Full Paper

Leifsonia lichenia sp. nov., isolated from lichen in Japan

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An actinobacterial strain, 2Sb^T, isolated from lichen was characterized taxonomically using a polyphasic approach. Strain 2Sb^T was Gram-positive, strictly aerobic, rod-shaped and non-motile. Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain 2Sb^T was located in the genus *Leifsonia*. Level of 16S rRNA gene sequence similarity between the isolated strains and the type strains of *Leifsonia* species were 95.0–99.2%. The value of DNA-DNA relatedness between strain 2Sb^T and *Leifsonia poae*, a phylogenetically related and phenotypically similar species, was 39.9/25.2%. The DNA G+C content of the strain 2Sb^T was 61.7 mol%. Cell wall peptidoglycan type (2,4-diaminobutyric acid), major cellular fatty acids (anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}) and quinone type (MK-11 and MK-10) of the isolate support their affiliation to the genus *Leifsonia*. On the basis of phylogenetic position, physiological and chemotaxonomic properties, strain 2Sb^T represent a novel species of the genus *Leifsonia*, for which the name *Leifsonia lichenia* sp. nov. is proposed. The type strain is 2Sb^T (=IAM 15426^T =JCM 23226^T =KCTC 13122^T).

Key Words—*Leifsonia lichenia* sp. nov.

Introduction

The genus *Leifsonia* was first described by Evtushenko et al. (2000). At present, genus *Leifsonia* comprises ten recognized species (An and Yokota, 2007; Dastager et al., 2008; Evtushenko et al., 2000; Qiu et al., 2007; Reddy et al., 2003, 2008; Suzuki et al., 1999), with *Leifsonia aquatica* as the type species. Members of the genus *Leifsonia* are Gram-positive, non-spore-forming, irregular rod- or filament-shaped and catalase-positive bacteria containing (DL)-2,4-diaminobutyric acid in the peptidoglycan, MK-11 as the major menaquinone and it has a high content of anteiso- and iso-branched fatty acids.

Here, we report the taxonomic study of a strain, 2Sb^T, isolated from lichen in Tokyo, Japan, in the

course of environmental investigation. On the basis of phenotypic characteristics, chemotaxonomic data and phylogenetic analysis of the 16S rRNA gene sequence, we propose *Leifsonia lichenia* sp. nov. for the isolate.

Materials and Methods

Bacterial strain. Strain 2Sb^T was isolated from lichen collected at the Botanical Garden of the University of Tokyo (Koishikawa, Bunkyo-ku, Tokyo, Japan). The lichen sample was washed five times with sterilized water and was homogenized in sterilized water. The suspension was diluted serially, spread on modified Detmer medium [1.0 g KNO₃, 0.25 g MgSO₄·7H₂O, 0.25 g K₂HPO₄, 0.1 g NaCl, 10 mg CaCl₂·2H₂O, 10 ml Fe solution (1.0 g FeSO₄·7H₂O, 0.5 L distilled water, and 2 drops conc. H₂SO₄), 1.0 ml A5 solution (2.86 g H₃BO₃, 2.5 g MnSO₄·7H₂O, 0.222 g ZnSO₄·7H₂O, 79.0 mg CuSO₄·5H₂O, 21.0 mg Na₂MoO₄, 1 L distilled water), in 1 L distilled water pH 8.0] and incubated at 30°C. The colony was selected and further purified us-

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ing nutrient agar medium.

Culture media and growth conditions. Growth tests of the temperature and pH were carried out in nutrient broth. The cell morphology and motility were examined by using phase-contrast microscopy (BX60 microscope; Olympus, Tokyo, Japan). The growth under anaerobic conditions was determined after a week of incubation in an AnaeroPack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Biochemical analyses. Catalase activity was tested by adding a drop of 3% H₂O₂ to a single colony and was recorded as positive when development of bubbles was observed. Oxidase activity was determined by cytochrome oxidase paper (Nissui Pharmaceutical Co., Inc., Tokyo, Japan). Temperature for growth was measured from 5 to 35°C. pH range for growth was determined in nutrient broth initially adjusted to various pH values (initial pH 4.5–9.5 at intervals of 0.5 pH units). Prior to sterilization the pH was adjusted to various levels by using HCl and NaOH. API 20E microtest galleries (bioMérieux, Marcy-l'Etoile, France) were used to determine the physiological and biochemical characteristics. The results were recorded after 48 h of incubation at 30°C.

Analysis of sequence data and construction of a phylogenetic tree. The DNA was prepared according to the method of Marmur (1961). The 16S rRNA gene was amplified and sequenced as previously described (Xie & Yokota, 2003). The DNA sequence of strain 2Sb^T was determined and used for a Blast search via NCBI (National Centre for Biotechnology Information). The obtained nucleotide sequences were aligned with the CLUSTAL X software package ver. 1.83 (Thompson et al., 1997), and the evolutionary distances and K_{nuc} value (Kimura, 1980) were generated. Alignment gaps and ambiguous bases were not taken into consideration when the 16S rRNA gene sequences were compared. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1,000 replicates. The similarity values were calculated using MEGA3 (Kumar et al., 2004).

Genomic DNA analysis. The G+C content of the total DNA was measured by HPLC according to the method described by Mesbah et al. (1989). DNA-DNA hybridization was performed by the photobiotin-labeling method of Ezaki et al. (1989) using a multi-well Plate Reader (CytoFluoR; PerSeptive Biosystems).

Chemotaxonomic investigation. Respiratory quinone analysis was performed according to the method described by Komagata and Suzuki (1987). The quinone fractions were separated with thin-layer chromatography (TLC) developed with hexane : diethyl ether (85 : 15, v/v). A quinone spot was detected under UV light (254 nm), the spot was scraped off, and quinones were extracted with acetone. After concentration the quinone samples were analyzed with high-performance liquid chromatography (HPLC), model LC-10AD VP (Shimadzu, Kyoto, Japan). The cell wall of strain 2Sb^T was prepared by the method described by Schleifer and Kandler (1972), and amino acid composition of complete wall hydrolysates was determined by two-dimensional chromatography on cellulose TLC (Harper and Davis, 1979) and by HPLC, as the phenylthiocarbamoyl derivatives (Wako Pure Chemical Ind., Osaka, Japan), with a model LC-10AD VP HPLC apparatus. Cellular fatty acid methyl esters were prepared, separated and identified with the Microbial Identification System (MIDI, Inc., Newark, DE, USA).

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence determined in this study is AB278552 (IAM 15426^T = JCM 23226^T = KCTC 13122^T).

Results

Morphological and physiological characteristics

The cells of strain 2Sb^T are Gram-positive, strictly aerobic, rod-shaped and non-motile. Morphological and physiological characteristics of the isolate are given in the species description.

Phylogenetic analysis

Almost complete 16S rRNA gene sequences of the strain 2Sb^T was determined and subjected to comparative analysis. The highest degree of 16S rRNA gene sequence similarity was found with *Leifsonia poae* (99.2%) followed by *Leifsonia shinshuensis* (99.1%), *Leifsonia xyli* (98.8%), *Leifsonia naganoensis* (98.6%), *Leifsonia cynodontis* (98.6%), *Leifsonia aquatica* (98.5%), *Leifsonia bigeumensis* (97.4%), *Leifsonia ginsengi* (97.0%), *Leifsonia pindariensis* (96.6%) and *Leifsonia aureus* (95.0%). The phylogenetic tree indicated that strain 2Sb^T is included in the genus *Leifsonia* (Fig. 1).

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain 2Sb^T and other related taxa.

The numbers at the nodes indicate the percentages of occurrence in 1,000 bootstrapped trees; only values greater than 50% are shown.

Genomic DNA analysis

The DNA G+C content of strain 2Sb^T was 61.7 mol%, which is lower than for the other species of genus *Leifsonia* (Table 1). DNA-DNA relatedness to *Leifsonia poae*, the one with the highest degree of 16S rRNA gene sequence similarity among phylogenetically related species, was 39.9/25.2%. DNA-DNA hybridization to other species of *Leifsonia* was not attempted because of a great difference in G+C content between these species and strain 2Sb^T (4.3–9.0%; Table 1).

Physiological and chemotaxonomic characteristics

The fatty acid composition of strain 2Sb^T was anteiso-C_{15:0} (32.0%), anteiso-C_{17:0} (31.9%), iso-C_{16:0} (27.5%), iso-C_{17:0} (3.7%), iso-C_{15:0} (3.6%), C_{16:0} (0.6%), iso-C_{14:0} (0.4%), iso-C_{18:0} (0.2%), and iso-C_{15:1} (0.1%). This fatty acid profile is quite similar to those of the species of *Leifsonia*. The cell wall of strain 2Sb^T contained glutamic acid, glycine, alanine, and 2,4-diaminobutyric acid in a molar ratio of about 1.0 : 2.0 : 1.1 : 1.3. The isoprenoid quinone type was MK-11 (84%) and MK-10 (16%).

Discussion

Strain 2Sb^T is able to be classified under the genus *Leifsonia*, but the isolate can be differentiated by some phenotypic characteristics (Table 1) such as motility, growth temperature, oxidase activity, H₂S production, major fatty acids, major menaquinone and G+C content. In particular, DNA G+C content is over 8.6% lower than the type species of genus *Leifsonia*. *Leifsonia poae* showed the highest degree of 16S rRNA gene sequence similarity, but DNA-DNA relatedness was 39.9/25.2%. These levels of DNA-DNA hybridization are lower than the threshold value of 70% for separate species delineation.

In summary, based on the physiological, chemotaxonomic, and phylogenetic characteristics, we conclude that strain 2Sb^T belong to a novel species of genus *Leifsonia*, for which the name *Leifsonia lichenia* sp. nov. is proposed.

Description of *Leifsonia lichenia* sp. nov.

Leifsonia lichenia (li.che'n.i.a. L. adj. *lichenia* lichen from which the organism was isolated).

Cells are Gram-positive, strictly aerobic, short single rods (0.5–1.0 × 1.5–2.5 µm) and non-motile. The colonies grown on nutrient agar medium are gummy and

Table 1. The differential characteristics of *L. lichenia* sp. nov. and phylogenetically related *Leifsonia* species.

Characteristics	1	2	3	4	5	6	7	8
Motility	–	+	–	–	–	–	+	+
Growth temperature (°C)	10–30	7–38	7–37	7–35	7–31	7–42	7–37	20–37
Oxidase	–	–	ND	–	–	ND	+	–
H ₂ S production	–	–	ND	–	–	ND	+	–
Acid production from:								
D-arabinose	+	+	ND	–	–	ND	+	–
galactose	–	+	–	–	–	+	+	–
glucose	–	–	–	+	+	+	+	–
Major fatty acids	ai-C _{15:0} ai-C _{17:0} i-C _{16:0}	ai-C _{17:0} ai-C _{15:0} i-C _{16:0}	ai-C _{15:0} ai-C _{17:0} i-C _{16:0}	ai-C _{17:0} ai-C _{15:0} i-C _{16:0}	ND	ai-C _{15:0} ai-C _{17:0} i-C _{16:0}	ai-C _{17:0} ai-C _{15:0} i-C _{16:0}	i-C _{16:0} ai-C _{15:0} ai-C _{17:0}
Major menaquinone(s)	MK-11	MK-11	MK-11, MK-12	MK-11 MK-12	ND	MK-11 MK-10	MK-11 MK-10	MK-11
G+C content (mol%)	61.7	69.5	70.7	69.4	66	70.5	70.3	69.0

Species: 1, *L. lichenia* sp. nov.; 2, *L. poae*; 3, *L. shinshuensis*; 4, *L. cynodontis*; 5, *L. xyli*; 6, *L. naganoensis*; 7, *L. aquatica*; 8, *L. bigeumensis*.

Data from Evtushenko et al., 2000; Suzuki et al., 1999; Reddy et al., 2003; Dastager et al., 2008; Qiu et al., 2007.

+, positive; –, negative; ND, not determined.

yellow. The conditions for growth are 10–30°C and pH 5.0–9.5, with optimum growth at 25°C and pH 7.0–8.0. NaCl is not required for growth but is tolerated up to 2% (w/v). Catalase, gelatinase and β-galactosidase activities, and citrate utilization are positive. H₂S, acetoin and indole are not produced. Oxidase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase activities are negative. Nitrate is reduced to nitrite. Nitrite is not reduced. Acid is produced from arabinose, but not from glucose, D-mannitol, inositol, D-sorbitol, L-rhamnose, sucrose, D-melibiose or D-amygdalin. Cell wall peptidoglycan type is 2,4-diaminobutyric acid. The quinone system is MK-11 and MK-10. The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, and iso-C_{16:0}. The G+C content of the DNA of the type strain is 61.7 mol%. The type strain is 2Sb^T (=IAM 15426^T =JCM 23226^T =KCTC 13122^T).

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