Saccharospirillum salsuginis sp. nov., a gammaproteobacterium from a subterranean brine

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A novel Gram-negative, non-sporulating, moderately halophilic, facultatively alkaliphilic, catalase- and oxidase-positive, obligately aerobic bacterium, strain YIM-Y25T, was isolated from a subterranean brine sample collected from a salt mine in Yunnan, south-west China. Cells were spirilla, motile by monopolar flagella, with meso-diaminopimelic acid in the cell-wall peptidoglycan. Growth occurred with 1–15 % (w/v) NaCl (optimum 5 %), at pH 6.0–10.0 (optimum pH 8.0) and at 15–50 °C (optimum 35–40 °C). Ubiquinone Q-8 was the predominant respiratory quinone. Polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmonomethylamine and an unidentified phospholipid. The major cellular fatty acids were C18:1ω7c, iso-C16:0, C16:0 and C16:1. The genomic DNA G+C content was 58.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain YIM-Y25T was most closely related to the type strain of the sole recognized species of the genus Saccharospirillum, Saccharospirillum impatiens EL-105T (sequence similarity 97.0 %), and these two strains formed a robust lineage in the phylogenetic tree. The level of DNA–DNA relatedness between them was 12.6 %. The combination of phylogenetic analysis, phenotypic differences, chemotaxonomic properties and DNA–DNA hybridization data supported the view that this strain represents a novel species of the genus Saccharospirillum, for which the name Saccharospirillum salsuginis sp. nov. is proposed, with YIM-Y25T (=CCTCC AA 207033T =KCTC 22169T) as the type strain.

The genus Saccharospirillum was proposed by Labrenz et al. (2003) with Saccharospirillum impatiens as the sole recognized species. The genus was defined as Gram-negative, motile by monopolar flagella, non-sporulating, catalase- and oxidase-positive, aerobic–microaerophilic spirilla, with ubiquinone Q-8 as the predominant respiratory quinone and diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylmonomethylamine as the major polar lipids. In a recent study of the microbial diversity in the ancient salt deposit of the Yipinglang salt mine (25°18′ N 101°54′ E) in Yunnan, south-west China (Chen et al., 2007a, b), a moderately halophilic, facultatively alkalophilic strain, YIM-Y25T, was isolated from a subterranean brine sample. The results of our polyphasic taxonomic study indicated that this strain represents a novel species of the genus Saccharospirillum. Strain YIM-Y25T was isolated from a subterranean brine sample by plating 1:10 serial dilutions of the sample on Difco marine agar 2216 (MA) supplemented with 3 % (w/v) NaCl [containing approximately 5 % (w/v) NaCl in total; MA5] at 37 °C for 2 weeks. After primary isolation and purification, the isolate was kept as serial transfer cultures on MA5 slants, lyophilized cultures at 4 °C or deep-frozen cultures at −80 °C in Difco marine broth 2216 (MB) supplemented with 20 % (v/v) glycerol. The reference strain S. impatiens DSM 12546T was obtained from the DSMZ. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on MA5 (pH 8.0) at 37 °C. Cell morphology was examined by light microscopy. Gram staining was carried out by using the standard Gram reaction (Doetsch, 1981) combined with the KOH lysis test method (Gregersen, 1978). Flagella were stained according to the method of Leifson (Smibert & Krieg, 1981). Growth was tested at various temperatures (5–55 °C, in increments of 5 °C) on MA5 and at different
pH values (pH 5.0–11.0, in increments of 0.5 pH units) on MA5 as well as in MB supplemented with 3 % (w/v) NaCl. For pH endurance experiments, the following buffer systems were used: pH 5.0, 0.1 M citric acid/0.1 M sodium citrate; pH 6.0–8.0, 0.1 M KH₂PO₄/0.1 M NaOH; pH 9.0–10.0, 0.1 M NaHCO₃/0.1 M Na₂CO₃; pH 11.0, 0.1 M Na₂HPO₄/0.1 M NaOH. Tolerance of and requirement for salts were determined in nutrient broth (Difco; 3.0 g beef extract and 5.0 g peptone l⁻¹) at various concentrations of NaCl, MgCl₂.6 H₂O or KCl [0.5 % (w/v) and 1.0–20.0 % (w/v), in increments of 1.0 %] supplemented with modified artificial seawater, containing (l⁻¹) 5.94 g MgSO₄.7 H₂O, 4.53 g MgCl₂.6 H₂O, 0.64 g KCl and 1.3 g CaCl₂ (Lim et al., 2005), and on some other media as controls [MA, trypticase soy agar (Difco) and ISP medium 2 agar (Shirling & Gottlieb, 1966)]. Aesculin hydrolysis, nitrate reduction and H₂S and indole production were determined as recommended by Smibert & Krieg (1994). Hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, urea and xanthine were determined as described by Cowan & Steel (1965). Motility, growth under anaerobic conditions and catalase and oxidase activities were assessed as described previously (Chen et al., 2007a). Other enzyme activities were tested by using the API ZYM system and acid production tests were performed by using API 50CH strips (bioMérieux), according to the manufacturer’s instructions. All suspension media were supplemented with 5 % (w/v) NaCl and artificial seawater. Nutritional assays were performed using classical methods (Ventosa et al., 1982) in a modified Koser medium (Koser, 1923), containing (l⁻¹) 50 g NaCl, 2 g KCl, 0.2 g MgSO₄.7 H₂O, 1 g KNO₃, 1 g (NH₄)₂HPO₄ and 0.5 g KH₂PO₄. When testing amino acids as substrates, the basal medium contained neither KNO₃ nor (NH₄)₂HPO₄. Tests for organic compounds as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy were performed at a concentration of 0.2 % (w/v). Morphological, physiological and biochemical characteristics of strain YIM-Y25T are given in the species description and in Table 1.

DNA was isolated according to Hopwood et al. (1985) and the G+C content was determined by using the HPLC method (Mesbah et al., 1989). Genomic DNA extraction, PCR-mediated amplification of 16S rRNA gene and purification of PCR products were done as described previously (Cui et al., 2001). The resulting 16S rRNA gene sequence was compared to sequences obtained from public databases (GenBank/EMBL/DDBJ) to find the most closely related species. Phylogenetic analysis was performed by using the software package MEGA version 3.1 (Kumar et al., 2004) after multiple alignment of sequence data by Table 1.

**Table 1. Characteristics that differentiate strain YIM-Y25T from S. impatiens and Reinekea species**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<tbody>
<tr>
<td>Cell morphology</td>
<td>Spirilla</td>
<td>Spirilla</td>
<td>Curved rods</td>
<td>Rods</td>
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<td>Temperature for growth (°C)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>15–50</td>
<td>&lt;2.5–43</td>
<td>15–42</td>
<td>4–37</td>
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<tr>
<td>Optimum</td>
<td>35–40</td>
<td>16–33</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>NaCl concentration for growth (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>1–15</td>
<td>&lt;1–15</td>
<td>0.3–12</td>
<td>0.5–5</td>
</tr>
<tr>
<td>Optimum</td>
<td>5</td>
<td>2–6</td>
<td>ND</td>
<td>ND</td>
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<td>Production of:</td>
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<tr>
<td>Indole</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>H₂S</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Casein</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Gelatin</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Tween 80</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>Utilization of:</td>
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<tr>
<td>L-Arabinose</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<td>Citrate</td>
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<td>+</td>
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<td>L-Phenylalanine</td>
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<td>–</td>
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<tr>
<td>L-Proline</td>
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<td>–</td>
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<td>–</td>
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<td>DNA G+C content (mol%)</td>
<td>58.5</td>
<td>54–55</td>
<td>52.4</td>
<td>51.1</td>
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</table>

*Data from Pinhassi et al. (2007).
CLUSTAL X (Thompson et al., 1997). Distances (corrected by Kimura’s two-parameter model; Kimura, 1980) were calculated and two-parameter model was used for the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and parsimony (Kluge & Farris, 1969) trees (not shown) were generated by using the treeing algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA–DNA hybridization was carried out by using photobiotin-labelled DNA probes and microdilution wells as described by Ezaki et al. (1989). A microplate spectrophotometer (Gemini XPS; Molecular Devices) was employed for fluorescence measurements.

The DNA G+C content of strain YIM-Y25T was 58.5 mol%. We amplified a fragment of 1416 bp of the 16S rRNA gene. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM-Y25T was most closely related to the type strain of the sole recognized species of the genus Saccharospirillum, S. impatiens EL-105T, with a comparatively high 16S rRNA gene sequence similarity value of 97.0%. These two strains constituted an independent lineage in the phylogenetic tree (100 % similarity value of 97.0 %). The DNA–DNA relatedness between strain YIM-Y25T and S. impatiens EL-105T was 12.6 %, which is far below the threshold value of about 70 % that was recommended by Wayne et al. (1987) for assigning strains to the same species. Thus, the results of phylogenetic analysis and DNA–DNA hybridization showed that strain YIM-Y25T could be a member of the genus Saccharospirillum but that it did not belong to the currently recognized species of this genus.

The amino acids of a whole-cell hydrolysate were determined as described by Hasegawa et al. (1983). Polar lipids were extracted by the method of Minnikin et al. (1984) and identified by two-dimensional TLC and spraying with appropriate detection reagents (Minnikin et al., 1984; Komagata & Suzuki, 1987). Isoprenoid quinones were analysed by HPLC as described by Groth et al. (1996). Fatty acids were determined as described by Sasser (1990) by using the Microbial Identification System (Microbial ID) with cells grown in MB supplemented with 3% NaCl (pH 8.0) in flasks on a rotary shaker at 200 r.p.m. at 37 °C for 3 days. The chemotaxonomic data for strain YIM-Y25T were compatible with its assignment to the genus Saccharospirillum (Labrenz et al., 2003). The peptidoglycan of strain YIM-Y25T contained meso-diaminopimelic acid. The polar lipids consisted of diphostatidglycerol, phosphatidylethanolamine, phosphatidylycerol, phosphatidylmethylamine and an unidentified phospholipid. The strain contained Q-8 (98.2 %) as the predominant respiratory quinone and Q-9 (1.8 %) in trace amounts. The fatty acid profile of strain YIM-Y25T was similar to that of the type strain of S. impatiens (Labrenz et al., 2003), in that it possessed the predominant non-polar fatty acids C18:1ω7c, C16:0 and C16:1ω7c. The major fatty acids were C18:1ω7c (53.4 %), iso-C16:0 (13.3 %), C16:0 (11.4 %) and C16:1ω7c/C16:1ω6c (5.2 %), with summed feature 2 (C14:0 3-OH/iso-C16:1 ω7c; 3.8%), C17:1ω8c (2.3 %), iso-C14:0 3-OH (1.4 %), cyclo-C19:0ω8c (1.4 %), C17:1ω6c (1.2 %), C17:0 (1.2 %) and iso-C18:1 ω7c present in minor amounts and iso-C12:0 3-OH, C12:0 3-OH, C12:0 2-OH, C14:0, iso-C18:0 and C20:0 10ω7c present at levels below 1 %.

The results of phylogenetic analysis and chemotaxonomic studies presented above supported the affiliation of strain YIM-Y25T to the genus Saccharospirillum (Labrenz et al., 2003). However, the novel isolate differed markedly from

Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis and constructed using the neighbour-joining method showing the phylogenetic position of strain YIM-Y25T among phylogenetic neighbours. Numbers at nodes indicate bootstrap values (>50 %) based on neighbour-joining analysis of 1000 resampled datasets. p, Branch also recovered with the maximum-parsimony algorithm (Kluge & Farris, 1969); * branch also recovered with the maximum-likelihood (Felsenstein, 1981) and parsimony (Kluge & Farris, 1969) algorithms. Bar, 2 substitutions per 100 nucleotide positions.
the sole recognized *Saccharospirillum* species by its comparatively higher growth temperature range (up to 50 °C) and higher DNA G+C content (58.5 versus 54–55 mol%), by its ability to produce indole and hydrolyse Tween 80 but not to produce H2S or hydrolyse casein or gelatin and by its distinct pattern of utilization of carbon sources (Table 1). In addition, strain YIM-Y25T and *S. impatiens* could be clearly distinguished from members of the genus *Reinekea* by a number of phenotypic properties including discriminative cell morphology and the noticeable differences in DNA G+C content (Table 1) as well as by the comparatively low 16S rRNA gene sequence similarity and the stably separated position occupied by the genus *Reinekea* in the phylogenetic tree (Fig. 1). Together with the low level of DNA–DNA relatedness between strain YIM-Y25T and the type strain of *S. impatiens*, these results supported the proposal of strain YIM-Y25T as representing a novel species of the genus *Saccharospirillum*, *Saccharospirillum salsuginis* sp. nov.

**Description of *Saccharospirillum salsuginis* sp. nov.**

*Saccharospirillum salsuginis* (sal.su’gi.nis. L. gen. fem. n. salsuginis of/from brine).

Cells are Gram-negative, non-sporulating, catalase- and oxidase-positive, obligately aerobic spirilla (0.5–0.7 x 3.5–7.5 µm), motile with monopolar flagella. Colonies are circular, smooth, convex, translucent, white-beige-coloured and 2–3 mm in diameter after incubation for 3 days on MA supplemented with 3 % (w/v) NaCl at 37 °C. No diffusible pigments are produced. Moderately halophilic, mesophilic and facultatively alkaliphilic, with growth occurring in 1–15 % (w/v) NaCl (optimum 5 %) and at 15–50 °C (optimum 37 °C) and pH 6.0–10.0 (optimum pH 8.0). NaCl cannot be replaced by MgCl2·6H2O or KCl in media. Aesculin and Tween 80 are hydrolysed, but casein, gelatin, hyaluronidase, starch, Tween 20, 40 and 60, urea and xanthine are not. Nitrate is reduced to nitrite. Indole is produced, but H2S is not. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, raffinose, L-rhamnose, D-ribose, sucrose, trehalose, myo-inositol, D-mannitol, D-sorbitol, acetate, malate, pyruvate, L-alanine, L-asparagine, L-phenylalanine, L-proline and L-tryptophan. The following substances are not utilized: melezitose, D-sallicin, D-xylene, adonitol, glycerol, butyrate, citrate, formate, fumarate, gluconate, malonate, propionate, succinate, L-arginine, L-cystine, L-glucose, L-histidine, hydroxy-L-proline, L-leucine, L-lysine, L-methionine and L-valine. Acids are produced from L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, glycogen, lactose, maltose, D-mannose, L-rhamnose, D-ribose, D-sorbitol, sucrose and trehalose (API 50CH). Constitutively expressed enzymes are acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), esterase lipase (C8), ß-galactosidase, ß-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and valine arylamidase (API ZYM). The peptidoglycan contains meso-diaminopimelic acid. The main respiratory quinone is Q-8 (98.2 % in the type strain); Q-9 is present in trace amounts. Polar lipids consist of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylmonomethylamine and an unidentified phospholipid. The major cellular fatty acids are C18:1ω7c, iso-C16:0, C16:0 and C16:1ω7c/C16:1ω6c. The DNA G+C content of the type strain is 58.5 mol%.

The type strain, YIM-Y25T (=CCTCC AA 207033T =KCTC 22169T), was isolated from a subterranean brine sample collected from the Yipinglang salt mine in Yunnan, south-west China.

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**References**


319–322.


J Bacteriol colon-aerogenes group.


