Hydrocarboniphaga daqingensis sp. nov., isolated from a freshwater lake

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A Gram-negative bacterium, designated B2-9^T, was isolated from water of a slightly alkaline lake, Longhu Lake in Daqing, north-east China. Strain B2-9^T was an aerobic rod, heterotrophic, catalase- and oxidase-positive and motile by means of a single polar flagellum. The isolate grew well on n-alkanes C₉–C₁₇ and weakly on C₆–C₈, C₁₈ and C₁₉. Growth occurred at 10–37 °C (optimum 20–25 °C), at pH 5.5–9.5 (optimum pH 7.0) and with ≤1.0 % (w/v) NaCl. The major fatty acids were C_{18:1}ω7c (41.4 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 18.5 %) and C_{16:0} (10.9 %). Phylogenetic analyses based on 16S rRNA gene sequences revealed that strain B2-9^T belonged to the genus *Hydrocarboniphaga* of the class *Gammaproteobacteria* and shared 97.0 % 16S rRNA gene sequence similarity with *Hydrocarboniphaga effusa* AP103^T. DNA–DNA relatedness between strain B2-9^T and *H. effusa* DSM 16095^T was 24 %. The DNA G + C content of strain B2-9^T was 66 mol%. Strain B2-9^T is considered to represent a novel species of the genus *Hydrocarboniphaga*, for which the name *Hydrocarboniphaga daqingensis* sp. nov. is proposed. The type strain is B2-9^T (=CGMCC 1.7049^T =NBRC 104238^T).

The genus *Hydrocarboniphaga* of the class *Gammaproteobacteria* was proposed by Palleroni *et al.* (2004) to accommodate aerobic, heterotrophic, Gram-negative bacteria that can grow on aliphatic hydrocarbons. At the time of writing, the genus *Hydrocarboniphaga* comprises one species with a validly published name, *Hydrocarboniphaga effusa* (Palleroni *et al.*, 2004), which was characterized by growing on n-alkanes. The phenotypic characteristics of *H. effusa* include good growth with complex and chemically defined media without organic growth factors and the formation of diffuse colonies on minimal agar.

In this study, we report the isolation and taxonomic characterization of an alkane-utilizing bacterium, strain B2-9^T, which was isolated from surface water of a slightly alkaline lake, Longhu Lake in Daqing, north-east China (124° 24' 3.2" E 46° 43' 48.6" N; depth 3.0 m, pH 8.9, 23 °C, chemical oxygen demand 25 mg l⁻¹, biological oxygen demand 15 mg l⁻¹, total nitrogen 1.1 mg l⁻¹, total phosphorus 0.10 mg l⁻¹). For isolation, serially diluted

water was spread onto low-organic Luria–Bertani agar [containing l⁻¹: 1.0 g tryptone (Difco), 0.5 g yeast extract and 10.0 g NaCl; pH 8.0] and incubated at 30 °C for 7 days. Strain B2-9^T was isolated from a single colony, purified by repeated subcultivation and stored in middle-organic trypticase soy broth [containing l⁻¹: 10 g trypticase soy broth (Difco)] supplemented with 15 % glycerol (v/v) at –80 °C.

All tests were performed in parallel on strain B2-9^T and *H. effusa* DSM 16095^T. Growth was tested on trypticase soy agar (TSA; Difco), low-organic TSA [containing l⁻¹: 3 g trypticase soy broth and 15 g agar], nutrient agar (NA; laboratory prepared), Luria–Bertani agar (LB; Difco) and R2A agar (Difco). Strain B2-9^T grew well on low-organic TSA and R2A and weakly on TSA; however, the viability of cells declined rapidly when growing on TSA. Unlike *H. effusa* DSM 16095^T, strain B2-9^T could not grow on LB and NA agar, did not form diffuse colonies on minimal agar and formed cell aggregates in R2A broth.

Routine cultivation was conducted at 25 °C with R2A medium. Cell morphology was observed using photomicroscopy (BH-2; Olympus). Flagellation was examined by transmission electron microscopy (JEM-1400; JEOL) after negative staining with 1 % (w/v) phosphotungstic acid. Motility was observed using phase-contrast microscopy

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain B2-9^T is EU313812.

A supplementary figure and a supplementary table are available with the online version of this paper.

(CH30; Olympus). Gram reaction was determined according to Gerhardt *et al.* (1994). Catalase and oxidase activities, hydrolysis of casein, gelatin, aesculin, L-tyrosine, starch and Tweens 20, 40, 60 and 80 were determined as described by Dong & Cai (2001). H₂S production was determined according to Bruns *et al.* (2001). Growth under anaerobic conditions was determined by incubation in an anaerobic chamber with anaerobically prepared R2A broth. Conditions for growth were tested in R2A broth with 0–5.0 % (w/v) NaCl (at intervals of 0.5 %), at pH 5.0–10.5 (at intervals of 0.5 pH unit) and at 5–35 °C (at intervals of 5 °C), 37 and 40 °C. Generation time at 25 °C was determined in R2A broth and calculated from the change in optical density during the exponential growth phase. Growth on n-alkanes (C₆–C₁₉) was performed according to Palleroni *et al.* (2004). Carbon source assimilation was determined according to Stanier *et al.* (1966). Additional biochemical properties and enzyme activities were determined using the API 20 NE and API ZYM systems (bioMérieux), according to the manufacturer's instructions. Susceptibility to antibiotics was determined using filter-paper discs (Beijing Pharmaceutical Company).

Cells of strain B2-9^T were Gram-negative, non-spore-forming rods (0.7–0.8 µm wide and 1.0–1.5 µm long). Growth occurred by binary fission. Cells were motile by means of a single polar flagellum (Supplementary Fig. S1a,

b, available in IJSEM Online). After growth on R2A agar for 3 days, cells of strain B2-9^T were covered with many vesicles and these vesicles were also seen free in the culture fluid (Supplementary Fig. S1b, c). No vesicles were observed for *H. effusa* DSM 16095^T under the same culture conditions (Supplementary Fig. S1d). High magnification revealed that the vesicles were formed by local extensions of the peripheral wall layers without involvement of the cytoplasmic membrane, which suggests a possible mechanism of vesicle formation (Forsberg *et al.*, 1981).

After 2 days at 30 °C on R2A agar, colonies of strain B2-9^T were transparent, non-pigmented, smooth, circular, convex and 1–2 mm in diameter. Growth occurred at 10–37 °C (optimum 20–25 °C) and pH 5.5–9.5 (optimum pH 7.0). NaCl was not required for growth but up to 1.0 % (w/v) NaCl was tolerated. In comparison with *H. effusa* DSM 16095^T, strain B2-9^T exhibited a higher maximum temperature and a lower maximum NaCl concentration for growth and a longer generation time (Table 1). Strain B2-9^T grew well on C₉–C₁₇ and weakly on C₆–C₈, C₁₈ and C₁₉, while *H. effusa* DSM 16095^T grew very well on all of the alkanes tested. The morphological, physiological and biochemical properties that differentiate strain B2-9^T from *H. effusa* DSM 16095^T are given in Table 1 and additional characteristics of strain B2-9^T are given in the species description.

Table 1. Differential properties of strain B2-9^T and *H. effusa* DSM 16095^T

Strains: 1, *Hydrocarboniphaga daqingensis* sp. nov. B2-9^T; 2, *H. effusa* DSM 16095^T. All data were taken from this study. R, Resistant; s, sensitive; +, positive; w, weakly positive; –, negative.

Characteristic	1	2
Colony/cell morphology		
Diffuse colonies on minimal agar	–	+
Cell aggregates in R2A broth	+	–
Vesicle-covered cells	+	–
Cell dimensions (µm)		
Width	0.7–0.8	0.75–0.85
Length	1.0–1.5	1.5–2.0
Growth on/at:		
LB, NA	–	+
37 °C	+	–
Maximum NaCl concentration (% w/v)	1.0	2.5
Generation time in R2A at 25 °C (h)	1.5	2.3
Utilization of:		
Isoamyl alcohol, L-arginine, L-glutamate, L-phenylalanine, L-rhamnose, D-mannitol, raffinose, L-histidine	–	+
n-Alkanes (C ₆ –C ₈ , C ₁₈ , C ₁₉)	w	+
API ZYM		
Esterase (C4)	+	–
Cystine arylamidase, α-glucosidase	w	–
Lipase (C14)	–	+
Antibiotic susceptibility (µg per disc):		
Ampicillin (10), cefazolin (30), erythromycin (15), gentamicin (10)	s	R
Clarithromycin (15)	R	s
DNA G + C content (mol%)	66	61

Genomic DNA extraction and sequencing of the 16S rRNA gene of strain B2-9^T were carried out according to the procedures by Kim *et al.* (1998). An almost-complete nucleotide sequence of the 16S rRNA gene (1487 nt) was obtained and compared with 16S rRNA gene sequences available in public databases using BLAST (Altschul *et al.*, 1990; <http://www.ncbi.nlm.nih.gov>). A multiple alignment with closely related members was performed using CLUSTAL X (Thompson *et al.*, 1994). Ambiguous and unalignable bases were manually omitted and phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood algorithms available in MEGA (Kumar *et al.*, 2004) and the maximum-parsimony algorithm available in PAUP (Swofford, 2002). Bootstrap analysis was performed according to the P-distance model. The phylogenetic analysis indicated that strain B2-9^T clustered with the genus *Hydrocarboniphaga* in the class *Gammaproteobacteria* (100 % bootstrap support; Fig. 1). Strain B2-9^T exhibited 97.0 % 16S rRNA gene sequence similarity with *H. effusa* AP103^T and <95.5 % 16S rRNA gene sequence similarity with its next closest phylogenetic neighbours, *Nevskia soli* GR15-1^T (95.1 %), *Nevskia ramosa* Soe1^T (94.6 %) and *Alkanibacter difficilis* MN154.3^T (93.4 %).

For the analysis of cellular fatty acids, strain B2-9^T and *H. effusa* DSM 16095^T were grown on low-organic TSA at 25 °C for 3 days, respectively. The fatty acids were determined using the MIDI Sherlock Microbial Identification System (Microbial ID), according to the manufacturer's instructions. Strain B2-9^T had a fatty acid profile similar to that of *H. effusa* DSM 16095^T (Supplementary Table S1). The major fatty acids of strain B2-9^T were C_{18:1}ω7c (41.4 %), summed feature 3 (iso-C_{15:0} 2-OH and/or C_{16:1}ω7c; 18.5 %) and C_{16:0} (10.9 %). Strain B2-9^T could be differentiated from *H. effusa* DSM 16095^T by higher levels of C_{18:1}ω7c and C_{14:0}, lower levels

of C_{16:0} and C_{12:0}, presence of C_{15:0} and absence of C_{14:0} 2-OH, C_{16:0} 2-OH and iso-C_{17:0} 3-OH.

The DNA G+C content was determined by the thermal denaturation method (Marmur & Doty, 1962). DNA from *Escherichia coli* K-12 was used as a control. The DNA G+C content of strain B2-9^T was 66 mol%, which was a little higher than that of *H. effusa* DSM 16095^T (61 mol%).

Strain B2-9^T shared several important characteristics with the genus *Hydrocarboniphaga*, such as being strictly aerobic, motile, oxidase- and catalase-positive, unable to reduce nitrate and, especially, able to utilize n-alkanes, which indicated that strain B2-9^T was a member of the genus *Hydrocarboniphaga*. However, several properties, including colony and cell morphology, growth on some media, temperature and NaCl concentration ranges for growth, carbon source utilization, enzyme activities, antibiotic susceptibility and DNA G+C content (Table 1), differentiated strain B2-9^T from *H. effusa* DSM 16095^T. To further distinguish strain B2-9^T from *H. effusa* DSM 16095^T, DNA–DNA hybridization was carried out using the thermal denaturation and renaturation method (De Ley *et al.*, 1970) with modifications by Huß *et al.* (1983) and a DU 800 spectrophotometer (Beckman). DNA–DNA relatedness was determined on the basis of the liquid reassociation rate according to the formula given by De Ley *et al.* (1970):

$$D\% = \frac{4V_m - (V_a + V_b)}{2\sqrt{V_a + V_b}} \times 100$$

DNA–DNA relatedness between strain B2-9^T and *H. effusa* DSM 16095^T was 24 %.

On the basis of phenotypic, genotypic and phylogenetic data, strain B2-9^T is considered to represent a novel species of the genus *Hydrocarboniphaga*, for which the name *Hydrocarboniphaga daqingensis* sp. nov. is proposed.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic positions of strain B2-9^T and representatives of some other related taxa. Bootstrap values (>70 %) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. *Acidithiobacillus albertensis* DSM 14366^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Description of *Hydrocarboniphaga daqingensis* sp. nov.

Hydrocarboniphaga daqingensis (da.qin.gen'sis. N.L. fem. adj. *daqingensis* pertaining to Daqing, where the type strain was isolated).

Strictly aerobic and heterotrophic. Cells are Gram-negative, non-spore-forming rods (0.7–0.8 µm wide and 1.0–1.5 µm long). Divides by binary fission. Motile by means of a single polar flagellum. After 2 days at 30 °C on R2A agar, colonies are transparent, non-pigmented, non-diffuse, smooth, circular, convex and 1–2 mm in diameter. Grows at 10–37 °C (optimum 20–25 °C), at pH 5.5–9.5 (optimum pH 7.0) and with ≤1.0 % (w/v) NaCl. Oxidase- and catalase-positive. Hydrolyses L-tyrosine and weakly hydrolyses aesculin, but does not hydrolyse casein, gelatin or starch. Negative for nitrate reduction, indole production, glucose fermentation and arginine dihydrolase, urease and β-galactosidase. Grows well on n-alkanes C₉–C₁₇ and weakly on C₆–C₈, C₁₈ and C₁₉. Utilizes L-isoleucine and L-leucine and weakly utilizes erythritol, but does not utilize isoamyl alcohol, L-arginine, L-glutamate, L-phenylalanine, lactic acid, *p*-aminobenzoic acid, benzoic acid, creatine, D-calcium pantothenate, asparagine, L-histidine, L-ornithine, L-serine or fumaric acid. With API ZYM, positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin and naphthol-AS-BI-phosphohydrolase and weakly positive for cystine arylamidase, acid phosphatase and α-glucosidase. Sensitive to (µg per disc unless otherwise stated) ampicillin (10), carbenicillin (100), cefazolin (30), chloramphenicol (30), ciprofloxacin (5), erythromycin (15), gentamicin (10), kanamycin (30), norfloxacin (10), novobiocin (5), penicillin (10 U), rifampicin (5), tetracycline (30) and vancomycin (30); resistant to cefradine (30), clarithromycin (15), oxacillin (1) and polymyxin B (300 U). The major fatty acids are C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH) and C_{16:0}. The DNA G+C content of the type strain is 66 mol%.

The type strain, B2-9^T (=CGMCC 1.7049^T =NBRC 104238^T), was isolated from water of Longhu Lake, Daqing, north-east China.

Acknowledgements

This work was supported by grants from the Ministry of Science and Technology of China (863 Program; grant number 2006AA06Z316)

and the Knowledge Innovation Program of the Chinese Academy of Sciences (grant number KSCS2-YW-G-055-01).

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