Hydrogenophaga caeni sp. nov., isolated from activated sludge

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A Gram-negative bacterium, designated strain EMB71\textsuperscript{T}, was isolated from activated sludge used for enhanced biological phosphorus removal in a sequencing batch reactor. The cells of the isolate were facultatively aerobic, motile rods with single polar flagella. Growth was observed to occur at 15–35 °C (optimally at 30 °C) and at pH 6.0–9.0 (optimally at pH 7.0–8.0). The predominant fatty acids of strain EMB71\textsuperscript{T} were C\textsubscript{16}:0 and summed feature 3 (C\textsubscript{16}:1\textit{ω}7c and/or iso-C\textsubscript{15}:0 2-OH), and the polar lipids comprised a large amount of phosphatidylethanolamine and a small amount of diphosphatidylglycerol. The G+C content of the genomic DNA was 61.6 mol % and the major quinone was Q-8. Comparative 16S rRNA gene sequence analyses showed that strain EMB71\textsuperscript{T} formed a phyletic lineage with the genus Hydrogenophaga within the family Comamonadaceae. The levels of 16S rRNA gene sequence similarity with respect to the type strains of Hydrogenophaga species ranged from 95.1 to 96.9 %. On the basis of the phenotypic, chemotaxonomic and molecular data, strain EMB71\textsuperscript{T} represents a novel species of the genus Hydrogenophaga, for which the name Hydrogenophaga caeni sp. nov. is proposed. The type strain is EMB71\textsuperscript{T} (=KCTC 12613\textsuperscript{T} = DSM 17962\textsuperscript{T}).

The genus Hydrogenophaga of the family Comamonadaceae was established, by Willems et al. (1989), as including four species: Hydrogenophaga flava, Hydrogenophaga pseudoflava, Hydrogenophaga taeniospiralis and Hydrogenophaga palleronii, all of which originally belonged to the genus Pseudomonas. Later, three more species, Hydrogenophaga intermedia, Hydrogenophaga defluvii and Hydrogenophaga atypica, were added to the genus (Contzen et al., 2000; Kämpfer et al., 2005). Initially, members of this genus were known as chemolithoautotrophic bacteria (using the oxidation of H\textsubscript{2} as an energy source and CO\textsubscript{2} as a carbon source), this being one of the main characteristics used to distinguish them from other genera of the family Comamonadaceae. However, it was reported that H. intermedia and H. atypica are unable to grow chemolithoautotrophically using hydrogen gas. Activated sludge processes for the removal of phosphate from wastewater have been used to reduce the eutrophication process in lakes. Therefore, during work designed to characterize the bacterial flora of activated sludge used for enhanced biological phosphorus removal, we isolated a novel non-chemolithoautotrophic strain (EMB71\textsuperscript{T}) subsequently identified as belonging to the genus Hydrogenophaga.

Strain EMB71\textsuperscript{T} was isolated from activated sludge used for enhanced biological phosphorus removal in a laboratory-scale sequencing batch reactor. Sodium acetate was supplied as a sole carbon source; the operation of the sequencing batch reactor has been described elsewhere (Jeon et al., 2003). To isolate the strain, a sludge sample was diluted serially with a 1 % (w/v) saline solution, spread on R2A agar (Difco) and incubated at 20 °C for 7 days. Subculturing was performed on R2A agar at 30 °C for 5 days. Gram staining was performed using a bioMérieux Gram stain kit according to the manufacturer’s instructions. Cell morphology and motility were studied using phase-contrast microscopy and transmission electron microscopy (JEM-1010; JEOL) as described by Jeon et al. (2005). The physiological characteristics of strain EMB71\textsuperscript{T} were examined by growing the isolate in R2A broth at various temperatures and pH values. R2A media with different pH values were prepared as described previously (Gomori, 1955). Oxidase activity was tested by assessing the oxidation of 1 % (w/v) tetramethyl-

p-phenylenediamine (Merck), and catalase activity was evaluated by determining the production of oxygen bubbles in a 3 % (v/v) aqueous hydrogen peroxide solution. The hydrolysis of casein, gelatin, Tweens 80 and 20, aesculin,

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain EMB71\textsuperscript{T} is DQ372983.

Figures showing the morphology of negatively stained cells of strain EMB71\textsuperscript{T} and phylogenetic trees generated using maximum-likelihood and maximum-parsimony and a table detailing the cellular fatty acid compositions of strain EMB71\textsuperscript{T} and related species are available as supplementary material in IJSEM Online.
urea, tyrosine and starch was investigated on R2A agar according to methods described by Lanyi (1987) and Gerhardt et al. (1994). The tests were read after 7 days incubation at 30 °C. Nitrate reduction was determined according to the method of Lanyi (1987), and acid production from carbohydrates was tested as described by Leifson (1963). The utilization of thiosulfate was tested in R2A broth supplemented with 10 mM Na₂S₂O₃·5H₂O, as described by Spring et al. (2004); the concentration of sulfate, the end product of thiosulfate oxidation, was quantified in spent R2A broth by using ion chromatography (ICS-1000; Dionex). Chemolithoautotrophic growth of strain EMB71T with hydrogen gas was tested on Medium 81 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) agar under the conditions described by Malik & Schlegel (1981). The oxidation of various substrates, additional enzymic activities and various biochemical features were determined using Biolog GN2 MicroPlate assay (Biolog) and API (API ZYM and API 20E; bioMérieux) kits at 30 °C as recommended by the manufacturers.

Growth of strain EMB71T was observed at temperatures between 15 and 35 °C; optimum growth occurred at 30 °C. The strain grew at pH 6.0–9.0, with an optimum at pH 7.0–8.0. The cells of the isolate were facultatively aerobic, Gram-negative, non-spore-forming, motile rods (0.8–1.2 μm wide and 1.4–2.0 μm long); the motility was by means of single polar flagella (see Supplementary Fig. S1, available in IJSEM Online). The strain grew on R2A agar, but not on Luria–Bertani agar, tryptic soy broth agar (Difco) or YPG agar (Contzen et al., 2000). Anaerobic growth was observed after 7 days incubation at 30 °C on R2A agar. The cells of strain EMB71T were oxidase- and catalase-positive and were able to reduce nitrate to nitrite, but they did not produce nitrogen gas. Strain EMB71T oxidized thiosulfate to sulfate, but no aerobic autotrophic growth with hydrogen as a substrate was detected. Other phenotypic features of strain EMB71T are presented in Table 1 and in the species description. Some of them are in accordance with characteristics of members of the genus Hydrogenophaga, while others allow the differentiation of strain EMB71T from closely related Hydrogenophaga species (Table 1).

For analysis of the fatty acid methyl esters, cells of strain EMB71T and related species were harvested from agar plates after incubation for 3 days on R2A agar. Analysis of the fatty acid methyl esters was performed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Analyses of polar lipids and isoprenoid quinones were carried out using the methods described by Komagata & Suzuki (1987). The DNA G+C content of strain EMB71T was determined by using HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; Grom) according to the method of Tamaoka & Komagata (1984). The major respiratory lipoquinone of strain EMB71T was found to be Q-8. The cellular membrane of the strain contained C₁₆:₀ (27.51 %) and summed feature 3 (C₁₆:₁₀7c and/or iso-C₁₅:₀ 2-OH, 44.77 %) as the major fatty acids and its profile resembles those determined for related Hydrogenophaga species. However, the fatty acid profile allows clear differentiation of strain EMB71T from the closely related species Xenophilus azovorans KF46T (Table 1 and Supplementary Table S1 in IJSEM Online). The polar lipids comprised a large amount of phosphatidylethanolamine and a small amount of diphasphatidylglycerol. The G+C content of the genomic DNA of strain EMB71T was 61.6 mol%. The G+C content, the predominant fatty acids and the major lipoquinone and polar lipid are in accordance with those of members of the genus Hydrogenophaga (Palleroni, 1984; Willems et al., 1989; Kämpfer et al., 2005).

Sequencing of the 16S rRNA gene was carried out as described previously (Lane, 1991). The resulting sequence (1446 nt) of strain EMB71T was compared with 16S rRNA gene sequences available from GenBank by using the BLAST program (http://www.ncbi.nlm.nih.gov/blast/) to determine an approximate phylogenetic affiliation, and the sequences were aligned with those of closely related species, namely the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in PHYLIP, version 3.6 (Felsenstein, 2002). Values for sequence similarity between the novel strain and related micro-organisms were computed using the FASTA3 program (European Bioinformatics Institute; http://www.ebi.ac.uk/fasta33/nucleotide.html). A bootstrap analysis was performed according to the algorithm of the Kimura two-parameter model (Kimura, 1980) of the neighbour-joining method in the PHYLP package. DNA–DNA hybridization was carried out to evaluate the genomic DNA–DNA relatedness between strain EMB71T and H. defluvii BSB 9.5T and H. atypica BSB 41.8T. Extracted genomic DNAs were fragmented with HaeIII for slot hybridization (Lim et al., 2005). Digested DNAs were diluted serially and loaded into slots, with three replications, and the DNA was used individually as a labelled DNA probe for cross-hybridization. Random primed DNA labelling with digoxigenin-dUTP and hybridization were performed using the DIG-High Prime DNA labelling kit (Roche Applied Science) according to the manufacturer’s instructions and standard procedures (Sambrook & Russell, 2001; Lim et al., 2005). Phylogenetic analysis based on the 16S rRNA gene sequences indicated that strain EMB71T formed a phylectic lineage with the genus Hydrogenophaga (Fig. 1). The topologies of phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms also supported the notion that the isolate belongs to the genus Hydrogenophaga (see Supplementary Fig. S2, available in IJSEM Online). Comparative 16S rRNA gene sequence analyses also showed that the isolate was most closely related to H. defluvii BSB 9.5T, H. atypica BSB 41.8T and H. palleronii DSM 63T, with sequence similarities of 96.9, 96.8 and 96.5 %, respectively. The DNA–DNA relatedness values obtained from hybridization experiments involving strain
Table 1. Comparison of characteristics of strain EMB71<sup>T</sup> and some related species

Strains: 1, strain EMB71<sup>T</sup> (data from this study); 2, *H. defluvii* BSB 9.5<sup>T</sup> (Kämpfer et al., 2005); 3, *H. atypica* BSB 41.8<sup>T</sup> (Kämpfer et al., 2005); 4, *H. flava* ATCC 33667<sup>T</sup> (Palleroni, 1984); 5, *H. intermedia* S1<sup>T</sup> (Contzen et al., 2000); 6, *H. palleronii* DSM 63<sup>T</sup> (Palleroni, 1984); 7, *H. taeniospiralis* ATCC 49743<sup>T</sup> (Willems et al., 1989); 8, *X. azovorans* KF46<sup>FT</sup> (Blümel et al., 2001). +, Positive; −, negative.

<table>
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<th>Characteristic</th>
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<td>30</td>
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<td>Denitrification†</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<td>−</td>
<td>+</td>
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<td>Nitrate reduction*</td>
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<td>+</td>
<td>+</td>
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<td>Oxidation of thiosulfate to sulfate</td>
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<td>−</td>
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<td>−</td>
<td>+</td>
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<td>+</td>
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<td>Major cellular fatty acids*</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, C&lt;sub&gt;18:1ω7c&lt;/sub&gt; summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, C&lt;sub&gt;17:0&lt;/sub&gt; cyclo, summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, C&lt;sub&gt;17:0&lt;/sub&gt; cyclo, summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt; summed feature 3</td>
<td>C&lt;sub&gt;16:0&lt;/sub&gt;, C&lt;sub&gt;17:0&lt;/sub&gt; cyclo</td>
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<td>DNA G+C content (mol%)</td>
<td>61.6</td>
<td>65.0</td>
<td>64.0</td>
<td>66.7</td>
<td>68.9*</td>
<td>67.3</td>
<td>64.8</td>
<td>70.4</td>
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*Results were obtained in this study and tests that were also performed by previous researchers gave congruent results.
†Growth under anoxic conditions on R2A agar with NO<sub>3</sub><sup>−</sup> as the terminal electron acceptor.
‡Summed feature 3 contained C<sub>16:1ω7c</sub> and/or iso-C<sub>15:0</sub> 2-OH.
EMB71\textsuperscript{T} with \textit{H. defluvii} BS9.5\textsuperscript{T} and \textit{H. atyica} BSB 41.8\textsuperscript{T} were about 28 and 25\%, respectively, which are clearly below the 70\% threshold generally accepted for species delineation (Stackebrandt \textit{et al.}, 2002). The physiological, biochemical and phylogenetic data for strain EMB71\textsuperscript{T} also support its description as a novel species within the genus \textit{Hydrogenophaga}, for which the name \textit{Hydrogenophaga caeni} sp. nov. is proposed.

\textbf{Description of \textit{Hydrogenophaga caeni} sp. nov.}

\textit{Hydrogenophaga caeni} (ca.e’ni. L. gen. n. caeni of mud, referring to isolation of the type strain from activated sludge).

Colonies on R2A agar are white, glistening, translucent, raised and circular with entire margins. Growth occurs optimally at 30\(^\circ\) C and pH 7.0–8.0. On R2A agar at 30\(^\circ\) C, cells are Gram-negative rods (0.8–1.2 \(\mu\)m wide and 1.4–2.0 \(\mu\)m long). Motile by means of single polar flagella. Nitrate is reduced to nitrite, but nitrogen gas is not produced. Thiosulfate is oxidized to sulfate. No aerobic chemoautotrophic growth occurs with hydrogen as a substrate. Catalase-positive and oxidase-positive. Anaerobic growth is observed. Tween 80 is hydrolysed. Tyrosine, aesculin, casein, Tween 20, starch, gelatin and urea are not hydrolysed. Acid is produced from D-raffinose and myo-inositol, but not from D-lactose, D-mannitol, melibiose, D-glucose, sorbitol, sucrose, rhamnose, amygdalin, D-fructose, D-galactose, D-mannose, L-arabinose, arbutin or salicin. Leucine arylamidase is produced, but esterase (C4), lipase (C14), acid phosphatase, trypsin, x-chymotrypsin, naphthol-AS-BI-phosphohydrolase, cystine arylamidase, \(\alpha\)-galactosidase, \(\beta\)-galactosidase, \(\alpha\)-glucosidase, \(\beta\)-glucosidase, N-acetyl-\(\beta\)-glucosaminidase, \(\alpha\)-mannosidase and \(\alpha\)-fucosidase are not produced. Weak enzymic activities are observed for alkaline phosphatase and esterase lipase (C8). The following are utilized (Biolog GN2): Tweens 40 and 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, adonitol, \(\alpha\)-D-glucose, myo-inositol, \(\alpha\)-D-lactose, maltose, L-rhamnose, D-sorbitol, succinic acid monomethyl ester, cis-aconitic acid, formic acid, D-galactonic acid lactone, \(\alpha\)-hydroxybutyric acid, \(\beta\)-hydroxybutyric acid, \(\alpha\)-keto-glutaric acid, DL-lactic acid, succinic acid, L-alaninamide, D-alanine, \(\alpha\)-leucine, phenylethylamine, putrescine and 2,3-butanediol. Other organic substrates included in Biolog GN2 microplates are not utilized. Cells contain a large amount of phosphatidylethanolamine and a small amount of diphasphatidylglycerol as the polar lipids. The major isoprenoid quinone is Q-8. Major fatty acids are C16:0 (27.51\%) and summed feature 3 (C16:1\(\omega7\)c and/or iso-C15:0 2-OH, 44.77\%). The DNA G+C content of the type strain is 116.6 mol\% (HPLC).

The type strain, EMB71\textsuperscript{T} (=KCTC 12613\textsuperscript{T} = DSM 17962\textsuperscript{T}), was isolated from sludge used for enhanced biological phosphorus removal in a laboratory-scale sequencing batch reactor.
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