Thiofaba tepidiphila gen. nov., sp. nov., a novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium of the *Gammaproteobacteria* isolated from a hot spring

Koji Mori and Ken-ichiro Suzuki

Correspondence

Koji Mori

mori@nbcrc.nite.go.jp

NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation (NITE), 2-5-8 Kazusakamatari, Kisarazu, Chiba 292-0818, Japan

A novel obligately chemolithoautotrophic, sulfur-oxidizing bacterium designated strain BDA453^T was isolated from a hot spring in Fukushima prefecture, Japan. The cells were short-rod-shaped and possessed an inclusion, a Gram-negative type cell wall and a single polar flagellum. Strain BDA453^T grew by sulfur-oxidizing respiration with thiosulfate, elemental sulfur, sulfide and tetrathionate as electron donors and used only carbon dioxide as a carbon source. The optimum growth conditions were 45 °C, pH 6.5 and the absence of NaCl. Analysis of the 16S rRNA gene revealed that the isolate was a member of the *Gammaproteobacteria* and related to the genera *Halothiobacillus* and *Thiovirga* in the family *Halothiobacillaceae*. However, the phylogenetic tree constructed using 16S rRNA gene sequences showed that strain BDA453^T was distant from any other known bacteria with sequence similarities of less than 90 %. On the basis of phenotypic features and phylogenetic analysis, strain BDA453^T is considered to represent a novel species of a new genus within the family *Halothiobacillaceae*, for which the name *Thiofaba tepidiphila* gen. nov., sp. nov. is proposed. The type strain of *Thiofaba tepidiphila* is BDA453^T (=NBRC 103218^T=DSM 19618^T).

In natural and artificial environments, many sulfide/oxygen interface layers exist (e.g. hot springs, hydrothermal vents, cold seep sediments, wastewater biofilms and anaerobic zones of lakes), which are inhabited by various chemolithoautotrophic, sulfur-oxidizing micro-organisms. These chemolithoautotrophic, sulfur-oxidizing micro-organisms play important roles in the sulfur cycle, as well as in the production of organic materials as primary producers. Various kinds of chemolithoautotrophic, sulfur-oxidizing micro-organisms have been detected at different sulfide concentrations, pH values, temperatures and under other chemical and physical conditions and they are phylogenetically widespread among the prokaryotes. Most of those identified to date belong to the phylum *Proteobacteria*, with other species belonging to a deep lineage in the *Bacteria* and the order *Sulfolobales* in the *Archaea* (Huber & Prangishvili, 2006; La Rivière & Schmidt, 2006; Robertson & Kuenen, 2006; Sorokin *et al.*, 2006; Takai *et al.*, 2003a).

Hot springs and hydrothermal vents are major sites with a constant supply of sulfide, where large biomasses of sulfur-oxidizing bacteria, called microbial mats, often develop (Elshahed *et al.*, 2003; Reysenbach *et al.*, 1994; Schulz *et al.*,

1999; Skirnisdottir *et al.*, 2000; Yamamoto *et al.*, 1998). The temperature of hot springs is very important for the community structures of microbial mats (Hiraishi *et al.*, 1999; Skirnisdottir *et al.*, 2000) and various thermophilic, chemolithoautotrophic, sulfur-oxidizing bacteria have been isolated from them depending on the *in situ* temperature, e.g. *Sulfurihydrogenibium subterraneum* (Takai *et al.*, 2003b), *Thermocrinis ruber* (Huber *et al.*, 1998), *Thiobacter subterraneus* (Hirayama *et al.*, 2005) and *Thiobacillus aquaesulis* (Wood & Kelly, 1988). Most thermophilic, chemolithoautotrophic, sulfur-oxidizing isolates belong to the orders *Aquificales* (Götz *et al.*, 2002; Huber *et al.*, 1992, 1998; Nakagawa *et al.*, 2004; Takai *et al.*, 2003b) and *Sulfolobales* (Huber & Stetter, 1991; Segerer *et al.*, 1986, 1991), although some strains have been reported to belong to the class *Gammaproteobacteria*. The only species of thermophilic (or thermotolerant), chemolithoautotrophic, sulfur-oxidizing bacteria that are known to belong to the *Gammaproteobacteria* are *Acidithiobacillus caldus* (Hallberg & Lindström, 1994; Kelly & Wood, 2000), *Thermithiobacillus tepidarius* (Kelly & Wood, 2000; Wood & Kelly, 1985), *Thiomicrospira thermophila* (Takai *et al.*, 2004) and *Sulfurivirga caldicurii* (Takai *et al.*, 2006).

Recently, a novel moderately thermophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium, designated

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BDA453^T is AB304258.

strain BDA453^T, was isolated from a hot spring in Fukushima prefecture, Japan. Based on phylogenetic analysis, this isolate has been classified in a new genus in the class *Gammaproteobacteria*. In this paper, a novel taxon is proposed for strain BDA453^T: *Thiofaba tepidiphila* gen. nov., sp. nov.

Akayu hot spring is a sulfur spring located in Fukushima prefecture, Japan. The temperature and pH of the water in this hot spring are 45 °C and 7.0, respectively. In order to enrich and isolate sulfur-oxidizing bacteria, AP96SO1 medium, i.e. basal medium supplemented with 5 mM Na₂S₂O₃ under an N₂/CO₂/O₂ [60:20:20 (v/v); 150 kPa] atmosphere, was used. The basal medium was composed of the following salts and solutions (l⁻¹): 1.2 g KH₂PO₄, 0.2 g K₂HPO₄, 0.75 g MgCl₂·6H₂O, 0.15 g CaCl₂·2H₂O, 0.5 g NH₄Cl, 0.5 g Na₂CO₃, 2 ml trace element solution of NBRC medium 377 (NBRC, 2005) and 2 ml vitamin solution of NBRC medium 377 (NBRC, 2005). The medium was prepared in vials and bottles sealed with butyl-rubber stoppers and aluminium caps under N₂/CO₂/O₂. For primary enrichment of sulfur-oxidizing bacteria, 2 ml hot spring water was inoculated into 20 ml AP96SO1 medium and cultivated at 45 °C. After incubation for 1 week, bacterial growth was observed in the culture microscopically and then the enrichment culture was transferred to fresh AP96SO1 medium several times. Because growth of the enriched bacteria could not be achieved successfully on solid AP96SO1 medium, an attempt was made to isolate bacteria by serial dilution using AP96SO1 medium. After repeating the maximum dilution several times, a sulfur-oxidizing bacterium, designated strain BDA453^T, was obtained. The purity of

the isolate was verified by microscopic observation, inoculation into the basal medium containing various heterotrophic substrates and determination of the 16S rRNA gene sequence amplified using the following primer sets: universal primer set 530f and 1392r, bacterial primer set 27f and 1492r (Lane, 1991) and archaeal primer set A109f (Grosskopf *et al.*, 1998) and Arch915 (Stahl & Amann, 1991).

Cells of strain BDA453^T were short rods (0.5–0.9 µm in length and 0.4 µm in width) with a single polar flagellum (Fig. 1a and b). Under the microscope, they appeared as single cells, in pairs or sometimes as aggregates. Cell motility was observed, but spore formation was not. For the observation of ultrathin sections, cells were fixed with 0.1 % (v/v) glutaraldehyde and 4 % (v/v) osmium tetroxide, stained with platinum blue (Inaga *et al.*, 2007) and lead citrate, and observed using an electron microscope (model H-7600; Hitachi) operating at 100 kV. Cells of strain BDA453^T possessed one or two inclusions like a carboxysome in the cell and a Gram-negative type of cell wall with an outer membrane (Fig. 1c). Cells were Gram-negative, catalase-negative and weakly oxidase-positive.

Cellular fatty acids were methylated by a 5 % HCl/methanol solution (Sasser, 1990) and analysed with the MIDI microbial identification system (Microbial ID; Agilent Technologies) and GC-MS (GC system model 6890 and MSD model 5973; Agilent Technologies). Strain BDA453^T possessed C_{18:1ω9c} (38 % of the total fatty acids) and C_{16:0} (34 %) as major fatty acids and C_{16:1ω7c} (18 %), C_{12:0} (7 %), C_{12:0} 3-OH (2 %) and C_{18:0} (2 %) as minor fatty acids. An isoprenoid quinone was extracted from the cells according to the protocol of Nakagawa & Yamasato

Fig. 1. Phase-contrast (a; bar, 3 µm) and transmission electron (b and c; bars, 0.5 µm) micrographs of strain BDA453^T. Negatively stained cells and ultrathin cell sections are shown in (b) and (c), respectively.

(1993) and analysed with an LCMS-QP 8000 α spectrometer (Shimadzu). Strain BDA453^T contained ubiquinone-8. The genomic DNA G+C content was determined by HPLC using a Shodex ODS pack F-411 (Showa Denko K.K.) after nuclease P1 treatment using a DNA-GC kit (Yamasa Shoyu) followed by alkaline phosphatase treatment (Kamagata & Mikami, 1991). The DNA G+C content of strain BDA453^T was 66.0 mol%.

In the basal medium containing sulfur compounds under an N₂/CO₂/O₂ atmosphere, strain BDA453^T oxidized thiosulfate (5 mM), elemental sulfur (5%), sulfide (2 and 5 mM) and tetrathionate (5 and 10 mM) as electron donors. The following substrates could not support growth of the strain: sulfite (2 and 5 mM), CH₄ [N₂/CO₂/O₂/CH₄, 50:20:20:10 (v/v); 150 kPa], H₂ [H₂/CO₂/O₂, 60:20:20 (v/v); 150 kPa], H₂+acetate (10 mM), methanol (2 and 5 mM), formate (10 and 30 mM), acetate (10 and 30 mM), butyrate (10 mM), citrate (10 mM), fumarate (10 mM), glutamate (10 mM), lactate (10 mM), pyruvate (10 mM), malate (10 mM), succinate (10 mM), L-arginine (10 mM), L-asparagine (10 mM), L-cysteine (10 mM), L-histidine (10 mM), L-leucine (10 mM), L-methionine (10 mM), arabinose (5 mM), fructose (5 mM), galactose (5 mM), glucose (5 mM), inositol (5 mM), mannose (5 mM), raffinose (5 mM), sucrose (5 mM), xylose (10 mM), Bacto yeast extract (2 g l⁻¹; Difco), polypeptone (2 g l⁻¹; Nihon Seiyaku) and Bacto Casamino acids (2 g l⁻¹; Difco). In the presence of thiosulfate, elemental sulfur or sulfide as an electron donor, strain BDA453^T used only oxygen as an electron acceptor. Strain BDA453^T could not grow by fermentation. Anaerobic growth with nitrate (10 mM) and fumarate (10 mM) was not observed. These results indicate that strain BDA453^T is an obligate chemolithoautotroph that obtains energy for growth by the oxidation of sulfur and carbon dioxide fixation.

The optimum temperature, pH and NaCl concentration ranges for growth with thiosulfate (5 mM) and oxygen as electron donor and acceptor, respectively, were determined by examining the time-course of optical density (temperature gradient incubator with a bio-photorecorder, model TN-2612; ADVANTEC), and the decrease in the level of thiosulfate and increase in that of sulfate. The concentration of thiosulfate and sulfate were measured by HPLC (model 2695 with conductivity detector model 432 and IC-Pak Anion column; Waters). Strain BDA453^T was able to grow at 20–51 °C, the optimum temperature being 45 °C. The initial pH range for growth was 6.0–9.0 and the optimum pH was 6.5. The pH in the medium decreased during the cultivation. The isolate grew optimally in the absence of NaCl and growth did not occur above 2% (w/v) NaCl. The growth curve of strain BDA453^T under optimum growth conditions (45 °C, pH 6.5 and 0% NaCl concentration) is shown in Fig. 2. Strain BDA453^T grew with a doubling time of 1.64 h and the final concentration of the cells was approximately 1 × 10⁶ cells ml⁻¹.

Fig. 2. Time-course of the growth of strain BDA453^T and concentrations of thiosulfate and sulfate under optimum growth conditions (45 °C, pH 6.5 and no NaCl). Cell density was measured by direct counting of cells stained with 4',6-diamidino-2-phenylindole on membrane filters (0.2 µm pore size polycarbonate membrane filter; ADVANTEC). ●, Cell density; □, thiosulfate concentration; △, sulfate concentration.

An almost-complete 16S rRNA gene sequence of strain BDA453^T was determined according to the procedure reported previously by Hattori *et al.* (2000). After alignment with the ARB program (Ludwig *et al.*, 2004), phylogenetic trees were constructed by the neighbour-joining method using the CLUSTAL_X program (Saitou & Nei, 1987; Thompson *et al.*, 1997) and the maximum-likelihood method using the NUCML program in MOLPHY (Adachi & Hasegawa, 1995; Hasegawa *et al.*, 1985; Mori *et al.*, 2003). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain BDA453^T belonged to the class *Gammaproteobacteria*. However, the sequence of strain BDA453^T was distant from those of all known genera in the *Gammaproteobacteria* and the similarities between strain BDA453^T and species with validly published names were less than 90%. Furthermore, the sequence of strain BDA453^T resembled those of '*Thiobacillus barengensis*' (sequence similarity 92.6%; GenBank accession number Y09280) and the following environmental clones: clone MO31 from a hot spring microbial mat in India (99.7%; EU037210); clone BPC028 from a hydrocarbon seep sediment (96.8%; AF154088); clone MS149BH1062003_5 from the subsurface water of the Kalahari Shield, South Africa (95.5%; DQ354745); clone zEL16 from a limestone-corroding stream biofilm in Frasassi Cave, Italy (95.1%; DQ415810) (Macalady *et al.*, 2006); and clone SILK74 from a sulfur-oxidizing biofilm in Frasassi Cave, Italy (95.2%; EF467495). Phylogenetic analyses using both neighbour-joining and maximum-likelihood methods of strain BDA453^T, related species in *Gammaproteobacteria* and related clones demonstrated that strain BDA453^T was associated with members of the family *Halothiobacillaceae* in the order *Chromatiales*, although the sequence

similarities between strain BDA453^T and species of the genera *Halothiobacillus* and *Thiovirga* were less than 90 %. The tree constructed using the neighbour-joining method is shown in Fig. 3.

Characteristics of strain BDA453^T and members of the genera *Halothiobacillus* and *Thiovirga* are summarized in Table 1. The genus *Halothiobacillus* comprises obligately chemolithoautotrophic, sulfur-oxidizing bacteria (Durand *et al.*, 1993; Hutchinson *et al.*, 1965, 1969; Kelly *et al.*, 1998; McDonald *et al.*, 1997; Sievert *et al.*, 2000; Smith & Kelly, 1979; Visser *et al.*, 1997; Wood & Kelly, 1991) and can be distinguished from *Thiobacillus* based on halophilicity and the results of a comparison of 16S rRNA gene sequences (Kelly & Wood, 2000). Strain BDA453^T preferred the absence of NaCl and this finding differentiated it from members of the genus *Halothiobacillus*. The sole species of the genus *Thiovirga* was isolated from wastewater biofilms (Ito *et al.*, 2004) and described as a new genus belonging to the *Halothiobacillaceae* (Ito *et al.*, 2005). *Thiovirga sulfuroxydans* was not halophilic and could not grow at

NaCl concentrations above 180 mM. Strain BDA453^T was also a non-halophilic, sulfur-oxidizing bacterium, but it clearly differed from *Thiovirga sulfuroxydans* with regard to the optimum growth temperature and pH and genomic DNA G + C content (Table 1). These phenotypic differences support the phylogenetically solitary position of strain BDA453^T in the family *Halothiobacillaceae* (Fig. 3). On the basis of physiological and phylogenetic findings, a novel taxon, *Thiofaba tepidiphila* gen. nov., sp. nov., belonging to the family *Halothiobacillaceae*, is proposed.

Description of *Thiofaba* gen. nov.

Thiofaba (Thi.o.fa'ba. Gr. n. *thion* sulfur; L. fem. n. *faba* bean; N.L. fem. n. *Thiofaba* sulfur bean).

Obligately aerobic and chemolithoautotrophic Gram-negative rods. Do not form spores. Grow by the oxidation of reduced sulfur compounds and the fixation of carbon dioxide. Non-halophilic. Contain ubiquinone-8 as a major respiratory quinone. Major cellular fatty acids are C_{18:1}ω₉c and

Fig. 3. Neighbour-joining tree based on 16S rRNA gene sequences of strain BDA453^T and relatives. After alignment with the ARB program, 1075 positions were used for analyses. The numbers at the nodes represent the bootstrap values. Two numbers are indicated if identical topology was given by both trees (neighbour-joining tree/maximum-likelihood tree); a single number represents the bootstrap value calculated by the neighbour-joining method. Bar, 0.03 substitutions per nucleotide site.

Table 1. Characteristics of strain BDA453^T and species of *Thiovirga* and *Halothiobacillus*

Strains: 1, *T. tepidiphila* BDA453^T; 2, *T. sulfuroxydans* SO07^T; 3, *H. neapolitanus* DSM 581; 4, *H. halophilus* DSM 6132^T; 5, *H. hydrothermalis* R3^T; 6, *H. kellyi* Milos-BIII^T. All strains grow chemolithoautotrophically using thiosulfate, sulfur and sulfide as electron donors in the presence of oxygen, they do not use nitrate as an electron acceptor and they contain ubiquinone-8. Reference data are from Hutchinson *et al.* (1965, 1969), Smith & Kelly (1979), Durand *et al.* (1993), Wood & Kelly (1991), McDonald *et al.* (1997), Visser *et al.* (1997), Kelly *et al.* (1998), Sievert *et al.* (2000), Kelly & Wood (2005) and Ito *et al.* (2005). ND, Not determined.

Characteristic	1	2	3	4	5	6
Conditions for optimum growth:						
Temperature (°C)	45	30–34	28–32	30–32	35–40	37–42
pH	6.5	7.5	6.5–6.9	7.0–7.3	7.5–8.0	6.5
NaCl (mM)	0	0	ND	800–1000	430	400–500
Upper NaCl concentration for growth (mM)	170	180	>860	4000	2000	2500
DNA G + C content (mol%)	66.0	47.1	56.0	64.2	67.4	62.0

C_{16:0}. Phylogenetic position based on 16S rRNA gene sequence is in the family *Halothiobacillaceae* of the class *Gammaproteobacteria*. The type species is *Thiofaba tepidiphila*.

Description of *Thiofaba tepidiphila* sp. nov.

Thiofaba tepidiphila (te.pi.di'phi.la. L. adj. *tepidus* moderately warm; Gr. adj. *philos* loving; N.L. fem. adj. *tepidiphila* loving lukewarm conditions).

Cells are short rods, approximately 0.4 µm wide and 0.5–0.9 µm long, with a single polar flagellum. Cells possess an inclusion and a Gram-negative type cell wall. Cells occur singly, in pairs or as aggregates. Motility is observed. Gram reaction is negative. Catalase-negative and weakly oxidase-positive. Obligately chemolithoautotrophic and grows aerobically by the oxidation of reduced sulfur compounds (thiosulfate, elemental sulfur, sulfide and tetrathionate) and the fixation of carbon dioxide. Anaerobic respiration and fermentation are not observed. Grows at 20–51 °C; optimum growth at 45 °C. The initial pH range for growth is 6.0–9.0, with optimum at pH 6.5; pH decreases during growth. Grows optimally in the absence of NaCl; does not grow at above 2% (w/v) NaCl. Ubiquinone-8 is a major quinone. Major cellular fatty acids are C_{18:1}ω9c and C_{16:0}; C_{16:1}ω7c, C_{12:0}, C_{12:0} 3-OH and C_{18:0} are also present as minor components. The DNA G + C content of the type strain is 66.0 mol%.

The type strain, BDA453^T (=NBRC 103218^T=DSM 19618^T), was isolated from water of a hot spring, Akayu, Fukushima Prefecture, Japan.

Acknowledgements

We thank Ms Kuniko Shimamura for technical support. We also thank Dr Kenji Tanaka and Ms Kaoru Yamaguchi for electron-microscopic observation.

References

Adachi, J. & Hasegawa, M. (1995). Improved dating of the human/chimpanzee separation in the mitochondrial DNA tree: heterogeneity among amino acid sites. *J Mol Evol* **40**, 622–628.

Durand, P., Reysenbach, A. L., Prieur, D. & Pace, N. (1993). Isolation and characterization of *Thiobacillus hydrothermalis* sp. nov., a mesophilic obligately chemolithoautotrophic bacterium isolated from a deep-sea hydrothermal vent in Fiji Basin. *Arch Microbiol* **159**, 39–44.

Elshahed, M. S., Senko, J. M., Najar, F. Z., Kenton, S. M., Roe, B. A., Dewers, T. A., Spear, J. R. & Krumholz, L. R. (2003). Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl Environ Microbiol* **69**, 5609–5621.

Götz, D., Banta, A., Beveridge, T. J., Rushdi, A. I., Simoneit, B. R. & Reysenbach, A. L. (2002). *Persephonella marina* gen. nov., sp. nov. and *Persephonella guaymasensis* sp. nov., two novel, thermophilic, hydrogen-oxidizing microaerophiles from deep-sea hydrothermal vents. *Int J Syst Evol Microbiol* **52**, 1349–1359.

Grosskopf, R., Janssen, P. H. & Liesack, W. (1998). Diversity and structure of the methanogenic community in anoxic rice paddy soil microcosms as examined by cultivation and direct 16S rRNA gene sequence retrieval. *Appl Environ Microbiol* **64**, 960–969.

Hallberg, K. B. & Lindström, E. B. (1994). Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. *Microbiology* **140**, 3451–3456.

Hasegawa, M., Kishino, H. & Yano, T. (1985). Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol* **22**, 160–174.

Hattori, S., Kamagata, Y., Hanada, S. & Shoun, H. (2000). *Thermacetogenium phaeum* gen. nov., sp. nov., a strictly anaerobic, thermophilic, syntrophic acetate-oxidizing bacterium. *Int J Syst Evol Microbiol* **50**, 1601–1609.

Hiraishi, A., Umezawa, T., Yamamoto, H., Kato, K. & Maki, Y. (1999). Changes in quinone profiles of hot spring microbial mats with a thermal gradient. *Appl Environ Microbiol* **65**, 198–205.

Hirayama, H., Takai, K., Inagaki, F., Nealson, K. H. & Horikoshi, K. (2005). *Thiobacter subterraneus* gen. nov., sp. nov., an obligately chemolithoautotrophic, thermophilic, sulfur-oxidizing bacterium from a subsurface hot aquifer. *Int J Syst Evol Microbiol* **55**, 467–472.

Huber, H. & Prangishvili, D. (2006). *Sulfolobales*. In *The Prokaryotes. A Handbook on the Biology of Bacteria*, 3rd edn, vol. 3, pp. 23–51. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.

Huber, G. & Stetter, K. O. (1991). *Sulfolobus metallicus*, sp. nov., a novel strictly chemolithoautotrophic thermophilic archaeal species of metal-mobilizers. *Syst Appl Microbiol* **14**, 372–378.

Huber, R., Wilharm, T., Huber, D., Trinccone, A., Burggraf, S., König, H., Rachel, R., Rockinger, I., Fricke, H. & Stetter, K. O. (1992). *Aquifex pyrophilus* gen. nov., sp. nov., represents a novel

- group of marine hyperthermophilic hydrogen-oxidizing bacteria. *Syst Appl Microbiol* **15**, 340–351.
- Huber, R., Eder, W., Heldwein, S., Wanner, G., Huber, H., Rachel, R. & Stetter, K. O. (1998). *Thermocrinis ruber* gen. nov., sp. nov., a pink-filament-forming hyperthermophilic bacterium isolated from Yellowstone National Park. *Appl Environ Microbiol* **64**, 3576–3583.
- Hutchinson, M., Johnstone, K. I. & White, D. (1965). The taxonomy of certain thiobacilli. *J Gen Microbiol* **41**, 357–366.
- Hutchinson, M., Johnstone, K. I. & White, D. (1969). Taxonomy of the genus *Thiobacillus*: the outcome of numerical taxonomy applied to the group as a whole. *J Gen Microbiol* **57**, 397–410.
- Inaga, S., Katsumoto, T., Tanaka, K., Kameie, T., Nakane, H. & Naguro, T. (2007). Platinum blue as an alternative to uranyl acetate for staining in transmission electron microscopy. *Arch Histol Cytol* **70**, 43–49.
- Ito, T., Sugita, K. & Okabe, S. (2004). Isolation, characterization, and in situ detection of a novel chemolithoautotrophic sulfur-oxidizing bacterium in wastewater biofilms growing under microaerophilic conditions. *Appl Environ Microbiol* **70**, 3122–3129.
- Ito, T., Sugita, K., Yumoto, I., Nodasaka, Y. & Okabe, S. (2005). *Thiovirga sulfuroxydans* gen. nov., sp. nov., a chemolithoautotrophic sulfur-oxidizing bacterium isolated from a microaerobic waste-water biofilm. *Int J Syst Evol Microbiol* **55**, 1059–1064.
- Kamagata, Y. & Mikami, E. (1991). Isolation and characterization of a novel thermophilic *Methanosaeta* strain. *Int J Syst Bacteriol* **41**, 191–196.
- Kelly, D. P. & Wood, A. P. (2000). Reclassification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. nov., *Halothiobacillus* gen. nov. and *Thermithiobacillus* gen. nov. *Int J Syst Evol Microbiol* **50**, 511–516.
- Kelly, D. P. & Wood, A. P. (2005). Family III. *Halothiobacillaceae* fam. nov. Kelly and Wood 2003. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 58–59. Edited by D. J. Brenner, N. R. Krieg, J. T. Staley & G. M. Garrity. New York: Springer.
- Kelly, D. P., Stackebrandt, E., Burghardt, J. & Wood, A. P. (1998). Confirmation that *Thiobacillus halophilus* and *Thiobacillus hydrothermalis* are distinct species within the gamma-subclass of the *Proteobacteria*. *Arch Microbiol* **170**, 138–140.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- La Rivière, J. W. M. & Schmidt, K. (2006). Morphologically conspicuous sulfur-oxidizing eubacteria. In *The Prokaryotes*, vol. 7, pp. 941–954. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Macalady, J. L., Lyon, E. H., Koffman, B., Albertson, L. K., Meyer, K., Galdenzi, S. & Mariani, S. (2006). Dominant microbial populations in limestone-corroding stream biofilms, Frasassi cave system, Italy. *Appl Environ Microbiol* **72**, 5596–5609.
- McDonald, I. R., Kelly, D. P., Murrell, J. C. & Wood, A. P. (1997). Taxonomic relationships of *Thiobacillus halophilus*, *Thiobacillus aquesulis*, and other species of *Thiobacillus*, as determined using 16S rRNA sequencing. *Arch Microbiol* **166**, 394–398.
- Mori, K., Kim, H., Kakegawa, T. & Hanada, S. (2003). A novel lineage of sulfate-reducing microorganisms: *Thermodesulfobiaceae* fam. nov., *Thermodesulfobium narugense*, gen. nov., sp. nov., a new thermophilic isolate from a hot spring. *Extremophiles* **7**, 283–290.
- Nakagawa, Y. & Yamasato, K. (1993). Phylogenetic diversity of the genus *Cytophaga* revealed by 16S rRNA sequencing and menaquinone analysis. *J Gen Microbiol* **139**, 1155–1161.
- Nakagawa, S., Nakamura, S., Inagaki, F., Takai, K., Shirai, N. & Sako, Y. (2004). *Hydrogenivirga caldilitoris* gen. nov., sp. nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field. *Int J Syst Evol Microbiol* **54**, 2079–2084.
- NBRC (2005). *NBRC Catalogue of Biological Resources: Microorganisms, Genomic DNA Clones, and cDNAs*, 1st edn. Chiba, Japan: National Institute of Technology and Evaluation (NITE).
- Reysenbach, A. L., Wickham, G. S. & Pace, N. R. (1994). Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Appl Environ Microbiol* **60**, 2113–2119.
- Robertson, L. & Kuenen, J. (2006). The genus *Thiobacillus*. In *The Prokaryotes*, 3rd edn, vol. 5, pp. 812–827. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*, MIDI Technical Note 101. Newark, DE: MIDI Inc.
- Schulz, H. N., Brinkhoff, T., Ferdelman, T. G., Mariné, M. H., Teske, A. & Jørgensen, B. B. (1999). Dense populations of a giant sulfur bacterium in Namibian shelf sediments. *Science* **284**, 493–495.
- Seegerer, A., Neuner, A., Kristjansson, J. K. & Stetter, K. O. (1986). *Acidianus infernus* gen. nov., sp. nov., and *Acidianus brierleyi* comb. nov.: facultatively aerobic, extremely acidophilic thermophilic sulfur-metabolizing archaeobacteria. *Int J Syst Bacteriol* **36**, 559–564.
- Seegerer, A. H., Trincone, A., Gahrtz, M. & Stetter, K. O. (1991). *Stygiolobus azoricus* gen. nov., sp. nov. represents a novel genus of anaerobic, extremely thermoacidophilic archaeobacteria of the order *Sulfolobales*. *Int J Syst Bacteriol* **41**, 495–501.
- Sievert, S. M., Heidorn, T. & Kuever, J. (2000). *Halothiobacillus kellyi* sp. nov., a mesophilic, obligately chemolithoautotrophic, sulfur-oxidizing bacterium isolated from a shallow-water hydrothermal vent in the Aegean Sea, and emended description of the genus *Halothiobacillus*. *Int J Syst Evol Microbiol* **50**, 1229–1237.
- Skirnisdottir, S., Hreggvidsson, G. O., Hjörleifsdottir, S., Marteinson, V. T., Petursdottir, S. K., Holst, O. & Kristjansson, J. K. (2000). Influence of sulfide and temperature on species composition and community structure of hot spring microbial mats. *Appl Environ Microbiol* **66**, 2835–2841.
- Smith, A. L. & Kelly, D. P. (1979). Competition in the chemostat between an obligately and a facultatively chemolithotrophic *Thiobacillus*. *J Gen Microbiol* **115**, 377–384.
- Sorokin, D. Yu., Banciu, H., Robertson, L. A. & Kuenen, J. G. (2006). Haloalkaliphilic sulfur-oxidizing bacteria. In *The Prokaryotes*, 3rd edn, vol. 2, pp. 969–984. Edited by M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer & E. Stackebrandt. New York: Springer.
- Stahl, D. A. & Amann, R. (1991). Development and application of nucleic acid probes in bacterial systematics. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 205–248. Edited by E. Stackebrandt & M. Goodfellow. Chichester: Wiley.
- Takai, K., Inagaki, F., Nakagawa, S., Hirayama, H., Nunoura, T., Sako, Y., Nealson, K. H. & Horikoshi, K. (2003a). Isolation and phylogenetic diversity of members of previously uncultivated epsilon-Proteobacteria in deep-sea hydrothermal fields. *FEMS Microbiol Lett* **218**, 167–174.

- Takai, K., Kobayashi, H., Nealson, K. H. & Horikoshi, K. (2003b). *Sulfurihydrogenibium subterraneum* gen. nov., sp. nov., from a subsurface hot aquifer. *Int J Syst Evol Microbiol* **53**, 823–827.
- Takai, K., Hirayama, H., Nakagawa, T., Suzuki, Y., Nealson, K. H. & Horikoshi, K. (2004). *Thiomicrospira thermophila* sp. nov., a novel microaerobic, thermotolerant, sulfur-oxidizing chemolithomixotroph isolated from a deep-sea hydrothermal fumarole in the TOTO caldera, Mariana Arc, Western Pacific. *Int J Syst Evol Microbiol* **54**, 2325–2333.
- Takai, K., Miyazaki, M., Nunoura, T., Hirayama, H., Oida, H., Furushima, Y., Yamamoto, H. & Horikoshi, K. (2006). *Sulfurivirga caldicuralii* gen. nov., sp. nov., a novel microaerobic, thermophilic, thiosulfate-oxidizing chemolithoautotroph, isolated from a shallow marine hydrothermal system occurring in a coral reef, Japan. *Int J Syst Evol Microbiol* **56**, 1921–1929.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Visser, J. M., Stefess, G. C., Robertson, L. A. & Kuenen, J. G. (1997). *Thiobacillus* sp. W5, the dominant autotroph oxidizing sulfide to sulfur in a reactor for aerobic treatment of sulfidic wastes. *Antonie Van Leeuwenhoek* **72**, 127–134.
- Wood, A. P. & Kelly, D. P. (1985). Physiological characteristics of a new thermophilic obligately chemolithotrophic *Thiobacillus* species, *Thiobacillus tepidarius*. *Int J Syst Bacteriol* **35**, 434–437.
- Wood, A. P. & Kelly, D. P. (1988). Isolation and characterisation of *Thiobacillus aquaesulis* sp. nov., a novel facultatively autotrophic moderate thermophile. *Arch Microbiol* **149**, 339–343.
- Wood, A. P. & Kelly, D. P. (1991). Isolation and characterisation of *Thiobacillus halophilus* sp. nov., a sulfur-oxidising autotrophic eubacterium from a Western Australian hypersaline lake. *Arch Microbiol* **156**, 277–280.
- Yamamoto, H., Hiraishi, A., Kato, K., Chiura, H. X., Maki, Y. & Shimizu, A. (1998). Phylogenetic evidence for the existence of novel thermophilic bacteria in hot spring sulfur-turf microbial mats in Japan. *Appl Environ Microbiol* **64**, 1680–1687.