Phenylobacterium aquaticum sp. nov., isolated from the reservoir of a water purifier

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A Gram-reaction-negative, strictly aerobic, non-motile, non-spore-forming, rod-shaped bacterial strain designated W2-3-4T was isolated from the reservoir of a water purifier. This bacterium was characterized to determine its taxonomic position by using a polyphasic approach. Strain W2-3-4T grew well at 25–30°C on nutrient and R2A agars. On the basis of 16S rRNA gene sequence similarity, strain W2-3-4T was shown to belong to the family Caulobacteraceae and to be related to Phenylobacterium conjunctum FWC21T (98.0% sequence similarity) and Phenylobacterium haematophilum CCUG 28751T (97.2%). Lower sequence similarities were found with the type strains of all other recognized members of the genus Phenylobacterium (95.7–97.1%). The G+C content of the genomic DNA was 68.7 mol%.

The major respiratory quinone was Q-10 and the major fatty acids were summed feature 8 (comprising C18 : 1ω7c and/or C18 : 1ω6c), C16 : 0, C18 : 1ω7c 11-methyl and summed feature 3 (comprising C16 : 1ω7c and/or C16 : 1ω6c). The polar lipids were phosphatidylglycerol, an unknown phospholipid, four unknown glycolipids and three unidentified polar lipids. DNA–DNA relatedness values between strain W2-3-4T and its closest phylogenetically neighbours were below 7%.

Strain W2-3-4T could be differentiated genotypically and phenotypically from recognized species of the genus Phenylobacterium. The isolate therefore represents a novel species, for which the name Phenylobacterium aquaticum sp. nov. is proposed, with the type strain W2-3-4T (=KACC 18308T=LMG 28593T).

The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of strain W2-3-4T is KT309097.

One supplementary figure and one supplementary table are available with the online Supplementary Material.

The genus Phenylobacterium was proposed by Lingens et al. (1985). Members of the genus Phenylobacterium were characterized as aerobic, Gram-stain-negative, non-motile, non-acid-fast, non-spore-forming straight to slightly curved rods, cocobacilli or cocci, occurring singly, in pairs or in short chains, and having a unique preference for phenyl moieties from heterocyclic compounds. Later, the description of the genus Phenylobacterium was emended several times. The range of DNA G+C content was described as 64–72 mol% (Zhang et al., 2007). Phylogenetic analysis revealed that the genus Phenylobacterium was closely related to the genera Caulobacter and Brevundimonas within the family Caulobacteraceae. At the time of writing, the genus Phenylobacterium comprises eight recognized species (Euzéby, 1997) isolated from various environments, including the recently described species Phenylobacterium conjunctum (Abraham et al., 2008), Phenylobacterium compositi (Weon et al., 2008), Phenylobacterium haematophilum (Abraham et al., 2008) and Phenylobacterium kunshanense (Chu et al., 2015).

During the course of a study on cultivable aerobic bacterial strains using the standard dilution-plating technique on R2A agar at room temperature from the reservoir of a water purifier, Daejeon city, South Korea (36° 36’ N 127° 36’ E), several novel bacterial strains were isolated including strain W2-3-4T, which appeared to be a member of the genus Phenylobacterium. Strain W2-3-4T was cultured routinely on nutrient agar at 30°C and preserved as a suspension in nutrient broth with 20% (w/v) glycerol, at –70°C.

The genomic DNA of strain W2-3-4T, extracted using a commercial DNA-extraction kit (Solgent), was used in a PCR with the universal bacterial primer pair 9F and 1512R to amplify the 16S rRNA gene (Weisburg et al., 1991). The purified PCR products were sequenced by Solgent (Im et al., 2010). The almost-complete sequence of the 16S rRNA gene was compiled using software (DNASTAR) and compared with the 16S rRNA gene sequences of related taxa, which were obtained from the GenBank database or EzTaxon [http://www.ezbiocloud.net/eztaxon; Kim et al. (2012)]. Multiple alignments were made using the CLUSTAL X program (Thompson et al., 1997) with gaps...
edited using the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were reconstructed by using the neighbour-joining method (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood method using the MEGA6 program (Tamura et al., 2013) with bootstrap values based on 1000 replications (Felsenstein, 1985).

The 16S rRNA gene sequence of strain W2-3-4 was determined in this study was a continuous stretch of 1404 bp (base positions 28–1491 with respect to the *Escherichia coli* numbering system), which has been deposited in the GenBank database (accession number KT309087). Sequence similarity calculations using the EzTaxon-e server indicated that the closest relatives of strain W2-3-4 (accession number KT309087). Sequence similarity calculations using the EzTaxon-e server indicated that the closest relatives of strain W2-3-4T were *P. conjunctum* LMG 24262T (98.0% sequence similarity) and *P. haematophilum* LMG 11050T (97.2%). Lower sequence similarities were found with the type strains of all other recognized species of the genus *Phenylobacterium* (95.7–97.1%). Phylogenetic analysis using the neighbour-joining method based on 16S rRNA gene sequences indicated that strain W2-3-4T clustered within the genus *Phenylobacterium*, which was also supported by the maximum-parsimony and maximum-likelihood trees (Fig. 1). Furthermore, comparison of the 16S rRNA gene sequence of strain W2-3-4T with those of other type strains of the genus *Phenylobacterium* revealed that strain W2-3-4T was also missing nucleotides 71–88, 183–190, 206–211 and 452–476 (*Escherichia coli* numbering) (Abraham et al., 2008). Strain W2-3-4T had 178-T, 1265-T and 1270-A, in agreement with all members of the genus *Phenylobacterium* except *Phenylobacterium falsum* AC-49T, and in disagreement with members of the related genera *Caulobacter*, *Brevundimonas* and *Aesticaulis*. In addition, other marker nucleotides in strain W2-3-4T were well conserved at 122-G, 359-A, 639-G, 823-G, 877-C and 1145-C as described by Abraham et al. (2008).

On the basis of these phylogenetic results, *P. conjunctum* FWC21T (=LMG 24262T) and *P. haematophilum* CCUG

![Phylogenetic tree reconstructed from a comparative analysis of 16S rRNA gene sequences showing the relationships of strain W2-3-4T with the type strains of related species. This tree was made using the neighbour-joining method with a Kimura two-parameter distance matrix and pairwise deletion. Filled circles, open circles and square indicate generic branches that were also recovered using the maximum-parsimony and maximum-likelihood algorithms, using the maximum-parsimony algorithm, and using the maximum-likelihood algorithm, respectively. Numbers at nodes indicate bootstrap values (%) as calculated by neighbour-joining maximum-parsimony maximum-likelihood probabilities; only values greater than 50% are shown. Bar, 0.01 substitutions per nucleotide position.](image-url)
26751<sup>T</sup> (=LMG 11050<sup>T</sup>) were selected as the closest recognized neighbours of strain W2-3-4<sup>T</sup> and were obtained from culture collections, grown under the same conditions and used as reference strains in most of the subsequent phenotypic tests.

The Gram reaction was tested by the non-staining method, as described by Buck (1982). Cell morphology was observed using an Optiphot-2 optical microscope (Nikon) at 1000×, using cells grown for 3 days at 30 °C on nutrient agar. Catalase activity was determined by bubble production in 3% (v/v) H<sub>2</sub>O<sub>2</sub> and oxidase activity was determined using 1% (w/v) tetramethyl p-phenylenediamine (Cappuccino & Sherman, 2002). Carbon source utilization and enzyme activities were tested using API 20NE, 32 GN and API ZYM strips (bioMérieux) according to the manufacturer’s instructions. Tests for the hydrolysis of casein, starch (Atlas, 1993) and DNA (using DNase agar from Scharlau, with DNase activity detected by flooding plates with 1 M HCl) were carried out over 3 days at 30 °C. Carbon assimilation tests for phenylalanine, antipyrin and chloridazon were carried out as described by Lingens et al. (1985). Growth at different temperatures (4, 10, 18, 25, 30, 37, 40, 45 and 50 °C) and various pH values (pH 4.5–10.0 at intervals of 0.5 pH units) was assessed after 7 days of incubation in nutrient broth. Three different buffers (final concentration, 50 mM) were used to adjust the pH of the nutrient broth. Three different buffers (final concentration, 50 mM) were used to adjust the pH of the nutrient broth. Three different buffers (final concentration, 50 mM) were used to adjust the pH of the nutrient broth. Three different buffers (final concentration, 50 mM) were used to adjust the pH of the nutrient broth.

Cells of strain W2-3-4<sup>T</sup> were Gram-reaction-negative, aerobic, non-spore-forming, non-motile and long rod-shaped (measuring 0.4–0.6 μm × 2.5–4.0 μm). Colonies grown on the nutrient agar plates for 3 days at 30 °C were smooth, opaque, circular, convex, light brown and 1–2 mm in diameter. The morphological, physiological and biochemical characteristics of strain W2-3-4<sup>T</sup> are given in the species description and Table 1, which lists the characteristics that serve to differentiate strain W2-3-4<sup>T</sup> from its closest phylogenetic relatives.

Genomic DNA of the novel strain and three reference strains was extracted and purified, as described by Moore & Dowhan (1995), so that the G+C content could be determined by reversed-phase HPLC (Mesbah et al., 1989). Cellular fatty acid profiles were determined using cells grown on nutrient agar for 3 days at 30 °C. The cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MID). They were then identified by capillary GLC, using version 6.0 of the Microbial Identification software package (MID), the TSBA60 database (Sasser, 1990) and a 6890 chromatograph (Hewlett Packard). For the chemotaxonomic analysis of isoprenoid quinones, strain W2-3-4<sup>T</sup> was grown in nutrient broth for 3 days at 30 °C. Collected cells were lyophilized for 24 h before the isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v), evaporated under vacuum and re-extracted in n-hexane/water (1:1, v/v). The crude n-hexane–quinone solution was purified using a Sep-Pak Vac silica cartridge (Waters) and subsequently analysed by HPLC, as previously described (Hiraiishi et al., 1996). Polar lipids were extracted and examined by two-dimensional TLC (Minnikin et al., 1984). DNA–DNA hybridization experiments were performed between strain W2-3-4<sup>T</sup> and the two reference strains P. conjunctum LMG 24262<sup>T</sup> and P. haematophilum LMG 11050<sup>T</sup> according to the method described by Ezaki et al. (1989) using photobiotin-labelled DNA probes and micro-dilution wells. Hybridizations were performed at 52.5 °C with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values.

The G+C content of the genomic DNA of strain W2-3-4<sup>T</sup> was determined to be 68.7 mol%, which was similar to those of P. conjunctum LMG 24262<sup>T</sup> and P. haematophilum LMG 11050<sup>T</sup>, 68.0 and 67.8 mol%, respectively (Table 1). The major respiratory quinone of strain W2-3-4<sup>T</sup> was ubiquinone 10 (Q-10), in line with all other members of the family Caulobacteraceae. The fatty acid profile of strain W2-3-4<sup>T</sup> was mainly composed of summed feature 8 (comprising C<sub>18:</sub>ω7c and/or C<sub>18:</sub>ω6c, 33.4%), C<sub>16:</sub>ω0 (27.3%), C<sub>18:</sub>ω7c 11-methyl (14.4%) and summed feature 3 (comprising C<sub>16:</sub>ω7c and/or C<sub>16:</sub>ω6c, 7.9%), C<sub>12:</sub> ω3-OH (2.1%) and C<sub>12:</sub> ω3-OH (9.9%), which were typical of members of the genus Phenylbacterium (Abraham et al., 2008; Oh et al., 2012). However, some minor qualitative and quantitative differences in fatty acid content could be observed between strain W2-3-4<sup>T</sup> and its phylogenetically closest relatives. (Table S1, available in the online Supplementary Material). Strain W2-3-4 had apolar lipid profile comprising phosphatidylglycerol, an unknown phospholipid, four unknown glycolipids and three unidentified polar lipids. This pattern was similar to that of the closely related type strain P. conjunctum LMG 24262<sup>T</sup>, but quite different from that of P. haematophilum LMG 11050<sup>T</sup> (Fig. S1).

DNA–DNA relatedness values between strain W2-3-4<sup>T</sup> and P. conjunctum LMG 24262<sup>T</sup> and P. haematophilum LMG 11050<sup>T</sup> were 7.0±1.0 and 2.5±0.6%, respectively. According to Wayne et al. (1987), DNA–DNA relatedness values lower than 70% are considered to be the threshold value for the delineation of genospecies, so the values obtained are low enough to assign strain W2-3-4<sup>T</sup> to a novel species of the genus Phenylbacterium.

The characteristics of strain W2-3-4<sup>T</sup> are consistent with the description of the genus Phenylbacterium with regard to morphological, biochemical and chemotaxonomic properties. However, the phylogenetic distance between strain W2-3-4<sup>T</sup> and recognized Phenylbacterium species, the unique phenotypic characteristics (Table 1) and the low level of DNA–DNA relatedness with the nearest neighbours identified by phylogenetic analysis
Table 1. Physiological characteristics of strain W2-3-4\textsuperscript{T} and related type strains of the genus *Phenylobacterium*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolation source</td>
<td>Reservoir of water purifier</td>
<td>Soil</td>
<td>Soil</td>
</tr>
<tr>
<td>Cell size (µm)</td>
<td>0.4–0.6×2.5–4.0</td>
<td>1.15–1.60×0.5–0.7</td>
<td>0.3–0.4×0.9–2.5</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Temperature range (optimum) (°C)</td>
<td>18–40</td>
<td>25–30</td>
<td>18–40</td>
</tr>
<tr>
<td>pH range</td>
<td>6.0–8.0</td>
<td>5.5–8.0</td>
<td>6.0–8.5</td>
</tr>
<tr>
<td>NaCl range (%)</td>
<td>0–0.5</td>
<td>0–2</td>
<td>0–2</td>
</tr>
</tbody>
</table>

**API 20 NE & ID32 GN tests:**

- Nitrate reduction to nitrite: + + –
- β-Glucosidase (aesculin hydrolysis): + – +
- Gelatin hydrolysis: – + –
- β-Galactosidase (PNPG): + – +
- Adipate: – + +
- Malate: – + +
- D-Mannitol: – + –
- Salicin: – – +
- Propionate: – – +
- Valerate: – + +
- 3-Hydroxy-butyrate: – + +
- L-Proline: – – +
- Suberate: – + +
- Acetate: – + +

**API ZYM results:**

- Valine arylamidase: + + –
- α-Chymotrypsin: + + –
- α-Galactosidase: + – –
- β-Glucuronidase: + – –
- α-Glucosidase: + + –
- α-Fucosidase: – – +
- DNA G+C content (mol%): 68.7 67.0 67.8

All data were from this study, except the G+C contents of the reference strains (taken from Abraham et al., 2008). All strains were negative for urease and arginine dihydrolase, but negative for acid production from glucose, indole production, and assimilation of L-arabinose, D-glucose, D-mannose, maltose, D-mannitol, gluconate, caprate, citrate, phenyl-acetate, melibiose, L-fucose, D-sorbitol, L-histidine, 2-ketogluconate, L-rhamnose, N-acetyl-D-glucosamine, D-ribose, inositol, sucrose, itaconate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, L-serine and malonate. In API ZYM kits, all the strains were positive for alkaline phosphatase, esterase lipase (C8), leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase, β-glucosidase, N-acetyl-β-glucosaminidase, acid phosphatase and esterase (C4), but negative for lipase (C14), crystalline arylamidase and α-mannosidase. +, Positive; –, negative.

### Description of *Phenylobacterium aquaticum* sp. nov.

*Phenylobacterium aquaticum* (aquaticum. L. neut. adj. aquaticum living in water, referring to the isolation of the type strain from water).

Cells are Gram-reaction-negative, oxidase-positive and catalase-negative, strictly aerobic, non-motile, non-spore-forming and rod shaped (0.4–0.6 µm in diameter and 2.5–4.0 µm in length) after culture on nutrient agar for 3 days. Colonies are smooth, opaque, convex, circular with regular margins, light brown and 1–2 mm in diameter. Growth occurs on nutrient agar at 18–40 °C (optimum, 25–30 °C), at pH 6.0–8.0 (optimum, 7.0) and with 0–0.5 % (w/v) NaCl. Grows well on nutrient agar without supplementary NaCl. Does not hydrolyse DNA, casein or starch. Assimilates phenylalanine but not antipyrin or chloridazon as a sole carbon source. Results of sole carbon source
assimilation tests (API ID 32 GN, API 20 NE) and enzyme activities (API ZYM) are listed in Table 1. Q-10 is the predominant respiratory quinone, and summed feature 8 (comprising C18:1ω7c and/or C18:1ω6c), C16:0, C18:1ω7c 11-methyl and summed feature 3 (comprising C16:1ω7c and/or C16:1ω6c) are the major cellular fatty acids (>7%). Hydroxylated fatty acids, C12:0 3-OH and C12:1 3-OH, are detected in small amounts. The polar lipids are phosphatidylethanolamine, phosphatidylglycerol, and unknown phospholipid, four unknown glycolipids and three unidentified polar lipids.

The type strain, W2-3-4T (=KACC 18306T=LMG 28593T), was isolated from the reservoir of a water purifier, South Korea. The G+C content of the genomic DNA of the type strain is 68.7 mol%.

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References


Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyri-
nucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39, 224–229.


Oh, Y. S. & Roh, D. H. (2012). Phenylbacterium muchangonense sp. nov., isolated from beach soil, and emended description of the genus Phenylbac-


Zhang, K., Han, W., Zhang, R., Xu, X., Pan, Q. & Hu, X. (2007). Phenylbacter-
ium zucineum sp. nov., a facultative intracellular bacterium isolated from a human erythroblastic cell line K562. Syst Appl Microbiol 30, 207–212.