Hydrogenophaga caeni sp. nov., isolated from activated sludge

Bok Sil Chung, Seung Hyun Ryu, Minjeong Park, Yeji Jeon, Young Ryun Chung and Che Ok Jeon

Correspondence
Che Ok Jeon
cojeon@gnu.ac.kr

Division of Applied Life Science, EB-NCRC, PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea

A Gram-negative bacterium, designated strain EMB71^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^T were C_{16:0} and summed feature 3 (C_{16:1}ω7c and/or iso-C_{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^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^T represents a novel species of the genus *Hydrogenophaga*, for which the name *Hydrogenophaga caeni* sp. nov. is proposed. The type strain is EMB71^T (=KCTC 12613^T = DSM 17962^T).

The genus *Hydrogenophaga* of the family *Comamonadaceae* was established, by Willems *et al.* (1989), as including four species: *Hydrogenophaga flava*, *Hydrogenophaga pseudo-flava*, *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 H₂ as an energy source and CO₂ as a carbon source), this being one of the main characteristics used to distinguish them from other genera of the family *Comamonadaceae* (Contzen *et al.*, 2000; Kämpfer *et al.*, 2005). 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^T) subsequently identified as belonging to the genus *Hydrogenophaga*.

Strain EMB71^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^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^T is DQ372983.

Figures showing the morphology of negatively stained cells of strain EMB71^T and phylogenetic trees generated using maximum-likelihood and maximum-parsimony and a table detailing the cellular fatty acid compositions of strain EMB71^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 EMB71^T 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 EMB71^T 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 EMB71^T were oxidase- and catalase-positive and were able to reduce nitrate to nitrite, but they did not produce nitrogen gas. Strain EMB71^T oxidized thiosulfate to sulfate, but no aerobic autotrophic growth with hydrogen as a substrate was detected. Other phenotypic features of strain EMB71^T 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 EMB71^T from closely related *Hydrogenophaga* species (Table 1).

For analysis of the fatty acid methyl esters, cells of strain EMB71^T 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 EMB71^T 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 EMB71^T was found to be Q-8. The cellular membrane of the strain contained C_{16:0} (27.51 %) and summed feature 3 (C_{16:1}ω7c

and/or iso-C_{15:0} 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 EMB71^T from the closely related species *Xenophilus azovorans* KF46F^T (Table 1 and Supplementary Table S1 in IJSEM Online). The polar lipids comprised a large amount of phosphatidylethanolamine and a small amount of diphosphatidylglycerol. The G + C content of the genomic DNA of strain EMB71^T 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 EMB71^T 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 using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed using three different methods, 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 PHYLIP package. DNA–DNA hybridization was carried out to evaluate the genomic DNA–DNA relatedness between strain EMB71^T and *H. defluvii* BSB 9.5^T and *H. atypica* BSB 41.8^T. Extracted genomic DNAs were fragmented with *Hae*III 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 EMB71^T formed a phyletic 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 9.5^T, *H. atypica* BSB 41.8^T and *H. palleronii* DSM 63^T, 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^T and some related species

Strains: 1, strain EMB71^T (data from this study); 2, *H. defluvii* BSB 9.5^T (Kämpfer *et al.*, 2005); 3, *H. atypica* BSB 41.8^T (Kämpfer *et al.*, 2005); 4, *H. flava* ATCC 33667^T (Palleroni, 1984); 5, *H. intermedia* S1^T (Contzen *et al.*, 2000); 6, *H. palleronii* DSM 63^T (Palleroni, 1984); 7, *H. taeniospiralis* ATCC 49743^T (Willems *et al.*, 1989); 8, *X. azovorans* KF46F^T (Blümel *et al.*, 2001). +, Positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8
Optimum temperature (°C)	30	25	25	30	30*	30	30	30
Denitrification†	+	–	–	–	–	–	+	–*
Nitrate reduction*	+	+	+	+	+	–	+	–
Colony pigmentation (yellow)*	–	+	+	+	+	+	+	+
Oxidation of thiosulfate to sulfate	+	–	–	–	+	+	+	–*
Utilization/assimilation of:*								
L-Arabinose	–	–	–	+	–	+	–	–
Sorbitol	+	–	–	+	–	+	–	–
Mannitol	–	–	–	+	+	–	+	+
Maltose	+	–	–	+	–	–	–	–
L-Histidine	+	+	–	+	–	–	–	+
Major cellular fatty acids*‡	C _{16:0} , summed feature 3	C _{16:0} , summed feature 3	C _{16:0} , summed feature 3	C _{16:0} , C _{18:1ω7c} , summed feature 3	C _{16:0} , C _{17:0} , C _{17:0} cyclo, summed feature 3	C _{16:0} , C _{17:0} cyclo, summed feature 3	C _{16:0} , summed feature 3	C _{16:0} , C _{17:0} cyclo
DNA G+C content (mol%)	61.6	65.0	64.0	66.7	68.9*	67.3	64.8	70.4

*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 NO₃[–] as the terminal electron acceptor.

‡Summed feature 3 contained C_{16:1ω7c} and/or iso-C_{15:0} 2-OH.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain EMB71^T and related taxa. Bootstrap percentages, based on 1000 replicates, are shown at branch points, when greater than 50%. *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. Bar, 0.01 changes per nucleotide position. Trees generated using maximum-likelihood and maximum-parsimony are available as Supplementary Fig. S2 in IJSEM Online.

EMB71^T with *H. defluvii* BSB 9.5^T and *H. atypica* BSB 41.8^T were about 28 and 25 %, respectively, which are clearly below the 70 % threshold generally accepted for species delineation (Stackebrandt *et al.*, 2002). The physiological, biochemical and phylogenetic data for strain EMB71^T also support its description as a novel species within the genus *Hydrogenophaga*, for which the name *Hydrogenophaga caeni* sp. nov. is proposed.

Description of *Hydrogenophaga caeni* sp. nov.

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 °C and pH 7.0–8.0. On R2A agar at 30 °C, cells are Gram-negative rods (0.8–1.2 µm wide and 1.4–2.0 µm 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, α -chymotrypsin, naphthol-AS-BI-phosphohydrolase, cystine arylamidase, α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -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, α -D-glucose, *myo*-inositol, α -D-lactose, maltose, L-rhamnose, D-sorbitol, succinic acid monomethyl ester, *cis*-aconitic acid, formic acid, D-galactonic acid lactone, α -hydroxybutyric acid, β -hydroxybutyric acid, α -ketoglutaric acid, DL-lactic acid, succinic acid, L-alaninamide, D-alanine, L-alanyl glycine, L-asparagine, L-histidine, hydroxy-L-proline, L-proline, L-threonine, γ -aminobutyric acid, 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 diphosphatidylglycerol as the polar lipids. The major isoprenoid quinone is Q-8. Major fatty acids are C_{16:0} (27.51 %) and summed feature 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH, 44.77 %). The DNA G+C content of the type strain is 61.6 mol % (HPLC).

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

Acknowledgements

This work was supported by grants from MOST/KOSEF to the Environmental Biotechnology National Core Research Center (grant R15-2003-012-02002-0) and to the 21C Frontier Microbial Genomics and Application Center Program (grant MG05-0104-4-0), Ministry of Science and Technology, Korea. S.H.R. and M.P. were supported by scholarships from the BK21 program of the Ministry of Education and Human Resources Development in Korea.

References

- Blümel, S., Busse, H.-J., Stolz, A. & Kämpfer, P. (2001). *Xenophilus azovorans* gen. nov., sp. nov., a soil bacterium that is able to degrade azo dyes of the Orange II type. *Int J Syst Evol Microbiol* **51**, 1831–1837.
- Contzen, M., Moore, E. R., Blümel, S., Stolz, A. & Kämpfer, P. (2000). *Hydrogenophaga intermedia* sp. nov., a 4-aminobenzenesulfonate degrading organism. *Syst Appl Microbiol* **23**, 487–493.
- Felsenstein, J. (2002). PHYLIP (phylogeny inference package), version 3.6a. Department of Genome Sciences, University of Washington, Seattle, USA.
- Gerhardt, P., Murray, R. G. E., Wood, W. A. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington, DC: American Society for Microbiology.
- Gomori, G. (1955). Preparation of buffers for use in enzyme studies. In *Methods in Enzymology*, vol. 1, pp. 138–146. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- Jeon, C. O., Lee, D. S. & Park, J. M. (2003). Microbial communities in activated sludge performing enhanced biological phosphorus removal in a sequencing batch reactor. *Water Res* **37**, 2195–2205.
- Jeon, C. O., Lim, J.-M., Lee, J.-M., Xu, L.-H., Jiang, C.-L. & Kim, C.-J. (2005). Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **55**, 1891–1896.
- Kämpfer, P., Schulze, R., Jäckel, U., Malik, K. A., Amann, R. & Spring, S. (2005). *Hydrogenophaga defluvii* sp. nov. and *Hydrogenophaga atypica* sp. nov., isolated from activated sludge. *Int J Syst Evol Microbiol* **55**, 341–344.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Lane, D. J. (1991). 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, pp. 115–175. Edited by E. Stackebrandt & M. Goodfellow. Chichester, UK: Wiley.
- Lanyi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Leifson, E. (1963). Determination of carbohydrate metabolism of marine bacteria. *J Bacteriol* **85**, 1183–1184.
- Lim, J.-M., Jeon, C. O., Park, D.-J., Kim, H.-R., Yoon, B.-J. & Kim, C.-J. (2005). *Pontibacillus marinus* sp. nov., a moderately halophilic bacterium from a solar saltern, and emended description of the genus *Pontibacillus*. *Int J Syst Evol Microbiol* **55**, 1027–1031.
- Malik, K. A. & Schlegel, H. G. (1981). Chemolithoautotrophic growth of bacteria able to grow under N₂-fixing conditions. *FEMS Microbiol Lett* **11**, 63–67.
- Palleroni, N. J. (1984). Genus I. *Pseudomonas* Migula 1894, 237^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 141–199. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Spring, S., Jäckel, U., Wagner, M. & Kämpfer, P. (2004). *Ottowia thiooxydans* gen. nov., sp. nov., a novel facultatively anaerobic, N₂O-producing bacterium isolated from activated sludge, and transfer of *Aquaspirillum gracile* to *Hylemonella gracilis* gen. nov., comb. nov. *Int J Syst Evol Microbiol* **54**, 99–106.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M., Grimont, P. A., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002). Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Willems, A., Busse, J., Goor, M., Pot, B., Falsen, E., Jantzen, E., Hoste, B., Gillis, M., Kersters, K. & other authors (1989). *Hydrogenophaga*, a new genus of hydrogen-oxidizing bacteria that includes *Hydrogenophaga flava* comb. nov. (formerly *Pseudomonas flava*), *Hydrogenophaga palleronii* (formerly *Pseudomonas palleronii*), *Hydrogenophaga pseudoflava* (formerly *Pseudomonas pseudoflava* and “*Pseudomonas carboxydoflava*”), and *Hydrogenophaga taeniospiralis* (formerly *Pseudomonas taeniospiralis*). *Int J Syst Bacteriol* **39**, 319–333.