Lebetimonas acidiphila gen. nov., sp. nov., a novel thermophilic, acidophilic, hydrogen-oxidizing chemolithoautotroph within the 'Epsilonproteobacteria', isolated from a deep-sea hydrothermal fumarole in the Mariana Arc

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A novel thermophilic, acidophilic bacterium, designated strain Pd55^T, was isolated from a self-temperature-recording *in situ* colonization system deployed in a hydrothermal diffusing flow (maximum temperature of 78 °C) at the TOTO caldera in the Mariana Arc. Cells of strain Pd55^T were motile, short rods with a single polar flagellum. Growth was observed between 30 and 68 °C (optimum growth at 50 °C; 120 min doubling time) and between (initial) pH 4.2 and 7.0 (optimum at pH 5.2). The isolate was a strictly anaerobic chemolithoautotroph capable of using molecular hydrogen as sole energy source and carbon dioxide as sole carbon source. Elemental sulfur served as the sole electron acceptor to support growth. The G + C content of the genomic DNA was 34.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the isolate was related to members of the genera *Nautilia* and *Caminibacter*, although it appeared to be a novel lineage prior to the divergence between *Nautilia* and *Caminibacter*. Strain Pd55^T could also be differentiated from *Nautilia* and *Caminibacter* species on the basis of its physiological properties. It is, therefore, proposed that strain Pd55^T (= JCM 12420^T = DSM 16356^T) represents the type strain of a novel species in a new genus, *Lebetimonas acidiphila* gen. nov., sp. nov.

An increasing number of successful isolations and characterizations of previously uncultivated 'Epsilonproteobacteria' in deep-sea hydrothermal environments has established a new insight into their physiological diversity as extremophiles (Alain *et al.*, 2002; Campbell *et al.*, 2001; Inagaki *et al.*, 2003, 2004; Miroshnichenko *et al.*, 2002, 2004; Takai *et al.*, 2003a, 2004a). *Nautilia lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002) and *Caminibacter hydrogeniphilus* strain AM1116^T (Alain *et al.*, 2002)

are strictly anaerobic, hydrogen-oxidizing, thermophilic chemolithomixotrophs isolated from nests of tube-dwelling polychaetes of deep-sea hydrothermal systems in the East Pacific Rise (EPR). Other isolates (strains Am-H and EX-18.2) from similar habitats have been reported as slightly thermophilic, anaerobic mixotrophs capable of using hydrogen as an energy source and elemental sulfur as a primary electron acceptor (Campbell *et al.*, 2001). Together with a further isolate, *Caminibacter profundus* strain CR^T, from the Rainbow site in the Mid-Atlantic Ridge (MAR) (Miroshnichenko *et al.*, 2004), a second order within the class 'Epsilonproteobacteria', in addition to the order 'Campylobacteriales', has been proposed – the *Nautiliales* (Miroshnichenko *et al.*, 2004). However, classification of 'Epsilonproteobacteria' at the order level is still unresolved since the previous phylogenetic analysis, justifying the paraphyletic lineages between 'Campylobacteriales' and *Nautiliales*, did not include all the recently isolated subgroups of 'Epsilonproteobacteria' such as Group A (e.g. *Hydrogenimonas thermophila* strain EP1-55-1%^T and others;

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Abbreviations: EPR, East Pacific Rise; MAR, Mid-Atlantic Ridge; STR-SCS, self-temperature-recording *in situ* colonization system.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Pd55^T is AB167820.

An electron micrograph of strain Pd55^T (Fig. A) and graphs showing the effects of temperature, pH and NaCl concentration on the growth rate of strain Pd55^T (Fig. Ba–c) are available as supplementary material in IJSEM Online.

Takai *et al.*, 2003a, 2004a), Group F (e.g. *Sulfurovum lithotrophicum* strain 42BKT^T and others; Inagaki *et al.*, 2004; Takai *et al.*, 2003a) and Group G (e.g. strain BKB25Ts-Y; Takai *et al.*, 2003a). These strains are aerobic to anaerobic, hydrogen- and/or sulfur-oxidizing, strict chemolithoautotrophs representing a variety of microhabitats in deep-sea hydrothermal systems. As these isolates and the numerous uncultivated rRNA gene clones were not included in this recent analysis, phylogenetic organization of the '*Epsilonproteobacteria*' is still uncertain. In addition, the taxonomic criteria of the orders *Nautiliales* and '*Campylobacteriales*' are unclear with respect to their coverage of the newly described genera and the numerous uncultivated phylotypes (Takai *et al.*, 2003a, 2004b).

Probably, the confident taxonomy and systematics of '*Epsilonproteobacteria*' require further exploration of as-yet-uncultivated members and their phylogenetic and physiological traits in naturally occurring microbial habitats, specifically in the deep-sea hydrothermal and cold seeping environments, and subsurface biota. In this study, a novel thermophilic, acidophilic bacterium was isolated from a self-temperature-recording *in situ* colonization system (STR-ISCS) deployed in a hydrothermal diffusing flow (maximum temperature of 78 °C) at the TOTO caldera in the Mariana Arc. Taxonomic characterization of this isolate, strain Pd55^T, is described and a new genus and novel species, *Lebetimonas acidiphila* gen. nov., sp. nov., are proposed.

The STR-ISCS, a microbial habitat consisting of a stainless steel pipe with many small holes (5 mm diameter) and substratum of very porous natural pumice (Takai *et al.*, 2003a), was deployed for 4 days in a diffusing hydrothermal flow with a maximum fluid temperature of 78 °C and a pH of 5.3 at the TOTO caldera in the Mariana Arc (12° 42' 8007' N; 143° 32' 3415' E), at a depth of 2922 m by means of the manned submersible *Shinkai 6500* (dive no. 772) in August 2003. After deployment, it was recovered to the sea surface in a sample box from the submersible (dive no. 776). The *in situ* temperature of the hydrothermal fluid was measured by a self-temperature-recording thermometer (Rigosya) and the pH of the hydrothermal fluid was obtained in the onboard laboratory immediately after recovery using a gas-tight fluid sampler 'WHATS' (Water Hydrothermal-fluid *Atsuryoku* Tight Sampler) (Tsunogai *et al.*, 2002).

The TOTO caldera deep-sea hydrothermal field in the Mariana Arc was discovered by Gamo *et al.* (2004) in 1999. Previous physical and chemical characterization of the hydrothermal fluid has suggested that the TOTO caldera deep-sea hydrothermal activity is accompanied by highly acidic hydrothermal fluids resulting from oxidation of volatile volcanic gas (H₂S) to sulfate (Gamo *et al.*, 2004). During a series of dive expeditions, a white-smoker hydrothermal vent with the lowest recorded pH value (pH 1.6) was found in the TOTO caldera, which reveals that the deep-sea hydrothermal activity in the TOTO caldera is a novel system driven by sub-seafloor mixing between the oxygenated sea water and the superheated volcanic gas,

as proposed previously in the DESMOS caldera in the Manus Basin (Gamo *et al.*, 1997). The STR-ISCS was deployed in one of the diffusing hydrothermal flows derived from the highly acidic hydrothermal fluid with further dilution of sea water. Thus, TOTO caldera hydrothermal activity is comparable to fumarole activity in terrestrial volcanoes and geothermal fields and this hydrothermal field may be described as a deep-sea hydrothermal fumarole. During the 4-day deployment, the temperature of the substratum in the colonization device shifted. The temperature fluctuated between 20 and 40 °C in the first 24 h, but gradually increased to 70 °C in the next 24 h. Finally, the temperature was stable at 65–70 °C for the last 2 days.

Immediately after recovery of the STR-ISCS on the ship, a portion of substratum (approx. 1 g wet weight) was suspended in 20 ml sterilized MJ synthetic sea water (Takai *et al.*, 1999) containing 0.05 % (w/v) sodium sulfide in a 100 ml glass bottle (Schott Glaswerke) and tightly sealed with a butyl-rubber cap under a gas phase of 100 % N₂ (100 kPa). The suspended slurry was used to inoculate a series of media, including MJ AIS-YTF medium [MJ AIS medium (Takai *et al.*, 2003c) supplemented with yeast extract, tryptone, formate and other components under a gas phase of H₂:CO₂ (80:20; 200 kPa); described below]. Cultures were incubated at 55 °C in a dry oven.

Growth of motile, short rods was observed in MJ AIS-YTF medium after 2 days incubation at 55 °C. A pure culture was obtained using the dilution-to-extinction technique at 55 °C with the same medium used for the enrichment (Takai & Horikoshi, 2000). The isolate was designated strain Pd55^T. Purity was confirmed routinely by microscopic examination and repeated partial sequencing of the 16S rRNA gene using several primers (Lane, 1991).

Cells were observed under a phase-contrast Olympus BX51 microscope with the SPOT RT Slider CCD camera system (Diagnostic Instruments). The Gram staining test was performed with a Gram Stain kit (Wako). Transmission electron microscopy of negatively stained cells was carried out as described by Zillig *et al.* (1990). Cells grown in MJ AIS-YTF medium under a gas phase of H₂:CO₂ (80:20; 200 kPa) at 55 °C in the mid-exponential phase of growth were used for microscopic observation. Cells of strain Pd55^T were Gram-negative short rods, about 0.6–0.8 µm in diameter, 1.5–2.5 µm in length and motile with a polar flagellum (see Fig. A available as supplementary material in IJSEM Online). Spore formation was not observed under any of the culture conditions. The morphological features of strain Pd55^T were similar to those of previously described thermophilic '*Epsilonproteobacteria*', *N. lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002), *C. hydrogeniphilus* strain AM1116^T (Alain *et al.*, 2002) and *H. thermophila* strain EP1-55-1%^T (Takai *et al.*, 2004a).

Strain Pd55^T was routinely cultivated in MJ AIS-YTF medium. To prepare MJ AIS-YTF medium, the MJ AIS components plus 0.1 % (w/v) yeast extract, 0.1 % (w/v)

tryptone and 0.02 % (w/v) formate were dissolved and the pH of the medium was adjusted to around pH 5.0 with HCl before autoclaving. After autoclaving under an air atmosphere, a concentrated vitamin solution (Balch *et al.*, 1979) and NaHCO₃, elemental sulfur and Na₂S (Takai *et al.*, 2003c) (pH adjusted to 7.0) were added to the medium under gas purging of 80 % H₂ and 20 % CO₂ and the pH was re-adjusted to pH 5.0 with HCl, unless otherwise noted. The four aforementioned components were separately sterilized by autoclaving except for the vitamin solution and elemental sulfur, which were filter- and steam-sterilized (three times at 95 °C for 3 h), respectively. The medium was dispensed at 20 % total bottle (Schott Glaswerke) or tube (Iwaki Glass) volume, tightly sealed with a butyl-rubber stopper under a gas phase consisting of 80 % H₂ and 20 % CO₂ at 200 kPa. All experiments described below were conducted in duplicate.

Growth of strain Pd55^T was measured by direct cell counting after staining with 4',6-diamidino-2-phenylindole (Porter & Feig, 1980) using a phase-contrast Olympus BX51 microscope. The cultures were grown in 100 ml glass bottles (Schott Glaswerke) each containing 20 ml medium with shaking (100 r.p.m.) in a temperature-controlled dry oven. With MJ AIS-YTF medium, strain Pd55^T grew over the temperature range of about 30–68 °C, showing optimal growth at 50 °C; the generation time at 50 °C, pH 5.0, was about 120 min (see Fig. Ba, available as supplementary material in IJSEM Online). The effect of initial pH on

growth was tested at 50 °C using MJ AIS-YTF medium adjusted to various pH levels with 10 mM acetate/acetic acid buffer (pH 3–5), MES (pH 5–6), PIPES (pH 6–7) and HEPES (pH 7.0–7.5) at room temperature (see Fig. Bb, available as supplementary material in IJSEM Online). Growth occurred at initial pH 4.2–7.0, with optimum growth at about pH 5.2 (see Fig. Bb). No growth was observed below initial pH 3.4 or above initial pH 7.2. When the initial pH was 4.2–5.2, the pH of the medium increased during cultivation (final pH 5.7) (see Fig. Bb); however, the growth rate was determined in the early-exponential phase of growth and the effect of pH shift on growth was negligible. Strain Pd55^T, when tested in MJ AIS-YTF medium with variable amounts of NaCl added, grew in 6–50 g NaCl l⁻¹, with optimum growth at 20 g NaCl l⁻¹, 50 °C and pH 5.0 (see Fig. Bc, available as supplementary material in IJSEM Online). In general, the temperature and salt requirements of Pd55^T were similar to those of *N. lithotrophica* strain 525^T, *C. hydrogeniphilus* strain AM1116^T and *H. thermophilus* strain EP1-55-1%^T (Table 1). However, the acidophilic growth of strain Pd55^T was a novel physiological feature within the previously described thermophilic epsilonproteobacterial genera.

The susceptibility of strain Pd55^T to molecular oxygen was tested with MJ AIS-YTF medium under gas mixtures of H₂:CO₂:O₂ (800:199:1, 800:195:5 and 800:190:10) at 200 kPa. Strain Pd55^T grew only under strict anaerobic culture conditions and was extremely sensitive to oxygen.

Table 1. Comparison of properties of strain Pd55^T with those of closely related species

Strains: 1, *Lebetimonas acidiphila* gen. nov., sp. nov. strain Pd55^T (data from this study); 2, *N. lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002); 3, *C. hydrogeniphilus* strain AM1116^T (Alain *et al.*, 2002); 4, *C. profundus* strain CR^T (Miroshnichenko *et al.*, 2004); 5, *H. thermophilus* strain EP1-55-1%^T (Takai *et al.*, 2004a).

Characteristic	1	2	3	4	5
Cell shape	Short rod	Short rod	Short rod	Short rod	Short rod or spherical
Temperature range for growth (°C)	30–68	37–68	50–70	45–65	35–65
Temperature optimum for growth (°C)	50	53	60	55	55
Doubling time under optimal conditions (min)	120	140	90	40	70
pH range for growth	4.2–7.0	6.4–7.4	5.5–7.5	6.5–7.4	4.9–7.2
pH optimum for growth	5.2	6.8–7.0	5.5–6.0	6.9–7.0	5.9
NaCl concentration range for growth (%)	0.6–5.0	0.8–5.0	1.0–4.0	0.5–5.0	1.6–5.6
NaCl concentration optimum for growth (%)	2	3	2.0–2.5	3	3.2
Microaerobic growth	–	–	–	+ (up to 2 % O ₂)	+ (up to 2 % O ₂)
Utilization of C source other than CO ₂ :					
Formate	–	+	–	–	–
Complex organic substrates	–	–	+	–	–
Utilization of electron donor other than H ₂ :					
Formate	–	+	–	–	–
Utilization of electron acceptor other than S ⁰ :					
O ₂	–	–	–	+	+
Nitrate	–	–	+	+	+
Sulfite	–	+	–	–	–
G + C content of genomic DNA (mol%)	34.0	34.7	29.0	32.1	34.6

Strictly autotrophic growth was examined in MJAIS-YTF medium lacking yeast extract, tryptone and formate under a gas phase of 80 % H₂ and 20 % CO₂ (200 kPa). Strain Pd55^T was able to grow under strict chemolithoautotrophic conditions with H₂ and S⁰ as the sole electron donor and acceptor, respectively. The maximal cell yield under strict chemolithoautotrophic conditions was almost the same as the yield in MJAIS-YTF medium (3–4 × 10⁹ cells ml⁻¹). However, when strain Pd55^T was grown under strict chemolithoautotrophic conditions with H₂ and S⁰, the culturability of the cells after growth was significantly reduced, i.e. the proportion of the culturable cell number after growth decreased to 10⁻⁵ of that in MJAIS-YTF medium. Culturability was examined in a series of 10⁻¹ dilution cultivations with the strictly autotrophic medium or MJAIS-YTF medium. An improvement in culturability was observed by addition of 0.1 % (w/v) yeast extract, 0.1 % (w/v) tryptone, 0.1 % (w/v) Casamino acids, 5 mM formate, 5 mM acetate, 5 mM citrate, 5 mM tartrate, 5 mM fumarate, 5 mM succinate, 5 mM malate, 5 mM lactate or 5 mM pyruvate to the strictly autotrophic medium. Heterotrophic growth was determined in MJAIS-YTF medium lacking NaHCO₃, yeast extract, tryptone and formate under a gas phase of 100 % H₂ (200 kPa), containing the following potential carbon sources: 0.1 % (w/v) yeast extract, 0.1 % (w/v) peptone, 0.1 % (w/v) tryptone, 0.1 % (w/v) Casamino acids, 1 and 5 mM formate, 5 mM acetate, 5 mM glycerol, 0.025 % (v/v) methanol, 0.05 % (v/v) ethanol, 0.1 % (v/v) 2-propanol, 5 mM citrate, 5 mM tartrate, 5 mM fumarate, 5 mM maleate, 5 mM succinate, 5 mM propionate, 5 mM malate, 5 mM lactate, 5 mM oxalate, 5 mM thioglycollate, 5 mM pyruvate, 5 mM each of 20 amino acids, 0.1 % (w/v) glucose, 0.1 % (w/v) galactose, 0.1 % (w/v) sucrose, 0.1 % (w/v) fructose, 0.1 % (w/v) lactose, 0.1 % (w/v) maltose, 0.1 % (w/v) arabinose, 0.1 % (w/v) trehalose and 0.1 % (w/v) starch. Strain Pd55^T was not able to grow with any of the heterotrophic substrates using H₂ as an energy source and S⁰ as an electron acceptor. Utilization of these organic compounds as an alternative energy source instead of H₂ was also examined in MJAIS-YTF medium lacking yeast extract, tryptone and formate under a gas phase of 80 % N₂ and 20 % CO₂ (200 kPa). None of the organic compounds sustained growth of strain Pd55^T. In an attempt to determine potential electron donors and acceptors other than a combination of H₂ and S⁰ for autotrophic growth, each of the potential electron donors such as thiosulfate (20 mM), sulfite (5 and 20 mM) or ferrous iron (20 mM) was tested with nitrate (10 mM) and fumarate (10 mM) as the electron acceptors, and each of the potential electron acceptors such as sulfite (2 and 10 mM), thiosulfate (10 mM), tetrathionate (10 mM), nitrate (10 mM), nitrite (1 and 5 mM), ferric citrate (20 mM), selenate (5 mM), arsenate (5 mM) or fumarate (10 mM) was tested with H₂ as the electron donor. The anaerobic cultivation procedure in the absence of Na₂S₉H₂O and in the presence of ferric citrate (20 mM), selenate (5 mM) or arsenate (5 mM) has been described previously (Takai *et al.*, 2003b). None of

the combinations other than H₂ and S⁰ supported growth of strain Pd55^T. The potential nutrients required for growth such as selenite, tungstate and vitamins were examined under strict chemoautotrophic conditions in the absence of the test materials and the nitrogen source for growth (NH₄Cl, NaNO₂, N₂, NaNO₃ or yeast extract) was also examined under strict autotrophic conditions. Strain Pd55^T utilized ammonium, nitrate and organic nitrogen compound as a nitrogen source, but could not utilize nitrite or molecular nitrogen. Selenium, tungsten and vitamins were not required for growth. These results indicate that strain Pd55^T was a strict chemolithoautotroph utilizing H₂ as the sole electron donor, S⁰ as the sole electron acceptor and CO₂ as the sole carbon source for growth. However, the presence of organic compounds strongly improved the culturability of strain Pd55^T although the mechanism is still unclear. Strain Pd55^T resembles *N. lithotrophica* strain 525^T, *C. hydrogeniphilus* strain AM1116^T and *H. thermophila* strain EP1-55-1%^T in utilization of molecular hydrogen as the primary electron donor, although it could not use formate as either energy or carbon source and it could not use any of the organic compounds tested as sole carbon source (Table 1). Other than S⁰, *N. lithotrophica* strain 525^T is able to utilize sulfite, *C. hydrogeniphilus* strain AM1116^T is able to utilize nitrate and *H. thermophila* strain EP1-55-1%^T is able to utilize nitrate and molecular oxygen as alternative electron acceptors (Table 1). Utilization of S⁰ as the sole electron acceptor of strain Pd55^T differs markedly from the energy metabolisms of the previously described thermophilic genera within the 'Epsilonproteobacteria' (Table 1).

The time-course of the reduction of elemental sulfur and concomitant bacterial growth of strain Pd55^T were examined in MJAIS-YTF medium under a gas phase of 80 % H₂ + 20 % CO₂ (200 kPa) (Fig. 1). The concentration of H₂S in the gas phase during growth was measured using a GC Micro GC CP2002 (GL Sciences). The gas phase was

Fig. 1. Time-course of reduction of hydrogen sulfide in the gas phase (■) and concomitant bacterial growth (●) of *Lebetimonas acidiphila* strain Pd55^T.

periodically sampled with a 1 ml gas-tight syringe, which was directly applied to the GC or diluted once with helium gas and then applied. The consumption of molecular hydrogen and elemental sulfur was not measured because of the large amounts of hydrogen and sulfur in the medium required for growth; however, the concentration of hydrogen sulfide increased during growth of strain Pd55^T (Fig. 1). Since the control medium that lacked a bacterial inoculum did not demonstrate reduction of elemental sulfur, bacterial reduction of elemental sulfur occurred during growth. Strain Pd55^T was found to be a respiratory hydrogen-oxidizing, sulfur-reducing chemolithoautotroph.

The sensitivity of strain Pd55^T to antibiotics such as chloramphenicol (50 and 100 µg ml⁻¹), streptomycin (50 and 100 µg ml⁻¹), kanamycin (50 and 100 µg ml⁻¹), ampicillin (50 and 100 µg ml⁻¹) and rifampicin (50 and 100 µg ml⁻¹) was tested at 55 °C. Strain Pd55^T was sensitive to all the antibiotics tested at a concentration of 50 µg ml⁻¹. Antibiotic susceptibility was similar in strain Pd55^T, *N. lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002) and *H. thermophila* strain EP1-55-1%^T (Takai *et al.*, 2004a); *C. hydrogeniphilus* strain AM1116^T (Alain *et al.*, 2002) was resistant to kanamycin (100 µg ml⁻¹).

The cellular fatty acid composition of cells grown in MJAIS-YTF medium at 55 °C in the late-exponential phase of growth was analysed. Lyophilized cells (100 mg) were placed in a Teflon-lined, screw-capped tube containing 3 ml anhydrous methanolic HCl and heated at 100 °C for 3 h. The extraction and analysis of fatty acid methyl esters were as described previously (Takai *et al.*, 2003b). The major cellular fatty acids of strain Pd55^T were C_{14:0} (4.3%), C_{14:0} 3-OH (9.8%), C_{16:0} (12.5%), C_{16:1} (9.7%), C_{17:0} (4.4%), C_{18:0} (26.5%), C_{18:1} (22.2%), anteiso-C_{19:0} (4.8%) and C_{19:1} (5.8%). This composition was generally similar to that of *H. thermophila* strain EP1-55-1%^T (Takai *et al.*, 2004a), although the cellular fatty acids anteiso-C_{19:0} (4.8%) and C_{19:1} (5.8%) were only present in strain Pd55^T.

Genomic DNA of strain Pd55^T was prepared as described by Marmur & Doty (1962). The G + C content of the DNA was determined by direct analysis of deoxyribonucleotides by HPLC (Tamaoka & Komagata, 1984). The DNA G + C content of strain Pd55^T was 34.0 mol%, which was similar to those of *N. lithotrophica* strain 525^T and *H. thermophila* strain EP1-55-1%^T (34.7 and 34.6 mol%, respectively), but higher than that of *C. hydrogeniphilus* strain AM1116^T (29 mol%) (Table 1).

The 16S rRNA gene was amplified by PCR using primers Bac27F and 1492R (DeLong, 1992; Lane, 1991), as described previously (Takai *et al.*, 2001). The nearly complete sequence (1431 bp) of the 16S rRNA gene from strain Pd55^T was directly sequenced using both strands by the dideoxynucleotide chain-termination method with a DNA sequencer model 3100 (Perkin Elmer/Applied Biosystems). The rRNA gene sequence was analysed using the gapped-BLAST search algorithm (Altschul *et al.*, 1997; Benson *et al.*,

1998) and was most closely related (94.4%) to the sequence of strain B455-1, isolated from a deep-sea hydrothermal environment in the Iheya North of the Okinawa Trough (Takai *et al.*, 2003a). Similarities with the sequences of *C. profundus* strain CR^T (Miroshnichenko *et al.*, 2004) and *N. lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002), isolated from deep-sea hydrothermal systems in the MAR and EPR, respectively, were 92.1 and 92.0%, respectively. The nearly complete sequence was manually re-aligned according to the secondary structures using ARB (Ludwig *et al.*, 2004). Phylogenetic analyses were restricted to nucleotide positions that could be chosen using the *ε-Proteobacteria* filter of Hugenholtz (2002). Evolutionary distance matrix analysis (using the Jukes–Cantor correlation method) and neighbour-joining analysis were performed using ARB (Fig. 2). Bootstrap analysis was performed to provide confidence estimates for phylogenetic tree topologies. The phylogenetic tree indicated that strain Pd55^T represented a novel lineage together with strain B455-1 prior to divergence of the genera *Nautilia* and *Caminibacter* (Fig. 2).

Phylogenetic analysis indicates that strain Pd55^T is associated with the genera *Nautilia* and *Caminibacter*, which include not only the type species of *N. lithotrophica* strain 525^T (Miroshnichenko *et al.*, 2002) and *C. hydrogeniphilus* strain AM1116^T (Alain *et al.*, 2002) from tube-dwelling polychaetes nests of deep-sea hydrothermal systems in the EPR, but also the recently described species *C. profundus* strain CR^T (Miroshnichenko *et al.*, 2004) and the potential *Nautilia* spp. strains Am-H and EX-18.2 (Campbell *et al.*, 2001). Similarity levels between the 16S rRNA gene sequences of strain Pd55^T and either *Nautilia* (92.0%) or *Caminibacter* (92.1%) are within the common index of 16S rRNA gene sequence similarities for genus-level differentiation (90–96%) (Gillis *et al.*, 2001). In addition, phylogenetic characterization demonstrates that strain Pd55^T represents a separate phylotype together with strain B455-1, which was isolated from the deep-sea hydrothermal environment in the Okinawa Trough (Takai *et al.*, 2003a), diverging prior to differentiation of the genera *Nautilia* and *Caminibacter*. These results suggest that strain Pd55^T can be genetically classified as representing a new genus within the 'Epsilonproteobacteria'. The physiological properties of strain Pd55^T reinforce differentiation from *Nautilia* and *Caminibacter* at the genus level (Table 1). Strain Pd55^T grows optimally at much lower pH (5.2) and is able to grow in a much more acidic pH range (pH 4.2–7.0) than *N. lithotrophica* strain 525^T, *C. hydrogeniphilus* strain AM1116^T and *C. profundus* strain CR^T (Table 1). Hydrogen-dependent energy metabolism is common among the previously described members of the genera *Nautilia* and *Caminibacter*, whereas strain Pd55^T is an obligate chemolithoautotroph and can utilize only elemental sulfur as an electron acceptor (Table 1). On the basis of these physiological and molecular properties of strain Pd55^T, a new genus, *Lebetimonas* gen. nov., is proposed. Strain Pd55^T (= JCM 12420^T = DSM 16356^T) is proposed as

Fig. 2. Phylogenetic tree of representative 'Epsilonproteobacteria' strains and environmental rRNA gene clones inferred from 16S rRNA gene sequences using the neighbour-joining method on the 1208 homologous sequence positions for each organism. The number at each node represents the bootstrap value (100 replicates). Bar, 5 substitutions per 100 nt.

the type strain of a novel species in this genus, *Lebetimonas acidiphila* sp. nov.

Description of *Lebetimonas* gen. nov.

Lebetimonas (Le.be.ti.mo'nas. L. n. *lebes* cauldron; L. fem. n. *monas* a unit, monad; N.L. fem. n. *Lebetimonas* cell from a cauldron).

Short rods, motile with a polar flagellum. Gram-negative. Strictly anaerobic. Thermophilic and acidophilic. Strictly chemolithoautotrophic. Able to utilize molecular hydrogen as an electron donor and elemental sulfur as an electron acceptor. NaCl absolutely required for growth. G+C content of genomic DNA is about 34 mol%. Major cellular fatty acids are $C_{18:0}$ and $C_{18:1}$. On the basis of 16S rRNA gene analysis, the genus *Lebetimonas* is related to the genera *Nautilia* and *Caminibacter* within the 'Epsilonproteobacteria'. Members of the genus *Lebetimonas* occur in global deep-sea hydrothermal systems.

The type species is *Lebetimonas acidiphila*.

Description of *Lebetimonas acidiphila* sp. nov.

Lebetimonas acidiphila (a.ci.di'phi.la. L. adj. *acidus*, -a, -um sour; N.L. n. *acidum* acid; Gr. adj. *philos* loving; N.L. fem. adj. *acidiphila* acid-loving).

Each cell is a highly motile rod with a polar flagellum and a mean length of 1.5–2.5 μm and width of approximately 0.6–0.8 μm . Cells occur singly. Gram-negative. Strictly anaerobic. The temperature range for growth is 30–68 °C (optimum 50 °C). The pH range for growth is 4.2–7.0 (optimum pH 5.2). NaCl at 6–50 g l^{-1} is an absolute

growth requirement; optimum growth occurs at 20 g l^{-1} . Strict chemolithoautotrophic growth occurs with molecular hydrogen as an electron donor and with elemental sulfur as an electron acceptor. Elemental sulfur is reduced to hydrogen sulfide during growth. Nitrate or ammonium is required as a nitrogen source. Vitamins, selenium and tungsten are not required for growth. The major cellular fatty acids are $C_{14:0}$ (4.3%), $C_{14:0}$ 3-OH (9.8%), $C_{16:0}$ (12.5%), $C_{16:1}$ (9.7%), $C_{17:0}$ (4.4%), $C_{18:0}$ (26.5%), $C_{18:1}$ (22.2%), anteiso- $C_{19:0}$ (4.8%) and $C_{19:1}$ (5.8%).

The type strain is Pd55^T (=JCM 12420^T=DSM 16356^T), isolated from an *in situ* colonization system deployed in a hydrothermal diffusing flow (maximum temperature of 78 °C) at the TOTO caldera in the Mariana Arc. The DNA G+C content of strain Pd55^T is 34.0 mol% (by HPLC).

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