Ancylobacter polymorphus sp. nov. and *Ancylobacter vacuolatus* sp. nov.

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The taxonomic positions of '*Renobacter vacuolatum*' DSM 1277 and '*Ancylobacter polymorphus*' DSM 2457 were investigated in this study. 16S rRNA gene sequence analysis indicated that both strains belonged to the genus *Ancylobacter*. DNA–DNA hybridization showed that they differed from *Ancylobacter aquaticus* DSM 101^T and *Ancylobacter rudongensis* AS 1.1761^T. According to molecular and phenotypic characteristics, strain DSM 1277^T (=AS 1.2807^T) is proposed as the type strain of *Ancylobacter vacuolatus* sp. nov. At the same time, valid publication of the name *Ancylobacter polymorphus* sp. nov. is proposed, with the type strain DSM 2457^T (=AS 1.2800^T=NCIMB 10516^T).

According to its curved, small rod shape, Ørskov named the genus *Microcycylus* in 1928 (Ørskov, 1928). However, because of the prior existence of a fungal genus of the same name [*Microcycylus* Saccardo, Sydow & P. Sydow 1904; Sydow & Sydow (1904)], Raj (1983) substituted the name *Ancylobacter*. Members of *Ancylobacter* are widespread in nature, particularly in aquatic environments, such as lowland marshes, water reservoirs and wastewater treatment ponds (Raj, 1989). Members of the genus seem to play an important ecological role in oligotrophic methylo-trophy (Raj, 1989). Before *Ancylobacter rudongensis* was described (Xin *et al.*, 2004), there was only one species in the genus, *Ancylobacter aquaticus*. In addition, various investigators have isolated several closely related bacteria from different countries, for instance '*Renobacter vacuolatum*' (Nikitin, 1971) and '*Ancylobacter polymorphus*' (Raj, 1989). However, these species names were neither included in the Approved Lists nor later validly published.

In this study, we studied the physiological, genetic and phylogenetic characteristics of '*R. vacuolatum*' DSM 1277 and '*A. polymorphus*' DSM 2457. Phylogenetic analysis showed that strains DSM 1277 and DSM 2457 belonged to the genus *Ancylobacter*. DNA–DNA hybridization indicated that each of these strains represents a distinct novel species.

A. aquaticus DSM 101^T and *Ancylobacter* spp. DSM 1227 and DSM 2457 were obtained from DSMZ. *A. rudongensis* AS 1.1761^T was obtained from China General Microbiological Culture Collection Center. These strains were

incubated at 30 °C in *Ancylobacter*–*Spirosoma* medium (DSMZ medium 7; DSMZ, 1998). In order to test the requirement for O₂, the strains were incubated in *Ancylobacter*–*Spirosoma* medium with 0.2 % agar. Growth with carbon sources was tested in the basal medium supplemented with vitamins at 0.1 % final concentrations of carbon sources. The production of acid and gas from carbohydrates was determined using the method of Hugh & Leifson (1953). Nitrogenase activity was tested as described by Hanson & Phillips (1981). DNase was tested as described by Schreier (1969). Indole production, methyl red and Voges–Proskauer tests, nitrate reduction and denitrification, H₂S production, milk reaction, starch hydrolysis, gelatin liquefaction, aesculin hydrolysis, urease, lipase (Tween 80), arginine dihydrolase and phenylalanine deaminase were tested according to the methods of Smibert & Krieg (1981).

Genomic DNAs were extracted according to the method of Marmur (1961). The G + C content of the DNA was tested by the thermal denaturation method (Marmur & Doty, 1962). Levels of DNA–DNA hybridization were determined from the initial DNA–DNA liquid reassociation rate as described by De Ley *et al.* (1970). Renaturation rates were determined in 2 × SSC on a model Lambda Bio 20 UV/Vis spectrophotometer equipped with a temperature program controller (Perkin-Elmer). The optimal renaturation temperature was 79 °C.

PCR amplification of the 16S rRNA gene used primers 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-TACGGCT-ACCTTGTTACGACTT-3'. PCR products were sequenced directly using an ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Perkin Elmer) on an ABI PRISM 377XL DNA Sequencer (Applied Biosystems). The

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains DSM 1277^T and DSM 2457^T are AY211515 and AY211516.

sequences of strains DSM 2457 and DSM 1277 and corresponding sequences retrieved from the GenBank database were aligned using the CLUSTAL X program (version 1.64b; Thompson *et al.*, 1997). Evolutionary distances were calculated using the program within the PHYLIP package (Felsenstein, 1993). The phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987) using the software package TREECON for Windows version 1.3b (Van de Peer & De Wachter, 1994).

Cells of strains DSM 1277 and DSM 2457 were Gram-negative, curved rods, 0.8–1.0 µm in diameter, non-motile, with a capsule and gas vesicles. Colonies were white, convex, round, entire and 0.5–1.0 mm in diameter after 7 days incubation on nutrient agar plates. When they grew in liquid medium, they produced pellicles. They produced poly-β-hydroxybutyrate granules. They could fix free nitrogen while growing on Döbereiner nitrogen-free medium (Döbereiner *et al.*, 1976). Strain DSM 1277 did not grow at 4 or 37 °C, and strain DSM 2457 did not grow at 4 or 50 °C. Optimum growth occurred at 28–30 °C and pH 7.0. Strain DSM 1277 showed no growth at pH 5.5 or 11.0, and strain DSM 2457 showed no growth at pH 4.5 but grew at pH 11.0. Strain DSM 1277 grew on medium containing 0–2.5 % (w/v) NaCl, whereas strain DSM 2457 grew on medium containing 0–3 % (w/v) NaCl. The two strains were positive for oxidase, catalase and urease activities and negative for DNase, phenylalanine deaminase, lipase (Tween 80) and arginine dihydrolase. They could not produce H₂S or indole. They could reduce nitrates but could not grow anaerobically with nitrate. Methyl red and Voges–Proskauer tests were negative. The strains hydrolysed aesculin and gelatin but not starch. Details of utilization of carbon sources and production of acid and gas from carbohydrates are given in the species descriptions. Differentiating phenotypic characteristics between these two strains and *A. aquaticus* DSM 101^T and *A. rudongensis* AS 1.1761^T are listed in Table 1.

Analysis of almost-complete 16S rRNA gene sequences (Fig. 1) showed that strains DSM 1277 and DSM 2457 belonged to the genus *Ancylobacter*. The sequence similarities of strain DSM 1277 with *A. aquaticus* DSM 101^T and *A. rudongensis* AS 1.1761^T were respectively 99.6 and 99.1 %, and the sequence similarities of strain DSM 2457 with *A. aquaticus* DSM 101^T and *A. rudongensis* AS 1.1761^T were respectively 99.2 and 98.98 %. The DNA–DNA relatedness values of strain DSM 1277 with strains DSM 101^T, AS 1.1761^T and DSM 2457 were respectively 51.4, 56.8 and 40.9 %, while the DNA–DNA relatedness values of strain DSM 2457 with strains DSM 101^T and AS 1.1761^T were respectively 43.7 and 38.8 %. These values were the means of three repeated experiments. The pooled standard deviations of all hybridization experiments for strains DSM 1277 and DSM 2457 were respectively 5.3 and 3.9 %. The DNA G + C content of both strains was 65.5 mol%.

The results of DNA–DNA hybridization suggested that these two strains represent novel species of *Ancylobacter*. According to the results of our study, we propose to classify

Table 1. Characteristics that differentiate strains DSM 1277 and DSM 2457 from *A. aquaticus* DSM 101^T and *A. rudongensis* AS 1.1761^T

Data for *A. aquaticus* DSM 101^T were taken from Raj (1976, 1981), Larkin *et al.* (1977) and Holt *et al.* (1994). Data for *A. rudongensis* AS 1.1761^T were taken from Xin *et al.* (2004).

Characteristic	DSM 1277	DSM 2457	DSM 101 ^T	AS 1.1761 ^T
Growth at/in:				
37 °C	–	+	–	+
pH 5.5	–	+	–	–
pH 10	+	+	–	–
3 % NaCl	–	+	–	+
Aesculin hydrolysis	+	+	–	+
Acid production from:				
Inulin	+	+	–	–
Cellobiose	+	+	–	+
Salicin	+	+	–	+
Maltose	–	+	–	+
Trehalose	–	–	–	+
Melibiose	+	+	–	+
Lactose	+	+	–	+
Utilization of:				
Maltose	+	+	–	+
Adonitol	+	+	–	+
α-Ketoglutarate	+	+	–	+
Fumarate	+	+	–	+
meso-Erythritol	–	+	–	–
Melezitose	+	+	–	–
Glucosate	+	–	ND	+
Arginine	+	+	ND	–
Propionate	+	+	–	+
Pyruvate	–	+	+	+
Salicin	+	+	–	–
Isolation source	Soil	River mud	Soil	Root of <i>Spartina anglica</i>

strain DSM 1277 as the type strain of *Ancylobacter vacuolatus* sp. nov. and strain DSM 2457 as the type strain of *Ancylobacter polymorphus* sp. nov.

Description of *Ancylobacter vacuolatus* sp. nov.

Ancylobacter vacuolatus (va.cu.o.la'tus. N.L. masc. adj. *vacuolatus* vacuolate).

The characters of type strain were originally reported by Nikitin (1971). Gram-negative, curved rods, 0.8–1.0 µm in diameter, non-motile, with a capsule and gas vesicles. Colonies are white, convex, round, entire and 0.5–1.0 mm in diameter after 7 days incubation on nutrient broth agar plates. Can produce pellicles when growing in liquid medium. Produces poly-β-hydroxybutyrate granules. Fixes free nitrogen while growing on Döbereiner nitrogen-free medium. No growth at 4 or 37 °C or at pH 5.5 or 11.0. Optimum

Fig. 1. Phylogenetic tree of strains DSM 1277 and DSM 2457 and related taxa based on 16S rRNA gene sequence comparisons using the neighbour-joining method (Saitou & Nei, 1987). Accession numbers of reference sequence are given after the strain names. Numbers represent confidence levels from 1000 replicate bootstrap samplings. Bar, 5 substitutions per 100 nucleotide positions.

growth at 28–30 °C and at pH 7.0. Grows in 0–2.5 % (w/v) NaCl. Oxidase-, catalase- and urease-positive. Negative for DNase, phenylalanine deaminase, lipase (Tween 80) and arginine dihydrolase. Does not produce H₂S or indole. Methyl red and Voges–Proskauer tests are negative. Reduces nitrates. Does not grow anaerobically with nitrate. Hydrolyses aesculin and gelatin but not starch. Utilizes the following substrates as carbon and energy sources: maltose, L-arabinose, fructose, glycerol, mannitol, sorbitol, starch, adonitol, ribose, mannose, aesculin, amygdalin, sodium gluconate, ketoglutaric acid, alanine, proline, fumaric acid, arginine, sodium acetate, sodium malonate, sodium citrate, sodium succinate, sodium propionate, sodium lactate, sodium malate and methanol. Utilizes the following substrates weakly as carbon and energy sources: cellobiose, galactose, glucose, xylose, methyl α -D-glucoside, melezitose, sodium formate and salicin. Does not utilize the following substrates as carbon and energy sources: rhamnose, dextrin, lactose, inulin, raffinose, sucrose, trehalose, sorbose, melibiose, dulcitol, inositol, *meso*-erythritol, sodium tartrate, sodium hippurate and sodium pyruvate. Forms acid but no gas from mannose, ribose, glycerol, mannitol, inulin, sorbitol, galactose, cellobiose, rhamnose, salicin, glucose, arabinose, xylose, melibiose, adonitol, fructose and lactose. Does not form acid or gas from inositol, raffinose, melezitose, starch, glycogen, methyl α -D-glucoside, sucrose, maltose, sorbose, trehalose, dulcitol, *meso*-erythritol or dextrin. The DNA G + C content of the type strain is 65.5 mol%.

The type strain is strain DSM 1277^T (= AS 1.2807^T), isolated from soil in Russia, previously described as '*Renobacter vacuolatus*' (Nikitin, 1971).

Description of *Ancylobacter polymorphus* sp. nov.

Ancylobacter polymorphus (po.ly.mor'phus. N.L. masc. adj. *polymorphus* polymorphic).

Gram-negative, curved rods, 0.8–1.0 μ m in diameter, non-motile, with a capsule and gas vesicles. Colonies are white, convex, round, entire and 0.5–1.0 mm in diameter after 7 days incubation on nutrient broth agar plates. Can produce pellicles when growing in liquid medium. Produces poly- β -hydroxybutyrate granules. Fixes free nitrogen while

growing on Döbereiner nitrogen-free medium. No growth at 4 or 50 °C or at pH 4.5. Optimum growth at 28–30 °C and at pH 7.0. Grows at pH 11.0. Grows in 0–3 % (w/v) NaCl. Oxidase-, catalase- and urease-positive. Negative for DNase, phenylalanine deaminase, lipase (Tween 80) and arginine dihydrolase. Does not produce H₂S or indole. Methyl red and Voges–Proskauer tests are negative. Reduces nitrates. Does not grow anaerobically with nitrate. Hydrolyses aesculin and gelatin but not starch. Utilizes the following substrates as carbon and energy sources: maltose, methyl α -D-glucoside, adonitol, glycerol, glucose, ribose, inulin, L-arabinose, fructose, galactose, mannitol, dextrin, *meso*-erythritol, xylose, sorbitol, amygdalin, ketoglutaric acid, alanine, proline, fumaric acid, arginine, sodium acetate, sodium malonate, sodium citrate, sodium succinate, sodium propionate, sodium lactate, sodium malate and methanol. Utilizes the following substrates weakly as carbon and energy sources: starch, sucrose, aesculin, melezitose, sodium pyruvate, sodium formate and salicin. Does not utilize the following substrates as carbon and energy sources: trehalose, sorbose, cellobiose, raffinose, melibiose, mannose, lactose, dulcitol, inositol, rhamnose, sodium gluconate, sodium tartrate and sodium hippurate. Forms acid but no gas from mannose, ribose, glycerol, mannitol, inulin, sorbitol, galactose, cellobiose, rhamnose, salicin, glucose, maltose, L-arabinose, xylose, melibiose, adonitol, fructose and lactose; forms neither acid nor gas from inositol, raffinose, melezitose, starch, glycogen, methyl α -D-glucoside, sucrose, sorbose, trehalose, dulcitol, *meso*-erythritol and dextrin. The DNA G + C content of the type strain is 65.5 mol%.

The type strain is strain DSM 2457^T (= AS 1.2800^T = NCIMB 10516^T), isolated from river mud (MacLennan *et al.*, 1974).

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