Filomicrobium insigne sp. nov., isolated from an oil-polluted saline soil

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Strain SLG5B-19T, isolated from an oil-polluted saline soil in Gudao in the coastal Shengli Oilfield, eastern China, was Gram-negative with monoprosthecae or bipolar prosthecae and buds on the prosthecal tips. Growth occurred at NaCl concentrations between 0 and 7 % (w/v), at temperatures between 4 and 45 °C, and at pH 6.0–9.0. Strain SLG5B-19T had Q-9 as the major respiratory quinone and unsaturated C18:1ω7c as the predominant cellular fatty acid. The G+C content of the genomic DNA was 59.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain SLG5B-19T belonged to a clade with the genera Filomicrobium and Hyphomicrobium in the class Alphaproteobacteria. However, 16S rRNA gene sequence similarities of strain SLG5B-19T to the phylogenetically most closely related strains, i.e. the type strains of Filomicrobium fusiforme and Hyphomicrobium zavarzinii, were 95.8 and 94.5 %, respectively. In addition, the 16S rRNA gene sequence of strain SLG5B-19T had 24 signature nucleotides that were identical to those of the type strain of F. fusiforme. Based on phylogenetic analysis of 16S rRNA gene sequences, strain SLG5B-19T could be allocated to the genus Filomicrobium. However, distinct phenotypic differences were observed between strain SLG5B-19T and the type strain of F. fusiforme. It is therefore proposed that strain SLG5B-19T represents a novel species in the genus Filomicrobium, Filomicrobium insigne sp. nov. The type strain is SLG5B-19T (=CGMCC 1.6497T=LMG 23927T).

Bacteria that are able to form buds and/or possess prosthecae have been investigated in detail in terms of their chemotaxonomic characteristics and by phylogenetic analysis (Sittig & Hirsch, 1992; Stackebrandt et al., 1988). These organisms belong mainly to the genera Hyphomicrobium, Hyphomonas, Pedomicrobium and Filomicrobium. The genus Filomicrobium was created in 1987 and comprises only one species, Filomicrobium fusiforme, which was isolated from the brackish water of the Kiel Bight in Germany, which is part of the Baltic Sea (Schlesner, 1987). F. fusiforme produced poor growth on solid media. Microcolonies of 0.1–0.2 mm in diameter with a rough surface were observed after 3 weeks incubation at 25 °C. Filomicrobium strains are non-motile and do not use C1 compounds as carbon sources, characteristics that enable them to be differentiated from members of the genus Hyphomicrobium (Rainey et al., 1998). The genus Hyphomicrobium currently contains 12 recognized species and can be divided into two phylogenetic clusters by the genus Filomicrobium in the phylogenetic tree based on 16S rRNA gene sequence analysis (Rainey et al., 1998; also shown in Fig. 1). Species of the genus Hyphomicrobium are distributed widely in diverse environments (Morgan & Dow, 1985; Poindexter, 1992), including soil and groundwater (Hirsch & Rades-Rohkohl, 1983), freshwater ponds and lakes, brackish water, marine environments (Montreal Biodome) (Labbé et al., 2003), hypersaline Antarctic lakes (Hirsch & Siebert, 1991), sewage treatment plants (Meyers & Meyers, 1986) and even in a fluidized bed reactor of a drinking water treatment plant (Liessens, 1993).

In this study, the characterization of a novel strain, SLG5B-19T, isolated from an oil-polluted saline soil in a coastal oilfield in eastern China is reported. Results indicate that the strain represents a novel species of the genus Filomicrobium.

An oil-polluted soil was sampled at Gudao Oil-Product in the coastal Shengli Oilfield, Shandong Province, eastern China (Gu et al., 2007). The soil sample was incubated in
inorganic salts medium (Gu et al., 2007) amended with 2% crude oil at 30 °C for 2 weeks. Strain SLG5B-19 T was isolated from this enrichment by a 10-fold dilution plating technique with inorganic salts agar. The isolate was then purified by restreaking on inorganic salts agar plates incubated for 7–10 days at 30 °C.

The strain was grown on Luria–Bertani (LB) agar for about 7 days at 30 °C to late exponential phase; cell morphology and hyphal type were then examined using transmission and scanning electron microscopy. Gram staining and poly-β-hydroxybutyrate (PHB) staining with Sudan black (according to Smibert & Krieg, 1994) and cell motility (Dong & Cai, 2001) were determined. Optimum pH and temperatures for growth and NaCl requirements/tolerance were tested using medium 166 containing 1.0 g (NH₄)₂SO₄, 0.2 g MgSO₄, 7H₂O, 0.5 g NaH₂PO₄•H₂O, 1.55 g K₂HPO₄, 3.4 g methylamine hydrochloride, 0.2 ml trace element solution, 15.0 g agar and 1 l distilled water, pH 7.2. Inorganic salts medium was used to determine carbon source assimilation. Each filter-sterilized carbon source was added at a concentration of 0.2% (w/v) after the mineral base solution had been autoclaved and growth was then examined after incubation at 30 °C for 1, 7, 10 and 14 days. Oxidase activity of strain SLG5B-19 T was tested according to Smibert & Krieg (1994) and catalase activity was determined by using 3% (v/v) hydrogen peroxide solution (Dong & Cai, 2001). Denitrification was tested by growing the strain anaerobically in the presence of NO₃⁻ (Zumft, 1992). Hydrolysis of starch, gelatin and Tween 80, and urease activity were examined according to the procedures of Williams et al. (1983). Methyl red and Voges–Proskauer tests were performed by using methyl red and Barritt’s reagent (Barritt, 1936; Mata et al., 2002).

Nitrate and nitrite reduction were assessed as described by Lányi (1987). The following tests were carried out according to the procedures recommended by Mata et al. (2002): exopolysaccharide (EPS) production, ONPG test, indole production, oxidation/fermentation of D-glucose, hydrolysis of casein and DNA, phenylalanine deaminase, and lysine and ornithine decarboxylases. Sensitivity to 5 μg rifampicin, 10 μg amoxicillin, kanamycin, carbenicillin, tobramycin and streptomycin, 15 μg erythromycin, 30 μg cefoxitin, cefotaxime, chloramphenicol, kanamycin and nalidixic acid, 300 μg nitrofurantoin, polymyxin B, sulfamethoxazole and streptomycin, and 23.75/1.25 μg sulfamethoxazole/trimethoprim was tested on LB plates by using the method described by Cho & Giovannoni (2003).

Strain SLG5B-19 T was Gram-negative and motile. Cells were bean-shaped, rod-shaped or oval, 0.5–1.0 μm in width and 1.0–2.5 μm in length with monopolar or bipolar prosthecae and buds at the end of the prosthecae (Fig. 2). Small milky white colonies were formed with a diameter of 0.1–0.2 mm on LB agar and clumps and pellicles with wall-growth were observed in medium 166 liquid culture. Cells
of strain SLG5B-19<sup>T</sup> accumulated PHB. Growth occurred at 4–45 °C (optimum 28–30 °C), at pH 6.0–9.0 (optimum pH 7.0–7.5) and in 0–7 % (w/v) NaCl (optimum 1–2 %). Strain SLG5B-19<sup>T</sup> was positive for oxidase, catalase, nitrate reduction activity, PHB production, hydrolysis of aesculin and oxidation of D-glucose, but negative for hydrolysis of sucrose, D-glucose, lactate, L-glutamic acid and dextrin. Growth was observed with methyl chloride/methanol (2 : 1, v/v) and analysed by reverse-phase HPLC (Shim-pack, VP-ODS; Shimadzu). Strain SLG5B-19<sup>T</sup> contained C<sub>18 : 1</sub> <i>v</i> (76.7 %), C<sub>16 : 0</sub> (9.26 %), C<sub>19 : 0</sub> cyclo<sub>ω8c</sub> (4.44 %) and C<sub>14 : 0</sub> 3-OH (3.2 %) as the main fatty acids; <i>Hyphomicrobium zavarzinii</i> DSM 1566<sup>T</sup> contained C<sub>18 : 1</sub> <i>v</i> (80.6 %), C<sub>16 : 0</sub> (4.5 %) and C<sub>14 : 0</sub> 3-OH (3.5 %), but lacked C<sub>19 : 0</sub> cyclo<sub>ω8c</sub>. Cells of strain SLG5B-19<sup>T</sup> contained ubiquinone-9 (Q-9) as the major quinone.

Genomic DNA was extracted and purified by the method of Marmur (1961). The DNA G + C content was determined by thermal denaturation (Marmur & Doty, 1962) using DNA from <i>Escherichia coli</i> K-12 as a control. The DNA G + C content was 59.5 mol%. The 16S rRNA gene was amplified using the bacterial universal primer pair 8<sup>f</sup> (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492<sup>r</sup> (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy plasmid (Promega) according to the manufacturers’ protocols. Positive clones were screened by PCR using the vector-specific primers SP6 (5′-ATTAGTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy plasmid (Promega) according to the manufacturers’ protocols. Positive clones were screened by PCR using the vector-specific primers SP6 (5′-ATTAGTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy plasmid (Promega) according to the manufacturers’ protocols. Positive clones were screened by PCR using the vector-specific primers SP6 (5′-ATTAGTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). PCR products were purified using a QIAquick PCR purification kit (Qiagen) and cloned into pGEM-T Easy plasmid (Promega) according to the manufacturers’ protocols. Positive clones were screened by PCR using the vector-specific primers SP6 (5′-ATTAGTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991).
sequence (1445 bp) analysis revealed that strain SLG5B-19\(^T\) was a member of the class *Alphaproteobacteria* and had a close phylogenetic relationship with the type strains of species of the genera *Filomicrobium* and *Hyphomicrobium* (Fig. 1); it was in a clade with the type strain of *F. fusiforme* (95.8 % 16S rRNA gene sequence similarity). In comparison, the 16S rRNA gene sequence similarities of strain SLG5B-19\(^T\) with the type strains of *Hyphomicrobium* Table 1. Differential characteristics of strain SLG5B-19\(^T\) and related species of the genera *Hyphomicrobium* and *Filomicrobium*

<table>
<thead>
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<th>2</th>
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<td>Cell size (µm)</td>
<td>0.5–1.0 × 1.0–2.5</td>
<td>0.5–0.7 × 1.0–4.0</td>
<td>0.3–1.2 × 1.0–3.0</td>
<td>0.3–0.6 × 1.0–3.0</td>
<td>0.5–0.9 × 0.7–2.5</td>
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<td>Bean-shaped mother cells</td>
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<td>−</td>
<td>−</td>
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<td>30</td>
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<td>−</td>
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<tr>
<td>45 °C</td>
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<td>−</td>
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<tr>
<td>Maximum growth at pH&gt;7.5</td>
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<td>−</td>
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<td>+</td>
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<td>Succinate</td>
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<tr>
<td>Trehalose</td>
<td>+</td>
<td>−</td>
<td>ND</td>
<td>−</td>
<td>ND</td>
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<tr>
<td>DNA G+C content (mol%; T(_m))</td>
<td>59.5</td>
<td>61.9</td>
<td>61</td>
<td>60–61</td>
<td>65</td>
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</tbody>
</table>
sulfonivorans and Hyphomicrobium species in cluster II and cluster I were 90.7%, 92.2–92.6% and 93.7–94.5%, respectively. There are 27 'signature nucleotides' in the 16S rRNA gene that are reported to define Hyphomicrobium species and F. fusiforme (Rainey et al., 1998). Among them, 24 'signature nucleotides' of strain SLG5B-19T were the same as those of the type strain of F. fusiforme, less than 14 signature nucleotides were identical between strain SLG5B-19T and the Hyphomicrobium species in clusters I and II (Table 2). These results also support the placement of strain SLG5B-19T in the genus Filomicrobium, rather than the genus Hyphomicrobium.

Although the phenotypic characteristics of strain SLG5B-19T and F. fusiforme were distinctly different, phylogenetic analysis based on 16S rRNA gene sequences suggested that strain SLG5B-19T could be allocated to the Filomicrobium clade. A novel species of the genus Filomicrobium is therefore proposed, Filomicrobium insigne sp. nov., to accommodate strain SLG5B-19T.

### Description of Filomicrobium insigne sp. nov.

*Filomicrobium insigne* (in.sig'ne. L. neut. adj. insigne distinguished by a mark, remarkable, extraordinary, referring to the remarkable phylogenetic relationship between the type strain and members of the genera *Hyphomicrobium* and *Filomicrobium*).

Cells are Gram-negative, aerobic, motile, non-pigmented, 0.5–1.0 μm in width and 1.0–2.5 μm long with mono-prosthecae or bipolar prosthecae and buds on the prosthecal tips. Colonies on LB agar are milky white and 0.1–0.2 mm in diameter after incubation at 30 °C for 5–7 days. Accumulates PHB and produces clumps and pellets with wall-growth, but does not produce EPS after incubation in medium 166 broth at 30 °C for 5–7 days. Growth occurs at pH 6.0–9.0 (optimum pH 7.0–7.5), at NaCl concentrations between 0 and 7% (w/v) (optimum 1–2%), and at 4–45 °C (optimum 28–30 °C). Positive for oxidase, catalase, nitrate reductase activity, PHB production, hydrolysis of aesculin and oxidation of D-glucose, but negative for hydrolysis of gelatin, Tween 80, starch, casein and DNA, urease, phenylalanine deaminase, nitrite reductase activity, indole production, lysis, ornithine and arginine decarboxylases, and the ONPG, Voges–Proskauer and methyl red tests. Grows on methanol, formate, methylamine, HCl, acetate, ethanol, D-fructose, cellobiose and trehalose as sole carbon and energy sources. The following organic compounds are not utilized as carbon sources: maltose, L-arabinose, sucrose, D-erythrose, L-sorbosone, D-sorbitol, malonate, succrose, myo-inositol, D-mannitol, α-D-lactose, pyruvate, D-ribose, propionate, glycine, L-alanine, D-arabinose, D-glucose, lactate, L-glutamic acid and dextrin. The major quinone is Q-9 and the major fatty acids are C₁₈:₁ω7c, C₁₆:₀, C₁₉:₀ cyclo ω8c and C₁₄:₀ 3-OH. Inhibited by 5 μg rifampicin, 10 μg amoxicillin, kanamycin, carbenicillin, tobramycin and streptomycin, 15 μg erythromycin, 30 μg cefoxitin, cefotaxime, chloramphenicol and kanamycin, and 300 μg polymyxin B and streptomycin, but resistant to 30 μg nalidixic acid, 300 μg nitrofurantoin and sulfamethoxazole, and 23.75/1.25 μg sulfamethoxazole/trimethoprim.

The type strain, SLG5B-19T (=CGMCC 1.6497 =LMG 23927T), was isolated from an oil-polluted saline soil at Gudaio Oil-Product in the coastal Shengli Oilfield, Shandong Province, eastern China. The DNA G+C content of the type strain is 59.5 mol%.

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References


