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Description of *Ancylobacter oerskovii* sp. nov. and two additional strains of *Ancylobacter polymorphus*

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A Gram-negative, pleomorphic, rod-shaped, non-spore-forming bacterium, designated strain NS05^T, was isolated from soil after enrichment with oxalate. On the basis of 16S rRNA gene sequence similarity, strain NS05^T was shown to be phylogenetically related to the genera *Ancylobacter*, *Starkeya* and *Angulomicrobium* (96.3–98.1 % sequence similarity), class *Alphaproteobacteria*. Strain NS05^T was most closely related to *Ancylobacter rudongensis* AS 1.1761^T (98.1 % sequence similarity). The whole-cell fatty acid pattern of strain NS05^T was typical of those found in members of the genus *Ancylobacter*. Its main components were $C_{18:1}\omega7c$ (60.4 %), $C_{19:0}\omega8c$ cyclo (28.3 %) and $C_{16:0}$ (7.4 %) and hydroxylated compounds were absent. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain NS05^T with respect to the four *Ancylobacter* species with validly published names. Therefore, it is concluded that NS05^T represents a novel species of the genus *Ancylobacter*, for which the name *Ancylobacter oerskovii* sp. nov. is proposed. The type strain is NS05^T (=DSM 18746^T =CCM 7435^T). Two other oxalate-utilizing strains, NS03 and NS04, isolated from paper-mill effluents, were shown by 16S rRNA gene sequence analysis to be affiliated to the species *Ancylobacter polymorphus*. The study of their physiological properties extends the knowledge of the physiological variability within this species.

Several strains of aerobic bacteria have been reported to be able to utilize oxalate as a sole carbon and energy source. Most of them are facultative methylotrophs and/or facultative hydrogen-oxidizing chemolithoautotrophs; some belong to the genera *Methylobacterium*, *Hyphomicrobium*, *Cupriavidus*, *Xanthobacter* and *Ancylobacter* (Sahin, 2003), but the exact taxonomic positions of many strains are unclear. In this study, we investigated the physiological, genetic and phylogenetic characteristics of three *Ancylobacter* strains, which were isolated by enrichment in mineral medium with potassium oxalate as described by Sahin *et al.* (2002). Members of the genus *Ancylobacter* play an important ecological role in oligotrophic methylotrophy and H_2-lithotrophy (Raj, 1989; Aragno & Schlegel, 1992). The genus *Ancylobacter* is assigned to the family *Hyphomicrobiaceae* according to the chapter by Garrity *et al.* (2005) in *Bergey's Manual*; however, Lee *et al.* (2005) found that the genera included in the *Hyphomicrobiaceae* in *Bergey's Manual* form two independent branches and thus established the families *Xanthobacteraceae* and *Hyphomicrobiaceae*, with *Ancylobacter* falling within the *Xanthobacteraceae*. The genus presently comprises four species with validly published names: *Ancylobacter aquaticus* (Ørskov, 1928; Raj, 1983), *Ancylobacter rudongensis* (Xin *et al.*, 2004), *Ancylobacter polymorphus* and *Ancylobacter vacuolatus* (Xin *et al.*, 2006). The present article deals with the taxonomic description of a novel species to accommodate soil isolate NS05^T and the description of two paper-mill isolates, NS03 and NS04, as additional strains of *Ancylobacter polymorphus*.

Among others, three strains of Gram-negative, non-motile, non-sporulating, strictly aerobic bacteria, designated NS03, NS04 and NS05^T, were isolated from enrichment cultures containing potassium oxalate (4 g l^{-1}) as the sole source of carbon and energy (Sahin *et al.*, 2002). The strains consisted of curved rods (NS03 and NS04) or pleomorphic rods (NS05^T; Fig. 1). Colonies of the strains were opaque, cream-coloured and convex.

Strains NS03, NS04 and NS05^T were deposited at the DSMZ with the accession numbers DSM 18745, DSM 19551 and DSM 18746^T, respectively. The following strains were used as references: *Ancylobacter rudongensis* DSM

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NS05^T (=DSM 18746^T) is AM778407.

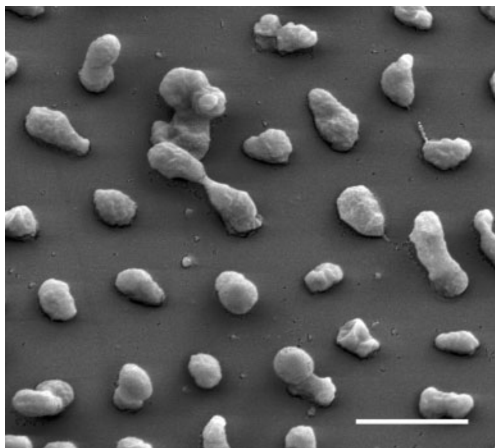

Fig. 1. Field-emission scanning electron micrograph of cells of strain NS05^T, grown on an R2A agar plate, showing the variable cellular morphology. Bacteria were fixed with 2 % glutaraldehyde and 5 % formaldehyde in the culture medium for 1 h on ice. This was followed by washing with TE buffer (20 mM Tris, 1 mM EDTA; pH 6.9) and dehydration in a graded acetone series (10, 30, 50, 70, 90, 100 %) on ice (15 min for each step), critical-point drying with liquid CO_2 (CPD 30; Balzers) and sputter-coating with a gold film (SCD 40; Balzers). The Zeiss microscope (DSM 982 Gemini) was used with an Everhart–Thornley secondary electron detector and the in-lens detector in a 50 : 50 ratio at an acceleration voltage of 5 kV. Bar, 2 μm.

17131^T, *Ancylobacter polymorphus* DSM 2457^T, *Ancylobacter vacuolatus* DSM 1277^T and *Ancylobacter aquaticus* DSM 101^T. The strains were grown routinely on nutrient agar (Difco) (containing 5 g peptone, 3 g beef extract and 15 g agar agar l^{-1}) at 28 °C.

16S rRNA gene sequences were generated and aligned as described by Somvanshi *et al.* (2006). Phylogenetic dendrograms were constructed using the algorithms of De Soete (1983) and Saitou & Nei (1987). Analysis of the 16S rRNA gene sequences resulted in the grouping of all three isolates within the family *Xanthobacteraceae*. The sequences of the 540 bp 5′ termini of the sequences of strains NS03 and NS04 were identical and the almost-complete sequence of strain NS04 was identical to the sequence of the type strain of *Ancylobacter polymorphus*, DSM 2457^T. This is in accord with the morphological appearance (curved rods) and the classification of these strains in the genus *Ancylobacter* on the basis of the physiological features determined in a previous study (Sahin *et al.*, 2002).

Strain NS05^T shared the highest 16S rRNA gene sequence similarity with *Ancylobacter rudongensis* AS 1.1761^T (98.1 %). Phylogenetic analysis using different distance-matrix algorithms consistently showed these two strains to be neighbours, while the positions of members of the genera *Ancylobacter*, *Starkeya* and *Angulomicrobium* depended somewhat on the selection of reference sequences included in the analysis. In several cases, the genus *Ancylobacter* did not emerge as a coherent genus, but members of the genera *Angulomicrobium* and *Starkeya* together with *Ancylobacter rudongensis* and strain NS05^T formed a separate clade. Although the relationships between the three genera have not been fully explored, the overall chemotaxonomic similarity of *Ancylobacter* strains supports the observation that the genus is phylogenetically coherent (Fig. 2; based on the algorithm of De Soete, 1983). This interpretation corresponds with the results of a 16S rRNA gene sequence analysis by Fritz *et al.* (2004), which confirmed the genus status of *Angulomicrobium*, clustering separately from *Ancylobacter aquaticus*. No 16S rRNA gene sequence is available for a strain of the species '*Ancylobacter natronum*' (Doronina *et al.*, 2001), and the name of this species has not yet been validly published.

For DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Hybridization was carried out in SSC buffer at 68 °C as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). For determination of the G + C content, DNA was degraded to nucleosides by using P1 nuclease and bovine intestinal mucosa alkaline phosphatase, as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC (Shimadzu apparatus) according to the method described by Tamaoka & Komagata (1984), and the G + C content was calculated from the ratio of deoxyguanosine to thymidine.

The DNA–DNA relatedness of strain NS05^T and *Ancylobacter rudongensis* DSM 17131^T, sharing a 16S rRNA gene sequence similarity of 98.1 %, was 17 %, which

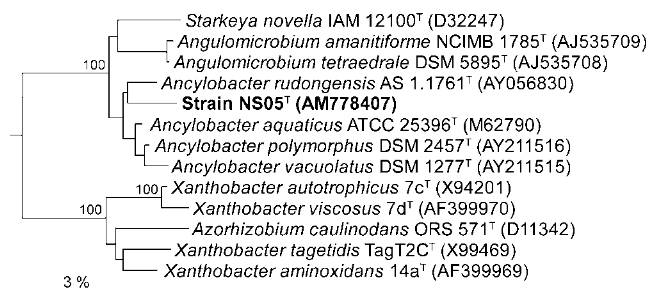

Fig. 2. Additive phylogenetic tree (De Soete, 1983), based on 16S rRNA gene sequences, showing the closest neighbours of strain NS05^T within the genus *Ancylobacter* and phylogenetically related genera. Bootstrap percentages (based on 500 datasets) are shown at branch points (if greater than 60 %). Bar, 3 % difference in nucleotide sequences, as determined by measuring the lengths of the horizontal lines connecting any two organisms.

confirms that strain NS05^{T} does not belong to the genospecies *Ancylobacter rudongensis*. The DNA G+C content of strain NS05^{T} was 68.0 mol%, which is within the range (65.5–68.2 mol%) previously obtained for *Ancylobacter* species (Urakami & Komagata, 1986; Xin *et al.*, 2004, 2006).

For the analysis of fatty acids, cells were grown on R2A agar (DSMZ medium 830; http://www.dsmz.de) for 48 h at 28 °C. This growth medium was used instead of the tryptic soy broth agar recommended for use with the MIDI system. The *Ancylobacter* reference strains did not grow well on the latter medium, and the composition of R2A agar is similar to the composition of the *Microcyclus–Spirosoma* agar used by Urakami & Komagata (1986) for fatty acid analysis. Whole-cell fatty acid methyl esters were obtained using methods described previously (Kämpfer & Kroppenstedt, 1996) and were separated using a gas chromatograph (model 5898A; Hewlett Packard). Peaks were automatically integrated and fatty acid names and percentages were determined using the Microbial Identification standard software package (MIDI) (Sasser, 1990). The fatty acid profile of strain NS05^{T} was dominated by $C_{18:1}\omega7c$ (60.4 %), $C_{19:0}\omega8c$ cyclo (28.3 %) and $C_{16:0}$ (7.4 %) (Table 1). The fatty acid compositions of members of the genus *Ancylobacter* were not included in earlier species descriptions. However, Urakami & Komagata (1986) included several strains of *Ancylobacter aquaticus* (analysed as *Microcyclus aquaticus*; the bacterial genus name *Microcyclus* is illegitimate), including ATCC 25396^{T}, and *Ancylobacter polymorphus* (analysed as '*Microcyclus polymorphum*') NCIB 10516^{T} in their studies. The patterns generated in the present study generally agree with those published by Urakami & Komagata (1986): while the major components are the same in both studies, they may differ in quantity. A major significant deviation is the absence of $C_{14:0}$ 3-OH in the present study (Table 1). This acid was described as being present in all '*Microcyclus*' strains (in amounts ranging from 1.5 to 2.8 %) by Urakami & Komagata (1986). It is possible that the growth medium selected and the age of the cells used could have influenced the fatty acid composition, as shown by Urakami & Komagata (1973). This suggestion is corroborated by the detection of minor amounts (0.2 %) of $C_{14:0}$ 3-OH in cells of NS05^{T} cultivated on tryptic soy broth agar (data not shown).

Table 1. Whole-cell fatty acid composition of strain NS05^{T} and *Ancylobacter* type strains

Strains: 1, strain NS05^{T}; 2, *Ancylobacter rudongensis* DSM 17131^{T}; 3, *Ancylobacter aquaticus* DSM 101^{T}; 4, *Ancylobacter vacuolatus* DSM 1277^{T}; 5, *Ancylobacter polymorphus* DSM 2457^{T}. Cells were grown on R2A agar at 28 °C for 2 days. Data shown are percentages of total fatty acids. –, Not detected; ECL, equivalent chain length.

Fatty acid	1	2	3	4	5
Unknown ECL 13.957	0.2	0.2	0.3	–	0.3
Summed feature 3*	0–0.5	1.3	1.4	0.7	0.4
$C_{16:0}$	7.4	4.8	6.4	4.9	6.8
$C_{17:0}$	0.4	0.5	1.0	1.1	0.9
$C_{18:1}\omega7c$	60.4	60.9	71.7	76.1	71.5
$C_{18:0}$	2.4	1.5	1.5	1.2	2.0
11-Methyl $C_{18:1}\omega7c$	–	2.2	–	1.0	0.4
Summed feature 7*	–	–	–	–	0.8
$C_{19:0}\omega8c$ cyclo	28.3	27.9	17.4	14.7	16.3
$C_{20:2}\omega6,9c$	–	0.35	–	–	0.2

*Summed feature 3 contains $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH; summed feature 7 contains one or more of $C_{19:0}\omega10c$ cyclo, $C_{19:1}\omega6c$ and unknown ECL 18.846.

Isoprenoid quinones were extracted from lyophilized cells according to the method of Collins *et al.* (1977) and the profile was analysed by reversed-phase HPLC (using the Shimadzu system based on solvent-delivery module LC-20AD) (Groth *et al.*, 1997). The main component was Q-10 and minor amounts of Q-9 were also present, the Q-10/Q-9 ratio being 9 : 1. This result was in accord with the grouping of strain NS05^{T} within the genus *Ancylobacter*, since *Microcyclus aquaticus* and '*Microcyclus polymorphus*' also contained Q-10 as the major component (Urakami & Komagata, 1986). However, these authors detected Q-11, in addition to Q-9, as a minor component.

Conventional biochemical tests were performed, according to standard methods (Smibert & Krieg, 1994), after incubation at 28 °C for up to 10 days. Chemolitho-autotrophy with H_2 was tested in mineral medium under a microaerobic atmosphere containing 60 % H_2 (Malik, 1988). API 20NE and API 50 CH strips (bioMérieux) were used according to the manufacturer's instructions. Utilization of carbohydrates (API 50 CH strips) was determined with modified AUX medium in which growth factors and amino acids had been replaced with yeast extract (0.1 g l^{-1}); results were read after 2 days.

All *Ancylobacter* strains tested were able to grow with oxalate as the sole source of carbon. Thus this capacity seems to be a common trait of the genus *Ancylobacter* rather than a unique feature of the strains isolated here using oxalate as the sole source of carbon. For NS05^{T}, clumpy growth was observed in liquid batch cultures with oxalate. Optimal growth occurred at pH 7 and 30 °C with potassium oxalate at 8 g l^{-1}; the maximum potassium oxalate concentration tolerated was 25 g l^{-1} if the pH was not corrected during growth. This limit was probably due to the pH increase in the medium that occurred during metabolism of the oxalic acid.

Physiologically, strains NS03 and NS04 showed the same properties as the type strain of *Ancylobacter polymorphus* (DSM 2457^{T}) except that they cleaved urea when incubated for 6 days and NS04 utilized glycerol (Table 2). This supports the view that strains NS03 and NS04 should be regarded as members of the species *Ancylobacter polymorphus*. The study of their physiological properties

Table 2. Morphological and physiological characteristics of strains NS03, NS04 and NS05^T and the type strains of the genus *Ancylobacter*

Strains: 1, NS05^T; 2, *Ancylobacter rudongensis* DSM 17131^T; 3, *Ancylobacter aquaticus* DSM 101^T; 4, *Ancylobacter vacuolatus* DSM 1277^T; 5, *Ancylobacter polymorphus* DSM 2457^T; 6, NS03; 7, NS04. All strains were positive for growth in the presence of 4 % NaCl and for utilization of oxalate, D-xylose and formate. In API 20NE strips, all strains were positive after 2 days for assimilation of glucose (except NS03) and mannitol and oxidase activity and all strains were negative for indole production, glucose acidification, arginine dihydrolase, gelatin hydrolysis and assimilation of mannose, *N*-acetylglucosamine, maltose, caprate, adipate, citrate and phenyl acetate. In API 50 CH strips, all strains were positive after 2 days for utilization of galactose, glucose, mannitol, xylitol, adonitol and D- and L-arabitol; all strains were negative for utilization of the other substrates included in the panel. ND, Not determined; W, weak reaction.

Characteristic	1	2	3	4	5	6	7
Rod morphology	Pleomorphic	Curved	Curved	Curved	Curved	Strongly curved	Strongly curved
Autotrophic growth with H_2	−	−	+	+	+	ND	ND
Maximum growth temperature (°C) on slant	40	40	34	37	42	40	40
Nitrate reduction	−	−	+	+	−	−	−
Urease	+	+	+	+	−	+	+
Utilization of (conventional tests):							
L-Fucose	+	−	−	+	−	−	−
D-Arabinose	+	+	−	+	−	−	−
D-Mannose	+	−	−	−	−	−	W
L-Rhamnose	+	−	−	−	−	−	−
D-Malate	+	W	+	−	+	+	+
Malonate	+	+	+	+	−	−	−
Gluconate	+	−	+	+	−	−	−
Citrate	−	+	+	−	−	−	−
API 20NE strip results							
Nitrate reduction	−	−	+	−	−	−	−
Urease	+	−	−	+	−	−	−
Aesculin hydrolysis	W	+	+	+	+	W	+
β-Galactosidase	−	+	+	+	+	+	+
Arabinose assimilation	+	+	−	+	+	+	+
Gluconate assimilation	+	−	−	+	−	−	−
Malate assimilation	+	−	−	−	+	+	+
Utilization of (API 50 CH):							
Glycerol	−	+	−	−	−	−	+
L-Arabinose	+	+	−	+	+	+	+
D-Xylose	W	+	W	−	+	W	+
Fructose	+	+	−	W	W	−	+
Rhamnose	+	−	−	−	−	−	−
Sorbitol	+	+	−	+	+	+	+
Aesculin	−	+	+	W	−	−	−
D-Fucose	+	−	−	+	−	−	−
L-Fucose	+	−	−	+	−	−	−
Gluconate	+	−	−	+	−	−	−
5-Ketogluconate	W	−	−	+	−	−	−

extends the knowledge of the physiological variability within this species.

The ability of strain NS05^T to utilize methanol and oxalate was shared with other members of the genus *Ancylobacter*. However, gas vesicles and chemolithoautotrophy with H_2, characteristics of the type species *Ancylobacter aquaticus* and of *Ancylobacter polymorphus*, were not detected in NS05^T. Growth occurred at temperatures up to 40 °C: this differentiated strain NS05^T from *Ancylobacter aquaticus* and *Ancylobacter vacuolatus*, which grew at temperatures up to 34 and 37 °C, respectively. Strain NS05^T utilized several carbohydrates and organic acids (Table 2). The results obtained using the API 50 CH panel confirmed the relatively poor metabolic versatility of *Ancylobacter aquaticus* with regard to carbohydrates, as stated previously (Xin *et al.*, 2006). Comparisons of results obtained in our work using different test methods revealed some deviations, e.g. for utilization of D-arabinose (strains NS03 and NS04 and *Ancylobacter polymorphus* DSM 2457^T) and urease (*Ancylobacter rudongensis* DSM 17131^T) and the reduction of nitrate (*Ancylobacter vacuolatus* DSM 1277^T):

these reactions were negative in the API panels but positive in standard tests. Perhaps the 2 day incubation period for the API strips was not sufficient to allow the development of positive reactions. Strain $NS05^T$ can be distinguished from members of the genus *Ancylobacter* using the following traits: pleomorphic cell morphology, the ability to utilize D-mannose and L-rhamnose and a negative result in the β-galactosidase test. Strain $NS05^T$ can be differentiated from its phylogenetically closest neighbour, *Ancylobacter rudongensis*, which also was incapable of chemolithoautotrophic growth in our hands, by its inability to utilize glycerol and citrate, its ability to utilize fucose, gluconate and malate and its ability to produce a positive urease reaction with the API 20NE strip. Strain $NS05^T$ differed from '*Ancylobacter natronum*' (as given by Doronina *et al.*, 2001) in being unable to hydrolyse gelatin, to grow chemolithoautotrophically with H_2 and to reduce nitrate and in being able to grow at 40 °C. The resistance of strain $NS05^T$ to antibiotics and heavy metal ions has been described by Sahin *et al.* (2002). The results are indicated in the species description.

Genomic and chemotaxonomic data confirm that strain $NS05^T$ belongs to the genus *Ancylobacter*. On the basis of low DNA–DNA hybridization with its closest phylogenetic neighbour and differences in biochemical and morphological traits with respect to all of the type strains described for the genus *Ancylobacter*, it can be concluded that strain $NS05^T$ represents a novel species within the genus, for which the name *Ancylobacter oerskovii* sp. nov. is proposed.

Description of *Ancylobacter oerskovii* sp. nov.

Ancylobacter oerskovii (oers.ko'vi.i. N.L. gen. masc. n. *oerskovii* of Ørskov, named in honour of J. Ørskov, who, in 1928, described *Microcyclus aquaticus*, now the type species of the genus *Ancylobacter*).

Gram-negative and non-motile. Cells are highly pleomorphic rods, $0.5–0.6 \times 0.9–1.7$ µm, and are often bone- or droplet-shaped; some coccoid cells are also found. Cells occur singly or in pairs in young cultures and often form clusters (presumably being held together by slime) in older cultures. No spores, gas vesicles or other cell inclusions are found. Non-pigmented. Forms round, cream-coloured, convex colonies, reaching 0.3–0.5 mm in diameter, on nutrient agar after 2 days incubation; colonies become slimy with age. Growth occurs on nutrient agar slopes at temperatures up to 40 °C. Growth occurs in nutrient broth containing 4 % NaCl. Oxidase- and urease-positive. Negative for chemolithoautotrophic growth with H_2, acid production from glucose, nitrate reduction, indole production, arginine dihydrolase, gelatin hydrolysis and β-galactosidase. Utilizes methanol, oxalate, glucose, galactose, D-fucose, L-fucose, fructose, L-arabinose, rhamnose, D-arabitol, mannitol, sorbitol, xylitol, gluconate, malate and malonate. Does not utilize glycerol, *N*-acetylglucosamine or 2-ketogluconate. The main fatty acids are $C_{18:1}\omega7c$ (60.4 %), $C_{19:0}\omega8c$ cyclo (28.3 %) and $C_{16:0}$ (7.4 %); minor amounts of $C_{17:0}$ and $C_{18:0}$ and an unknown component are present. Contains ubiquinone Q-10 and minor amounts of Q-9. The DNA G+C content is 68.0 mol%. The type strain is resistant to ampicillin and bacitracin (each at 10 µg per disc), erythromycin (15 µg) and chloramphenicol (30 µg) and susceptible to gentamicin and streptomycin (10 µg each). Sensitive to $HgCl_2$ (2.5 µg on disc) and resistant to $ZnSO_4 . 7H_2O$, $NiCl_2 . 6H_2O$, $CoCl_2 . 6H_2O$, $CuSO_4 . 5H_2O$, lead acetate and K_2CrO_7 (each at 2.5 µg per disc).

The type strain, $NS05^T$ (=DSM 18746^T =CCM 7435^T), was isolated from soil using mineral medium with oxalate for enrichment.

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