**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 KP1-19T, was isolated from a 22-year-old volcanic deposit at a site lacking vegetation on the island of Miyake, Japan. Strain KP1-19T was able to use thiosulfate (optimum concentration 10 mM) as an additional energy source. 16S rRNA gene sequence analysis indicated that strain KP1-19T was closely related to *Limnobacter thiooxidans* CS-K2T 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 C16:0, C16:1ω7c and C18:1ω7c and minor amounts of C10:0 3-OH were also found. DNA–DNA relatedness between strain KP1-19T and *L. thiooxidans* LMG 19593T was 18 %. Therefore, strain KP1-19T represents a novel species, for which the name *Limnobacter litoralis* sp. nov. is proposed. The type strain is KP1-19T (=LMG 24869T =NBRC 105857T =CIP 109929T).

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-spor-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 C18:1ω7c, C16:1ω7c, C16:0 and C10:0 3-OH. Until now, only the type species, *Limnobacter thiooxidans*, has been described. Strain KP1-19T 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 KP1-19T did not grow chemolithoautotrophically with H2, O2 or CO2 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 KP1-19T 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.
such as recently formed volcanic deposits (King, 2007; King et al., 2008).

For phenotypic tests and chemotaxonomic and molecular systematic characterization, strain KP1-19\textsuperscript{T} was cultured in 10-fold-diluted nutrient broth (NB) [containing 0.1 % (w/v) meat extract (Kyokuto Seiyaku), 0.1 % (w/v) polypeptide (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\textsuperscript{−1}) at different temperatures and NaCl concentrations were determined by measuring optical density (OD\textsubscript{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, \textit{L. thiooxidans} LMG 19593\textsuperscript{T} was obtained from the BCCMLMG 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 ARVOx 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\textsuperscript{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\textsuperscript{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\textsuperscript{T} did not grow chemo-lithoautotrophically 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 \textit{Limnobacter} (Spring et al., 2001). Strain KP1-19\textsuperscript{T} did not grow at 8 or 46 °C and its optimum growth temperature was 38–42 °C, suggesting that strain KP1-19\textsuperscript{T} is more mesophilic than \textit{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\textsuperscript{T} could grow with 8 % but not with 9 % NaCl. Other characteristics of strain KP1-19\textsuperscript{T} are given in the species description and differences between strain KP1-19\textsuperscript{T} and \textit{L. thiooxidans} LMG 19593\textsuperscript{T} are shown in Table 1.

The cellular fatty acid profiles of strain KP1-19\textsuperscript{T} and \textit{L. thiooxidans} LMG 19593\textsuperscript{T} were very similar, the fatty acids in both strains being C\textsubscript{16:0} (39.9 and 24.2 %, respectively), C\textsubscript{16:1ω7c} (21.4 and 38.8 %), C\textsubscript{18:1ω7c} (20.0 and 27.3 %), C\textsubscript{10:0} 3-OH (4.9 and 2.7 %), C\textsubscript{18:0} (3.4 and 4.8 %) and C\textsubscript{14:0} (1.1 and 2.3 %). Strain KP1-19\textsuperscript{T} also contained two additional fatty acids, which were tentatively identified as C\textsubscript{17:0} cyclo (6.9 %) and C\textsubscript{19:0} (2.5 %). The major respiratory quinone of strain KP1-19\textsuperscript{T} was ubiquinone Q-8 and the DNA G+C content was 59 mol%, which was slightly higher than that reported for \textit{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\textsuperscript{T} and revealed that the isolate was associated with the family \textit{Burkholderiaceae} by phylogenetic analysis. In addition, Lu et al. (2008) showed that strain KP1-19\textsuperscript{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<sup>T</sup> and *L. thiooxidans* LMG 19593<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>KP1-19&lt;sup&gt;T&lt;/sup&gt;</th>
<th><em>L. thiooxidans</em> LMG 19593&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td>Growth at 4 °C</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Growth at 44 °C</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Arginine dihydrolyase</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Urease</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Assimilation of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Aspartate</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formate</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>Fumarate</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>–&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>β-Hydroxybutyrate</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>w</td>
<td>–</td>
</tr>
<tr>
<td>DNA G + C content (mol%)</td>
<td>59</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
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</table>

*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<sup>T</sup>. 16S rRNA gene sequence similarity between strain KP1-19<sup>T</sup> and *L. thiooxidans* LMG 19593<sup>T</sup> was 97.7%. DNA–DNA relatedness between strain KP1-19<sup>T</sup> and *L. thiooxidans* LMG 19593<sup>T</sup> was 18% (mean of two independent determinations: 20% with strain KP1-19<sup>T</sup> 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 genus species (Wayne et al., 1987).

On the basis of phenotypic, genotypic and phylogenetic analysis, it is concluded that strain KP1-19<sup>T</sup> 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.ri.a.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. Chemolithotrophic 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 dihydrolyase 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, di-lactate, succinate, formate (weakly) and 2-oxoglutarate (weakly) are assimilated, but fumarate is not.

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<sup>T</sup> was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.
assimilated. The major ubiquinone is Q-8. The major fatty acids are C_{16:0}, C_{16:1ω7c}, C_{18:1ω7c} and C_{10:0} 3-OH. The DNA G+C content of the type strain is 59 mol%.

The type strain is KPI-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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**References**


