Hydrogenophaga defluvii sp. nov. and *Hydrogenophaga atypica* sp. nov., isolated from activated sludge

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Two Gram-negative, oxidase-positive rods (strains BSB 9.5^T and BSB 41.8^T) isolated from wastewater were studied using a polyphasic approach. 16S rRNA gene sequence comparisons demonstrated that both strains cluster phylogenetically within the family *Comamonadaceae*: the two strains shared 99·9 % 16S rRNA gene sequence similarity and were most closely related to the type strains of *Hydrogenophaga palleronii* (98·5 %) and *Hydrogenophaga taeniospiralis* (98·0 %). The fatty acid patterns and substrate-utilization profiles displayed similarity to the those of the five *Hydrogenophaga* species with validly published names, although clear differentiating characteristics were also observed. The two strains showed DNA–DNA hybridization values of 51 % with respect to each other. No close similarities to any other *Hydrogenophaga* species were detected in hybridization experiments with the genomic DNAs. On the basis of these results, two novel *Hydrogenophaga* species, *Hydrogenophaga defluvii* sp. nov. and *Hydrogenophaga atypica* sp. nov. are proposed, with BSB 9.5^T (=DSM 15341^T=CIP 108119^T) and BSB 41.8^T (=DSM 15342^T=CIP 108118^T) as the respective type strains.

The genus *Hydrogenophaga* was described by Willems *et al.* (1989) and, at present, comprises five species: *Hydrogenophaga flava*, *Hydrogenophaga pseudoflava*, *Hydrogenophaga palleronii*, *Hydrogenophaga taeniospiralis* (Willems *et al.*, 1989) and *Hydrogenophaga intermedia* (Contzen *et al.*, 2000). Members of this genus are chemo-organotrophic or chemolithoautotrophic, using the oxidation of H_2 as an energy source and CO_2 as a carbon source, these being major differentiating characteristics for distinguishing between them and other genera of the family *Comamonadaceae* (Wen *et al.*, 1999; Spring *et al.*, 2004).

Numerous studies applying cultivation-independent methods have revealed that members of the β1-group of the *Proteobacteria* are abundant in activated sludge from wastewater treatment plants (e.g. Amann *et al.*, 1996b; Snaidr *et al.*, 1997). Since members of this group, although closely related, are physiologically diverse, it is almost impossible to infer the metabolic phenotype of members of this group by 16S rRNA gene sequence comparison studies. To determine their physiological traits, members of this important bacterial group were isolated, by a directed cultivation procedure, from activated sludge of the wastewater treatment plant München I (Großlappen, Germany) as described by Schulze *et al.* (1999). The isolates obtained were screened by whole-cell hybridization with specific probes directed against signature regions of 16S rRNA sequences. Isolates that hybridized to probe BONE23a (Amann *et al.*, 1996b) directed against members of the β1-group of the *Proteobacteria* were further grouped using the probes LDI (Wagner *et al.*, 1994) and SNA8b (Amann *et al.*, 1996b). Two strains (BSB 9.5^T and BSB 41.8^T) that hybridized to probe LDI were also detected by the probe HYD208 directed against members of the genus *Hydrogenophaga*, which seemed to represent an abundant group of wastewater bacteria (Amann *et al.*, 1996a). Therefore, these two isolates were chosen for further investigation. Both strains were maintained on nutrient agar (Difco). Type strains of all *Hydrogenophaga* species with validly published names were used for comparison.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BSB 9.5^T and BSB 41.8^T are AJ585993 and AJ585992, respectively.

Phylogenetic trees and a table showing fatty acid compositions are available as supplementary material in IJSEM Online.

Table 1. Physiological characteristics of the type strains of *Hydrogenophaga* species

Strains: 1, *H. defluvii* sp. nov. BSB 9.5^T; 2, *H. atypica* sp. nov. BSB 41.8^T; 3, *H. flava* DSM 619^T; 4, *H. pseudoflava* LMG 5945^T; 5, *H. taeniospiralis* DSM 2082^T; 6, *H. palleronii* DSM 63^T; 7, *H. intermedia* S1^T. +, Positive; −, negative; (+), weakly positive. Data for reference taxa are from Contzen *et al.* (2000).

Characteristic	1	2	3	4	5	6	7
Utilization/assimilation of:*							
L-Arabinose, sucrose, D-galactose, D-fructose, D-mannose, sorbitol, D-cellobiose	−	−	+	+	−	+	−
Formate	−	−	−	−	−	+	−
Mannitol	−	−	+	+	+	−	+
Maltose	−	−	+	+	−	−	−
L-Histidine	(+)	−	+	+	−	−	−
Azelate	−	−	−	+	+	+	−
2-Oxoglutarate	−	+	−	−	−	−	−
D-Xylose	−	−	−	+	+	−	−
Autotrophic growth with H_2	+	−	+	+	+	+	−
Oxidation of thiosulfate to sulfate	−	−	−	−	−	+	+
Denitrification†	−	−	−	+	+	−	−
Reduction of nitrate	+	+	+	+	+	−	+

*Tests (based on a different method) were also performed by Willems *et al.* (1989) with *H. flava*, *H. pseudoflava*, *H. taeniospiralis* and *H. palleronii* and gave congruent results.
†For strains BSB 9.5^T, BSB 41.8^T and *H. intermedia*, no growth was observed under anoxic conditions on R2A agar with NO_3 as terminal electron acceptor. Data for *H. flava*, *H. pseudoflava*, *H. taeniospiralis* and *H. palleronii* are from Willems *et al.* (1989).

Cell morphology was examined by using phase-contrast microscopy (Leitz). Cell dimensions were measured with an ocular (× 10) and an objective (× 100/1·25). Gram-staining was performed by using Hucker's modification (Gerhardt *et al.*, 1994). Colony morphology was studied using a stereo microscope (model SZ 11; Olympus).

The effects of different temperatures on growth were determined on Bacto nutrient agar (Oxoid) incubated at 5, 10, 28, 37, 45 and 50 °C. Physiological tests in microtitre plates were performed as described previously (Kämpfer *et al.*, 1991). Tests were read after 7 days at 30 °C. Chemolithoautotrophic growth of both strains was tested under the conditions described by the DSMZ. Both strains were grown on medium 81 (Malik & Schlegel, 1981) under an atmosphere of $O_2/CO_2/H_2/N_2$ (approximately 2 : 10 : 60 : 28, by vol.) (Malik & Schlegel, 1981). Nitrate reduction and denitrification were tested in R2A broth (Difco) supplemented with 10 mM nitrate under aerobic and anaerobic conditions. The obligately aerobic heterotrophic strains BSB 9.5^T and BSB 41.8^T grew as circular, entire, slightly convex, smooth, pale-yellow colonies on R2A agar (Oxoid). The cells were Gram-negative, non-spore-forming, motile, rod-shaped organisms. Both strains grew at 28 °C on nutrient agar, R2A agar and tryptone soy broth agar (Oxoid), but only very weak growth was observed on MacConkey agar (Oxoid). They did not grow on nutrient agar at 5 or 10 °C, but grew well in a temperature range from 20 to 37 °C. Neither strain grew at 40 or 45 °C. Both strains were oxidase- and catalase-positive. Only a few organic compounds could be used as sole sources of carbon (see Table 1 and the species descriptions). Differentiation from all five *Hydrogenophaga* species is possible on the basis of the results of several tests. Both strains could be differentiated on the basis of the utilization of 2-oxoglutarate and L-histidine. A detailed comparison was made with all previously published data (Willems *et al.*, 1989; Contzen *et al.*, 2000). Table 1 shows only those tests for which identical results were obtained with the method used in this study and the method used by Willems *et al.* (1989). Only strain BSB 9.5^T showed good chemolithotrophic growth. Strain BSB 41.8^T was not able to grow chemolithoautotrophically under the conditions described – an additional important feature enabling differentiation between the two strains. The utilization of thiosulfate was tested with all type strains of *Hydrogenophaga* in R2A medium supplemented with 10 mM $Na_2S_2O_3.5H_2O$, as described by Spring *et al.* (2004). Only the type strains of *H. palleronii* (DSM 63^T) and *H. intermedia* (DSM 5680^T) were positive and oxidized thiosulfate to sulfate.

The 16S rRNA gene was analysed as described by Kämpfer *et al.* (2003). Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004) as well as the software package MEGA version 2.1 (Kumar *et al.*, 2001) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Calculation of distances (with distance options according to the Kimura-2 model) and clustering with the neighbour-joining method and maximum parsimony was performed. Bootstrap values based on 1000 replications were determined to show support for branching points (results are available as supplementary figures in IJSEM

Online). Nearly complete 16S rRNA gene sequences of BSB 9.5^T and BSB 41.8^T, respectively comprising 1503 and 1527 nucleotide positions, were determined by PCR amplification and sequencing of the PCR-amplified 16S rRNA genes. A comparative analysis and estimation of the phylogenetic relationships demonstrated that the two strains show 99·9 % sequence similarity and cluster within the genus *Hydrogenophaga*, being most closely related to *H. palleronii* (98·5 %) and *H. taeniospiralis* (98·0 %).

The G + C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989).

For fatty acid analysis, cells were grown on YPG agar (Contzen *et al.*, 2000). The fatty acid methyl esters were prepared and analysed as described elsewhere (Kämpfer & Kroppenstedt, 1996). When grown on YPG agar, the two strains were very similar with regard to their fatty acid patterns and, as with the type strains of all *Hydrogenophaga* species, contained the fatty acids 15 : 0, 16 : 0, summed feature 4 (16 : 1ω7*c* and/or 15 : 0 iso 2-OH) and summed feature 7 (18 : 1ω7*c*, 18 : 1ω9*t* and/or 18 : 1ω12*t*) (see the supplementary table available in IJSEM Online). These fatty acids have also been detected in previous studies and are characteristic of all *Hydrogenophaga* species with validly published names (Willems *et al.*, 1989). The presence of the hydroxy fatty acid 8 : 0 3-OH in all *Hydrogenophaga* species could be confirmed, although this fatty acid could be detected only in trace amounts in *H. pseudoflava* LMG 5945^T and *H. taeniospiralis* DSM 2082^T. *H. intermedia* S1^T (when grown on YPG agar) contained 8 : 0 3-OH in larger amounts (> 2·5 %), a feature shared only by *H. palleronii* DSM 63^T. Additionally, both isolates (BSB 9.5^T and BSB 41.8^T) and the type strains *H. palleronii* DSM 63^T and *H. intermedia* S1^T produced large amounts of the cyclopropane fatty acid, 17 : 0 cyclo, in agreement with data in the literature (Willems *et al.*, 1989).

DNA–DNA hybridization experiments were performed with both isolates and with the type strains of all *Hydrogenophaga* species by using the method described by Ziemke *et al.* (1998) except that, for nick translation, 2 µg DNA was labelled during a 3 h incubation at 15 °C. Strains BSB 9.5^T and BSB 41.8^T showed a DNA–DNA hybridization value of 51 % (mean value from four experiments, including reciprocal analyses), indicating that they belong to different species, despite their close 16S rRNA gene sequence similarity. The following DNA–DNA hybridization values between BSB 9.5^T and other *Hydrogenophaga* type strains were found: *H. flava*, 30 %; *H. pseudoflava*, 26 %; *H. palleronii*, 29 %; *H. taeniospiralis*, 13 %; *H. intermedia*, 12 %. The DNA–DNA hybridization values between BSB 41.8^T and other *Hydrogenophaga* type strains were as follows: *H. flava*, 32 %; *H. pseudoflava*, 28 %; *H. palleronii*, 31 %; *H. taeniospiralis*, 26 %; *H. intermedia*, 9 %.

Although both strains showed a very high degree of 16S rRNA gene sequence similarity, they could be differentiated from each other and from other species of the genus *Hydrogenophaga* by means of DNA–DNA hybridization and a few physiological tests. In conclusion, we propose the names *Hydrogenophaga defluvii* sp. nov. for strain BSB 9.5^T and *Hydrogenophaga atypica* sp. nov. for strain BSB 41.8^T.

Description of *Hydrogenophaga defluvii* sp. nov.

Hydrogenophaga defluvii (de.flu′vi.i. L. n. *defluvium* sewage; L. gen. n. *defluvii* of sewage).

Cells are Gram-negative, rod-shaped, motile and 1·5 µm long by 0·5 µm wide, with rounded ends. Metabolism is oxidative. Oxidase-positive. Able to grow chemolithoautotrophically on H_2 under the conditions described. Chemolithoautotrophic growth of *H. defluvii* (in contrast to that of *H. flava*) is not inhibited by high levels of oxygen in the atmosphere (5 % O_2 or more). Thiosulfate is not oxidized to sulfate. The fatty acid pattern is characterized by the presence of the fatty acids typical of the genus *Hydrogenophaga*, as well as the hydroxylated fatty acid 8 : 0 3-OH and also small amounts of 9 : 0 3-OH. Phylogenetically, the species is a member of the genus *Hydrogenophaga*. On YPG agar at 25 °C, colonies are circular, entire, slightly convex, smooth and pale yellow. Growth occurs at 37 °C but not at 10 °C. Only a few organic compounds can be used as sole sources of carbon: gluconate, glutarate, lactate, 3-hydroxybutyrate, pyruvate, suberate, L-alanine, L-leucine, L-aspartate, L-histidine, phenylalanine, L-proline, 3-hydroxybenzoate and 4-hydroxybenzoate. Furthermore, tests for hydrolysis of L-alanine *p*-nitroanilide (pNA) are positive. Does not utilize *N*-acetyl-D-glucosamine, L-arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, D-mannitol, adipate, D-melibiose, L-rhamnose, ribose, sucrose, salicin, trehalose, D-xylose, adonitol, inositol, sorbitol, putrescine, acetate, propionate, *cis*-aconitate, *trans*-aconitate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, itaconate, D-malate, mesaconate, 2-oxoglutarate, β-alanine, L-ornithine, L-serine, L-tryptophan or phenylacetate as sole sources of carbon. Tests for hydrolysis of *p*-nitrophenyl (pNP) α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP β-D-galactopyranoside, pNP β-D-glucuronide, pNP phenylphosphonate, pNP phosphorylcholine, 2-deoxythymidine-5′-pNP phosphate, glutamate-γ-3-carboxy pNP-ester and L-proline pNA are negative. The G + C content of the genomic DNA is 65 mol%. Characteristics used for differentiation from other *Hydrogenophaga* species are given in Table 1.

The type strain, BSB 9.5^T (= DSM 15341^T = CIP 108119^T), was isolated from activated sludge in Munich, Germany.

Description of *Hydrogenophaga atypica* sp. nov.

Hydrogenophaga atypica (Gr. pref. *a-*, *an-* not; L. adj. *typicus*, *-a*, *-um* from Gr. adj. *tupikos* typical; N.L. fem. adj. *atypica* atypical).

Cells are Gram-negative, rod-shaped, motile and 1·5 µm long by 0·5 µm wide, with rounded ends. Oxidative metabolism. Oxidase-positive. Unable to grow chemolithoautotrophically

on H_2 under the conditions described. Thiosulfate is not oxidized to sulfate. The fatty acid pattern is characterized by the presence of the fatty acids typical of the genus *Hydrogenophaga* with the hydroxylated fatty acid 8:0 3-OH and also 9:0 3-OH. Phylogenetically, the species is a member of the genus *Hydrogenophaga*. On YPG agar at 25 °C, colonies are circular, entire, slightly convex, smooth and pale yellow. Growth occurs at 37 °C but not at 10 °C. Only a few organic compounds can be used as sole sources of carbon: gluconate, glutarate, lactate, 3-hydroxybutyrate, 2-oxoglutarate, pyruvate, phenylalanine, L-proline, 3-hydroxybenzoate and 4-hydroxybenzoate. Furthermore, tests for hydrolysis of L-alanine pNA are positive. Does not utilize *N*-acetyl-D-glucosamine, L-arabinose, arbutin, D-cellobiose, D-fructose, D-galactose, D-glucose, D-mannose, D-mannitol, adipate, D-melibiose, L-rhamnose, ribose, sucrose, salicin, trehalose, D-xylose, adonitol, inositol, sorbitol, putrescine, acetate, propionate, *cis*-aconitate, *trans*-aconitate, suberate, 4-aminobutyrate, azelate, citrate, fumarate, glutarate, itaconate, D-malate, mesaconate, L-alanine, β-alanine, L-leucine, L-aspartate, L-histidine, L-ornithine, L-serine, L-tryptophan or phenylacetate as sole sources of carbon. Tests for hydrolysis of pNP α-D-glucopyranoside, pNP β-D-glucopyranoside, pNP β-D-galactopyranoside, pNP β-D-glucuronide, pNP phenylphosphonate, pNP phosphorylcholine, 2-deoxythymidine-5′-pNP phosphate, glutamate-γ-3-carboxy pNP-ester and L-proline pNA are negative. The G+C content of the genomic DNA is 64 mol%. Characteristics used for differentiation from the other *Hydrogenophaga* species are given in Table 1.

The type strain, BSB 41.8^T (=DSM 15342^T=CIP 108118^T), was isolated from activated sludge in Munich, Germany.

Acknowledgements

We thank Jean Euzéby for his nomenclatural advice and Peter Schumann for determining the G+C values.

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