) containing DAPI (10 µg/ml), and incubated at room temperature for 30 min. After
140 the mixture was filtered, each filter was rinsed twice with 2 ml of filter-sterilized PBS.
141 The filters were examined under epifluorescence using a phase-contrast Olympus BX51
142 microscope with the Olympus DP71 digital camera system. An average total cell count
143 was obtained from more than 100 microscopic fields from three separate filters.

144 **Liquid serial dilution cultures:** To estimate the abundance of culturable
145 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%

170 H₂ + 20% CO₂ (0.2 MPa) with every 1/5 dilution. A 1 ml portion of the inoculated
171 culture was taken from each of the serial dilution of test tubes into a piezophilic
172 cultivation syringe and then, a 250 µl of 100% H₂ was added to the syringe
173 (corresponding to approx. 10 mM of soluble H₂ and 30 mM of ΣCO₂ under the
174 piezophilic condition) (Takai *et al.*, 2008a). The syringe was sealed by a needle sticking
175 into a butyl rubber stopper (Takai *et al.*, 2008a). Finally, the pressure vessels containing
176 the cultivation syringes were compressed by a hydraulic pump at 36 MPa and incubated
177 at 50 °C for two weeks. The serial dilution culture under the piezophilic condition was
178 conducted twice.

179 After two weeks of incubation, the possible enrichment was examined by a
180 microscopic observation. Up to a certain dilution of culture, two morphotypes of
181 microorganisms were identified; one was a motile, thin, long spiral cell and the other
182 was a non-motile, short, oval rod. The short, oval rods were also observed in two more
183 dilution steps of culture than the one containing two morphotypes. Thus, the highest
184 dilution of culture of oval rods was further conducted to the extinction-dilution culture
185 for the purification under the same condition. For the purification of long spiral cells,
186 the highest dilution of culture including the long spiral and short oval cells were
187 inoculated into a serial dilution of MMJHS medium under a gas phase of 80% H₂ +
188 20% CO₂ (0.2 MPa) and incubated under the non-piezophilic condition at 50 °C. Under
189 the non-piezophilic culture condition, only the long spiral cells were grown. The highest
190 dilution obtained from the non-piezophilic cultivation was again applied to the serial
191 dilution cultivation under the piezophilic cultivation condition (36 MPa). This
192 procedure provided only the growth of long spiral cells under the high pressure. Thus,
193 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 H₂ and 30 mM of ΣCO₂ 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

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

226 The potential nutrients required for growth such as selenite, tungstate and
227 vitamins were examined with Piezo-MMJHS medium under an optimal pressure with
228 and without the specified nutrients. The nitrogen source (NH₄Cl, NaNO₂, N₂, NaNO₃
229 and yeast extract) for growth was also examined with Piezo-MMJHS medium including
230 none of the nitrogen sources under an optimum pressure. Antibiotics susceptibility was
231 tested with Piezo-MMJHS medium at an optimal pressure by using a 50 µg/ml of
232 ampicilin, kanamycin, chloramphenicol, streptomycin or vancomycin.

233 **Energy and carbon sources:** In an attempt to determine potential electron donors
234 for autotrophic growth of both strains, each of the H₂ (10 mM), elemental sulfur (3%,
235 w/v), thiosulfate (10 mM), sulfide (1.25 mM), cystein-HCl (2.5 mM), tetrathionate (5
236 mM), and sulfite (2.5 mM) was tested instead of a combination of H₂, elemental sulfur
237 and thiosulfate as a sole electron donor in Piezo-MMJHS medium (nitrate as a sole
238 electron acceptor) at 50 °C and an optimal pressure. In contrast, to examine potential
239 electron acceptors for autotrophic growth of both strains, each of nitrate (20 mM) and
240 O₂ (0.04, 0.12, 0.4, 1.2, 2 or 4 mM) was tested instead of a combination of nitrate,
241 elemental sulfur and thiosulfate as a sole electron acceptor in MMJHS medium

242 (thiosulfate as a sole electron donor) at 50 °C and an optimal pressure. If H₂ (10 mM)
243 was used as a sole electron donor, each of elemental sulfur (3% w/v), thiosulfate (10
244 mM), sulfite (2.5 mM) and ferrihydrite (5 mM) was also tested.

245 Heterotrophic growth was tested for both strains using Piezo-MMJHS medium
246 including yeast extract (0.1%, w/v), tryptone (0.1%, w/v), Casamino acid (0.1 %, w/v),
247 formate (5 mM), acetate (5 mM) or pyruvate (5 mM) instead of 30 mM of ΣCO₂ at 50
248 °C and under an optimum pressure. The pH of the media was adjusted at 6.5 in advance.
249 In addition, organotrophic growth was tested using Piezo-MMJHS medium including
250 yeast extract (0.1%, w/v), tryptone (0.1%, w/v), Casamino acid (0.1 %, w/v), formate (5
251 mM), acetate (5 mM) or pyruvate (5 mM) at pH 6.5 instead of H₂, thiosulfate, elemental
252 sulfur and 30 mM of ΣCO₂ at 50 °C and under an optimal pressure.

253 Time course of oxidations of H₂ (strain 108) and thiosulfate (strains 106 and 108)
254 by nitrate-reduction and concomitant growth of both strains were examined with
255 Piezo-MMJHS medium as H₂ or thiosulfate as a sole electron donor, of which all the
256 sulfate salts were replaced by the chloride salts, at 50 °C and under an optimal pressure.
257 The concentrations of nitrate, thiosulfate and sulfate were analyzed by ion
258 chromatography using a Shim-pack IC column (Shimadzu, Kyoto, Japan) and the
259 concentration of H₂, N₂O and N₂ was measured by a gas chromatography Micro GC
260 CP2002 (GL Sciences, Tokyo, Japan) with a thermal conductivity detector. Nessler's
261 reagent was employed to monitor the production of ammonium ion.

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

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

272 **Nucleic acid analyses:** DNA was prepared as described by Marmur & Doty
273 (1962). The G+C content of DNA was determined by direct analysis of
274 deoxyribonucleotides on HPLC (Tamaoka and Komagata, 1984). The 16S rRNA gene
275 was amplified by PCR using Bac 27F and 1492R primers (DeLong, 1992; Lane, 1985).
276 The nearly complete sequences of the 16S rRNA genes from both strains were directly
277 sequenced by both strands using the dideoxynucleotide chain termination method with a
278 DNA sequencer Model 3100 (Perkin Elmer/Applied Biosystems Co., Foster City, CA,
279 USA). The nearly complete sequences were manually aligned to the representative
280 sequences according to the secondary structures using ARB (Ludwig *et al.*, 2004).
281 Phylogenetic analyses were restricted to unambiguously aligned nucleotide positions.
282 Evolutionary distance matrix analysis (using the Jukes & Cantor correlation method)
283 and neighbor-joining analysis were performed using PHYLIP package
284 (<http://evolution.genetics.washington.edu/phylip.html>). Bootstrap analysis was
285 performed to provide confidence estimates for phylogenetic tree topologies.

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

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

290 FISH analysis was conducted to estimate the cellular abundance of strains 106 and 108
291 and their relatives in the *in situ* chimney habitat by using the 16S rRNA gene sequences.
292 The targeted microbial populations were the whole microbial cells (DAPI-stained cell
293 fraction and both bacterial- and archaeal-probes-binding cell fraction), the strain 106
294 cells (a specific probe-binding cell fraction) and the strain 108 cells (a specific
295 probe-binding fraction), respectively. The EUB338 (Stahl and Amann, 1991) and the
296 ARC915 (Amann *et al.*, 1990) probes were used for the detection of both bacterial and
297 archaeal populations. The ribosomal RNA-targeted oligonucleotide probes specifically
298 binding to the 16S rRNAs of strains 106 and 108, respectively, were designed using
299 ARB (Ludwig *et al.*, 2004) based on the multiply aligned sequences including those of
300 strains 106 and 108. Among several potential sites for the probes, the probes designated
301 as TF1110 (5'-CTCCATCTCTGGAGCCTTCC-3') and PB1001
302 (5'-TGGTAACTGAGGGCGTGGGT-3') were finally chosen through the sequence
303 specificity analysis using the BLAST program to all the nucleic acid sequences in the
304 DDBJ/EMBL/GenBank nucleotide sequence databases. In the *in silico* analysis, the
305 TF1110 probe was found to have at least three bases of mismatch with any other 16S
306 rRNA sequences and the PB1001 probe also had more than three bases of mismatch
307 with any other 16S rRNA sequences except for the 16S rRNAs of *Methanobrevibacter*
308 spp. (one mismatch).

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

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

325

325 **RESULTS AND DISCUSSION**

326

327 **Chimney habitats and total, viable and FISH cell counts:** At the present, it is still
328 very difficult to determine directly the physical and chemical conditions of the
329 microbial habitats in the chimney structure by using some of the *in situ* hardware such
330 as temperature-, pH- and other chemical-sensors at the deep seafloor. Thus, the potential
331 chemical conditions for a given temperature range of chimney habitat have been often
332 extrapolated by the mixing between the hot endmember hydrothermal fluid and the cold
333 ambient seawater (McCollom and Shock, 1997; Shock and Holland, 2004; Tivey,
334 2004).

335 According to the chemical composition of the endmember hydrothermal fluid in
336 the BSC of the TAG field already reported (Campbell *et al.*, 1988; Charlou *et al.*, 1996;
337 Chiba *et al.*, 2001), the potential concentration ranges of gaseous energy and carbon
338 substrates in the chimney habitats were estimated. If the microbially habitable
339 temperature range was assumed to be up to ~130 °C that could be brought by a simple
340 mixing of approx. 1:2 ratio between the hydrothermal fluid and the seawater in the case
341 of the TAG field, the available concentrations of dissolved H₂, N₂, CO₂, CH₄ and H₂S
342 are estimated to be up to 0.12, 0.3, 3.4, 0.05 and 2.3 mM, respectively, based on the
343 highest values reported by Campbell *et al.* (1988) and Charlou *et al.* (1996). The total
344 dissolved concentrations of H₂, ΣCO₂ and reduced sulfur compounds were about 10, 30
345 and 10 mM in Piezo-MMJHS medium and <1.3, 30 and 10 mM in non-piezophilic
346 MMJHS medium at 50 °C, respectively. Thus, the enrichment by using Piezo-MMJHS
347 medium might provide a larger chemical dissimilarity than that under the
348 non-piezophilic condition, particularly in dissolved H₂ concentration, between the *in*

349 *situ* habitats and the laboratory experiments. The dissolved H₂ concentration and
350 hydrostatic pressure could be significantly different under piezophilic and
351 non-piezophilic enrichment culture conditions.

352 The total cell counts in the chimney inside and surface habitats were $3.2 \times 10^5 \pm$
353 7.2×10^4 and $9.6 \times 10^6 \pm 4.0 \times 10^5$ cell/g (wet weight), respectively. These total cell
354 counts were very comparable to those in other black smoker chimneys studied so far in
355 different deep-sea hydrothermal systems (e.g., Takai *et al.*, 2008b). In addition to the
356 total cell counts in the chimney habitats, the viable counts of the representative
357 cultivated populations in the habitats were also determined under the conventional
358 cultivation conditions. From the chimney surface habitat, members of the genera
359 *Thermococcus*, *Deferribacter*, *Persephonella* and *Sulfurimonas* were cultivated as the
360 predominant populations. The viable cell counts of these members were 10^6 - 10^7 cells/g
361 for *Thermococcus*, 10^3 - 10^4 cells/g for *Deferribacter*, 10^5 - 10^6 cells/g for *Persephonella*,
362 and 10^5 - 10^6 cells/g for *Sulfurimonas*, respectively. Based on these viable cell counts by
363 the serial dilution cultivation experiments under the non-piezophilic condition, the
364 predominant H₂ and/or S-oxidizing chemolithoautotrophic members in the TAG black
365 smoker chimney surface were represented by *Persephonella* spp. within the *Aquificae*
366 and *Sulfurimonas* spp. within the *Epsilonproteobacteria*, which has been commonly
367 observed in other chimney habitats of the different hydrothermal systems (e.g., Takai *et*
368 *al.*, 2008b).

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

373 cultivation condition, but represented 0.01-0.3% (for strain 106) and 0.2-7.5% (for
374 strain 108) of the sum of viable cell counts in the chimney surface habitat. In the highest
375 dilution culture of a serial dilution experiment using the non-piezophilic MMJHS
376 medium at 50 °C, a minor but certain proportion of thin, long spiral cells were observed
377 together with abundant, short rod cells of the *Persephonella* members. This morphotype
378 of microorganism was successfully cultivated and isolated from the highest dilution of
379 culture using Piezo-MMJHS medium. The partial 16S rRNA gene sequence of the
380 isolate was 99.8% identical to that of strain 106. Thus, the viable cell count of strain 106
381 under the non-piezophilic cultivation condition was considered to be $10^5 - 10^6$ cells/g,
382 which was equivalent to that of the *Persephonella* members and 1-50% of the sum of
383 viable cell counts. These results suggested that both strains 106 and 108 were novel
384 microbial components previously uncultivated from other deep-sea hydrothermal
385 systems but potentially represented the functionally significant H₂- and/or S-oxidizing
386 chemolithoautotrophic components in the chimney structure of the TAG field.

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

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

400 **Morphological, physiological and metabolic properties of strain 106:** Cells of
401 strain 106 were motile (not all the cells but some), long, thin spiral and approximately
402 6–20 μm long and 0.4–0.6 μm wide, with a polar flagellum under the piezophilic
403 cultivation condition (Fig. 1a). Under the conventional gas pressure condition (0.2 MPa),
404 however, most of the cells became shorter (up to 4 μm long) with motility (Fig. 1b). As
405 compared to other deep-sea chemolithoautotrophs having similar growth temperature
406 ranges with the *Aquificae* and *Epsilonproteobacteria*, strain 106 was morphologically
407 novel.

408 Strain 106 autotrophically grew at a temperature range between 30 and 55 $^{\circ}\text{C}$
409 (optimally 50 $^{\circ}\text{C}$) (Fig. 2a), at a pH range between 5.8 and 7.6 (optimally pH 7.0) (Fig.
410 2b) and at a NaCl range from 1.2 to 4.2 (%; w/v) (optimally 3%) (Fig. 2c) under the
411 piezophilic cultivation condition. In addition, the growth of strain 106 was observed at a
412 hydrostatic pressure range between 0.2 and 36 MPa, and the highest growth rate was
413 identified at 16 MPa (Fig. 3). Thus, strain 106 was piezophilic but not a strict
414 piezophile.

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

421 addition, strain 106 was able to utilize O₂ as the electron acceptor but its O₂ utilization
422 represented the microaerophilic mode (up to 0.4 mM of dissolved O₂ concentration or
423 5% of O₂ partial pressure) (Table 2). Strain 106 utilized nitrate, ammonium and yeast
424 extract as a nitrogen source. Molecular nitrogen (N₂) could also support the growth as a
425 sole nitrogen source, suggesting the possible N₂ fixation, although the existence of
426 genetic components of N₂ fixation enzymes (*nif* genes) and the ¹⁵N₂ incorporation into
427 the cellular nitrogens were not experimentally examined. Selenium, tungsten and
428 vitamins were not required for the growth.

429 A time course of oxidation of thiosulfate by nitrate-reduction and concomitant
430 growth under the piezophilic condition were characterized (Fig. 4a). During the growth,
431 thiosulfate and nitrate were consumed, and sulfate, N₂ and N₂O were accumulated (Fig.
432 4a). None of the other potentially intermediate products such as sulfite and nitrite from
433 the thiosulfate-oxidation and the nitrate-reduction was detected (Fig. 4a). The
434 consumption of thiosulfate and nitrate was stoichiometrically equivalent to the
435 accumulation of sulfate and gaseous nitrogen compounds (Fig. 4a). These results clearly
436 demonstrated that strain 106 was a strictly chemolithoautotrophic, facultatively
437 anaerobic piezophile oxidizing the reduced sulfur compounds to sulfate by O₂- or
438 nitrate-reduction.

439 Strain 106 was found to be sensitive to a variety of antibiotics, including
440 chloramphenicol (50 µg ml⁻¹), streptomycin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹),
441 ampicillin (50 µg ml⁻¹) and vancomycin (50 µg ml⁻¹).

442 **Morphological, physiological and metabolic properties of strain 108:** Cells of
443 strain 108 were non-motile, short, oval and approximately 1–1.5 µm long and 0.6–0.7
444 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 *Thermococales* 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

469 of strain 106 (Table 2). It was capable of both chemolithoautotrophic and organotrophic
470 growth (Table 2). The possible inorganic electron donor to support the growth was H₂,
471 elemental sulfur, thiosulfate, cystein-HCl, tetrathionate or sulfite, and various organic
472 compounds such as yeast extract, tryptone, acetate and pyruvate also served as the
473 energy sources (Table 2). All these organic compounds and Casamino acid were utilized
474 as the carbon source instead of the inorganic carbons. Both the chemolithoautotrophic
475 and organotrophic growth were based on the electron acceptors of nitrate and O₂ (Table
476 2), and the O₂-dependent growth was fully aerobic (Table 2). These results demonstrate
477 that strain 108 is a piezophilic, facultatively anaerobic and chemolithoautotrophic,
478 thermophile. Strain 108 utilized nitrate and ammonium as a nitrogen source. Similarly
479 as strain 106, molecular nitrogen (N₂) could be utilized as a sole nitrogen source
480 although the existence of genetic components of N₂ fixation enzymes (*nif* genes) and the
481 ¹⁵N₂ incorporation into the cellular nitrogens were not experimentally checked.
482 Selenium, tungsten and vitamins were not required for the growth.

483 A time course of oxidation of either H₂ or thiosulfate by nitrate-reduction and
484 concomitant growth under the piezophilic condition were characterized (Figs. 4b and
485 4c). During the growth, either H₂ or thiosulfate and nitrate were consumed, and either
486 H₂O (not identified) or sulfate and N₂O were accumulated (Figs. 4b and 4c). The
487 consumptions of thiosulfate and nitrate were stoichiometrically equivalent to the
488 accumulation of sulfate and N₂O during the thiosulfate-dependent growth (Fig. 4c),
489 while H₂ consumption was too much as expected by the extent of nitrate-reduction (Fig.
490 4b). As mentioned in the previous study for development of this piezophilic cultivation
491 technique (Takai *et al.*, 2008a), it is inevitable that a certain proportion of H₂ gas leaks
492 from the piezophilic cultivation syringe during the experiment. Probably, the excess

493 amount of H₂ consumption observed was due to the excess amount of H₂ leak as
494 compared to the control experiment without the microbial inoculum. These results
495 demonstrated that strain 108 was a piezophilic, N₂O-producing H₂- and S-oxidizer
496 under the chemolithoautotrophic growth condition.

497 Strain 108 was also sensitive to a variety of antibiotics, including chloramphenicol
498 (50 µg ml⁻¹), streptomycin (50 µg ml⁻¹), kanamycin (50 µg ml⁻¹), ampicillin (50 µg ml⁻¹)
499 and vancomycin (50 µg ml⁻¹).

500 **Cellular fatty acid composition:** Cellular fatty acid compositions of strains 106
501 and 108 grown at the optimal pressures and at the conventional pressures are shown in
502 Table 3. Each of the fatty acid components was typical as the components observed in
503 the *Proteobacteria* except for small amounts of odds number of carbon chains (C₁₅ and
504 C₁₇) found in strain 106 (Table 3). The different patterns in the compositions of both
505 strains were notable between the cells grown under the piezophilic and non-piezophilic
506 conditions (Table 3). Since the first discovery (DeLong and Yayanos, 1985; 1986), it
507 has been well known that the fatty acid compositions of many deep-sea piezophilic
508 bacteria vary as a function of pressure. At the present, it has become evident that the
509 pressure-controlled variability in the fatty acid compositions of the deep-sea piezophilic
510 bacteria is not simply generalized (e.g., Mangelsdorf *et al.*, 2005). Nevertheless, an
511 increasing ratio of total unsaturated fatty acids to saturated fatty acids with an increasing
512 hydrostatic pressure initially suggested by DeLong and Yayanos (1985; 1986) still
513 works as a very practical principle in most cases. Indeed, the pressure-induced
514 enrichment of the unsaturated fatty acids was identified in our new piezophilic,
515 chemolithoautotrophic bacteria (Table 3). This is the first evidence showing that the
516 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 H₂-
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

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

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

587 **Description of *Thiopfundum* gen. nov.:** *Thiopfundum* (Thi.o.pro’fu.n.dum.
588 Gr. n. *thios* sulfur; L. n. *profundus* deep; N.L. neut. n. *Thiopfundum* 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 C₁₆, anteiso-C₁₇, C₁₈ and C_{18:1}. Based on 16S rRNA gene
594 analysis, the genus *Thiopfundum* is related to the endosymbionts of the deep-sea
595 animals within the *Gammaproteobacteria*. The type species is *Thiopfundum*
596 *lithotrophica*.

597 **Description of *Thiopfundum lithotrophica* sp. nov.:** *Thiopfundum*
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 N₂ and N₂O 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 C₁₂ (1.2%), C₁₄ (7.2%),
610 C₁₅ (2.1%), C₁₆ (41.3%), C_{16:1} (14.3%), anteiso-C₁₇ (14.3%), C₁₇ (1.2%), C₁₈ (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.

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

617 **Description of *Piezobacter* gen. nov.:** *Piezobacter* (Pie.zo.bac.ter. Gr. v. *piezo* to
618 press; N.L. masc. n. *bacter* rod; N.L. masc. n. *Piezobacter* piezophilic rod). Oval rod.
619 Facultatively anaerobic. Piezophilic. Thermophilic and neutrophilic. Facultatively
620 chemolithoautotrophic. Able to utilize H₂ or reduced sulfur compounds as an electron
621 donor and nitrate or molecular oxygen as an electron acceptor. NaCl absolutely required
622 for growth. G + C content of genomic DNA is about 66%. Major cellular fatty acids are
623 C₁₆, C₁₈ and C_{18:1}. Based on 16S rRNA gene analysis, the genus *Piezobacter* is related
624 to members of the family *Rhodobacteraceae* within the *Alphaproteobacteria*. The type
625 species is *Piezobacter thermophila*.

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

637 strictly piezophilic. Thiosulfate is oxidized to sulfate and nitrate is reduced to N₂O
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 C₁₄
640 (3.4%), C₁₆ (21.4%), C₁₈ (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 <i>et al.</i> , 2000
MMJHS	80% H ₂ + 20% CO ₂ (0.2 MPa)	30, 50, 55, 70, 85, 95	6.5	H ₂ , S ⁰ , S ₂ O ₃	S ⁰ , S ₂ O ₃ , NO ₃ , CO ₂	Without sodium sulfide	Takai <i>et al.</i> , 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 ₂ O ₃	O ₂ , S ⁰ , S ₂ O ₃ , NO ₃ , CO ₂	Without sodium sulfide	Takai <i>et al.</i> , 2003
Piezo-MMJHS	10 mM H ₂ , 30 mM ΣCO ₂ (36 MPa)	50	6.5	H ₂ , S ⁰ , S ₂ O ₃	S ⁰ , S ₂ O 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 <i>et al.</i> , 2008b

799 *YE means yeast extract.

800 †TT means tryptone.

801

802

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 ₂ O ₃ (10 mM)	N.G.	N.A.	N.G.	N.A.
S ⁰ (3%; w/v)/NO ₃ (20 mM)	++	++	++	N.G.
S ₂ O ₃ (10 mM)/NO ₃ (20 mM)	++	++	++	N.G.
S ₂ O ₃ (10 mM)/NO ₂ (2.5 mM)	N.G.	N.G.	N.G.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (0.04 mM)	++	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (0.12 mM)	+	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (0.4 mM)	+	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (1.2 mM)	N.G.	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (2 mM)	N.G.	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (4 mM)	N.G.	N.A.	++	N.A.
S ₂ O ₃ (10 mM)/O ₂ (0.1%; v/v)	N.A.	++	N.A.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (0.3%; v/v)	N.A.	++	N.A.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (1%; v/v)	N.A.	+++	N.A.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (3%; v/v)	N.A.	+++	N.A.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (5 %; v/v)	N.A.	+++	N.A.	N.G.
S ₂ O ₃ (10 mM)/O ₂ (10 %; v/v)	N.A.	N.G.	N.A.	N.G.
H ₂ S+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 ₄ O 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 10⁸ cells/ml, ++; between 1.0 x 10⁸ and 5.0 x 10⁸, +++; >1.0 x 10⁹.

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811

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 ω7}	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 ω2}	n.d.	n.d.	16.5%	12.2%
C _{18:1 ω7}	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

816 FIGURE LEGENDS

817

818 **Figure 1.** Electron micrographs of negatively stained cell of strain 106 grown under the
819 piezophilic cultivation condition at 50 °C and 16 MPa (a) and under the non-piezophilic
820 cultivation condition at 50 °C and 0.2 MPa of gas pressure (80% H₂ + 20% CO₂) (b),
821 and of strain 108 grown under the piezophilic cultivation condition at 50 °C and 16 MPa
822 (c). Bar indicates 2.0 μm (a) and 1.0 μm (b and c), respectively.

823

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

832

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

835

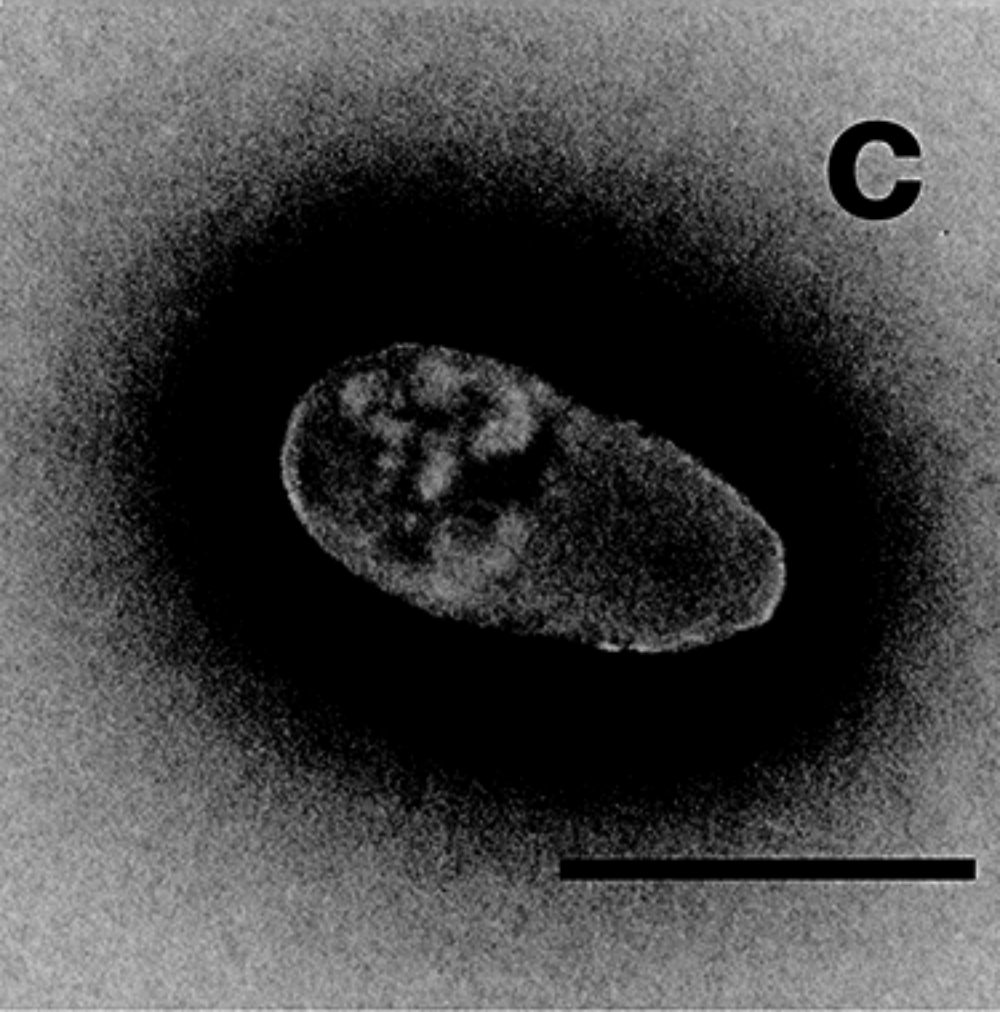
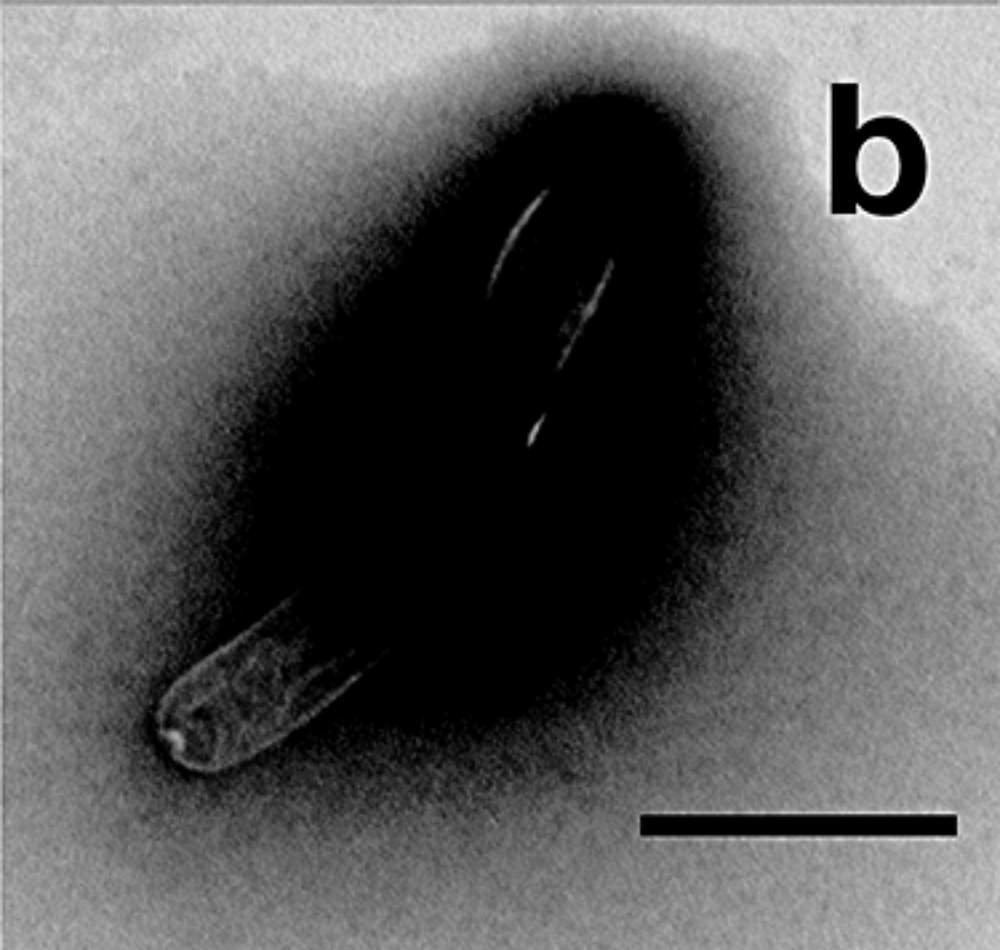
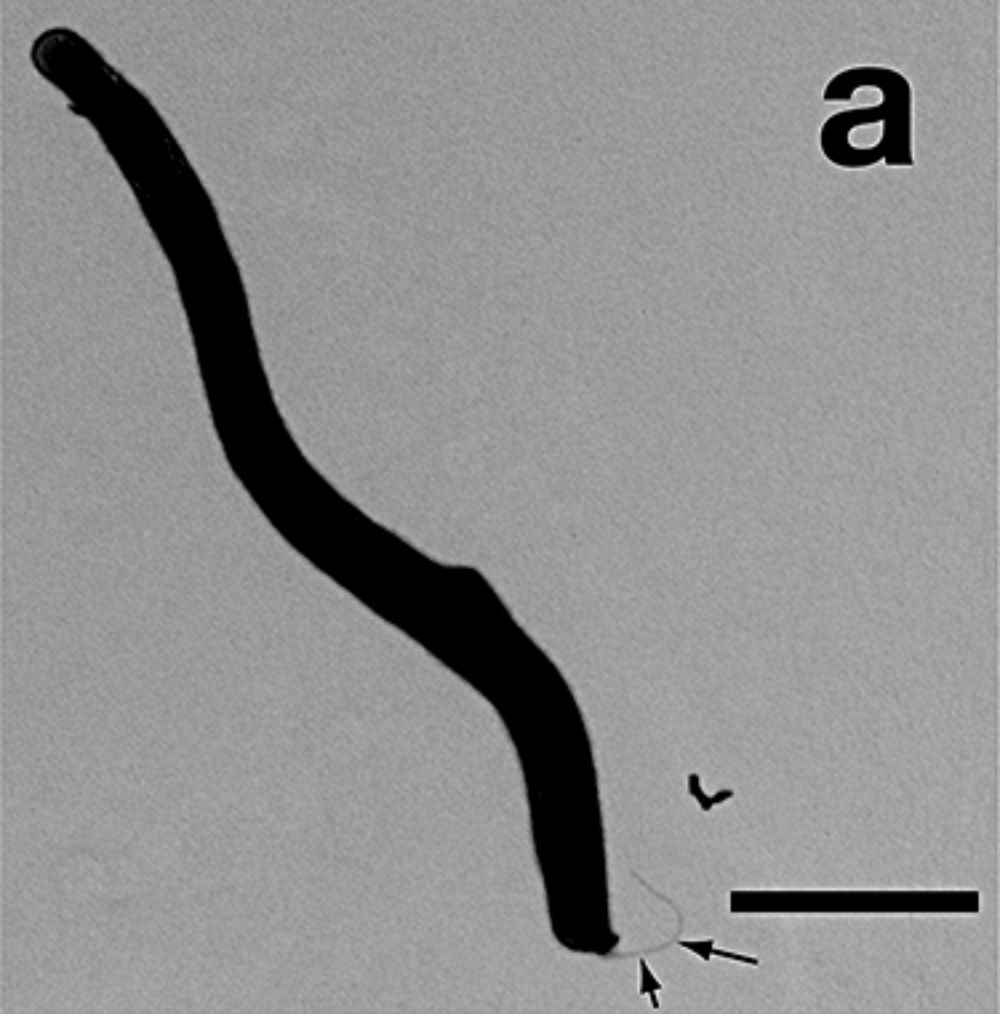
836 **Figure 4.** Time course of oxidation of electron donor, reduction of electron acceptor
837 and concomitant growth of strain 106 grown with thiosulfate and nitrate (a), strain 108
838 grown with H₂ and nitrate (b) or strain 108 grown with thiosulfate and nitrate (c). (a)
839 Consumption of thiosulfate and nitrate, production of sulfate and nitrogenous gases and
840 cellular proliferation of strain 106 were determined at 50 °C, pH 6.5 and 16 MPa. (b)
841 Consumption of H₂ and nitrate, production of nitrogenous gases and cellular
842 proliferation of strain 108 were determined at 50 °C, pH 6.5 and 36 MPa. The
843 concentration N₂O is indicated as 5 times higher value for easy perception. (c)
844 Consumption of thiosulfate and nitrate, production of nitrogenous gases and cellular
845 proliferation of strain 108 were determined at 50 °C, pH 6.5 and 36 MPa.

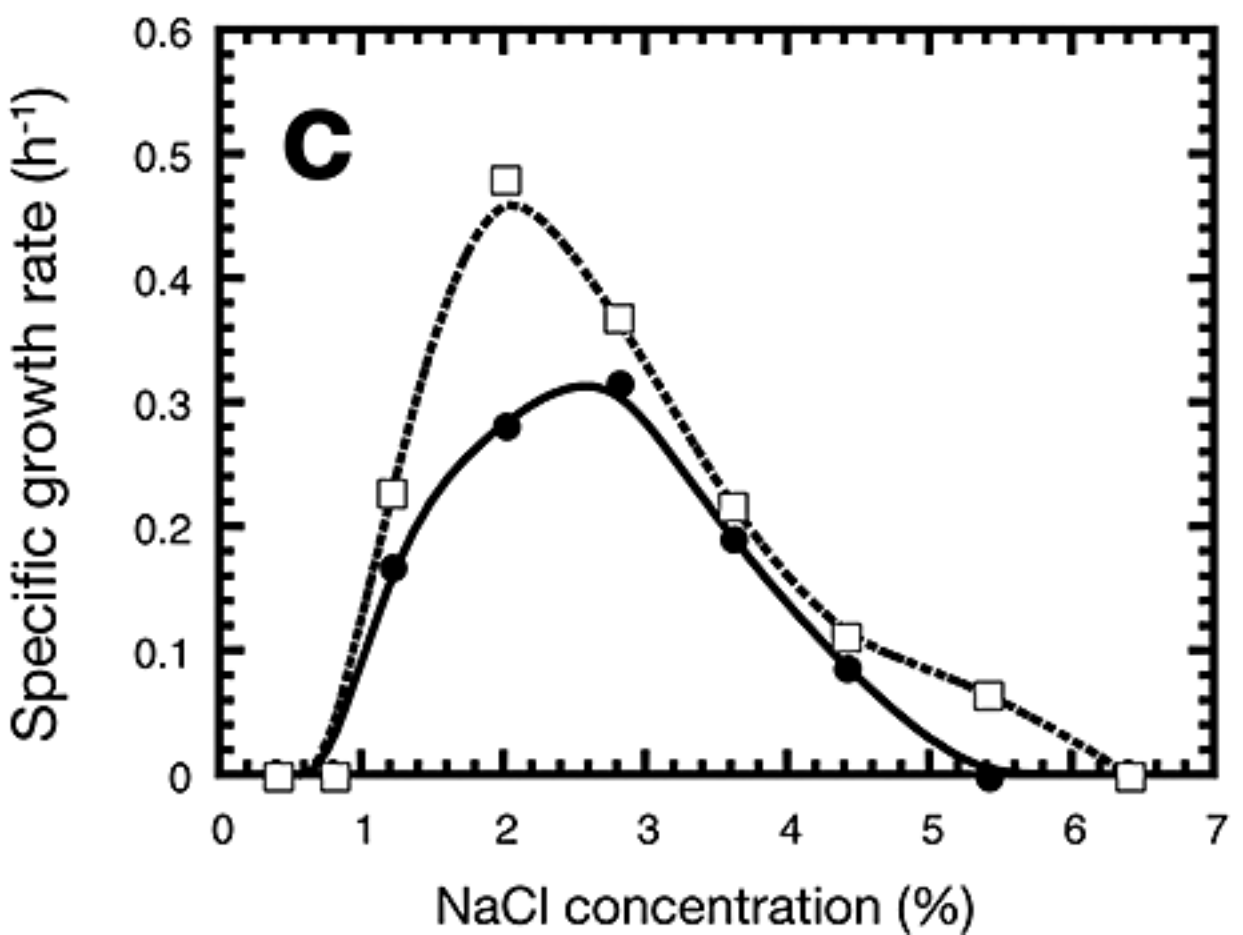
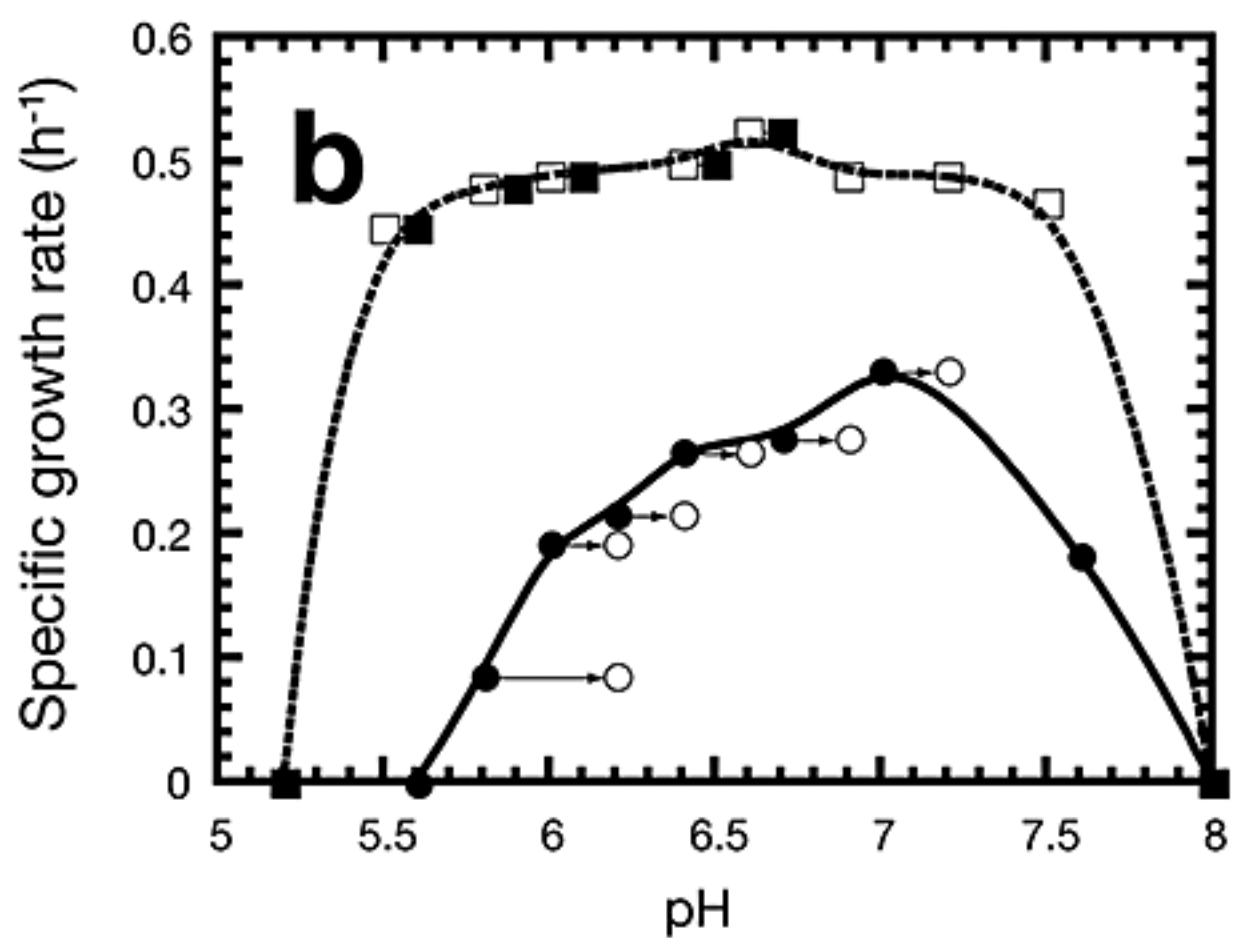
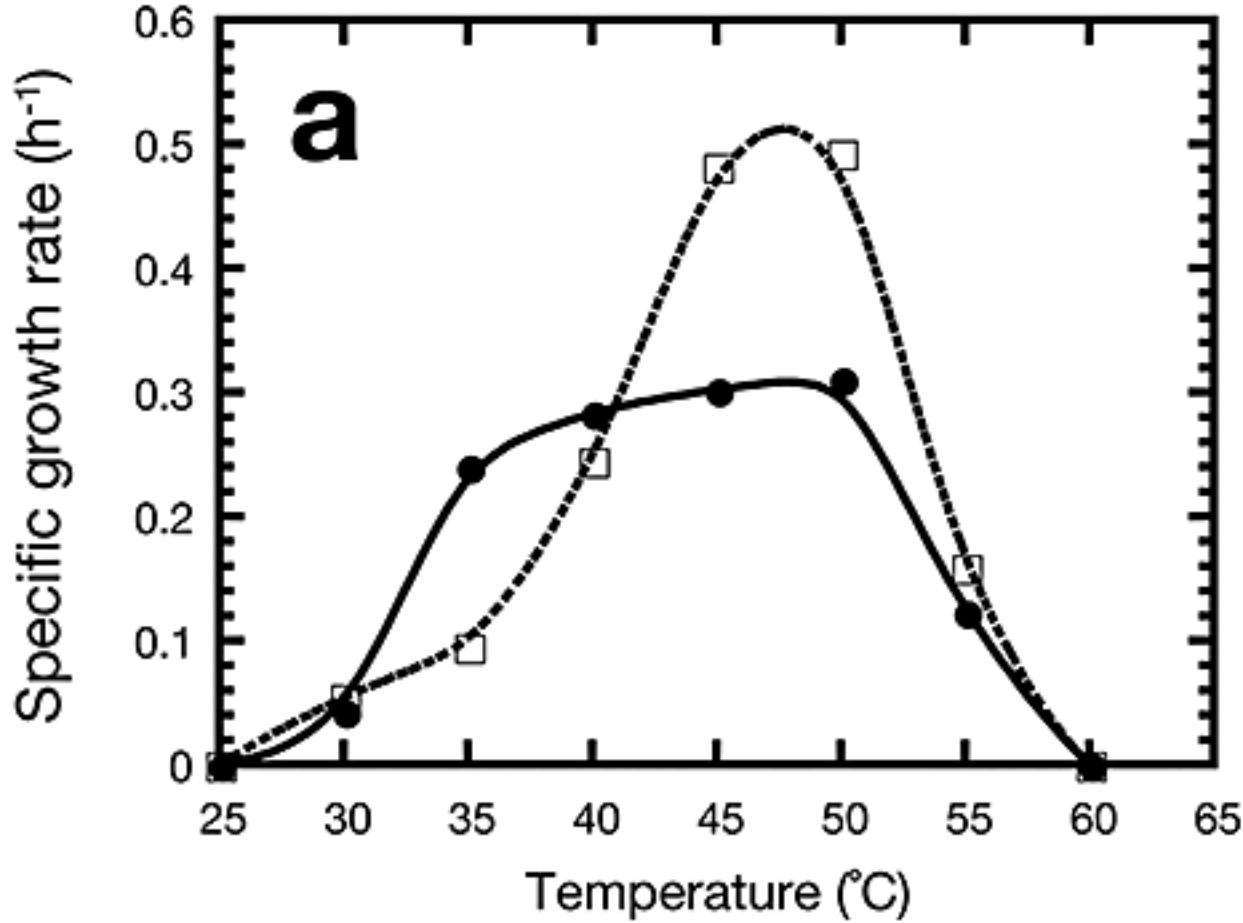
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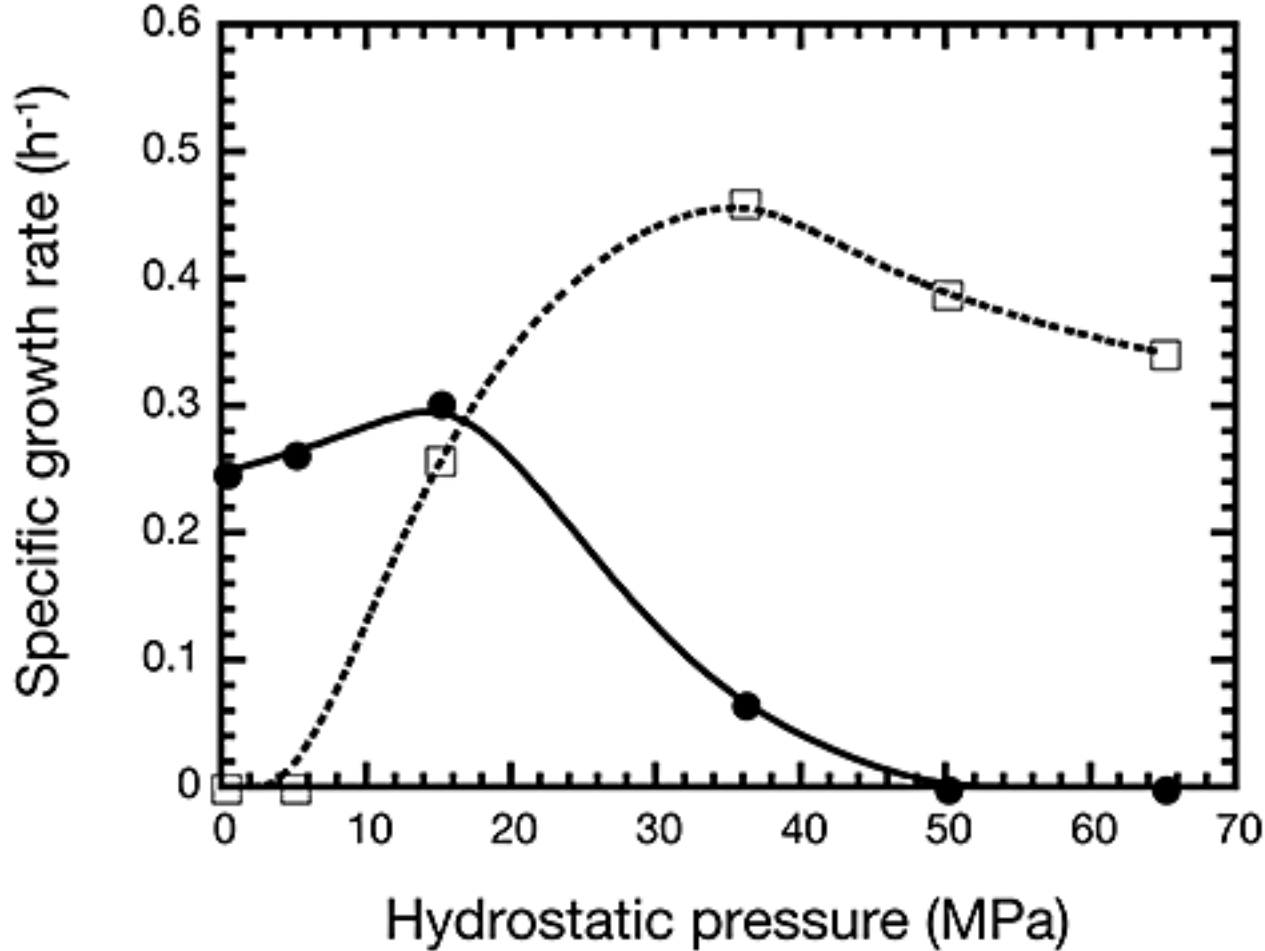
847 **Figure 5.** Phylogenetic tree of representative species and endosymbionts within the
848 *Alphaproteobacteria* and *Gammaproteobacteria* including strain 106 and 108. The tree
849 was inferred from 16S rRNA gene sequences using the neighbor-joining method on the
850 1022 homologous sequence positions for each sequence. The number at each node
851 represents the bootstrap value (in percent) determined by 1000 replicates. The scale bar

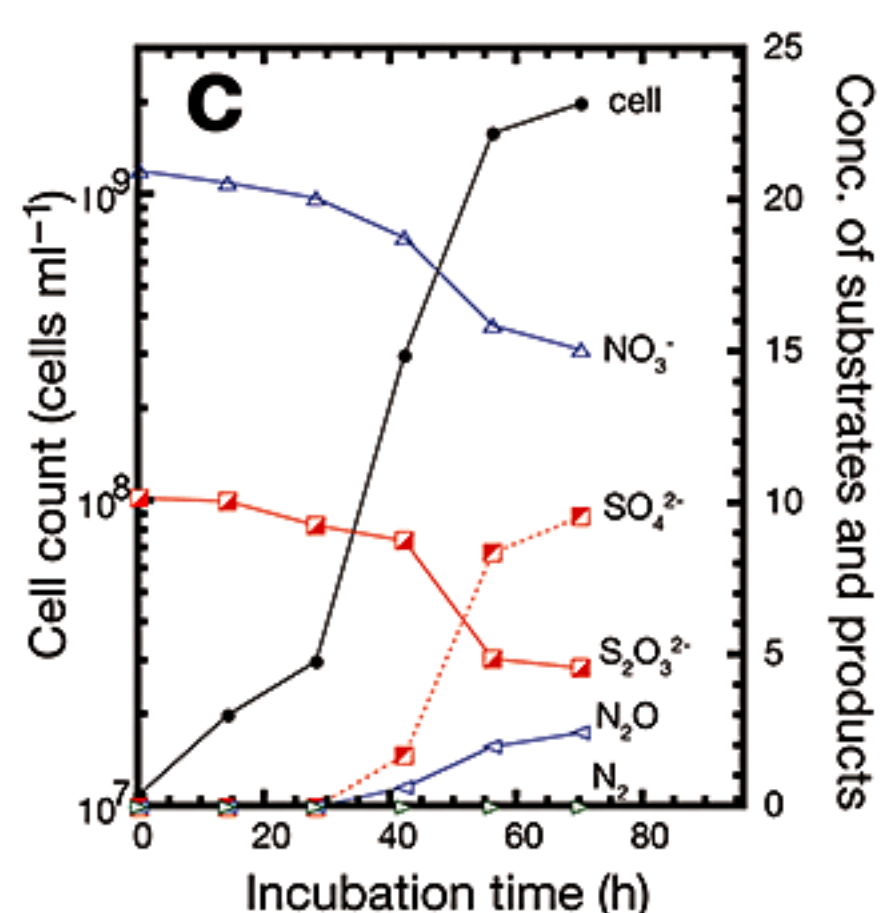
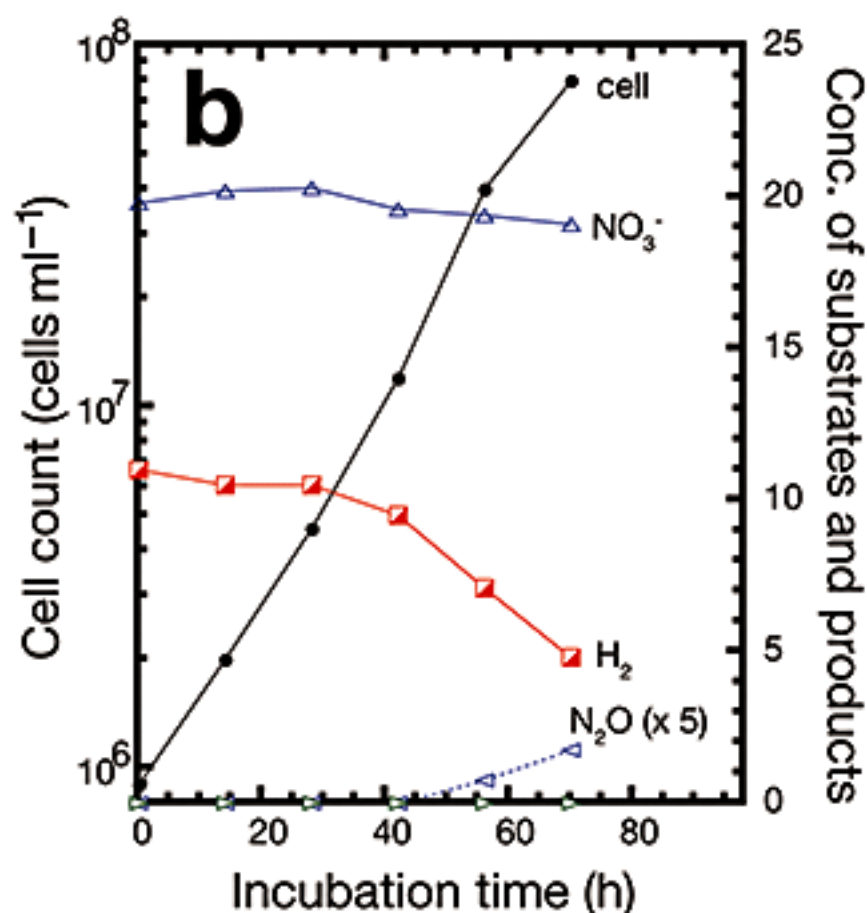
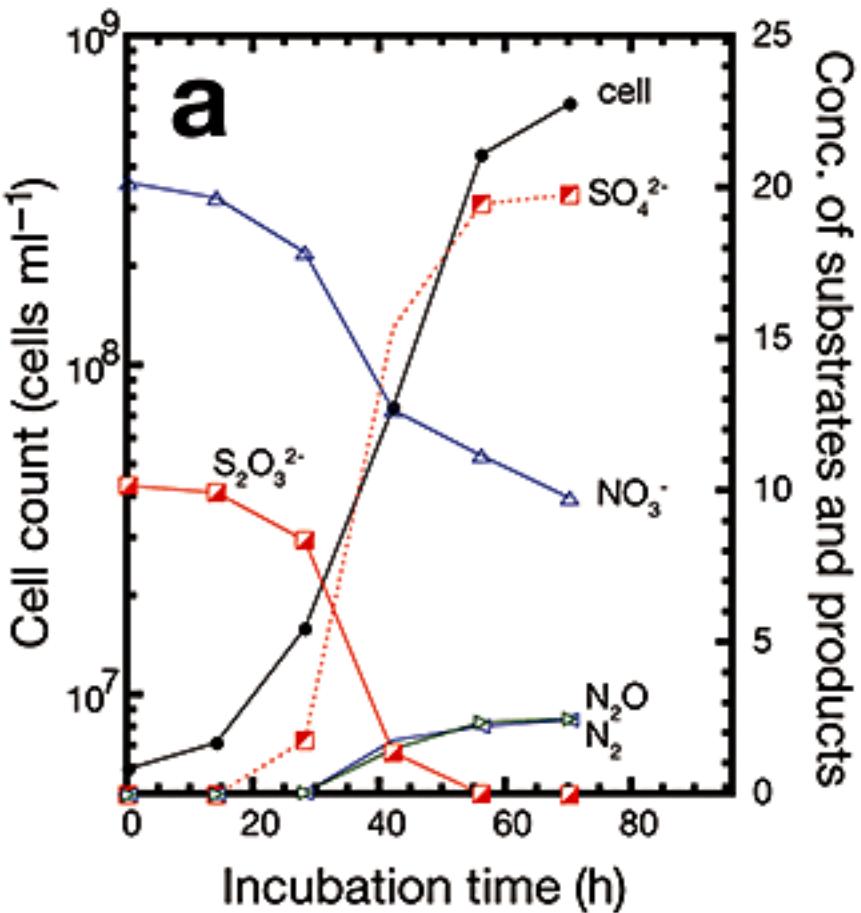
852 indicates 5 substitutions per 100 nucleotides.

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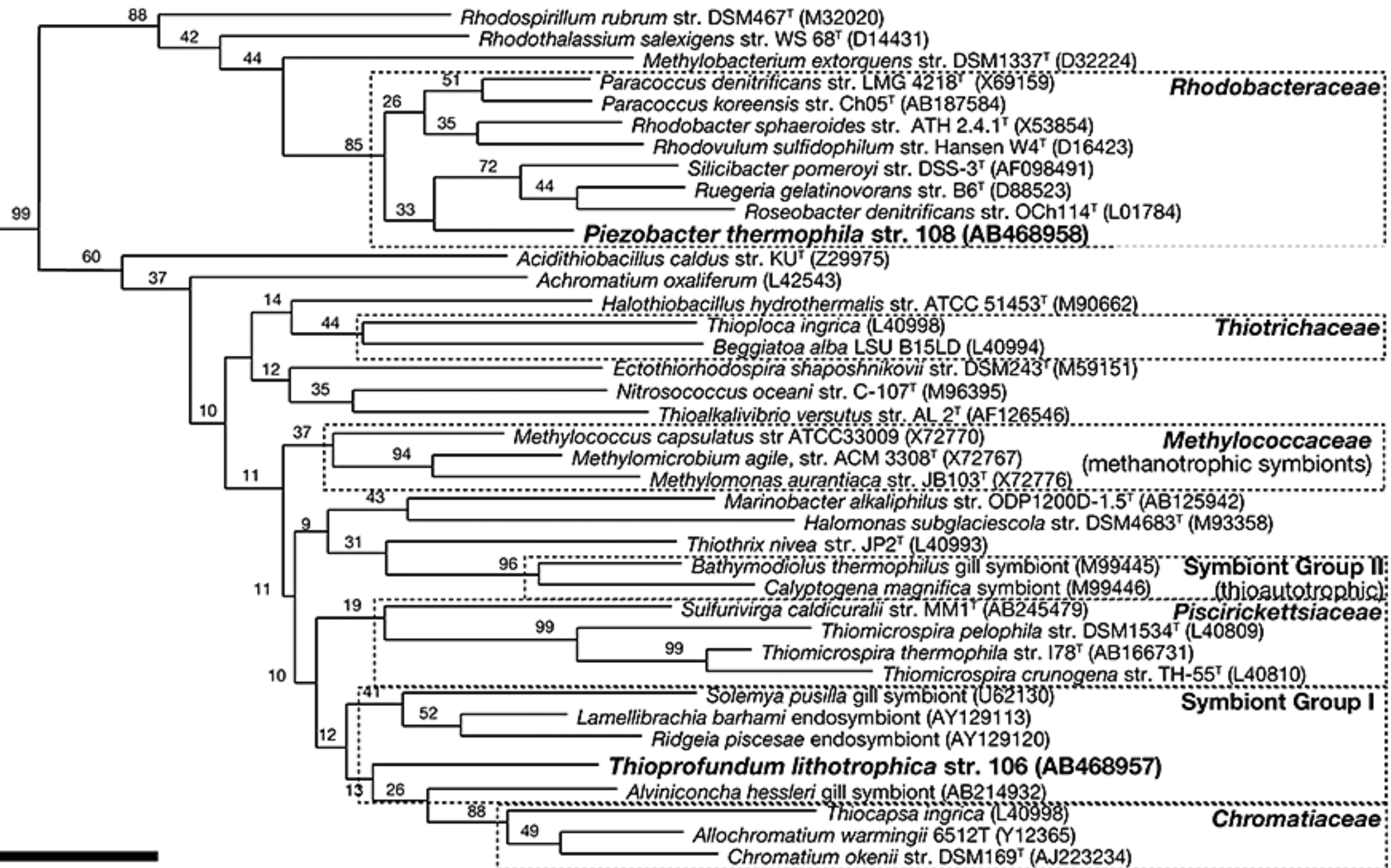








Epsilon-proteobacteria



Alpha-proteobacteria
Gamma-proteobacteria