Denitrobaculum tricleocarpace gen. nov., sp. nov., a marine bacterium from coralline algae Tricleocarpa sp

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Abstract

A Gram-stain-negative, non-spore-forming, aerobic, motile, curved rod-shaped bacterium, designed strain R148T was isolated from a coralline algae Tricleocarpa sp. collected from Weizhou island, PR China. The optimal growth of R148T occurred at 25 °C, pH 8–9 in the presence of 0.5% (w/v) NaCl on the basis of amended marine broth 2216. The genomic DNA G+C content was 59.5 mol%. The only detected respiratory quinone was Q-10. The major polar lipids were phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and three unidentified ninhydrin-positive lipids. The major cellular fatty acids were C18:1ω7c, C16:0, C19:0 cyclo 9, 10 DMA and C18:0. The results of 16S rRNA gene-based global alignment indicated that the closest neighbour of strain R148T was Pelagibius litoralis DSM 21314T (93.1% similarity), the second is Limibacillus halophilus KCTC 42420T (92.2%). The results of phylogenetic analysis indicated that R148T forms a distinct branch in the robust clade of R148T and Pelagibius litoralis DSM 21314T (93.1% similarity), the second is Limibacillus halophilus KCTC 42420T (92.2%). The results of phylogenetic analysis indicated that R148T forms a distinct branch in the robust clade of R148T and Pelagibius litoralis DSM 21314T, while the taxonomic position of this clade in the family Rhodospirillaceae is ambiguous among phylogenetic approaches. The low 16S rRNA gene similarity and distinct polar lipid and cellular fatty acid profile could readily distinguish R148T from closely related type strains. So R148T is suggested to represent a novel species in a novel genus, for which the name Denitrobaculum tricleocarpace gen. nov., sp. nov. is proposed. The type strain is R148T (=MCCC 1K03781T=KCTC 72137T).
2 weeks. A cream colony, designed strain R148^T, was isolated and purified on marine R2A plates, then stored at –70°C with R2A broth (Halbo)–glycerol (4:1, v/v). Type strains Pelagibius litoralis DSM 21314^T and Limibacillus halophilus KCTC 42420^T were used as references and were obtained from the German Collection of Microorganisms and Cell Cultures (DSM) and the Korean Collection for Type Cultures (KCTC), respectively.

The 16S rRNA gene of R148^T was obtained by using PCR amplification with the universal primers 27F and 1492R [5] and sequenced using the Sanger method. Determination of the 16S rRNA gene sequence similarities were performed using EzBioCloud [6] and the NCBI database. Alignment of 16S rRNA gene sequences was performed using the Silva software package [7] and Silva rRNA database. Phylogenetic trees were reconstructed using the maximum likelihood [8], neighbor-joining [9] and maximum-parsimony [10] algorithms in the software package Mega version 7.0 [11]. The phylogenetic distance matrices were estimated by the Kimura two-parameter model [12]. The topology of the phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein [13] with 1000 replicates. Whole-genome sequencing was performed on the HiSeq PE150 platform (Illumina). A-tailed, ligated to paired-end adaptors and PCR amplified DNA with a 350 bp insert was used for the library construction at Beijing Novogene Bioinformatics Technology (Beijing, PR China). All good quality paired reads were assembled using SOAP denovo [14, 15] (http://soap.genomics.org.cn/soapdenovo.html) into a number of scaffolds. Genome information was extracted according to the protocols of Chun et al. [16]. The average nucleotide identity was calculated as described by Yoon et al. [17]. The phylogenomic tree was reconstructed using the up-to-date bacterial core gene set (UBCG v.3) according to its manual [18].

The genome sequencing depth of R148^T was 156x, the N50 was 492275 bp. A total of 36 contigs were obtained, the obtained genome size was 6.44 Mb, and the genome DNA G+C content was 59.5 mol%. The full length 16S rRNA gene sequence was 1479 bp, which was similar to the Sanger result (1360 bp, MN094886). The genome of P. litoralis DSM 21314^T was also sequenced and deposited in the GenBank under the accession number JAAQPH000000000. The global alignment based on 16S rRNA gene sequence in the EzBioCloud database indicated that R148^T shares 93.1 and 92.2% sequence similarity with the most closely related type strains P. litoralis DSM 21314^T and L. halophilus KCTC 42420^T, respectively. The ANI of R148^T to P. litoralis DSM 21314^T and L. halophilus KCTC 42420^T was 71.2 and 67.9%, respectively. Phylogenetic analysis based on the maximum likelihood algorithm indicated that R148^T forms a distinct branch in a stable clade that consists of R148^T and P. litoralis DSM 21314^T, then forms a large loose clade with L. halophilus KCTC 42420^T and members of the genus Fodinicurvata (Fig. 1). The neighbor-joining algorithm also supported this tree topology (Fig. S1, available in the online version of this article). However, the maximum-parsimony algorithm indicated a large loose clade consisting of R148^T, P. litoralis DSM 21314^T, L. halophilus KCTC 42420^T and members of genera Fodinicurvata, Kiloniella and Aestuariispira (Fig. S2). The phylogenomic tree based on the up-to-date bacterial core gene set also indicated the close relationship between R148^T, P. litoralis DSM 21314^T and L. halophilus KCTC 42420^T, also forming a robust clade with members of genus Kiloniella (Fig. S3). These phylogenetic results indicated the close relationship of R148^T, P. litoralis DSM 21314^T and L. halophilus KCTC 42420^T and also the possibility that R148^T may represent a novel genus [16, 19], but reveal the uncertainty of the taxonomic position of the R148^T clade in current phylogenetic analysis systems.

Cell morphology was observed by using a transmission electron microscope (HT7700; Hitachi). Cell mobility was tested by using an optical microscope (BX53; Olympus) with the hanging drop technique [20]. Gram staining was determined on marine R2A plates following the protocols of Gerhardt et al. [21]. Catalase activity was determined by gas production upon exposure to 3% (v/v) hydrogen peroxide solution. Oxidase activity was determined by using oxidase test strains (Huankai). NaCl requirement and tolerance were tested at 25°C for 7 days in amended marine broth 2216 with NaCl concentrations ranging from 0 to 16% (w/v), namely 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14 and 16% (w/v). Growth at different pH values was tested for seven days at 25°C in marine broth 2216 (BD) amended with different buffers (0.5 pH unit intervals, pH 5–8, 0.1 M KH2PO4/K2HPO4; pH 8.5–10, 0.1 M NaHCO3/Na2CO3; pH 10.5–11, 0.1 M Na2CO3/NaOH). Optimal growth temperature was determined on marine agar 2216 (BD) plates after 7–30 days of growth at temperatures of 5, 10, 15, 20, 25, 30, 33, 37, 40 and 45°C. The ability to form endospores and hydrolysis of starch, Tween 20, 40, 60 and 80 was tested described by Dong and Cai [22]. Carbohydrate metabolism were tested by using API 20NE and API ZYM strips according to the manufacturer’s protocols except that cells were suspended in sterile aged nature seawater. Anaerobic fermentation was determined using API 50CH strips according to the manufacturer’s protocol, the inoculum medium was R2A liquid medium. H2S production was tested by following standard procedures compiled by Tindall et al. [23].

Cells of R148^T were Gram-stain-negative, non-sporo-forming, aerobic, slightly curved rods, motile with a monopolar flagellum or bipolar flagella (Fig. S4). The position of the flagellum could readily be used to differentiate strain R148^T from L. halophilus KCTC 42420T, L. halophilus KCTC 42420^T had no flagellum [24]. In the API 20NE test, arginine dihydrolase in R148^T was negative, while P. litoralis DSM 21314^T and L. halophilus KCTC 42420^T were positive. Other characteristics of R148^T are listed in Table 1 and the species description.

Biomass of R148^T and reference strains for cellular fatty acid analysis was acquired from the third quadrant of the quadrant-streaked (exponential state) marine agar 2216 (BD) plate incubated at 25°C for 3–5 days; P. litoralis DSM 21314^T required 5 days. Cellular fatty acid composition was analysed by gas chromatography (G6890N; Agilent) and identified by using the Sherlock Microbial Identification System (Version
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6.0) according to the manufacturer's instructions. Biomass for the analyses of quinones and DNA extraction was obtained from marine R2A liquid medium. Respiratory quinones were extracted as described by Collins [25] and analysed using reversed-phase HPLC [26]. The isoprenoid quinones were eluted with a mixture of methanol and 2-propanol (2:1, v/v) using a flow rate of 1 ml min⁻¹ at room temperature and detected by UV absorbance at 270 nm. Biomass for polar lipid analysis was obtained from marine R2A liquid medium. Polar lipids were extracted as described by Kamekura [27], and identified by spraying with ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin, α-naphthol/sulphuric acid and Dragendorff’s reagent after two-dimensional TLC [28].

The major cellular fatty acids of R148ᵀ were C₁₈₀₇ω₇c (57.4% of total content), C₁₆₀₇c (12.7%), C₁₉₀₉cyclo 9, 10 DMA (12.3%) and C₁₈₀ (7%) (Table S1). This fatty acid profile was much different from those of L. halophilus KCTC 42420ᵀ and P. litoralis DSM 21314ᵀ, both of them lacked C₁₆₀₇c, L. halophilus KCTC 42420ᵀ had a high C₁₈₀₇c₁₀-methyl (10%) content (Table S1). The detected respiratory quinone of R148ᵀ was ubiquinone 10 (Q-10). The major polar lipids were phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, and three unidentified ninhydrin-positive lipids (Fig. S5). L. halophilus KCTC 42420ᵀ and P. litoralis DSM 21314ᵀ had the same major polar lipids, both of them lacked an unidentified ninhydrin-positive lipid (NL3) compared with R148ᵀ (Fig. S5).

In summary, low 16S rRNA gene similarities to (93.1%) and different polar lipids (possession of NL3) and cellular fatty acid profiles (C₁₆₀₇c or C₁₈₀₇c₁₀-methyl) from closely related type strains indicated that R148ᵀ should represent a novel species in a novel genus in the family Rhodospirillaceae, for which the name Denitrobaculum tricleocarpae gen. nov., sp. nov. is proposed.

**DESCRIPTION OF DENITROBACULUM GEN. NOV.**

Denitrobaculum (De.niтро.bа’cu.lum. L. pref. de, from; N.L. pref. nitro-, pertaining to nitrate; L. neut. n. baculum, a stick; N.L. neut. n. Denitrobaculum, nitrate-reducing rod)

Cells are Gram-stain-negative, non-spore-forming, aerobic curved rods, motile by means of a single polar flagellum. Catalase is positive while oxidase is negative. The major respiratory quinone is Q-10. The major polar lipids are
phosphatidylmethylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, and three unidentified ninhydrin-positive lipids. The major cellular fatty acids are C\(_{18:1}\)\(\omega_7\)c, C\(_{16:1}\)\(\omega_7\)c, C\(_{19:0}\) cyclo 9,10 DMA, and C\(_{18:0}\).

The type species is *Denitrobaculum tricleocarpae*.

### DESCRIPTION OF *DENITROBACULUM TRICLEOCA RPÆS* NOV.

*Denitrobaculum tricleocarpae* (tri.cle.o.car’pae. N.L. gen. n. *tricleocarpæ*, of/from *Tricleocarpa*, isolated from the red alga *Tricleocarpa* sp.)

The description is as for the genus with the following additional properties. Cells are usually 0.5–0.7 \(\mu\)m wide and 0.9–3.2 \(\mu\)m long. Colonies are cream and circular on marine R2A plates. Cells can grow at 15–37°C (optimum 25°C), pH 8–9 in 0.5–5% (w/v) NaCl (optimum 0.5%) on the basis of marine broth 2216 medium. Production of H\(_2\)S does not occur. Starch and gelatin are hydrolysed. In the API 20NE test, nitrate reduction, urease, \(\beta\)-glucosidase and protease are positive. In the API ZYM test, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, \(\alpha\)-chymotrypsin, acid phosphatase, naphthol-AS-BI phosphohydrolase, \(\alpha\)-galactosidase, \(\beta\)-galactosidase, \(\beta\)-glucuronidase, \(\alpha\)-glucosidase, \(\beta\)-glucosidase, \(N\)-acetyl-\(\beta\)-glucosaminidase and \(\alpha\)-mannosidase are positive.

The type strain, R148\(^T\) (=MCCC 1K03781\(^T\) =KCTC 72137\(^T\)), was isolated from a coralline alga *Tricleocarpa* sp. collected from Weizhou island, in the Beibu Gulf, PR China. The genomic DNA G+C content is 59.5 mol%. The 16S rRNA gene sequence of R148\(^T\) has been deposited in GenBank under the accession number MN094886. The Whole Genome Shotgun project of R148\(^T\) has been deposited at DDBJ/ENA/GenBank.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source of isolate</td>
<td><em>Tricleocarpa</em> sp.</td>
<td>Coastal seawater*</td>
<td>Reclaimed land†</td>
<td>Salt mine sediment</td>
<td>Marine macroalga</td>
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<tr>
<td>Cell size ((\mu)m)</td>
<td>0.5–0.7×0.9–3.2</td>
<td>0.5–1.0×1.2–2.5*</td>
<td>0.3–0.5×1.0–2.0†</td>
<td>0.3–0.5×0.7–1.5</td>
<td>0.5–0.6×2.5–5.0</td>
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<tr>
<td>Flagellum</td>
<td>Monopolar</td>
<td>Monopolar*</td>
<td>–†</td>
<td>+</td>
<td>Monopolar</td>
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<tr>
<td>Cell shape</td>
<td>Slightly curved rod</td>
<td>Slightly curved rod*</td>
<td>Short rod†</td>
<td>Vibrio and rod</td>
<td>Curved spirilla</td>
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<tr>
<td>NaCl tolerance (w/v, %)</td>
<td>0.5–5 (0.5)</td>
<td>1–6 (1)</td>
<td>0–8 (1–4)</td>
<td>1.5–20</td>
<td>0.3–10 (3)</td>
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<td>pH</td>
<td>8–9</td>
<td>7–10 (8–9)</td>
<td>6–9 (7)</td>
<td>6.5–8.5 (7.5)</td>
<td>3.5–9.5 (5.5)</td>
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<tr>
<td>Temperature (°C)</td>
<td>15–37</td>
<td>15–37</td>
<td>15–40</td>
<td>15–42</td>
<td>4–40</td>
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<td>Urease</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Arginine dihydrolyase</td>
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<td>+</td>
<td>+</td>
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<td>(\beta)-glucosidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Genomic DNA G+C content (mol%)</td>
<td></td>
<td></td>
<td>59.5</td>
<td>62.7</td>
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<td>ANI to R148(^T) (%)</td>
<td>100</td>
<td>71.2</td>
<td>67.9</td>
<td>70.0</td>
<td>67.9</td>
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<td>Major polar lipids(|$</td>
<td>PME, PE, PG, DPG, NL(1-3)</td>
<td>PME, PE, PG, DPG, NL(1-2)</td>
<td>PME, PE, PG, DPG, NL(1-2)</td>
<td>DPG, PE, PG, NL, PL</td>
<td>PE, PG, NPLs, Ls</td>
</tr>
<tr>
<td>Major cellular fatty acids</td>
<td>C(<em>{18:1})(\omega_7)c, C(</em>{16:1})(\omega_7)c, C(<em>{19:0}) cyclo 9,10 DMA, C(</em>{18:0})</td>
<td>C(<em>{18:1})(\omega_7)c, C(</em>{16:1})(\omega_7)c, C(<em>{19:0}) cyclo 9,10 DMA, C(</em>{18:0})</td>
<td>C(<em>{18:1})(\omega_7)c, C(</em>{16:1})(\omega_7)c, C(<em>{19:0}) cyclo 9,10 DMA, C(</em>{18:0})</td>
<td>C(<em>{18:1})(\omega_7)c, C(</em>{16:1})(\omega_7)c, C(<em>{19:0}) cyclo 9,10 DMA, C(</em>{18:0})</td>
<td>C(<em>{18:1})(\omega_7)c, C(</em>{16:1})(\omega_7)c, C(<em>{19:0}) cyclo 9,10 DMA, C(</em>{18:0})</td>
</tr>
</tbody>
</table>

*Data from [29].
†Data from [24].
‡Data from [30].
§Data from [31,32].
||Data from genome sequencing.
¶PME, phosphatidylmethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; NPL, unidentified ninhydrin-positive phospholipid; NL, unidentified ninhydrin-positive lipid; PL, unidentified phospholipid; PC, phosphatidylcholine; L, unidentified lipid.

Table 1. Characteristics that differentiate strain R148\(^T\) from type strains of related species

Strains: 1, R148\(^T\); 2, *Pelagibius litoralis* DSM 21314\(^T\); 3, *Limibacillus halophilus* KCTC 42420\(^T\); 4, *Fodinicurvata sediminis* YIM D82\(^T\); 5, *Kiloniella laminariae* LD81\(^T\). All the data were obtained from this study except where indicated. +, Positive; –, negative or not detected. All strains were catalase-positive.
under the accession number VHSH0000000. The version described in this paper is version VHSH0000000.

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

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