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Acinetobacter soli sp. nov., Isolated from Forest Soil

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A non-motile and rod shaped bacterium, designated strain B1^T, was isolated from forest soil at Mt. Baekwoon, Republic of Korea. Cells were Gram-negative, catalase-positive, and oxidase-negative. The major fatty acids were 9-octadecenoic acid ($C_{18:1}$ *ω9c*; 42%) and hexadecanoic acid ($C_{16:0}$; 25.9%) and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*; 10.0%). The DNA G+C content was 44.1 mol%. A phylogenetic tree based on 16S rRNA gene sequences showed that strain B1^T formed a lineage within the genus *Acinetobacter* and was closely related to *A. baylyi* DSM 14961^T (98.6% sequence similarity), followed by *A. baumannii* DSM 30007^T (97.4%), *A. calcoaceticus* DSM 30006^T (97.0%) and 3 genomic species (96.8~7.6%). Phenotypic characteristics, *gyrB* gene sequence analysis and DNA-DNA relatedness data distinguished strain B1^T from type strains of *A. baylyi*, *A. baumannii*, and *A. calcoaceticus*. On the basis of the evidence presented in this study, strain B1^T represents a novel species of the genus *Acinetobacter*, for which the name *Acinetobacter soli* sp. nov. is proposed. The type strain is B1^T (= KCTC 22184^T= JCM 15062^T).

***Keywords*:** *Acinetobacter soli*, *gyrB* gene, DNA-DNA relatedness

Members of the genus *Acinetobacter* are ubiquitously distributed in nature. The genus *Acinetobacter* comprises non-motile, strictly aerobic, oxidase-negative, and Gram-negative bacteria that grow well on simple media. Thirty-two (genomic) species are currently recognized within the genus (Bouvet and Grimont, 1986; Bouvet and Jeanjean, 1989; Tjernberg and Ursing, 1989; Gerner-Smidt and Tjernberg, 1993; Vaneechoutte *et al.*, 1999; Nemec *et al*., 2001; Carr *et al.*, 2003; Nemec *et al.*, 2003). Seventeen of these have been provided with valid species names. Among them, nine species were the clinical isolates, i.e. *Acinetobacter baumannii* DSM 30007^T, *Acinetobacter junii* DSM 6964^T and *Acinetobacter johnsonii* DSM 6963^T, *Acinetobacter haemolyticus* DSM 6962^T and *Acinetobacter lwoffii* DSM 2403^T described or emended by Bouvet and Grimont (1986), *Acinetobacter schindleri* LMG 19576^T and *Acinetobacter ursingii* LUH 3792^T described by Nemec *et al*. (2001) and *Acinetobacter parvus* LMG 21765^T described by Nemec *et al*. (2003). Two species were isolated from the soil, i.e. *Acinetobacter radioresistens* DSM 6976^T described by Nishimura *et al*. (1988) and *Acinetobacter calcoaceticus* DSM 30006^T described or emended by Bouvet and Grimont (1986). Seven species were isolated from the activated sludge (Carr *et al*., 2003), i.e. *Acinetobacter baylyi* DSM 14961^T, *Acinetobacter bouvetii* DSM 14964^T, *Acinetobacter grimontii* DSM 14968^T, *Acinetobacter tjernbergiae* DSM 14971^T, *Acinetobacter towneri* DSM 14962^T, *Acinetobacter tandoii* DSM 14670^T, and *Acinetobacter gerneri* DSM 14967^T.

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In the course of our study on the microbial diversity in forest soil, a bacterium, designated strain B1^T, was isolated and could not be identified as any known (genomic) species. On the basis of polyphasic evidence, strain B1^T represents a novel species in the genus *Acinetobacter*, for which the name *Acinetobacter soli* sp. nov. is proposed.

Materials and Methods

Collection of microorganisms

Strain B1^T was isolated from forest soil collected from Mt. Baekwoon (35°06' N, 127°37' E) near Gwangyang located in the Republic of Korea, during May 2007, using the standard dilution plating technique. Isolation was achieved using Plate Count Agar (PCA, Difco) at 25°C for 7 days. The isolate was routinely cultured on Tryptic Soy Agar (TSA, Difco) and maintained as a glycerol suspension (20%, w/v) at -80°C.

Reference strains, *Acinetobacter baylyi* KACC 12224^T (= DSM 14961^T), *Acinetobacter baumannii* KACC 12454^T (= DSM 30007^T), and *Acinetobacter calcoaceticus* KACC 11541^T (= DSM 30006^T) were obtained from the Korean Agricultural Culture Collection (KACC), Suwon, Republic of Korea.

Phylogenetic analyses

Bacterial DNA preparation, PCR amplification and sequencing of 16S rRNA gene were carried out as described previously (Chun and Goodfellow, 1995). The resultant sequence of strain B1^T (1,422 nt) was aligned manually against sequences obtained from the GenBank database. Calculation of pairwise 16S rRNA gene sequence similarity was achieved

using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007).

Phylogenetic trees were inferred from the regions available in all sequences (positions 39∼1450; *Escherichia coli* numbering system) using the Fitch-Margoliash (Fitch and Margoliash, 1967) and neighbor-joining (Saitou and Nei, 1987) methods. Evolutionary distance matrices were generated according to Jukes and Cantor (1969). The resultant neighbor-joining tree topology was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings. Alignment and phylogenetic analyses were carried out using the jPHYDIT program (available at http://plaza.snu.ac.kr/∼jchun/) and PAUP 4.0 (Swofford, 1998) as described previously (Chun *et al*., 2000).

PCR amplification and sequencing of *gyrB* gene were carried out as described previously (Yamamoto and Harayama, 1995). UP-1 and UP-2r were used for PCR primers and UP-1S and UP-2Sr were used for sequencing primers. The *gyrB* gene sequences of strain B1^T (927 nt) were aligned using the CLUSTAL W computer program (Thompson *et al*., 1994). Sequence comparisons and phylogenetic analyses were carried out using the methods described above.

Phenotypic characterization

Growth on various standard bacteriological media was tested by using Nutrient Agar (NA, Difco), TSA (Difco), Marine Agar (MA, Difco), Glucose Yeast Extract Agar (GYEA, Gordon and Mihm, 1962), MacConkey agar and R2A agar (Difco) according to the manufacturer's instructions. Cells of strain B1^T grown on TSA at 30°C for 1∼2 days were used for the physiological and biochemical tests. Motility was examined by observing the cells grown in wet mounts using phase-contrast microscopy (TMS-F, Nikon). Growth at various NaCl concentrations (0∼10%, w/v, using increments of 1.0%) was investigated in Tryptic Soy Broth (TSB, Difco) prepared according to the formula of Difco medium except that no NaCl was used. The pH range for growth was determined in TSB. The medium adjusted to various pH values (pH 4∼11, using increments of 1 pH units) by the addition of HCl and NaOH was sterilized by filtration. Growth temperature (4∼42°C) and growth in an anaerobic chamber ($CO_2/H_2/N_2$, 10:10:80; Sheldon Manufacturing) were checked using TSA in one week incubation time. Catalase and oxidase activities were determined using 3% (v/v) hydrogen peroxide and Kovac's reagent (Kovacs, 1956), respectively. Haemolysis of sheep blood was recorded after 2 days on TSA containing 5% sheep blood. Production of acid from glucose was detected by using the method A described previously (Bouvet and Grimont, 1986). Nitrate reduction was tested on nitrate broth containing 0.2% KNO_3

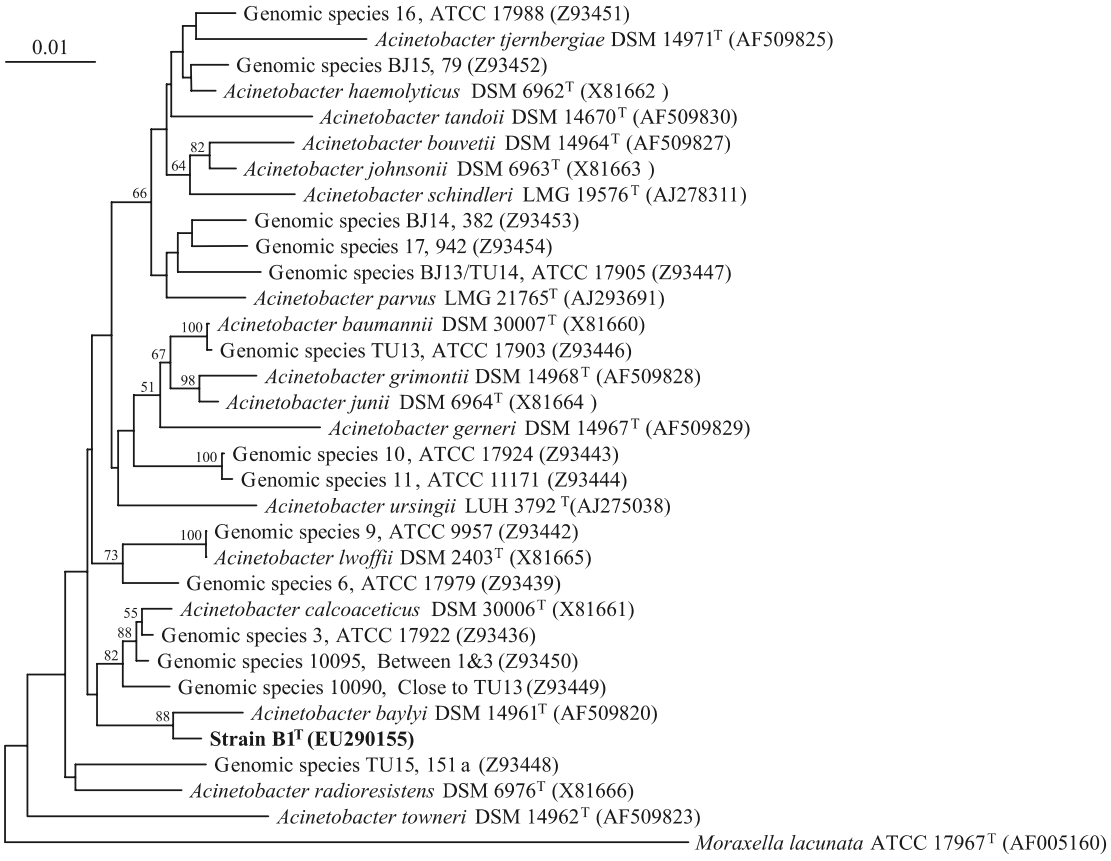

Fig. 1. Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between strain B1^T and the other members of the genus *Acinetobacter*. The percentage numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets. The sequence of *Moraxella lacunata* ATCC 17967^T (AF005160) was used as an outgroup. Bar, 0.01 nucleotide substitution per position.

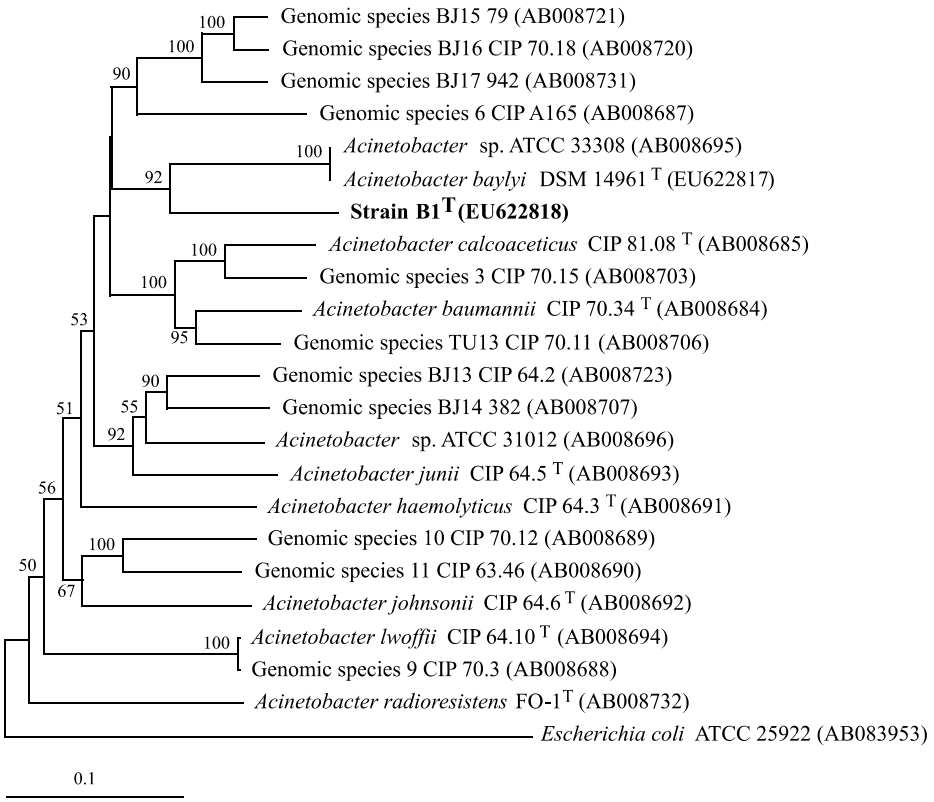

Fig. 2. Neighbor-joining tree based on *gyrB* gene sequences showing relationships between strain B1^T and the other members of the genus *Acinetobacter*. The percentage numbers at the nodes are the levels of bootstrap support based on neighbor-joining analyses of 1,000 resampled data sets. The sequence of *Escherichia coli* ATCC 25922 (AB083953) was used as an outgroup. Bar, 0.1 nucleotide substitution per position.

(Skerman, 1967). Citrate utilization was tested on Simmons' Citrate agar (Sigma). Indole production was determined with Kovac's indole reagent in 1% Tryptone broth. H_2S production was determined on Kligler Iron agar (Difco). L-phenylalanine deamination was examined with the method of Richard and Kiredjian (1995). Urease activity was tested on Christensen's medium (Oxoid). Degradation of casein, starch, xylan, carboxymethylcellulose (CMC), and L-tyrosine was examined on TSA containing 2% skim milk, 0.2% soluble starch, 1% xylan, 1% sodium carboxymethylcellulose, and 0.5% L-tyrosine (Smibert and Krieg, 1994), respectively. Voges-Proskauer test was analyzed as described by Smibert and Krieg (1994).

Additional physiological and biochemical tests were performed using API 20E, API 20NE, API 50CH, and API ZYM systems (bioMérieux) and the GN2 MicroPlate (Biolog) according to the manufacturers'.

Antibiotic resistance was determined with disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton-Dickinson). The results were interpreted according to the guidelines set forth by the CLSI (2003).

Chemotaxonomy

Fatty acid profiles of strain B1^T and the type strains were determined on the identical conditions. Cells grown on TSA for 24 h at 30°C were prepared and analyzed as methyl esters by GLC according to the instructions of the Microbial Identification System (MIDI, 1999).

Isoprenoid quinones were extracted according to the method of Minnikin *et al.* (1984) and analyzed by reverse-phase thin-layer partition chromatography using Merck HPTLC RP18F_{254} as described (Collins and Jones, 1981).

The G+C content of the DNA was determined by using the thermal denaturation method of Marmur and Doty (1962).

DNA-DNA hybridization

The taxonomic relationships between strain B1^T and the type strains of *A. baylyi*, *A. calcoaceticus*, and *A. baumanii* were further examined using DNA-DNA hybridization. Genomic relatedness was determined using a membrane filter technique (Seldin and Dubnau, 1985) according to the method described by Baik *et al*. (2006).

Results and Discussion

Phylogenetic analyses

Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that strain B1^T was closely related to the genus *Acinetobacter.* The newly determined sequence was then aligned manually against representatives of the genus *Acinetobacter.* Strain B1^T showed the highest 16S rRNA gene sequence similarity with *Acinetobacter baylyi* DSM 14961^T (98.6%), followed by *Acinetobacter baumannii*

DSM 30007^T (97.4%), *Acinetobacter calcoaceticus* DSM 30006^T (97.0%), and 3 genomic species (96.8~97.6%). Each of the related species of strain B1^T was originated from different sources, i.e. *A. baylyi* DSM 14961^T, from activated sludge (Carr *et al.*, 2003), *A. baumannii* DSM 30007^T, from clinical specimen (Bouvet and Grimont, 1986) and *A. calcoaceticus* DSM 30006^T, from soil (Bouvet and Grimont, 1986).

To elucidate the phylogenetic relationship between the novel isolate and other species of the genus *Acinetobacter*, phylogenetic trees were constructed using two different tree-making algorithms. The neighbor-joining tree (Fig. 1) showed that strain B1^T was closely related to *A. baylyi* DSM 14961^T with 88% bootstrap support. Also, strain B1^T occupied a distinct position in the neighbour-joining tree. The tree based on the Fitch-Margoliash method showed essentially similar topology (data not shown).

Strain B1^T showed the highest *gyrB* gene sequence similarity with *Acinetobacter baylyi* DSM 14961^T (83.6%), followed by *Acinetobacter baumannii* DSM 30007^T (80.5%) and *Acinetobacter calcoaceticus* DSM 30006^T (79.2%). The neighbor-joining tree (Fig. 2) showed that strain B1^T was closely related to *A. baylyi* DSM 14961^T with 92% bootstrap support. Also, strain B1^T occupied a distinct position in the neighbour-joining tree, as was like in the 16S rRNA gene sequence analysis.

Phenotypic characteristics

Strain B1^T was aerobic, Gram-negative and non-motile. Cells from overnight broth culture were predominantly short rods (0.8~1.0 by 1.2~1.5 μm) and occurred in pairs. Occasionally short chains (three to six cells) occurred. Colonies grown on TSA were circular, convex, entire margin, smooth, and slightly opaque. On TSA, the colonies of 24 and 48 h incubation at 30°C were 0.5~0.8 and 1~2 mm in diameter, respectively. The novel strain grew well on NA, MA, TSA, PCA, R2A, MacConkey agar, and GYEA. On TSA medium, strain B1^T was able to grow at 10~37°C with the optimum at 30°C. Detailed results of morphological, physiological and biochemical tests are given in the species description

Table 1. Characteristics that differentiate strain B1^T from related *Acinetobacter* species

Characteristic	1	2	3	4
Habitat	Soil	Human clinical specimen[a]	Activated sludge[a]	Soil[a]
Growth temperature	10~41	15~44[a]	37~41[a]	15~37[a]
Urease activity	+	-	+	-
Assimilation of (API 20NE)				
Malate	+	+	+	-
Caprate	+	+	+	-
Phenyl acetate	+	+	-	-
Utilization of (API 50CH)				
L-Arabinose	+	-	+	+
Ribose	+	-	+	+
D-Xylose	+	-	+	+
Galactose	+	-	+	+
Fructose	+	-	-	-
Mannose	+	-	+	+
Melibiose	+	-	-	-
D-Fucose	+	-	-	-
Enzyme activity (API ZYM)				
Alkaline aphosphatase	-	-	-	+
Esterase (C4)	-	-	-	+
Lipase (C14)	+	+	+	-
Acid phosphatase	-	+	+	w
DNA G+C content (mol%)	44.1	40~43[a]	NA	40~42[a]

[a] Data from Bouvet and Grimont (1986) and Carr *et al*. (2003)

Species: 1, B1^T; 2, *A. baumannii* KACC 12454^T; 3, *A. baylyi* KACC 12224^T; 4, *A. calcoaceticus* KACC 11541^T. Data are from this study unless otherwise indicated. All species are positive for catalase, utilization of citrate (Simmons), glucose, DL-lactic acid, malonic acid, quinic acid, L-aspartic acid and adipate and hydrolysis of Tween 20 and Tween 80 and acid production from glucose. All species are negative for haemolysis, and production of L-phenylalanine deamiase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane deaminase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-glucosaminidase, α-mannosidase and α-fucosidase, and assimilation of mannose, glycerol, erythritol, D-arabinose, L-xylose, adonitol, β-methyl-D-xyloside, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, α-methyl- D-mannoside, α-methyl-D-glucoside, N-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, raffinose, xylitol, β-gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto-gluconate, 5-keto-gluconate, inulin, starch and glycogen, and degradation of starch, xylan, CMC, esculin, gelatin, and production of H_2S, indole and acetoin, and reduction of nitrates to nitrites. +, positive; -, negative; w, weak positive; NA, data not applicable.

Table 2. Fatty acid composition (%) of strain B1^T and related *Acinetobacter* species

Fatty acid	1	2	3	4
$C_{12:0}$	5.6	5.7	5.7	5.1
$C_{12:0}$ 2-OH	1.8	2.3	2.0	1.9
$C_{12:0}$ 3-OH	3.8	3.5	4.2	4.0
$C_{16:0}$ *N* alcohol	1.5	1.2	Tr	1.2
$C_{16:1}$ *ω7c* alcohol	-	-	-	2.5
$C_{16:0}$	25.9	21.3	23.5	16.8
$C_{17:1}$ *ω8c*	-	1.7	-	1.4
$C_{17:0}$	-	1.2	Tr	1.1
$C_{18:3}$ *ω6c*	1.5	1.8	-	1.1
$C_{18:1}$ *ω9c*	42.0	38.0	38.4	31.1
$C_{18:1}$ *ω7c*	Tr	Tr	1.0	2.5
$C_{18:0}$	4.0	3.4	1.4	3.5
Sum In Feature 2[a]	1.8	3.4	1.8	2.2
Sum In Feature 3[a]	10.0	13.7	18.7	23.3

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained $C_{14:0}$ 3-OH and/or iso-$C_{16:1}$ I. Summed feature 3 contained iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*.
Species: 1, B1^T; 2, *A. baumannii* KACC 12454^T; 3, *A. baylyi* KACC 12224^T; 4, *A. calcoaceticus* KACC 11541^T. Data are from this study. Tr, trace amount (<1%); -, not detected.

and in Table 1. It is evident from Table 1 that there are several phenotypic characters that readily separate strain B1^T from the type strains of phylogenetically related species, namely *A. baylyi*, *A. baumannii*, and *A. calcoaceticus*.

Chemotaxonomy

The major fatty acids of strain B1^T are shown in Table 2. The predominant fatty acids of strain B1^T were 9-octadecenoic acid ($C_{18:1}$ *ω9c*; 42%) and hexadecanoic acid ($C_{16:0}$; 25.9%) as were the cases for the type strains of *A. baylyi*, *A. calcoaceticus*, and *A. baumanii*. Ubiquinone 9 was the only isoprenologue found in strain B1^T. The DNA G+C ratio of strain B1^T was 44.1 mol%.

DNA-DNA relatedness

DNA relatedness of strain B1^T with the type strains of *A. calcoaceticus*, *A. baumanii*, and *A. baylyi* were 16, 29, and 39%, respectively.

It is clear from the phylogenetic analyses based both on 16S rRNA gene and *gyrB* gene sequences and DNA-DNA hybridization data that strain B1^T represents a novel species in the genus *Acinetobacter* (Wayne *et al*., 1987). In addition, a number of physiological and chemotaxonomic characteristics clearly distinguished strain B1^T from other phylogenetically related species (Table 1 and 2). Therefore, strain B1^T should be classified in a novel species within the genus *Acinetobacter*, for which the name *Acinetobacter soli* sp. nov. is proposed.

Description of *Acinetobacter soli* sp. nov.

Acinetobacter soli (so'li. L. gen. neut. n. *soli*, of/from soil). Cells are rod-shaped, Gram-negative, catalase-positive, oxidase-negative, and aerobic bacterium. Cells grow best on media such as MA, TSA, PCA, NA, MacConkey agar, and R2A. Colonies on TSA agar are circular, low-convex, entire margin, smooth, slightly opaque, and approximately 0.5~0.8 mm in diameter after 24 h incubation at 30°C (pH 7). Cells are non-motile short rods and 0.8~1.0×1.2~1.5 μm in size. Growth occurs in 0~5% (w/v) NaCl (optimum 0.5~1.0%). Growth occurs in pH 5~10 (optimum pH 6~8) and at 10~40°C (optimum 30°C). Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities are absent. Degrades L-tyrosine, but not casein and starch. Citrate is utilized. Positive for acid production from glucose. Negative for haemolysis of sheep blood and nitrate reduction. Does not produce H_2S, acetoin and indole. Utilizes the following substrates as sole carbon and energy sources: glucose, gluconate, caprate, adipate, citrate, and phenyl acetate. Does not utilize arabinose. In the API 50CH tests, acid is produced from L-arabinose, frucose, D-fucose, galactose, mannose, melibiose, ribose, and D-xylose. In the API ZYM gallery, lipase (C14) is present, but acid phosphatase, alkaline phosphatase, and esterase (C4) activities are absent. Cells are sensitive to (μg per disc, unless otherwise indicated) ampicillin (10), penicillin (10 IU) and vancomycin (30), but resistant to amikacin (30), chloramphenicol (30), erythromycin (15), gentamicin (10), kanamycin (30), nalidixic acid (30), polymyxin B (300 IU), streptomycin (10), and tetracycline (30). Other physiological and biochemical characteristics are given in Table 1. Major fatty acids are 9-octadecenoic acid ($C_{18:1}$ *ω9c*; 42.0%), hexadecanoic acid ($C_{16:0}$; 25.9%) and summed feature 3 (comprising iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*; 10.0%), and complete fatty acid composition is given in Table 2. The predominant respiratory lipoquinone was Q-9. The DNA G+C content is 44.1 mol%. Other physiological and biochemical characteristics are given in Table 1 and Table 2.

The type strain is B1^T (= KCTC 22184^T= JCM 15062^T) and isolated from a forest soil collected from the Mt. Baekwoon, Republic of Korea.

Acknowledgements

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