Galenea microaerophila gen. nov., sp. nov., a mesophilic, microaerophilic, chemosynthetic, thiosulfate-oxidizing bacterium isolated from a shallow-water hydrothermal vent

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A mesophilic, strictly microaerophilic, chemosynthetic bacterium, designated strain P2D^T, was isolated from the sediment of an active shallow-water hydrothermal vent in Paleochori Bay, on the Greek island of Milos. The cells were Gram-staining-negative rods that measured approximately 0.8–1.3 μm in length and 0.4–0.5 μm in width. Strain P2D^T grew at 20–50 °C (optimum 35 °C), with 1.0–5.0 % (w/v) NaCl (optimum 3.0 %), and at pH 4.5–8.0 (optimum pH 5.5). The generation time under optimal conditions was 1.1 h. Growth occurred under chemolithoautotrophic conditions with $S_2O_3^{2-}$ and CO_2 as the energy and carbon sources, respectively. Oxygen (5 %) was used as sole terminal electron acceptor. No growth was observed in the presence of acetate, formate, lactate, tryptone or peptone. Chemolithoheterotrophic growth occurred when D-glucose or sucrose were present as carbon sources. None of the organic compounds tested was used as an electron donor. The genomic DNA G+C content of the novel strain was 44.9 mol%. In a phylogenetic analysis based on 16S rRNA gene sequences, strain P2D^T was found to be most closely related to *Thiomicrospira psychrophila* DSM 13453^T (92.8% sequence similarity). Based on the phylogenetic, physiological and chemotaxonomic evidence, strain P2D^T represents a novel species of a new genus within the class *Gammaproteobacteria* of the family *Piscirickettsiaceae*, for which the name *Galenea microaerophila* gen. nov., sp. nov. is proposed. The type strain of the type species is P2D^T (=DSM 24963^T=JCM 17795^T).

Sulfur-oxidizing bacteria, which are widely represented within the class *Gammaproteobacteria*, are found in a variety of marine environments, both as free-living bacteria and in symbiotic associations with invertebrates (Brinkhoff & Muyzer, 1997; Dando *et al.*, 1998; Brinkhoff *et al.*, 1999a, b; Schulz *et al.*, 1999; Takai *et al.*, 2004; Nakagawa & Takai, 2008; Crespo-Medina *et al.*, 2009). The most common sulfur-oxidizing members of the class *Gammaproteobacteria* belong to the families *Thiotrichaceae* and *Piscirickettsiaceae* in the order *Thiotrichales*. They include the genera *Achromatium*, *Beggiatoa*, *Thiobacterium*, *Thiomargarita*, *Thioploca*, *Thiospira* and *Thiomicrospira* (Jannasch *et al.*, 1985; Muyzer *et al.*, 1995; Brinkhoff & Muyzer, 1997; Dando *et al.*, 1998; Wirsen *et al.*, 1998; Brinkhoff *et al.*, 1999b; Schulz *et al.*, 1999; Sievert *et al.*, 1999, 2000; Takai *et al.*, 2004). The aerobic, chemosynthetic, thiosulfate-oxidizing bacteria commonly isolated from samples collected in or near hydrothermal vents often include representatives of the genus *Thiomicrospira* (Ruby & Jannasch, 1982; Jannasch *et al.*, 1985; Teske *et al.*, 2000).

In this study, we describe a novel mesophilic, strictly microaerophilic, chemosynthetic, thiosulfate-oxidizing member of

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Three supplementary figures and two supplementary tables are available with the online version of this paper.

the class *Gammaproteobacteria* that was isolated from the sediment of an active hydrothermal vent in Paleochori Bay, on the Greek island of Milos.

Core samples of the sediments near active hydrothermal vents were collected by scuba divers, during the summer of 2010, from both Paleochori Bay, Milos, Greece (36° 40.351′ N 24° 31.108′ E; at a depth of 12 m) and Faial, Azores, Portugal (38° 32.401′ N 28° 35.338′ W; at a depth of 44 m). Although the Paleochori Bay site has been described in detail by Sievert *et al.* (1999) and Dando *et al.* (2000), the Faial site has not yet been characterized. The samples were transferred to a laboratory where subsamples from the different sediment layers were prepared before being stored at 4 °C under nitrogen gas.

For the isolation of mesophilic, chemolithoautotrophic organisms, a 1 g subsample of sediment was suspended in 1 ml anaerobic artificial seawater that contained (l^{-1}): 28 g NaCl, 0.77 g KCl, 1.6 g $CaCl_2 . 2H_2O$, 0.11 g $NaHCO_3$ and 3.5 g $MgSO_4 . 7H_2O$. The suspension was then inoculated into 10 ml modified medium 1011 (Inagaki *et al.*, 2004) supplemented with 10 % (w/v) nitrate under CO_2/O_2 (95 : 5; 200 kPa). The modified medium 1011 contained (l^{-1}): 30 g NaCl, 0.14 g K_2HPO_4, 0.14 g $CaCl_2 . 2H_2O$, 3.4 g $MgSO_4 . 7H_2O$, 4.18 g $MgCl_2 . 6H_2O$, 0.33 g KCl, 0.5 mg $NiCl_2 . 6H_2O$, 0.5 mg $Na_2SeO_3 . 5H_2O$, 0.01 g $Fe(NH_4)_2(SO_4)_2 . 6H_2O$, 0.25 g NH_4Cl, 1.5 g $NaHCO_3$, 1.5 g $Na_2S_2O_3 . 5H_2O$, 10 ml trace mineral solution 151 and 1 ml trace vitamins 197 (http://www.dsmz.de/). Aliquots (0.1 ml) of the primary enrichments were subsequently transferred to fresh medium, and pure cultures were isolated by plating dilutions on modified medium 1011 solidified with 1 g Phytagel (Sigma) l^{-1} and picking single colonies. Plates were incubated in an anaerobic jar (Oxoid) pressurized with CO_2/O_2 (95 : 5, v/v; at 200 kPa). Although no colonies were obtained when agar was used to solidify medium 1011, two pure cultures – one from a Paleochori Bay sample and the other from a Faial sample – were obtained using Phytagel and designated strains P2D^T and P3D, respectively. A long-term stock of each novel strain was prepared by adding 150 µl sterile glycerol (Fisher Scientific) to 850 µl culture and storing the resultant suspension at −80 °C.

Preliminary phylogenetic analysis of 16S rRNA gene sequences indicated that strains P2D^T and P3D were closely related, as they showed 99.5 % sequence similarity. Strain P2D^T was chosen for further characterization.

Cell morphology was investigated under a light microscope (BX 60; Olympus) after staining with 0.1 % (w/v) acridine orange. Transmission electron micrographs were obtained as previously described (Vetriani *et al.*, 2004). The cells of strain P2D^T were Gram-staining-negative short rods that measured approximately 0.8–1.3 µm in length (mean 1.1 µm) and 0.4–0.5 µm in width (mean 0.45 µm; Fig. 1a). They were motile and possessed one polar flagellum per cell (Fig. 1b). In liquid cultures, the cells grouped together at the liquid/gas interface, to form a biofilm. When biofilm flakes were sampled and observed in a scanning electron microscope, what appeared to be large sulfur crystals were observed (data not shown). On solidified medium 1011, strain P2D^T formed circular colonies that were cream to yellow in colour.

Growth rates (μ; h^{-1}) were estimated as $(\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_2 and N_1 represent the cell densities (in cells ml^{-1}) in cultures at times (in h of incubation) t_2 and t_1, respectively. Generation times (t_g; measured in h) were then calculated as $(\ln 2)/\mu$. Unless stated otherwise, all growth experiments were carried out in duplicate in modified liquid medium 1011 under CO_2/O_2 gas (95 : 5, v/v; at 200 kPa). Samples were collected at regular intervals and used for direct cell counts.

When cultures of strain P2D^T were incubated at temperatures between 20 and 55 °C (at 5 °C intervals), growth was observed at 20–50 °C, with optimal growth at 35 °C (Fig. S1a, available in IJSEM Online). All subsequent experiments were carried out at 35 °C. When the concentration of NaCl in the growth medium was varied between 1.0 and 5.0 % (w/v) (at intervals of 0.5 %), strain P2D^T was found to grow with NaCl at 1.5–4.5 % (w/v) (optimum 3.0 %; Fig. S1b). The pH of the growth medium was varied between pH 4.0 and pH 8.5 (at intervals of 0.5 of a pH unit) as previously described (Voordeckers *et al.*, 2005). Growth of

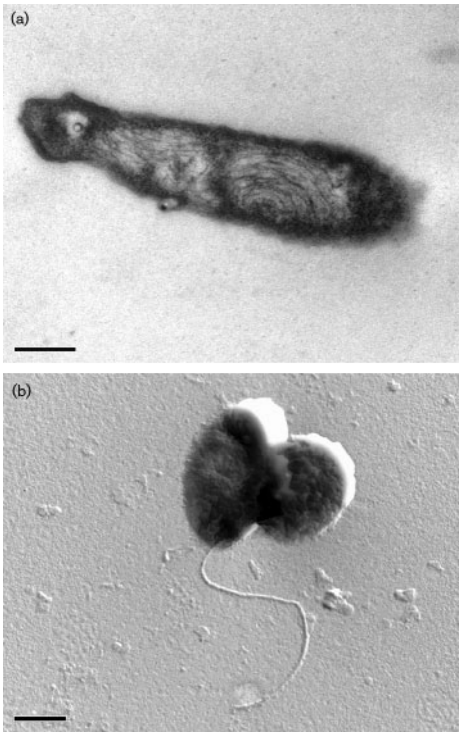

Fig. 1. Electron micrograph of an ultrathin section of cells of strain P2D^T, showing cell morphology (a), and an electron micrograph of platinum-shadowed P2D^T cells, showing a single polar flagellum (b). Bars, 0.2 µm (a) and 0.5 µm (b).

strain P2D^T occurred between pH 4.5 and pH 8.0, with optimal growth at pH 5.5 (Fig. S1c). Under optimal conditions, the generation time of isolate P2D^T was 1.1 h. Strain P2D^T was a microaerophilic, chemolithoautotrophic bacterium that used thiosulfate as an electron donor and oxygen as a terminal electron acceptor.

Antibiotic resistance was tested in liquid cultures containing ampicillin, chloramphenicol, streptomycin or kanamycin (each at 100 μg ml^{-1}). All antibiotics were added aseptically before incubation at 35 °C. An ethanol control was used in the tests of chloramphenicol resistance. Strain P2D^T was inhibited by chloramphenicol, ampicillin, streptomycin and kanamycin. The novel strain appeared to be catalase-negative; no gas bubbles were observed when concentrated cells were resuspended in 3 % (v/v) H_2O_2 at room temperature.

The effect of organic substrates on the growth of strain P2D^T was investigated by adding lactate, peptone, tryptone, acetate, formate, D-glucose or sucrose (each at 2 g l^{-1}) or yeast extract (at 0.1 or 1 g l^{-1}) to cultures of the strain and incubating under a CO_2/O_2 gas phase (95 : 5, v/v; at 200 kPa). Under these conditions, no growth of P2D^T occurred in the presence of lactate, peptone, tryptone, acetate or formate or with yeast extract at 1 g l^{-1} but weak growth (with a generation time of several hours) occurred with yeast extract at 0.1 g l^{-1}. No inhibition of growth was observed in the presence of D-glucose or sucrose. Lactate, peptone, tryptone, acetate, formate, D-glucose, sucrose and yeast extract were also each tested as possible energy and/or carbon sources, either with a CO_2/O_2 gas phase (95 : 5, v/v; 200 kPa) and no thiosulfate added to the medium or with an N_2/O_2 gas phase (95 : 5, v/v; 200 kPa) and no sodium bicarbonate present in the medium. Under these conditions, strain P2D^T only grew in the presence of D-glucose or sucrose under an N_2/O_2 gas phase; in the presence of D-glucose or sucrose, no growth was observed when thiosulfate was absent from the medium. Strain P2D^T also did not grow when the thiosulfate was replaced, as an energy source, by either elemental sulfur (at 3 %, w/v) or H_2 (as 80 % of the gas phase). The ability of strain P2D^T to use electron acceptors other than oxygen was tested by adding sulfate (7 mM), sulfite (4.1 mM), arsenate (5 mM), selenate (5 mM), sulfur (3 % w/v) or potassium nitrate (9.9 mM) to oxygen-depleted medium under N_2/CO_2 (95 : 5, v/v; 200 kPa). Strain P2D^T did not grow in the presence of any electron acceptor other than O_2 (at 5 %, v/v) and did not grow in the presence of 0.5 % or 21 % (v/v) O_2. However, cultures of strain P2D^T survived exposure to air for several hours, indicating that the organism was aerotolerant. In conclusion, strain P2D^T is a strict microaerophile and a facultative mixotroph, capable of using CO_2, D-glucose or sucrose as carbon sources and thiosulfate as the sole electron donor.

The genomic DNA G+C content of strain P2D^T (44.9 mol%) was determined by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig, Germany, by the HPLC analysis of deoxyribonucleosides (Mesbah *et al.*, 1989). The analyses of the cellular fatty acids, polar lipids and respiratory quinones of the novel strain were carried out by the Identification Service and Dr Brian Tindall (both of the DSMZ), using 300 mg of freeze-dried cells that had been grown to early stationary phase, under optimal culture conditions, by the authors. The major cellular fatty acids of strain P2D^T were analysed, as the methyl ester derivatives (Kuykendall *et al.*, 1988), using the standard protocol of version 6.1 of the Sherlock Microbial Identification System (MIDI) with version 4.10 of the TSBA40 database (MIDI), version 6.10 of the TSBA6 database (MIDI) and a 6890N gas chromatograph (Agilent). The predominant fatty acids were identified as $C_{16:1}\omega7c$ (39.8 %), $C_{16:0}$ (19.1 %), $C_{18:1}\omega7c$ (18.3 %) and $C_{18:0}$ (10.5 %), with smaller amounts of $C_{12:0}$ (2.3 %), $C_{12:0}$ alde (2.4 %), $C_{10:0}$ 3-OH (1.8 %) and $C_{17:0}$ (2.0 %). This fatty acid profile was markedly different from those of the novel strain's closest phylogenetic neighbours in the genera *Thiomicrospira*, *Thioalkalimicrobium* and *Sulfurivirga* (Table S1). The known fatty acid profiles that most closely matched that of strain P2D^T were that of *Grimontia hollisae* (which gave a similarity index of 0.263 in calculations based on TSBA6) and those of *Pseudomonas huttiensis*, *Photobacterium leiognathi* and *Vibrio aestuarianus* (which gave similarity indexes of 0.111, 0.089 and 0.089, respectively, in calculations based on TSBA40).

The two predominant polar lipids of strain P2D^T, identified by their staining behaviour on TLC plates (Tindall, 1990a, b), were phosphatidylethanolamine and phosphatidylglycerol, with minor amounts of an unidentified phospholipid and an unidentified aminolipid also detected (Fig. S2).

Analysis of the respiratory lipoquinones of strain P2D^T, by TLC followed by HPLC of the eluted products (Tindall, 1990a, b), revealed that strain P2D^T had ubiquinone-8 (Q-8) as its sole respiratory quinone, like most members of the class *Gammaproteobacteria*.

Genomic DNA was extracted from cells of strain P2D^T by using the UltraClean microbial DNA isolation kit (MoBio). The 16S rRNA gene was selectively amplified from the genomic DNA by PCR and sequenced as described previously (Vetriani *et al.*, 2004). The strain's closest phylogenetic relatives were then identified using BLASTN algorithms (against the public non-redundant database) followed by the BLAST and megaBLAST programs (against the database of prokaryotic type strains with validly published names) (Chun *et al.*, 2007). Each of the 50 sequences with the highest similarity scores was then compared with the 16S rRNA gene sequence of strain P2D^T, using a global alignment algorithm implemented at the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Sequences were aligned automatically using CLUSTAL_X before the alignment was manually refined using SEAVIEW (Galtier *et al.*, 1996; Thompson *et al.*, 1997). A neighbour-joining

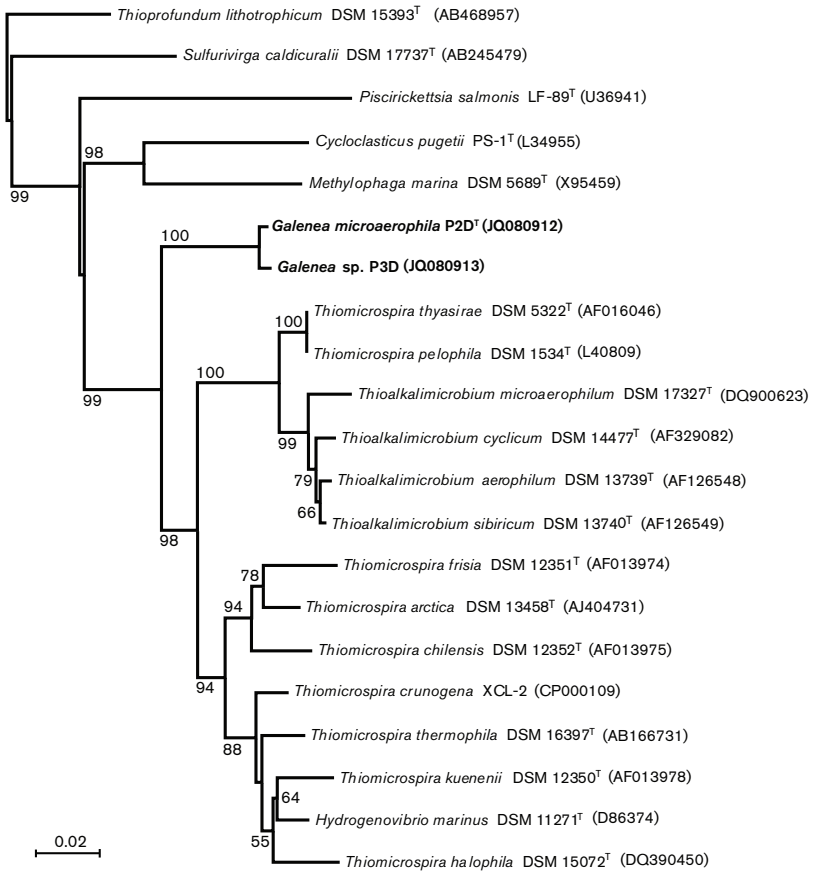

Fig. 2. A neighbour-joining tree based on 16S rRNA gene sequences, showing the positions of strains P2D^T and P3D and related taxa. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

Table 1. Differentiating features of strain P2D^T and type strains of the type species of the genera *Thiomicrospira* and *Thioalkalimicrobium*

Strains: 1, P2D^T (data from this study); 2, *Thiomicrospira pelophila* DSM 1534^T (Kuenen & Veldkamp, 1972); 3, *Thioalkalimicrobium aerophilum* DSM 13739^T (Sorokin *et al.*, 2001). +, Positive; −, negative.

Characteristic	1	2	3
Cell size (μm)	1.1 × 0.45	2.5 × 0.2	1.3 × 0.5
Growth on solid media	+ (Phytagel), − (agar)	+ (agar)	+ (agar)
DNA G+C content (mol%)	44.9	48	49.5
Isolation area	Shallow hydrothermal vents Milos, Greece	Shallow mud flat, Waddenzee, The Netherlands	Soda lake sediment, Buryatia, Siberia, Russia
Temperature range for growth (°C)*	20–50 (35)	28	4–40 (10)
NaCl concentration for growth (%, w/v)*	1.5–4.5 (3.0)	1.5–3.0 (2.5)	1.0–6.5 (4.0)
pH range for growth*	4.5–8.0 (5.5)	5.0–8.5 (7.2)	7.5–10.6 (10)
Terminal electron acceptors	O_2 (5 %)	O_2	O_2
Electron donors	$S_2O_3^{2-}$	$S_2O_3^{2-}$, S_0, H_2S, tetrathionate	$S_2O_3^{2-}$, H_2S
Carbon source	CO_2, D-glucose, sucrose, yeast extract 0.1 g l^{-1}	CO_2	CO_2
Optimum generation time (h)	1.1	0.3	0.3

*Optimum values are given in parentheses.

tree (Fig. 2) was then constructed, from an evolutionary-distance matrix, using PHYLO_WIN, the least-squares algorithm of De Soete and the Jukes–Cantor correction (De Soete, 1983; Perrière & Gouy, 1996). The same sequences, PhylML (Guindon *et al.*, 2010) and the general time reversible (GTR) model were used to construct a maximum-likelihood tree (Fig. S3). The topologies of the neighbour-joining and maximum-likelihood trees were evaluated by bootstrap analysis, with 1000 and 500 replications, respectively. In both trees, strain P2D^T (like strain P3D) was placed within the family *Piscirickettsiaceae*, with high bootstrap support (Figs 2 and S3). Strains P2D^T and strain P3D were closely related (99.5 % 16S rRNA gene sequence similarity) and were placed in a discrete branch outside of the *Thiomicrospira/Thioalkalimicrobium* cluster. The type strains of the type species of the genera *Thiomicrospira* and *Thioalkalimicrobium*, *Thiomicrospira pelophila* DSM 1534^T (Kuenen & Veldkamp, 1972) and *Thioalkalimicrobium aerophilum* DSM 13739^T (Sorokin *et al.*, 2001), showed 92.1 % and 91.5 % sequence similarity with strain P2D^T, respectively (Table S2). The closest phylogenetic neighbour of the novel strain was identified as *Thiomicrospira psychrophila* DSM 13453^T (92.8% sequence similarity). These sequence similarities are below the minimum range of 95% for which a new genus is considered (Tindall *et al.*, 2010). Based on the physiological characteristics that permit the novel strain to be distinguished from its closest cultured relatives (Table 1) and the results of the phylogenetic and chemotaxonomic analyses, strain P2D^T represents a novel species of a new genus, for which the name *Galenea microaerophila* gen. nov., sp. nov. is proposed. The type strain is strain P2D^T (=DSM 24963^T=JCM 17795^T).

Galenea microaerophila gen. nov., sp. nov. strain P2D^T is a strict microaerophile, obligate lithotroph and facultative mixotroph that depends on the oxidation of thiosulfate. The narrow metabolic versatility of this strain is unexpected in a bacterium that is, presumably, able to survive in such an unstable, fluctuating environment as a hydrothermal system in shallow water. The physical and chemical conditions of shallow-water vents tend to vary much more widely than those of deep-sea hydrothermal systems because of several factors: variable loads of organic matter, temperature shifts due to the seasonal formation of thermoclines, hydrodynamism, fluctuations in hydrothermal emission, the input of freshwater from the land, and perturbation of the sediment/water interface as the result of currents and wave action (Wenzhöfer *et al.*, 2000). These conditions create a challenging environment in which ecological niches are often unstable on both temporal and spatial scales (Wenzhöfer *et al.*, 2000). However, it remains possible that the ecological niche from which strain P2D^T was collected is more stable than expected, at least in terms of its physico-chemical gradients (Stockdale *et al.*, 2009). The temperature, pH and salinity ranges for growth of strain P2D^T are fairly broad and the strain is aerotolerant. These characteristics, along with its ability to form biofilms, may afford strain P2D^T the ability to survive the sudden changes in environmental conditions that are typical of shallow-water geothermal environments.

Description of *Galenea* gen. nov.

Galenea (Ga.len′ea. N.L. fem. n. *Galenea* named after Gr. fem. n. *Galene*, one of the Nereids, generally associated with the Aegean Sea. *Galene* was known as the goddess of calm seas and shallow water).

Cells are Gram-staining-negative, short rods that are motile by means of a single polar flagellum. Mesophilic, strict microaerophiles that require thiosulfate for growth. Facultative mixotrophs. The major respiratory quinone is Q-8, the major fatty acids are $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{18:1}\omega7c$, and the predominant polar lipids are phosphatidylglycerol and phosphatidylethanolamine. Phylogenetic analyses placed the genus within the family *Piscirickettsiaceae* in the class *Gammaproteobacteria*. The type species of the genus is *Galenea microaerophila*.

Description of *Galenea microaerophila* sp. nov.

Galenea microaerophila (mi.cro.a.e.ro′phi.la. Gr. adj. *mikros* small, little; Gr. n. *aer* gas; Gr. adj. *philos -ê, -on* loving; N.L. fem. adj. *microaerophila* low-air-loving).

Shows the following characteristics in addition to those given in the genus description above. Cells are ~1 × 0.45 μm (on average) and carry a single polar flagellum. Under optimal growth conditions [35 °C, 3 % (w/v) NaCl, pH 5.5 and CO_2/O_2 at 95 : 5 (v/v)] the generation time is 1.1 h. Catalase-negative. Growth occurs between 20 and 55 °C, with 1.5–4.5 % (w/v) NaCl, and between pH 4.5 and pH 8.0. Growth occurs under strictly microaerophilic, chemolithoautotrophic conditions, in the presence of O_2 and $S_2O_3^{2-}$ as sole terminal electron acceptor and donor, respectively. Aerotolerant. No growth occurs in the presence of lactate, peptone, tryptone, acetate or formate (each at 2 g l^{-1}) or with yeast extract at 1 g l^{-1}. D-Glucose, sucrose and yeast extract (0.1 g l^{-1}) are utilized as carbon sources. Sensitive to chloramphenicol, ampicillin, streptomycin and kanamycin (each at 100 mg ml^{-1}).

The type strain, P2D^T (=DSM 24963^T=JCM 17795^T), was isolated from the sediment of an active shallow-water hydrothermal vent in Paleochori Bay, Milos, Greece. The genomic DNA G+C content of the type strain is 44.9 mol%.

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