Defluviimonas nitratireducens sp. nov., isolated from surface seawater

Yang Liu, Qiliang Lai, Wanpeng Wang and Zongze Shao*

Abstract

A bacterial strain, designated DL5-4T, was isolated from the surface seawater of Dalian Bay and characterized using a polyphasic taxonomy approach. Cells of DL5-4T were Gram-staining-negative, non-motile and short-rod-shaped. Growth was observed at 8–40 °C (optimum 28–30 °C), at pH 6–9 (optimum pH 7) and in 0–7 % NaCl (optimum 1–3 %, w/v). The results of the phylogenetic analysis based on 16S rRNA gene sequences indicated that DL5-4T formed an independent branch with members of the genus Defluviimonas, sharing high similarities with five related type strains, Defluviimonas aquaemixtae DSM-7T (96.6 %), Defluviimonas denitrificans DSM 18921T (96.0 %), Defluviimonas indica 20V17T (95.8 %), Defluviimonas aestuarii BS14T (95.8 %) and Defluviimonas alba cai42T (94.5 %). The predominant fatty acid was summed feature 8 (C18:1ω6c and/or C18:1ω7c). The isoprenoid quinone was identified as Q-10. The polar lipids were diphasphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four phospholipids, an aminolipid and an unknown lipid. The DNA G+C content was 63.8 mol%. The results of the phenotypic, phylogenetic and chemotaxonomic analyses clearly indicated that DL5-4T represents a novel species of the genus Defluviimonas, for which the name Defluviimonas nitratireducens sp. nov. is proposed, with type strain DL5-4T (=MCCC 1A06955T=LMG 29616T).

In an attempt to investigate the diversity of oil-degrading bacteria from the surface seawater of Dalian Bay, a large number of strains have been isolated and characterized taxonomically [1]. This study focused on one of these isolates, designated DL5-4T. The results of the 16S rRNA gene sequence analysis indicated that DL5-4T may represent a putative novel species within the genus Defluviimonas, which has been proposed [2] and affiliated to the family Rhodobacteraceae within the class Alphaproteobacteria. At the time of writing, the genus Defluviimonas comprises five species with validly published names, Defluviimonas denitrificans [2], Defluviimonas aestuarii [3], Defluviimonas indica [4], Defluviimonas aquaemixtae [5] and Defluviimonas alba [6]. Most members of this genus have been isolated from marine environments, such as marine aquaculture, marine tidal flat and deep-sea hydrothermal vent chimney; while some were isolated from terrestrial environments, for example D. alba cai42T from oil-production water of Xinjiang Oilfield, PR China [6], which may play an important role in degradation of crude oil. The aim of the present study was to determine the phylogenetic position of DL5-4T using a polyphasic taxonomic approach.

DL5-4T was isolated from the surface seawater of Dalian Bay (38° 58′ N, 124° 46′ E), PR China [1]. The purified strain was then stored at −80 °C in water containing 20 % glycerol (v/v). All of five type strains of species of the genus Defluviimonas, D. denitrificans DSM 18921T (=MCCC 1A00643T), D. aestuarii BS14T (=MCCC 1A11752T), D. indica 20V17T (=MCCC 1A01802T), D. aquaemixtae CDM-7T (=MCCC 1A11691T) and D. alba cai42T (=MCCC 1A11677T), were obtained from the Marine Culture Collection of China (MCCC) and used as references. Unless otherwise noted, for all tests of morphological, physiological and chemotaxonomic characteristics, DL5-4T and five reference strains were incubated on Marine Agar 2216 (MA; BD Difco) or in Marine Broth 2216 (MB; BD Difco) at 28 °C.

Cell morphology was observed using transmission electron microscopy (JEM-2100, JEOL) (Fig. S1, available in the online Supplementary Material). Motility was determined by the hanging drop test [7]. Gram staining was performed using a Gram stain kit (Hangzhou Tianhe Microorganism Reagent) according to the manufacturer’s instructions. Catalase activity was determined by adding a drop of 3 % hydrogen peroxide (v/v) to colonies. Oxidase activity was

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Keywords: Defluviimonas nitratireducens; the surface seawater; taxonomy.

Abbreviations: ME, minimum evolution; ML, maximum likelihood; NJ, neighbor-joining.

The GenBank/EMBL/DDJB accession number of 16S rRNA gene sequence for Defluviimonas nitratireducens DL5-4T is KF146513.

Two supplementary figures and one supplementary table are available with the online Supplementary Material.
tested using tetramethyl-p-phenylenediamine. The ranges and optima of temperature, pH and NaCl were determined according to previously described methods [8]. Anaerobic growth was tested on MA in an anaerobic jar with the Anoxomat Mark II Anaerobic System (Mart Microbiology) at 28 °C for 2 weeks. Other biochemical tests were carried out using API ZYM, API 20NE, API 20E and API 50CHB strips and GN2 MicroPlate system (Biolog) according to the manufacturer’s instructions, with the modification of adjusting the NaCl concentration to be 3.0 % in all tests. Antibiotic susceptibility tests were performed using a disc diffusion method as previously described [9]. DL5-4 T is sensitive to (all values µg/per disc unless noted) ampicillin (10), Oxoixid, chloromycetin (30), carbenicillin (100), cephradin (30), cefobid (30), ciprofloxacin (5), ceftaxim (30), gentamicin (10), rocephin (30), vibramycin (30), kanamycin (30), cefazolin (30), ofloxacin (5), oxacillin (1), penicillin G (10), polymyxin B (30 IU), pipercillin (100), rifampicin (5), tetracycline (30) and furazolidone (15); and is resistant to clindamycin (2), erythromycin (15), norfloxacin (10), streptomycin (10), co-trimoxazole (25) and vancomycin (30). More phenotypic characteristics of DL5-4 T are listed in Tables 1 and S1 and in the species description.

Genomic DNA was extracted using the AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences) according to the manufacturer’s instructions. The amplification of the 16S rRNA gene was performed using universal bacterial-specific primers 27F and 1492R. The PCR reagent and amplification systems, detection and sequencing of PCR amplicons were carried out following the previously published methods [10]. The similarities of 16S rRNA gene sequences between DL5-4 T and the closely related type strains were determined using the EzTaxon-e server [11]. Sequences of related taxa were obtained from the GenBank database. Phylogenetic trees based on 16S rRNA gene sequences were reconstructed using the software MEGA version 5.05 [12] with distance option according to the default parameter models and clustering with the neighbour-joining (NJ) [13], minimum evolution (ME) [14] and maximum likelihood (ML) [15] methods, with bootstrap values based on 1000 replications [16].

An almost complete 16S rRNA gene sequence (1433 bp) of DL5-4 T was determined in this study. DL5-4 T shared less than 97 % similarities of 16S rRNA gene sequences with five related type strains, D. aquaeamitae CDM-7 T (96.6 %), D. denitrificans DSM 18921 T (96.0 %), D. indica 20V17 T (95.8 %), D. aestuarii BS14 T (95.8 %) and D. alba cai42 T (94.5 %). The results of the phylogenetic analysis of the 16S rRNA gene indicated that DL5-4 T formed an independent lineage within the genus Defluviimonas, and separated from other genera in the NJ tree, with a support bootstrap value of 88 % (Fig. 1). The ME and ML trees showed similar topology to the NJ tree.

To determine whole-cell fatty acid composition, cells of DL5-4 T and the five above-mentioned type strains were harvested from the third quadrants on MA agar at 28 °C. The cells were saponified, methylated and extracted using the standard MIDI (Sherlock Microbial Identification System, version 6.0B) protocol [17]. The fatty acids were then analyzed by gas chromatography (model 6850; Agilent Technologies) and identified using the TSBA6.0 database of the Microbial Identification System. The respiratory quinone of DL5-4 T was determined by HPLC according to the previously reported method [18]. Polar lipids of DL5-4 T were extracted using a chloroform/methanol system and analysed using two-dimensional TLC. Merck silica gel 60 F254 aluminium-backed thin-layer plates were used in two-dimensional TLC analysis, with the first solvent of chloroform/methanol/acetate acid/water (65 : 25 : 4, v/v) followed by second solvent of chloroform/methanol/acetate acid/water (85 : 12 : 15 : 4, v/v). The polar lipids were analyzed according to a previously described method [19].

The compositions of fatty acids for DL5-4 T and five reference strains are listed in Table 2. The major fatty acid of DL5-4 T was summed feature 8 (C18:1ω6c and/or C18:1ω7c) (90.3 %), while the major fatty acids were C18:0 (3.4 %), C16:0 (1.5 %), summed feature 3 (C16:0ω7c and/or C16:0ω6c) (2.2 %) and summed feature 7 (unknown equivalent chain length 18.846 and/or C19:0ω6c) (1.0 %). As shown in Table 2, although the kinds of fatty acids of DL5-4 T and the five reference strains were similar, their proportions of them were remarkably different, indicating the distinctness of DL5-4 T from the reference strains. The respiratory quione of DL5-4 T was identified as Q-10, which was in accordance with those of the five reference strains. The polar lipids of DL5-4 T comprised diphostydiacylglycerol, phosphatidylethanolamin, phosphatidylglycerol, four phospholipids, an unidentified amino lipid and an unknown lipid (Fig. S2), which were in accordance with those of the reference strains. The distinct differences were the presence of phosphatidylcholine in strain D. aquaeamitae CDM-7 T and D. alba cai42 T and the presence of glycolipid in D. indica 20V17 T and D. alba cai42 T, according to the original identification results for each type strain. The DNA G+C content of DL5-4 T was determined from the midpoint value (Tm) of the thermal denaturation profile [20] with Escherichia coli K-12 as reference. The DNA G+C content of the type strain is 63.8 mol%.

In conclusion, on the basis of phylogenetic evidence, physiological and biochemical characteristics, DL5-4 T clearly represents a member of the genus Defluviimonas, but is distinguishable from the described species of this genus. Therefore, DL5-4 T is considered to represent a novel species of the genus Defluviimonas, for which the name Defluviimonas nitratireducens sp. nov. is proposed.

**DESCRIPTION OF DEFLUVIIMONAS NITRATIREDUCENS SP. NOV.**

Defluviimonas nitratireducens (n.tra.ti.re.du’cens. N.L. n. nitratum, nitrate; L. pres. part. reducens, bringing back to a state or condition; N.L. part. adj. nitratireducens, reducing nitrate).
Table 1. Characteristics that differentiate DL5-4T from the type strains of species of the genus Defluviimonas

Strains: 1, DL5-4T; 2, D. denitrificans D9-3T; 3, D. aestuarii BS14T; 4, D. indica 20V17T; 5, D. aquamixtae CDM-7T; 6, D. alba cai42T. Data for API ZYM, API 20NE and API 20E were obtained from this study. All strains were positive for catalase and oxidase. In the API ZYM tests, all strains were positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and acid phosphatase; negative for N-acetyl-β-glucosaminidase, α-galactosidase, β-galactosidase, α-mannosidase and α-fucosidase. In API 20NE, all strains were negative for β-galactosidase and utilization of capric acid. In API 20E, all strains were negative for β-galactosidase, H₂S production, tryptophane deaminase and acid production from glucose, inositol, sorbitol, sucrose, melibiose, amygdalin and arabinose. +, Positive; —, negative; w, weakly positive.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell width (µm)</td>
<td>0.7-0.8</td>
<td>0.5</td>
<td>0.5-0.8</td>
<td>0.5-0.7</td>
<td>0.2-0.3</td>
<td>0.5-0.7</td>
</tr>
<tr>
<td>Cell length (µm)</td>
<td>0.9-1.3</td>
<td>1.1-1.9</td>
<td>1.6-2.7</td>
<td>1.2-1.8</td>
<td>0.2-1.2</td>
<td>1.0-1.5</td>
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<td>Motility</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>8-40</td>
<td>10-40</td>
<td>5-40</td>
<td>2.0-37</td>
<td>15-37</td>
<td>20-37</td>
</tr>
<tr>
<td>Optimum temperature (°C)</td>
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<td>30-40</td>
<td>30</td>
<td>25-28</td>
<td>30</td>
<td>30</td>
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<tr>
<td>pH range</td>
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<td>5.5-9.0</td>
<td>6.5-9.5</td>
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<td>5.0-8.5</td>
<td>7.0-9.0</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.0</td>
<td>6.5-7.0</td>
<td>7.0-7.5</td>
<td>7.0</td>
<td>7.0-8.0</td>
<td>8.0</td>
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<tr>
<td>NaCl range (% w/v)</td>
<td>0-7.0</td>
<td>0-5.0</td>
<td>0-10.0</td>
<td>0-5.0</td>
<td>0.5-11.0</td>
<td>0-5.0</td>
</tr>
<tr>
<td>Optimum NaCl (% w/v)</td>
<td>1.0-3.0</td>
<td>0.5-3.0</td>
<td>1.0-1.5</td>
<td>1.5-2.0</td>
<td>2.0-3.0</td>
<td>1.0-3.0</td>
</tr>
</tbody>
</table>

**API ZYM**

- Lipase (C14) + + + + — +
- Valine arylamidase + + + + + w +
- Cystine arylamidase + + w + w +
- Trypsin — w — w — w
- α-Chymotrypsin — w — w — +
- Naphthol-AS-BI-phosphohydrolase w + w + + w
- β-Glucuronidase — w — — — —
- α- and β-Glucosidase — + — — — w

**API 20NE**

- Reduction of nitrate + + — + — —
- Denitrification + + — — — —
- Indole production — — — — — —
- D-glucose fermentation w — — — — —
- Arginine dihydrolase — — — — — — w
- Urease — — — — + — w
- β-Glucosidase (aesculin hydrolysis) + + — — — —
- Gelatin hydrolysis — — + — — —

**Utilization of**

- D-glucose, trisodium citrate + + + — — +
- L-arabinose — + — — — +
- D-mannose, phenylacetic acid — + — — — —
- D-mannitol + + + + — +
- N-acetyl-glucosamine — + + — — —
- Maltose + + — — — +
- Potassium gluconate — w — — — +
- Adipic acid — w — + — w
- Malic acid + w + — + +

**API 20E**

- Arginine dihydrolase, ornithine decarboxylase — — — — — w
- Lysine decarboxylase — — — + — —
- Citrate utilization + + + — — —
- Urease — — — + — w
- Indole production, acid production from mannitol — — — — — +
- Acetoin production (Voges–Proskauer) + + + + + +
- Gelatinase — — + — — —
- Acid production from rhamnose — + — — — +

**DNA G+C content (mol%)**

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|
|  | 63.8 | 65.7* | 61.6 | 66.3* | 66.8 | 66.7* |

aData were obtained from the respective genome sequences [21, 22].
Cells are Gram-stain-negative, facultatively aerobic, short-rod-shaped, non-motile, 0.7–0.8 µm wide and 0.9–1.3 µm long (Fig. S1). Colonies are brown, circular, non-translucent, convex and approximately 0.5–1 mm in diameter on MA agar after 3 days at 28 °C. Growth occurs at 8–40 °C (optimum 28–30 °C), at pH 6–9 (optimum pH 7) and in 0–7% NaCl (optimum 1–3%, w/v). Catalase and oxidase are positive. In the API ZYM tests, positive for lipase (C14), cystine arylamidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase; weakly positive for naphthol-AS-BI-esterase lipase (C8), leucine arylamidase, valine arylamidase and acid phosphatase; negative for indole production, arginine dihydrolase, lysine decarboxylase, H₂S production, urease, tryptophan deaminase, indole production, gelatinase, acid production of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. In the API 50CHB tests, positive for acid production from D-glucose, D-fructose, D-sorbitol, cellobiose, maltose, D-sucrose, trehalose, starch, turanose, D-fucose and D-arabitol; weakly positive for acid production from D-mannitol and salicin; negative for acid production from other substrates. The following substrates in the Biolog GN2 MicroPlate are utilized: dextrin, glycogen, Tween 80, D-arabitol, cellobiose, D-fructose, α-D-glucose, myo-inositol, lactose, maltose, D-mannitol, D-psicose, D-sorbitol sucrose, trehalose, turanose, methyl pyruvate, monomethyl succinate, acetic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, α-ketoglutaric acid, DL-lactic acid, propionic acid, quinic acid, succinic acid, succinamic acid, L-alanine, L-proline and glycerol. The other substrates in the GN2 MicroPlate are not utilized. The major fatty acid is summed feature 8 (C₁₈:1ω₆c and/or C₁₈:1ω7c). The respiratory quinone is Q-10. The polar lipids comprise diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, four phospholipids, an unidentified aminolipid and an unknown lipid.

Fig. 1. Neighbour-joining tree showing the phylogenetic positions of DL5-4T and the closely related strains within the family Rhodobacteraceae based on 16S rRNA gene sequences. *Rhodospirillum rubrum* ATCC 11170T (CP000230) was used as an outgroup. Bootstrap values (greater than 70%) expressed as percentages of 1000 replications are shown at branch points. Bar, 0.02 nucleotide substitution rate (Knuc) units.
Strains: 1, DL5-4; 2, D. denitrificans D9-3; 3, D. aestuarii BS14; 4, D. indica 20V17; 5, D. aquamixtse CDM-7; 6, D. alba ca42. All data were obtained in this study. TR, Trace amount (less than 1 %); ND, not detected. Major components (greater than 5.0 %) are highlighted in bold type.

<table>
<thead>
<tr>
<th>Fatty acid</th>
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<th>4</th>
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<td>C_{16:0}</td>
<td>1.5</td>
<td>2.3</td>
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<td>1.7</td>
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<td>8.1</td>
</tr>
<tr>
<td>C_{18:0}</td>
<td>3.4</td>
<td>4.6</td>
<td>2.8</td>
<td>6.4</td>
<td>7.6</td>
<td>4.4</td>
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<tr>
<td>C_{10:0}  3-OH</td>
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<td>4.3</td>
<td>3.0</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>C_{12:0}  3-OH</td>
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<td>TR</td>
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<tr>
<td>C_{18:0}  3-OH</td>
<td>TR</td>
<td>ND</td>
<td>ND</td>
<td>4.1</td>
<td>2.0</td>
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<td>C_{17:1} w9c</td>
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<td>TR</td>
<td>ND</td>
<td>TR</td>
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<tr>
<td>C_{18:1} w7c 11-methyl</td>
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<td>ND</td>
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<td>ND</td>
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<td>C_{16:0}  cyclo w8c</td>
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<td>TR</td>
<td>1.0</td>
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<tr>
<td>Summed feature 3*</td>
<td>2.2</td>
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<td>Summed feature 8*</td>
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<td>85.2</td>
<td>68.9</td>
<td>81.4</td>
<td>74.0</td>
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</table>

*Summed features represent groups of two fatty acids which could not be separated by GLC with the MIDI system. Summed feature 2: C_{17:0} 3-OH and/or iso-C_{16:1} w5c; summed feature 3: C_{16:1} w7c and/or C_{16:1} w6c; summed feature 7: unknown equivalent chain length 18.846 and/or C_{19:1} w6c; summed feature 8: C_{18:1} w7c and/or C_{18:1} w6c.

The type strain is DL5-4\textsuperscript{T} (=MCCC 1A06955\textsuperscript{T}=LMG 29616\textsuperscript{T}), isolated from the surface seawater of Dalian Bay, PR China. The DNA G+C content of the type strain is 63.8 mol%.

Funding information
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Acknowledgements
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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

Table 2. Cellular fatty acids compositions of DL5-4\textsuperscript{T} and the type strains of species of the genus Defluviimonas

| Fatty acid | Strains: 1, DL5-4; 2, D. denitrificans D9-3; 3, D. aestuarii BS14; 4, D. indica 20V17; 5, D. aquamixtse CDM-7; 6, D. alba ca42. All data were obtained in this study. TR, Trace amount (less than 1 %); ND, not detected. Major components (greater than 5.0 %) are highlighted in bold type. |
