

**Pseudacidovorax intermedius** gen. nov., sp. nov., a novel nitrogen-fixing betaproteobacterium isolated from soil

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A Gram-negative, short rod-shaped, nitrogen-fixing bacterium (CC-21T) was isolated from a soil sample collected from the regional agricultural research station in Kaohsiung County (Taiwan). Using 16S rRNA gene sequence analysis, it could be clearly demonstrated that this isolate was novel: it showed <97% similarity to species of the genera *Acidovorax*, *Alicyclobacillus*, *Giesbergeria*, *Simplicispira* and *Diaphorobacter*. The organism used several organic acids, but only a few sugars as substrates. The fatty acid profile differed from those reported for members of the genera *Acidovorax*, *Alicyclobacillus*, *Giesbergeria*, *Simplicispira* and *Diaphorobacter*. On the basis of 16S rRNA gene sequence analysis in combination with physiological data, strain CC-21T represents a novel species in a new genus, for which the name *Pseudacidovorax intermedius* gen. nov., sp. nov. is proposed; the type strain is CC-21 (=CCUG 54492T = CIP 109510T).

Strain CC-21T was isolated from a soil sample from an experimental field soil sample of the regional agricultural research station in Kaohsiung County (Taiwan) that was regularly cultivated with soybean. Surface soil samples were collected and plated on nutrient agar after appropriate serial dilutions. Strain CC-21T was maintained on nutrient agar after incubation at 32 °C for 2 days. Subcultivation for physiological, biochemical and chemotaxonomic analyses was done on tryptone soy agar (TSA; Oxoid) at 28 °C for 24 h up to 7 days. On this agar, strain CC-21T was able to grow at 15–36 °C, but not at 10 or 45 °C. The organism was able to grow on nutrient agar, TSA and R2A agar (all Oxoid). It stained Gram-negative using the modified Hucker method described by Burris (1972) and the *nifH* gene was amplified from genomic DNA of strain CC-21T by nested PCR using degenerate primers Pol-F and Pol-R (Poly et al., 2001). Reaction products were separated by agarose gel electrophoresis and stained with ethidium bromide; the band size was calculated by comparison with the 100 bp ladder length standard (Gibco-BRL). The purified *nifH* PCR product was directly sequenced. A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by the neighbour-joining method (Saitou & Nei, 1987), was used for phylogenetic analysis.

DNA for the determination of the G+C content was isolated by using the UltraClean microbial DNA isolation kit (MOBIO) according to the manufacturer’s instructions. The DNA G+C content was determined by HPLC using a Waters Symmetry Shield C8 column as described by Mesbah et al. (1989). Non-methylated lambda phage DNA (Sigma) was used as the calibration reference. Cell morphology was observed under a Zeiss light microscope at ×1000 using cells that had been grown for 3 days at 28 °C on nutrient agar (Oxoid). Cell morphology results

**Abbreviations**: pNA, para-nitroanilide; pNP, para-nitrophenyl.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifH* gene sequences of strain CC-21T are EF469609 and EU008822, respectively.

A *nifH* gene sequence-based phylogenetic tree generated by using the neighbour-joining method, showing the relationship between strain CC-21T and other dinitrogen-fixing bacteria, is available as a supplementary figure with the online version of this paper.

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are given in the species description. The 16S rRNA gene was analysed as described previously (Kämpfer et al., 2003; Young et al., 2005).

Analysis of the sequence data was performed by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar et al., 2001) after multiple alignments of data using CLUSTAL_X (Thompson et al., 1997). A distance matrix method (distance options according to the Kimura two-parameter model), including clustering by neighbour-joining (Saitou & Nei, 1987), and a discrete character-based maximum-parsimony method were used. In each case, bootstrap values were calculated based on 1000 replications. The 16S rRNA gene sequence of strain CC-21T was a continuous stretch of 1491 bp. Based on 16S rRNA gene sequence analysis, strain CC-21T was classified in the family Comamonadaceae of the class Betaproteobacteria. Sequence similarity calculations indicated that strain CC-21T showed the greatest degree of similarity to Acidovorax avenae subsp. avenae (GenBank accession no. AB021421; 96.9%), Alicyclobacillus denitrificans (GenBank accession no. AJ418042; 96.2%).

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences available from the GenBank/EMBL/DDJB database (accession numbers are given in parentheses) constructed after multiple alignments of data using CLUSTAL_X (Thompson et al., 1997). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining method were determined by using the software package MEGA (Molecular Evolutionary Genetics Analysis) version 2.1 (Kumar et al., 2001). Percentage bootstrap values based on 1000 replications are given at branch points. Bar, 0.005 nucleotide substitutions per nucleotide position.
and Acidovorax temperans (GenBank accession no. AF078766; 96.1%). Lower sequence similarities (<96%) were found with members of all other genera shown in Fig. 1. Maximum-parsimony analysis (data not shown) confirmed the separate position of strain CC-21\textsuperscript{T}.

The nifH gene of strain CC-21\textsuperscript{T} showed the greatest degree of similarity to those of Pelomonas saccharophila (GenBank accession no. AB188120; 92.0%) and Burkholderia vietnamiensis (GenBank accession no. AJ512206; 89.7%) (see Supplementary Fig. S1, available in IJSEM Online). In the nifH gene-based phylogenetic tree, strain CC-21\textsuperscript{T} is placed among other members of the class Betaproteobacteria that are capable of dinitrogen fixation, thus further confirming the nitrogen-fixing ability of strain CC-21\textsuperscript{T}.

Fatty acid analyses were performed according to Kämpfer & Kroppenstedt (1996) after subcultivating the strain in TSA for 48 h at 28 °C. The fatty acid profile of strain CC-21\textsuperscript{T} (given in the species description) was similar to those of members of the genera listed in Table 1, but also showed significant differences. Strain CC-21\textsuperscript{T} showed high amounts of C\textsubscript{16:0} summed feature 3 (C\textsubscript{16:0} i/C\textsubscript{15:0} i iso 2-OH) and C\textsubscript{18:1}ω7c as reported for strains of the genera Acidovorax, Alicyclobifluvi, Giesbergeria, Simplicispira and Diaphorobacter, but also showed relatively high amounts (>8%) of C\textsubscript{17:0} cyclo, which is not reported in Giesbergeria or Simplicispira species (Grabovich et al., 2006), and only in small amounts in species of Acidovorax (Gardan et al., 2003; Schulze et al., 1999; Willems et al., 1990) and Alicyclobifluvi (Mechichi et al., 2003). In addition, C\textsubscript{8:0} 3-OH was found, a hydroxylated fatty acid that is not detected in Alicyclobifluvi, Giesbergeria, Simplicispira or Diaphorobacter, but found in Acidovorax (Gardan et al., 2003; Grabovich et al., 2006; Willems et al., 1990). The presence of the hydroxylated fatty acids C\textsubscript{16:1} 2-OH and C\textsubscript{18:1} 2-OH distinguished strain CC-21\textsuperscript{T} from all other related genera.

Results of the physiological characterization, determined using previously described methods (Kämpfer et al., 1991), are given in the species description and Table 1. Denitrification and autotrophic growth with H\textsubscript{2} were negative. Strain CC-21\textsuperscript{T} used several organic acids, but only a few sugars as substrates.

On the basis of these results, it is proposed that strain CC-21\textsuperscript{T} represents a novel species in a new genus, Pseudacidovorax intermedius gen. nov., sp. nov.

**Description of Pseudacidovorax intermedius sp. nov.**

Pseudacidovorax intermedius (in.ter.me’di.us. L. masc. adj. intermedius intermediate, because of the intermediate phylogenetic position of the species).

Forms short rod-shaped, motile cells (0.5 × 1.5–2.0 μm). Cells contain poly-β-hydroxybutyrate granules after Sudan Black staining. Gram-negative. Oxidase- and catalase-positive. Aerobic or facultatively aerobic metabolism. Unable to denitrify. Capable of dinitrogen fixation. Colonies are beige pigmented on nutrient agar. Good growth occurs after 96 h incubation on TSA and nutrient agar at 30 °C. Fatty acid profile of the type strain comprises C\textsubscript{16:0} (22.6%), C\textsubscript{18:1}ω7c (26.3%), summed feature 3 (C\textsubscript{16:1} i/C\textsubscript{15:0} i iso 2-OH; 21.5%), C\textsubscript{14:0} (4.7%), C\textsubscript{17:0} cycl (8.9%), C\textsubscript{18:0} (2.1%), C\textsubscript{16:0} 3-OH (1.3%), C\textsubscript{10:0} 3-OH (4.3%), C\textsubscript{16:1} 2-OH (1.8%) and C\textsubscript{18:1} 2-OH (2.0%).

Aesculin is not hydrolysed. Negative for acid production from glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, adonitol, inositol, rhamnose, maltose, trehalose, cellobiose, D-arabitol, D-mannose, sorbitol, L-arabinose, raffinose, D-xylene, methyl-D-glucoside, erythritol and melibiose. The following carbon sources are utilized (method according to Kämpfer et al., 1991): acetate, propionate, cis-aconitate (weak), trans-aconitate (weak), 4-aminobutyrate (weak), azelate, citrate (weak), fumarate, glutarate, DL-3-hydroxybutyrate, DL-lactate, L-alanine, L-aspartate, L-proline, L-arabinose (weak), D-glucose (weak) and D-fructose (weak).

The following carbon substrate utilization tests are negative: N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, P-arbutin, D-cellobiose, D-glucosanate, maltose, D-mannose, melibiose, L-rhamnose, D-ribose, sucrose, salicin, trehalose, D-xylene, adonitol, L-inositol, maltitol, D-mannitol, sorbitol, putrescine, adipate, itaconate, mesaconate, 2-oxoglutarate, suberate, β-alanine, L-leucine, L-ornithine, L-phenylalanine, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate.


The type strain is CC-21\textsuperscript{T} (=CCUG 54492\textsuperscript{T}=CIP 109510\textsuperscript{T}), isolated from soil. The DNA G+C content of the type strain is 70.1 mol%.

**Description of Pseudacidovorax intermedius gen. nov.**

Pseudacidovorax (Pseu.da.ci.do.vo’rax. Gr. adj. pseudes false; N.L. masc. n. acidovorax a bacterial genus name, Acidovorax; N.L. masc. n. Pseudacidovorax the false Acidovorax).

Cells are Gram-negative, short rods that are motile by a polar flagellum. Facultatively aerobic. Growth occurs after 96 h incubation on TSA and nutrient agar at 30 °C. Major fatty acids are C\textsubscript{16:0}0 C\textsubscript{18:1}ω7c and summed feature 3 (C\textsubscript{16:1ω7c} C\textsubscript{15:0} i iso 2-OH). The hydroxylated fatty acids C\textsubscript{8:0} 3-OH, C\textsubscript{10:0} 3-OH, C\textsubscript{16:1} 2-OH and C\textsubscript{18:1} 2-OH are also present. The type and only species is Pseudacidovorax intermedius.
Table 1. Differential characteristics of the genus *Pseudacidovorax* and related genera within the family *Comamonadaceae*


<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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<tbody>
<tr>
<td>Cell shape</td>
<td>Rods</td>
<td>Spirilla</td>
<td>Spirilla, curved rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Spirilla</td>
<td>Rods or spirilla</td>
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<tr>
<td>Flagellation</td>
<td>Polar monotrichous</td>
<td>Bipolar tufts</td>
<td>Bipolar tufts</td>
<td>Polar monotrichous or absent</td>
<td>Polar monotrichous</td>
<td>Monotrichous</td>
<td>+</td>
<td>Bipolar tufts</td>
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<td>Autotrophic growth with H₂</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>d</td>
<td>NA</td>
<td>−</td>
<td>−</td>
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<td>Reduction of nitrate</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>d</td>
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<tr>
<td>Denitrification</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>d</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Assimilation of:</td>
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<td>D-Fructose</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>−</td>
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<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
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<td>Glycerol</td>
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<td>−†</td>
<td>+</td>
<td>NA</td>
<td>+</td>
<td>−</td>
<td>d</td>
</tr>
<tr>
<td>β-Alanine</td>
<td>−</td>
<td>−</td>
<td>−†</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
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<tr>
<td>3-Hydroxy fatty acids‡</td>
<td>C₁₀:₀, C₈:₀</td>
<td>C₁₀:₀, (C₈:₀ *)</td>
<td>C₁₀:₀</td>
<td>C₁₀:₀, (C₈:₀ §)</td>
<td>C₁₀:₀</td>
<td>C₁₀:₀, C₁₂:₀</td>
<td>C₁₀:₀</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>70.1</td>
<td>63–65</td>
<td>56.5–60</td>
<td>62–66</td>
<td>64–65</td>
<td>66</td>
<td>65</td>
<td>59.7–68.7</td>
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<tr>
<td>Source</td>
<td>Soil</td>
<td>Freshwater, wastewater, Antarctic mosses</td>
<td>Sulfide spring, freshwater, wastewater, pond water</td>
<td>Soil, freshwater, clinical isolates</td>
<td>Activated sludge</td>
<td>Wastewater</td>
<td>Pond water, stream water</td>
<td>Soil, freshwater, wastewater, clinical isolates</td>
</tr>
</tbody>
</table>

*Positive for *S. psychrophila.*
†Positive for *G. kuznetsovi.*
‡Data from this study and Sakane & Yokota (1994).
§Present in phytopathogenic species.
Acknowledgements

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References


