Hydrogenophaga bisanensis sp. nov., isolated from wastewater of a textile dye works

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A Gram-negative, non-spore-forming, rod-shaped, *Hydrogenophaga*-like bacterial strain, K102^T, was isolated from wastewater collected from a textile dye works in Korea and subjected to a polyphasic taxonomic study. Strain K102^T grew optimally at pH 7.0–8.0 and 30–37 °C in the presence of 0.5 % (w/v) NaCl. It contained Q-8 as the predominant ubiquinone and C_{16:0}, C_{16:1 ω 7c} and/or iso-C_{15:0} 2-OH and C_{18:1 ω 7c} as the major fatty acids. The DNA G+C content was 64.8 mol%. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain K102^T fell within the radiation of the cluster comprising species of the genus *Hydrogenophaga*. Strain K102^T exhibited 16S rRNA gene sequence similarity values of 95.9–98.9 % to the type strains of recognized *Hydrogenophaga* species. Levels of DNA–DNA relatedness between strain K102^T and the type strains of its four phylogenetically most closely related species, together with differential phenotypic properties, revealed that strain K102^T could be distinguished from all recognized species of the genus *Hydrogenophaga*. On the basis of phenotypic, phylogenetic and genetic data, strain K102^T is considered to represent a novel species of the genus *Hydrogenophaga*, for which the name *Hydrogenophaga bisanensis* sp. nov. is proposed. The type strain is K102^T (=KCTC 12980^T =CCUG 54518^T).

The genus *Hydrogenophaga* was proposed by Willems *et al.* (1989) with the reclassification of *Pseudomonas* species as *Hydrogenophaga flava*, *H. palleronii*, *H. pseudoflava* and *H. taeniospiralis*. Subsequently, four further species, *Hydrogenophaga intermedia* (Contzen *et al.*, 2000), *H. atypica* and *H. defluvii* (Kämpfer *et al.*, 2005) and *H. caeni* (Chung *et al.*, 2007), have been described. Phylogenetic analysis based on 16S rRNA gene sequences showed that the genus *Hydrogenophaga* falls within the family *Comamonadaceae*, class *Betaproteobacteria* (Anzai *et al.*, 2000; Kämpfer *et al.*, 2005). In the present study, we report on the taxonomic characterization of a *Hydrogenophaga*-like bacterial strain, designated K102^T, which was isolated from wastewater of a textile dye works in Bisan, Daegu city, Korea.

Strain K102^T was isolated by means of the standard dilution plating technique on nutrient agar (NA; Difco) at 30 °C. *H. atypica* DSM 15342^T, *H. defluvii* DSM 15341^T,

H. flava DSM 619^T and *H. pseudoflava* DSM 1034^T, which were used as reference strains for DNA–DNA hybridization experiments, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. The morphological, physiological and biochemical characteristics of strain K102^T were investigated by using routine cultivation on NA at 37 °C. Cell morphology was examined by light microscopy (Nikon E600) and transmission electron microscopy with cells from exponentially growing cultures. Flagellation was determined by using a Philips CM-20 transmission electron microscope with cells from exponentially growing cultures: for this purpose, cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. The Gram reaction was determined by using the bioMérieux Gram stain kit according to the manufacturer's instructions. Growth at various temperatures (4–50 °C) was measured on NA. The pH range for growth was determined in nutrient broth (NB; Difco) that was adjusted to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) by the addition of HCl and Na₂CO₃. Growth in the absence of NaCl and at various NaCl concentrations [0.5 % (w/v) and 1.0–5.0 % (w/v) at intervals of 1.0 %] was investigated by using trypticase soy broth prepared according to the formula of the Difco

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K102^T is EF532793.

A table detailing the cellular fatty acid compositions of strain K102^T and the type strains of recognized species of the genus *Hydrogenophaga* is available as supplementary material with the online version of this paper.

medium except that NaCl was excluded. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on NA and on NA supplemented with potassium nitrate (0.1%, w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities and hydrolysis of casein, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were studied as described by Lanyi (1987). Utilization of thiosulfate was tested in NB supplemented with 10 mM $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ as described by Spring *et al.* (2004), and the concentration of sulfate and the end product of thiosulfate oxidation were quantified in spent NB by using an ion chromatograph (ICS-1000; Dionex). Chemolithoautotrophic growth of strain K102^T with hydrogen gas was tested on a solid medium (DSMZ medium no. 81) under the conditions described by Malik & Schlegel (1981). Susceptibility to antibiotics was tested on NA plates by using discs containing the following antibiotics: polymyxin B, 100 U; streptomycin, 50 µg; penicillin G, 20 U; chloramphenicol, 100 µg; ampicillin, 10 µg; cephalothin, 30 µg; gentamicin, 30 µg; novobiocin, 5 µg; tetracycline, 30 µg; kanamycin, 30 µg; lincomycin, 15 µg; oleandomycin, 15 µg; neomycin, 30 µg; and carbenicillin, 100 µg. Utilization of various substrates, enzyme activities and other physiological and biochemical properties were tested by using the API 20E, API 20NE, API 50CH and API ZYM systems (bioMérieux); utilization of various substrates was determined by inoculating the API 50CH strip with cells suspended in AUX medium (bioMérieux). Utilization of various substrates was also tested as described by Kämpfer *et al.* (1991).

Cell biomass for DNA extraction and for isoprenoid quinone analysis was obtained by cultivation in NB at 37 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR by using two universal primers (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed by using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain K102^T was harvested from NA plates after incubation for 3 days at 37 °C and from YPG agar (Contzen *et al.*, 2000) after incubation for 2 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with a modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization experiments were performed

fluorometrically according to the method of Ezaki *et al.* (1989) by using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values are quoted as DNA–DNA relatedness values.

Morphological, cultural, physiological and biochemical characteristics of strain K102^T are given in the species description or are shown in Table 1. Strain K102^T grew under anoxic conditions on NA with nitrate as a terminal electron acceptor. The almost-complete 16S rRNA gene sequence of strain K102^T determined in this study comprised 1484 nucleotides, representing approximately 96% of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analysis showed that strain K102^T was phylogenetically related most closely to members of the genus *Hydrogenophaga* (Fig. 1). In the neighbour-joining tree based on 16S rRNA gene sequences, strain K102^T fell within the genus *Hydrogenophaga* by joining the cluster comprising *H. flava* and *H. pseudoflava* at a bootstrap resampling value of 100% (Fig. 1). Strain K102^T exhibited 16S rRNA gene sequence similarity values of 98.9, 98.8, 97.3 and 97.2% to the type strains of *H. flava*, *H. pseudoflava*, *H. atypica* and *H. defluvii*, respectively, and of 95.9–96.8% to the type strains of other recognized species of the genus. 16S rRNA gene sequence similarity values to the type strains of other species included in the phylogenetic analysis were less than 95.1%.

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain K102^T and some related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms, while open circles indicate that the corresponding nodes were also recovered in the tree generated with one of these algorithms. *Burkholderia cepacia* ATCC 25416^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Table 1. Differential phenotypic characteristics between strain K102^T and recognized species of the genus *Hydrogenophaga*

Taxa: 1, strain K102^T; 2, *H. flava* (data from Palleroni, 1984; Willems *et al.*, 1989; Contzen *et al.*, 2000; Kämpfer *et al.*, 2005); 3, *H. pseudoflava* (Palleroni, 1984; Willems *et al.*, 1989; Contzen *et al.*, 2000; Kämpfer *et al.*, 2005); 4, *H. taeniospiralis* (Lalucat *et al.*, 1982; Willems *et al.*, 1989; Kämpfer *et al.*, 2005); 5, *H. palleronii* (Palleroni, 1984; Willems *et al.*, 1989; Contzen *et al.*, 2000; Kämpfer *et al.*, 2005); 6, *H. intermedia* (Contzen *et al.*, 2000; Kämpfer *et al.*, 2005); 7, *H. defluvii* (Kämpfer *et al.*, 2005); 8, *H. atypica* (Kämpfer *et al.*, 2005); 9, *H. caeni* EMB71^T (Chung *et al.*, 2007). +, Positive; –, negative; ND, no data available. All organisms are Gram-negative, motile rods and are positive for oxidase but negative for hydrolysis of starch (not determined for *H. intermedia*, *H. defluvii* or *H. atypica*).

Characteristic	1	2	3	4	5	6	7	8	9
Optimum temperature	30–37	30	35–38	33 or 37*	30	30	25	25	30
Growth at 41 °C	+	–	+	ND	–	ND	ND	ND	–
Yellow pigmentation	+	+	+	+	+	+	+	+	–
Denitrification	+	–	+	+	–	–	–	–	+
Nitrate reduction	+	+	+	+	–	+	+	+	+
Nitrite reduction	–	–	+	+	–	ND	ND	ND	–
Autotrophic growth with H ₂	–	+	+	+	+	–	+	–	–
Oxidation of thiosulfate to sulfate	+	–	–	–	+	+	–	–	+
Hydrolysis of:									
Aesculin	–	+	ND	–†	ND	ND	ND	ND	–
Gelatin	–	–	–	+	–	ND	ND	ND	–
Tween 80	–	–	+	+	+	ND	ND	ND	+
Utilization/assimilation of:‡									
L-Arabinose, sucrose, D-galactose, D-mannose, cellobiose	–	+	+	–	+	–	–	–	–
D-Fructose	+	+	+	–	+	–	–	–	–
Sorbitol	–	+	+	–	+	–	–	–	+
Mannitol	–	+	+	+	–	+	–	–	–
Maltose	–	+	+	–	–	–	–	–	+
D-Xylose	–	–	+	+	–	–	–	–	ND

*No growth at 37 °C according to Willems *et al.* (1989).

†Reported as positive by Willems *et al.* (1989).

‡Data for strain K102^T were obtained according to the method of Kämpfer *et al.* (1991); congruent results were obtained with the API 50CH system. Data for *H. taeniospiralis* are from Kämpfer *et al.* (2005).

The chemotaxonomic properties of strain K102^T confirmed the phylogenetic result that this strain represented a member of the genus *Hydrogenophaga*. The predominant isoprenoid quinone detected in strain K102^T was ubiquinone-8 (Q-8), which has been reported as the predominant ubiquinone in species of the genus *Hydrogenophaga* (Willems *et al.*, 1989; Chung *et al.*, 2007). The cellular fatty acid profile of strain K102^T is detailed in Supplementary Table S1 (available in IJSEM Online), together with those of the type strains of recognized *Hydrogenophaga* species; the major fatty acids of strain K102^T were C_{16:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{18:1}ω7c. This fatty acid profile was similar to those of recognized *Hydrogenophaga* species, although there were differences in the proportions of some components (Supplementary Table S1). The DNA G+C content of strain K102^T was 64.8 mol%.

Strain K102^T exhibited DNA–DNA relatedness levels of 11, 17, 28 and 25 %, respectively, to the type strains of *H. atypica*, *H. defluvii*, *H. flava* and *H. pseudoflava*, which showed 16S rRNA gene sequence similarity values of greater than 97 % to the novel strain. Strain K102^T differed

from recognized *Hydrogenophaga* species based on several phenotypic characteristics (Table 1). Phylogenetic distinctiveness together with DNA–DNA relatedness data and differential phenotypic properties were sufficient to allocate strain K102^T in a species that is distinct from recognized species of the genus *Hydrogenophaga* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994). Therefore, on the basis of the data presented, strain K102^T is considered to represent a novel species of the genus *Hydrogenophaga*, for which the name *Hydrogenophaga bisanensis* sp. nov. is proposed.

Description of *Hydrogenophaga bisanensis* sp. nov.

Hydrogenophaga bisanensis (bi.san.en'sis. N.L. fem. adj. *bisanensis* of Bisan, Daegu, Korea, from where the type strain was isolated).

Cells are Gram-negative, non-spore-forming rods (0.4–0.6 × 1.0–5.0 µm). Colonies on NA are circular to slightly irregular, raised, smooth, glistening, moderate yellow in colour and 1.5–3.0 mm in diameter after 3 days incubation

at 37 °C. The optimal temperature range for growth is 30–37 °C. Growth occurs at 15 and 46 °C, but not at 10 or 47 °C. The optimal pH range for growth is 7.0–8.0; growth occurs at pH 6.5 and 9.5, but not at pH 6.0 or 10.0. Growth occurs in the presence of 0–1.0 % (w/v) NaCl; optimal growth occurs in the presence of 0.5 % (w/v) NaCl. Anaerobic growth does not occur on NA, but does occur on NA supplemented with potassium nitrate. Weakly positive for catalase. Casein, hypoxanthine, xanthine, L-tyrosine, urea and Tweens 20, 40 and 60 are not hydrolysed. H₂S and indole are not produced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase are absent. In the API 20NE and API 50CH systems, adipate, glycerol, glucose and gluconate are utilized, but caprate, malate, citrate, phenylacetate, erythritol, D-arabinose, ribose, L-xylose, adonitol, methyl β-D-xyloside, sorbose, rhamnose, dulcitol, inositol, methyl α-D-mannoside, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, lactose, melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, 2-ketogluconate and 5-ketogluconate are not utilized. Susceptible to ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, oleandomycin, penicillin G, polymyxin B, streptomycin and tetracycline, but not to lincomycin. In assays with the API ZYM system, esterase (C4), esterase lipase (C8) and leucine arylamidase are present, but alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. The predominant ubiquinone is Q-8. The major fatty acids (>10 % of the total) are C_{16:0} and summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH). The DNA G + C content is 64.8 mol% (determined by HPLC). Other phenotypic characteristics are given in Table 1.

The type strain, K102^T (=KCTC 12980^T =CCUG 54518^T), was isolated from wastewater of a textile dye works at Bisan, Daegu city, Korea.

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