# *Saccharospirillum aestuarii* sp. nov., isolated from tidal flat sediment, and an emended description of the genus *Saccharospirillum*

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A Gram-reaction-negative, chemoheterotrophic, non-motile, facultatively anaerobic, curved rod-shaped bacterial strain, IMCC4453$^T$, was isolated from tidal flat sediment and subjected to a taxonomic study using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain IMCC4453$^T$ belonged to the genus *Saccharospirillum*, forming a robust clade with members of the genus, and was most closely related to the type strains of *Saccharospirillum salsuginis* (97.6 % similarity) and *Saccharospirillum impatiens* (95.9 %). The DNA–DNA relatedness value between strain IMCC4453$^T$ and *S. salsuginis* YIM-Y25$^T$ was 23–30 %. Differences in several physiological and biochemical characteristics between strain IMCC4453$^T$ and the two recognized species of the genus *Saccharospirillum*, together with phylogenetic and genomic distinctiveness, differentiated the novel strain from members of the genus *Saccharospirillum*. On the basis of the data from the present study, it is concluded that strain IMCC4453$^T$ represents a novel species of the genus *Saccharospirillum*, for which the name *Saccharospirillum aestuarii* sp. nov. is proposed. The type strain is IMCC4453$^T$ (=KCTC 22684$^T$=KCCM 42930$^T$=NBRC 105825$^T$). An emended description of the genus *Saccharospirillum* is provided.

The genus *Saccharospirillum* (Labrenz *et al.*, 2003) belongs to the order *Oceanospirillales* (Garrity *et al.*, 2005) and, at the time of writing, comprises two recognized species, *Saccharospirillum impatiens* (Labrenz *et al.*, 2003) and *Saccharospirillum salsuginis* (Chen *et al.*, 2009). The genus accommodates Gram-negative, chemoheterotrophic, aerobic, motile by monopolar flagella, spirillum-shaped bacteria, which have Q-8 as the major respiratory quinone. The two recognized species of the genus *Saccharospirillum* were isolated from hypersaline environments; *S. impatiens* was isolated from a hypersaline Antarctic meromictic lake and *S. salsuginis* was cultured from a subterranean brine sample. During a survey of bacterial diversity in tidal flat sediments of the Yellow Sea, a *Saccharospirillum*-like bacterial strain, designated IMCC4453$^T$, was isolated and subjected to a taxonomic study using a polyphasic approach. The combined results of the present study showed that strain IMCC4453$^T$ represents a novel species of the genus *Saccharospirillum*.

Strain IMCC4453$^T$ was isolated as a pure culture from a tidal flat sediment sample collected off the coast of Kanghwa island (37° 36′ 07″ N 126° 29′ 10″ E), Korea. The sample was homogenized with 100 ml sterile seawater and spread onto an oligotrophic medium, R2A agar (BD Difco), diluted 1 : 10 (v/v) with aged seawater (hereafter 1/10R2A). Strain IMCC4453$^T$, initially grown on 1/10R2A, was further purified on marine agar 2216 (MA; BD Difco) after incubation of the agar plates at 25 °C for 5 days. For phenotypic comparisons between strain IMCC4453$^T$ and the two recognized species of the genus *Saccharospirillum*, *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany) and obtained from the Yunnan Institute of Microbiology (Kunming, China), respectively.

Genomic DNA was extracted by using a DNeasy tissue kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene was amplified by using primers 27F-B and 1492R, and was sequenced as described by Cho & Giovannoni (2004). The almost-complete 16S rRNA gene sequence (1457 bp) of strain IMCC4453$^T$ was compared with those available in GenBank (Benson *et al.*, 2008) by BLASTN searches (Altschul *et al.*, 1997) and it was apparent that the novel strain belonged to the order *Oceanospirillales*. The 16S rRNA gene sequence of strain IMCC4453$^T$ was aligned against those of its nearest neighbours in the order *Oceanospirillales* by using the ARB software package (Ludwig *et al.*, 2004) and sequence similarities were calculated based on this alignment in the

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC4453$^T$ is GQ250189.

A supplementary table and two supplementary figures are available with the online version of this paper.

ARB software and were confirmed on the EzTaxon server (Chun *et al.*, 2007). Based on 16S rRNA gene sequence similarity analyses, strain IMCC4453$^T$ was most closely related to *S. salsuginis* YIM-Y25$^T$ (97.6 %), *S. impatiens* DSM 12546$^T$ (95.9 %) and *Reinekea blandensis* MED297$^T$ (95.1 %).

To clarify the phylogenetic position of the novel strain further, unambiguously aligned nucleotide positions identified in the ARB package were employed for phylogenetic analyses in PAUP* 4.0 beta 10 (Swofford, 2002). Phylogenetic trees were generated by using the neighbour-joining method (Saitou & Nei, 1987), with Jukes–Cantor correction (Jukes & Cantor, 1969), and the maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. The robustness of the neighbour-joining and maximum-parsimony trees was confirmed by bootstrap analyses based on 1000 randomly generated trees. In all the phylogenetic trees generated in this study with the three treeing algorithms (Fig. 1), strain IMCC4453$^T$, *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ formed a robust clade showing high bootstrap values (100 % in the neighbour-joining tree and 97 % in the maximum-parsimony tree), indicating that strain IMCC4453$^T$ was a member of the genus *Saccharospirillum*.

As the level of 16S rRNA gene sequence similarity (97.6 %) between strain IMCC4453$^T$ and *S. salsuginis* YIM-Y25$^T$ exceeded the recommended threshold (97 %) for the delineation of bacterial genomic species (Stackebrandt & Goebel, 1994), DNA–DNA relatedness between the two strains was determined by membrane-based dot-blot hybridization. Pre-hybridization, hybridization, stringency washing and detection were performed by using a DIG-High Prime DNA Labelling and Detection Starter kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The level of DNA–DNA relatedness between strain IMCC4453$^T$ and *S. salsuginis* YIM-Y25$^T$ was 22 % (IMCC4453$^T$ as the probe) and 30 % (YIM-Y25$^T$ as probe). The level of 16S rRNA gene sequence similarity (95.9 %) between strain IMCC4453$^T$ and *S. impatiens* DSM 12546$^T$ and the low level of DNA–DNA relatedness between strain IMCC4453$^T$ and *S. salsuginis* YIM-Y25$^T$ indicated that the novel strain represented a separate genomic species in the genus *Saccharospirillum* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006).

To compare the phenotypic characteristics of strain IMCC4453$^T$, *S. impatiens* DSM 12546$^T$ and *S. salsuginis*

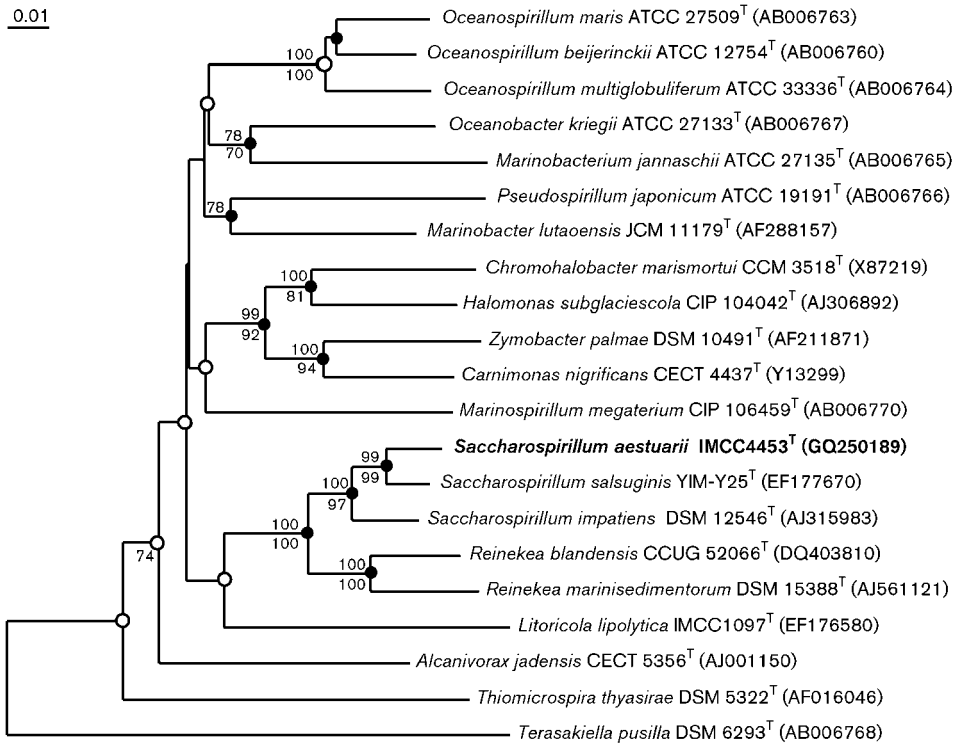


**Fig. 1.** Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain IMCC4453$^T$ and its neighbours in the order *Oceanospirillales*. Bootstrap values ($\geqslant$70 %) from both the neighbour-joining (above nodes) and maximum-parsimony (below nodes) methods are presented. Nodes recovered reproducibly by the neighbour-joining, maximum-parsimony and maximum-likelihood methods (filled circles) or by two of the three treeing methods (open circles) are indicated. Bar, 0.01 substitutions per nucleotide position.

YIM-Y25$^T$, all three strains were routinely grown on MA at 25 °C. Unless indicated otherwise, standard methods for phenotypic characterization were employed as described by Smibert & Krieg (1994). Morphology and size of the cells and colonies, Gram reaction, presence of flagella and intracellular granules, motility, temperature, pH and salinity ranges and optima for growth, catalase and oxidase activities, and ability to grow anaerobically were determined by the methods described by Yang *et al.* (2008) except that strain IMCC4453$^T$ was grown on MA or in marine broth (BD Difco) at 25 °C. Production of $H_2S$ was determined by using triple-sugar iron agar (BD Difco) with the salinity adjusted to 3.0 %. Hydrolysis of casein (10 % skimmed milk, w/v), starch (0.2 %, w/v), chitin (0.5 %, w/v), carboxymethylcellulose (CMC; 0.2 %, w/v) and Tween 80 (1.0 %, v/v) was tested by using MA as the basal medium. Degradation of DNA was tested by using DNase test agar (Difco) amended with 2.0 % NaCl. Other biochemical tests and substrate oxidation tests were carried out by using API 20NE, API ZYM and API 50CH test strips (bioMérieux) and Biolog GN2 microplates (Biolog) by inoculating cells into artificial seawater medium (Choo *et al.*, 2007). The following antibiotics (all purchased from Sigma-Aldrich) were tested by using the diffusion plate method: ampicillin (10 μg), chloramphenicol (25 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 μg), rifampicin (50 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg).

Morphological, physiological and biochemical characteristics of strain IMCC4453$^T$ are presented in the species description below and in Table 1. Strain IMCC4453$^T$ was a Gram-reaction-negative, chemoheterotrophic, non-motile, facultatively anaerobic, curved rod-shaped bacterium (see Supplementary Fig. S1 in IJSEM Online). Strain IMCC4453$^T$ could be clearly differentiated from the two recognized species of the genus *Saccharospirillum* by the absence of flagella, ability to grow anaerobically and low optimum temperature for growth. In addition, a number of phenotypic characteristics, including hydrolysis of macromolecules, enzyme activities and carbon source oxidation, differentiated strain IMCC4453$^T$ from the two recognized species of the genus *Saccharospirillum* (Table 1). The results of the phenotypic characterization of *S. salsuginis* YIM-Y25$^T$ and *S. impatiens* DSM 12546$^T$ observed in this study that were different from the original descriptions (Labrenz *et al.*, 2003; Chen *et al.*, 2009) are shown in Supplementary Table S1. These discrepancies might be due to the different incubation temperature and different suspension media used for the API ZYM, API 50CH and Biolog GN2 tests. Detailed information on the basal media for hydrolysis of macromolecules, incubation temperature and suspension media applied in the present study and the original studies are provided in Supplementary Table S1 (see IJSEM Online).

The DNA G+C content was determined by using HPLC with a Discovery C18 column (5 μm, 15 cm × 4.6 mm; Supelco) according to Mesbah *et al.* (1989). Fatty acid methyl esters of strain IMCC4453$^T$, *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ were extracted from fresh cultures grown on MA at 25 °C for 3 days and were identified by the Sherlock Microbial Identification System (MIDI). Polar lipids of strain IMCC4453$^T$ were extracted by using an integrated approach (Minnikin *et al.*, 1984) for co-extracting isoprenoid quinones and were identified by using two-dimensional TLC on silica gel thin layers as also described by Minnikin *et al.* (1984). The TLC plates were developed in chloroform/methanol/water (65 : 25 : 4, by volume) in the first direction, followed by chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by volume) in the second direction. Total polar lipids were detected by spraying with 10 % ethanolic molybdophosphoric acid followed by heating at 150 °C for 30 min. Specific functional group-containing lipids were detected with the following spraying reagents: ninhydrin for free amino groups, α-naphthol for sugars, periodate-Schiff for α-glycols and Dragendorff for quaternary nitrogen. The isoprenoid quinones were extracted by TLC according to Minnikin *et al.* (1984) and were analysed by HPLC (Collins, 1985). The DNA G+C content of strain IMCC4453$^T$ was 56.5 mol%, a 2.0–2.5 mol% difference from those of *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$. The respiratory quinones detected were ubiquinones, with Q-8 predominating and Q-9 present in minor amounts, in accordance with data for members of the genus *Saccharospirillum*. Strain IMCC4453$^T$ contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, lyso-phosphatidylethanolamine and several unidentified polar lipids (Table 1; Supplementary Fig. S2). The polar lipid profile of strain IMCC4453$^T$ was similar to those of *S. impatiens* and *S. salsuginis* (Labrenz *et al.*, 2003; Chen *et al.*, 2009) except for the presence of lyso-phosphatidylethanolamine. The cellular fatty acid profile of strain IMCC4453$^T$ showed large amounts of mono-unsaturated or saturated fatty acids, with summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$; 46.6 %) and $C_{16:0}$ (24.3 %) as the predominant components (Table 2). The fatty acid profiles of strain IMCC4453$^T$, *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ obtained in this study were similar in the proportions of the predominant components, although a larger amount (10.7 %) of $C_{19:0}$ cyclo $\omega8c$ was found in *S. salsuginis* YIM-Y25$^T$.

Based on phylogenetic data showing the robust clade formed by strain IMCC4453$^T$, *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ (Fig. 1) and similar chemotaxonomic characteristics (DNA G+C content, isoprenoid quinone content, profiles of polar lipids and fatty acids), strain IMCC4453$^T$ is assigned to the genus *Saccharospirillum*. However, strain IMCC4453$^T$ could clearly be differentiated from the two recognized species of the genus *Saccharospirillum* based on phenotypic, phylogenetic and genomic characteristics. Therefore, on the basis of the data presented, strain IMCC4453$^T$ is considered to represent a novel species of the genus *Saccharospirillum*,

**Table 1.** Characteristics that differentiate strain IMCC4453$^T$ from recognized species of the genus *Saccharospirillum*

Strains: 1, IMCC4453$^T$; 2, *S. salsuginis* YIM-Y25$^T$; 3, *S. impatiens* DSM 12546$^T$. All data are from this study except where indicated otherwise. None of the strains hydrolysed DNA, chitin or Tween 80. In API 20NE tests, all were positive for nitrate reduction, aesculin hydrolysis, gelatin liquefaction and β-galactosidase, but negative for indole production, acid production from glucose, and arginine dihydrolase and urease activities. In API ZYM tests, all were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and valine arylamidase, but negative for α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase activities. In API 50CH tests, all three strains produced acid from D-ribose, D-xylose, D-galactose, D-fructose, D-mannose, salicin, lactose, trehalose and turanose, but not from erythritol, D-arabinose, L-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, methyl α-D-mannopyranoside, inulin, raffinose, xylitol, D-tagatose, D-fucose, L-arabitol or potassium gluconate. In GN2 Biolog microplates, all three strains oxidized glycogen, *N*-acetyl-D-galactosamine, melibiose, turanose, D-galacturonic acid, D-alanine, L-threonine and inosine, but not Tween 40, Tween 80, *N*-acetyl-D-glucosamine, adonitol, D-arabitol, i-erythritol, *myo*-inositol, succinic acid monomethyl ester, formic acid, D-gluconic acid, α-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α-ketobutyric acid, malonic acid, D-saccharic acid, sebacic acid, L-alanyl glycine, L-aspartic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, DL-carnitine, γ-aminobutyric acid, urocanic acid, thymidine, DL-α-glycerol phosphate, α-D-glucose 1-phosphate and D-glucose 6-phosphate. All were susceptible to ampicillin, erythromycin, gentamicin, kanamycin, penicillin G, rifampicin, streptomycin and vancomycin, but resistant to tetracycline. +, Positive; −, negative; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; LPE, lyso-phosphatidylethanolamine; PG, phosphatidylglycerol.

| Characteristic | 1 | 2 | 3 |
|---|---|---|---|
| Cell morphology* | Curved rod | Spirillum | Spirillum |
| Temperature range (optimum, °C)* | 10–42 (25) | 15–50 (37) | 2.5–43 (16–27) |
| Flagella and motility* | − | + | + |
| Relation to oxygen* | Facultatively anaerobic | Obligately aerobic | Microaerophilic |
| $H_2S$ production† | − | − | + |
| Hydrolysis of:† | | | |
| Casein | + | − | + |
| CMC | + | − | − |
| Starch | − | + | + |
| Enzyme activities (API ZYM)† | | | |
| Acid phosphatase, α-galactosidase, β-glucosidase | + | + | − |
| Lipase, naphthol-AS-BI-phosphohydrolase | + | − | + |
| Cystine arylamidase, trypsin, α-chymotrypsin, α-glucosidase | − | + | − |
| Acid production from carbohydrates (API 50CH)† | | | |
| Glycerol, D-mannitol, D-sorbitol, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, ferric citrate, maltose, sucrose, melezitose, potassium 2-ketogluconate, potassium 5-ketogluconate | + | + | − |
| Glycogen, L-fucose | + | − | + |
| D-Glucose, inositol, *N*-acetylglucosamine, melibiose, D-lyxose | − | + | + |
| Cellobiose, D-arabitol | − | + | − |
| L-Rhamnose, starch, gentiobiose | − | − | + |
| Oxidation of (Biolog GN2):† | | | |
| α-Cyclodextrin, dextrin, L-fucose, methyl β-D-glucoside, acetic acid, citric acid, D-galactonic acid lactone, DL-lactic acid, succinamic acid, L-alaninamide, L-alanine, L-leucine, L-proline, uridine | + | + | − |
| Cellobiose, sucrose, propionic acid | + | − | + |
| D-Mannose, trehalose, succinic acid, putrescine, 2-aminoethanol | + | − | − |
| D-Galactose, lactulose, maltose, γ-hydroxybutyric acid, α-ketovaleric acid, L-ornithine, L-pyroglutamic acid, D-serine | − | + | + |
| L-Arabinose, D-fructose, gentiobiose, α-D-glucose, α-lactose, D-mannitol, D-psicose, raffinose, L-rhamnose, D-sorbitol, xylitol, pyruvic acid methyl ester, *cis*-aconitic acid, D-glucuronic acid, β-hydroxybutyric acid, α-ketoglutaric acid, bromosuccinic acid, glucuronamide, L-asparagine, L-histidine, hydroxy-L-proline, L-phenylalanine, L-serine, glycerol | − | + | − |
| D-Glucosaminic acid, quinic acid, L-glutamic acid, phenylethylamine, 2,3-butanediol | − | − | + |
| Susceptibility to chloramphenicol† | + | + | − |
| Polar lipids* | DPG, PE, PG, LPE | DPG, PE, PG | DPG, PE, PG |
| DNA G+C content (mol%)* | 56.5 | 54–55 | 58.5 |

*Data for *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ obtained from Labrenz *et al.* (2003) and Chen *et al.* (2009), respectively.
†Data for *S. impatiens* DSM 12546$^T$ and *S. salsuginis* YIM-Y25$^T$ obtained from this study.

for which the name *Saccharospirillum aestuarii* sp. nov. is proposed.

### Emended description of the genus *Saccharospirillum* Labrenz *et al.* 2003

The description of the genus *Saccharospirillum* is as given by Labrenz *et al.* (2003) with the following amendments. Cells are obligately aerobic, microaerophilic or facultatively anaerobic heterotrophs. Motility and presence of flagella are species-dependent.

### Description of *Saccharospirillum aestuarii* sp. nov.

*Saccharospirillum aestuarii* (aes.tu.a′ri.i. L. gen. n. *aestuarii* of a tidal flat).

Cells are Gram-reaction-negative, chemoheterotrophic, oxidase- and catalase-positive, non-motile, non-flagellated, facultatively anaerobic, non-pigmented, curved rods (0.5–1.0 × 1.4–2.6 μm). Cells do not contain poly-$\beta$-hydroxybutyrate granules. Colonies grown on MA at 25 °C for 5 days are 2–4 mm in diameter, circular, convex, opaque, smooth and whitish beige. Grows anaerobically, but aerobic growth is stronger. Grows at 10–42 °C (optimum, 25 °C), at pH 5.0–12.0 (optimum, pH 8.0) and in the presence of 0.5–10.0 % NaCl (optimum, 2.0–5.0 % NaCl). Hydrolyses casein and CMC, but not DNA, starch, chitin or Tween 80. $H_2S$ is not produced. Positive for nitrate reduction, aesculin hydrolysis, gelatin liquefaction and PNPG ($\beta$-galactosidase) in API 20NE tests, but negative for indole production, acid production from glucose, and arginine dihydrolase and urease activities. The major cellular fatty acids are summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$), $C_{16:0}$, iso-$C_{16:0}$ and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$). The major respiratory quinone is ubiquinone Q-8; Q-9 is present in minor amounts. The predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lyso-phosphatidylethanolamine. Other phenotypic characteristics are detailed in Table 1.

The type strain, IMCC4453$^T$ (=KCTC 22684$^T$=KCCM 42930$^T$=NBRC 105825$^T$), was isolated from tidal flat sediment of the Yellow Sea in Korea. The DNA G+C content of the type strain is 56.5 mol%.

**Table 2.** Cellular fatty acid composition (%) of strain IMCC4453$^T$ and the type strains of recognized species of the genus *Saccharospirillum*

Strains: 1, IMCC4453$^T$; 2, *S. salsuginis* YIM-Y25$^T$; 3, *S. impatiens* DSM 12546$^T$. −, Not detected; tr, trace (<1.0 %). All strains were grown on MA at 25 °C for 3 days. Fatty acids that represented <1.0 % in all the strains are not shown.

| Fatty acid | 1 | 2 | 3 |
|---|---|---|---|
| $C_{14:0}$ | tr | tr | 1.1 |
| $C_{16:0}$ | 24.3 | 18.0 | 23.0 |
| $C_{17:0}$ | tr | 1.6 | tr |
| $C_{18:0}$ | 1.1 | tr | tr |
| iso-$C_{16:0}$ | 7.9 | 4.5 | 2.9 |
| iso-$C_{18:0}$ | 1.5 | tr | tr |
| $C_{17:1}\omega6c$ | tr | 1.4 | − |
| $C_{17:1}\omega8c$ | tr | 3.5 | tr |
| $C_{17:0}$ cyclo | tr | 1.5 | 2.0 |
| $C_{19:0}$ cyclo $\omega8c$ | tr | 10.7 | 1.2 |
| Summed features* | | | |
| 2 | 4.2 | 3.7 | 6.9 |
| 3 | 6.7 | 8.0 | 11.6 |
| 7 | 1.4 | tr | tr |
| 8 | 46.6 | 41.4 | 46.0 |

*Summed features represent groups of two or more fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 comprised $C_{12:0}$ aldehyde, summed feature 3 comprised $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$, summed feature 7 comprised $C_{19:1}\omega6c$ and/or $C_{19:1}\omega7c$, and summed feature 8 comprised $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$.

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