Mucilaginibacter puniceus sp. nov., isolated from wetland freshwater

Ji Hee Lee,¹ Mi Sun Kim,¹ Joo Won Kang,¹ Keun Sik Baik² and Chi Nam Seong¹

¹Department of Biology, College of Life Science and Natural Resources, Sunchon National University, Sunchon 57922, Republic of Korea
²CNCNATURE, Gwangju 61008, Republic of Korea

A non-motile, rod-shaped and pale-pink bacterium, designated strain WS71ᵀ, was isolated from freshwater collected from the Woopo Wetland (Republic of Korea). Cells were Gram-stain-negative, aerobic, catalase-positive and oxidase-negative. The major fatty acids were summed feature 3 (comprising C₁₆:1ω-6c and/or C₁₆:1ω-7c), iso-C₁₇:0 3-OH, C₁₈:0 and iso-C₁₅:0. The strain contained menaquinone 7 (MK-7) and the DNA G+C content was 39.4±0.4 mol%. The major polar lipids were phosphatidylethanolamine and an unknown aminophospholipid.

The phylogenetic tree based on 16S rRNA gene sequences showed that WS71ᵀ forms an evolutionary lineage within the radiation enclosing the members of genus Mucilaginibacter with M. soyangensis HME6664ᵀ as its nearest neighbor (98.3 % sequence similarity). DNA–DNA relatedness between WS71ᵀ and M. soyangensis HME6664ᵀ was 61.3±1.0 %. A number of phenotypic characteristics distinguished WS71ᵀ from the other members of the genus Mucilaginibacter. On the basis of the evidence presented in this study, WS71ᵀ represents a novel species, for which the name Mucilaginibacter puniceus sp. nov. is proposed. The type strain is WS71ᵀ (=KCTC 32270ᵀ=JCM 19495ᵀ).

The genus Mucilaginibacter, a member of the family Sphingobacteriaceae (Steyn et al., 1998), was first proposed by Pankratov et al. (2007). Members of the genus Mucilaginibacter are Gram-stain-negative, non-motile, rod-shaped and produce large amounts of extracellular polymeric substances and contained MK-7 and phosphatidylethanolamine and several unknown aminophospholipids, phospholipids and aminolipids and DNA G+C contents were 39.1–47.8 mol% (Pankratov et al., 2007; Urai et al., 2008; Baik et al., 2010; Chen et al., 2014). At the time of writing, the genus comprises 39 species with validly published names, with Mucilaginibacter paludis as the type species (Pankratov et al., 2007). Most of the members were mainly isolated from terrestrial environments such as acidic peat (Pankratov et al., 2007), rhizosphere (Madhaiyan et al., 2010; Kim et al., 2010), soil (Urai et al., 2008; Ji et al., 2012), compost (Cui et al., 2011), rice straw and rotten wood (An et al., 2009; Khan et al., 2013), lichen (Männistö et al., 2010), freshwater (Baik et al., 2010; Joung et al., 2014) and wastewater (Hwang et al., 2014). A small number originated from tidal flat sediment (Kim et al., 2012a; Yoon et al., 2012). In the course of our study on microbial diversity of Woopo wetland, a rod-shaped bacterial strain, designated WS71ᵀ was isolated from a freshwater sample and subjected to a polyphasic taxonomic investigation.

Woopo wetland (35°33′ N, 128°25′ E), located to the east of the Nakdong river, Republic of Korea, is surrounded by low-level elevated mountains and a levee has been built to protect the agricultural areas. This wetland was declared a Nature Conservation Area in 1997, and designated a Ramsar site (http://www.wetlands.org). At the time of water sampling, this wetland was in full of vegetation and blooming. WS71ᵀ was isolated using R2A agar (Becton Dickinson) at 25° C for 7 days. The isolate was routinely cultured on R2A agar (Becton Dickinson) and preserved at −80° C as a suspension in distilled water containing 20 % glycerol (w/v).

Bacterial DNA preparation, PCR amplification and sequencing of the 16S rRNA gene were carried out as described previously (Chun & Goodfellow, 1995). Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012b). Multiple sequence alignments were
performed by using the CLUSTAL W program (Thompson et al., 1994) integrated in the BioEdit program (Hall, 1999). Phylogenetic analysis was performed by using the software package MEGA version 6 (Tamura et al., 2013). Phylogenetic trees were inferred using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1993) and maximum-parsimony (Fitch, 1971) algorithms. The distance matrix of the neighbour-joining method was generated according to the model of Jukes & Cantor (1969). The robustness of the topology in the neighbour-joining phylogenetic tree was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

Preliminary sequence comparison with 16S rRNA gene sequences held in GenBank indicated that the isolate was related closely to the members of the genus Mucilaginibacter. The newly determined sequence was then aligned against related species. Growth of WS71<sup>T</sup> was closely related to the type strain of *M. soyangensis* HME6664<sup>T</sup> (96.2 % sequence similarity), *Mucilaginibacter boryangensis* BDR-9<sup>T</sup> (96.1 %), *Mucilaginibacter gossypii* Gc-48<sup>T</sup> and *Mucilaginibacter gossypii* Gs-67<sup>T</sup> (96.0 %). Sequence similarity with other members of the genus *Mucilaginibacter* was less than 96.0 %. The neighbor-joining tree (Fig. 1) showed that WS71<sup>T</sup> was closely related to the type strains of species of the genus *Mucilaginibacter*, occupying a distinct position. WS71<sup>T</sup> was clustered with *M. soyangensis* HME6664<sup>T</sup> with 96 % bootstrap support. The trees based on maximum-likelihood and maximum-parsimony methods showed similar topology.

DNA–DNA hybridization was performed by the membrane filter technique using the DIG High Prime DNA Labelling and Detection Starter kit II (Roche Molecular Biochemicals) according to the method described in detail by Lee et al. (2003), with the modification that the hybridization temperature was 45 °C. DNA–DNA relatedness between WS71<sup>T</sup> and *M. soyangensis* HME6664<sup>T</sup> was 61.3±1.0 % (mean±SD of three determinations). Thus, levels of genetic relatedness according to DNA–DNA hybridization experiments were less than 70 %, which leads to the conclusion that WS71<sup>T</sup> and *M. soyangensis* HME6664<sup>T</sup> represent two distinct species (Wayne et al., 1987).

Growth was tested on nutrient agar (NA, Becton Dickinson), plate count agar (PCA, Becton Dickinson), R2A agar (Becton Dickinson) and tryptic soy agar (TSA; Becton Dickinson). Cells of WS71<sup>T</sup> grown on R2A agar at 25 °C for 3 days were used for the physiological and biochemical tests. Cell morphology was observed by phase-contrast microscopy (TMS-F; Nikon) and transmission electron microscopy (CM-20; Philips) using cells grown for 3 days at 25 °C on R2A agar. Gliding motility was assessed by examining wet mounts of a 48 h R2A broth culture under a phase-contrast microscope (Bernardet et al., 2002). The pH range for growth was determined in R2A broth adjusted to pH 4–11 (using increments of 1 pH unit) by using 100 mM acetate buffer (pH 4–5), 100 mM NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> buffer (pH 6–8) and 100 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer (pH 9–11). Growth temperature [4, 37 °C, 10–50 °C (at 5 °C intervals)] and NaCl tolerance [1–10 % (at 1 % intervals)] were tested on R2A agar. Anaerobic growth on R2A agar was tested using the AnaeroPack-Anaero (Mitsubishi Gas Chemical). Catalase and oxidase activities were determined using 3 % (v/v) hydrogen peroxide and Kovac's reagent (Kovac, 1956), respectively. Nitrate reduction was tested on nitrate broth containing 0.1 % KNO<sub>3</sub> (Tindall et al., 2007). H<sub>2</sub>S production was determined on Kligler iron agar (Becton Dickinson) according to the method of Smibert & Krieg (1994). Hydrolysis of casein [2 % (w/v) skimmed milk], carboxymethyl cellulose (CMC) (0.5 %, w/v), starch (0.2 %, w/v), Tween 20 (1 %, w/v), Tween 20 (1 %, w/v) and xylan (1 %, w/v) was tested using R2A agar according to the protocols of Smibert & Krieg (1994). Decomposition of hypoxanthine (0.5 %, w/v), L-tyrosine (0.5 %, w/v) and xanthine (0.4 %, w/v) was tested using R2A agar according to the methods of Gordon et al. (1974). Hydrolysis of aesculin (0.1 %, w/v) and urea (2 %, w/v) were determined as described by Lányi (1987). DNase activity was determined using DNase test agar (Becton Dickinson). Other biochemical tests and tests of enzyme activities were performed using the API 20NE and API ZYM kits (bioMérieux) and Biolog GN2 MicroPlate. Antibiotic sensitivity was determined by the disc diffusion method using commercial antibiotic-impregnated discs (BBL Becton Dickson). After 5 days of incubation at 25 °C on R2A, the results were interpreted according to the guidelines set forth by the CLSI (2009).

Cells were Gram-stain-negative, non-motile and rod-shaped (0.2–0.4×0.8–1.2 µm in size; see Fig. S1, available in the online Supplementary Material). Cells were sensitive to (µg per disc unless otherwise stated) ampicillin (10), chloramphenicol (30), erythromycin (15), nalidixic acid (30), penicillin G (10 IU), streptomycin (10), tetracycline (30) and vancomycin (30) but resistant to amikacin (30), gentamicin (10), kanamycin (30) and polymyxin B (300 IU). The detailed results of physiological and biochemical analyses are given in Table 1 and the species description. There were several phenotypic characters such as optimum growth temperature, nitrate reduction, oxidase activity and hydrolysis of Tween 80 that separate WS71<sup>T</sup> from phylogenetically related species.

For cellular fatty acid analysis, WS71<sup>T</sup> and two reference strains were grown on R2A agar for 3 days at 25 °C. Extraction of fatty acid methyl esters (FAME) and separation by gas chromatography were performed by using the Instant FAME method of the Microbial Identification System (MIDI) version 6.1 and the RTSB6 database. For polar lipid and isoprenoid quinone analyses cells of strain WS71<sup>T</sup> grown in R2A broth for 3 days at 25 °C were harvested and freeze-dried. Polar lipids were analyzed by using standard procedures (Minnikin et al., 1984). Extracted lipids were separated by two-dimensional TLC and identified by spraying with appropriate detection reagents (Minnikin et al., 1977; Embley & Wait, 1994). Isoprenoid quinones were

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extracted and purified according to the method of Minnikin et al. (1984) and analyzed by TLC as described by Collins (1994). For genomic DNA G+C content calculations, the DNA sample was prepared in triplicate and determined by the thermal denaturation method of Marmur & Doty (1962).

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic position of strain WS71<sup>T</sup>. Evolutionary distances, generated using the Jukes and Cantor model are based on 1244 unambiguously aligned nucleotides. Bootstrap values greater than 50% (1000 resamplings) for nodes conserved among neighbour-joining analyses are shown. Sphingobacterium spiritivorum NCTC 11386<sup>T</sup> (EF090267) was used as an outgroup. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.01 substitutions per nucleotide position.
Table 1. Phenotypic characteristics that differentiate strain WS71\textsuperscript{T} from phylogenetically related species of the genus Mucilaginibacter

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td><strong>Isolation source</strong></td>
<td>Freshwater</td>
<td>Freshwater</td>
</tr>
<tr>
<td><strong>Colony colour</strong></td>
<td>Pale pink</td>
<td>Yellow-orange</td>
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<tr>
<td><strong>Growth at 37°C</strong></td>
<td>+</td>
<td>–</td>
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<tr>
<td><strong>Flexirubin-type pigments</strong></td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>Nitrate reduction</strong></td>
<td>+</td>
<td>–</td>
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<tr>
<td><strong>Oxidase</strong></td>
<td>–</td>
<td>+</td>
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<tr>
<td><strong>Hydrolysis of:</strong></td>
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<tr>
<td>Aesculin</td>
<td>–</td>
<td>+</td>
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<tr>
<td>CMC</td>
<td>–</td>
<td>W</td>
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<tr>
<td>DNA</td>
<td>–</td>
<td>NG</td>
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<tr>
<td>L-Tyrosine</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Tween 20</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Tween 80</td>
<td>+</td>
<td>–</td>
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<td><strong>Enzyme activity (API ZYM)</strong></td>
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<tr>
<td>Esterase (C4)</td>
<td>–</td>
<td>–</td>
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<tr>
<td>α-Fucosidase</td>
<td>–</td>
<td>–</td>
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<tr>
<td>α-Galactosidase</td>
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<td>–</td>
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<tr>
<td>β-Galactosidase</td>
<td>–</td>
<td>+</td>
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<tr>
<td>α-Glucosidase</td>
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<td>–</td>
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<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Naphthol-AS-BI-phosphohydrolase</td>
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<td>+</td>
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<tr>
<td><strong>Biolog GN2 plate substrate response for:</strong></td>
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<tr>
<td>L-Alanine</td>
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<td>+</td>
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<tr>
<td>γ-Aminobutyric acid</td>
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<td>–</td>
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<tr>
<td>Cellbiose</td>
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<tr>
<td>Gentiobiose</td>
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<tr>
<td>α-D-Glucose</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Glucose 1-phosphate</td>
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<td>–</td>
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<tr>
<td>T-Glutaric acid</td>
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<td>–</td>
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<tr>
<td>Methyl β-D-glucoside</td>
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<td>–</td>
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<td>β-Hydroxybutyric acid</td>
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<td>–</td>
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<td>α-Ketobutyric acid</td>
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<td>α-Lactose</td>
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<tr>
<td>Lactulose</td>
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<td>L-Leucine</td>
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<tr>
<td>D-Mannose</td>
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<td>+</td>
</tr>
<tr>
<td>D-Melibiose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L-Proline</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Quinic acid</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Raffinose</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Succinic acid</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Succinic acid monomethyl ester</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>–</td>
</tr>
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Table 1. cont.

<table>
<thead>
<tr>
<th>1</th>
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<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotic susceptibility (µg):</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin (10)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Chloramphenicol (30)</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin (15)</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>Penicillin G (10 IU)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Streptomycin (10)</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>Vancomycin (30)</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td><strong>DNA G+C content (mol%):</strong></td>
<td>39.4</td>
<td>40.8\textsuperscript{*}</td>
</tr>
</tbody>
</table>

*Data from Joung et al. (2014).
\textsuperscript{T}Data from Luo et al. (2009).

The fatty acid profile of WS71\textsuperscript{T} (>10.0% of total fatty acids) included summed feature 3 (C\textsubscript{16:1}ω6c and/or C\textsubscript{16:1}ω7c) (31.6%), iso-C\textsubscript{17:0} 3-OH (11.3%), C\textsubscript{16:0} (10.8%) and iso-C\textsubscript{15:0} (10.7%) (Table 2). This fatty acid profile was similar to those of related species of the genus Mucilaginibacter. However, WS71\textsuperscript{T} contained more C\textsubscript{16:0} but less iso-C\textsubscript{15:0} than the reference strains. The predominant polar lipids of WS71\textsuperscript{T} were phosphatidylethanolamine and an unknown aminophospholipid; smaller amounts of unknown polar lipids, unknown aminophospholipids, unknown phospholipids and an unknown aminolipid were also detected (see Fig. S2). The polar lipid profile of WS71\textsuperscript{T} was similar to those of members of the genus Mucilaginibacter (Chen et al., 2014). The predominant respiratory quinone detected was menaquinone-7 (MK-7), which is the characteristic respiratory quinone of the members of the genus Mucilaginibacter (Baik et al., 2010). The DNA G+C content of WS71\textsuperscript{T} was 39.4±0.4 mol%, which is in the range recorded for members of the genus Mucilaginibacter (Baik et al., 2010; Chen et al., 2014).

Therefore, on the basis of the data presented, WS71\textsuperscript{T} represents a novel species within the genus Mucilaginibacter, for which the name Mucilaginibacter puniceus sp. nov. is proposed.

**Description of Mucilaginibacter puniceus sp. nov.**

*Mucilaginibacter puniceus* (pu.ni’ce.us. L. masc. adj. puni-ceus pink).

Cells are Gram-stain-negative, non-endospore-forming, non-motile, non-gliding, aerobic and rod-shaped (0.2–0.4×8.0–1.2 µm in size). Colonies on R2A agar are circular, convex with entire margins, pale pink-pigmented and approximately 1.0 mm in diameter after 3 days at 25°C. Growth occurs on NA, PCA, R2A agar and TSA (optimally...
in the GN2 gallery, all activities are negative. The major activities are present, but other enzyme activities are absent. phosphatase, leucine arylamidase and acid phosphatase urea, xanthine and xylan. In the API ZYM gallery, alkaline in all strains are not shown. traces (<1%). Fatty acids amounting to <1% of the total fatty acids unknown aminophospholipids, unknown phospholipids phospholipid, with small amounts of unknown polar lipids, are phosphatidylethanolamine and an unknown amino-

\[
\begin{array}{|c|c|c|c|}
\hline
& 1 & 2 & 3 \\
\hline
\text{Straight-chain saturated} & & & \\
C_{12}:0 & TR & 1.0 & 1.2 \\
C_{14}:0 & TR & 1.2 & 1.0 \\
C_{15}:0 & 1.6 & 3.5 & 2.3 \\
C_{16}:0 & 10.8 & 5.4 & 6.1 \\
C_{18}:0 & 3.4 & 5.1 & 4.9 \\
C_{16}:0 3-OH & TR & TR & 1.2 \\
\hline
\text{Branched saturated} & & & \\
iso-C_{15}:0 & 10.7 & 15.7 & 25.8 \\
isoy-C_{17}:0 & 1.6 & TR & 1.2 \\
isoy-C_{15}:0 3-OH & 3.5 & 2.8 & 1.9 \\
isoy-C_{17}:0 3-OH & 11.3 & 8.6 & 9.5 \\
anteiso-C_{18}:0 & TR & 1.4 & 2.1 \\
anteiso-C_{15}:0 & 1.2 & TR & TR \\
\hline
\text{Monounsaturated} & & & \\
C_{15}:1ω6c & TR & 2.0 & 1.0 \\
C_{16}:1ω5c & 4.8 & 7.2 & 3.2 \\
C_{18}:1ω9c & 1.7 & 2.7 & 1.8 \\
\hline
\text{Summed feature*} & & & \\
3 & 31.6 & 29.2 & 20.0 \\
4 & TR & TR & 1.0 \\
8 & 1.2 & 1.7 & 1.2 \\
9 & 6.0 & 3.1 & 6.7 \\
\hline
\end{array}
\]

\*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consists of C_{16}:1ω6c and/or C_{16}:1ω7c. Summed feature 4 consists of anteiso-C_{17}:1ω6c and/or iso-C_{17}:1ω7c. Summed feature 8 consists of C_{18}:1ω6c and/or C_{18}:1ω7c. Summed feature 9 consists of iso-C_{17}:1ω9c and/or C_{16}:1ω6c.

The type strain is WS71$^T$ (=KCTC 32270$^T$=JCM 19495$^T$), isolated from the freshwater of Woopo wetland in Gyeongnam Province, Republic of Korea. The genomic DNA G+C content of the type strain is 39.4±0.4 mol%.

### Acknowledgements

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Mucilaginibacter lutimaris sp. nov., isolated from a tidal flat sediment. 

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