Flavihumibacter solisilvae sp. nov., isolated from forest soil

Hyo Jung Lee,1 Sang Eun Jeong,1 Myong-Suk Cho,2 SeonHee Kim,2 Sang-Suk Lee,3 Byoung-Hee Lee4 and Che Ok Jeon1

1Department of Life Science, Chung-Ang University, Seoul, 156-756, Republic of Korea
2Department of Biological Sciences, Sungkyunkwan University, Suwon, 440-746, Republic of Korea
3Department of Animal Science & Technology, Sunchon National University, Sunchon 540-742, Republic of Korea
4National Institute of Biological Resources, Hwangyeong-ro 42, Seo-gu, Incheon, 404-708, Republic of Korea

A Gram-stain-positive, strictly aerobic, yellow colony-forming bacterium, designated strain 3-3T, was isolated from forest soil of Bac Kan Province in Vietnam. Cells were non-motile rods without gliding motility, showing oxidase- and catalase-positive reactions. Growth was observed at 20–37 °C (optimum, 28 °C) and pH 5.5–9.5 (optimum, pH 7.5). The major cellular fatty acids were iso-C15 : 0, iso-C15 : 1 G and summed feature 3 (comprising C16 : 1³7c and/or C16 : 1⁴6c). Strain 3-3T contained phosphatidylethanolamine, three unidentified aminolipids and three unidentified lipids. The G+C content of the genomic DNA was 49.5 mol% and the only isoprenoid quinone detected was menaquinone 7 (MK-7). Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain 3-3T formed a tight phylogenetic lineage with Flavihumibacter petaseus T41T with a bootstrap value of 100 %. Strain 3-3T was related most closely to F. petaseus T41T with 97.3 % 16S rRNA gene sequence similarity and the level of DNA–DNA relatedness between the two was 9.4 ± 1.2 %. Based on phenotypic, chemotaxonomic and molecular features, strain 3-3T represents a novel species of the genus Flavihumibacter, for which the name Flavihumibacter solisilvae sp. nov. is proposed. The type strain is 3-3T (=KACC 17917T=JCM 19891T).

The genus Flavihumibacter, forming an evolutionary lineage within the family Chitinophagaceae of the phylum Bacteroidetes, was first proposed by Zhang et al. (2010). At the time of writing, the genus Flavihumibacter comprises only one recognized species, Flavihumibacter petaseus, isolated from a soil sample of a subtropical rainforest. Cells of F. petaseus are Gram-positive, aerobic rods producing flexirubin-type pigments, show catalase- and oxidase-positive reactions, and contain iso-C15 : 0 and iso-C15 : 1 G as major fatty acids and menaquinone 7 (MK-7) as the isoprenoid quinone (Zhang et al., 2010, 2013). In the course of a study investigating microbial communities of subtropical rainforest soil (Lee et al., 2014), we isolated a novel strain, designated 3-3T, belonging to the genus Flavihumibacter and here we describe its taxonomic characterization.

A wet forest soil sample (less than 5 cm depth) from Bac Kan Province in Vietnam was resuspended and serially diluted in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2), and the diluted samples were spread on R2A agar (Difco) and incubated at 30 °C for 5 days under aerobic conditions. The genomic DNA from colonies grown on R2A agar was extracted using Chelex-100 (Bio-Rad) and the 16S rRNA genes were PCR-amplified using universal primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Lane, 1991) as described previously (Lee et al., 2012). The PCR amplicons were double-digested with HaeIII and HhaI and the fragment patterns were analysed on 2.5 % MetaPhore agarose (BioWhittaker) gel with a 100 bp ladder (Bioneer), which was used as a guide to classify the colonies (Jin et al., 2012). Representative PCR products showing unique fragment patterns

Abbreviations: ML, maximum-likelihood; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 3-3T is KC569790.

One supplementary table and two supplementary figures are available with the online version of this paper.
were partially sequenced using the 27F primer and the resulting 16S rRNA gene sequences were compared with those of validly reported type strains using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). A putative novel strain belonging to the genus *Flavihumibacter*, designated 3-3T, was selected for further phenotypic and phylogenetic analyses. Strain 3-3T was routinely grown aerobically on R2A agar at 28 °C for 2 days, unless otherwise indicated. Strain 3-3T was preserved at −80 °C in R2A broth with 15% (v/v) glycerol. *Flavihumibacter petaseus* NBRC 106054T was purchased from NBRC (Japan) and was used as a reference strain for the phenotypic characterizations and DNA–DNA hybridization experiments.

The PCR amplicon from strain 3-3T was sequenced with the 27F and 1492R primers. The resulting 16S rRNA gene sequences were compared with closely related type strains using the EzTaxon-e server (Kim et al., 2012). Multiple alignments of the 16S rRNA gene sequences of strain 3-3T and closely related taxa were carried out using the secondary-structure aware Infernal aligner of the Ribosomal Database Project (RDP) (https://pyro.cme.msu.edu/login.spr; Wang et al., 2007). Phylogenetic analyses using the neighbour-joining (NJ) and maximum-parsimony (MP) algorithms were performed by the PHYLIP software (version 3.68, Felsenstein, 2002). Evolutionary distances in the NJ tree were calculated based on Kimura’s two-parameter model (Kimura, 1983). The resulting trees were evaluated with calculation of bootstrap values based on 1000 resampled datasets using the PHYLIP software. A maximum-likelihood (ML) tree with bootstrap values was reconstructed using RAxML-HPC BlackBox (version 7.6.3) of the Cyber-Infrastructure for Phylogenetic Research project (CIPRES, www.phylo.org; Stamatakis et al., 2005). An additional analysis for the taxonomic assignment of strain 3-3T was performed using the RDP naïve Bayesian rRNA Classifier tool at an 80% confidence threshold (http://rdp.cme.msu.edu/classifier; Wang et al., 2007). DNA–DNA hybridization between strain 3-3T and *F. petaseus* NBRC 106054T was performed in triplicate to evaluate their DNA–DNA relatedness according to a previously described procedure (Lee et al., 2012) using the DIG High Prime DNA Labelling kit (Roche Applied Science). Hybridization signals on nylon membrane were analysed with the program Bio-Rad Quantity One (ver. 4.6.2). The signal intensities produced by self-hybridization of the serial dilutions were used for calculation of the DNA–DNA relatedness value between strain 3-3T and *F. petaseus* NBRC 106054T. The experiment was confirmed using reciprocal hybridization.

On the basis of 16S rRNA gene sequence similarities, strain 3-3T was related most closely to *Flavihumibacter petaseus* T41T (97.3%); levels of 16S rRNA gene sequence similarity with other validly reported type strains were less than 93.3%. The phylogenetic tree using the NJ algorithm showed that strain 3-3T formed a tight phyletic lineage with *F. petaseus* T41T with 100% bootstrap support (Fig. 1). The phylogenetic trees reconstructed using the MP and ML algorithms also supported the proposition that strain 3-3T could represent a member of the genus *Flavihumibacter*, which was also confirmed by the classification using the RDP Classifier program. Mean (± SD) DNA–DNA relatedness between strain 3-3T and *F. petaseus* NBRC 106054T was 9.4 ± 1.2%, which was clearly below the 70% cut-off value generally accepted for novel species delineation (Rossello-Mora & Amann, 2001).

Growth of strain 3-3T on R2A agar (BD), nutrient agar (NA; BD), trypticase soy agar (TSA; BD), and Luria–Bertani (LB)
agar (per litre: 10 g tryptone, 5 g yeast extract, 10 g NaCl and 15 g agar, prepared in the laboratory) was evaluated after 3 days of incubation at 28 °C. Growth temperatures and pH ranges for strain 3-3T were examined by growing the strain at 5, 10, 15, 20, 25, 28, 30, 35, 37 and 40 °C on R2A agar and at pH 5–10 with 0.5 pH unit intervals in R2A broth, respectively. R2A broths with different pH were prepared using the Na2HPO4–NaH2PO4 and Na2CO3–NaHCO3 buffers for pH below 8 and pH 8–10, respectively (Gomori, 1955). pH was adjusted again after sterilization by the addition of HCl or NaOH when necessary. Growth at various NaCl concentrations was tested in R2A broth with different NaCl concentrations [0–2 % (w/v), 0.5 % intervals] prepared in the laboratory according to the formula of the BD medium. Gram-staining of strain 3-3T was tested using the bioMérieux Gram Stain kit according to the instructions of the manufacturer. Anaerobic growth was assessed on R2A agar using the GasPak Plus system (BBL) at 28 °C for 20 days. Oxidase and catalase activities were tested by the oxidation of 1 % (w/v) tetramethyl-p-phenylenediamine (Merck) and the production of oxygen bubbles in 3 % (v/v) aqueous hydrogencarbonate solution, respectively (Smibert & Krieg, 1994). Production of flexirubin-type pigments was determined using the KOH test (Bernardet et al., 2002). Cell morphology, gliding motility and the presence of flagella were studied using phase-contract microscopy and transmission electron microscopy (JEM-161 1010, JEOL) (Bowman, 2000; Bernardet et al., 2002). The following properties of strain 3-3T and the reference strain, F. petaseus NBRC 106054T, were tested in parallel under the same conditions. Hydrolysis of casein, Tween 20, Tween 80, tyrosine, aesculin, gelatin and starch was determined on R2A agar according to the methods of Lányi (1987) and Smibert & Krieg (1994). Utilization of various substrates for growth and acid production from various carbon sources were determined as described by Yurkov et al. (1994) and Leifson (1963), respectively. Additional enzyme activities, biochemical properties and carbohydrate utilization were determined using the API ZYM and API 20NE kits (bioMérieux) and the GN2 MicroPlate system (Biolog) according to the instructions of the manufacturers, respectively. Antibiotic susceptibility of strain 3-3T and F. petaseus NBRC 106054T was tested on R2A agar according to the method of Jeong et al. (2013).

Strain 3-3T grew on R2A agar (optimally) and NA, but not on TSA or LB agar. Growth of strain 3-3T occurred with 0–0.5 % (w/v) NaCl, but not with 1 % NaCl. Cells of strain 3-3T were Gram-stain-positive, strictly aerobic, non-motile, curved long rods (0.4–0.6 μm in width and 1.7–3.4 μm in length) without gliding motility (Fig. S1, available in the online Supplementary Material). Additional physiological and biochemical characteristics of strain 3-3T are summarized in Table 1, the species description and Table S1 and are compared with those of F. petaseus NBRC 106054T. Most properties of strain 3-3T such as colony colour, growth temperatures, catalase and oxidase activities and aerobic growth were in accordance with those of F. petaseus, whereas other properties such as cell size, optimum pH, NaCl tolerance and utilization of D-mannose and glycerol allowed the differentiation of strain 3-3T from F. petaseus.

Isoprenoid quinones of strain 3-3T were analysed using a HPLC system (model LC-20A; Shimadzu) equipped with a diode array detector (SPD-M20A; Shimadzu) and a reversed-phase column (250 × 4.6 mm, Kromasil; Akzo Nobel) as described by Komagata & Suzuki (1987). Polar lipids of strain 3-3T and F. petaseus NBRC 106054T were determined by TLC as described by Minnikin et al. (1977).
The following reagents were used to detect the different polar lipids: phosphomolybdic acid (for total polar lipids), ninhydrin (for amino lipids), α-naphthol/sulfuric acid (for glycolipids) and Dittmer–Lester reagent (for phospholipids). For the analysis of cellular fatty acids, strain 3-3T and F. petaseus NBRC 106054T were cultivated in R2A broth at 28 °C and their cells were harvested at the same growth phase (exponential growth stage, OD600 of 0.8). The cellular fatty acids were extracted, saponified and methylated using the standard MIDI protocol. The fatty acid methyl esters were analysed by GC (Hewlett Packard 6890) and identified by using the TSBA6 database of the Microbial Identification System (Sherlock Version 6.0B) (Sasser, 1990). The DNA G+C content of strain 3-3T was determined by the fluorometric method (Gonzalez & Saiz-Jimenez, 2002) using SYBR Green I and a real-time PCR thermocycler (Bio-Rad). Strain 3-3T contained menaquione-7 (MK-7) as the sole respiratory isoprenoid quinone. The major polar lipid of strain 3-3T was phosphatidyethanolamine, and three unidentified aminolipids and three unidentified lipids were also identified as minor polar lipids (Fig. S2). The polar lipid analysis of F. petaseus NBRC 106054T showed that the overall profiles of strain 3-3T and F. petaseus were almost similar (Fig. S2). The major cellular fatty acids (>10% of the total) of strain 3-3T were iso-C15:0 (39.8%), iso-C15:1 G (24.2%) and summed feature 3 (comprising C16:1ω6c and/or C16:1ω7c, 11.1%), which were in common with those of F. petaseus (Table 2). Although the overall fatty acid profiles of strain 3-3T and F. petaseus were similar, the proportions and the presence or absence of some fatty acids distinguished strain 3-3T from F. petaseus. The G+C content of strain 3-3T was 49.5 mol%, which was similar to that of F. petaseus NBRC 106054T (Table 1). Physiological and chemotaxonomic features and phylogenetic inference support the proposition that strain 3-3T represents a novel species of the genus Flavihumibacter, for which the name Flavihumibacter solisilvae sp. nov. is proposed.

**Description of Flavihumibacter solisilvae sp. nov.**

Flavihumibacter solisilvae (so.li.sil’vae. L. n. solum soil; L. n. silva forest; N.L. gen. n. solisilvae oil/from forest soil).

Colonies are yellow, circular and convex with smooth margins on R2A agar. Growth occurs at 20–37 °C (optimum, 28 °C) and pH 5.5–9.5 (optimum, pH 7.5) and in R2A broth with 0–0.5 % (w/v) NaCl (optimum, 0 %). Cells are Gram-stain-positive, strictly aerobic, curved long rods without flagella. Gliding motility is not observed. Oxidase- and catalase-positive. Flexirubin-type pigments are produced. Casein, aesculin, gelatin and tyrosine are hydrolysed, but starch, Tween 20 and Tween 80 are not. Sucrose, D-glucose, lactose, D-galactose, L-arabinose and glyceral are utilized as a sole carbon and energy source, but D-mannose, mannitol, D-xylene, D-sorbitol and D-fructose are not. Acids are produced from glucose, sucrose, L-arabinose, D-galactose, D-sorbitol, lactose, D-fructose and D-xylene, but not from glyceral, D-mannitol or D-mannose. In API 20NE tests, β-galactosidase activity, assimilation of N-acetylglucosamine and maltose are positive, but nitrate reduction, indole production, fermentation of glucose, activities of arginine dihydrolase and urease, and assimilation of potassium gluconate, caprate, adipate, malate, trisodium citrate and phenylacetate are negative. In API ZYM strips, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are positive and lipase (C14), trypsin and α-chymotrypsin are weakly positive. The following substrates in the Biolog GN2 MicroPlate are utilized: Tween 40, α-D-glucose, maltose, L-alanine, L-serine, succinic acid monomethyl ester, L-aspartic acid, melibiose, D-galacturonic acid, D-glucosaminic acid, D-glucuronic acid, β-hydroxybyuteric acid, p-hydroxyphenylacetic acid, malonic acid, quinic acid, D-saccharic acid, bromosuccinic acid, succinic acid, glucuronamide, D-alanine, L-histidine, L-prolylglutamic acid, γ-aminobutyric acid, urocanic acid, glyceral, L-asparagine, D-galactose, D-alanyl glycine and L-alaninamide. Weakly positive for L-arabinose and dextrin.

**Table 2. Comparison of the cellular fatty acid compositions of strain 3-3T and the type strain of F. petaseus**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C15:0</td>
<td>–</td>
<td>1.7</td>
</tr>
<tr>
<td>C16:0</td>
<td>1.7</td>
<td>TR</td>
</tr>
<tr>
<td><strong>Unsaturated:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:105c</td>
<td>6.1</td>
<td>7.4</td>
</tr>
<tr>
<td>C17:105c</td>
<td>–</td>
<td>1.0</td>
</tr>
<tr>
<td>** Branched:**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>1.3</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C15:1 G</td>
<td>24.2</td>
<td>22.2</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>39.8</td>
<td>29.4</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>2.5</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Hydroxy:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iso-C16:0 3-OH</td>
<td>3.7</td>
<td>4.1</td>
</tr>
<tr>
<td>iso-C17:0 3-OH</td>
<td>3.0</td>
<td>1.1</td>
</tr>
<tr>
<td>C16:0 3-OH</td>
<td>TR</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>Summed feature 3</strong></td>
<td>11.1</td>
<td>17.5</td>
</tr>
<tr>
<td><strong>Unknown:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECL 11.543</td>
<td>1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>ECL 13.565</td>
<td>2.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprises C16:1ω6c and/or C16:1ω7c.*

*Unknown fatty acids are designated by their equivalent chain-lengths (ECL).*
Other organic substrates included in the Biolog GN2 MicroPlate are not utilized. Susceptible to ampicillin, carbenicillin, kanamycin, novomycin, lincomycin, neomycin, oleandomycin and gentamicin, but resistant to tetracycline. Phosphatidylethanolamine, three unidentified aminolipids and three unidentified lipids are detected. iso-C_{15:0}, iso-C_{15:1} G and summed feature 3 (comprising C_{16:1} 9c and/or C_{16:1} 10c7c) are the major cellular fatty acids. Contains MK-7 as the sole isoprenoid quinone.

The type strain is 3-3^T (＝KACC 17917^T＝JCM 19891^T), which was isolated from forest soil of Bac Kan Province in Vietnam. The DNA G+C content of the type strain is 49.5 mol%.

Acknowledgements

This work was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment (MOE) of the Republic of Korea (NIBR No. 2013-02-004). S. E. J. was supported by Chung-Ang University Research Scholarship Grants in 2014.

References


