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 MB18T, 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 2286T (96 % similarity). The DNA G+C content of strain MB18T was 64.6 mol%. Chemotaxonomic data (MK-8(H2) as the major menaquinone, galactose as the sole cell-wall sugar, meso-diaminopimelic acid as the diagnostic cell-wall diamino acid, phosphatidylglycerol and diphosphatidylglycerol as constituents of the polar lipids, anteiso-C15:0 3-OH, anteiso-C17:0 and iso-C15:0 as the major fatty acids) supported the affiliation of strain MB18T 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 MB18T 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 MB18T (=DSM 23676T = LMG 25772T).

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 an industrial wastewater treatment plant (Cui et al., 2012), was added to the existing list. In the present study, strain MB18T, 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 MB18T 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 21455T 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 MB18T is AM937247.

Supplementary figures and tables are available with the online version of this paper.
method of Claus (1992). Growth of the strain MB18T 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 1⁻¹, 5 g yeast extract 1⁻¹) 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) H₂O₂. 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). Pyrazinamidase activity and acid production from 2,3-butyric acid 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, lysozyme utilization, ornithine utilization, urease activity, deamination of phenylalanine, reduction of nitrate, H₂S production, citrate utilization, VP reaction, methyl red test, malonate utilization and carbon source utilization/fermentation tests were carried out using HiBioID/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 MB18T 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 B. epidermidis DSM 21455T 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 F₂₅₄ plates (Merck) by one- and two-dimensional TLC (Counsell & Murray, 1986). Polar lipids, phosphatidylglycerol and diphosphatidylglycerol phospholipids were characteristically found in strain MB18T. The absence of mycolic acids was demonstrated with TLC (Minnikin et al., 1980). The cell walls were prepared (Boone & Pine, 1968) from 200 mg cell mass, and sugars and meso-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 MB18T. 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. The DNA G+C content was calculated from the ratio of deoxyguanosine (dG) and deoxythymidine (dT) according to the method of Mesbah et al. (1980). The DNA G+C content of the strain

### Table 1. Differential phenotypic characteristics between strain MB18T and B. epidermidis LMG 21455T, the nearest phylogenetic neighbour

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>MB18T</th>
<th>B. epidermidis LMG 21455T</th>
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<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 D-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>

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MB18\(^T\) was found to be 64.6 mol\%, while the DNA G+C content of \(B.\) epidermidis DSM 20660\(^T\) 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\(^T\) 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\(^T\) [anteiso-C\(_{15:0}\) (50.82%), anteiso-C\(_{17:0}\) (26.73%), iso-C\(_{15:0}\) (14.04%), iso-C\(_{16:0}\) (4.07%); iso-C\(_{17:0}\) (3.16%); and trace amount of C\(_{16:0}\) (0.48%); iso-C\(_{14:0}\) (0.47%) and C\(_{14:0}\) (0.22%)] were typical of members of the genus Brevibacterium, but the proportions differed from those reported for \(B.\) epidermidis DSM 20660\(^T\) [anteiso-C\(_{15:0}\) (70.0%), anteiso-C\(_{17:0}\) (21.5%), iso-C\(_{15:0}\) (3.0%); and trace amount of anteiso-A C\(_{17:1}\) (2.0%), C\(_{18:0}\) (1.5%) and C\(_{16:0}\) (1.0%); Collins et al., 1983].

The 16S rRNA gene of strain MB18\(^T\) was amplified from the genomic DNA, purified and sequenced according to Kumar et al. (2010). An almost complete 16S rRNA gene sequence comprising 1433 bp was obtained. 16S rRNA sequences, the closest relative of strain MB18\(^T\) was \(B.\) epidermidis NCDO 2286\(^T\) (=DSM 20660\(^T\) =LMG 21455\(^T\)), 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 hybridization. To determine the phylogenetic affiliation, the 16S rRNA gene sequence of strain MB18\(^T\) was aligned with the sequences of members of the genus Brevibacterium with the CLUSTAL W program (Thompson et al., 1994). An approximate 1360 bp-long stretch of the 16S rRNA gene sequences present in all member of the genus Brevibacterium 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 Brevibacterium 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 et al., 2007). According to the comparison of 16S rRNA gene sequences, the closest relative of strain MB18\(^T\) was \(B.\) epidermidis NCDO 2286\(^T\) (=DSM 20660\(^T\) =LMG 21455\(^T\)), 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 hybridization.

**Fig. 1.** Consensus neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences showing the position of strain MB18\(^T\) within the genus Brevibacterium. 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 replications are given at each branch point. *Dermabacter hominis* DSM 7083\(^T\) was used as outgroup. Bar, 5 nt substitutions per 1000 nt.
hybridization experiments. A tree depicting the phylogenetic position of strain MB18\(^T\) within the genus *Brevibacterium* is shown in Fig. 1. Based on 16S rRNA gene sequence comparison, strain MB18\(^T\) forms a distinct subclade with *B. epidermidis* NCDO 2286\(^T\).

Strain MB18\(^T\) can be readily differentiated from the closest relative, *B. epidermidis* NCDO 2286\(^T\) 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 MB18\(^T\) 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-la-pha-pro arylamidase, α-asparrtate, arylamidase, α-mannosidase, phosphatase, β-glucuronidase, H\(^2\)S production, indole production, L-tryptophan-arylamidase and fermentation of D-xylene, D-sorbitol, D-galactose, D-ribose, lactose, N-acetyl D-glucosamine, maltose, raffinose, saccharose, trehalose and D-amygaldin are negative. Additional phenotypic characteristics and the antibiogram are given in Tables S1a and S1b. Contains mDAP in the cell-wall peptidoglycan. Phosphatidylglycerol and diphosphatidylglycerol are the major polar lipids. The major menaquinone is MK-8(H\(^2\)) and fatty acid profile contains predominantly anteiso-C\(_{15:0}\) anteiso-C\(_{17:0}\) and iso-C\(_{15:0}\) gagged.

The type strain MB18\(^T\) (=DSM 23676\(^T\) =LMG 25772\(^T\)) was isolated from a water sample from the river Mahananda, Siliguri, Darjeeling district, West Bengal, India. The DNA G+C content of the type strain is 64.6 mol%.

**Acknowledgements**

This work was supported by the Department of Biotechnology (DBT), Government of India via a PhD studenthip (A. Kumar, award no. DBT-JRF/05-06/71/29.08.05) and a major research grant (R. Chakraborty, grant number, BT/PR-7164/BCE/08/448/2006). We express our gratitude to M. D. Adhikari (Indian Institute of Technology Guwahati) for providing the SEM micrograph of *Brevibacterium siliguriense* sp. nov. strain MB18\(^T\). Dr B. Bhadra (CCMB Hyderabad, now at Dupont, Hyderabad) is acknowledged for providing the mDAP standard. We thank the following for providing chemicals: B. K. Tiwary, R. Kumar, S. Mandal and K. K Singh. Strain *B. epidermidis* LMG 2145\(^T\) was a kind gift from the BCCM/LMG Bacteria collection, University of Ghent, Belgium.

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