

Robiginitomaculum antarcticum gen. nov., sp. nov., a member of the family *Hyphomonadaceae*, from Antarctic seawater

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A seawater bacterium, designated IMCC3195^T, was isolated from the Antarctic coast. Cells of the novel strain were Gram-negative, rusty-coloured, strictly aerobic, chemoheterotrophic, non-budding and non-motile rods or vibrioids that possessed a thin prostheca. Based on 16S rRNA gene sequence comparisons, the novel strain was most closely related to the genera *Hyphomonas* (89.4–90.9%), *Maricaulis* (90.1–90.4%), *Hirschia* (89.0%) and *Oceanicaulis* (87.9%) of the family *Hyphomonadaceae*. Phylogenetic analyses also showed the Antarctic isolate to be only distantly related to the genera of stalked bacteria of marine origin in the family *Hyphomonadaceae*. The DNA G + C content of the novel strain was 60.3 mol% and the predominant cellular fatty acids were C_{18:1ω7c} (41.9%), C_{17:1ω8c} (21.4%) and C_{17:0} (14.3%). The major quinone was Q-10. Several phenotypic and chemotaxonomic characteristics, including optimum temperature and salinity range for growth, cell morphology, pigmentation and fatty acid content, differentiated the novel strain from other related genera in the family *Hyphomonadaceae*. From the taxonomic evidence collected in this study, it is suggested that strain IMCC3195^T (=KCCM 42687^T=NBRC 103098^T) represents a new genus and novel species in the family *Hyphomonadaceae*, for which the name *Robiginitomaculum antarcticum* gen. nov., sp. nov. is proposed.

The family *Hyphomonadaceae* in the order *Rhodobacterales* was proposed by Lee *et al.* (2005) based on phylogenetic analyses of 16S rRNA gene sequences. The family encompasses several members of prostheca-bearing, motile, obligately aerobic, chemoheterotrophic bacteria that have been isolated from diverse marine environments, including surface seawater, brackish water, deep-sea, warm water hydrothermal vents and dinoflagellates (Abraham *et al.*, 1999; Moore *et al.*, 1984; Schlesner *et al.*, 1990; Strömpl *et al.*, 2003). The family is currently composed of four well-defined genera: *Hyphomonas* (Moore *et al.*, 1984), *Maricaulis* (Abraham *et al.*, 2002), *Hirschia* (Schlesner *et al.*, 1990) and *Oceanicaulis* (Strömpl *et al.*, 2003). The present study focuses on the taxonomy of strain IMCC3195^T, isolated from Antarctic surface seawater. Data obtained from the polyphasic approach adopted in this study indicated that strain IMCC3195^T represents a novel genus and species. Therefore, we propose the inclusion of this novel isolate in a fifth genus of the family *Hyphomonadaceae*.

Strain IMCC3195^T was isolated from a seawater sample collected from the coast of King George Island, Weaver Peninsula, West Antarctica (62° 14' S 58° 47' E), using a standard dilution-plating method on an oligotrophic medium, R2A agar (Difco), diluted 1:10 in aged seawater (v/v, 1/10R2A). Strain IMCC3195^T, initially grown on 1/10R2A, was further purified on marine agar 2216 (MA; Difco) after culture at 20 °C for 2 weeks. After the optimum growth temperature for the novel strain had been determined, cultures were maintained routinely on MA at 20 °C for characterization studies and preserved as a glycerol suspension (10%, v/v) at –75 °C.

The 1415 bp sequence of the 16S rRNA gene for strain IMCC3195^T was obtained as described previously (Cho & Giovannoni, 2003). Phylogenetic analyses, including multiple alignment of 16S rRNA gene sequences, determination of sequence similarity and generation of phylogenetic trees, were performed with the ARB (Ludwig *et al.*, 2004) and PAUP software packages (Swofford, 2002) as described by Cho & Giovannoni (2006). Reference sequences of more than 1300 bp were included in the phylogenetic analysis and 1171 unambiguously aligned nucleotide positions were used. Sequence comparisons in the ARB database and

Abbreviation: PHA, poly-β-hydroxyalkanoate.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMCC3195^T is EF495229.

BLASTN search results showed that strain IMCC3195^T was only distantly related to members of the family *Hyphomonadaceae*. The novel strain showed the highest sequence similarity with coastal alphaproteobacterium 26III/A02/215 (93.9%, GenBank accession no. AY576758, Agogue *et al.*, 2005). The most closely related members of the family *Hyphomonadaceae* with validly published names were the genera *Hyphomonas* (89.4–90.9%), *Maricaulis* (90.1–90.4%), *Hirschia* (89.0%) and *Oceanicaulis* (87.9%). No other recognized species showed greater than 91% 16S rRNA gene sequence similarity with strain IMCC3195^T. To further reveal the phylogenetic position of the strain, phylogenetic trees were generated by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods. The robustness of the neighbour-joining and maximum-parsimony trees was evaluated by bootstrap analyses based on 1000 resamplings. In all the phylogenetic trees generated in this study, strain IMCC3195^T did not form any robust phylogenetic clade with other genera of the order *Rhodobacterales*, but formed an independent monophyletic clade within the family *Hyphomonadaceae* (Fig. 1). The low gene sequence similarity and distinct phylogenetic relationship between the novel strain and other genera of the family *Hyphomonadaceae* revealed that the novel strain could not be assigned to any of the recognized genera. Consequently, strain IMCC3195^T was considered to represent a new genus in the family *Hyphomonadaceae*.

Phenotypic and physiological characterization was carried out according to a previous study (Choo *et al.*, 2007) and

standard methods (Smibert & Krieg, 1994) using MA as the basal medium at 20 °C, unless otherwise specified. The morphology and sizes of cells and colonies were examined from cultures grown aerobically on MA for 5 days. The presence of poly- β -hydroxyalkanoate (PHA) granules was checked by epifluorescence microscopy after staining the cells with Nile blue A (Ostle & Holt, 1982). For detecting bacteriochlorophyll *a* and carotenoids, pigments of strain IMCC3195^T were extracted with acetone/methanol (1:1, v/v) and the absorption spectra were determined using a scanning UV/visible spectrophotometer (Optizen 2120UV; Mechasis). Additionally the genetic potential for anoxygenic phototrophy was determined by PCR amplification of the photosynthetic reaction centre genes (*pufLM*) using the *pufL*f and *pufM*r primer set (Béjà *et al.*, 2002). Growth temperature range and optimum temperature were tested at 3–42 °C. The pH range and optimum for growth were examined on MA adjusted to pH values of 4.0–12.0 with 0.1 M HCl and 0.1 M NaOH. The NaCl concentration range and optimum level for growth were determined in NaCl-free artificial seawater medium (ASW; Choo *et al.*, 2007), supplemented with 5.0 g peptone, 1.0 g yeast extract and various concentrations of NaCl (0–15%, w/v). The temperature, pH and NaCl concentrations ranges for growth were monitored for 2 weeks on MA or MA adjusted to various pH and NaCl concentrations. Biochemical and carbon source utilization tests were carried out with API 20NE and API ZYM strips (bioMérieux) and in GN2 microplates (Biolog), according to the manufacturer's instructions, and inoculated with bacterial suspensions in ASW. Ten different antimicrobial agents (listed in the species description) were tested by the

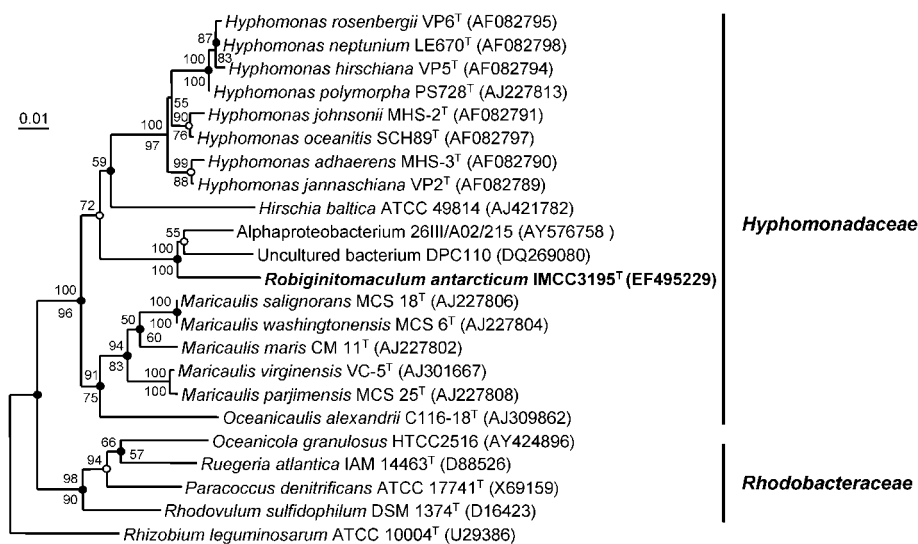


Fig. 1. Neighbour-joining 16S rRNA gene sequence phylogenetic tree showing the relationships between strain IMCC3195^T and representatives of the families *Hyphomonadaceae* and *Rhodobacteraceae*. Bootstrap proportions over 50% from both neighbour-joining (above nodes) and maximum-parsimony (below nodes) are shown. Closed circles indicate the nodes that were recovered reproducibly by all treeing methods. Open circles represent nodes that were recovered by two treeing methods. Bar, 0.01 substitutions per nucleotide position.

diffusion plate method (Jorgensen *et al.*, 1999). The DNA G+C content was analysed by using HPLC (Mesbah *et al.*, 1989). Cellular fatty acid methyl esters were prepared from cultures grown on MA 20 °C for 5 days and analysed according to the MIDI Microbial Identification System by the Korean Culture Center of Micro-organisms (KCCM). Respiratory quinones were analysed by the KCCM using a reverse-phase HPLC.

The morphological, physiological and biochemical characteristics of strain IMCC3195^T are listed in the genus and species descriptions and in Table 1. In summary, cells of strain IMCC3195^T were Gram-negative, rusty-coloured, obligately aerobic, chemoheterotrophic, non-motile, non-budding, prostheca-possessing and were rod or vibrioid-shaped (Fig. 2). The novel strain did not produce bacteriochlorophyll *a* or PHA granules. The absence of anoxygenic phototrophy was also supported by the PCR results indicating that *pufLM* genes were not amplified. The DNA G+C content of strain IMCC3195^T was 60.3 mol%.

The only respiratory quinone detected was Q-10. The major fatty acids found in strain IMCC3195^T, C_{18:1}ω7c (41.9%), C_{17:1}ω8c (21.4%), C_{17:0} (14.3%) and C_{17:1}ω6c (7.7%), showed moderate differences when compared with the other genera in the family *Hyphomonadaceae* (Table 1). Several phenotypic characteristics, including cell morphology, pigmentation, optimum growth temperature and salinity range for growth, clearly differentiated the strain from other related genera in the family *Hyphomonadaceae* (Table 1).

As shown by the low 16S rRNA gene sequence similarity (<91%) with other genera, the distinct phylogenetic relationship (Fig. 1) and several differential phenotypic characteristics, strain IMCC3195^T could not be assigned to any of the known genera in the family *Hyphomonadaceae*. These data, obtained by a polyphasic approach, demonstrate conclusively that the novel strain belongs to a new genus and species in the family *Hyphomonadaceae* of the order *Rhodobacterales*, for which the name *Robiginitomaculum* gen. nov., sp. nov. is proposed.

Table 1. Differential characteristics of strain IMCC3195^T and related genera of the family *Hyphomonadaceae*

Taxa: 1, IMCC3195^T (data from this study); 2, *Hyphomonas* (Moore *et al.*, 1984; Weiner *et al.*, 1985, 2000); 3, *Hirschia* (Schlesner *et al.*, 1990); 4, *Maricaulis* (Abraham *et al.*, 1999, 2002); 5, *Oceanicaulis* (Strömpl *et al.*, 2003). +, Positive reaction; -, negative reaction; v, variable data; ND, not determined. For cellular fatty acid analyses, all species of the genus *Hyphomonas* were grown on MA. *Hirschia baltica* was grown on M13 medium (Schlesner *et al.*, 1990). All species of the genera *Maricaulis* and *Oceanicaulis* were grown on SPYEM medium (Abraham *et al.*, 1999).

Characteristic	1	2	3	4	5
Cell morphology:					
Shape	Rod or vibrioid	Ovoid or rod	Ovoid or rod	Rod or vibrioid	Rod or vibrioid
Budding	-	+	+	-	-
Flagella	-	+*	+	+†	+
Prostheca	+‡	+	+	+	+
Pigmentation	Rusty orange	-, Grey, reddish brown	Yellow	-	-
Optimum temperature	20	20-45	22-28	30-40§	30
Growth at:					
6.0% NaCl	-	v	ND	+	+
0.5% NaCl	+	v¶	ND	+ #	-
Oxidase	-	+	ND	ND	+
Nitrate reduction	+	v	-	v	+
Hydrolysis of gelatin	-	-	+	ND	-
Major fatty acid composition (%):					
C _{17:0}	14	0-23	1	7-22	10
C _{17:1}	29	0-24	1	10-39	1
C _{18:0}	4	-	2	1-8	22
C _{18:1}	45	15-80	52	16-54	30
Major quinone	Q-10	Q-10/Q-11	Q-10	Q-10	ND
DNA G+C content (mol%)	60.3	57-64	45.6	62.5-65.2	61.8

*Data for *Hyphomonas adhaerens*, *Hyphomonas johnsonii* and *Hyphomonas rosenbergii*.

†Data for *M. maris*. The other species are not determined.

‡Some cells have a prostheca with thin-tapered end.

§Data for *M. parjimensis* and *M. salignorans*.

||All species are positive except *Hyphomonas polymorpha*.

¶All species are negative except *Hyphomonas polymorpha*.

#Data for all species except *M. maris*.

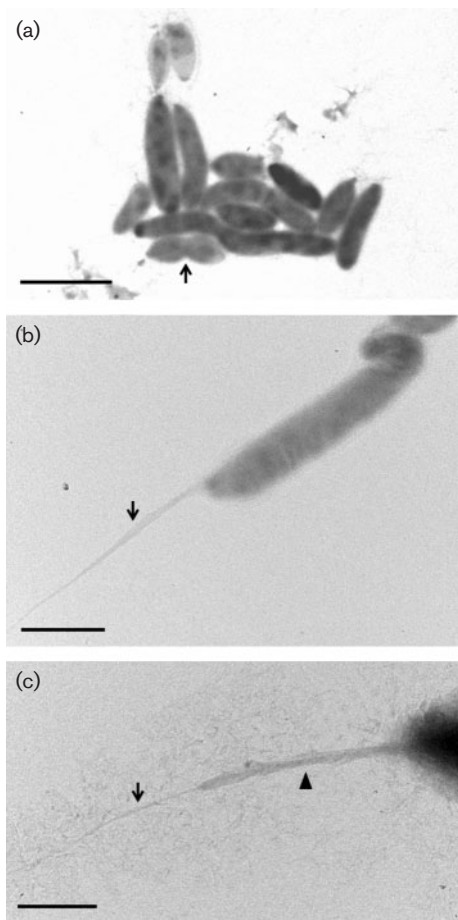


Fig. 2. Transmission electron micrographs of negatively stained cells of strain IMCC3195^T. (a) Rod or vibrioid-shaped cells showing multiplication by binary fission (arrow). (b) A cell possessing a thin prostheca (arrow); (c) A magnified view of a prostheca. The relatively thick part (triangle) of the prostheca is linked to the cell. The prostheca has a very thin and tapered end (arrow). Bars, 2 μm (a), 1 μm (b) and 0.5 μm (c).

Description of *Robiginitomaculum* gen. nov.

Robiginitomaculum (Ro.bi.gi.ni.to.ma.cul'um. L. n. *robigo* -inis rust; L. neut. n. *tomaculum* a kind of sausage; N.L. neut. n. *Robiginitomaculum* a rust-coloured sausage).

Cells are Gram-negative, non-motile, non-budding, thin prostheca-producing, obligately aerobic rods or vibrioids. Multiplication occurs by binary fission. Flagella and holdfast are not present. Some cells possess a thin prostheca that extends along the long cell axis from one pole. Carotenoid pigments are found. Bacteriochlorophyll *a* and the genes (*pufLM*) for anoxygenic photosynthesis are not found. Chemoheterotrophic. The predominant fatty acids are C_{18:1 ω 7c}, C_{17:1 ω 8c}, C_{17:0} and C_{17:1 ω 6c}. The major respiratory quinone is Q-10. The DNA G+C content is 60.3 mol%. The genus is phylogenetically assigned to the family *Hyphomonadaceae* in the order

Rhodobacterales. The type species of the genus is *Robiginitomaculum antarcticum*.

Description of *Robiginitomaculum antarcticum* sp. nov.

Robiginitomaculum antarcticum (ant.arc'ti.cum. L. neut. adj. *antarcticum* of the Antarctic environment, from where the organism was isolated).

In addition to the characteristics reported for the genus, cells are 1.3–4.5 μm long and 0.4–1.0 μm wide, with a tapered end. Colonies on MA are circular, smooth, convex, viscous, rusty-coloured and 0.3–1.0 mm in diameter. Growth occurs at 3–25 $^{\circ}\text{C}$, optimally at 20 $^{\circ}\text{C}$, but not above 30 $^{\circ}\text{C}$. Growth occurs at pH 5–10 and 0.5–5.0% NaCl, optimally at pH 7 and at 2.0–2.5% NaCl. Oxidase-negative and catalase-positive. In API 20NE strips, positive for nitrate reduction, aesculin hydrolysis and β -galactosidase activity (substrate, *p*-nitrophenyl- β -D-galactopyranoside). Negative for urea hydrolysis, indole production, acid production from glucose, gelatin liquefaction and arginine dihydrolase. In the API ZYM system, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase and cystine arylamidase activities are present. Negative for esterase (C4), acid phosphatase, β -glucosidase, *N*-acetyl- β -glucosaminidase and α -mannosidase, lipase (C14), trypsin, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, β -galactosidase (substrate, 2-naphthyl- β -D-galactopyranoside), β -glucuronidase, α -galactosidase, α -glucosidase and α -fucosidase activities. In tests with GN2 microplates (Biolog), oxidizes the following carbon substrates: Tween 40 and Tween 80, D-galactose, *myo*-inositol, trehalose, turanose, succinic acid monomethyl ester, D-gluconic acid, D-glucuronic acid, α -ketoglutaric acid, quinic acid, succinamic acid, L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, L-ornithine, L-proline, L-pyrroglutamic acid, D-serine, L-serine, glycerol and DL- α -glycerol phosphate. Does not utilize the following carbon substrates: α -cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, i-erythritol, D-fructose, L-fucose, gentiobiose, α -D-glucose, α -D-lactose, lactulose, maltose, D-mannitol, D-mannose, D-melibiose, methyl β -D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, xylitol, pyruvic acid methyl ester, acetic acid, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, α -hydroxybutyric acid, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketobutyric acid, α -ketovaleric acid, DL-lactic acid, malonic acid, propionic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, glucuronamide, L-alaninamide, D-alanine, L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-threonine, DL-carnitine, γ -aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, α -

D-glucose 1-phosphate and D-glucose 6-phosphate. Susceptible to chloramphenicol (25 µg), erythromycin (15 µg), rifampicin (50 µg) and tetracycline (30 µg), but resistant to ampicillin (10 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 µg), streptomycin (10 µg) and vancomycin (30 µg). The cellular fatty acids comprise C_{18:1}ω7c (41.9%), C_{17:1}ω8c (21.4%), C_{17:0} (14.3%), C_{17:1}ω6c (7.7%), C_{18:0} (3.5%), C_{16:1} ω7c and/or iC_{15:0} 2-OH (3.0%) and C_{18:1}ω9c (2.8%). Traces (<1%) of the following fatty acids are also present: C_{16:0}, C_{10:0} 3-OH, C_{11:0} 3-OH, C_{15:1}ω8c, C_{16:1}ω9c, C_{15:0}, C_{9:0} 3-OH and C_{15:1}ω6c.

The type strain, IMCC3195^T (=KCCM 42687^T=NBRC 103098^T), was isolated from surface seawater of Maxwell Bay, King George Island, West Antarctica.

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