Arcicella aurantiaca sp. nov., isolated from stream water

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A bacterial strain designated TNR-18^T was isolated from stream water in southern Taiwan and characterized using a polyphasic taxonomic approach. Cells of strain TNR-18^T were Gram-stain-negative, strictly aerobic, non-motile and vibrioid, and the strain formed orange colonies. Growth occurred at 10–30 °C, with 0–0.2 % NaCl and at pH 6.0–10.0. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain TNR-18^T belonged to the genus *Arcicella*; its closest neighbours were *Arcicella rosea* TW5^T and *Arcicella aquatica* NO-502^T, with respective sequence similarities of 97.9 and 97.8 %. Predominant cellular fatty acids (>10 %) were summed feature 3 (C_{16:1}ω7c/C_{16:1}ω6c; 37.7 %), C_{16:1}ω5c (13.0 %) and iso-C_{15:0} (11.1 %). The major respiratory quinone was MK-7. The polar lipid profile contained phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and several uncharacterized polar lipids. The DNA G+C content was 39.9 mol%. On the basis of the phylogenetic and phenotypic data, strain TNR-18^T should be classified as representing a novel species, for which the name *Arcicella aurantiaca* sp. nov. is proposed. The type strain is TNR-18^T (=LMG 25207^T =BCRC 17969^T).

The genus *Arcicella*, first proposed by Nikitin *et al.* (2004), belongs to the family *Cytophagaceae* of the order *Cytophagales*, class ‘*Cytophagia*’, phylum *Bacteroidetes* (Ludwig *et al.*, 2008). At the time of writing, the genus contains only two species with validly published names, the type species *Arcicella aquatica*, isolated from a neuston film on a freshwater lake (Nikitin *et al.*, 2004), and *Arcicella rosea*, from tap water (Kämpfer *et al.*, 2009). The present study was carried out to clarify the taxonomic position of the *Arcicella*-like bacterial strain TNR-18^T by a polyphasic taxonomic approach.

Stream water (approx. 200 ml) was collected in a sterile bag from Maolin township (GPS location 22° 54′ 12.5″ N 120° 40′ 59.9″ E) in Kaohsiung Prefecture, southern Taiwan, stored at 4 °C and transported to the laboratory within 2 h. The water sample was spread-plated on R2A agar (BD Difco) following the standard dilution plating

method. After incubation of the plates at 25 °C for 5 days, strain TNR-18^T was purified as a single colony. The strain was preserved at –80 °C as a 20 % (v/v) glycerol suspension in R2A broth or by lyophilization with 20 % (w/v) skimmed milk. *A. aquatica* LMG 21963^T and *A. rosea* CCUG 55942^T were obtained from the Belgian Coordinated Collections of Microorganisms and the Culture Collection of the University of Göteborg, respectively, for detailed comparisons.

Bacterial cells were observed by phase-contrast microscopy (DM 2000; Leica) in the lag, exponential and stationary phases of growth to ascertain their morphology. Flagellar and gliding motility were tested using the hanging drop method. Spot Test flagella stain (BD Difco) was used to stain any flagella that might be present. The Gram stain set (BD Difco) kit and the Ryu non-staining KOH method (Powers, 1995) were used to test Gram-staining behaviour. Colony morphology was examined on R2A agar using a stereoscopic microscope (SMZ 800; Nikon). The presence of flexirubin-type pigments and carotenoids was investigated as described by Reichenbach (1992), Bernardet *et al.* (2002) and Schmidt *et al.* (1994).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TNR-18^T is FJ593908.

Results of 2D TLC of polar lipids of strain TNR-18^T are available as supplementary material with the online version of this paper.

The pH range for growth was determined by measuring the OD₆₀₀ of R2A broth cultures (BD Difco). The medium was adjusted to pH 4.0–10.0 (at intervals of 0.5 pH units) using appropriate biological buffers (Chung *et al.*, 1995). To investigate the tolerance of NaCl, R2A broth was prepared according to the formula of the BD Difco medium with the NaCl concentration adjusted to 0–1.0% (w/v) (at intervals of 0.1%). The temperature range for growth was determined in R2A broth at 4 and 10–45 °C (at intervals of 5 °C). Growth under anaerobic conditions was determined after incubating strain TNR-18^T in the Oxoid AnaeroGen system.

Strain TNR-18^T was examined for a broad range of phenotypic properties. Activities of catalase, oxidase and lipase (corn oil) and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined according to standard methods (Gerhardt *et al.*, 1994). Additional biochemical tests were performed using the API ZYM and API 20NE kits (bioMérieux) and carbon source utilization was evaluated using the Biolog GN2 microplate. Phenotypic tests using commercial kits were performed according to the manufacturers' recommendations.

Sensitivity of strain TNR-18^T to antibiotics was tested by the disc diffusion method after spreading cell suspensions (0.5 McFarland) on R2A agar (BD Difco) plates. The discs (Oxoid) contained the following antibiotics: ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 µg), rifampicin (5 µg), streptomycin (10 µg), sulfamethoxazole plus trimethoprim (23.75/1.25 µg) and tetracycline (30 µg). The effect of antibiotics on cell growth was assessed after 3 days of incubation at 25 °C. The strain was considered susceptible when the diameter of the inhibition zone was >13 mm, intermediate at 10–12 mm and resistant at <10 mm. Detailed results from the phenotypic and biochemical analyses of strain TNR-18^T are provided in Table 1 and the species description. Phenotypic characteristics that serve to differentiate strain TNR-18^T from the type strains of the genus *Arcicella* are presented in Table 1.

The 16S rRNA gene sequence was analysed as described previously by Chen *et al.* (2001). Analysis of the sequence was performed by using the software BioEdit (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (corrected according to Kimura's two-parameter model; Kimura, 1983) were calculated and clustering was performed with the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated by using the treeing algorithms contained in the PHYLIP software package (Felsenstein, 1993). In each case, bootstrap values were calculated based on 1000 replications. An almost-complete 16S rRNA gene sequence (1411 bp) of strain TNR-18^T was compared against 16S rRNA gene sequences available from

Table 1. Differential characteristics of type strains of *Arcicella* species

Strains: 1, *A. aurantiaca* sp. nov. TNR-18^T; 2, *A. aquatica* LMG 21963^T; 3, *A. rosea* CCUG 55942^T. Data were acquired in this study. +, Positive; –, negative; w, weakly positive. All strains stain Gram-negative and are positive for C8 esterase lipase, leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, α-glucosidase and N-acetyl-β-glucosaminidase activities and negative for indole production, glucose fermentation, arginine dihydrolase, urease, C14 lipase, β-glucuronidase, α-mannosidase and lipase (corn oil) activities and hydrolysis of Tweens 20, 40, 60 and 80.

Characteristic	1	2	3
Colony pigmentation*	O	LO	PP
Mucus formation	–	+	+
NaCl range for growth (%)	0–0.2	0–0.5	0–0.7
Catalase	+	–	+
Oxidase	w	w	–
Hydrolysis of:			
Casein	+	–	–
Aesculin	+	+	–
Oxidation of (GN2):			
L-Fucose	+	–	–
Methyl β-D-glucoside	+	w	+
Raffinose	+	–	–
Pyruvic acid methyl ester	+	–	+
Acetic acid	+	–	w
D-Galactonic acid lactone	+	–	–
D-Glucosaminic acid	+	–	w
α-Ketobutyric acid	+	–	–
α-Ketoglutaric acid	+	–	–
α-Ketovaleric acid	+	–	–
DL-Lactic acid	+	–	+
Quinic acid	+	–	–
L-Alaninamide	+	–	+
L-Alanine	+	–	+
L-Alanyl glycine	+	–	+
L-Aspartic acid	+	–	+
L-Glutamic acid	+	–	+
Glycyl L-aspartic acid	+	–	w
Glycyl L-glutamic acid	+	–	w
L-Serine	+	–	+
Inosine	+	–	–
Uridine	+	–	–
α-D-Glucose 1-phosphate	+	–	+
D-Glucose 6-phosphate	+	–	–
Enzyme activity (API ZYM)			
Alkaline phosphatase	+	+	–
C4 esterase	–	+	–
Acid phosphatase	+	+	–

*LO, Light orange; O, orange; PP, pale pink.

the EzTaxon server (Chun *et al.*, 2007), the Ribosomal Database Project (Maidak *et al.*, 2001) and the GenBank database (<http://www.ncbi.nlm.nih.gov/>). 16S rRNA gene sequence analysis indicated that strain TNR-18^T belonged

to the family *Cytophagaceae* (order *Cytophagales*, class 'Cytophagia', phylum *Bacteroidetes*). Strain TNR-18^T formed a distinct subline within the genus *Arcicella* in the neighbour-joining tree (Fig. 1). The overall topologies of the phylogenetic trees retrieved by the maximum-likelihood and maximum-parsimony methods were similar. Sequence similarity calculations (over 1400 bp) indicated that strain TNR-18^T was closely related to *A. rosea* TW5^T (97.9% 16S rRNA gene sequence similarity) and *A. aquatica* NO-502^T (97.8%). Lower sequence similarities (<93.1%) were found with representative members of all other genera listed in Fig. 1.

Whole genome DNA–DNA hybridization experiments were carried out with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The degree of hybridization was calculated as the mean of triplicate experiments. Separate species status of strain TNR-18^T was confirmed by the hybridization values obtained with *A. aquatica* LMG 21963^T and *A. rosea* CCUG 55942^T (59.5 ± 3.1 and 23.1 ± 2.4 %, respectively). The DNA–DNA relatedness between strain TNR-18^T and its closest phylogenetic neighbours was well below the 70 % cut-off point recommended for the assignment of strains to the same genomic species (Wayne *et al.*, 1987). Based on the above data, strain TNR-18^T warrants separate species status in the genus *Arcicella*.

Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification System (Microbial ID; Sasser, 1990). The fatty acid profiles of strain TNR-18^T, *A. aquatica* LMG 21963^T and *A. rosea* CCUG 55942^T were determined using cells grown on R2A agar at 25 °C for 3 days. The fatty acid profile of strain TNR-18^T was similar to those of the other two *Arcicella* type strains, although there were differences in the proportions of some components (Table 2). Cellular fatty acids present in strain TNR-18^T at ≥1 % included summed feature 3 (C_{16:1}ω7c and/or C_{16:1}ω6c), C_{16:1}ω5c, iso-C_{15:0}, iso-C_{15:0} 3-OH, C_{16:0}, C_{16:0} 3-OH, iso-C_{17:0} 3-OH, C_{14:0}, anteiso-C_{15:0}, C_{15:1}ω6c, iso-C_{17:1}ω9c and C_{18:0}. Isoprenoid quinones were extracted and purified according to the method of Collins (1985) and analysed by HPLC; the major respiratory quinone of strain TNR-18^T

was MK-7. The DNA G+C content of strain TNR-18^T, determined by HPLC according to Mesbah *et al.* (1989), was 39.9 ± 1.0 mol%.

Polar lipids were extracted and analysed by two-dimensional TLC according to Lechevalier *et al.* (1977). Strain TNR-18^T exhibited a complex polar lipid profile consisting of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and several uncharacterized polar lipids (see Supplementary Fig. S1, available in IJSEM Online). Strain TNR-18^T exhibited a polar lipid profile very similar to that of its closest relative, *A. rosea* TW5^T (Kämpfer *et al.*, 2009), in which the predominant compounds were also phosphatidylethanolamine and an unknown aminophospholipid.

On the basis of 16S rRNA gene sequence similarity, strain TNR-18^T occupies a distinct position within the genus *Arcicella*. This result was supported by the unique combination of chemotaxonomic characteristics and biochemical traits of the strain (Table 1). Based on 16S rRNA gene sequence similarity, DNA–DNA relatedness, fatty acid profile, major respiratory quinone, DNA G+C content and phenotypic properties, it is evident that strain TNR-18^T should be classified as the type strain of a novel species in the genus *Arcicella*, for which the name *Arcicella aurantiaca* sp. nov. is proposed.

Description of *Arcicella aurantiaca* sp. nov.

Arcicella aurantiaca (au.ran.ti.a.'ca. N.L. fem. adj. *aurantiaca* orange-coloured).

Cells are Gram-stain-negative, strictly aerobic, vibrioid and non-motile. After 72 h of incubation on R2A agar at 25 °C, mean cell dimensions are 0.5–0.6 µm diameter and 1.0–3.0 µm length. Colonies on R2A agar are orange, non-mucous, circular, smooth and convex with entire edges. Colonies are approximately 1.0–1.5 mm in diameter on R2A agar after 72 h of incubation at 25 °C. Growth occurs at 10–30 °C, with 0–0.2 % NaCl and at pH 6.0–10.0. Optimum growth occurs at 25 °C, in the absence of NaCl and at pH 7.0. Carotenoid/flexirubin pigments are absent. Positive for catalase and oxidase activities and hydrolysis of

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Arcicella aurantiaca* sp. nov. TNR-18^T and related taxa in the class 'Cytophagia'. Numbers at nodes are bootstrap percentages (>70 %) based on the neighbour-joining (above nodes) and maximum-parsimony (below nodes) tree-making algorithms. Filled circles indicate branches of the tree that were also recovered using the maximum-likelihood and maximum-parsimony algorithms. *Flexibacter litoralis* ATCC 23117^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 2. Cellular fatty acid compositions of the type strains of *Arcicella* species

Strains: 1, *A. aurantiaca* sp. nov. TNR-18^T; 2, *A. aquatica* LMG 21963^T; 3, *A. rosea* CCUG 55942^T. Data were acquired in this study. Values are percentages of total fatty acids. Fatty acids that made up <1% of the total in all strains are not shown; —, <1%. For unsaturated fatty acids, the position of the double bond is located by counting from the methyl (ω) end of the carbon chain; *cis* isomers are indicated by the suffix *c*.

Fatty acid	1	2	3
C _{10:0}	—	1.5	—
iso-C _{10:0}	—	1.1	—
C _{14:0}	1.5	1.9	1.9
anteiso-C _{15:0}	1.4	1.0	1.1
iso-C _{15:0}	11.1	7.8	11.1
iso-C _{15:0} 3-OH	8.1	5.4	4.7
C _{15:1} ω 6 <i>c</i>	1.4	—	—
C _{16:0}	5.0	8.0	6.9
C _{16:0} 3-OH	4.6	5.0	3.8
C _{16:1} ω 5 <i>c</i>	13.0	22.2	21.0
iso-C _{17:0} 3-OH	3.0	2.0	3.4
iso-C _{17:1} ω 9 <i>c</i>	1.3	—	—
C _{18:0}	1.0	—	1.1
Summed feature 3*	37.7	37.6	36.0

*Summed feature 3 are groups of two or three fatty acids that cannot be separated by GLC using the MIDI system. Summed feature 3 contains C_{16:1} ω 7*c* and/or C_{16:1} ω 6*c*.

casein and starch. Negative for lipase activity and hydrolysis of Tweens 20, 40, 60 and 80. In API 20NE tests, positive for nitrate reduction, aesculin hydrolysis, gelatin hydrolysis and β -galactosidase activity and negative for indole production, D-glucose fermentation and arginine dihydrolase and urease activities. In API ZYM tests, alkaline phosphatase, C8 esterase lipase, leucine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase, α -glucosidase and N-acetyl- β -glucosaminidase activities are present and C4 esterase, C14 lipase, valine arylamidase, cystine arylamidase, α -chymotrypsin, α -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase and α -fucosidase activities are absent. The following compounds in the Biolog GN2 microplate are utilized as sole carbon sources: α -cyclodextrin, dextrin, glycogen, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, α -D-glucose, lactose, lactulose, maltose, D-mannose, melibiose, methyl β -D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, pyruvic acid methyl ester, succinic acid monomethyl ester, acetic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α -ketobutyric acid, α -ketoglutaric acid, α -ketovaleric acid, DL-lactic acid, propionic acid, quinic acid, succinic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-aspartic acid,

L-glutamic acid, glycyl L-aspartic acid, glycyl L-glutamic acid, hydroxy-L-proline, L-serine, inosine, uridine, 2,3-butanediol, α -D-glucose 1-phosphate and D-glucose 6-phosphate. Other substrates in the GN2 microplate are not utilized. Sensitive to tetracycline, sulfamethoxazole, rifampicin, novobiocin, streptomycin, chloramphenicol, nalidixic acid, penicillin G and ampicillin and resistant to gentamicin and kanamycin. The predominant cellular fatty acids (>10%) are summed feature 3 (C_{16:1} ω 7*c*/C_{16:1} ω 6*c*), C_{16:1} ω 5*c* and iso-C_{15:0}. The major respiratory quinone is MK-7. The polar lipid profile consists of a mixture of phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol and several uncharacterized polar lipids; the predominant polar lipids are phosphatidylethanolamine and an unknown aminophospholipid. The DNA G + C content of the type strain is 39.9 mol%.

The type strain is TNR-18^T (=LMG 25207^T =BCRC 17969^T), isolated from stream water in Kaohsiung Prefecture, southern Taiwan.

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