Bacillus oleivorans sp. nov., a diesel oil-degrading and solvent-tolerant bacterium

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The genus Bacillus was described by Cohn in 1872 with Bacillus subtilis as the type species (Cohn 1872; Skerman et al., 1980). The genus Bacillus encompasses Gram-stain-positive, rod-shaped, endospore-forming, aerobic or facultatively anaerobic bacteria (Rheims et al., 1999). Members of the genus Bacillus are phenotypically and genotypically heterogeneous (Priest, 1993; Slepecky & Hemphill, 2006), and thereby exhibit a vast metabolic diversity and hence thrive in wide variety of habitats with variable ecological conditions (Takami, 2006; Subhash et al., 2014; Reddy et al., 2015). In the present study, two colonies designated JC228T and JC279, were characterized using a polyphasic taxonomic approach based on the minimal standards proposed for the aerobic endospore-formers (Logan et al., 2009) for description of new taxa.

Two colonies were observed on an uninoculated nutrient agar plate which was left undisturbed for 16 h after dispensing media. The source of contamination may be air at the time of dispensing medium into the Petri dishes. Both colonies were creamish-white. Axenic cultures of both isolates were designated strains JC228T and JC279 and were preserved as glycerol stocks and by lyophilization. Unless otherwise mentioned, both strains and Bacillus carboniphilus LMG 18001T (nearest phylogenetic neighbour) were grown in nutrient broth or on agar.

Genomic DNA was extracted and purified from strains JC228T and JC279 according to the method of Marmur (1961) and the DNA G+C content was 39 and 38.4 mol%, respectively, as determined by HPLC (Mesbah et al., 1989). Amplification and sequencing of the 16S rRNA gene was done as described previously (Subhash et al., 2014). Identification of phylogenetic neighbours and calculation of pairwise 16S
rRNA gene sequence similarity were achieved using the EzTaxon-e server (Kim et al., 2012). EzTaxon-e server search analysis revealed that strains JC228<sup>T</sup> and JC279 were most closely related to members of the genus Bacillus, and the highest 16S rRNA gene sequence similarity was observed with <i>B. carboniphilus</i> JCM 9731<sup>T</sup> (98.1 %) and <i>&lt;96.0 %</i> similarity was observed with other members of the genus Bacillus. The 16S rRNA gene sequence similarity between strains JC228<sup>T</sup> and JC279 was 99.2 %.

The SINA alignment service (http://www.arb-silva.de/) and CLUSTAL W algorithm of MEGA 6 software (Tamura et al., 2013) was used for phylogenetic analysis of the individual sequences. Distances were calculated by using the Kimura correction in a pairwise deletion manner (Kimura, 1980). Neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods in the MEGA 6 software package were used to reconstruct phylogenetic trees. Percentile support values were obtained using a bootstrap procedure based on 1000 replications. The results of phylogenetic analysis of the 16S rRNA gene sequences suggested that strains JC228<sup>T</sup> and JC279 clustered consistently with the genus <i>Bacillus</i> and formed a separate clade along with the type strain of <i>B. carboniphilus</i> (Fig. 1).

The taxonomic relationship between strains JC228<sup>T</sup>, JC279 and <i>B. carboniphilus</i> LMG 18001<sup>T</sup> was examined using DNA–DNA hybridization studies. The membrane-filter technique was used to determine genomic relatedness using a DIG high prime DNA labelling kit (Roche). A sample containing 0.5 µg DNA in 170 µl 100 mM Tris/HCl (pH 7.5), 30 µl 2 M NaOH and 100 µl 20× SSC buffer was added and then heated at 80 °C for 10 min and immediately cooled to room temperature. After denaturation, DNA was loaded on to a nitrocellulose membrane, as previously described (Seldin & Dubnau, 1985; Chakravarty et al., 2012). Hybridization was performed with three replications for each sample and the mean values quoted as DNA–DNA relatedness. The DNA–DNA reassociation value between strains JC228<sup>T</sup> and JC279 was 88 ± 2 %, while strain JC228<sup>T</sup> was only 23.4 ± 1 % related (based on DNA–DNA hybridization) to <i>B. carboniphilus</i> LMG 18001<sup>T</sup>; these hybridization values are within the recommended standards to delineate a bacterial species (Stackebrandt & Goebel, 1994).

Cellular fatty acids and polar lipids composition were analysed from cultures at the late exponential phase of growth. Fatty acid analysis was performed as described previously (Sasser, 1990; Subhash et al., 2014) and results are shown in Table S1 (available in the online Supplementary Material). The major fatty acids (≥6.3 %) were iso-C<sub>15 : 0</sub>, anteiso-C<sub>15 : 0</sub>, iso-C<sub>17 : 0</sub> and C<sub>16 : 0</sub> with minor amounts of anteiso-C<sub>17 : 0</sub>, iso-C<sub>14 : 0</sub> and C<sub>14 : 0</sub> and iso-C<sub>16 : 0</sub>. Though the fatty acid profile of strain JC279 was qualitatively identical to that of strain JC228<sup>T</sup>, minor differences in the relative amounts of iso-C<sub>16 : 0</sub> and iso-C<sub>17 : 0</sub> were observed. Strains JC228<sup>T</sup> and JC279 shared the presence of major fatty acids with the closely related type strain of <i>B. carboniphilus</i>. However, significant differences

![Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing phylogenetic relationships between strains JC228<sup>T</sup> and JC279 and closely related members of the genus Bacillus. The tree was reconstructed by the neighbour-joining method using MEGA 6 software and rooted by using Aneurinibacillus migulanus DSM 2895<sup>T</sup> as the outgroup. Numbers at nodes represent bootstrap values (based on 1000 resamplings); bootstrap percentages refer to NJ/ML/MP analysis. GenBank accession numbers are shown in parentheses. Bar, 1 nt substitution per 100 nt.](http://ijs.sgmjournals.org)
in the relative amounts of anteiso-C_{15:0} and iso-C_{16:0} were found between strains JC228<sup>T</sup> and JC279 and the type strain of <i>B. carboniphilus</i>.

Polar lipids were extracted from 1 g freeze-dried cells with methanol/chloroform/saline (2:1:0.8, by vol.) as described by Kates (1986). The lipids were separated using silica gel TLC (Kieselgel 60 F254; Merck) by two-dimensional chromatography using chloroform/methanol/water (65:25:4, by vol.) in the first dimension and chloroform/methanol/acetic acid/water (80:12:15:4, by vol.) in the second dimension (Tindall, 1990; Tindall et al., 1987; Oren et al., 1996). Total polar lipid profiles were detected by spraying with 5% ethanolic molybdophosphoric acid and further characterized by spraying with ninhydrin (specific for amino groups), molybdenum blue (specific for phosphates), Dragendorff’s reagent (for quaternary nitrogen) or x-naphthol (specific for sugars) (Kates, 1972; Oren et al., 1996).

Polar lipids of strain JC228<sup>T</sup> and <i>B. carboniphilus</i> LMG 18001<sup>T</sup> contained diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and an unidentified phospholipid PL2 as major lipids (Fig. S1), with minor amounts of unidentified phospholipid PL3. The polar lipids of strain JC228<sup>T</sup> differ from those of <i>B. carboniphilus</i> LMG 18001<sup>T</sup> in the presence of unidentified lipids L1 and L2, and the absence of an unidentified phospholipid PL1 and unidentified lipids L3, L4, L5 and L6 (Figs S1A and S1B). The polar lipid profiles of strains JC228<sup>T</sup> and JC279 are identical.

The peptidoglycan of cells of strains JC228<sup>T</sup>, JC279 and <i>B. carboniphilus</i> LMG 18001<sup>T</sup> were isolated after disruption of the cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The cell wall was hydrolysed for amino acid analyses as described by Schleifer & Kandler (1972). Amino acids in cell-wall hydrolysates were analysed by HPLC (McKerrow et al., 2000). Cell-wall amino acids of strains JC228<sup>T</sup>, JC279 and <i>B. carboniphilus</i> LMG 18001<sup>T</sup> were L-alanine, D-alanine, D-glutamic acid and meso-diaminopimelic acid. The diagnostic meso-diaminopimelic acid corresponds to those of other members of the genus Bacillus (Priest et al., 1988; Carrasco et al., 2007; Zhai et al., 2012; Amoozegar et al., 2013).

Morphological properties (cell shape, size and motility) of strains JC228<sup>T</sup> and JC279 grown in nutrient broth (Hi-media) were observed directly or after Gram-staining using an Olympus BH-2 phase-contrast microscope. Malachite green was used for spore staining as described by Schaeffer & Fulton (1933). On nutrient agar, colonies of JC228<sup>T</sup> and JC279 were round, 1–3 mm in diameter and creamish-white. Cells were straight to curved rods (2–8 μm long and 0.1–0.2 μm wide; Fig. S2A), Gram-stain-positive, divided by binary fission and formed oval-shaped terminal endospores. Strains JC228<sup>T</sup> and JC279 were non-motile, while the closest phylogenetic neighbour, <i>B. carboniphilus</i> LMG 18001<sup>T</sup>, was motile. The absence of motility was confirmed through the hanging-drop method, and negatively stained cells observed by transmission electron microscopy (H-7500; Hitachi) indicated the absence of flagella (Fig. S2B).

The pH range for growth was tested using nutrient broth, adjusted to different pH values (pH 4.0–11.0, intervals of 0.5 pH units) by using the appropriate biological buffers as described by Xu et al. (2005). The buffer systems used were: 0.1 M citric acid/0.1 M sodium citrate (for pH 4.0–5.0); 0.1 M KH<sub>2</sub>PO<sub>4</sub>/0.1 M NaOH (for pH 6.0–8.0); 0.1 M NaHCO<sub>3</sub>/0.1 M Na<sub>2</sub>CO<sub>3</sub> (for pH 9.0–10.0); 0.05 M Na<sub>2</sub>HPO<sub>4</sub>/0.1 M NaOH (for pH 11.0). Final pH was determined by using a pH indicator (Fisher scientific). The NaCl (0–10% (w/v) at 0.5% intervals) and temperature (4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C) ranges for growth were examined in nutrient broth and growth was measured turbidimetrically at 540 nm in a colorimeter (Systronics). Strains JC228<sup>T</sup> and JC279 grew at pH 7.0–9.5 (optimum pH 7.0) and differ from <i>B. carboniphilus</i> LMG 18001<sup>T</sup> which has a pH range of 6.0–9.0. NaCl was not required for growth of the two novel strains and they were able to tolerate up to 8% (w/v) NaCl, while <i>B. carboniphilus</i> LMG 18001<sup>T</sup> was less tolerant (Table 1). The optimum temperature for growth of strains JC228<sup>T</sup> and JC279 was 35 °C (range 25–40 °C); this temperature range further differentiates strains JC228<sup>T</sup> and JC279 from the closest phylogenetic neighbour.

Various biochemical tests such as hydrolysis of starch, casein, gelatin and Tween 80; oxidase, catalase and urease activity; nitrate and nitrite reduction; H<sub>2</sub>S production; acid and gas production from carbohydrates; and methyl red and Voges–Proskauer tests were performed by the procedures outlined in Cappuccino & Sherman (1998). Phenylalanine deaminase, ornithine decarboxylase, lysine decarboxylase and aesculin hydrolysis were determined as described by Smibert & Krieg (1981). Arginine dihydrolase activity was determined by applying method one as described by Smibert & Krieg (1981). Oxidation of various organic substrates for growth was tested by using GP2 MicroPlates (Biolog) in accordance with the manufacturer’s instructions. Utilization of organic carbon compounds as carbon and energy sources for organo-heterotrophic growth was also tested in a mineral medium as previously described (Lakshmi et al., 2011) containing specific organic compound (0.35%, w/v or v/v) to replace sodium pyruvate. The organic compounds tested include acetate, arabinose, benzoate, capric acid, citrate, ethanol, formate, fructose, fumarate, glucose, glycerol, glycolate, gluconate, glutamate, lactate, malate, mannitol, N-acetylcysteine, proline, pyruvate, succinate, sorbitol, sucrose, tartrate and valerate. Growth was measured turbidimetrically (OD at 540 nm) after 48 h. Nitrogen source utilization was tested by replacing ammonium chloride with different nitrogen sources (NaNO<sub>3</sub>, NaNO<sub>2</sub>, glutamate, aspartate, glutamine and urea). Utilization of glutamate, glutamine, methionine, aspartate, peptone, Casamino acids and urea as sole source of carbon, nitrogen and energy was also determined.

Strains JC228<sup>T</sup> and JC279 grew chemoorganoheterotrophically. In contrary to <i>B. carboniphilus</i> LMG 18001<sup>T</sup>,
strains JC228\textsuperscript{T} and JC279 were negative for hydrolysis of starch and Tween 80. All three strains (JC228\textsuperscript{T}, JC279 and \textit{B. carboniphilus} LMG 18001\textsuperscript{T}) were positive for oxidase and catalase activities, and hydrolysis of casein and gelatin. Arginine dihydrolase, phenylalanine deaminase, lysine decarboxylase and ornithine decarboxylase were absent in all three strains. All strains were negative for nitrate and nitrite reduction, indole and H$_2$S production, and acid production from galactose, ribose, glucose, cellobiose, tagatose and salicin. Acetate, benzoate, ethanol, formate, fructose, fumarate, glycerol, glycolate, glutamate, mannitol, proline, succinate, sucrose and tartrate were not used as sole sources of carbon and energy by all three strains. Both strains JC228\textsuperscript{T} and JC279 could grow using citrate, glucose, sorbitol, valerate, pyruvate and capric acid as sole carbon and energy sources. Malate and gluconate could support the growth of strain JC279 and \textit{B. carboniphilus} LMG 18001\textsuperscript{T} but not strain JC228\textsuperscript{T} when used as sole source of carbon and energy. Other phenotypic and biochemical characteristics of strains JC228\textsuperscript{T} and JC279 are presented in the species description and Table 1.

Solvent tolerance of strains JC228\textsuperscript{T} and JC279 was monitored in the presence of solvents with varied log $P$-values (log $P$-value is the partition coefficient of the given solvent in an equimolar mixture of octanol and water, which is a measure of solvent toxicity), including acetophenone (log $P=1.5$), benzene (log $P=2.0$), toluene (log $P=2.5$), xylene (log $P=3.2$) and hexane (log $P=3.4$). Log $P$-values between 1.5 and 4 are considered exceptionally toxic to micro-organisms (Ramos et al., 2002). Strains were grown in 250 ml Erlenmeyer flasks containing 100 ml nutrient liquid medium overlaid with 5 % (v/v) respective organic solvents and incubated for 60 h at 35 °C. The flasks were plugged with butyl rubber stoppers to prevent solvent evaporation. Bacterial cultures growing in the absence of organic solvent under similar conditions served as a positive control. Cell growth was monitored by measuring the dry cell weight. For measuring the dry cell mass, solvent-grown culture broth was centrifuged at 10 000 g at 4 °C for 10 min to pellet the cell mass. The pellet was then washed twice with sterile distilled water and dried under vacuum. Despite the toxicity exerted by the solvents, strains JC228\textsuperscript{T} and JC279 could grow in the presence of acetophenone, benzene, toluene, xylene and hexane. However, growth in solvent medium with organic solvents was considerably less and the dry cell mass of strain JC228\textsuperscript{T} grown in the presence and absence of solvents is depicted in Fig. S3.

Strains JC228\textsuperscript{T} and JC279 grown in 250 ml conical flasks containing 100 ml mineral salts media [consisting (g l$^{-1}$): Table 1. Differential characteristics between strains JC228\textsuperscript{T} and JC279 and their closest phylogenetic neighbour \textit{B. carboniphilus} LMG 18001\textsuperscript{T}

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.1–0.2 × 2–8</td>
<td>0.5–0.9 × 3.0–5.0</td>
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<tr>
<td>Motility</td>
<td>−</td>
<td>+</td>
<td></td>
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<tr>
<td>NaCl tolerance range (optimum) (%)</td>
<td>0–8 (0–1)</td>
<td>0–6 (0.5)</td>
<td></td>
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<tr>
<td>Temperature range for growth (optimum) (°C)</td>
<td>25–40 (35)</td>
<td>20–50 (40)</td>
<td></td>
</tr>
<tr>
<td>pH range for growth (optimum)</td>
<td>7.0–9.5 (7.0)</td>
<td>6.0–9.0 (8.0)</td>
<td></td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Starch</td>
<td>−</td>
<td>+</td>
<td></td>
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<tr>
<td>Tween 80</td>
<td>−</td>
<td>+</td>
<td></td>
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<tr>
<td>Organic substrate utilization for growth</td>
<td></td>
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<tr>
<td>L-Arabinose</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Capric acid</td>
<td>+</td>
<td>−</td>
<td></td>
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<tr>
<td>Citrate</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Glucose</td>
<td>+</td>
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<tr>
<td>Gluconate</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Lactate</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Malate</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<tr>
<td>N-Acetylglucosamine</td>
<td>−</td>
<td>+</td>
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<tr>
<td>Sorbitol</td>
<td>+</td>
<td>−</td>
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<tr>
<td>Valerate</td>
<td>+</td>
<td>−</td>
<td></td>
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<tr>
<td>Acid production from D-mannose</td>
<td>+</td>
<td>−</td>
<td></td>
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<tr>
<td>Gas production from D-mannose</td>
<td>+</td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>39</td>
<td>38.4</td>
<td>36</td>
</tr>
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</table>
Cells were harvested by centrifugation (10,000 g for 15 min at 4 °C). The diesel oil in the supernatant was extracted with 50 ml dichloromethane (DCM) and collected by centrifugation at 10,000 g for 10 min at 4 °C. The DCM fraction containing diesel oil was concentrated and the extracted constituents were analysed by GC-MS. A control containing diesel oil and no cells was used for determination of background baseline of growth and degradation. GC-MS analysis was done using a Pegasus HT TOF-MS system (Leco) equipped with an Agilent series (7890) gas chromatograph. One microtitre of sample was injected into HP-5 column (30 m, internal diameter 0.32 mm, thickness 0.25 μm), with helium as carrier gas at a constant flow of 1.2 ml min⁻¹. The initial oven temperature of 60 °C was ramped to 300 °C at 3 °C min⁻¹ and held for 5 min. Inlet temperature was 250 °C, ion source temperature 250 °C and ionization energy −70 eV. Mass spectra were recorded at 50–1000 m/z. LecoChromaTOF software (version 4.21) was used to process the chromatograms and the metabolites were identified based on mass spectral comparison to a standard NIST (National Institutes of Standards and Technology) 98 library. Mass spectral peaks showing similarity above 700 were accepted, with maximum match equal to 1000 for statistical analysis. Based on the GC-MS analysis of diesel layer, various hydrocarbons were seen to decrease in abundance sequentially based on their carbon chain length and branching. Strains JC228ᵀ and JC279 degraded almost all the constituents of diesel oil (C10–C28) which suggests that they can be efficiently used for the treatment processes for various diesel oil-contaminated sites. GC-MS profiles of various hydrocarbons present in diesel oil and its degradation observed after 5 days by strain JC228ᵀ is depicted in Fig. S4.

Strains JC228ᵀ and JC279 were distinct from their closest phylogenetic neighbour, B. carbonihilus LMG 18001ᵀ, with respect to cell size, motility, NaCl tolerance range, temperature and pH range for growth, organic substrate utilization, fatty acid and polar lipid profiles, organic hydrocarbon degradation and genomic DNA G+C content (Table 1). Hence these two strains are considered to represent a novel species of the genus Bacillus, for which we propose the name Bacillus oleivorans sp. nov.

### Description of Bacillus oleivorans sp. nov.

Bacillus oleivorans [o.lei.vor.ans. L. n. oleum oil; L. v. vorare to devour; N.L. part. adj. oleivorans capable of utilizing oil (diesel oil i.e. hydrocarbons)].

Colonies are translucent, circular with entire margin and texture is moist with raised elevation. Forms creamish-white colonies on nutrient agar, measuring 1–3 mm in diameter. Cells are straight to curved rods (0.1–0.2 μm x 2–8 μm), Gram-stain-positive, non-motile and multiply by binary fission. Forms terminal endospore of oval shape (0.5–0.6 μm x 0.9–1.0 μm) in a swollen sporangium. Obligate aerobe. Able to degrade diesel oil and thrives in presence of various organic solvents with varied log P-values (the partition coefficient of the given solvent in an equimolar mixture of octanol and water). The temperature range for growth is 25–40 °C (optimum 35 °C). Growth occurs in the presence of NaCl at concentrations up to 8 % (w/v) (optimum 0–1 %) and at pH 7.0–9.5 (optimum pH 7.0). Positive result in tests for catalase and oxidase activities, and hydrolysis of casein and gelatin, but negative result for hydrolysis of aesculin, starch, Tween 80 and urea. Arginine dihydrolase, phenylalanine deaminase, lysine decarboxylase and ornithine decarboxylase activities, methyl red and Voges–Proskauer tests, and production of H₂S are negative. Indole is not produced from tryptophan. Negative result in tests for acid production from galactose, ribose, glucose, cellobiose, tagatose and salicin. Acid and gas are produced from D-mannose. Can grow using citrate, glucose, sorbitol, valerate, pyruvate and capric acid as sole carbon and energy sources. Oxidizes 2, 3-butanediol, β-hydroxybutyric acid, γ-hydroxybutyric acid, methyl pyruvate and monomethyl succinate. L-Alanine glycine, α-cyclodextrin, dextrin, glycy1-glutamic acid, inosine, myo-inositol, α-lactose, lactulose, palatinose, sedoheptulose, stachyose, trehalose, turanose, uridine and xylitol are not oxidized. Ammonium chloride is used as sole nitrogen source; cannot use glutamine or glutamate as sole carbon, nitrogen and energy source. Polar lipid profile includes D PG, PG, PE, unidentified phospholipids (PL2, PL3) and unidentified lipids (L1, L2). Predominant fatty acids are iso-C₁₅:₀, anteiso-C₁₅:₀, iso-C₁₇:₀ and C₁₆:₀ along with anteiso-C₁₇:₀, iso-C₁₄:₀, C₁₄:₀ and iso-C₁₆:₀ in minor quantities. Cell-wall peptidoglycan contains L-alanine, D-glutamic acid and meso-diaminopimelic acid.

The type strain JC228ᵀ (=LMG 28084ᵀ=CCTCC AB 2013353ᵀ) was isolated from a contaminated laboratory Petri dish. The DNA G+C content of the type strain is 39 mol%. An additional strain of the species, JC279, was isolated from the same source and has a DNA G+C content of 38.4 mol%.

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### References

Bacillus oleivorans sp. nov.


