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Algoriphagus machipongonensis sp. nov., co-isolated with a colonial choanoflagellate

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A Gram-negative, non-motile, non-spore-forming bacterial strain, PR1^T, was isolated from a mud core sample containing colonial choanoflagellates near Hog Island, Virginia, USA. Strain PR1^T grew optimally at 30 °C and with 3 % (w/v) NaCl. Strain PR1^T contained MK-7 as the major menaquinone as well as carotenoids but lacked pigments of the flexirubin-type. The predominant fatty acids were iso-$C_{15:0}$ (29.4 %), iso-$C_{17:1}\omega9c$ (18.5 %) and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; 11.3 %). The major polar lipids detected in strain PR1^T were phosphatidylethanolamine, an unknown phospholipid, an aminophospholipid, an aminolipid and two lipids of unknown character. The DNA G+C content was 38.7 mol%. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain PR1^T fell within the cluster comprising the genus *Algoriphagus* and was most closely related to *Algoriphagus halophilus* JC 2051^T (95.4 % sequence similarity) and *Algoriphagus lutimaris* S1-3^T (95.3 % sequence similarity). The 16S rRNA gene sequence similarity between strain PR1^T and the type strains of other species of the genus *Algoriphagus* were in the range 91–95 %. Differential phenotypic properties and phylogenetic and genetic distinctiveness of strain PR1^T demonstrated that this strain was distinct from other members of the genus *Algoriphagus*, including its closest relative, *A. halophilus*. Based on phenotypic, chemotaxonomic, phylogenetic and genomic data, strain PR1^T should be placed in the genus *Algoriphagus* as a representative of a novel species, for which the name *Algoriphagus machipongonensis* sp. nov. is proposed. The type strain is PR1^T (=ATCC BAA-2233^T =DSM 24695^T).

The *Algoriphagus* genus belongs to the phylum *Bacteroidetes*. The type species, *Algoriphagus ratkowskyi* IC025^T, was first described by Bowman *et al.* (2003). At the time of writing, the genus consists of 23 species predominantly isolated from aquatic habitats (Bowman *et al.*, 2003; Copa-Patiño *et al.*, 2008; Lee *et al.*, 2012; Li *et al.*, 2011; Liu *et al.*, 2009; Nedashkovskaya *et al.*, 2004, 2007; Oh *et al.*, 2012; Park *et al.*, 2010; Rau *et al.*, 2012; Tao *et al.*, 2006; Tiago *et al.*, 2006; Van Trappen *et al.*, 2004; Yoon *et al.*, 2005a, b, 2006; Young *et al.*, 2009). In this study, we report the taxonomic characterization of an *Algoriphagus*-like bacterial strain, PR1^T, which was co-isolated with the choanoflagellate *Salpingoeca rosetta* ATCC 50818 in May 2000 from a mud core sample near Hog Island, Virginia, USA, part of the coastal barrier system of the Virginia Coast Reserve (Dayel *et al.*, 2011).

Strain PR1^T was isolated by the standard dilution-plating technique on modified ZoBell medium at 25 °C (Carlucci & Pramer, 1957). *Algoriphagus halophilus* JC 2051^T, obtained from the Korean Collection for Type Cultures, was used as a reference strain for phenotypic characterization and fatty acid analysis.

The morphological characteristics of strain PR1^T were investigated after cultivation on seawater complete media (SWC; Atlas, 2004) at 25 °C for 5 days. Cell morphology was examined by light microscopy (DMIL; Leica). The Gram reaction and absorption maximum of crude extracts were determined as described by Tindall *et al.* (2007). Organic cell extracts of strain PR1^T showed an absorption peak maximum at 487 nm, which indicated the presence of

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Three supplementary figures are available with the online version of this paper.

carotenoids. Flexirubin-type pigments were not produced, as shown by a negative KOH test result (Reichenbach, 1989). Growth with 0, 0.5, 1.0, 2.0 and 4.0 % (w/v) NaCl was examined in trypticase soy broth (Difco) that had been prepared according to the manufacturer's instructions except that NaCl was added to the desired final concentration and 0.45 % (w/v) $MgCl_2 . 6H_20$ or 0.06 % (w/v) KCl were used as supplements. Growth with 2–10 % (w/v) NaCl (in increments of 1 %) was investigated in marine broth 2216 (MB; Difco), containing a base of 2 % NaCl and supplemented with additional NaCl. Growth at pH 4.5–9.5 (in increments of 0.5 pH units) was investigated in MB by addition of HCl or Na_2CO_3. Growth was detected by changes in OD_{600} for 3 days. Growth at 4, 10, 20, 25, 28, 30, 35, 37, 40 and 45 °C was measured on marine agar 2216 (MA; Difco). Carbon source assimilation was determined using the GN2 MicroPlate system (Biolog), according to the manufacturer's instructions. Acid production from carbohydrates was determined using API 50 CH test trips and CH B/E medium (bioMérieux) according to the manufacturer's recommendations. Evaluation of growth was performed after 2 and 5 days. Susceptibility to antibiotics was determined by streaking strain PR1^T on modified ZoBell agar containing the following (μg ml^{-1}, unless otherwise stated): polymyxin B (100 U), streptomycin (50), chloramphenicol (100), ampicillin (100), gentamicin (30), neomycin (50), tetracycline (30) or kanamycin (30). Other physiological tests were performed with the API ZYM system (bioMérieux).

Cell biomass of strain PR1^T for analysis of cellular fatty acids was obtained from cultures grown for 1 day in SWC medium at 30 °C. *A. halophilus* JC 2051^T was used as a reference strain. Cellular fatty acid methyl ester content was determined using the MIDI Sherlock Microbial Identification System (Microbial ID, MidiLabs; Sasser, 1990). In addition, GC-MS analysis was performed to resolve ambiguities in fatty acid identification (Jahnke *et al.*, 2001). For isoprenoid quinone and polar lipid analysis, strain PR1^T was grown for 1 day in SWC medium at 30 °C, harvested, lyophilized and analysed by the Identification Service and Dr Brian Tindall of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) Braunschweig, Germany.

DNA was extracted from cell biomass of strain PR1^T grown in modified ZoBell medium using a Bacterial Genomic DNA Mini-prep kit (Bay Gene, Burlingame, CA, USA) according to the manufacturer's specifications. The 16S rRNA gene was amplified using universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-ACCTTGTTACGRCTT-3′; Weisburg *et al.*, 1991). A global sequence alignment of members of the genus *Algoriphagus* with validly published names and closely related taxa was computed using iterative pairwise methods by the MAP software program (Huang, 1994). Poorly aligned regions were removed by the software program Gblocks (version 0.91b) using default block parameters (Fig. S1 available in IJSEM Online; Castresana, 2000; Talavera & Castresana, 2007). Sequence alignments have also been deposited online at Treebase (www.treebase.org). A distance-matrix method (distance options according to the Kimura two-parameter model), including clustering with the neighbour-joining, maximum-likelihood and discrete character-based maximum-parsimony algorithms, was applied using the PHYLIP version 3.67 software package (Felsenstein, 1989) based on comparison of 1244 base pairs. In each case, the stability of the groups was estimated by bootstrap analysis based on 1000 replications.

The morphological, physiological and biochemical characteristics of strain PR1^T are given in the species description and Table 1. Strain PR1^T was distinguishable from *A. halophilus* JC 2051^T and *Algoiphagus lutimaris* S1-3^T by differences in several phenotypic characteristics, most of which were determined under the same conditions and methods (Table 1; Park *et al.*, 2010). The distinctive characteristics of strain PR1^T were as follows: D-galactose, mannitol and glycerol were positive in assays with the Biolog system, but cellobiose, D-fructose, D-mannose, salicin, sucrose, trehalose, L-arabinose, D-glucose, lactose and maltose were negative; alkaline phosphatase, esterase (C4) (weak), valine arylamidase (weak), β-galactosidase (weak), α-glucosidase and β-glucosidase (weak) were present in the API ZYM test, whereas *N*-acetyl-β-glucosaminidase was absent; acids were not produced from L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melezitose and raffinose in the API 50 CH kit; and sensitivity to gentamicin and tetracycline. The chemotaxonomic analysis was in agreement with the result of phylogenetic classification of strain PR1^T as a member of the genus *Algoriphagus* (Bowman *et al.*, 2003; Copa-Patiño *et al.*, 2008; Liu *et al.*, 2009; Nedashkovskaya *et al.*, 2004, 2007; Park *et al.*, 2010; Tiago *et al.*, 2006; Van Trappen *et al.*, 2004; Yoon *et al.*, 2005a, b, 2006; Young *et al.*, 2009).

Strain PR1^T had a cellular fatty acid composition similar to its closest phylogenetic neighbours but showed minor differences (Table 2). The major fatty acids (>10 % of total) in strain PR1^T were iso-$C_{15:0}$ (29.4 %) and summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$; 11.3 %), which are also the dominant fatty acids in other members of the genus *Algoriphagus* (Table 2; Park *et al.*, 2010), and a third fatty acid, summed feature 9 ($C_{16:0}$ 10-methyl and/or iso-$C_{17:1}\omega9c$; 18.5 %), which was predominant only in strain PR1^T. GC-MS analysis, together with comparison with an authentic standard, showed that 10-methyl fatty acids were below the detection limit and summed feature 9 was, therefore, inferred to be iso-$C_{17:1}\omega9c$. The polar lipid profile consisted of phosphatidylethanolamine, one unknown phospholipid, one unknown aminolipid, one unknown aminophospholipid and two unknown lipids (Fig. S2).

A nearly full-length 16S rRNA gene sequence of strain PR1^T, comprising 1420 nt, was determined in this study.

Table 1. Differential phenotypic characteristics of strain PR1^T and its closest phylogenetic relatives in the genus *Algoriphagus*

Strains: 1, *Algoriphagus machipongonensis* sp. nov. PR1^T; 2, *A. halophilus* JC 2051^T; 3, *A. lutimaris* S1-3^T. Data for columns 2 and 3 were taken from Yi & Chun (2004), Young *et al.* (2009), Park *et al.* (2010) and Oh *et al.* (2012). All strains are Gram-negative rods and positive for catalase and oxidase, acid production from aesculin, activity of alkaline phosphatase, esterase lipase (C8), leucine arylamidase, cysteine arylamidase, trypsin, α-chymotrypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase and susceptibility to chloramphenicol, neomycin and polymyxin B. All strains are negative for flagellation, gliding motility, flexirubin-type pigment production, acid production from inositol, D-mannitol, D-ribose and D-sorbitol, activity of α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase and susceptibility to ampicillin, kanamycin and streptomycin. +, Positive; W, weakly positive; −, negative; ND, data not reported.

Characteristic	1	2	3
Absorption peak maximum (nm)	487	475	467
NaCl requirement for growth	−*	+	−*
Optimal growth temperature (°C)	30	35	30
Utilization of:			
Cellobiose	−	+	+
D-Fructose	−	+	+
D-Mannose	−	+	+
Salicin	−	+	+
Sucrose	−	+	+
Trehalose	−	+	+
L-Arabinose	−	W	−
D-Galactose	+	+	−
D-Glucose	−	+	+
Lactose	−	+	+
Maltose	−	+	+
D-Mannitol	+	−	ND
Glycerol	+	−	ND
Acid production from:			
L-Arabinose	−	+	+
D-Xylose	−	+	−
D-Galactose	−	−	+
D-Glucose	−	+	+
D-Fructose	−	+	−
D-Mannose	−	+	+
L-Rhamnose	−	+	+
Cellobiose	−	+	−
Maltose	−	+	+
Lactose	−	+	+
Melibiose	−	+	−
Sucrose	−	+	−
Trehalose	−	−	+
Melezitose	−	+	−
Raffinose	−	+	+
Enzyme activity (API ZYM)			
Esterase (C4)	W	+	−
Valine arylamidase	W	W	−
β-Galactosidase	W	+	−
α-Glucosidase	+	+	−
β-Glucosidase	W	+	−
N-acetyl-β-glucosaminidase	−	+	+
Susceptibility to:			
Gentamicin	+	−	−
Tetracycline	+	+	−
DNA G+C content (% mol)	38.7	37.0	41.4

*Growth in the absence of NaCl occurs when Mg^{2+} ions are added.

Table 2. Cellular fatty acid compositions of strain PR1^T and its closest phylogenetic relatives in the genus *Algoriphagus*

Strains: 1, *Algoriphagus machipongonensis* sp. nov. PR1^T; 2, *A. halophilus* JC 2051^T; 3, *A. lutimaris* S1-3^T. Data for columns 1 and 2 were taken from this study and for column 3 were from Park *et al.* (2010). Fatty acids representing <0.5 % in all strains are omitted.

Fatty acid (%)	1	2	3
Straight-chain			
$C_{15:0}$	−	−	2.2
$C_{16:0}$	3.4	2.1	1.1
Branched			
iso-$C_{11:0}$	−	0.7	−
anteiso-$C_{11:0}$	−	2.6	1.5
iso-$C_{13:0}$	0.2	0.7	−
anteiso-$C_{15:0}$	2.3	4.4	2.6
iso-$C_{15:0}$	29.4	30.4	25.1
iso-$C_{15:1}$ F	1.7	−	−
iso-$C_{16:0}$	4.4	7.7	4.6
iso-$C_{16:1}$ H	1.8	1.7	1.2
iso-$C_{17:0}$	2.1	0.9	−
anteiso-$C_{17:1}\omega9c$	1.1	0.4	−
Unsaturated			
$C_{15:1}\omega6c$	0.5	0.7	0.7
$C_{15:1}\omega8c$	0.3	0.7	−
$C_{16:1}\omega5c$	2.9	5.1	3.9
$C_{17:1}\omega6c$	1.3	1.1	−
$C_{17:1}\omega8c$	0.9	0.3	−
Hydroxy			
iso-$C_{15:0}$ 3-OH	2.6	2.7	2.2
iso-$C_{16:0}$ 2-OH	1.0	2.2	−
$C_{16:0}$ 3-OH	1.6	1.1	−
iso-$C_{17:0}$ 3-OH	4.6	5.9	9.3
Summed features*			
3	11.3	15.8	19.4
4	2.6	1.4	3.9
9	18.5	6.9	−
Unknown ECL 13.565	−	−	2.0

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$. Summed feature 4 consisted of iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B. Summed feature 9 consisted of $C_{16:0}$ 10-methyl and/or iso-$C_{17:1}\omega9c$.

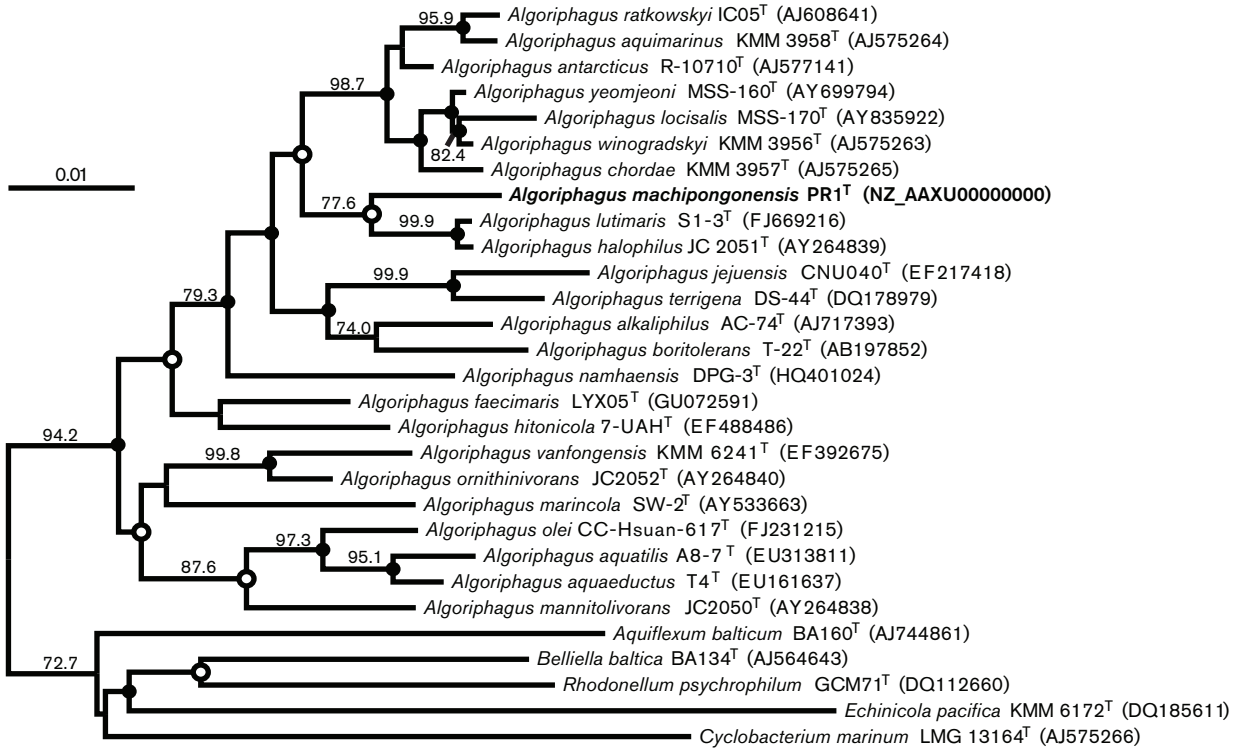

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain PR1^T relative to members of the genus *Algoriphagus* and the family *Cyclobacteriaceae*. Bootstrap values (>70 %) based on 1000 replicates are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony analyses. Open circles indicate that the corresponding nodes were also recovered in either the maximum-likelihood or maximum-parsimony analyses. *Cytophaga hutchinsonii* ATCC 33406^T (accession no. M58768) was used as an outgroup (not shown). Bar, 0.01 substitutions per nucleotide position.

Strain PR1^T was most closely related to *A. halophilus* JC 2051^T (95.4 % 16S rRNA gene sequence similarity) and *A. lutimaris* S1-3^T (95.3 %). Lower levels of 16S rRNA gene sequence similarity (91–95 %) were found between strain PR1^T and the type strains of all other species of the genus *Algoriphagus*, and <88 % sequence similarity was found with the type strains of the other species used in the phylogenetic analysis. A 16S rRNA gene sequence similarity threshold range of 98.7–99 % is recommended as the point above which DNA–DNA hybridization experiments should be mandatory (Stackebrandt & Ebers, 2006). Since strain PR1^T displayed 16S rRNA gene sequence similarity to *A. halophilus* JC 2051^T and *A. lutimaris* S1-3^T below the threshold, DNA–DNA hybridization experiments were not performed. The DNA G+C content of strain PR1^T was 38.7 mol%, as determined by genomic sequencing by the J. Craig Venter Institute and Broad Institute (Alegado *et al.*, 2011).

In the neighbour-joining tree based on 16S rRNA gene sequences (Fig. 1), strain PR1^T fell within the cluster comprising the genus *Algoriphagus* and formed a coherent subcluster with *A. halophilus* JC 2051^T and *A. lutimaris* S1-3^T with a bootstrap resampling value of 77.6 %. The close relationship of strain PR1^T, *A. halophilus* JC 2051^T and *A. lutimaris* S1-3^T was also found when the maximum-likelihood algorithm was used (Fig. S3).

The phylogenetic distinctiveness of strain PR1^T together with differential phenotypic properties are sufficient to demonstrate that this strain is distinct from previously recognized *Algoriphagus* species, including *A. halophilus* and *A. lutimaris* (Stackebrandt & Goebel, 1994). Therefore, on the basis of phenotypic, chemotaxonomic and phylogenetic data, strain PR1^T is considered to represent a novel species of the genus *Algoriphagus*, for which the name *Algoriphagus machipongonensis* sp. nov. is proposed.

Description of *Algoriphagus machipongonensis* sp. nov.

Algoriphagus machipongonensis (ma.chi.pon.go.nen′sis. N.L. masc. adj. *machipongonensis* of or belonging to Machipongo, the Algonquin name for Hog Island).

Cells are Gram-negative, non-spore-forming, non-flagellated short rods between 2 and 3 μm in length and 0.5 μm in width; motility not detected. Colonies on MA, modified ZoBell medium and SWC medium are circular, convex, smooth, glistening, light pink and 1–2 mm in diameter after incubation for 5 days at 25 °C. Growth occurs at 10–40 °C, with weak growth at 4 °C (optimum 30 °C). Growth occurs at 5.0, but not at pH 4.5 or 9.5 (optimum pH 7.0–8.0). Growth occurs with 0 and 10 % (weak) NaCl (optimum 3 % NaCl). Mg^{2+} ions are required for growth. Growth does not occur under anaerobic conditions on MA. With GN2 MicroPlates, utilizes D-galactose, mannitol and glycerol, but not cellobiose, D-fructose, D-mannose, salicin, sucrose, trehalose, L-arabinose, D-glucose, lactose or maltose. With API 50 CH, acids are produced aerobically from aesculin, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Positive for alkaline phosphatase, esterase (C4) (weak), esterase lipase (C8), leucine arylamidase, valine arylamidase (weak), cysteine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase (weak), α-glucosidase and β-glucosidase (weak), but negative for *N*-acetyl-β-glucosaminidase, α-galactosidase, β-glucuronidase, α-mannosidase and α-fucosidase. Sensitive to neomycin, tetracycline, polymyxin B and gentamicin, but not to ampicillin, streptomycin or kanamycin. The predominant menaquinone is MK-7. The major fatty acids (>10 % of total) are iso-$C_{15:0}$, iso-$C_{17:1}\omega 9c$ and summed feature 3 ($C_{16:1}\omega 6c$ and/or $C_{16:1}\omega 7c$). The major polar lipids are phosphatidylethanolamine, an aminophospholipid, an aminolipid, a phospholipid and two lipids of unknown character.

The type strain, PR1^T (=ATCC BAA-2233^T =DSM 24695^T), was co-isolated with the colonial choanoflagellate *Salpingoeca rosetta* from a mud core sample taken from Hog Island, Virginia, USA. The DNA G+C content is 38.7 mol % (Alegado *et al.*, 2011).

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