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Acinetobacter brisouii sp. nov., Isolated from a Wetland in Korea

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A bacterial strain 5YN5-8^T was isolated from peat layer on Yongneup in Korea. Cells of strain 5YN5-8^T were strictly aerobic, Gram-negative, coccobacilli, non-spore forming, and non-motile. The isolate exhibited optimal growth at 28°C, pH 7.0, and 0-1% NaCl. Results of 16S rRNA gene sequence analyses indicated a close relationship of this isolate to *Acinetobacter calcoaceticus* (97.8% similarity for strain DSM 30006^T). It also exhibited 94.4-97.8% 16S rRNA gene sequence similarities to the validly published *Acinetobacter* species. The value for DNA-DNA hybridization between strain 5YN5-8^T and other members of the genus *Acinetobacter* ranged from 16 to 28%. Predominant cellular fatty acids were $C_{18:1}$ *ω9c*, summed feature 4 containing $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ *ω7c*, and $C_{16:0}$. The DNA G+C content was 43.9 mol%. Phylogenetic, phenotypic, and chemotaxonomic data accumulated in this study revealed that the isolate could be classified in a novel species of the genus *Acinetobacter*. The name *Acinetobacter brisouii* sp. nov. is proposed for the novel species, with 5YN5-8^T (=KACC 11602^T =DSM 18516^T) as the type strain.

Keywords: *A. brisouii*, 16S rRNA gene sequence, fatty acid, DNA-DNA hybridization

The genus *Acinetobacter* was first proposed by Brisou and Prévot (1954) and comprises non-motile, aerobic, Gram-negative bacteria. *Acinetobacter* spp. represented a well-defined genus by 16S rRNA gene sequence analysis (Juni, 1984; Lee *et al*., 2009). *Acinetobacter* spp. was mainly isolated from clinical specimens. However, some species with validly published names were isolated from environmental sources such as soil, cotton, and activated sludge (Brisou and Prévot, 1954; Nishimura *et al*., 1988; Carr *et al*., 2003). At present, the genus included 20 species (*A. calcoaceticus*, *A. lwoffii*, *A. baumannii*, *A. haemolyticus*, *A. johnsonii*, *A. junii*, *A. radioresistens*, *A. ursingii*, *A. schindleri*, *A. baylyi*, *A. tjernbergiae*, *A. towneri*, *A. bouvetii*, *A. grimontii*, *A. gerneri*, *A. tandoii*, *A. parvus*, *A. beijerinckii*, *A. gyllenbergii*, and *A. venetianus*) with validly published names, and the type species is *Acinetobacter calcoaceticus* (http://www.bacterio.cict.fr/a/acinetobacter.html; Professor. J.P. Euzeby's list of prokaryotic names with standing nomenclature). In addition to species with the recognized names, there are many genomospecies and also invalidly published species ('*A. marinus*', '*A. seohaensis*', '*A. soli*', and '*A. antiviralis*') are existing in the genus *Acinetobacter* (Yoon *et al*., 2007; Kim *et al*., 2008; Lee *et al*., 2009). Yongneup (38°12′53″N 128°07′30″E) is a wetland located at above 1,200 meters above sea level, which is the only high moor in Korea. The peat layers are about 150 cm thick and they have been formed over 4,000-5,000 years. In the course of the bacterial population study of Yongeup, we have isolated a bacterial strain designated as 5YN5-8^T. The aim of the present study was to elucidate the taxonomic position of strain 5YN5-8^T using a polyphasic approach.

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Materials and Methods

Isolation, morphological, and physiological characterization

In order to isolate the culturable bacteria from wetland, peat sample was serially diluted with 0.85% (w/v) NaCl and appropriate 10-fold dilutions were plated on R2A agar (Difco, USA) (Reasoner and Geldreich, 1985). The plates were incubated at 30°C for 4 days. The 5YN5-8^T was one of the isolates that appeared on the R2A medium and subjected to taxonomic investigation. *Acinetobacter calcoaceticus* DSM 30006^T, *Acinetobacter haemolyticus* DSM 6962^T, *Acinetobacter johnsonii* DSM 6963^T, and *Acinetobacter schindleri* DSM 16038^T were used as reference strains. Phenotypic characteristics such as Gram staining, catalase, oxidase, and hydrolysis of carboxymethylcellulose, casein, chitin from crab shells, DNA, pectin, tyrosine, Tween 80, and starch were performed using the methods of Smibert and Krieg (1994). The pH range (pH 4.0-10.0 at intervals of 1.0 pH units) for growth was determined in R2A broth which was adjusted with citrate-phosphate buffer or tris-hydrochloride buffer (Breznak and Costilow, 1994). Growth at 0, 1, 2, 3, and 5% NaCl (w/v) was investigated in R2A broth. Growth at various temperatures (4-50°C) was measured on R2A agar medium. Growth under anaerobic condition was tested in GasPak (BBL, USA) jar at 30°C for 15 days. Physiological and biochemical properties were further determined with API ZYM, API 20NE, and API ID 32 GN (bioMérieux, France). Tests in the commercial systems were generally performed according to the manufacturer's instructions. The API ZYM test strip was read after 4 h incubation at 37°C and API test strips were examined after 48 h at 28°C.

Molecular characterization

The 16S rRNA gene sequence was determined as described previously (Kwon *et al*., 2003). The nearly complete 16S rRNA sequence of strain 5YN5-8^T (1,405 nucleotides) was obtained. The 16S rRNA sequences were aligned by using CLUSTAL W program (Thompson *et al*., 1994). The evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed with neighbor joining (Saitou and Nei, 1987), maximum parsimony (Fitch, 1971) and maximum likelihood (Felsenstein, 1981) methods using the program MEGA3 (Kumar *et al.*, 2004), with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Chemotaxonomic characterization

Fatty acid methyl esters were extracted and prepared by the standard protocol of the Microbial Identification System (MIDI; Microbial ID) after cells were grown on R2A for 48 h at 30°C. The determination of DNA G+C contents were performed based on the method described by Mesbah *et al*. (1989) using a reverse-phase column (Supelcosil LC-18-S; Supelco, USA).

DNA-DNA hybridization

DNA-DNA hybridization was carried out as filter hybridization method described as Seldin and Dubnau (1985). Probe labeling was conducted by using the non-radioactive DIG-High prime system (Roche, Swiss), and hybridized DNA was visualized using the DIG luminescent detection kit (Roche). DNA−DNA relatedness was quantified by using a densitometer (Bio-Rad, USA).

The GenBank accession number for the 16S rRNA gene sequence of strain 5YN5-8^T is DQ832256.

Results and Discussion

Cells of strain 5YN5-8^T were Gram-negative, coccobacilli, non-spore forming, and non-motile. Colonies of strain 5YN5-8^T is smooth, glistening, slightly convex, milky-white in color on R2A agar. The strain 5YN5-8^T can be differentiated from the closely related species by physiological and biochemical properties. For example, it can be differentiated from *Acinetobacter calcoaceticus* DSM 30006^T, its closest phylogenetic neighbor, based on the growth at 41°C and production of alkaline phosphatase, and incapability of assimilating adipic acid, trisodium citrate, suberic acid, and L-histidine (Table 1). Strain 5YN5-8^T exhibited 16S rRNA gene similarity levels of 94.4-97.8% and 94.0-96.7% to the type strains of *Acinetobacter* species with validly published names and genomospecies, respectively. The 16S rRNA gene sequence similarity values between strain 5YN5-8^T and its closest relatives, *Acinetobacter calcoaceticus*, *Acinetobacter schindleri*, *Acinetobacter haemolyticus*, and *Acinetobacter johnsonii*, were 97.8, 96.9, 96.8, and 96.6%, respectively. Phylogenetic analysis based on the neighbor-joining method revealed that strain 5YN5-8^T formed a monophyletic clade from the cluster containing *Acinetobacter calcoaceticus* and '*A. antiviralis*' (Fig. 1). The same clustering pattern was also observed in maximum-likelihood tree; however in maximum-parsimony tree strain 5YN5-8^T formed a separate branch with '*A. antiviralis*' (data not shown). In this study, *A. antiviralis* was not included since it is not validly published and also this strain was submitted for patent and not available for experiments (Jung-Sook Lee, Personal communication).

Table 1. Characteristics that differentiate strain 5YN5-8^T and closely related *Acinetobacter* species

1, 5YN5-8^T; 2, *Acinetobacter calcoaceticus* DSM 30006^T; 3, *Acinetobacter haemolyticus* DSM 6962^T; 4, *Acinetobacter johnsonii* DSM 6963^T; 5, *Acinetobacter schindleri* DSM 16038^T. All data from this study.

All strains assimilate capric acid, malic acid, sodium acetate, L-alanine, propionic acid, valeric acid, and L-proline. All strains do not assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-maltose, potassium gluconate, L-rhamnose, D-ribose, inositol, D-saccharose, itaconic acid, potassium 5-ketogluconate, glycogen, salicin, D-melibiose, L-fucose, D-sorbitol, and potassium 2-ketogluconate.

+, positive; –, negative; W, weakly positive

Characteristics	1	2	3	4	5
Growth at 41°C	+	–	–	–	+
Glucose fermentation	–	–	+	–	–
Urease	+	+	–	–	–
Gelatin hydrolysis	–	–	+	–	–
Assimilation of					
Adipic acid	–	+	–	–	–
Trisodium citrate	–	+	+	–	+
Phenylacetic acid	+	+	–	–	–
Suberic acid	–	+	–	–	–
Sodium malonate	+	+	–	–	–
Lactic acid	+	+	–	+	+
3-Hydroxybenzoic acid	–	–	–	–	+
L-Serine	–	–	+	–	–
L-Histidine	–	+	+	–	–
3-Hydroxybutyric acid	+	+	+	–	–
4-Hydroxybenzoic acid	+	+	+	–	+
Enzyme activity					
Alkaline phosphatase	+	–	+	–	–
Lipase (C14)	–	–	–	+	–
Cystine arylamidase	–	–	+	+	–
Acid phosphatase	+	W	–	+	+

The G+C content (43.9 mol%) of 5YN5-8^T was in the range 38-47 mol% reported for *Acinetobacter* species (Juni, 1984). All the *Acinetobacter* species shown in Table 2 contained $C_{18:1}$ $\omega 9c$ and summed feature 4 and $C_{16:0}$ as the common major fatty acids; however, strain 5YN5-8^T and *Acinetobacter haemolyticus* DSM 6962^T contained the highest amount of $C_{18:1}$ $\omega 9c$ (42.4 and 30.8%). *Acinetobacter calcoaceticus* DSM 30006^T, *Acinetobacter johnsonii* DSM6963^T, and *Acinetobacter schindleri* DSM16038^T had the highest proportion of summed feature 4 (30.7-40.2%). Strain 5YN5-8^T especially had a small amount of $C_{16:1}$ $\omega 9c$ (2.1%) as the characteristic fatty acid (Table 2). Strain 5YN5-8^T showed a DNA-DNA hybridization value of 28% with *Acinetobacter calcoaceticus* DSM 30006^T, 20% with *Acinetobacter haemolyticus* DSM 6962^T, 20% with *Acinetobacter johnsonii* DSM 6963^T, and 16% with *Acinetobacter schindleri* DSM 16038^T. These hybridization values strongly support that strain 5YN5-8^T represent a novel species within the genus *Acinetobacter* (Wayne *et al*., 1987). Based on the above results, a new species *Acinetobacter brisouii* with type strain 5YN5-8^T (=KACC 11602^T =DSM 18516^T) is proposed.

Description of *Acinetobacter brisouii* sp. nov.

Acinetobacter brisouii (bri.so.u´ii. N.L. gen. n. *brisouii* in honour

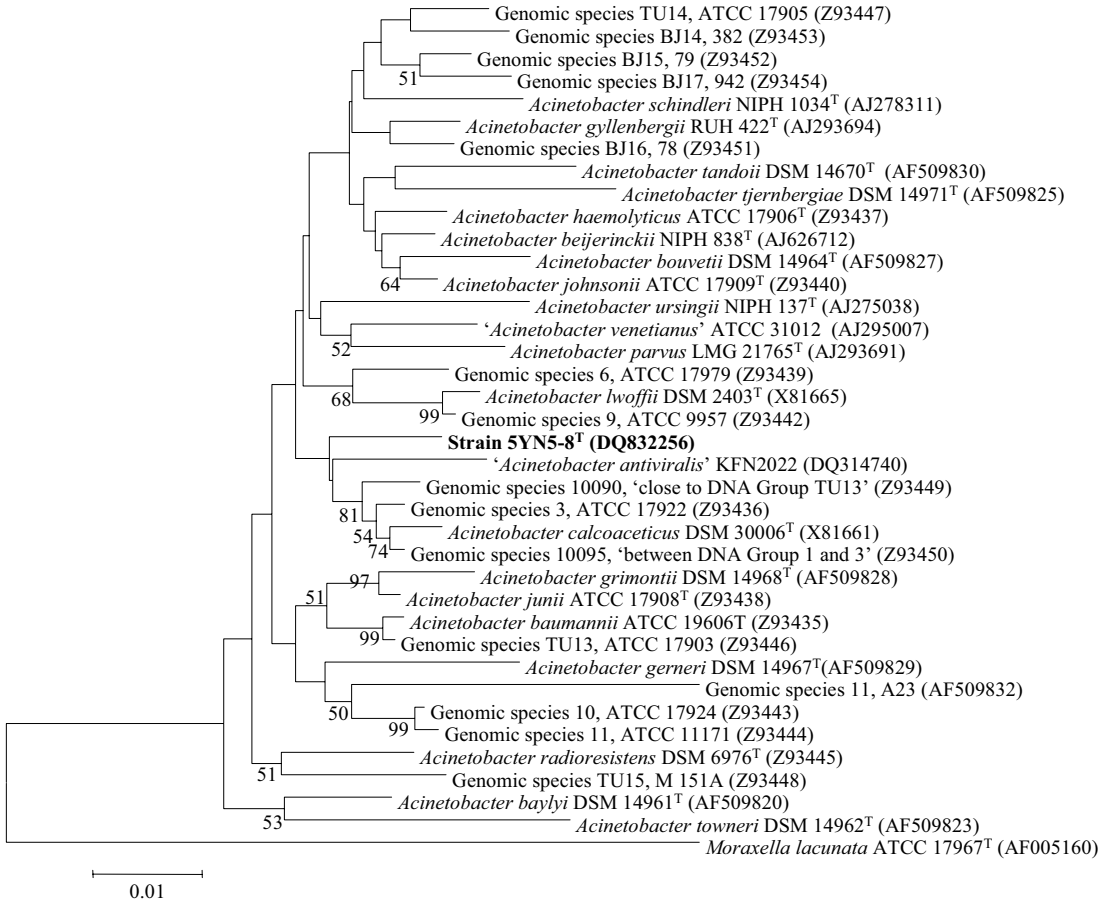

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences. The position of strain 5YN5-8^T is shown with respect to other closely related species. The tree was generated using the neighbor-joining method. Numbers at the nodes indicate bootstrap values, expressed as percentages of 1,000 replications; only values >50% are shown. Bar, 0.01 changes per nucleotide position.

Table 2. Fatty acid profiles of strain 5YN5-8^T and other closely related *Acinetobacter* species

1, 5YN5-8^T; 2, *Acinetobacter calcoaceticus* DSM 30006^T; 3, *Acinetobacter haemolyticus* DSM 6962^T; 4, *Acinetobacter johnsonii* DSM6963^T; 5, *Acinetobacter schindleri* DSM16038^T. Only fatty acids that represent more than 1% of total fatty acids are indicated. Prior to fatty acid extraction all the strains were cultivated in R2A medium for 48 h at 30°C.

Fatty acid	1	2	3	4	5
$C_{10:0}$	–	–	–	1.6	–
$C_{12:0}$	4.8	7.5	7.7	10.7	7.9
$C_{12:0}$ 2-OH	4.5	3.4	6.2	1.2	–
$C_{12:0}$ 3-OH	3.0	5.8	9.3	7.9	4.7
$C_{14:0}$	1.1	1.1	–	–	1.3
$C_{16:1}$ $\omega 9c$	2.1	–	–	–	–
$C_{16:0}$	16.7	14.3	18.2	17.1	18.3
$C_{17:0}$	–	1.6	–	–	–
$C_{17:1}$ $\omega 8c$	1.0	3.7	–	–	–
$C_{18:1}$ $\omega 9c$	42.4	23.7	38.0	19.6	20.1
$C_{18:1}$ $\omega 7c$	1.4	2.2	–	4.6	2.4
$C_{18:0}$	–	–	1.3	–	1.1
$C_{20:0}$	–	1.0	–	–	–
Summed feature 3[a]	2.7	2.4	–	–	1.3
Summed feature 4[a]	17.1	30.7	14.1	33.7	40.2

[a] Summed features contain the following fatty acids: summed feature 3, $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I; summed feature 4, $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ $\omega 7c$.

of Jean Brisou, a French microbiologist who has contributed to the understanding of the taxonomy of this genus).

In addition to the results given in the Table 1, strain 5YN5-8^T displays the following properties; Cells of strain 5YN5-8^T are Gram-negative, non-spore forming, non-motile, and coccobacilli with the size of 0.8-1.5 μm. Colonies of strain 5YN5-8^T are smooth, glistening, slightly convex, and milky-white in color. It is found to be positive for catalase and negative for oxidase tests. Growth occurs on R2A, TSA, and NA, but not on MacConkey agar. Growth occurs at 10-40°C (optimum 30°C). pH range for growth is 4.0-9.0 (optimum pH 7.0). Growth is inhibited by >2% NaCl. It hydrolyzes Tween 80, but not casein, chitin, carboxymethylcellulose, DNA, pectin, starch, and tyrosine. Strain 5YN5-8^T assimilates capric acid, malic acid, sodium acetate, L-alanine, propionic acid, valeric acid, and L-proline. Does not assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-maltose, potassium gluconate, L-rhamnose, D-ribose, inositol, D-saccharose, itaconic acid, potassium 5-ketogluconate, glycogen, salicin, D-melibiose, L-fucose, D-sorbitol, and potassium 2-ketogluconate. Strain 5YN5-8^T showed positive activities for esterase (C4), esterase lipase (C8), leucine arylamidase, and naphthol-AS-BI-phosphohydrolase. Strain 5YN5-8^T exhibited negative response for nitrate reduction,

indole production, arginine dihydrolase, aesculin hydrolysis, valine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Predominant cellular fatty acids are $C_{18:1}$ $\omega 9c$, summed feature 4 containing $C_{15:0}$ iso 2-OH and/or $C_{16:1}$ $\omega 7c$, and $C_{16:0}$. The DNA G+C content of the type strain is 43.9 mol%.

The type strain is 5YN5-8^T (=KACC 11602^T =DSM 18516^T), which was isolated from Yongneup, Republic of Korea.

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