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Chitinimonas naiadis sp. nov., Isolated from a Freshwater River

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A rod shaped, aerobic, Gram-stain-negative, and motile bacterium, strain AR2^T, was isolated from a water sample of Yeongsan river, Republic of Korea. Strain AR2^T clustered closely with the members of the genus *Chitinimonas* and showed the highest 16S rRNA gene sequence similarity with *Chitinimonas prasina* LY03^T (96.4%), *Chitinimonas viridis* HMD2169^T (96.4%), *Chitinimonas taiwanensis* cf^T (96.2%), and *Chitinimonas koreensis* R2A43-10^T (94.2%). The predominant fatty acids of strain AR2^T were identified to be summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$, and $C_{10:0}$3-OH. Diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine were found to be the major polar lipids. The genomic DNA G+C content was 60.4 mol%. Based on the polyphasic characterization, the isolated strain AR2^T is described as a representative of a novel species in the genus *Chitinimonas*, for which the name *Chitinimonas naiadis* sp. nov. (type strain =AR2^T =KCTC 42755^T =JCM 31504^T) is proposed.

Keywords: *Chitinimonas*, polyphasic taxonomy

Introduction

The genus *Chitinimonas* [1] was classified to accommodate Gram-stain-negative strains having chitinolytic activity, showing distant relatedness to members of the genera *Propionivibrio*, *Burkholderia*, *Pandoraea*, and others, and forming a distinct clade within the class Betaproteobacteria. Members of this genus are rod-shaped and motile by gliding or by means of a polar flagellum. The major fatty acids are summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and $C_{16:0}$. The G+C content of the genomic DNA ranges from 59.8 to 65.0 mol%. Currently, the genus *Chitinimonas* consists of four species (http://www.bacterio.net/chitinimonas.html), *Chitinimonas taiwanensis* isolated from a fresh water shrimp pond [1], *Chitinimonas koreensis* isolated from a greenhouse soil [2], *Chitinimonas viridis* isolated from a mesotrophic artificial lake [3], and *Chitinimonas prasina* isolated from lake water [4]. Owing to its chitinolytic activity, the type strain of *Chitinimonas prasina* was reported to have algicidal activity on marine diatoms [5]. During the search for antibiotic-resistant bacteria from various freshwater bodies, strain AR2^T was isolated and proposed to be the type strain of a novel species in the genus *Chitinimonas* based on polyphasic characterization.

Materials and Methods

Strains and Culture Conditions

Strain AR2^T was isolated from an ampicillin-containing (16 μg/ml) Mueller Hinton agar plate inoculated with a surface water sample from Yeongsan river, Republic of Korea. The purity of the isolate was confirmed by repeated streaking on Mueller-Hinton agar plates at 30°C and maintained by regular subculturing and also preserved as stocks in 10% skim milk or sucrose at -80°C. Based on 16S rRNA gene sequence similarity, the type strains of *C. taiwanensis* cf^T (=DSM 18899^T), *C. viridis* HMD2169^T (=KCTC 22839^T), and *C. prasina* LY03^T (=KCTC 32574^T =MCCC 1F01209^T) were selected for comparative taxonomic studies and procured from the respective culture collections. However, the type strain of *C. prasina* obtained twice as a lyophilized vial and also on agar plate from the Marine Culture Collection of China (MCCC) and also as a lyophilized vial from the Korean Collection for Type Cultures (KCTC) could not be revived in the laboratory even after following the growth conditions prescribed by the culture collection centers. Currently, the strain is not available from KCTC owing to the same reason. Hence, the comparative polyphasic characterization of strain AR2^T was performed only with *C. taiwanensis* cf^T and *C. viridis* HMD2169^T in the laboratory following the recommended standards proposed by Tindall *et al*. [6], and the characters of *C. prasina* were taken from Li *et al.* [4]. In addition, it was observed that *C. viridis* HMD2169^T could grow only on R2A medium, whereas strain AR2^T

showed optimum growth on tryptone soy medium but could not grow on R2A medium. Therefore, comparative characterization of strain AR2^T and *C. taiwanensis* cf^T was carried on tryptone soy medium, but for *C. viridis* HMD2169^T, R2A medium was used.

Phenotypic Characterization

Morphological properties (cell shape, cell size, and motility) of strain AR2^T were observed by phase contrast light microscopy (Zeiss Primo Star, USA) after 24 h incubation in tryptone soy broth. Following the protocol described earlier [7], a transmission electron microscope (H-7650, Hitachi, Japan) was used for viewing the position and number of flagella. The Gram reaction was performed by the standard staining method and also by the KOH lysis method according to Buck [8]. Chitinolytic activity was tested according to the method of Chang *et al*. [1]. Growth at different concentrations of NaCl was tested in tryptone soy broth supplemented with varying amounts of NaCl (0–6% (w/v) with an interval of 0.5%). Ability to grow at different pH values (4.0–12.0 at an interval of 0.5) was tested in tryptone soy broth adjusted to varying pHs using phosphate-citrate buffer (4.0–6.5), Tris-HCl buffer (7.0–9.0), $NaHCO_3$-NaOH buffer (9.5–11.0), and Na_2HPO_4-NaOH buffer (11.5–12.0). Similarly, the growth temperature range was tested in tryptone soy broth with incubation at different temperatures (5–50°C with an interval of 5°C). Utilization of various substrates as the sole carbon source and growth in the presence of inhibitory compounds were tested using the GEN III MicroPlate test panel (BIOLOG, USA) following the manufacturer's instructions. Catalase and oxidase tests were performed according to standard protocols [9]. In addition, the production of acid from carbohydrates, enzyme activities, and other biochemical tests were performed using API 50CH, API 20E, API 20NE, and API ZYM strips (bioMérieux, France). Antibiotic sensitivity was tested by the disc diffusion method using antibiotic discs (Liofilchem, Italy) containing (μg) gentamicin (10), amoxicillin (30), cephalexin (30), meropenem (10), tetracycline (30), erythromycin (15), tylosin (30), ciprofloxacin (5), clindamycin (2), vancomycin (5), sulfamethoxazole (50), trimethoprim (5), linezolid (10), rifampicin (5), colistin (10), and fosfomycin (200).

Chemotaxonomy

Following the integrated procedure by Minnikin *et al*. [10], polar lipids were extracted from 250 mg of freeze-dried cells. Separation of the lipids was achieved according to Tindall *et al*. [11] by two-dimensional chromatography on silica gel TLC plates (Kieselgel 60 F254; Merck, Germany) using chloroform:methanol:water (65:25:4 (v/v/v)) and chloroform:methanol:acetic acid:water (80:12:15:4 (v/v/v/v)) as the mobile phase in the first and second dimensions, respectively. To detect the total polar lipids, the plates were sprayed with 5% ethanolic molybdophosphoric acid, and specific functional groups of amino, phosphates, or sugars were detected using ninhydrin, molybdenum blue, or α-naphthol spray reagents, respectively [12, 13]. Quinone analysis was performed according to Tamaoka *et al*. [14], which was outsourced through the Korean Culture Centre of Microorganisms (Republic of Korea). Quinones were extracted from the wet culture pellet using chloroform:methanol (2:1) and shaking for 3–4 h. The suspension was filtered (Whatman No. 2 filter paper, USA) and the filtrate was concentrated and suspended in 100 μl of chloroform:methanol (8.5:1.5). The suspension was centrifuged (12,000 ×*g*, 5 min) and the supernatant was analyzed by HPLC (YOUNG LIN YL9100 (YL9111 Binary pump, Korea); Solvent system, methanol:isopropyl ether (4:1); flow rate 1.0 ml/min; Spherisorb 5 μm ODS2, 4.6 mm × 150 mm, Waters column; YOUNG LIN YL9120 UV/Vis Detector (Korea); detection wavelength 254 mm). Data analysis was carried out using YOUNG LIN Autochro-3000 software (Korea). For determination of the cellular fatty acid composition, strain AR2^T was grown on tryptone soy agar (Difco, USA) at 30°C for 48 h. Following the instructions for the Microbial Identification System (Microbial ID; MIDI 6.0 version) [15], fatty acid methyl esters were prepared, separated, and identified. Genomic DNA was extracted and purified according to the method of Marmur [16] and the mol% G+C of the DNA was estimated using the fluorimetric procedure described by Gonzalez and Saiz-Jimenez [17].

Phylogenetic Analysis

For 16S rRNA gene sequencing, biomass from 1.5 ml of well-grown liquid culture of strain AR2^T was taken and genomic DNA was extracted using the GeneAll Exgene Cell SV kit. PCR amplification of the 16S rRNA gene and sequencing were performed as described previously [18]. The gene was amplified using universal primers 27F (5′-AGA GTT TGA TCA TGG CTC AG-3′), and 1522R (5′-AAG GAG GTG ATC CAG CCG AC-3′). Sequencing was performed by thes chain termination reaction using the BigDye Terminator ver. 3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, USA). The 16S rRNA gene sequences obtained were compiled using SeqMan software (DNASTAR, USA) and the almost complete sequence (1,485 bp) of strain AR2^T was compared with available 16S rRNA gene sequences of cultured species by NCBI-BLAST search [19] and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) [20]. For phylogenetic analysis, 16S rRNA gene sequences of the representatives of closely related taxa and of strain AR2^T were aligned using the CLUSTAL_W algorithm, and the MEGA6 software [21] was used for phylogenetic analyses. In a pairwise deletion procedure, evolutionary distances were calculated by Kimura 2-parameter correction [22]. Neighbor-joining, maximum-parsimony (MP), and minimum evolution (ME) [23–25] methods were used to construct phylogenetic trees. Percentage support values were obtained using a bootstrap procedure with 1,000 replications.

The obtained 16S rRNA gene sequence of strain AR2^T was deposited under the GenBank/EMBL/DDBJ accession number KT184596.

Results and Discussion

Strain AR2^T was observed to be a rod-shaped bacterium that was 2.0–4.0 μm long and 0.5 μm wide, aerobic, gram-stain-negative, and motile by means of a single polar flagellum (Figs. S1 and S2). Colonies of strain AR2^T on tryptone soy agar were observed to be circular and convex, with entire margins. Strain AR2^T did not require NaCl for its growth but could tolerate NaCl concentrations up to 0.5% (w/v). Growth was observed at a pH range of 7.0–7.5 (optimum at 7.0) and temperature of 15–35°C (optimum at 30°C). Comparison of different morphological, physiological, and biochemical characteristics is shown in Table 1. Inability to grow over a wide range of pH and NaCl concentrations, absence of valine arylamidase activity, and ability to utilize arabinose as the sole carbon source differentiated strain AR2^T from other members of the genus *Chitinimonas*.

Phylogenetic analysis based on the 16S rRNA gene sequence of strain AR2^T established its relationship with members of the genus *Chitinimonas*, showing highest 16S rRNA gene sequence similarity with strain LY03^T (96.4%) followed by strains HMD2169^T (96.4%), cf^T (96.2%), and R2A43-10^T (94.2%). The neighbor-joining phylogenetic tree indicated that strain AR2^T clustered with the members of genus *Chitinimonas* with high bootstrap support (Fig. 1). Similar topology was also observed in the MP and ME trees (Figs. S3 and S4). The G+C content of strain AR2^T was 60.4 mol% as determined by the fluorimetric method.

The major polar lipids of strain AR2^T were identified as diphosphatidylglycerol, phosphatidylglycerol, and phosphatidylethanolamine. The presence of two unidentified phospholipids (PL1 and 2), three amino phospholipids (APL1, 2, and 3),

Table 1. Comparison of phenotypic characteristics of strain AR2^T with those of members of the genus *Chitinimonas*.

Characteristic	1[a]	2[a]	3[a]	4[b]	5[b]
Growth temperature range and optimum (°C)	15-35 (30)	4-37 (25-37)	20-37 (30)	15-39 (37)	10-40 (28-37)
pH range (optimum)	7-7.5 (7)	4-10 (6-8)	5-10 (6)	4-10 (7-9)	5-8 (ND)
NaCl tolerance % (w/v)	0.5%	1%	3%	1%	1%
Hydrolysis of					
Gelatin	+	(+)	-	-	+
Esculin	-	(+)	(+)	+	-
Tween 40	+	+	-	+	+
API ZYM tests					
Esterase (C4)	+	+	-	(+)	+
Valine arylamidase	-	+	+	(+)	+
Trypsin	-	-	+	(+)	+
α-Chymotrypsin	-	+	-	+	+
Acid phosphatase	+	-	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	-	+	+
β-Galactosidase	-	+	-	-	-
α-Glucosidase	+	-	-	-	+
Utilization of					
Glucose	+	+	-	+	+
Arabinose	+	-	-	-	-
Mannose	+	-	-	-	+
Mannitol	+	+	-	-	-
N-Acetyl-glucosamine	-	+	-	-	-
Malate	(+)	+	-	-	+
DNA G+C content (mol%)	60.4	62.8	59.8	63.6	65.0

Strains: 1, AR2^T; 2, cf^T; 3, HMD2169^T; 4, LY03^T; 5, R2A43-10^T. [a]Data obtained from the present study. [b]Data take from Li *et al*. [4]. All strains are positive for hydrolysis of chitin, utilization of maltose, alkaline phosphatase, esterase lipase (C8), and leucine arylamidase activities but negative for indole production, arginine dihydrolase, urea hydrolysis, cystine arylamidase, α-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase activities, and utilization of potassium gluconate, capric acid, adipic acid, citrate, and phenyl acetic acid. +, substrate utilized/present; -, substrate not utilized/absent; (+), weak growth/activity.

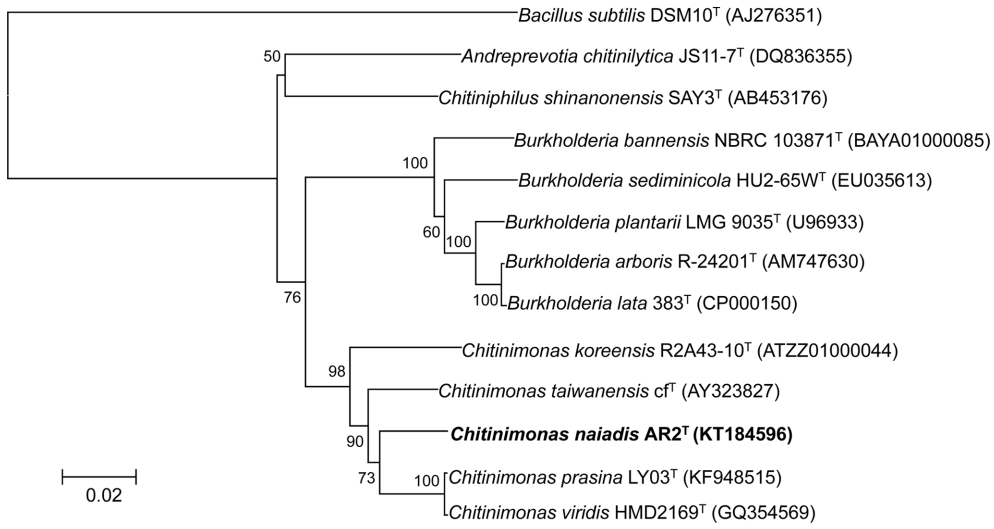

Fig. 1. Phylogenetic analysis showing the relationship of strain AR2^T to closely related species based on 16S rRNA gene sequences. Accession numbers are given in parentheses. Multiple alignment, distance calculations (distance options according to the Kimura 2-parameter model), and clustering with the neighbor-joining method were performed by using the software MEGA ver. 6. Bootstrap values based on 1,000 replications are listed as percentages at the branching points. Bar, 0.02 nucleotide substitutions per nucleotide position.

and six unidentified lipids (UL1-6) in minor amounts was also observed (Fig. S5). Ubiquinone-8 (Q-8) is the sole isoprenoid quinone in strain AR2^T, which is in accordance with the description of the genus *Chitinimonas*. Whole-cell fatty acid analysis revealed that summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$, and $C_{10:0}$3-OH are the predominant fatty acids (Table 2).

Along with the phylogenetic interpretation based on 16S rRNA gene sequence similarities below the threshold for delineation of species [26] (*i.e.* 96.4–94.2%) between strain AR2^T and the other members of genus *Chitinimonas*, distinct physiological characters were also observed during the present study (Tables 1 and 2, and Fig. S5). These results indicated that the strain should be assigned as a representative of a novel species within the genus *Chitinimonas*, for which the name *Chitinimonas naiadis* sp. nov. is proposed.

Description of *Chitinimonas naiadis* sp. nov.

Chitinimonas naiadis (na.ia'dis. L. fem. gen. n. *naiadis* of a Naiad, a water-nymph of the Greek mythology living in the flowing water)

Cells are rod shaped, 2.0–4.0 μm long and 0.5 μm wide, aerobic, Gram-stained-negative, and motile by means of a single polar flagellum. Growth occurs over a pH range of 7.0–7.5 (optimum 7.0) and at temperature range 15–35°C (optimum 30°C). NaCl is not required for growth but can tolerate NaCl concentrations up to 0.5% (w/v). Positive for catalase, oxidase, alkaline phosphatase, esterase (C4),

Table 2. Cellular fatty acid contents (%) of strain AR2^T and closely related strains.

Fatty Acid	1	2[a]	3[a]	4[a]	5[b]
$C_{10:0}$	TR	TR	3.9	TR	3.4
$C_{10:0}$ 3-OH	12.1	13.9	9.2	8.6	5.8
$C_{11:0}$	1.6	ND	ND	ND	ND
$C_{11:0}$ 2-OH	1.0	ND	ND	ND	ND
$C_{12:0}$	6.2	12.4	2.8	9.3	2.2
$C_{12:0}$ 2-OH	4.0	5.0	ND	6.9	ND
$C_{12:0}$ 3-OH	TR	ND	ND	ND	2.0
$C_{14:0}$	1.7	1.7	4.3	3.3	2.6
$C_{14:1}\omega5c$	ND	TR	2.5	TR	ND
$C_{15:1}\omega6c$	6.4	ND	ND	ND	ND
$C_{16:0}$	22.4	20.9	26.9	19.5	26.4
$C_{16:0}$ N-alcohol	ND	TR	TR	6.0	ND
$C_{16:1}\omega7c$-alcohol	ND	1.7	TR	5.2	ND
$C_{17:0}$	1.6	ND	ND	ND	ND
$C_{17:1}\omega6c$	2.8	ND	ND	ND	ND
anteiso- $C_{17:1}\omega9c$	ND	ND	ND	1.9	ND
Summed feature 3	29.1	33.9	41.6	30.0	43.7
Summed feature 8	4.3	4.3	4.8	2.1	9.8

Strains: 1, AR2^T; 2, cf^T; 3, LY03^T; 4, R2A43-10^T; 5, HMD2169^T.
TR, trace (<1%); ND, not detected; Summed feature 3 comprised $C_{16:1}\omega7C$ and/or $C_{16:1}\omega6C$, and summed feature 8 comprised $C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$. [a,b]Data taken from Li *et al.* [4] and Joung *et al.* [3], respectively.

esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, VP test, nitrate reduction, hydrolysis of gelatin, chitin, and Tween 40. Negative for α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, valine arylamidase, cysteine arylamydase, trypsin, α-chymotrypsin, MR, starch hydrolysis, esculin hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, urea hydrolysis, H_2S production, and indole production. Assimilation of the following carbon sources is positive: dextrin, D-maltose, D-cellobiose, gentiobiose, D-turanose, D-salicin, *N*-acetyl-β-D-mannosamine, *N*-acetyl-D-galactosamine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-pyroglutamic acid, L-serine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, D-sorbitol, D-mannitol, D-arabitol, myoinositol, glycerol, D-glucose-6-PO_4, D-fructose-6-PO_4, D-aspartic acid, methyl pyruvate, D-lactic acid methyl ester, L-lactic acid, α-keto-glutaric acid, D-malic acid, L-malic acid, bromo-succinic acid, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, mucic acid, quinic acid, D-saccharic acid, D-xylose, γ-amino-butyric acid, β-hydroxy-D,L-butyric acid, acetoacetic acid, propionic acid, acetic acid, formic acid, L-arabinose, and glycogen. Can tolerate and grow in the presence of 1% sodium lactate, tetrazolium violet, and tetrazolium blue. Resistant to amoxicillin, clindamycin, vancomycin, trimethoprim, fusidic acid, lincomycin, rifamycin SV, aztreonam, and linezolid. Sensitive to gentamicin, cephalexin, meropenem, tetracycline, erythromycin, tylosin, ciprofloxacin, sulfamethoxazole, and fosfomycin. Predominant fatty acids include summed feature 3 (comprising $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$), $C_{16:0}$, and $C_{10:0}$3-OH. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, and phosphatidyl-ethanolamine, along with two unidentified phospholipids, three amino phospholipids, and six unidentified lipids in minor quantities. Ubiquinone-8 (Q-8) is the sole isoprenoid quinone. The DNA G+C content of the type strain is 60.4 mol%.

The type strain AR2^T (=KCTC 42755^T =JCM 31504^T) was isolated from a water sample collected from Yeongsan river, Republic of Korea.

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