Rhizobium subbaraonis sp. nov., an endolithic bacterium isolated from beach sand

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Two strains (JC85T and JC108) of Gram-stain-negative, motile bacteria were isolated from endolithic beach sand samples on an oligotrophic medium. Based on the 16S rRNA gene sequence analysis, both strains were identified as belonging to the genus Rhizobium. Strain JC108 had 16S rRNA gene sequence similarity of 100% with Rhizobium pusense NRCPB10T and formed a cluster with this strain. Strain JC85T had 96.9% 16S rRNA gene sequence similarity and was 18% related (based on DNA–DNA hybridization) to Rhizobium borbori DN316T. With other strains of the genus Rhizobium, the 16S rRNA gene sequence similarity was less than 96.3%. Strain JC85T could tolerate up to 3% salinity, fix N2, was resistant to ampicillin (10 μg) and was positive for catalase and oxidase. The major fatty acid was C18:1ω7c (69%) with minor amounts of C19:0 cyclo ω8c (8.9%), C16:0 (6.9%), C12:0 (5.7%) and C19:1ω7c/C19:1ω6c (2.2%). Polar lipids of strain JC85T include two unidentified aminophospholipids (APL1,2), two unidentified phospholipids (PL1,2), phosphatidylcholine and four unidentified lipids (L1–4). Q-10 is the major quinone of strain JC85T. Based on polyphasic taxonomic analysis, strain JC85T represents a novel species for which, the name Rhizobium subbaraonis JC85T is proposed. The type strain is JC85T (=DSM 24765T=KCTC 23614T).

Abbreviations: ME, minimum evolution; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16s rRNA, recA and gltA gene sequences of strain JC85T are FR714938, HE572579 and HE572578, respectively.

Six supplementary figures and a supplementary table are available with the online version of this paper.
Satpada, where a mouth to the Chilika lake, Orrisa, India is situated (GPS location: 19°66' N 85°51' E). Both the strains grew very well on nutrient agar. Pure cultures were lyophilized and preserved at 4 °C.

Genomic DNA was extracted and purified from strains JC85T and JC108 according to the method of Marmur (1961) and the mol% G+C of the DNA was 67.7 and 62.1, respectively, as determined by HPLC (Mesbah et al., 1989). Cell material for 16S rRNA gene sequencing was taken from a colony. DNA was extracted and purified by using a Qiagen genomic DNA extraction kit. Recombinant Taq polymerase (Genei) was used for PCR. The complete length of the 16S rRNA gene sequence was obtained by polymerase (Genei) was used for PCR. The complete length of the 16S rRNA gene sequence was obtained by polymerase chain reaction (PCR) amplification as described previously (Imhoff & Pfennig, 1977) using an automated laser fluorescence sequencer (Pharmacia).

Identification of phylogenetic neighbours and calculation of pair wise 16S rRNA gene sequence similarity were achieved using the NCBI-BLAST search (Altschul et al., 1990) and EzTaxon server (Chun et al., 2007). The CLUSTAL W algorithm of MEGA4 was used for sequence alignments and MEGA 4 (Tamura et al., 2007) software was used for phylogenetic analysis. Distances were calculated by using the Kimura correction in a pair-wise deletion manner (Tamura et al., 2007). Neighbour-joining (NJ) and minimum evolution (ME) methods in the MEGA4 software were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure. The results of phylogenetic analysis of the 16S rRNA gene sequence (~1385 bp) suggested that strain JC85T and JC108 belonged to the genus Rhizobium and have sequence similarity to Rhizobium borbore DN316T of 96.9 % and less than 96.3 % with other members of Rhizobium and to R. pusense NRCBP10T (~100 %), respectively [the NJ tree is shown as Fig. 1 and the ME tree has similar tree topology (Fig. S1, available at IJSEM online); a full-length NJ tree is shown as Fig. S2]. Since strain JC108 shared 100 % 16S rRNA gene sequence similarity with R. pusense NRCBP10T and had very similar DNA G+C content, this strain was not further characterized. Furthermore, strains JC108 and R. pusense NRCBP10T were isolated from closely related geographical locations; however, the only difference is that

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**Fig. 1.** Phylogenetic analysis of strain JC85T and its closest relatives based on 16S rRNA gene sequences available from the EMBL database (accession numbers are given in parentheses). Multiple alignment, distance calculations (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were performed by using the software package MEGA version 4 (Tamura et al., 2007). Bootstrap values based on 500 replications are listed as percentages at the branching points. Bar, 0.01 nt substitutions per nucleotide position.
the former was isolated as an endolith, while the latter was from a rhizosphere soil (Panday et al., 2011).

Phylogenetic analysis of the housekeeping genes, gltA (Fig. S3) and recA (Fig. S4) was performed using the protocol described by Martens et al. (2007) and these results further confirmed the phylogenetic affiliation of strain JC85<sup>T</sup> to the type strains of the genus Rhizobium. The taxonomic relationship between strain JC85<sup>T</sup> and <i>Rhizobium</i> was examined using genome DNA–DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Tourouva & Antonov, 1988) using a DIG High Prime DNA Labelling and Detection Starter kit II (Roche). Hybridization was performed with three replications for each sample (control: reversal of strains was used for binding and labelling) and a mean value of 18% relatedness was observed between strain JC85<sup>T</sup> and <i>Rhizobium</i>.

Colonies of strain JC85<sup>T</sup> grown on nutrient agar (Hi-media ultrapure agar) medium are yellow coloured, circular (0.8–2.0 mm in diameter) and convex with entire margins. Morphological properties such as cell shape, cell size and motility were observed by phase-contrast light microscopy (Olympus BH-2). Nutrient-broth-grown cells of strain JC85<sup>T</sup> are rod-shaped (length 2.5–4.5 μm, width 0.8–1.0 μm) and motile. The pH range for growth was tested using nutrient broth, adjusted to different pH values (pH 5.0–11.0, at intervals of 0.5 units) by using the appropriate biological buffers as described by Xu et al. (2005). Salinity and temperature ranges for growth were examined in nutrient broth and growth was measured turbidometrically at 540 nm in a colorimeter (Systronics). Strain JC85<sup>T</sup> grew at a pH range of 6.0–7.5 with an optimum at pH 7.0 and differs from <i>Rhizobium</i> which has much higher pH tolerance (Table 1). NaCl was not required for growth of strain JC85<sup>T</sup> but could be tolerated up to 3% (w/v), while <i>Rhizobium</i> is less tolerant. Temperature range further differentiated strain JC85<sup>T</sup> from <i>Rhizobium</i> (Table 1).

Nitrate reduction as determined according to the method of Lányi (1987) was not observed with both JC85<sup>T</sup> and <i>Rhizobium</i>. Various biochemical tests such as hydrolysis of starch and gelatin, oxidase, chitinase, lipase, coagulase and urease activity were performed by the procedures as outlined in Cappuccino & Sherman (1998). Casein was not hydrolysed and gelatin was not liquefied by

### Table 1. Differential characteristics of strain JC85<sup>T</sup> and <i>Rhizobium borbori</i> DN316<sup>T</sup>

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
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</thead>
<tbody>
<tr>
<td>Cell size (width × length; μm)</td>
<td>0.8–1.0 × 2.5–4.5</td>
<td>1.0–1.5 × 3.0–5.5</td>
</tr>
<tr>
<td>Temperature range (optimum; °C)</td>
<td>25–30 (25)</td>
<td>4–37 (28)</td>
</tr>
<tr>
<td>pH range (optimum)</td>
<td>6.0–7.5 (7.0)</td>
<td>5.0–9.0 (6.5–7.0)</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>0–3</td>
<td>0–0.5</td>
</tr>
<tr>
<td>Aniline degradation</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Organic substrate utilization for growth:</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Acetate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Citrate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Antibiotic sensitivity</td>
<td>Ampicillin (10 μg)</td>
<td>RS</td>
</tr>
<tr>
<td>Fermentative growth on pyruvate</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Major cellular fatty acids (%)</td>
<td>C&lt;sub&gt;12:0&lt;/sub&gt;</td>
<td>5.7</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;</td>
<td>6.9</td>
<td>19.0</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1ω5&lt;/sub&gt;</td>
<td>0.5</td>
<td>18.8</td>
</tr>
<tr>
<td>C&lt;sub&gt;16:1ω6&lt;/sub&gt;</td>
<td>1.9</td>
<td>3.8</td>
</tr>
<tr>
<td>C&lt;sub&gt;17:0 cyclo&lt;/sub&gt;</td>
<td>0.1</td>
<td>4.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;18:1ω7&lt;/sub&gt;</td>
<td>69.2</td>
<td>29.9</td>
</tr>
<tr>
<td>C&lt;sub&gt;19:0 cyclo&lt;/sub&gt;</td>
<td>8.9</td>
<td>14.8</td>
</tr>
<tr>
<td>C&lt;sub&gt;19:1ω6&lt;/sub&gt;</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>67.7</td>
<td>61.2</td>
</tr>
</tbody>
</table>

http://ijs.sgmjournals.org
both JC85<sup>T</sup> and <i>R. borbori</i> DN316<sup>T</sup>. Oxidase and catalase were positive for both the strains. Chitinase, lipase, coagulase, amylase and urease activities were negative for both strains. Resistance to antibiotics was determined according to the method described by Gao et al. (1994). Strain JC85<sup>T</sup> is resistant to ampicillin (10 μg), while <i>R. borbori</i> DN316<sup>T</sup> is sensitive. Diazotrophic growth (determined in a medium devoid of combined nitrogen) and acetylene reduction activity (Fig. S5) as determined according to the method of Sasikala et al. (1990) were positive for both strains.

Strain JC85<sup>T</sup> is a facultative anaerobe (tested by agar shake; Widdel, 1983) having chemo-organoheterotrophy [aerobic (respiration) and anaerobic (fermentation); only with glucose and not pyruvate and thus differs from <i>R. borbori</i> DN316<sup>T</sup>)] growth, while chemolithoautotrophy [aerobic, Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> (1 mM) and HCO<sub>3</sub> (0.1 %)] could not be demonstrated. The utilization of various organic substrates for growth as sole carbon sources was tested by using GN2 MicroPlates (Biolog) in accordance with the manufacturer’s instructions. Organic substrates which support growth of strain JC85<sup>T</sup> are given in Table S1. Aniline is not degraded by strain JC85<sup>T</sup>, which further differentiates it from <i>R. borbori</i> DN316<sup>T</sup>. Ammonium chloride, NO<sub>3</sub>, glutamate and glutamine supported growth of strain JC85<sup>T</sup> as sole source of nitrogen, while urea and NO<sub>2</sub> did not.

Cells of JC85<sup>T</sup> and <i>R. borbori</i> DN316<sup>T</sup> growing in nutrient broth were harvested by centrifugation (10000 g for 15 min at 4 °C) on reaching a cell density of 70 % of the maximum optical density and the pellet was used for fatty acid analysis. Cellular fatty acids were methylated, separated and identified according to the instructions for the Microbial Identification System [Microbial ID; MIDI 6.0 version; Agilent: 6850; peak identification was done based on RTSBA6 database; Sasser (1990); revised-www.midi-inc.com] which was outsourced through Royal Research Laboratories, Secunderabad, India. Whole-cell fatty acid analysis of strain JC85<sup>T</sup> revealed that C<sub>18:1ω7c</sub> (69 %) is predominant with minor amounts of C<sub>19:0 cyclo ω8c</sub> (8.9 %), C<sub>16:0</sub> (6.9 %), C<sub>12:0</sub> (5.7 %) and C<sub>19:1ω7c/C<sub>19:1ω6c</sub> (2.2 %).

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 F<sub>254</sub>; 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 (quaternary nitrogen) or α-napthol (specific for sugars) (Kates, 1972; Oren et al., 1996). Polar lipids of strain JC85<sup>T</sup> include two unidentified aminophospholipids (APL1,2), two unidentified phospholipids (PL1,2), phosphatidyl choline (PC) and four unidentified lipids (L1–4). Polar lipids of strain JC85<sup>T</sup> differ from <i>R. borbori</i> DN316<sup>T</sup> in the presence of unidentified lipid L1 and absence of unidentified lipid L5 (Fig. S6). Quinones were extracted with a chloroform: methanol (2:1 v/v) mixture, purified by TLC and analysed by HPLC as reported by Tamaoka et al. (1983). Both strains have ubiquinone-10 (>97%) as the major respiratory quinone.

Based on the clear phenotypic and genotypic distinctiveness (Table 1) from its phylogenetic neighbour, <i>R. borbori</i> DN316<sup>T</sup>, strain JC85<sup>T</sup> is considered to represent a novel species of the genus <i>Rhizobium</i>, for which we propose the name <i>Rhizobium subbaraonis</i> sp. nov.

### Description of <i>Rhizobium subbaraonis</i> sp. nov.

<i>Rhizobium subbaraonis</i> (sub.ba.ra.o.nis N.L. gen. n. subbaraonis of Subba Rao, named after Professor N. S. Subba Rao, an eminent microbiologist who significantly contributed to the knowledge of <i>Rhizobium</i> biofertilizers in India).

Forms yellow coloured colonies on nutrient agar. Cells are rod-shaped, 0.8–1.2 μm in diameter. Gram-stain-negative. Oxidase- and catalase-positive. Growth mode is chemooorganoheterotrophic (respiration/fermentation). Good growth occurs after 48 h of incubation on nutrient agar at 30 °C. Growth occurs between pH 6.0–7.5 (optimum 7.0). NaCl is not required and tolerated up to 3 % (w/v). Utilizes a number of carbon sources for growth. Good carbon sources for growth are D-fructose, α-D-glucose, D-sorbitol, glycerol, malate, succinate and glutamate. Major fatty acid is C<sub>18:1ω7c</sub> with minor amounts of C<sub>19:0 cyclo ω8c</sub> C<sub>16:0</sub>, C<sub>12:0</sub> and C<sub>19:1ω7c/C<sub>19:1ω6c</sub> Q-10 is the major quinone.

The type strain is JC85<sup>T</sup> (=DSM 24765<sup>T</sup>=KCTC 23614<sup>T</sup>). The type strain was isolated as an endolith from a beach sand sample collected from Diamond Harbour, Kolkata, West Bengal, India. The DNA G+C content of the type strain is 67.7 mol%.

### Acknowledgements

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### References


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Epilithic and endolithic bacterial communities


