Chitinophaga polysaccharea sp. nov., an exopolysaccharide-producing bacterium isolated from the rhizoplane of Dioscorea japonica

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A novel strain designated MRP-15T, belonging to the class Sphingobacteriia (phylum Bacteroidetes), was isolated from the rhizoplane of Dioscorea japonica in South Korea and was characterized taxonomically using a polyphasic approach. The strain was found to comprise Gram-stain-negative, aerobic, non-motile, non-spore-forming rods. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain belonged to the genus Chitinophaga but was clearly separated from established species of the genus Chitinophaga. 16S rRNA gene sequence similarities between strain MRP-15T and type strains of established species of the genus Chitinophaga ranged from 90.3 to 97.8%. Phenotypic and chemotaxonomic data (major menaquinone, MK-7; major fatty acids, iso-C15:0 and C16:1ω5c) supported the affiliation of strain MRP-15T with the genus Chitinophaga. Therefore strain MRP-15T represents a novel species of the genus Chitinophaga, for which the name Chitinophaga polysaccharea sp. nov. is proposed. The type strain is MRP-15T (=KACC 17184T=NCAIMB 02530T).

The genus Chitinophaga, a member of the family Chitinophagaceae in the phylum Bacteroidetes, was originally proposed by Sangkhobol & Skerman (1981). Later, four species [Flexibacter] sancti, [Flexibacter] filiformis, [Flexibacter] japonensis and [Cytophaga] arvensicola, were transferred to the genus Chitinophaga (Kämpfer et al., 2006). The description of Chitinophaga arvensicola was emended by Pankratov et al. (2006). At the time of writing, there are fifteen species of the genus Chitinophaga with validly published names. We have been investigating the distribution of exopolysaccharide (EPS)-producing bacterial species in the rhizosphere soil of domestic traditional medicinal herbs in Geumsan, South Korea. Several strains isolated from the rhizoplane of Dioscorea japonica have been characterized based on their production of exopolysaccharides. A novel bacterial strain, designated MRP-15T, isolated from the rhizoplane of Dioscorea japonica, was shown to belong to the genus Chitinophaga according to 16S rRNA gene sequence analysis. In this study, its detailed taxonomic position was investigated using polyphasic analyses.

Strain MRP-15T was originally isolated from rhizoplane of Dioscorea japonica obtained from Geumsan (36° 11’ 11” N 127° 54’ 32” E). The rhizoplane of Dioscorea japonica was thoroughly suspended and the suspension was spread on potato dextrose agar (Difco) plates. The plates were incubated at 28°C for 3 days. Single colonies were purified by transferring onto R2A agar (Difco) plates and incubating at 28°C once again. Strain MRP-15T was routinely cultured on R2A agar at 28°C and maintained as a glycerol suspension (20%, w/v) at −80°C. In order to characterize strain MRP-15T phenotypically, the isolate was routinely grown aerobically on R2A agar for 3 days at 28°C and pH 7.0, except where indicated otherwise.

The morphology of the isolate was observed by Gram staining and scanning electron microscopy, and motility was observed by phase-contrast microscopy (Eclipse 80i; Nikon) using cells from exponentially growing cultures. Gram staining was performed by the Burke method (Murray et al., 1994). Catalase activity was determined by assessing bubble production in 3% (v/v) H₂O₂, and oxidase activity was determined using 1% (w/v) tetramethyl-p-phenylenediamine. Carbon-source utilization and enzyme activities were tested by using API 20NE, API 50CH and API ZYM test kits (bioMérieux). Growth at 4, 10, 15, 20, 25, 30, 37, 40, 45 and 50°C and at pH 3.0–10.0 (in increments of 0.5 pH unit) was assessed in R2A broth after 48 h of incubation at each temperature.

Abbreviation: EPS, exopolysaccharide.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MRP-15T is KC430923.

Three supplementary figures and a supplementary table are available with the online version of this paper.
5 days. The pH of the medium was adjusted with 1 M NaOH and HCl. The pH of a subsample of autoclave-sterilized media was measured before inoculation to check pH change; there were no significant pH changes in the media after autoclave sterilization. Tolerance to NaCl was tested in R2A broth supplemented with 0–10 % (w/v) NaCl (in increments of 1 %) after incubation for 5 days. Growth on R2A agar, nutrient agar, tryptase soy agar, potato dextrose agar and Luria–Bertani agar (all Difco) was evaluated at 28 °C.

Strain MRP-15T was found to comprise Gram-stain-negative, aerobic, non-motile, non-spore-forming rods. Colonies grown on R2A agar for 3 days were smooth, circular and yellowish. The strain grew well on R2A agar, nutrient agar, trypticase soy agar, potato dextrose agar and Luria–Bertani agar. The physiological characteristics of strain MRP-15T are summarized in the species description, and differential characteristics are compared with other species of the genus Chitinophaga in Table 1.

Extraction of genomic DNA from strain MRP-15T was performed with a commercial genomic DNA extraction kit (Solgent). The 16S rRNA gene was amplified by PCR with the forward primer Eubac 27F and the reverse primer 1492R (Lane, 1991). Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer (model ABI 3730XL; Applied Biosystems). The almost-complete 16S rRNA gene sequence (1401 bp) of strain MRP-15T was obtained and used for initial BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov/) and for phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved by using the EzTaxon-e server (http://www.eztaxon-e.ezcloud.net/; Kim et al., 2012).

Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain MRP-15T belonged to the genus Chitinophaga. Strain MRP-15T showed the highest level of 16S rRNA gene sequence similarity to Chitinophaga ginsengisegetis Gsoil 040T (97.8 %), followed by Chitinophaga niastensis KACC 12954T (Weon et al., 2009); 5, C. terrae KACC 12755T (Kim & Jung, 2007). All strains produce yellow colonies and are positive for aesculin hydrolysis and β-galactosidase activity, but are negative for indole production and arginine dihydrolase activity. All strains assimilate D-glucose, N-acetylglucosamine, maltose, sucrose and melibiose, but do not assimilate capric acid, adipic acid, phenylacetic acid, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, D-sorbitol, propionic acid, valeric acid, potassium 2-ketogluconate, glycogen, D-sorbitol, propionic acid, valeric acid, potassium 2-ketogluconate or 3-hydroxybutyric acid. According to the API ZYM test strips, all strains are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucosidase and N-acetyl-β-glucosaminidase activities, but are negative for lipase (C14) and β-glucuronidase activities. †, Positive; –, negative; ND, not determined.

### Table 1. Differential characteristics of strain MRP-15T and type strains of related members of the genus Chitinophaga

<table>
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<td>4.5–8.0</td>
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<td>43.0</td>
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arvensicola DSM 3695T (97.7 %), Chitinophaga niastensis JS16-4T (96.9 %) and Chitinophaga terrae KP01T (96.5 %). The 16S rRNA gene sequence of strain MRP-15T was aligned with the published sequences of closely related bacteria using CLUSTAL W 2.0 software (Larkin et al., 2007). Phylogenetic trees were reconstructed by using three different methods: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) algorithms within the MEGA5 program (Tamura et al., 2011). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Kimura’s two-parameter model (Kimura, 1983). To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed (Felsenstein, 1985). The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1. Maximum-likelihood and maximum-parsimony methods resulted in highly similar tree topologies (Figs S1 and S2 available in IJSEM Online), with strain MRP-15T forming a distinct phylectic line with C. ginsengisgetis Gsoil 040T (97.8 %) and C. arvensicola DSM 3695T (97.7 %).

To determine genomic relatedness, DNA–DNA hybridization was performed using the modified method of Ezaki et al. (1989). Probe labelling for DNA–DNA hybridization was conducted by using the non-radioactive DIG–High prime system (Roche); hybridized DNA was visualized using the DIG luminescent detection kit (Roche) and the level of DNA–DNA relatedness was quantified by using a densitometer (Bio-Rad). The DNA–DNA relatedness values between strain MRP-15T and C. ginsengisgetis Gsoil 040T and C. arvensicola DSM 3695T, were 15.2 % and 11.3 %, respectively.

Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum conditions and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane-quinone solution was purified using Sep-Pak Vac silica cartridges (Waters) and subsequently analysed by HPLC as described previously (Hiraishi et al., 1996). The major respiratory quinone of strain MRP-15T was MK-7, as is the case for recognized species of the genus Chitinophaga (Kämpfer et al., 2006).

For polar lipid analysis, strain MRP-15T was grown in TSB at 28 °C for 3 days. Polar lipids were extracted with chloroform/methanol (2:1, v/v) and loaded onto thin-layer silica gel 60 plates (Merck) according to Bligh & Dyer (1959). Two-dimensional migration was performed on each plate using chloroform/methanol/water (65:25:14, by vol.) as the first solvent and chloroform/acetoc acid/methanol/water (80:15:12:4, by vol.) as the second solvent. Total polar lipids were revealed by spraying with 5 % ethanolic molybdatophosphoric acid, and other plates were sprayed with ninhydrin for amino lipids or molybdenum blue for phospholipids. The total polar lipids of strain MRP-15T were phosphatidylethanolamine (PE), unidentified aminophospholipids (AL1–3), and unidentified polar lipids (L1–3) (Fig. S3).

Cellular fatty acid profiles were determined for strains grown on TSB agar at 28 °C for 2 days. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed by gas chromatography (6890; Hewlett Packard) and identified using the Microbial Identification software package (Sasser, 1990). The major fatty acids of strain MRP-15T were iso-C15 : 0 (37.68 %) and C16 : 1ω5c (34.18 %) (Table S1).

For the measurement of the G+C content of the chromosomal DNA, genomic DNA of the novel strain was extracted and purified as described by Moore & Dowhan (1995) and enzymically degraded into nucleosides and the G+C content was determined as described by Mesbah et al. (1989) using reversed-phase HPLC. The DNA G+C content of strain MRP-15T was 47.9 mol%.

On the basis of the data and observations described above, strain MRP-15T should be assigned to the genus Chitinophaga as the type strain of a novel species, for which the name Chitinophaga polysaccharea sp. nov. is proposed.

Description of Chitinophaga polysaccharea sp. nov.

Chitinophaga polysaccharea (po.ly.sac.cha’re.a. Gr. adj. polu many; Gr. n. sakchár sugar; L. fem. suff. -ea suffix denoting made of or belonging to; N.L. fem. adj. polysaccharea with many saccharides).

Cells are Gram-stain-negative, aerobic, non-motile rods that are 0.3–0.4 μm in width and 0.6–0.9 μm in length after 3 days of culture on R2A agar. Colonies grown on R2A agar for 3 days are smooth, circular, convex and yellowish. Grows at 4–45 °C and at pH 4.0–10.0, but grows slowly at 45 °C and pH 10.0. Catalase- and oxidase-negative. Can reduce nitrate to nitrate and nitrate to nitrogen gas. Produces N-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, cystine arylamidase, esterase (C4), esterase (C8), α-fucosidase, β-galactosidase, β-glucosidase, leucine arylamidase, α-mannosidase, naphthol-AS-BI-phosphorylase, trypsin and valine arylamidase, but does not produce arginine dihydrolase, β-glucuronidase, lipase (C14), protease (gelatin hydrolysis) or urease. Assimilates trisodium citrate, L-arabinose, D-ribose, L-fucose, D-glucose, maltose, melibiose, L-rhamnose, sucrose, D-mannitol, N-acetyl-D-glucosamine and salicin, but does not assimilate D-mannose, acetate, adipate, caprate, citrate, gluconate, 3-hydroxybenzoate, 4-hydroxybenzoate, 3-hydroxybutyrate, itaconate, 2-ketogluconate, 5-ketogluconate, lactate, L-malate, malonate, phenylacetate, propionate, suberate, n-valerate, myo-inositol, D-sorbitol, L-alanine, L-histidine, L-proline, L-serine or glycogen. MK-7 is the predominant menaquinone and iso-C15 : 0 and C16 : 1ω5c are the predominant cellular fatty acids.

The type strain, MRP-15T (=KACC 17184T=NCAIMB 02530T), was isolated from rhizoplane of Dioscorea japonica
in Geumsan, Korea. The G+C content of the genomic DNA of the type strain is 47.9 mol%.

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References


Chitinophaga polysaccharea sp. nov.


