Ancylobacter rudongensis sp. nov., isolated from roots of *Spartina anglica*

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A curved, ring-like bacterium, strain AS 1.1761^T, isolated from the roots of *Spartina anglica*, was studied by a polyphasic approach. According to phylogenetic analysis, strain AS 1.1761^T belongs to the genus *Ancylobacter*, with 99.21 % 16S rDNA sequence similarity to *Ancylobacter aquaticus*, the only species described so far in this genus. However, strain AS 1.1761^T had no significant DNA–DNA binding with the type strain of *A. aquaticus*. In addition, strain AS 1.1761^T differed from *A. aquaticus* in many phenotypic features. Based on molecular and phenotypic data, a novel species, *Ancylobacter rudongensis* sp. nov., is proposed. The type strain is AS 1.1761^T (=JCM 11671^T).

The genus *Microcycylus* was created by Ørskov (1928) for bacteria with a ring-like shape; such bacteria had been isolated from wood-like waters, freshwater ponds and occasionally from soil. Because of their mostly aquatic habitat, Ørskov named them *Microcycylus aquaticus* (Raj, 1981). Other bacteria with a similar morphology had been described; Gromov (1963) proposed '*Microcycylus major*', which was isolated from *Scenedesmus* sp., an algae collected from lake water in Leningrad. Raj (1970) described '*Microcycylus flavus*', which was isolated from a deep, freshwater well in Long Beach, California, in 1964. Consequently, in the eighth edition of *Bergey's Manual of Determinative Bacteriology*, this genus included three species: *M. aquaticus*, '*M. flavus*' and '*M. major*' (Staley, 1974). However, on the basis of phenotypic characteristics and genomic DNA G+C content, '*M. flavus*' became a heterotypic synonym of *Spirosoma linguale* and '*M. major*' was transferred to a novel genus, *Flectobacillus*, as *Flectobacillus major* (Claus *et al.*, 1968; Larkin *et al.*, 1977). A novel species that was isolated from a marine environment, '*Microcycylus marinus*', was described by Raj (1976); because of its similarity to *F. major*, Borral & Larkin (1978) reclassified it into the genus *Flectobacillus* as '*Flectobacillus marinus*'. After further studies, Raj & Maloy (1990) proposed that '*F. marinus*' should be transferred to the novel genus *Cyclobacterium*. At the time of writing, the genus *Microcycylus* comprises only one species, *M. aquaticus* (Staley & Konopka, 1984).

The genus *Microcycylus* had been recognized formally from 1928 to 1983. However, the same name had been used before

Ørskov for some fungi, one of which had been known as a plant pathogen for a long time (Saccardo, 1905). To avoid confusion and to conform to the rules of the Bacteriological Code (Lapage *et al.*, 1975), Raj (1983) suggested that the name *Microcycylus* should be replaced by *Ancylobacter*.

In this study, we describe a curved, ring-like bacterium, strain AS 1.1761^T, which was isolated from the roots of *Spartina anglica* on the beach of Rudong County, Jiangsu Province, P.R. China. Phylogenetic analysis indicated that it was a member of the genus *Ancylobacter*. DNA–DNA hybridization with DNA from *Ancylobacter aquaticus* DSM 101^T revealed that it represented a novel genospecies. Phenotypic analysis showed that this genospecies could be differentiated clearly from *A. aquaticus* and, consequently, a novel species, *Ancylobacter rudongensis* sp. nov., is proposed.

S. anglica is a grass that grows on beaches, originally in Europe. It can protect beaches and dykes from damage by waves, and its stalks and leaves can be used as fodder, green manure and fuel. It is now planted widely on beaches in China. Some tender roots (3–5 cm) of *S. anglica* were sterilized with 0.1 % HgCl₂ and washed with sterile water. After grinding in a mortar, the juice was spread-plated onto Döbereiner nitrogen-free agar medium (Döbereiner *et al.*, 1976) that contained 0.3 % NaCl. A ring-like bacterium was isolated and deposited in the China General Microbiological Culture Collection Center under the accession number AS 1.1761^T. *A. aquaticus* DSM 101^T was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). To study its morphological characteristics, strain AS 1.1761^T was incubated at 30 °C in *Ancylobacter*–*Spirosoma* medium (DSMZ, 1998). Staining procedures were performed as described by Doetsch (1981). Inoculum was incubated in Döbereiner nitrogen-free

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medium that contained 0.2 % agar to test for nitrogenase ability by the acetylene (C_2H_2) reduction method, as described by Hanson & Phillips (1981). To test utilization of carbon sources, a basal medium [containing 0.2 % $(NH_4)_2SO_4$, 0.05 % $NaH_2PO_4 \cdot H_2O$, 0.05 % K_2HPO_4 , 0.02 % $MgSO_4 \cdot 7H_2O$ and 0.01 % $CaCl_2 \cdot 2H_2O$] was supplemented with vitamins, as described by Green & Bousfield (1982). Production of acid or gas from carbohydrates was determined by using the medium of Hugh & Leifson (1953). DNase was tested as described by Schreier (1969) and the KCN test was performed as described by Farmer *et al.* (1980). The following characteristics were tested as described by Smibert & Krieg (1981): indole production, methyl red and Voges–Proskauer tests, nitrate reduction and denitrification, H_2S production, milk reactions, starch hydrolysis, gelatin liquefaction, aesculin hydrolysis, urease, lipase (Tween 80), arginine dihydrolase, phenylalanine deaminase, ornithine decarboxylase, arginine decarboxylase and lysine decarboxylase.

Chromosomal DNA was extracted by using a modified version of the method of Marmur (1961). The 16S rRNA gene was amplified by PCR using the following primers: 5'-CGggatccAGAGTTTGATCCTGGCTCAGAACGAACGCT-3' and 5'-CGggatccTACGGCTACCTTGTACGACTTCACCCC-3' (positions 8–37 and 1479–1506, respectively, according to *Escherichia coli* 16S rDNA sequence numbering). Small letters indicate *Bam*HI recognition sequences. Amplified products were purified and ligated into plasmid pUC18. Recombinants were transformed into *E. coli* DH 5 α (Sambrook *et al.*, 1989). DNA from clones was purified by using the Wizard Plus Minipreps DNA Purification system (Promega). Automated sequencing was carried out by using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) on an ABI PRISM 377XL DNA sequencer (Applied Biosystems). The 16S rDNA sequence of strain AS 1.1761^T was aligned manually by using the CLUSTAL X program (version 1.64b; Thompson *et al.*, 1997) against corresponding sequences that were retrieved from GenBank. Evolutionary distance matrices were calculated by employing programs within the PHYLIP package (Felsenstein, 1993). An unrooted phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) by using the software package TREECON for Windows, version 1.3b (Van de Peer & De Wachter, 1994).

DNA G + C content was determined by thermal denaturation (Marmur & Doty, 1962). *E. coli* K-12 was used as the reference. DNA–DNA hybridization between strains AS 1.1761^T and *A. aquaticus* DSM 101^T was determined on the basis of the initial DNA–DNA liquid reassociation rate method, as described by De Ley *et al.* (1970). Renaturation was achieved at 75 °C. The procedure was performed on a model λ Bio 20 UV/VIS spectrometer equipped with a temperature program controller (Perkin Elmer).

The almost-complete 16S rDNA sequence of strain AS 1.1761^T (1457 bp) was compared with those of related

Fig. 1. Phylogenetic tree of strain AS 1.1761^T and related taxa, based on 16S rDNA sequence comparison by using the neighbour-joining method (Saitou & Nei, 1987). GenBank/EMBL accession numbers of reference sequences are given after strain names. Numbers at branch-points represent confidence levels from 1000 replicate bootstrap resamplings. Bar, 2 nucleotide substitutions per 100 positions.

strains (Fig. 1). The result showed that strain AS 1.1761^T was closest to *A. aquaticus* DSM 101^T, with 99.21 % 16S rDNA sequence similarity. This indicated that strain AS 1.1761^T should be assigned to the genus *Ancylobacter*. The DNA G + C content of strain AS 1.1761^T was 68.2 mol%, which is within the range of the genus *Ancylobacter* (66.3–68.8 mol%) (Raj, 1981). No (0 %) DNA–DNA hybridization was found between isolate AS 1.1761^T and *A. aquaticus* DSM 101^T and, consequently, strain AS 1.1761^T can be considered as a distinct genospecies of the genus *Ancylobacter*.

Phenotypically, strain AS 1.1761^T can be differentiated from *A. aquaticus* by several characteristics (Table 1). Based on these genomic and phenotypic data, a novel species of the genus *Ancylobacter* is proposed, with the name *Ancylobacter rudongensis* sp. nov.

Description of *Ancylobacter rudongensis* sp. nov.

Ancylobacter rudongensis (ru.dong.en'sis. N.L. masc. adj. *rudongensis* referring to Rudong County, Jiangsu Province, P.R. China, where the strain was isolated).

Gram-negative curved rods, 0.6–0.8 μ m in diameter, non-motile, without capsule and gas vesicles. Intracellular granules of poly- β -hydroxybutyrate are formed. Colonies are white, convex, round, opaque, entire and 0.5–1.0 mm in diameter after 7 days incubation on *Ancylobacter*–*Spirosoma* agar. Optimum temperature for growth is 28–30 °C; no growth occurs at 4 or 45 °C. Optimum pH for growth is 6.8–7.0; no growth occurs at pH 5.5 or 10.0. Growth occurs in the presence of 0–5.0 % (w/v) NaCl. Microaerophilic in semi-solid, nitrogen-free medium to fix free nitrogen. Delayed growth occurs in the presence of KCN. Fluorescent pigment is not produced. Urease-, arginine decarboxylase- and lysine decarboxylase-positive. Arginine dihydrolase, phenylalanine deaminase, ornithine

Table 1. Differential phenotypic characteristics of strain AS 1.1761^T and *A. aquaticus*

Data for *A. aquaticus* DSM 101^T were taken from Raj (1976), Larkin *et al.* (1977), Raj (1981) and Holt *et al.* (1994). Both taxa can utilize formate and methanol. ND, Not determined.

Characteristic	<i>A. rudongensis</i> AS 1.1761 ^T	<i>A. aquaticus</i> DSM 101 ^T
Cell shape	Curved rods	Curved rods
Encapsulated	—	+
Poly- β -hydroxybutyrate present	+	ND
Growth at 37 °C	+	—
Growth in presence of 3 % NaCl	+	—
Nitrogenase	+	ND
Lysine decarboxylase	+	—
Ornithine decarboxylase	—	+
Aesculin hydrolysis	+	—
Acid production from:		
Melibiose	+	—
Salicin	+	—
Cellobiose	+	—
Lactose	+	—
Maltose	+	—
Trehalose	+	—
Utilization of:		
Fumarate	+	—
2-Oxoglutarate	+	—
Malate	+	—
Propionate	+	—
Adonitol	+	—
Maltose	+	—
Habitat	Roots of <i>Spartina anglica</i>	Soil, freshwater

decarboxylase, DNase and lipase (Tween 80) are negative. Alkali is produced from litmus milk. H₂S and indole are not produced. Methyl red and Voges–Proskauer tests are negative. Nitrates are reduced. Growth does not occur anaerobically with nitrate. Aesculin and gelatin are hydrolysed, but starch is not. The following carbohydrates are used as sole carbon sources: starch, maltose, methyl α -D-glucoside, adonitol, glycerol, glucose, ribose, inulin, L-arabinose, fructose, galactose, mannitol, xylose, aesculin, sorbitol, sodium gluconate, α -ketoglutaric acid, alanine, proline, sodium acetate, fumaric acid, sodium malonate, sodium citrate, sodium succinate, sodium lactate, sodium malate, sodium propionate, methanol, sodium formate, sodium pyruvate, amaroid, sucrose, dextrin and cellobiose; trehalose, sorbose, raffinose, melibiose, mannose, lactose, dulcitol, inositol, *meso*-erythritol, rhamnose, melizitose, arginine, sodium tartrate, sodium hippurate and salicin are not used. Acid is formed from mannose, ribose, glycerol, mannitol, sorbitol, galactose, cellobiose, rhamnose, salicin, glucose, maltose, L-arabinose, trehalose, xylose, melibiose, adonitol, fructose and lactose, but not from inositol, raffinose, inulin, melizitose, starch, glycogen, methyl α -D-glucoside, sucrose, sorbose, sodium gluconate, dulcitol, *meso*-erythritol or dextrin. No gas is formed from these carbohydrates. DNA G+C content is 68.2 mol%.

The type strain is AS 1.1761^T (=JCM 11671^T), which was isolated from the roots of *Spartina anglica* in Rudong County, Jiangsu Province, P.R. China.

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