**Brevibacterium siliguriense** sp. nov., a facultatively oligotrophic bacterium isolated from river water

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A Gram-positive-staining, rod-shaped, facultatively oligotrophic bacterial strain, designated MB18\(^T\), was isolated from a water sample collected from the River Mahananda at Siliguri (26° 44' 23.20" N, 88° 25' 22.89" E), West-Bengal, India. On the basis of 16S rRNA gene sequence similarity, the closest relative of this strain was *Brevibacterium epidermidis* NCDO 2286\(^T\) (96% similarity). The DNA G+C content of strain MB18\(^T\) was 64.6 mol%. Chemotaxonomic data (MK-8(H\(_2\)) as the major menaquinone, galactose as the sole cell-wall sugar, meso-diaminopimelic acid as the diagnostic cell-wall diamino acid, phosphatidylglycerol and diphasatidylglycerol as constituents of the polar lipids, anteiso-C\(_{15}:0\) anteiso-C\(_{17}:0\) and iso-C\(_{15}:0\) as the major fatty acids) supported the affiliation of strain MB18\(^T\) to the genus *Brevibacterium*. The results of DNA G+C content, 16S rRNA gene sequence analysis and biochemical and physiological analyses allowed genotypic and phenotypic differentiation of strain MB18\(^T\) from its nearest neighbour *B. epidermidis*. The isolate therefore represents a novel species, for which the name *Brevibacterium siliguriense* sp. nov. is proposed; the type strain is MB18\(^T\) (=DSM 23676\(^T\)=LMG 25772\(^T\)).

The genus *Brevibacterium* was established by Breed (1957) for some non-sporulating, non-branching, Gram-positive rods, which were earlier assigned to the genus 'Bacterium'. Eventually, the genus *Brevibacterium* was described after the type species, *Brevibacterium linens* (Collins et al., 1980). At present, 44 species isolated from diverse habitats, ranging from human body parts to soil and water, have been reported in this genus (http://www.bacterio.cict.fr/b/brevibacterium.html). Very recently, a novel species, *Brevibacterium daeguense*, isolated from a industrial wastewater treatment plant (Cui et al., 2012), was added to the existing list. In the present study, strain MB18\(^T\), a novel member of the genus *Brevibacterium*, was isolated from the facultatively oligotrophic bacterial population of Mahananda River water, recovered on nutrient-poor medium [10\(^{-3}\) diluted Luria broth (LB) amended with 1.5% agar (0.001 × LA)].

Sampling, isolation and identification of oligotrophic bacteria were conducted according to the methods described previously (Kumar et al., 2010). Strain MB18\(^T\) was facultatively oligotrophic because it had the ability to grow and multiply in both nutrient-poor (0.001 × LA) and nutrient-rich (undiluted LA) media. In diluted (10\(^{-3}\)) LB, an increase of ~12 times the initial cell number took place after 4 days incubation at 28 °C. In rich (undiluted LB) medium, an increase of 10 times the initial cell number was noted after 10 h of incubation at 28 °C (see Fig. S1 in IJSEM Online). The strain was also capable of growing in other diluted (10\(^{-3}\)) and undiluted media such as nutrient broth (NB; HiMedia, India) and tryptone soy broth (TSB; HiMedia). Luria agar (M575; HiMedia) was used for the maintenance of the strain and for the determination of phenotypic, including chemotaxonomic, characteristics. *Brevibacterium epidermidis* LMG 21455\(^T\) was used as a reference strain. Cell morphology and motility were determined with a phase-contrast microscope (Olympus); details of the cell shape (Fig. S2) were ascertained with help of a scanning electron microscope (LEO 1430 VP). The Gram test was performed by the KOH lysis method (Murray et al., 1999) and further confirmed by the Gram-staining.

Abbreviations: FAMES, fatty acid methyl esters; mDAP, meso-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of MB18\(^T\) is AM937247.
method of Claus (1992). Growth of the strain MB18<sup>T</sup> was tested at 10, 20, 28, 30, 37, 40 and 45 °C (± 1). For salt tolerance tests, 2, 4, 6, 8, 10, 15 and 20% (w/v) NaCl or KCl was added to peptone-yeast extract (PY) medium (composition: 10 g peptone l<sup>−1</sup>, 5 g yeast extract l<sup>−1</sup>) devoid of NaCl or KCl. To assess growth at different pH levels, the pH of the sterile LB medium was adjusted from pH 3.0 to 12.0 by using either 0.1 M HCl or 0.1 M NaOH. Results were scored after 48 h incubation at 28 °C. Catalase activity was examined by bubble production after the addition of few drops of 3% (v/v) H<sub>2</sub>O<sub>2</sub>. The ability to hydrolyse starch was determined by assessing the development of clear zones around the streaked culture. Lipase production was determined by standard procedures. Haemolytic activity and gelatin hydrolysis were tested according to the method described by Bouvet & Grimont (1986). Pyrrozinamidase activity and acid production from 2,3-butyrate glycol were detected as described by Wauters et al. (2001). Acid production from ethylene glycol and phenylacetate and alkali production from sodium-formate were detected by using previously described methods (Wauters et al., 1998, 2003). Hydrolysis of casein, tyrosine and xanthine were examined using the method described by Gordon et al. (1974). Oxidase, lysine utilization, ornithine utilization, urease activity, deamination of phenylalanine, reduction of nitrate, H<sub>2</sub>S production, citrate utilization, VP reaction, methyl red test, malonate utilization and carbon source utilization/fermentation tests were carried out using HiBio-ID/HiCarbo system (HiMedia) and GP cards (VITEK 2 system, bioMérieux) according to the manufacturers’ instructions. Results were scored after 7 days at 28 °C (Tables S1a and b). The biochemical characteristics of strain MB18<sup>T</sup> were also determined using the Biolog GP2 MicroPlate system (bioMérieux). Bacterial suspensions, prepared in GP sterile inoculation fluid, were transferred to GP2 Microplates as described by the manufacturer. Incubation was carried out in an aerobic atmosphere for 24 h and the reactions were read using the fully automated OmniLog system (Tables S1a and b). Antibiotic susceptibility (specific for oligotrophic bacteria) was determined according to the method described by Kumar et al. (2010). Susceptibilities to some of the drugs were also tested using a GP card (VITEK 2 system) and the results were interpreted according to the manufacturer’s instructions (Table S1a). The novel strain differed from <i>B. epidermidis</i> DSM 21455<sup>T</sup> with respect to many phenotypic characteristics (Table 1).

Menaquinones were extracted from lyophilized cells and analysed by HPLC following methods described by Collins et al. (1977) and Groth et al. (1997), respectively. Polar lipids were isolated following the procedure of Minnikin et al. (1984). The polar lipids were detected on aluminium backed silica gel 60 F<sub>254</sub> plates (Merck) by one- and two-dimensional TLC (Counsell & Murray, 1986). Polar lipids, phosphatidylglycerol and diphasphatidylglycerol phospholipids were characteristically found in strain MB18<sup>T</sup>. The absence of mycolic acids was demonstrated with TLC (Minnikin et al., 1980). The cell walls were prepared

### Table 1. Differential phenotypic characteristics between strain MB18<sup>T</sup> and <i>B. epidermidis</i> DSM 21455<sup>T</sup>, the nearest phylogenetic neighbour

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MB18&lt;sup&gt;T&lt;/sup&gt;</th>
<th>B. epidermidis DSM 21455&lt;sup&gt;T&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth at:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>37 °C</td>
<td>Poor</td>
<td>Good</td>
</tr>
<tr>
<td>40 °C</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of casein</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Hydrolysis of xanthine</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Acid from phenylacetate</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Carbon utilization:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Arabinose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Xylose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Inositol</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Mannose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Raffinose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Glycerol</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Galactose</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Gluconate</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Enzymes:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Fermentation of o-mannitol</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>64.6</td>
<td>63.5</td>
</tr>
<tr>
<td>Origin of isolation</td>
<td>River water</td>
<td>Human skin</td>
</tr>
</tbody>
</table>

(Boone & Pine, 1968) from 200 mg cell mass, and sugars and <i>meso</i>-diaminopimelic acid (mDAP) in acid hydrolysates were identified by single-dimensional TLC following a previously described method (Staneck & Roberts, 1974). The characteristic presence of mDAP in the peptidoglycan and galactose as the sole sugar was identified in the cell wall of strain MB18<sup>T</sup>. Genomic DNA (for the determination of the DNA G+C content) was prepared by disrupting cells with a French pressure cell and was purified on hydroxyapatite following standard procedures (Cashion et al., 1977). The purified DNA was hydrolysed with p1 nuclease and the nucleotides dephosphorylated with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleoside were analysed by HPLC (Shimadzu) with a LC-20AD solvent delivery module, DGU-3A online degasser, CTO-10AC column oven, SIL-20A automatic sample injector and a SPD-6A UV spectrophotometric detector. The system was calibrated with non-methylated lambda DNA (Sigma; 49.86 mol% G+C) and three reference sample DNAs from <i>Bacillus subtilis</i> DSM 402 (43.52 mol% G+C), <i>Xanthomonas campes tris</i> pv. <i>campes tris</i> DSM 3586<sup>T</sup> (65.07 mol% G+C) and <i>Streptomyces violaceoruber</i> DSM 40783 (72.12 mol% G+C). The G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah et al. (1989). The DNA G+C content of the strain
MB18<sup>T</sup> was found to be 64.6 mol%, while the DNA G+C content of <i>B. epidermidis</i> DSM 20660<sup>T</sup> was 63.5 mol%.

For analysis of fatty acids, fatty acid methyl esters (FAMEs) were extracted from 36 h-old (exponentially growing) cells grown in tryptone-soy-agar (M290; HiMedia) at 28 °C (the growth curve for strain MB18<sup>T</sup> is shown in Fig. S1). They were then analysed by GC (Hewlett Packard 5890 II plus) and the Sherlock Microbial Identification System using version 4.10 of the TSBA40 library (Microbial ID). Fatty acids of strain MB18<sup>T</sup> [anteiso-C<sub>15:0</sub> (50.82 %), anteiso-C<sub>17:0</sub> (26.73 %), iso-C<sub>15:0</sub> (14.04 %), iso-C<sub>16:0</sub> (4.07 %); iso-C<sub>17:0</sub> (3.16 %); and trace amount of C<sub>16:0</sub> (0.48 %); iso-C<sub>14:0</sub> (0.47 %) and C<sub>14:0</sub> (0.22 %)] were typical of members of the genus <i>Brevibacterium</i>, but the proportions differed from those reported for <i>B. epidermidis</i> DSM 20660<sup>T</sup> [anteiso-C<sub>15:0</sub> (70.0 %), anteiso-C<sub>17:0</sub> (21.5 %), iso-C<sub>15:0</sub> (50.82 %), anteiso-C<sub>17:0</sub> (21.5 %), iso-C<sub>16:0</sub> (3.0 %); and trace amount of anteiso-A C<sub>17:1</sub> (2.0 %), C<sub>18:0</sub> (1.5 %) and C<sub>16:0</sub> (1.0 %); Collins <i>et al.</i>, 1983].

The 16S rRNA gene of strain MB18<sup>T</sup> was amplified from the genomic DNA, purified and sequenced according to Kumar <i>et al.</i> (2010). An almost complete 16S rRNA gene sequence comprising 1433 bp was obtained. 16S rRNA gene sequence comparisons with entries in the updated GenBank and EMBL databases were performed with the FASTA and BLAST programs (Pearson, 1990; Altschul <i>et al.</i>, 1990, 1997). To determine the phylogenetic affiliation, the 16S rRNA gene sequence of strain MB18<sup>T</sup> was aligned with the sequences of members of the genus <i>Brevibacterium</i> with the CLUSTAL W program (Thompson <i>et al.</i>, 1994). An approximate 1360 bp-long stretch of the 16S rRNA gene sequences present in all member of the genus <i>Brevibacterium</i> between positions 13 and 1371 were selected from the nucleotide database of the EMBL European Bioinformatics Institute (http://www.ebi.ac.uk) and used for further analysis. The rest of the flanking nucleotide sequences were omitted due to alignment ambiguities. Evolutionary relationships of members of genus <i>Brevibacterium</i> were inferred using two different tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971). Evolutionary distances were computed using the Kimura two-parameter method (Kimura, 1980). Phylogenetic analyses and the fidelity of the tree topologies were evaluated by bootstrap analysis with 1000 replicates using MEGA4 software (Felsenstein, 1985; Tamura <i>et al.</i>, 2007). According to the comparison of 16S rRNA gene sequences, the closest relative of strain MB18<sup>T</sup> was <i>B. epidermidis</i> NCDO 2286<sup>T</sup> (=DSM 20660<sup>T</sup>) =LMG 21455<sup>T</sup>) showing 96 % sequence similarity. In the light of earlier studies done by Stackebrandt & Goebel (1994) and Lee (2006), the 16S rRNA gene sequence comparisons showed sufficient differences so that the isolate could be allocated to a separate genospecies without the need for DNA–DNA

![Fig. 1. Consensus neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain MB18<sup>T</sup> within the genus <i>Brevibacterium</i>. The tree was reconstructed using maximum-parsimony method and common clusters obtained in both the neighbour-joining and maximum-parsimony trees are indicated by the hash symbol (#). Bootstrap values (>70 %) expressed as percentages of 1000 replicates are given at each branch point. <i>Dermabacter hominis</i> DSM 7083<sup>T</sup> was used as outgroup. Bar, 5 nt substitutions per 1000 nt.](http://ijs.sgmjournals.org)
hybridization experiments. A tree depicting the phylogenetic position of strain MB18T within the genus *Brevibacterium* is shown in Fig. 1. Based on 16S rRNA gene sequence comparison, strain MB18T forms a distinct subclade with *B. epidermidis* NCDO 2286T.

Strain MB18T can be readily differentiated from the closest relative, *B. epidermidis* NCDO 2286T with reference to some physiological and biochemical characteristics (Table 1), cellular fatty acids, 16S rRNA gene sequence and DNA G+C content. On the basis of the data obtained from our study using a polyphasic taxonomic approach, strain MB18T merits recognition as a member of a novel species of the genus *Brevibacterium*, for which we propose the name *Brevibacterium siliguriense* sp. nov.

**Description of *Brevibacterium siliguriense* sp. nov.**

*Brevibacterium siliguriense* (si.li.gu.ri.en’se. N.L. neut. adj. siliguriense of or pertaining to the town Siliguri, the location from where the water sample was collected).

Cells stain Gram-positive and are non-motile, non-spore-forming rods, 2.0±0.23 μm long and 0.4±0.06 μm wide. Catalase-positive and oxidase-negative. Colonies are off-white, circular and low convex with entire margin. Growth is observed at 20–37 °C (optimum 28 °C; no growth at 40 °C), pH 5–12 (optimum 7.0), and 0–15 % (w/v) KCl and NaCl (optimum 2 %). The supplementation of KCl in PY media produced better growth than NaCl. Urease, gelatinase, arginine dihydrolase, β-galactosidase, β-galactopyranosidase, leucine arylamidase, L-proline arylamidase, α-galactosidase, alanine arylamidase and tyrosine arylamidase activities are positive. Nitrate is reduced to nitrite. Tests for α-glucosidase, α-l-threo-pro arylamidase, L-asparrate, arylamidase, α-mannosidase, phosphatase, β-glucuronidase, H2S production, indole production, L-lyprolidinyl-arylamidase and fermentation of D-xylene, D-sorbitol, D-galactose, D-galactose, D-ribose, lactose, N-acetyl D-glucosamine, maltose, raffinose, saccharose, trehalose and D-amylodalan are negative. Additional phenotypic characteristics and the antibiogram are given in Tables S1a and S1b.

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**References**


