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EXPERIMENTAL ARTICLES

Ancylobacter abiegnus sp. nov., an Oligotrophic Member of the Xylotrophic Mycobacterial Community

M. V. Zaichikova[a], Yu. Yu. Berestovskaya[a], V. N. Akimov[b], A. K. Kizilova[a], and L. V. Vasilieva[a, 1]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

[b] *Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

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Abstract—A new species of the genus *Ancylobacter*, exemplified by strain Z-0056, was isolated from dystrophic humified waters formed by xylotrophic fungi grown on decaying spruce wood. The cells of strain Z-0056 (0.65–0.9 μm) are coccoid, gram-negative, with fimbriae, and nonmotile. The strain is pleomorphic and reproduces by nonuniform division. Strain Z-0056 is an aerobic organoheterotroph and a mesophilic and moderately acidophilic oligotrophic microorganism. As an inhabitant of dystrophic ultrafresh waters, strain Z-0056 is sensitive to NaCl. The bacterium utilizes organic acids (acetate, pyruvate, oxalate, gluconate, malate, succinate, and citrate), as well as xylose and xylan. The microorganism grows in a pH range of 4.0–8.0, with an optimum at pH 5.5. The temperature range for growth is 15–25°C, with an optimum at 20°C. According to its ecophysiological properties, strain Z-0056 belongs to the group of ombrophilic dissipotrophs. The DNA G+C base content is 66.8 mol %. According to phylogenetic analysis, strain Z-0056 belongs to the genus *Ancylobacter*. Strain Z-0056 showed the highest similarity (98.3%) with the type strain of the species *A. oerskovii*. The phenotypic and phylogenetic properties of strain Z-0056 support classification of this microorganism within the genus *Ancylobacter* as the novel species *Ancylobacter abiegnus* sp. nov.

Key words: xylotrophic community, oligotrophic bacteria, dissipotrophs, acidotrophic microorganisms, ombrophils, wood decomposition, oligotrophic waters.

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An aquatic bacterium with a circular morphology was first isolated by Örskov in 1928 and described as a member of the genus *Microcyclus, M. aquaticus* [1]. The generic name *Microcyclus* was used before Örskov in the classification of fungi [2]. Thus, for ease of reference, the bacterial genus *Microcyclus* was renamed *Ancylobacter* in 1983 [3]. Today, the genus *Ancylobacter* of the family *Xanthobacteriaceae* is represented by the following five validated species: *A. aquaticus* [1, 3], *A. rudongensis* [4], *A. polymorphus* and *A. vacuolatus* [5], and *A. oerskovii* [6]. The representatives of this genus were isolated from soil, aquatic ecosystems, and *Spartina anglica* roots. As oligotrophic methylotrophs [7], hydrogen-utilizing lithotroph [7, 8], and oxalotrophic microorganisms [6], they play an important ecological role.

The aim of the present work was to study strain Z-0056, an oligotrophic heterotroph isolated from dystrophic acidic water formed by the myco–bacterial community in the course of spruce wood degradation, as well as to determine its phylogenetic position.

[1] Corresponding author; e-mail: lvasilyeva@mail.ru

MATERIALS AND METHODS

Isolation and cultivation of strain Z-0056. Strain Z-0056 was isolated on agarized PC medium (according to the previously described procedure) from dystrophic, acidic (pH 4.3) water of a microlysimeter in which spruce wood was degraded by a xylotrophic fungal community [9].

Microscopy. Cell morphology was studied under a light microscope with a phase-contrast device (Amplival, Germany), as well as by electron microscopy (JEM 100C, Japan) of negatively stained preparations and ultrathin sections. The preparations were stained with 1% uranyl acetate. To obtain ultrathin sections, the cells were fixed with glutaraldehyde with subsequent additional fixation with osmium tetroxide in cacodylate buffer and then embedded in Epon. Ultrathin sections were obtained with an LKB ultramicrotome, stained with lead citrate, and then additionally stained with a 3% aqueous solution of uranyl acetate.

Physiological properties of strain Z-0056 were studied using generally accepted techniques [10]. The range of substrates utilized by strain Z-0056 as carbon sources was determined on liquid low-mineral olig-

otrophic PC medium with yeast extract (0.05 g/l) as a growth factor. Sugars (arabinose, xylose, glucose, fructose, galactose, mannose, lactose, maltose, sucrose, raffinose, starch, and xylan), polyalcohols (glycerol, sorbitol, and mannitol), salts of organic acids (formate, acetate, butyrate, propionate, pyruvate, fumarate, succinate, oxalate, oxaloacetate, citrate, malate, and benzoate), primary alcohols (methanol and ethanol), amino acids (methionine, glutamate, leucine, cysteine, and aspartate), and methylamines were tested as carbon and energy sources. With the exception of methanol, all tested substrates were added to a concentration of 0.25 g/l. Growth of Z-0056 on methanol was investigated within a concentration range of 0.01–1%. Bacterial growth was assessed by optical density (OD_{600}) of the cell suspension measured on a UNICO 2100 spectrophotometer.

The growth rate of strain Z-0056 at a pH range of pH 4.8–8.0 was determined by the addition of 0.05 M solutions of Na_2HPO_4 and KH_2PO_4 to the medium. Growth in a pH range of 3.0–4.8 was determined in the medium acidified with 0.1 N HCl to the required pH level. The medium pH was determined potentiometrically using an Expert 001 pH/ion meter (Russia).

Growth of Z-0056 was investigated within a temperature range of 2–37°C.

The effect of NaCl concentrations on growth was determined in medium supplemented with NaCl (0.5–30.0 g/l).

The temperature and pH optima for growth of strain Z-0056, as well as the effect of NaCl concentrations on growth, were determined with succinate as a substrate.

The capacity for lithoautotrophic growth was assessed by measuring optical density (OD_{600}) of the cell suspension and by monitoring hydrogen utilization during cultivation of strain Z-0056 in liquid medium with the gas phase $H_2 : O_2 : CO_2$ (7 : 2 : 1). The hydrogen concentration was measured on an LKhM-80 gas chromatograph (Russia) with a katharometer detector. The separation was carried out on a column packed with a 5 A molecular sieve.

The nitrogen-fixing activity of the strain was determined by the acetylene method on semisolid nitrogen-free medium NFb with sodium malate (1 g/l) [11].

Two primer systems, F1/R6 [12] and PolF/PolR [13], designed for the *nifH* gene, were used to detect the presence of the *nifH* gene in the DNA.

The ability of the culture to grow on various nitrogen sources was tested using inorganic salts (ammonium sulfate, potassium nitrate, nitrite, and urea), as well as amino acids (phenylalanine, methionine, serine, tyrosine, valine, lysine, aspartate, tryptophan, and glutamate).

The sensitivity of strain Z-0056 to antibiotics was determined by the diameter of growth inhibition zones surrounding antibiotic discs (Oxoid) on the agar surface. Antibiotic concentrations per disk were as follows: lincomycin, 10 μg; novobiocin, 30 μg; ampicillin, 10 μg; chloramphenicol, 30 μg; neomycin, 10 μg; gentamycin, 10 μg; kanamycin, 30 μg; and streptomycin, 10 μg.

Catalase activity was assayed by monitoring the formation of gas bubbles on addition of a 3% hydrogen peroxide solution to the cells; the presence of oxidase was detected by changes in the colony pigmentation when the reagent REF-55635 was applied.

The fatty acid (FA) composition of the lipids of strain Z-0056 was determined on a Microbial Identification System (Sherlock) chromatograph (MIDI Inc., Newark, United States) according to the protocol described in [14]. The separated fatty acids were identified using an Agilent Technologies AT-5971 SMART mass spectrometer.

Molecular genetic analysis. DNA isolation and purification, as well as determination of the DNA G+C content, were performed as described earlier [15].

Determination of the nucleotide sequence of the 16S rRNA gene was performed as follows. DNA was extracted by the phenol method [16]. PCR amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27f and 1492r on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, United States). Sequencing of the amplified 16S rRNA gene fragment was performed on a CEQ2000 XL automatic sequencer (Beckman Coulter, United States) according to the manufacturer's instructions. To determine the strains closely related to strain Z-0056, the GenBank database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) was used. The phylogenetic tree was constructed by the methods implemented in the TREECON software package [17]. The obtained 16S rRNA gene sequence of strain Z-0056 was deposited in the GenBank under the accession number GU247895.

RESULTS

Isolation source. Strain Z-0056 was isolated from dystrophic, low-humified, acidic (pH 4.3) low-mineral oligotrophic (conductivity 140 μS) water of a microlysimeter in which spruce wood was degraded by the xylotrophic fungal community.

Cell morphology and ultrastructure. When grown under optimal cultivation conditions on the medium with succinate as a substrate (0.25 g/l), the cells of strain Z-0056 were nonmotile cocci (0.65–0.9 μm) (Fig. 1a). The cells reproduced by nonuniform division. During reproduction, the cell elongated (Fig. 1b) and a rod-shaped cell (0.65–0.9 × 1.35–1.50 μm) was formed (Fig. 1c). This cell then divided nonuniformly into two cells (Figs. 1d–f). The cell surface is covered with fimbriae and slime. The cells of strain Z-0056 did not produce dormant forms. Ultrathin sections of the

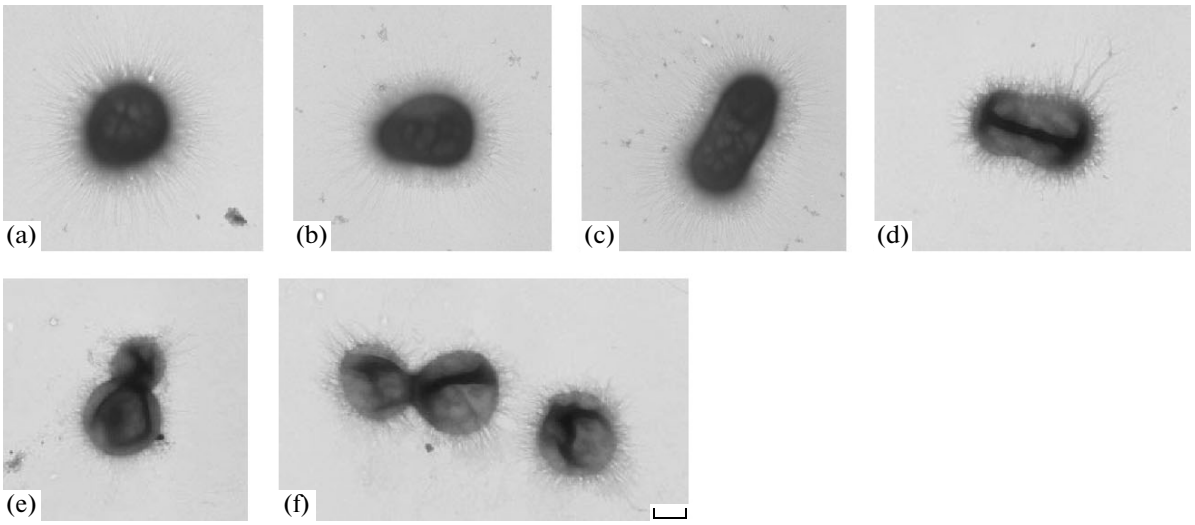

Fig. 1. Electron microphotographs of the cells of strain Z-0056 at various stages of division (scale bar, 0.5 μm). The preparations were stained with 1% uranyl acetate.

cells of strain Z-0056 revealed the gram-negative structure of their cell wall (Fig. 2). No vesicles were detected.

Cultural properties. On agarized PC medium, the strain produced milky, dense, rounded, convex, slimy, opaque, smooth colonies (up to 4 mm in diameter) with even edges.

Physiological properties. Strain Z-0056 was an obligately aerobic and mesophilic bacterium. The bacterium grew within a temperature range of 15–25°C with an optimum at 20°C. The microorganism was moderately acidophilic and grew within a pH range from 4.0 to 8.0 with an optimum at 5.5.

The strain was NaCl-sensitive: the NaCl content in the media above 1.0 g/l inhibited growth; this suggests that this microorganism is a typical inhabitant of low-mineral waters.

Strain Z-0056 utilized salts of organic acids (acetate, succinate, citrate, malate, oxalate, and gluconate), as well as xylose and xylan, as carbon and energy sources. The strain did not grow on methanol as a substrate within the range of tested concentrations (Table 1).

The bacterium was oligotrophic and grew at substrate concentrations ranging from 0.05 to 2 g/l. The optimal substrate concentration in the medium was 0.25 g/l. The maximum growth rate was observed on succinate (μ_{max} 0.024 h^{-1}). The cells retained their typical morphological properties within the range of substrate concentrations of 0.05–0.25 g/l. Further increase in the substrate concentration resulted in the appearance of pleomorphic forms.

Strain Z-0056 did not grow on nitrogen-free medium. Nitrogenase activity was not revealed under the experimental conditions. Since, under certain conditions, some species of the genus *Ancylobacter* are capable of dinitrogen fixation, we tested strain Z-0056 for the presence of the *nifH* gene. In the DNA of strain Z-0056, the *nifH* gene fragment was not detected. Strain Z-0056 utilized ammonium sulfate, potassium nitrate, urea, phenylalanine, methionine, tyrosine, and serine as nitrogen sources. The organism was catalase- and oxidase-positive.

Antibiotics. The bacterium was resistant to novobiocin, chloramphenicol, lincomycin, and ampicillin. Strain Z-0056 was sensitive to streptomycin, neomycin, and gentamycin.

Fatty acid (FA) composition. The fatty acid composition of the cell membrane lipids of strain Z-0056 (% of the total fatty acid content) is shown in Table 2. As in the other representatives of the genus *Ancylobacter*, the principal fatty acid of strain Z-0056 was $C_{18:1\omega7}$ (11-octadecenoic acid).

Molecular genetic analysis. The content of the G+C base pairs in the DNA of strain Z-0056 was 66.8 mol %.

The sequence of the 16S rRNA gene fragment (1310 bp) was determined for strain Z-0056. Phylogenetic analysis confirmed affiliation of strain Z-0056 within the *Ancylobacter–Starkeya* cluster. The levels of 16S rRNA similarity between strain Z-0056 and the type strains of these genera were 98.3, 97.7, 97.6, 97.4, 96.5, 97.2, and 96.6% similarity with *A. oerskovii, A. rudongensis, A. vacuolatus, A. polymorphus, A. aquaticus, S. koreensis*, and *S. novella*, respectively (Table 3).

DISCUSSION

Comparative phylogenetic analysis of the 16S rRNA gene sequence of strain Z-0056 revealed a high level of similarity between the new isolate and representatives of the genera *Ancylobacter* and *Starkeya*

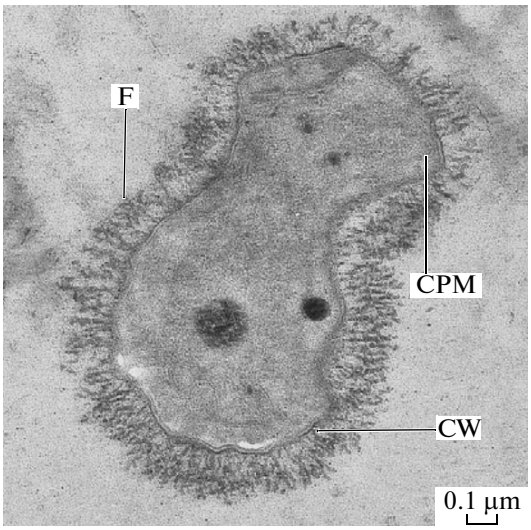

Fig. 2. Ultrathin section of a cell of strain Z-0056. F, fimbriae; CW, cell wall; CPM, cytoplasmic membrane. Scale bar, 0.1 μm.

(Table 3). The highest level of similarity (98.3%) was observed between the 16S rRNA gene sequence of strain Z-0056 and that of *A. oerskovii* (Fig. 3). The phenotypic, physiological, and biochemical properties of strain Z-0056 indicated significant differences between this microorganism and the known species of both genera (Table 1). Strain Z-0056 was isolated from dystrophic, acidic, low-mineral oligotrophic water and was well adapted to these conditions. Unlike all species of the genera *Ancylobacter* and *Starkeya*, the bacterium was found to be acid-tolerant with a growth optimum at pH 5.5 [1, 4–6, 18, 19]. Strain Z-0056 grew within a narrow temperature range from 15 to 25°C; the growth optimum (20°C) was lower than that of other representatives of both genera. The new isolate was NaCl-sensitive; growth ceased at NaCl concentrations that were one order of magnitude lower than in the case of the closely related species. Strain Z-0056, like most other members of the genus *Ancylobacter*, was an obligate heterotroph (unlike species of the genus *Starkeya*, which are capable of utilizing sulfur compounds as energy sources). The bacterium was incapable of methylotrophic growth, unlike all species of the genera *Starkeya* and *Ancylobacter*. Strain Z-0056 was unable to grow under autotrophic conditions utilized organic acids and carbohydrates as carbon and energy sources. The new isolate was oligotrophic; the maximum growth rate (μ_{max} 0.024 h^{-1}) was observed at a substrate concentration of 0.25 g/l.

The principal fatty acids of strain Z-0056 were $C_{18:1\omega7}$ (11-octadecenoic), 72.5%; C_{19cyc} (cyclopropan-nonadecanoic), 15.86%; and $C_{16:0}$ (hexadecanoic), 7.91%. This is typical of all *Ancylobacter* species (Table 2) [6]. Minor amounts of the following fatty acids were detected: $C_{16:1\omega7c}$ (9-hexadecenoic), $C_{17:0}$ (heptadecanoic), $C_{18:1\omega9}$ (9-octadecenoic), $C_{18:0}$ (octadecanoic), and $C_{11Me18:1\omega7c}$ (11-methyl-octadecenoic acid). The presence of $C_{18:1\omega9}$ (9-octadecenoic acid) was detected only in the case of strain Z-0056 and was not found in any species of the genus *Ancylobacter* [6].

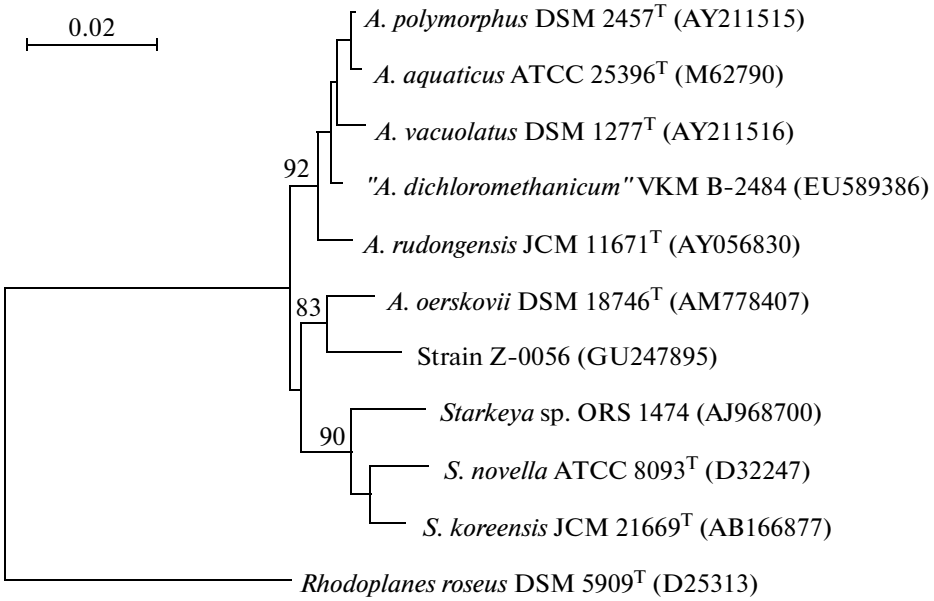

Fig. 3. Phylogenetic position of strain Z-0056 among the type strains of the genera *Starkeya* and *Ancylobacter*. The numerals show the results of the bootstrap analysis.

Table 1. Differentiating characteristics of strain Z-0056 and members of the genera *Ancylobacter* and *Starkeya*

	Strain Z-0056	*A. oerskovii* NS 05^T	*A. aguaticus* DSM 101^T	*A. polymorphus* DSM 2457^T	*A. vacuolatus* DSM 1277^T	*A. rudongensis* JCM 11671^T	*S. koreensis* KCTC 12212^T	*S. novella* DSM 506^T
Cell shape	Cocci	Rods	Rods	Rods	Rods	Rods	Rods	Rods
Cell size, μm	0.65–0.9	0.5–0.6 × 0.9–1.7	0.3–1 × 1–3	0.8–1.0	0.8–1.0	0.6–0.8	0.4–0.8 × 1.2–2.0	0.4–0.8 × 0.8–2.0
pH range	4.8–8.0	N/D	6.8–7	4.5–11.0	5.5–11.0	5.5–10.0	6.5–8.5	5.7–9.0
Optimal pH	5.5	7	N/D	7.0	7.0	6.8–7.0	7.0–8.0	7.0
T range, °C	15–25	40	5–43	Above 4 and below 50	4–37	4–45	4–40	10–37
Optimal T°C	20	30	22–37	28–30	28–30	28–30	28–30	25–30
G+C base content, mol %	66.8	68	66.3–67.7	65.5	65.5	68.2	69	67.3–68.4
Nitrogenase activity	–	–	–	+	+	+	–	–
NaCl range, %	0–0.1	0–4.0	N/D	0–3.0	0–2.5	0–5.0	0–3.0	
Autotrophic growth	–	–	+	–	–	–	+	+
Growth on C_1 compounds	–	+	+	+	+	+	+	+
Galactose	–	+	N/D	+	N/D	+	N/D	N/D
Rhamnose	–	+	–	–	–	–	N/D	N/D
Fucose	–	+	–	+	–	–	N/D	N/D
Arabinose	–	+	–	–	+	+	N/D	N/D
Mannose	–	+	–	–	–	–	–	N/D
Glucose	–	+	+	+	+	+	+	+
Maltose	–	N/D	–	+	N/D	+	–	–
Glycerol	–	+	–	+	+	N/D	N/D	N/D
Sorbitol	–	+	–	+	+	+	N/D	N/D
Acetate	+	N/D	N/D	N/D	N/D	N/D	N/D	N/D
Pyruvat	–	N/D	+	N/D	–	+	N/D	N/D
Propionate	–	N/D	–	+	+	+	+	N/D
Gluconate	+	+	+	–	+	–	–	N/D
Citrate	+	–	+	–	+	+	+	–
Malate	+	+	+	+	–	–	–	–
Succinate	+	N/D	–	+	+	+		–
Oxalate	+	+	N/D	N/D	N/D	N/D	+	+
Gluconate	+	+	+	–	+	–	–	N/D
Xylose	+	–/+	+\–	+	+	+	N/D	N/D
Xylan	+	N/D	N/D	N/D	N/D	N/D	N/D	N/D

Note: N/D stands for “not determined.”

Table 2. The fatty acid composition of strain Z-0056 and other representatives of the genus *Ancylobacter* (% of total fatty acid content)

Fatty acid	Abbreviation	1	2	3	4	5	6
9-Hexadecenoic	$C_{16:1\omega7c}$	0.55	0–0.5	1.4	0.4	0.7	1.3
Hexadecanoic	$C_{16:0}$	7.91	7.4	6.4	6.8	4.9	4.8
Heptadecanoic	$C_{17:0}$	0.20	0.4	1.0	0.9	1.1	0.5
9-Octadecenoic	$C_{18:1\omega9}$	0.52	–	–	–	–	–
11-Octadecenoic	$C_{18:1\omega7c}$	72.5	60.4	71.7	71.5	76.1	60.9
Octadecanoic	$C_{18:0}$	1.88	2.4	1.5	2.0	1.2	1.5
11-Methyl-octadecenoic	$C_{11Me\ 18:1\omega7c}$	0.58	–	–	0.4	1.0	2.2
Cyclopropan-nonadecanoic	$C_{19:0\omega8c\ cyclo}$	15.86	28.3	17.4	16.3	14.7	27.9

Designations: 1, Strain Z-0056; 2, *Ancylobacter oerskovii* NS 05^T [6]; 3, *A. aquaticus* DSM 101^T [6]; 4, *A. polymorphus* DSM 2457^T [5]; 5, *A. vacuolatus* DSM 1277^T [5]; 6, *A. rudongensis* JCM 11671^T [4]; –, FA were not detected.

Table 3. The levels of 16S rDNA similarity between strain Z-0056 and the type strains of the genera *Ancylobacter* and *Starkeya*

Strain	16S rDNA similarity levels, %								
Strain Z-0056									
A. oerskovii DSM18746^T	98.3								
A. aquaticus ATCC 25396^T	96.5	97.0							
A. polymorphus DSM 2457^T	97.4	97.8	98.8						
A. vacuolatus DSM 1277^T	97.6	97.9	98.4	99.3					
"*A. dichloromethanicum*" VKM B-2484	97.7	97.9	98.6	99.4	99.4				
A. rudongensis JCM 11671^T	97.7	98.1	97.8	99.1	98.9	99.2			
S. koreensis JCM 21669^T	97.2	97.2	96.6	97.5	97.6	97.6	97.7		
S. novella ATCC 8093^T	96.6	96.7	96.4	97.2	97.2	97.3	97.4	98.6	
Starkeya sp. ORS 1474	97.4	97.5	96.2	97.0	97.1	97.1	97.4	98.1	98.0

Notes: Designations for the genera *Ancylobacter* and *Starkeya* are *A.* and *S.*, respectively; the species "*Ancylobacter dichloromethanicum*" was proposed, but has not yet been validly described; strain *Starkeya* sp. ORS 1474 has not been proposed as a representative of a new species.

The content of the G+C base pairs in the DNA of strain Z-0056 66.8 mol %) is close to the values for other *Ancylobacter* species (65.5–68.2 mol %).

Thus, the phenotypic properties of strain Z-0056 differ considerably from those of the closely related species of the genera *Ancylobacter* and *Starkeya*.

The high 16S rRNA similarity (98.3%) between strain Z-0056 and *A. oerskovii* allowed us to assign the new isolate to the genus *Ancylobacter*. On the basis of its clear-cut phenotypic distinctions from *A. oerskovii*, we propose that strain Z-0056 should be described as a novel species of this genus, *A. abiegnus* sp. nov. The description of *A. abiegnus* sp. nov. expands the description of the genus *Ancylobacter*, which now includes not only methylotrophic microorganisms.

The ecophysiological properties of strain Z-0056 suggest that this microorganism is a typical ombrophilic [20] oligotrophic dissipotroph inhabiting low-mineral dystrophic waters and utilizing primarily organic acids formed during hydrolysis of spruce wood by the xylotrophic fungal community.

Description of *Ancylobacter abiegnus* sp. nov. *abiegnus*, Gr. f. *abies*, spruce; M. L. masc. *abiegnus*, (spruce).

The cells are gram-negative, coccoid, 0.65–0.9 μm, pleomorphic, with fimbriae, and nonmotile. The bacterium reproduces by nonuniform division and does not form spores. During division, rod-shaped cells (1.35–1.50 μm) are formed, which then split nonuniformly into two cells. No gas vesicles were detected.

The colonies are milky-white, dense, rounded, convex, slimy (up to 4 mm in diameter), with even edges. No pigments were detected.

The pH range for growth is 4.0–8.0 with an optimum at 5.5. The bacterium is a mesophile growing in a temperature range from 15 to 25°C with a growth optimum at 20°C. Active growth occurs at NaCl concentrations not exceeding 1.0 g/l.

The organism is an obligate aerobe. Organic acids (acetate, succinate, citrate, malate, oxalate, and gluconate) and carbohydrates (xylose and xylan) are utilized as carbon and energy sources. The strain does not utilize C_1 compounds, mono- and disaccharides, and amino acids and is not capable of chemolithoautotrophic growth. The bacterium is an oligotroph. The typical cell morphology is retained at substrate concentrations not exceeding 0.25 g/l. Yeast extract is required for growth.

The organism is catalase- and oxidase-positive and is incapable of dinitrogen fixation.

The principal fatty acids of strain Z-0056 are $C_{18:1\omega}$ (11-octadecenoic), 72.5%; C_{19cyc} (cyclopropan-nonadecanoic), 15.86%; and $C_{16:0}$ (hexadecanoic), 7.91%.

The DNA G+C base content is 66.8 mol %.

The type strain is resistant to novobiocin, chloramphenicol, lincomycin, and ampicillin; it is sensitive to streptomycin, neomycin, and gentamycin.

The type strain is Z-0056 (VKM B-2563).

The organism was isolated from acidic (pH 4.3), low-mineral oligotrophic water formed by the xylotrophic fungal community grown on decaying spruce wood.

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