Microlunatus ginsengisoli sp. nov., isolated from soil of a ginseng field

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A Gram-positive, aerobic, coccus-shaped, non-endospore-forming bacterium (Gsoil 633T) was isolated from soil from a ginseng field in Pocheon province in South Korea. The novel isolate was characterized in order to determine its taxonomic position. On the basis of 16S rRNA gene sequence similarities, strain Gsoil 633T was shown to belong to the family Propionibacteriaceae. The closest phylogenetic relative was Microlunatus phosphovorus DSM 19555T, with 96.1 % sequence similarity; the sequence similarity to other members of the family was less than 95.4 %. The isolate was characterized chemotaxonomically as having LL-2,6-diaminopimelic acid in the cell-wall peptidoglycan, MK-9(H4) as the predominant menaquinone and anteiso-C15:0, iso-C15:0 and iso-C16:0 as the major fatty acids. The G+C content of the genomic DNA was 69.8 mol%. The morphological and chemotaxonomic properties of the isolate were consistent with those of M. phosphovorus, but the results of physiological and biochemical tests allowed the phenotypic differentiation of strain Gsoil 633T from this species. Therefore, strain Gsoil 633T represents a novel species, for which the name Microlunatus ginsengisoli sp. nov. is proposed. The type strain is Gsoil 633T (=KCTC 13940T = DSM 17942T).

The genus Microlunatus was first described by Nakamura et al. (1995), and currently comprises just one species, Microlunatus phosphovorus. This species, which was isolated from activated sludge, is a Gram-positive, strictly aerobic, chemoorganotrophic, coccus-shaped bacterium that can accumulate polyphosphate. It possesses LL-2,6-diaminopimelic acid (LL-DAP) in the cell-wall peptidoglycan, MK-9(H4) as the predominant menaquinone and anteiso-C15:0, iso-C15:0 and iso-C16:0 as the major fatty acids.

Strain Gsoil 633T, which is phylogenetically close to Microlunatus phosphovorus, was isolated in the course of a study on the culturable aerobic bacteria community in soil from a ginseng field in Pocheon province in South Korea (Im et al., 2005). For the isolation of bacteria, a soil sample was suspended in 50 mM phosphate buffer (pH 7.0) and the suspension was spread on plates of one-fifth-strength MR2A agar [containing (l−1): 0.25 g tryptone, 0.25 g peptone, 0.25 g yeast extract, 0.125 g malt extract, 0.125 g beef extract, 0.25 g Casamino acids, 0.25 g soytone, 0.5 g glucose, 0.3 g soluble starch, 0.2 g xylan, 0.3 g sodium pyruvate, 0.3 g K2HPO4, 0.05 g MgSO4, 0.05 g CaCl2, 15 g agar] after being serially diluted with the same buffer. The plates were incubated at 30 °C for 1 month and single colonies on the plates were purified by transferring them onto new MR2A agar plates. Strain Gsoil 633T was routinely cultured on MR2A agar plates at 30 °C and maintained as a glycerol suspension (20 %, w/v) at −70 °C.

The Gram reaction was tested using the non-staining method, as described by Buck (1982). Cell morphology was observed with a light microscope (Nikon) (×1000 magnification) using cells grown for 24 and 72 h at 30 °C on MR2A broth. Catalase activity was determined by assessing bubble production in 3 % (v/v) H2O2, and oxidase activity was determined using 1 % (w/v) tetramethyl-p-phenylenediamine. To study the assimilation of sole carbon sources, a basal liquid medium was used, comprising the following (l−1): 1.8 g K2HPO4, 1.08 g KH2PO4, 0.5 g NaNO3, 0.5 g NH4Cl, 0.1 g KCl, 0.1 g MgSO4 and 0.05 g CaCl2. A vitamin solution (Widdel & Bak, 1992), trace element solution SL-10 (Widdel et al., 1983) and selenite/tungstate solution (Tschech & Pfennig, 1984) were added to

Abbreviation: LL-DAP, LL-2,6-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Gsoil 633T is AB245389.
this medium and the pH was adjusted to 6.8. Aliquots of this liquid medium were added to 96-well trays and then a different filter-sterilized carbon source was added to each well (0.1 %, w/v, in each case). Growth in 96-well plates incubated at 30 °C for up to 7 days was examined visually. The negative-control well did not contain any carbon sources, and the positive-control well contained MR2A broth. Some physiological characteristics were determined with API 20E galleries according to the instructions of the manufacturer (bioMérieux). The ability to grow under anaerobic conditions was tested in serum bottles containing MR2A broth supplemented with thioglycolate (1 g l⁻¹) under nitrogen gas. An anaerobic nitrate-reduction test designed to determine the final electron acceptor was performed in serum bottles, containing MR2A broth supplemented with thioglycolate (1 g l⁻¹) and KNO₃ (10 mM), in which the contents of the headspace had been replaced with nitrogen gas. Aerobic reduction of nitrate and nitrite was measured subsequently by inoculating, in each case, three serum bottles (25 ml) containing 12 ml R2A medium, and adding nitrate and nitrite (as KNO₃ and NaNO₂) at concentrations of 10 mM. The reduction of nitrate and nitrite was monitored with an ion chromatography (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion exchange column (Metrosep Anion Supp 4; Metrohm). Tests for the degradation of DNA, in which DNase agar (Scharlau) plates were flooded with 1 M HCl, casein, chitin, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987), xylan and cellulose (Ten et al., 2004), were performed and then evaluated after 15 days. Growth at different temperatures (4, 15, 20, 25, 30, 37, 42 and 50 °C) and at various pHs (pH 5.0–10.0, with increments of 0.5 pH units) was assessed after incubation for 5 and 15 days. Salt tolerance was tested after 5 days incubation in MR2A broth supplemented with 1–10 % (w/v) NaCl. Growth on nutrient agar, trypticase soy agar (Difco) and MacConkey agar was also evaluated, at 30 °C.

Extraction of genomic DNA was performed with a commercial genomic DNA-extraction kit (Solgent) and PCR-mediated amplification of the 16S rRNA gene and sequencing of the purified PCR product were carried out according to Kim et al. (2005). Full sequences of the 16S rRNA gene were compiled using SeqMan software (DNASTAR). The 16S rRNA gene sequences of representatives of related taxa were obtained from GenBank. Multiple alignments were performed by using the CLUSTAL_X program (Thompson et al., 1997). Gaps were edited in the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using a neighbour-joining method (Saitou & Nei, 1987) and maximum parsimony (Fitch, 1971), using the MEGA3 program (Kumar et al., 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985).

To measure the G+C content of the chromosomal DNA, genomic DNA from the novel strain was extracted and purified as described by Moore & Dowhan (1995) and then enzymically degraded into nucleosides; the determination was performed as described by Mesbah et al. (1989), using reversed-phase HPLC. Isoprenoid quinones were extracted with chloroform/methanol (2 : 1, v/v), evaporated under vacuum conditions and then re-extracted in n-hexane/water (1 : 1, v/v). The crude n-hexane–quinone solution was purified using silica Sep-Pak Vac cartridges (Waters) and subsequently analysed by HPLC as described previously (Hiraishi et al., 1996). The cellular fatty acids were determined for strain Gsoil 633T grown on trypticase soy agar for 7 days: saponification, methylation and extraction were performed according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed using a gas chromatograph (model 6890; Hewlett Packard) and identified using the Microbial Identification software package (Sasser, 1990). Diaminopimelic acid isomers in the cell-wall peptidoglycan were determined using TLC after hydrolysis with 6 M HCl at 100 °C for 18 h, as described by Komagata & Suzuki (1987).

The cells of strain Gsoil 633T were aerobic, Gram-positive, non-endospore-forming, non-motile and coccus-shaped. Colonies grown on MR2A agar plates for 3 days were smooth, circular, non-glossy, yellowish in colour and 1–2 mm in diameter. Physiological and biochemical characteristics of strain Gsoil 633T are summarized in the species description, and the distinguishing characteristics are compared with those of related type strains in Table 1.

The 16S rRNA gene sequence of strain Gsoil 633T determined in this study consisted of a continuous stretch of 1471 bp. Sequence-similarity calculations performed after the neighbour-joining analysis indicated that the closest relative of strain Gsoil 633T was M. phosphovorus DSM 10555T (96.1 %). Lower levels of similarity (<95.4 %) were found with sequences from all species in the family Propionibacteriaceae that have validly published names. This relationship between strain Gsoil 633T and members of the family Propionibacteriaceae was also evident in the phylogenetic tree (Fig. 1).

DNA–DNA hybridization between strain Gsoil 633T and the nearest phylogenetic neighbours was not attempted since strains differing by >3.0 % at the 16S rRNA gene level are unlikely to exhibit >70 % relatedness at the whole-genome level (Stackebrandt & Goebel, 1994).

The G+C content of the genomic DNA of strain Gsoil 633T was 69.8 mol%. MK-9(H₄) was the predominant respiratory menaquinone. The fatty acid content of strain Gsoil 633T was mainly composed of anteiso-C₁₅:0 (38.7 %), iso-C₁₅:0 (27.2 %), iso-C₁₆:0 (12.9 %), anteiso-C₁₇:0 (4.5 %), C₁₆:0 (3.4 %), iso-C₁₄:0 (2.4 %), iso-C₁₇:0 (2.3 %), a C₁₈:1 isomer (1.5 %) and anteiso-C₁₇:0 (1.5 %). The cell-wall peptidoglycan of strain Gsoil 633T contained LL-DAP.

In summary, the characteristics of strain Gsoil 633T are consistent with the description of the genus Microlunatus.
Table 1. Characteristics that differentiate strain Gsoil 633\textsuperscript{T} from \textit{M. phosphovorus}

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<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Gsoil 633\textsuperscript{T}</th>
<th>\textit{M. phosphovorus} NM-1\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature range for growth (°C)</td>
<td>20–30</td>
<td>5–35</td>
</tr>
<tr>
<td>Oxidase</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction under anaerobic conditions</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Utilization as a sole carbon source of:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Lactose</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Glycogen</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Inositol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Mannitol</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>69.8</td>
<td>67.9</td>
</tr>
</tbody>
</table>

with regard to morphological, biochemical and chemotaxonomic properties. On the basis of the relatively low 16S rRNA gene sequence similarities (<97%) and the combination of unique phenotypic characteristics (Table 1), Gsoil 633\textsuperscript{T} represents a novel species of the genus \textit{Microlunatus}, for which the name \textit{Microlunatus ginsengisoli} sp. nov. is proposed.

\textbf{Description of \textit{Microlunatus ginsengisoli} sp. nov.}

\textit{Microlunatus ginsengisoli} (gin.seng.i.so’li. N.L. n. ginsengum ginseng; L. n. solum soil; N.L. gen. n. ginsengisoli of soil of a ginseng field, the source of the type strain).

Cells are Gram-positive, strictly aerobic, non-motile, coccus-shaped and 0.5–0.8 \textmu m in diameter after 24 h culture on MR2A agar plates. Colonies grown on MR2A agar for 3 days are very small, smooth, circular, non-glossy, yellowish and convex. Grows well at 20–30 °C and at pH 5.5–8.5, but does not grow at, or below, 15 °C or above 37 °C. Grows on nutrient agar but not MacConkey agar. Growth on MR2A agar occurs in the absence of NaCl and in the presence of 4.0 % (w/v) NaCl, weakly with 5.0 % NaCl (w/v), but not with 6.0 % (w/v) NaCl. Catalase-positive and oxidase-negative. H\textsubscript{2}S is not produced. Nitrate, as a nitrogen source, is reduced under aerobic conditions, but nitrite is not. Anaerobic growth does not occur; nitrate is not reduced under anaerobic conditions. In API 20E tests, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase results are negative; results for \beta-galactosidase and gelatinase activities and for the Voges–Proskauer reaction are positive. No acid is produced from L-arabinose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose or amygdalin. D-Glucose is utilized as a sole carbon source, but neither acid nor gas is produced. The following compounds are utilized as sole carbon sources: L-rhamnose, D-fructose, D-lyxose, D-ribose, L-xylose, propionate, valerate, fumarate, salicin, lactate, malate, tartrate, sucrose, D-trehalose, D-raffinose, gluconate, D-adonitol, D-sorbitol, xylitol, amygdalin, inulin, dextran, alanine, asparagine, aspartate, histidine, phenylalanine,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{phylogenetic_tree.png}
\caption{Phylogenetic tree, based on a comparative analysis of 16S rRNA gene sequences, showing the relationships between strain Gsoil 633\textsuperscript{T} and related species. The tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter distance matrix (Kimura, 1983) and pairwise deletion. Filled circles indicate generic branches that were also recovered with the maximum-parsimony algorithm. Bootstrap values (expressed as percentages of 1000 replications) greater than 60% are shown at branch points. Bar, 2 substitutions per 100 nucleotide positions.}
\end{figure}
proline and tyrosine. The following compounds are not utilized as sole carbon sources: D-fucose, L-sorbose, D-arabinose, N-acetylglucosamine, formate, 3-hydroxybutyrate, caprate, maleic acid, phenylacetate, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, citrate, malonate, succinate, glutarate, itaconate, adipate, suberate, oxalic acid, arginine, cysteine, glutamate, glycine, glutamine, isoleucine, leucine, lysine, methionine, serine, threonine, tryptophan and valine. Xylan, chitin, cellulose, casein, olive oil, starch and DNA are not degraded. MK-9(H4) is the predominant menaquinone and anteiso-C15:0, iso-C15:0 and iso-C16:0 are the major cellular fatty acids. The G+C content of genomic DNA is 69.8 mol% (as determined by HPLC). The cell-wall peptidoglycan contains LL-DAP.

The type strain, Gsoil 633T ( = KCTC 13940T = DSM 17942T), was isolated from soil from a ginseng field in Pocheon province, South Korea.

Acknowledgements

This work was supported by the Key Project of the Chinese Ministry of Education (no. 206039) and by the 2006 research programme of the Rural Development Administration, Republic of Korea. We thank Jean Euzéby for his help with the etymology of the species epithet.

References


