Diaphorobacter polyhydroxybutyrativorans sp. nov., a novel poly(3-hydroxybutyrate-co-3-hydroxyvalerate)-degrading bacterium isolated from biofilms

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A novel Gram-stain-negative, facultatively aerobic and rod-shaped strain, designated SL-205T, was isolated from the biofilms of a denitrifying reactor using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) as the sole carbon source in Beijing, PR China. A polyphasic taxonomic characterization was performed on the novel isolate. Phylogenetic analyses based on the 16S rRNA gene sequence revealed that strain SL-205T is a member of the genus Diaphorobacter. High levels of 16S rRNA gene sequence similarity were found between strain SL-205T and Diaphorobacter nitroreducens NA10BT (99.4 %) and Diaphorobacter oryzae RF3T (98.5 %), respectively. However, the DNA–DNA relatedness values between strain SL-205T and D. nitroreducens NA10BT and D. oryzae RF3T were 57 ± 1 % and 45 ± 1.5 %, respectively. The G + C content of the genomic DNA of strain SL-205T was 66.8 mol%. The major fatty acids consisted of summed feature 3 (including C16 : 1v7c and/or iso-C15 : 02-OH), C16 : 0 and C18 : 1v7c. Ubiquinone Q-8 was the only respiratory quinone; the polar lipid profile comprised phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and one uncharacterized phospholipid. We conclude that strain SL-205T represents a novel species of the genus Diaphorobacter for which the name Diaphorobacter polyhydroxybutyrativorans is proposed; the type strain is SL-205T (=ACCC 19739T=DSM 29460T).

The genus Diaphorobacter was first proposed for strains isolated from activated sludge (Khan & Hiraishi, 2002). The genus was described as accommodating aerobic, Gram-negative, catalase-positive, denitrifying bacteria. At the time of writing, there are four recognized species of the genus Diaphorobacter: Diaphorobacter nitroreducens (Khan & Hiraishi, 2002), Diaphorobacter oryzae (Pham et al., 2009), Diaphorobacter aerolatus (Kim et al., 2014) and ‘Diaphorobacter ruginosibacter’ (Wei et al., 2015), which were isolated from activated sludge, paddy soils, air and soybean root nodule, respectively. In the present study, a novel strain, designated SL-205T, was isolated from a solid-phase denitrifying reactor and some of its genotypic and phenotypic properties were investigated. The results of a polyphasic taxonomic characterization indicated that this strain represents a novel species of the genus Diaphorobacter.

Strain SL-205T was isolated from the biofilms of a denitrifying reactor using poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) as carbon source, in which the inoculum (active sludge) was collected from a sequencing batch reactor for treating swine manure wastewater in the Fangshan District of Beijing, PR China. The cylindrical plexiglass reactor (6 cm inner diameter and 50 cm height) packed with PHBV granules was inoculated with 20 ml activated sludge. Continuous experiments operated with an up-flow mode using tap water containing approximately 50 mg nitrate-nitrogen l−1 as the inlet water (Xu et al.,

Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); PM, phenotype microarray

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SL-205T is JX974341.

Three figures and one table are available with the online Supplementary Material.
2013). After 48 days of continuous running, the supernatant in the reactor was discarded and approximately 10 g of wet PHBV granules were aseptically transferred to a flask with 100 ml sterile water and 50 small glass balls. To suspend the biofilms attached on the granules, the flask was shaken on a rotary shaker (THZ-C) at 150 r.p.m. for 30 min. Serial dilutions were plated on denitrifying medium (DM; 1 L: 2 g KNO₃, 0.2 g MgSO₄·7H₂O, 0.5 g K₂HPO₄, 0.3 g NH₄Cl, 15 g agar, pH 7.2, supplemented 5 g PHBV powder). Inoculated plates were incubated at 28 ℃ for 1 week. Single colonies with transparent circles were purified by streaking onto Luria–Bertani (LB) medium plates, which were then incubated for 3 days at 28 ℃. Among the isolates, a colony which was circular, yellow, smooth-surfaced and 1–2 mm in diameter was designated SL-205ᵀ. The purified isolate was preserved at −80 ℃ as suspensions in DM containing 30 % (v/v) glycerol. The type strains D. nitroreducens DSM 15985 (NA10BTᵀ), D. oryzae DSM 22780 (RF3ᵀ) and D. aerolatus KACC 16536 (8604S-37ᵀ) were used as reference strains for comparative studies. D. nitroreducens NA10BTᵀ and D. oryzae RF3ᵀ were bought from DSMZ, while D. aerolatus 8604S-37ᵀ was kindly supplied by Professor Kwon (National Academy of Agricultural Science, Republic of Korea).

For phylogenetic analysis, the genomic DNA of strain SL-205ᵀ was extracted, from 800 ml of a 24 h culture at 28 ℃ in LB medium, by the method of Marmur (1961). The 16S rRNA genes were amplified by using genomic DNA as a template and the universal primers 27F and 1492R (Weisburg et al., 1991). Purified PCR products of approximately 1.5 kb in length were cloned to pGEM-T (Promega) and sequenced by Sangon Biotech (Beijing, PR China). Partial 16S rRNA gene sequences were compiled by using SeqMan software (DNASTAR) and these were compared with those retrieved from the DDBJ/EMBL/GenBank databases. Multiple alignments of the sequence were performed using SINA 1.2.11 (SILVA) (Pruesse et al., 2012), then phylogenetic analysis was performed using MEGA 6.06 software (Tamura et al., 2013). Phylogenetic trees were reconstructed based on the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods by using the bootstrap test of phylogeny with 1000 replicates. Phylogenetic analysis of strain SL-205ᵀ revealed that it belongs to the class Betaproteobacteria. Pairwise 16S rRNA gene sequence similarities were calculated using the EzTaxon-e sever (Kim et al., 2012). The highest level of 16S rRNA gene sequence similarity was found with D. nitroreducens NA10BTᵀ (99.4 %). The 16S rRNA gene sequence of strain SL-205ᵀ also showed a similarity of 98.5 % to D. oryzae RF3ᵀ, 96.5 % to D. aerolatus 8604S-37ᵀ and 96.8 % to ‘Diaphorobacter ruginosibacter’ BN30. Strain SL-205ᵀ showed less than 96.8 % (<97 %) sequence similarities with the type strains of other species in family Comamonadaceae. In the neighbour-joining, maximum-likelihood and maximum-parsimony phylogenetic trees (Fig. 1), strain SL-205ᵀ was grouped in the genus Diaphorobacter with a high bootstrap value (99 %).

Gram staining was determined by using the bioMérieux Colour Gram 2 kit (bioMérieux), according to the manufacturer’s instructions. Cell morphology of colonies grown on LB agar for 2 days was examined with a light microscope (CF21FS1, Olympus), scanning electron microscope (S-3400N, Hitachi) (Fig. S1, available in the online Supplementary Material) and by transmission electron microscopy (JEM-1400, JEDL) (Fig. S2). Cells were rods of variable size (0.35–0.9 μm × 1.1–1.9 μm) with a Gram-stain-negative cell wall. Cells were motile with a single polar flagellum (Fig. S2). Catalase activity was determined by bubble production in 3 % (v/v) H₂O₂ and oxidase activity was determined by using 1 % (w/v) tetramethyl p-phenylenediamine (bioMérieux). Anaerobic growth was determined by incubation in screw-capped test tubes (20 ml capacity) with 10 ml DM and a 100 % helium gas phase for 5 days at 28 ℃. PHBV degradation was tested on DM plates, and the amount of PHBV degraded in liquid medium was estimated from the direct measurement of dry weight (Khan & Hiraishi, 2001). Casein, starch and tyrosine degradation were tested on LB medium containing milk powder (5 %, w/v), starch (1 %, w/v) or tyrosine (0.1 %, w/v), respectively. Growth was tested at 4, 7, 10, 25, 30, 37, 40 and 45 ℃ on LB medium. The pH of LB medium was adjusted to the desired value by using sterile solutions of citric acid/Na₂HPO₄ (pH 4.0 to 5.0), MES (pH 5.5 to 6.0), PIPES (pH 6.5 to 7.0), Tricine (pH 7.5 to 8.5), CAPSO (pH 9.0 to 9.5) or CAPS (pH 10.0 to 11), with final concentrations of 30 mM. Growth in the absence of NaCl and in the presence of 1–5.0 % (w/v) NaCl at 1 % intervals was investigated at 28 ℃ in LB medium. Other physiological and biochemical characteristics were tested using API 20NE and API ZYM kits (bioMérieux) and the Biolog GEN III microtest system with three replicates, according to the manufacturers’ protocols. API 20NE test strips and Biolog GEN III microplates were checked after incubation for 7 days (Kim et al., 2014) and 5 days (Barbeyron et al., 2001; Lenaerts et al., 2014), respectively, and API ZYM kits were checked after incubation for 4 h at 28 ℃. Phenotype MicroArray (PM) tests (Biolog) were performed using PM Plates 1 and 2A, containing 190 different single carbon sources in addition to a negative control (Bochner et al., 2001). Plates were incubated for five days at 28 ℃ in the OmniLog automated incubator-reader (Biolog) and were read every 15 min. Interpretation of the results was performed using Omnilog PM software according to the manufacturer’s instructions. To test antibiotic susceptibility, the following antimicrobial compound discs (Hangzhou Binhe Micro-organism Reagent) were used: amikacin (30 μg), ampicillin (10 μg), cefotaxime (30 μg), cefalotin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), netilmicin (30 μg), penicillin (10 μg), polymyxin...
For determination of their cellular fatty acid composition, when the diameter of the inhibition zone was 8 mm. A strain was recorded as susceptible. The genomic DNA of SL-205T was extracted with the method of Marmur (1961) and used in the determination of G+C contents by the thermal denaturation method using a Lambda 35 spectrometer (PerkinElmer) with Escherichia coli K-12 as a control (Marmur & Doty, 1962). DNA–DNA relatedness between SL-205T and the three reference strains was determined in triplicate by the initial renaturation rate method in 2 × SSC by using a Lambda 35 spectrometer (PerkinElmer) (De Ley, 1970).

The morphological, cultural, physiological and biochemical characteristics of strain SL-205T are given in the species description, and the distinctive phenotypic features of strain SL-205T and the other three reference strains are shown in Table 1. Compared with the closest relatives, it was observed that strain SL-205T could grow at 40 °C, but the reference strains could not grow at such a high temperature. Moreover, strain SL-205T was resistant to nalidixic acid and troleandomycin, but the other strains were sensitive to them.

Fig. 1. (a), Neighbour-joining, (b) maximum-likelihood and (c), maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences showing the position of strain SL-205T and closely related type strains. Bootstrap values based on 1000 replicates are shown at branch nodes. Bars, 0.005 (a) or 0.01 (b) changes per nucleotide position.

(30 µg), rifampicin (5 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (30 µg). The diameter of the antibiotic discs was 8 mm. A strain was recorded as susceptible when the diameter of the inhibition zone was >10 mm, and resistant when it was <10 mm.

For determination of their cellular fatty acid composition, SL-205T and the three reference strains were incubated on LB agar plates for 3 days at 28 °C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). These were analysed using an Agilent 6890N gas chromatograph. The fatty acid methyl esters were identified by separating with two-dimensional TLC and spraying chromatograms with phosphomolybdic acid (total polar lipids), ninhydrin (amino lipids) and molybdenum blue (phospholipids) (Collins & Jones, 1980). Respiratory quinones were extracted according to the method of Collins et al. (1977), purified by TLC and analysed by HPLC as described by Collins & Jones (1980).

The genomic DNA of SL-205T was extracted with the method of Marmur (1961) and used in the determination of G+C contents by the thermal denaturation method using a Lambda 35 spectrometer (PerkinElmer) with Escherichia coli K-12 as a control (Marmur & Doty, 1962). DNA–DNA relatedness between SL-205T and the three reference strains was determined in triplicate by the initial renaturation rate method in 2 × SSC by using a Lambda 35 spectrometer (PerkinElmer) (De Ley, 1970).

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Strain SL-205T contained ubiquinone Q-8 as the only quinone, which was common to other members of the genus Diaphorobacter. The polar lipids were diphasphatidglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and one uncharacterized phospholipid (Fig. S3). The polar lipid pattern of strain SL-205T was similar to that of D. aerolatus, which also has PE, DPG and PG as the major polar lipids (Kim et al., 2014). The fatty acids of strain SL-205T included summed feature 3 (C₁₆ : 1ω7c and/or iso-C₁₅ : 0 2-OH) (45.4 %), C₁₆ : 0
Table 1. Differential phenotypic properties between strain SL-205<sup>T</sup> and species of the genus Diaphorobacter

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter (µm)</td>
<td>0.35–0.9</td>
<td>0.7–0.9*</td>
<td>0.5–0.8†</td>
<td>0.8–0.9‡</td>
</tr>
<tr>
<td>Assimilation of:</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>PHBV</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Glycerol</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adipic acid</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Propionic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Methyl pyruvate</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>( + )</td>
</tr>
<tr>
<td>α-Ketobutyric acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>α-Ketoglutaric acid</td>
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<td>γ-Aminobutyric acid</td>
<td>+</td>
<td>+</td>
<td>–</td>
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</tr>
<tr>
<td>α-Hydroxybutyric acid</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Asparagine</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>L-Proline</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>D-Alanine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Threonine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Enzymic activity</td>
<td></td>
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<td></td>
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<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Trypsin</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Susceptibility to antibiotics</td>
<td></td>
<td></td>
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<tr>
<td>Nalidixic acid</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Ampicillin</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Penicillin</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Cefalotin</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Temperature range for growth (°C)</td>
<td>7–40</td>
<td>7–37</td>
<td>7–35</td>
<td>10–37</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>66.8 (T&lt;sub&gt;m&lt;/sub&gt;)</td>
<td>65.8 (T&lt;sub&gt;m&lt;/sub&gt;)</td>
<td>62.9† (HPLC)</td>
<td>65‡ (Fluorometric)</td>
</tr>
</tbody>
</table>

*Data from Khan & Hiraishi (2002).
†Data from Pham et al. (2009).
‡Data from Kim et al. (2014).

(24.2 %) and C<sub>18 : 1ω7c</sub> (16.8 %) as the major fatty acids (>10 % of the total fatty acids), and had C<sub>10 : 0</sub> 3-OH as the predominant hydroxyl fatty acid (Table S1). All four strains shared similar overall fatty acid compositions. The DNA G+C content (T<sub>m</sub>) of SL-205<sup>T</sup> was 66.8 mol%, which is different from that of the closely related strain, <i>D. nitroreducens</i> NA10B<sup>T</sup> (65.8 %, in this study). The DNA–DNA relatedness values of strain SL-205<sup>T</sup> with <i>D. nitroreducens</i> NA10B<sup>T</sup> and <i>D. oryzae</i> RF3<sup>T</sup> were 57 ± 1 % (mean ± stand deviation) and 45 ± 1.5 % (mean ± stand deviation), respectively.

In conclusion, SL-205<sup>T</sup> could be classified as a member of the genus <i>Diaphorobacter</i> on the basis of the phylogenetic tree and the quinone, polar lipid and fatty acid compositions. However, strain SL-205<sup>T</sup> could be differentiated from the other three members of the genus <i>Diaphorobacter</i> on the basis of phenotypic properties, such as susceptibility to antibiotics, enzymic activity and the assimilation patterns of substrates (Table 1), together with DNA–DNA relatedness data. Therefore, strain SL-205<sup>T</sup> is considered to represent a novel species of the genus <i>Diaphorobacter</i>, for which the name <i>Diaphorobacter polyhydroxybutyrativorans</i> sp. nov. is proposed.

Description of <i>Diaphorobacter polyhydroxybutyrativorans</i> sp. nov.

<i>Diaphorobacter polyhydroxybutyrativorans</i> (poly.hy.dro.x-y.by.ty.ra.ti.vo-rans. N.L. neut. n. polyhydroxybutyrtatum polyhydroxybutyrate; L. part. adj. vorans devouring; N.L. part. adj. polyhydroxybutyrativorans polyhydroxybutyrate-devouring).

Cells are Gram-stain-negative, oxidase-positive, facultatively aerobic, denitrifying, rod-shaped (0.35–0.9 µm × 1.1–1.9 µm) and possess a single polar flagellum. After 3 days of aerobic incubation at 28 °C, colonies are 1–2 mm in diameter,
circular and ivory (R2A agar) or yellow (LB medium agar). Growth occurs at 7–40 °C (optimum 28 °C; no growth occurs at 4 or 45 °C) and at pH 5–9 (optimum pH 7; no growth occurs at pH 4 or 10). The NaCl tolerance range is 0–3 % (w/v). Catalase-positive. PHBV is degraded under aerobic and anaerobic denitrifying conditions. The mean PHBV degrading rate is 0.14 ± 0.12 g PHBV day−1 under anaerobic conditions. Hydrolysis of casein is positive. Reduces nitrate and nitrite to N2. Negative in tests for indole production, glucose fermentation, arginine dihydrolase, urease, ascelin hydrolysis and gelatin hydrolysis (API 20NE test strips). The carbon sources utilized are glycerol, succinic acid, fumaric acid, D-glucconic acid, D-saccharic acid, methyl pyruvate, monomethyl succinate, L-lactic acid, z-ketoglutaric acid, L-malic acid, bromosuccinic acid, D-serine, L-serine, D-alanine, L-alanine, L-proline, L-aspartic acid, L-glutamic acid, L-isoleucine, L-leucine, L-phenylalanine, L-α-alanine, Glycine, L-lysine, L-proline, L-phenylalanine, L-glutamic acid, L-aspartic acid, threonine, and D-proline (API 20NE test strips). The carbon sources utilized are glycerol, succinic acid, fumaric acid, D-glucconic acid, D-saccharic acid, methyl pyruvate, monomethyl succinate, L-lactic acid, z-ketoglutaric acid, L-malic acid, bromosuccinic acid, D-serine, L-serine, D-alanine, L-alanine, L-proline, L-aspartic acid, L-glutamic acid, L-isoleucine, L-leucine, L-phenylalanine, L-α-alanine, Glycine, L-lysine, L-proline, L-phenylalanine, L-glutamic acid, L-aspartic acid, threonine, and D-proline (API 20NE test strips).

The type strain, SL-205T (=KACC 19739T =DSM 29460T), was isolated from the biofilms of a denitrifying reactor using PHBV as the carbon source in Beijing, PR China. The DNA G+C content of the type strain is 66.8 mol% (Tm).

Acknowledgements

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References


