Limnobacter litoralis sp. nov., a thiosulfate-oxidizing, heterotrophic bacterium isolated from a volcanic deposit, and emended description of the genus *Limnobacter*

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A Gram-negative, aerobic, heterotrophic bacterium, designated KP1-19^T, was isolated from a 22-year-old volcanic deposit at a site lacking vegetation on the island of Miyake, Japan. Strain KP1-19^T was able to use thiosulfate (optimum concentration 10 mM) as an additional energy source. 16S rRNA gene sequence analysis indicated that strain KP1-19^T was closely related to *Limnobacter thiooxidans* CS-K2^T within the class *Betaproteobacteria* (97.7 % 16S rRNA gene sequence similarity). The cellular fatty acid profile was characteristic of the genus *Limnobacter*: the major fatty acids (>5 %) were $C_{16:0}$, $C_{16:1}\omega7c$ and $C_{18:1}\omega7c$ and minor amounts of $C_{10:0}$ 3-OH were also found. DNA–DNA relatedness between strain KP1-19^T and *L. thiooxidans* LMG 19593^T was 18 %. Therefore, strain KP1-19^T represents a novel species, for which the name *Limnobacter litoralis* sp. nov. is proposed. The type strain is KP1-19^T (=LMG 24869^T =NBRC 105857^T =CIP 109929^T).

The genus *Limnobacter*, a member of the class *Betaproteobacteria*, was established to accommodate thiosulfate-oxidizing heterotrophic bacteria isolated from sediment of the littoral zone of a freshwater lake (Spring *et al.*, 2001). The genus *Limnobacter* comprises Gram-negative, polyhydroxybutyrate (PHB)-containing, non-spore-forming, strictly aerobic, oxidase- and catalase-positive, slightly curved rods, motile by single polar flagella. Carboxylic acids and amino acids are used as energy and carbon sources, but carbohydrates and polyols are not used. Thiosulfate is oxidized to sulfate in the presence of an organic carbon source, but autotrophic growth is not exhibited. The major fatty acids are $C_{18:1}\omega7c$, $C_{16:1}\omega7c$, $C_{16:0}$ and $C_{10:0}$ 3-OH. Until now, only the type species, *Limnobacter thiooxidans*, has been described.

Strain KP1-19^T was isolated from a 22-year-old volcanic deposit at the Nippana site (34° 02′ 50.7″ N 139° 30′ 02.1″ E) on the island of Miyake, Japan, on the western rim of the Pacific Ocean (Lu *et al.*, 2008). The isolate was cultured on 100-fold-diluted nutrient agar and characterized as a member of the genus *Limnobacter* by comparative 16S rRNA gene sequence analysis (Lu *et al.*, 2008). Strain KP1-19^T did not grow chemolithoautotrophically with H_2, O_2 or CO_2 but grew chemolithoheterotrophically with thiosulfate (optimum concentration 10 mM) in succinate/mineral medium [containing 0.27 % (w/v) sodium succinate, 0.01 % (w/v) yeast extract and various mineral salts; Lu *et al.*, 2008], which has been described for the genus *Limnobacter* (Spring *et al.*, 2001). Strain KP1-19^T was characterized as an oligotroph (Ohta & Hattori, 1983; Ohta *et al.*, 2004) by its ability to grow in very low-nutrient media such as 10 000-fold-diluted nutrient broth. This growth characteristic appears to represent a selective advantage in organic substrate-deficient environments

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Abbreviation: PHB, polyhydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KP1-17, KP1-18, KP1-19^T, KP1-22 and KP1-23 are AB366172–AB366174, AB366177 and AB366178, respectively.

A supplementary figure is available with the online version of this paper.

such as recently formed volcanic deposits (King, 2007; King *et al.*, 2008).

For phenotypic tests and chemotaxonomic and molecular systematic characterization, strain KP1-19^T was cultured in 10-fold-diluted nutrient broth (NB) [containing 0.1 % (w/v) meat extract (Kyokuto Seiyaku), 0.1 % (w/v) polypeptone (Nihon Seiyaku) and 0.05 % (w/v) NaCl, pH 7.0]. Conditions for growth were determined at 30 °C and pH 7.0, unless otherwise stated, for 2 weeks at 4, 8, 10, 28–36 (at intervals of 4 °C) and 36–46 °C (at intervals of 2 °C), with 0.05, 0.1, 0.5, 1.0 and 5–10 % (w/v) NaCl (at intervals of 1 % NaCl) and at pH 5.5–10.5 (at intervals of 0.5 pH units). Growth at different pH and the maximum specific growth rate (h^{-1}) at different temperatures and NaCl concentrations were determined by measuring optical density (OD_{660}). For the examination of autotrophic growth, the strain was cultured at 35 °C in mineral salt medium (Sato *et al.*, 2004) supplemented with either sodium thiosulfate (10 or 50 mM) or elemental sulfur (1 %, w/v). Thiosulfate- and sulfur-oxidizing autotrophic growth was determined by following optical density and pH change of the medium. The presence of PHB was examined microscopically by staining with a basic oxazine dye, Nile blue A (Ostle & Holt, 1982). Oxidase and catalase tests were carried out as described previously (Ohta & Hattori, 1983). Morphology of cells from the exponential growth phase was examined under a light microscope (BX-51; Olympus) and a transmission microscope (JEM-2000 FX II; JEOL) as described previously (Ushiba *et al.*, 2003). The ability to grow anaerobically was tested on R2A medium (van der Linde *et al.*, 1999) at 30 °C for 1 week using a BBL GasPak anaerobic system. Additional phenotypic characteristics were determined using API 20NE (bioMérieux) and Biolog GN2 MicroPlates (Hayward), according to the manufacturers' instructions. As a reference strain for DNA–DNA hybridization tests and cellular fatty acid profiling, *L. thiooxidans* LMG 19593^T was obtained from the BCCM/LMG culture collection, Gent, Belgium.

Cellular fatty acid methyl esters were prepared by heating dried cells in anhydrous methanolic HCl at 100 °C for 3 h (Ikemoto *et al.*, 1978) and then analysed by GLC with a GC-14A gas chromatograph and a ULBON HR-SS-10 capillary column (0.23 mm × 50 m; Shimadzu). Fatty acid methyl ester peaks were identified using a bacterial acid methyl ester mixture (Supelco) and comparing retention times against those of standard compounds. Isoprenoid quinones were extracted and analysed by HPLC as described by Komagata & Suzuki (1987) with conditions and preparation of standards as described previously by Ohta *et al.* (2003). G+C content was determined by hydrolysing the DNA enzymically and quantifying the nucleotides by HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization tests were carried out with photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989) using a Wallac 1420 ARVOsx multilabel counter for chemiluminescence measurements. For enzymic development, alkaline phosphatase–streptavidin conjugate (Vector) was used with CDP-Star (Tropix) as the substrate. Nearly complete 16S rRNA gene sequences of strain KP1-19^T and four additional strains (KP1-17, KP1-18, KP1-22 and KP1-23) were obtained as described previously (Sato *et al.*, 2006). For phylogenetic analysis, closely related 16S rRNA gene sequences were retrieved from public databases using BLAST (Pearson & Lipman, 1988). The sequences were aligned and a phylogenetic tree was produced with the neighbour-joining method (Saitou & Nei, 1987) in CLUSTAL W (Thompson *et al.*, 1994). The tree was visualized using TreeView version 1.6.6 (Page, 1996).

Cells of strain KP1-19^T were found to be Gram-negative, catalase- and oxidase-positive, non-spore-forming, slightly curved rods (0.4–0.6 × 1–3 μm) that were motile with a polar flagellum (Supplementary Fig. S1, available in IJSEM Online). Cells contained PHB granules and fluoresced with a bright orange colour when stained with Nile blue A. The strain did not grow anaerobically on R2A medium and did not reduce nitrate. Strain KP1-19^T did not grow chemolithoautotrophically with thiosulfate and sulfur and did not use any of the 31 tested carbohydrates (Table 1). These phenotypic characteristics are identical to those reported for the genus *Limnobacter* (Spring *et al.*, 2001). Strain KP1-19^T did not grow at 8 or 46 °C and its optimum growth temperature was 38–42 °C, suggesting that strain KP1-19^T is more mesophilic than *L. thiooxidans* (range 4–38 °C; Spring *et al.*, 2001). The optimum NaCl concentration for growth was 0.5 % NaCl, and the growth rate with 5 % NaCl was about half that with 0.5 % NaCl. Strain KP1-19^T could grow with 8 % but not with 9 % NaCl. Other characteristics of strain KP1-19^T are given in the species description and differences between strain KP1-19^T and *L. thiooxidans* LMG 19593^T are shown in Table 1.

The cellular fatty acid profiles of strain KP1-19^T and *L. thiooxidans* LMG 19593^T were very similar, the fatty acids in both strains being $C_{16:0}$ (39.9 and 24.2 %, respectively), $C_{16:1}\omega7c$ (21.4 and 38.8 %), $C_{18:1}\omega7c$ (20.0 and 27.3 %), $C_{10:0}$ 3-OH (4.9 and 2.7 %), $C_{18:0}$ (3.4 and 4.8 %) and $C_{14:0}$ (1.1 and 2.3 %). Strain KP1-19^{T}also contained two additional fatty acids, which were tentatively identified as $C_{17:0}$ cyclo (6.9 %) and $C_{19:0}$ (2.5 %). The major respiratory quinone of strain KP1-19^T was ubiquinone Q-8 and the DNA G+C content was 59 mol%, which was slightly higher than that reported for *L. thiooxidans* (55 mol%; Spring *et al.*, 2001).

Our previous study (Lu *et al.*, 2008) determined the nearly complete 16S rRNA gene sequence (1495 nt) of strain KP1-19^T and revealed that the isolate was associated with the family *Burkholderiaceae* by phylogenetic analysis. In addition, Lu *et al.* (2008) showed that strain KP1-19^T and 36 other related isolates from the volcanic deposit shared ⩾99.3 % 16S rRNA gene sequence similarity (about 600 nt). Therefore, in this study, four other isolates (KP1-17, KP1-18, KP1-22 and KP1-23) were randomly selected

Table 1. Differential characteristics of strain KP1-19^T and *L. thiooxidans* LMG 19593^T

Data were taken from this study unless otherwise stated. Both strains are positive for β-glucosidase and β-galactosidase and assimilation of Tweens 40 and 80, acetate, DL-lactate and succinate. Both strains are negative for protease, indole production and assimilation of α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl α-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-alaninamide, D-alanine, L-alanine, L-alanyl glycine, L-asparagine, glycyl L-aspartic acid, glycyl L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglutamic acid, D-serine, L-serine, L-threonine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate, n-capric acid and adipic acid. +, Positive; W, weakly positive; −, negative.

Characteristic	KP1-19^T	*L. thiooxidans* LMG 19593^T
Growth at 4 °C	−	+
Growth at 44 °C	+	−
Arginine dihydrolase	+	−
Urease	+	−
Assimilation of:		
L-Aspartate	−[a]*	+[a]
Formate	W	−
Fumarate	−[a]	+[a]
L-Glutamate	−[a]	+[a]
β-Hydroxybutyrate	+	W
2-Oxoglutarate	W	−
DNA G+C content (mol%)	59	55[b]

*Data from: *a*, Lu *et al.* (2008); *b*, Spring *et al.* (2001).

and their almost full-length 16S rRNA gene sequences were determined. The five isolates shared ⩾99.7 % 16S rRNA gene sequence similarity (1412 nt). As shown in Fig. 1, the five isolates formed a clade that represented a sibling taxon of *L. thiooxidans* CS-K2^T. 16S rRNA gene sequence similarity between strain KP1-19^T and *L. thiooxidans* CS-K2^T was 97.7 %. DNA–DNA relatedness between strain KP1-19^T and *L. thiooxidans* LMG 19593^T was 18 % (mean of two independent determinations: 20 % with strain KP1-19^T as the probe and 16 % with the reciprocal experiment). This value is below the 70 % cut-off for DNA–DNA relatedness that is a criterion for the assignment of bacterial strains to different genomic species (Wayne *et al.*, 1987).

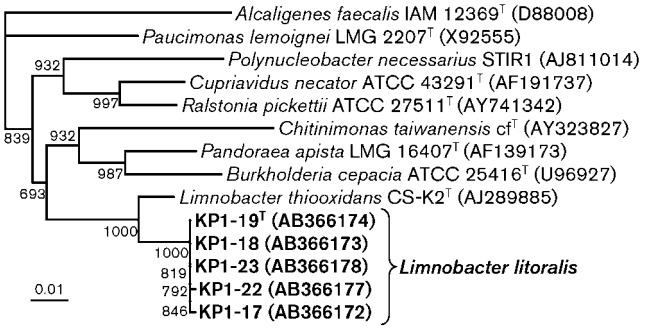

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences (1319–1328 nt) showing the position of strains of *Limnobacter litoralis* sp. nov. within the family *Burkholderiaceae*. Bootstrap values (>600) based on 1000 replicates are shown at branch nodes. The sequence of *Alcaligenes faecalis* IAM 12369^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

On the basis of phenotypic, genotypic and phylogenetic analysis, it is concluded that strain KP1-19^T represents a novel species of the genus *Limnobacter*, for which the name *Limnobacter litoralis* sp. nov. is proposed.

Emended description of the genus *Limnobacter* Spring *et al.* 2001

Main characteristics are as given by Spring *et al.* (2001), with the following amendments. Growth occurs between 4 and 44 °C. The major ubiquinone is Q-8.

Description of *Limnobacter litoralis* sp. nov.

Limnobacter litoralis (li.to.ra′lis. L. masc. adj. *litoralis* of or belonging to the seashore, referring to the supralitoral habitat from which the type strain was isolated).

Cells are Gram-negative, aerobic, non-sporulating, slightly curved rods (0.4–0.6 μm wide and 1–3 μm long). Motile by a single polar flagellum. Colonies on 10-fold-diluted nutrient agar after 1 week at 30 °C are 0.5–1.0 mm in diameter, circular, complete, convex, opaque and white. Grows at 10−44 °C (optimum 38−42 °C) and pH 6.5−9.0 (optimum pH 7.0−7.5). Slightly halotolerant; grows with 0−8 % (w/v) NaCl (optimum 0.5 % NaCl). Oligotrophic; grows in 10- to 10 000-fold-diluted nutrient broth but not in undiluted nutrient broth. Chemolithoheterotrophic growth occurs by oxidizing thiosulfate to sulfate as an additional energy source in the presence of organic substrates such as succinate; the optimum concentration of thiosulfate is about 10 mM. Catalase, oxidase, arginine dihydrolase and urease activities are present. Nitrate is not reduced to nitrite. Carbohydrates and amino acids, including L-aspartate and L-glutamate, are not used. Several carboxylic acids are assimilated: acetate, β-hydroxybutyrate, DL-lactate, succinate, formate (weakly) and 2-oxoglutarate (weakly) are assimilated, but fumarate is not

assimilated. The major ubiquinone is Q-8. The major fatty acids are $C_{16:0}$, $C_{16:1}\omega7c$, $C_{18:1}\omega7c$ and $C_{10:0}$ 3-OH. The DNA G+C content of the type strain is 59 mol%.

The type strain is KP1-19^T (=LMG 24869^T =NBRC 105857^T =CIP 109929^T), isolated from a 22-year-old volcanic deposit at the Nippana onshore site on the island of Miyake, Japan.

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