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Siphonobacter intestinalis sp. nov., a bacterium isolated from the feces of *Pseudorhynchus japonicus*[§]

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Strain 63MJ-2^T was isolated from the feces of broad-winged katydid (*Pseudorhynchus japonicus*) collected in Korea. The 16S rRNA gene sequence of this strain showed the highest sequence similarity with that of *Siphonobacter aquaeclarae* P2^T (96.1%) and had low similarities (below 86.3%) with those of other members of family '*Flexibacteraceae*'. The strain 63MJ-2^T is a strictly aerobic, Gram-stain-negative, non-motile, rod-shaped bacterium. The strain grew at 4–35°C (optimum, 25–30°C), pH of 5.0–9.0 (optimum, 6.0–7.0), and 0–2.0% (optimum, 1.0–2.0) (w/v) NaCl. The DNA G+C content of strain 63MJ-2^T was 43.5 mol%. The major fatty acids were $C_{16:1}$ *ω5c* (42.5%), iso-$C_{17:0}$ 3-OH (18.7%), and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*, 18.0%). The major menaquinone was MK-7 and polar lipids were phosphatidylethanolamine, six unknown aminolipids, and five unknown lipids. Based on the evidence from our polyphasic taxonomic study, we conclude that strain 63MJ-2^T should be classified as a novel species of the genus *Siphonobacter*, and propose the name *Siphonobacter intestinalis* sp. nov. The type strain is 63MJ-2^T (=KACC 18663^T =NBRC 111883^T).

Keywords: *Siphonobacter*, strain 63MJ-2, novel species, polyphasic taxonomy

Introduction

The genus *Siphonobacter* has been proposed by Táncsics *et al.* (2010) as a new genus of the family '*Flexibacteraceae*' in the phylum *Bacteroidetes*. The only species is *Siphonobacter aquaeclarae*, which was isolated from the biofilm developed on the inner surface of an ultrapure cooling water pipeline system (Táncsics *et al.*, 2010). This species characterized as Gram-negative, facultatively anaerobic, non-motile rods. Its predominant fatty acids are $C_{16:1}$ *ω5c*, iso-$C_{15:0}$ 2-OH, iso-$C_{17:0}$ 3-OH, and iso-$C_{15:0}$, whereas MK-7 is the major isoprenoid quinone and phosphatidylethanolamine is the main polar lipid.

Insect guts harbor a wide variety of microorganisms, which play essential roles in the growth and health of the hosts (Dillon and Dillon, 2004). It is likely that the number of microbes in the insect gut outnumber the total numbers of insect cells (Dillon and Dillon, 2004). During an investigation of bacterial diversity in insect gut, we isolated and characterized bacterial strains from the gut content i.e., feces. Based on the phenotypic and phylogenetic analyses, one of the fecal isolates from broad-winged katydid (*Pseudorhynchus japonicus*) was found to represent a novel species of the genus *Siphonobacter*. In this paper, we designate this novel species as *Siphonobacter intestinalis* sp. nov.

Materials and Methods

Bacterial strains

Broad-winged katydids were collected in Jinan-gun, Jeollabuk-do, the Republic of Korea (127°25′10″ E, 35°46′19″ N). The samples of fresh feces from the insects were diluted serially in 0.85% (w/v) saline solution, spread on R2A agar (Difco), and incubated for 7 days at 28°C. Bacterial colonies with unique morphologies were selected and subjected to 16S rRNA gene sequence analysis, and one of them, designated as strain 63MJ-2^T, was selected for polyphasic characterization. *Siphonobacter aquaeclarae* KACC 18773^T was obtained from the Korean Agricultural Culture Collection (KACC) for comparative taxonomic analysis.

Phylogenetic analysis

Chromosomal DNA was isolated with a Wizard Genomic DNA Purification kit (Promega). The 16S rRNA gene of strain 63MJ-2^T was amplified by polymerase chain reaction (PCR) and sequenced as described by Ahn *et al.* (2014). The near-complete 16S rRNA gene sequence of strain 63MJ-2^T (1,445 nucleotides) and those of the selected type strains were aligned using the online tool SINA Alignment Service, version 1.2.11 (Pruesse *et al.*, 2012) (www.arb-silva.de/aligner). The aligned sequences were exported to the Molecular Evolutionary Genetics Analysis (MEGA) program, version 6.06 (Tamura *et al.*, 2011) and phylogenetic trees were constructed with the maximum-likelihood, neighbor-joining, and maximum-parsi-

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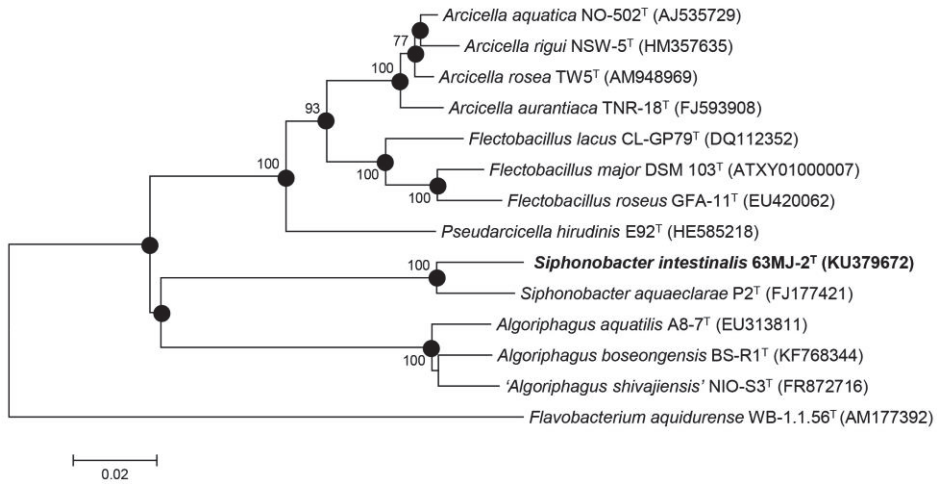

Fig. 1. Neighbour-joining tree based on a comparative analysis of 16S rRNA gene sequences, showing the phylogenetic relationships of strain 63MJ-2^T and some other related taxa. Filled circles indicate that the corresponding nodes were also recovered in trees generated with maximum-parsimony and maximum-likelihood algorithms. Percentage bootstrap values (from 1,000 replications) greater than 70% are shown at nodes. Bar, 0.02 substitutions per nucleotide position. *Flavobacterium aquidurense* WB-1.1.56^T was used as an outgroup.

mony algorithms. The robustness of the phylogenetic trees was calculated by bootstrap analysis based on 1000 random resamplings of the sequences. The similarities between the sequence of strain 63MJ-2^T and the sequences of the type strains of the closely related taxa were calculated using the EzTaxon-e server (www.ezbiocloud.net/eztaxon; Kim *et al.*, 2012).

Nucleotide sequence accession number

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 63MJ-2^T is KU379672.

Determination of DNA G+C content

The DNA G+C content was determined as described by Gonzalez and Saiz-Jimenez (2002) using the CFX96 real-time PCR system (Bio-Rad). Four bacterial strains were used to construct the calibration curve: *Bacillus cereus* KACC 11240^T, *Bacillus velezensis* KACC 17177^T, *Pseudomonas stutzeri* KACC 10290^T, and *Micrococcus luteus* KACC 10488^T.

Morphological, physiological, and biochemical characterization

The cell morphology and motility of strain 63MJ-2^T, cultured for 1 day on R2A agar at 28°C, were examined by oil-immersion phase-contrast microscopy (Axioplan 2; Zeiss). Gram staining was performed using the Difco Gram staining kit (Difco), according to the manufacturer's instructions. The temperature range for growth was tested at 4, 10, 15, 20, 25, 30, 35, 37, and 40°C. To investigate tolerance to NaCl, R2A broth was prepared with NaCl concentrations adjusted to 0–3.0%, w/v (at intervals of 1.0 %). The pH range for growth was assessed after 10 days of incubation in R2A broth at pH 4.0–10.0, with increments of one pH unit. The pH of the R2A broth was adjusted using citrate/phosphate buffer (pH 3.0–7.0), Tris/hydrochloride buffer (pH 8.0–9.0), and carbonate/bicarbonate buffer (10.0–11.0). After sterilization (121°C, 15 min), the pH of the R2A broth was checked and adjusted if necessary. Catalase activity was detected by dispersing colonies in 3% (v/v) hydrogen peroxide and checking for bubble formation, and oxidase activity was determined by using the Oxidase Reagent (bioMérieux). Hydrolyses of carboxymethyl cellulose (CM-cellulose, 0.1%, w/v), casein (skimmed milk, 10% w/v), chitin (0.5%, w/v), hypoxanthine (0.5%, w/v), tyrosine (0.5%, w/v), Tween 80 (1.0%, v/v), starch (1.0%, w/v), and xanthine (0.5%, w/v) was examined after incubation for 7 days using the protocol described by Smibert and Krieg (1994). DNase activity was determined using DNase test agar (Difco). Growth under anaerobic conditions was tested by incubating R2A agar plates in AnaeroGen sachets (Oxoid Ltd.) for 7 days at 28°C. Additional enzyme activities, biochemical features, and acid production from carbon sources were determined by using API ZYM, API 20NE, and API 50CH kits (bioMérieux).

Chemotaxonomy

Cells of strain 63MJ-2^T and *Siphonobacter aquaeclarae* KACC 18773^T were grown on R2A agar for 1 day at 28°C to the exponential phase (Sasser, 1990). Fatty acid methyl esters were prepared from these strains, analyzed according to the standard protocol of MIDI (Sherlock Microbial Identification System, version 5.0), and identified using the TSBA 50 database of the Microbial Identification System.

Polar lipids were extracted and separated by two-dimensional silica-gel thin-layer chromatography (TLC) of the cell extract, as described by Tindall (1990a and 1990b). Isoprenoid quinones were analyzed by HPLC as described by Groth *et al.* (1996). Menaquinones and phospholipids were extracted and purified according to the protocol of Minnikin *et al.* (1984). After the two-dimensional TLC, the following reagents were sprayed for the detection of polar lipids (Collins *et al.*, 1980): ninhydrin for free amino groups, molybdenum blue for phosphorus-containing lipids, α-naphthol for sugars and Dragendorff's solution for quaternary nitrogen.

Results and Discussion

Phylogenetic characteristics

The 16S rRNA gene sequence analysis revealed that strain 63MJ-2^T shared the highest similarity with *Siphonobacter aquaeclarae* P2^T (96.1%) and had low sequence similarities

(below 86.3%) with other members of the family '*Flexibacteraceae*' in the phylum *Bacteroidetes*. Phylogenetic analysis based on the neighbor-joining tree revealed that strain 63MJ-2^T formed a clade with *S. aquaeclarae* P2^T, which was also supported by the maximum-parsimony and maximum-likelihood trees (Supplementary data Figs. S2 and S3). The DNA G+C content of strain 63MJ-2^T was 43.5 mol%.

Morphological, physiological, and biochemical characteristics

Following culture on R2A agar for 1 day at 28°C, strain 63MJ-2^T formed light yellow, convex, regular, round (0.65–0.85 mm in diameter) colonies. The cells were strictly aerobic, Gram-negative, non-motile rods with 0.6–1.0 μm width, and 4.5–6.1 μm length. The temperature, pH, and NaCl ranges for growth were 4–35°C (optimum, 25–30°C), pH 5.0–9.0 (optimum, 6.0–7.0), and 0–2.0% (optimum, 1.0–2.0) (w/v) NaCl, respectively. Cells were positive for catalase activity and negative for oxidase activity. Other phenotypic characteristics distinguishing strain 63MJ-2^T from *Siphonobacter aquaeclarae* KACC 18773^T are shown in Table 1.

Chemotaxonomy

The major fatty acids of strain 63MJ-2^T were $C_{16:1}$ *ω5c* (42.5%), iso-$C_{17:0}$ 3-OH (18.7%), and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*, 18.0%). The fatty acid profile of strain 63MJ-2^T was similar to that of *S. aquaeclarae* KACC 18773^T.

Table 1. Phenotypic comparison between strain 63MJ-2^T and *Siphonobacter aquaeclarae* KACC 18773^T

Strains: 1, 63MJ-2^T; 2, *Siphonobacter aquaeclarae* KACC 18773^T. +, positive; -, negative. Both strains are positive for asculin hydrolysis, β-galactosidase (PNPG), and assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, D-maltose, and potassium gluconate (API 20NE), acid production of L-arabinose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, gentiobiose, and D-turanose (API 50CH), but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, gelatin hydrolysis and assimilation of D-mannitol, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid (API 20NE), acid production of glycerol, erythritol, D-ribose, D-adonitol, L-sorbose, dulcitol, inositol, D-mannitol, sorbitol, glycogen, xylitol, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate (API 50CH).

Characteristics	1	2
Isolation source	Insect feces	Biofilm
Anaerobic growth	-	+[a]
Temperature for growth (°C)	4–35	4–37
NaCl range for growth (%)	0–2	0–1
Urease (API 20NE)	-	+
Acid production from:		
D-Arabinose	-	+
L-Xylose	-	+
D-Rhamnose	-	+
Arbutin	-	+
D-Lyxose	-	+
L-Fucose	-	+
DNA G+C content (mol%)	43.5	54.5[a]

[a] Data was taken from Táncsics *et al.* (2010).

The complete fatty acid profile is provided in Table 2. Polar lipids of strain 63MJ-2^T were phosphatidylethanolamine (PE), six unknown aminolipids (AL), and five unknown lipids (L) (Supplementary data Fig. S1). Consistent with data from *S. aquaeclarae* P2^T, the predominant isoprenoid quinone and polar lipid of strain 63MJ-2^T were MK-7 and phosphatidylethanolamine, respectively.

Taxonomic conclusion

In the phylogenetic trees, strain 63MJ-2^T was clustered with *Siphonobacter aquaeclarae* P2^T. In addition, the chemotaxonomic data, such as the polar lipid and fatty acid profiles and the major isoprenoid quinone, of strain 63MJ-2^T were highly similar to those of *S. aquaeclarae* P2^T. These results support the hypothesis that strain 63MJ-2^T is a representative of a novel taxon within the genus *Siphonobacter*. Moreover, 63MJ-2^T can be distinguished from *Siphonobacter aquaeclarae* P2^T by its low 16S rRNA gene sequence similarity, disparate DNA G+C content, and the several differentiating physiological traits (anaerobic growth, temperature, and NaCl range for growth, glucose fermentation, urease activity, and acid production pattern of 6 carbon sources). Thus, strain 63MJ-2^T represents a novel species in the genus *Siphonobacter*, for which the name *Siphonobacter intestinalis* sp. nov. is proposed.

Emended description of the genus *Siphonobacter* Táncsics *et al.* 2010

The description of the genus *Siphonobacter* is as given by Táncsics *et al.* (2010) with the following amendments. Cells are aerobic or facultatively anaerobic.

Description of *Siphonobacter intestinalis* sp. nov.

Siphonobacter intestinalis (in.tes.tin.a'lis. L. masc. adj. intestinalis, pertaining to the intestine)

Cells are strictly aerobic, Gram-staining-negative, non-motile, non-spore-forming rods, 0.6–1.0 μm wide, and 4.5–6.1

Table 2. Cellular fatty acid composition of strain 63MJ-2^T and *Siphonobacter aquaeclarae* KACC 18773^T

Strains: 1, 63MJ-2^T; 2, *Siphonobacter aquaeclarae* KACC 18773^T. All data are from this study. All strains were grown on R2A agar at 28°C for 1 day. Values are percentages of total fatty acids. -, Not detected.

Fatty acids	1	2
$C_{14:0}$	0.6	-
iso-$C_{15:0}$	7.6	9.1
anteiso-$C_{15:0}$	-	0.7
$C_{16:1}$ *ω5c*	42.5	39.8
$C_{16:0}$	2.4	3.6
iso-$C_{15:0}$ 3-OH	2.7	3.9
Unknown 16.582	1.3	1.6
iso-$C_{17:0}$	0.7	0.7
anteiso-$C_{17:0}$	-	0.5
iso-$C_{16:0}$ 3-OH	1.3	1.9
$C_{16:0}$ 3-OH	2.4	3.4
iso-$C_{17:0}$ 3-OH	18.7	17.2
Unknown 18.814	-	1.5
Summed feature 3[a]	18.0	15.0

[a] Summed features are groups of two or three fatty acids that cannot be separated by the MIDI system. Summed feature 3 comprised iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*.

μm long. Grows at 4–35°C (optimum, 25–30°C) and pH 5.0–9.0 (optimum, 6.0–7.0). Tolerates up to 2% (w/v) NaCl. Forms light-yellow, round, convex, and regular colonies (0.65–0.85 mm in diameter) on R2A medium. Positive for catalase and negative for oxidase. Hydrolyzes DNA and Tween 80, but does not hydrolyze casein, chitin, CM-cellulose, hypoxanthine, starch, tyrosine, and xanthine. Positive for asculin hydrolysis, β-galactosidase (PNPG), and assimilation of D-glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, D-maltose, and potassium gluconate but negative for glucose fermentation, indole production, arginine dihydrolase, urea and gelatin hydrolysis, and assimilation of D-mannitol, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid (API 20NE).

In the API 50CH system, produces acids from L-arabinose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetyl-glucosamine, amygdalin, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, gentiobiose, and D-turanose, but not from glycerol, erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, L-sorbose, D-rhamnose, dulcitol, inositol, D-mannitol, sorbitol, arbutin, glycogen, xylitol, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, and potassium 5-ketogluconate. Positive activities (in API ZYM system) for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase, but negative activities for lipase (C14) and β-glucuronidase.

The major isoprenoid quinone is menaquinone MK-7. Polar lipids of strain 63MJ-2^T are phosphatidylethanolamine, six unknown aminolipids, and five unknown lipids (L). The predominant fatty acids are $C_{16:1}$ *ω5c* (42.5%), iso-$C_{17:0}$ 3-OH (18.7%), and summed feature 3 (iso-$C_{15:0}$ 2-OH and/or $C_{16:1}$ *ω7c*, 18.0%). The DNA G+C content of the type strain is 43.5 mol%. The type strain, 63MJ-2^T (=KACC 18663^T =NBRC 111883^T), was isolated from the feces of *Pseudorhynchus japonicus*.

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