**Mucilaginibacter litoreus** sp. nov., isolated from marine sand

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A Gram-staining-negative, non-spore-forming, facultatively anaerobic, non-flagellated, non-gliding, rod-shaped bacterium, designated strain BR-18T, was isolated from marine sand collected on the western coast of South Korea. The taxonomic position of the novel strain was determined using a polyphasic approach. Strain BR-18T grew optimally at 25 °C, at pH 6.5–7.0 and in the absence of NaCl. In phylogenetic analyses based on 16S rRNA gene sequences, the novel strain fell within a clade comprising members of the genus *Mucilaginibacter* and appeared most closely related to *Mucilaginibacter lutimaris* BR-3T (96.6 % sequence similarity) and *Mucilaginibacter rigui* WPCB133T (95.9 %). The novel strain showed lower levels of 16S rRNA gene sequence similarity with the type strains of other members of the genus *Mucilaginibacter* (93.4–95.5 %) and those of other species included in the phylogenetic analyses (<91.6 %). Strain BR-18T contained MK-7 as its predominant menaquinone, summed feature 3 (C16 : 1ω7c and/or iso-C15 : 0 2-0H) and iso-C15 : 0 as its major fatty acids, phosphatidylethanolamine and an unidentified aminophospholipid as its major polar lipids, and sphingolipids. The genomic DNA G+C content of the novel strain was 42.4 mol%. Based on the phylogenetic and phenotypic data, strain BR-18T represents a novel species of the genus *Mucilaginibacter*, for which the name *Mucilaginibacter litoreus* sp. nov. is proposed. The type strain is BR-18T (=KCTC 23697T =CCUG 61484T).

The genus *Mucilaginibacter*, a member of the family Sphingobacteriaceae (phylum Bacteroidetes), was created by Pankratov et al. (2007). At the time of writing, the genus *Mucilaginibacter* comprised 17 species: *Mucilaginibacter paludis* (type species) and *Mucilaginibacter gracilis* (Pankratov et al., 2007), *Mucilaginibacter kameinonensis* (Urai et al., 2008), *Mucilaginibacter daejeonensis* (An et al., 2009), *Mucilaginibacter ximonensis* (Luo et al., 2009), *Mucilaginibacter oryzae* (Jeon et al., 2009), *Mucilaginibacter rigui* (Baik et al., 2010), *Mucilaginibacter gossypii* and *Mucilaginibacter gossypicola* (Madhaiyan et al., 2010), *Mucilaginibacter frigortolerans*, *Mucilaginibacter lappiensis* and *Mucilaginibacter mallensis* (Männistö et al., 2010), *Mucilaginibacter dorajii* (Kim et al., 2010, 2011), *Mucilaginibacter myungsuensis* (Joung & Jho, 2011), *Mucilaginibacter boryungensis* (Kang et al., 2011), *Mucilaginibacter angelicae* (B.-C. Kim et al., 2012) and *Mucilaginibacter lutimaris* (J.-H. Kim et al., 2012). Members of the genus *Mucilaginibacter* have been isolated from various habitats, including peat bog, soil, dried rice straw, wetland freshwater and the rhizospheres of plants (Pankratov et al., 2007; Urai et al., 2008; An et al., 2009; Baik et al., 2010; Madhaiyan et al., 2010; Kim et al., 2010). In this study, we describe a *Mucilaginibacter*-like bacterial strain, designated BR-18T, that was isolated from marine sand collected at Boryung, on the western coast of South Korea. The aim of the present work was to determine the exact taxonomic position of this novel strain using a polyphasic approach.

Strain BR-18T was isolated by the dilution plating technique on R2A agar (Difco) at 25 °C and was cultivated routinely under the same conditions. Cell morphology was investigated under a light microscope (DP70; Olympus) and by transmission electron microscopy (CM-20; Philips). The presence of flagella was also investigated by transmission electron microscopy. For this purpose, cells from an exponentially growing culture on R2A agar were negatively stained with 1 % (w/v) phosphotungstic acid, and the grids were examined after being air-dried. Gliding motility was investigated as described by Bowman (2000). Gram staining was determined by using a Gram staining kit (bioMérieux) according to the manufacturer’s instructions. Growth under anaerobic conditions was determined after...
incubation in an anaerobic chamber (1029; Forma), in an atmosphere of \( \text{N}_2/\text{CO}_2/\text{H}_2 \) (86:7:7, by vol.) and on R2A agar that had been prepared anaerobically, under nitrogen gas. Growth in the absence of NaCl and in the presence of 0.5, 1.0, 2.0 and 3.0 % (w/v) NaCl was investigated by using R2A broth (prepared according to the Difco formula for R2A agar except that agar was excluded). Growth at 4, 10, 15, 20, 25, 28, 30, 37, 40 and 45 °C was measured on R2A agar. The pH range for growth was determined in R2A broth that had been adjusted to pH 4.0–9.5 (at intervals of 0.5 pH unit) with sodium acetate/acetic acid or Na\(_2\)CO\(_3\) buffers. Initial pH values were verified after autoclaving. Catalase and oxidase activities were determined as described by Cowan & Steel (1965). The presence of flexirubin-type pigments was investigated as described previously (Reichenbach, 1992; Bernardet et al., 2002). Hydrolysis of casein (skim milk; BD), hypoxanthine (Sigma), L-tyrosine (Sigma) and xanthine (Sigma) was described by Cowan & Steel (1965). The presence of Catalase and oxidase activities were determined as described previously (La ´nyı´, 1987). Degradation of various polysaccharides was tested on basal agar that had been adjusted to pH 4.0–9.5 (at intervals of 0.5 pH unit; Forma), in an anaerobic chamber (1029; Forma), in an atmosphere of N\(_2/\text{CO}_2/\text{H}_2\) (86 : 7 : 7, by vol.) and on R2A agar, using the substrate concentrations described by Cowan & Steel (1965). Hydrolysis of gelatin and urea was investigated by using nutrient gelatin and urea agar base media (BD), respectively. Nitrate reduction and hydrolysis of aesculin (Sigma) and Tween 80 (Junsei) were investigated as described previously (Lányi, 1987).

Degradation of various polysaccharides was tested on basal medium agar containing [1L artificial seawater]\(^{-1}\) 12 g gellan gum, 1 g yeast extract, 0.5 g NH\(_4\)Cl, 50 ml 1 M Tris/HCl (pH 7.4), 5 g low-melting agarose (Lonza) and 5 g low-viscosity alginate (Wako Chemicals), carboxymethyl-cellulose (CMC; Sigma), \( \kappa \)-carrageenan (Sigma), colloidal chitin, curdlan (Wako Chemicals), pectin from apple peel (Sigma), starch (Sigma) or birchwood xylan (Sigma). Colloidal chitin was prepared from shrimp shells by following a modification of the method of Rodriguez-Kabana et al. (1983). For this, shrimp shells (5 g) were dissolved in concentrated HCl (50 ml) and stirred at 4 °C overnight. The resultant chitin solution was slowly poured into 50 % (v/v) chilled ethanol with vigorous stirring. The pH of the mixture was neutralized with NaOH and, after centrifugation for 30 min at 6000 r.p.m., the supernatant solution was decanted off, and the colloid was dried in a desiccator. Alginate lyase activity was revealed by flooding with 10 % (w/v) cetylpyridinium chloride while degradation of CMC and chitin was revealed by flooding with 0.1 % (w/v) Congo red. Degradation of agarose, carrageenan, pectin and xylan was revealed by flooding with 10-fold-diluted Lugol’s iodine solution. For the detection of curdlanase activity, aniline blue was added to the medium before autoclaving, to give a final concentration of 0.005 % (w/v); activity was then revealed as clear haloes around the bacterial colonies (Mahasneh & Stewart, 1980).

Susceptibility to antibiotics was investigated, on R2A agar plates, using antibiotic discs (Advantec) containing ampicillin (10 \( \mu \)g), carbenicillin (100 \( \mu \)g), cephaplatin (30 \( \mu \)g), chloramphenicol (100 \( \mu \)g), gentamicin (30 \( \mu \)g), kanamycin (30 \( \mu \)g), lincomycin (15 \( \mu \)g), neomycin (30 \( \mu \)g), novobiocin (5 \( \mu \)g), oleandomycin (15 \( \mu \)g), penicillin G (20 U), polymyxin B (100 U), streptomycin (50 \( \mu \)g) or tetracycline (30 \( \mu \)g). Utilization of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20NE, API 50CH and API ZYM systems (bioMérieux). Utilization of various substrates was determined by inoculating the API 50CH and API 20NE strips with cells suspended in AUX medium (bioMérieux). The morphological, cultural, physiological and biochemical characteristics of strain BR-18\(^T\) are summarized in the species description (see below) and Table 1.

Cell biomass for DNA extraction and the analyses of isoprenoid quinones and polar lipids was obtained by the culture of strain BR-18\(^T\) in R2A broth for 3 days at 25 °C. Genomic DNA was isolated and purified by the method of Yoon et al. (1996), with the exception that RNase T1 was used, in combination with RNase A, to minimize contamination with RNA. The 16S rRNA gene of the novel strain was amplified by PCR using two universal primers, as described previously (Yoon et al., 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon et al. (2003). In a neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, the almost-complete 16S RNA gene sequence of strain BR-18\(^T\) determined in this study (1445 nt) was clustered with that of \( M. \) rigui WPCB133\(^T\) and \( M. \) lutimaris BR-3\(^T\), within a clade comprising \( Mucilaginibacter \) species (Fig. 1). The corresponding maximum-likelihood and maximum-parsimony trees showed similar topologies (Fig. 1). In the analyses based on 16S rRNA gene sequences, strain BR-18\(^T\) appeared most closely related to \( M. \) lutimaris BR-3\(^T\) (96.6 %), \( M. \) rigui WPCB133\(^T\) (95.9 %) and \( M. \) paludis TPT56\(^T\) (94.5 %). The novel strain showed lower levels of 16S rRNA gene sequence similarity with the type strains of other members of the genus \( Mucilaginibacter \) (93.4–95.5 %) and those of other species included in the phylogenetic analyses (<91.6 %).

Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and an ODS-A (250 × 4.6 mm) column (YMC). The isoprenoid quinones were eluted at room temperature with a mixture of methanol/2-propanol (2 : 1, v/v) at a flow rate of 1 ml min\(^{-1}\) and were detected by the measurement of absorbance at 270 nm. The predominant isoprenoid quinone detected in strain BR-18\(^T\), as well as all members of the family \textit{Sphingobacteriaceae} (Pankratov et al., 2007; Baik et al., 2010; Madhaiyan et al., 2010; Männistö et al., 2010; Kim et al., 2010), was menaquinone-7 (MK-7), although a minor amount of MK-6 was also detected.

Fatty acids were saponified, methylated and extracted using the standard protocol of version 4.0 of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed on a 6890 GC (Hewlett Packard) and identified by using the TSBA40 database of the Microbial Identification System (Sasser, 1990). In Table 2, the
complete cellular fatty acid profile of strain BR-18<sup>T</sup> is compared with those of the type strains of M. paludis (the type species of the genus Mucilaginibacter), M. rigui and M. lutinaris (J.-H. Kim et al., 2012). The fatty acid profiles of the four strains were similar, even though there were differences in the proportions of some fatty acids, particularly anteiso-C<sub>15:0</sub> (Table 2). The major fatty acids found in strain BR-18<sup>T</sup> were summed feature 3 (C<sub>16:1</sub>ω7c and/or iso-C<sub>15:0</sub> 2-OH) and iso-C<sub>15:0</sub> (Table 2).

The polar lipids of strain BR-18<sup>T</sup> were extracted according to the procedures described by Minnikin et al. (1984) and identified by two-dimensional TLC followed by spraying with ethanolic molybdophosphoric acid, molybdenum blue, ninhydrin and α-naphthol reagents (Minnikin et al., 1984; Komagata & Suzuki, 1987) or with Dragendorff’s reagent (Sigma). The polar lipid profile of strain BR-18<sup>T</sup>, in which phosphatidylethanolamine and an unidentified aminophospholipid predominated (see Fig. S1, available in IJSEM Online), was similar to those reported for M. rigui KCTC 12534<sup>T</sup>, M. lutinaris BR-3<sup>T</sup> and M. paludis KACC 13450<sup>T</sup> (J.-H. Kim et al., 2012). However, several additional minor polar lipids were detected only in strain BR-18<sup>T</sup>, and an unidentified aminolipid detected as a major polar lipid of M. paludis KACC 13450<sup>T</sup> (J.-H. Kim et al., 2012) was not detected in strain BR-18<sup>T</sup>.

For the analysis of sphingolipids, cell mass of strain BR-18<sup>T</sup> and Sphingobacterium multivorum KCTC 22892<sup>T</sup> (used as a positive control) was harvested from R2A agar after incubation for 5 days at 25 °C. The sphingolipids were extracted and analysed by TLC (Yano et al., 1982), with dihydro sphingosine (Sigma) used as a standard. For both strains investigated, extraction of the long-chain bases with hexane/diethyl ether from the alkaline solution yielded a ninhydrin-positive spot that migrated close to the
The sphingolipid profile of *S. multivorum* KCTC 22892^T^ obtained in this study was the same as that reported by Yano *et al.* (1982). Hence, strain BR-18^T^ contained a sphingolipid with the same chromatographic behaviour as DL-dihydrosphingosine. Other unidentified spots were also present in the long-chain base profile of strain BR-18^T^.

The genomic DNA G+C content of strain BR-18^T^ was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC on an ODS-A (250 × 4.6 mm) column. The nucleotides were eluted at room temperature with a mixture of 0.55 M NH₄H₂PO₄ (pH 4.0) and acetonitrile (40:1, v/v), using a flow rate of 1 ml min⁻¹, and detected by the measurement of absorbance at 270 nm. The genomic DNA G+C content of strain BR-18^T^, 42.4 mol%, fell at the low end of the range of values described for members of the genus *Mucilaginibacter* and was markedly lower than the values reported for *M. lutimaris* (49.8 mol%; J.-H. Kim *et al.*, 2012) and *M. paludis* (46.1 mol%; Pankratov *et al.*, 2007). In terms of its phylogeny and chemotaxonomy, strain BR-18^T^ appears to resemble members of the genus *Mucilaginibacter* but can be separated from the existing species in this genus (see Table 2 and Fig. S1; Pankratov *et al.*, 2007; Baik *et al.*, 2010; Madhaiyan *et al.*, 2010; Männistö *et al.*, 2010; Kim *et al.*, 2010). Strain BR-18^T^ was distinguishable from *M. rigui* KCTC 12534^T^ and *M. lutimaris* BR-3^T^ by differences in some phenotypic characteristics (Table 1). Based on these differences and the phylogenetic evidence, the isolate represents a novel species of the genus *Mucilaginibacter* (Stackebrandt & Goebel, 1994), for which the name *Mucilaginibacter litoreus* sp. nov. is proposed.

**Description of *Mucilaginibacter litoreus* sp. nov.**

*Mucilaginibacter litoreus* (li.tो.re.u.s. L. masc. adj. *litoreus* belonging to the seashore).
Cells are Gram-staining-negative, non-spore-forming, facultatively anaerobic, non-flagellated and non-gliding rods that measure approximately 0.4–0.8 x 1.0–3.5 µm. No cyclic change in cell shape is observed during culture. Cells produce numerous outer-membrane vesicles. Colonies on R2A agar are circular, slightly convex, mucoid, smooth, glistening, light pink in colour and 1.0–1.5 mm in diameter after incubation for 3 days at 25 °C. Large amounts of extracellular polymeric substance are produced, as shown by the mucoid consistency of colonies and adherence to the agar. Growth occurs at 10 and 40 °C but not at 4 or 45 °C (optimum about 25 °C). The optimal pH value for growth lies between 6.5 and 7.0; growth occurs at pH 4.5 and 9.0 but not at pH 4.0 or 9.5. Grows with 0–2.0 % (w/v) NaCl (optimum 0 %). Catalase- and oxidase-positive. Flexirubin-type pigments are not produced. Nitrate is not reduced to nitrite. Aesculin, agarose, t-carrageenan, chitin, CMc, starch, pectin and xylan are hydrolysed but alginate, casein, curdlan, gelatin, hyoxanthine, xanthine, Tween 80, L-tyrosine and urea are not. On API 50CH and API 20NE strips, galactose, glucose, fructose, mannose, methyl α-D-mannoside, methyl α-D-gluconoside, N-acetyl-D-glucosamine, arbutin, ascuclin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, mellizetose, raffinose, starch, gentiobiose and turanose are utilized, and D-xylose, rhamnose, amygdalin and salicin are weakly utilized, as carbon and energy sources but glycerol, erythritol, D-arabinose, L-arabinose, ribose, L-xylene, adonitol, methyl β-D-xylloside, sorbose, dulcitol, inositol, mannotol, sorbitol, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, adipate, caprate, malate, citrate and phenylacetate are not utilized. The predominant menaquinone is MK-7 but a minor amount of MK-6 is also present. The major fatty acids are summed feature 3 (C16:1ω7c and/or iso-C15:0 2-OH) and iso-C15:0. The major polar lipids are phosphatidylethanolamine and an unidentified aminophospholipid. Sphingolipids are present. Other phenotypic properties of the type strain are shown in Table 1.

The type strain, BR-18T (=KCTC 23697T =CCUG 61484T) was isolated from marine sand collected on the seashore at Boryung, South Korea. The genomic DNA G+C content of the type strain is 42.4 mol%.

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References


*Summed feature 3 contained C16:1ω7c and/or iso-C15:0 2-OH.
†ECL, Equivalent chain-length.


