ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 1, pp. 59–66. © Pleiades Publishing, Ltd., 2012.
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EXPERIMENTAL ARTICLES

Hoeflea siderophila sp. nov., a New Neutrophilic Iron-Oxidizing Bacterium

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Received May 30, 2011

Abstract—A new representative of neutrophilic iron-oxidizing bacteria was isolated from the iron-containing sediments of the brackish low-temperature iron-rich spring of the Staraya Russa Resort (Novgorod region, Russia). The cells of strain Hf1 were thin, slightly curved rods, motile by means of a single polar flagellum. The bacterium reproduced by binary division and was capable of producing rosettes. Optimal growth was observed within the pH range of 6.2–8.5 (with an optimum at 7.5), at 9–38°C (with an optimum at 30°C), and in the salinity range of 0.1–8.5% NaCl (with an optimum at 1%). The organism was a facultative anaerobe. The strain was capable of mixotrophic and organoheterotrophic growth. Fe(II) oxidation occurred under anaerobic conditions via reduction of NO_3^- and N_2O, or under microaerobic conditions with oxygen as an electron acceptor. According to phylogenetic analysis based on the comparison of the 16S rRNA gene sequences, the strain was closest to the organotrophic marine bacterium *Hoeflea phototrophica* (98.5% similarity). The level of DNA–DNA homology with the type species of the genus *Hoeflea* was 19%. The DNA G + C base content was 57.5 mol %. According to its phenotypic and chemotaxonomic properties, as well as to the results of phylogenetic analysis, strain Hf1 was classified into the genus *Hoeflea* of the family *Phyllobacteriaceae*, order *Rhizobiales* of the phylum *Alphaproteobacteria* as a novel species, *Hoeflea siderophila* sp. nov. The type strain is Hf1^T (=DSM 21587 = VKM A7094). The GenBank accession number for the 16S rRNA gene sequences of strain Hf1^T is EU670237.

Keywords: neutrophilic iron-oxidizing bacteria, genus *Hoeflea, Hoeflea siderophila* sp. nov.

DOI: 10.1134/S0026261712010146

The capacity for utilizing Fe(II) as an inorganic electron donor in the electron transport chain is shared by a small number of bacteria belonging to several taxonomic groups. Up to the end of the 1980s, lithotrophic iron-oxidizing bacteria were represented only by acidophilic microorganisms growing at a pH of less than 4.5, when Fe(II) is resistant to chemical oxidation with the oxygen of air. Since the end of the 1980s, some attempts have been made to apply the radioisotope method with $^{14}CO_2$ to confirm that the previously described iron-oxidizing bacterium *Gallionella ferruginea* [1] could grow chemolithoautotrophically with Fe(II) as an energy source under neutral ambient conditions [2]. Few representatives of iron-oxidizing bacteria are able to oxidize Fe(II) under anaerobic conditions via reduction of NO_3^- as an electron acceptor. Under natural conditions, growth is usually detected at the boundary of the redox zone in such aquatic ecosystems as sediments formed where groundwater is brought to the surface [3, 4], deep-water marine hydrotherms [5, 6], and plant rhizosphere [7].

Due to the difficulties in both isolation and cultivation, the physiological properties of neutrophilic lithotrophic iron-oxidizing bacteria are poorly understood. Over the past 20 years, only two species of *Betaproteobacteria* [8] and the only representative of the new phylum *Zetaproteobacteria* [5, 9] have been described. The taxonomic position of some recently isolated strains remains underdetermined [3, 4, 6, 10, 11].

All isolates are capable of Fe(II) oxidation under anaerobic conditions coupled to reduction of oxidized nitrogen compounds (NO_3^-, NO_2^-, and N_2O). All known strains are mixotrophs; capacity for chemolithoautotrophic growth was strictly confirmed only for two species [2, 8].

In the present work, the results of our study of the new strain of neutrophilic facultatively anaerobic iron-oxidizing bacteria isolated from the brackish, low-temperature, iron-rich spring of the Staraya Russa Resort (Novgorod region, Russia) are presented.

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MATERIALS AND METHODS

Isolation source. Samples of water and newly formed ochreous precipitates from the low-temperature mineral iron-rich spring no. 8 of the Staraya Russa Resort (Novgorod region, Russia) were the subjects of this study. The spring is allied with the water-bearing bed of the ancient Devon sea. Total mineralization of the water was 20 g l^{-1}; the water temperature varied from 11 to 14°C; pH was 7.0; oxygen was not detected near the spring outlet. The Fe(II) content in the water was 3 mg l^{-1}.

Quantitative assessment and conditions of isolation and cultivation of iron-oxidizing bacteria. The numbers of iron-oxidizing bacteria were determined with tenfold dilutions in Hungate tubes filled to capacity with agarized medium. The cultivation medium contained the following (g L^{-1}): NaCl, 20; NH_4Cl, 0.3; $CaCl_2 \cdot 6H_2O$, 0.3; $MgCl_2 \cdot 7H_2O$, 3; $NaHCO_3$, 0.5; 10% phosphate buffer (pH 7.0), 0.1; Hepes buffer (pH 7.2), 3.0; KNO_3, 0.3; CH_3COONa, 0.15; vitamins and trace elements [12]; Difco agar, 5.0; pH 7.0. The cultivation temperature was 28°C. Before inoculation, the medium was supplemented with the fresh sterile FeS suspension [13] (0.2 mL per 10 mL of medium). Inoculated media were incubated for 2–3 weeks.

The pure culture of strain Hf1 was isolated with tenfold dilutions on the above-described agarized medium; the obtained ochreous colonies were transferred to liquid medium.

Cell morphology and ultrastructure. Cell morphology was studied under an Olympus CX31 phase contrast microscope (Japan). Ultrathin sections were obtained using the standard methods. The cells grown on iron-free medium were stained with phosphotungstic acid; iron-encrusted cells were not stained. Whole cell preparations and ultrathin sections were examined under a JEM-100C electron microscope (JEOL, Japan) at an accelerating voltage of 80 kV.

Cultural and analytical methods. The ability of iron-oxidizing bacteria to utilize various carbon sources was tested using the above-described mineral medium. The capacity for utilizing various nitrogen sources was tested using the above-described mineral medium with sodium acetate and without NH_4Cl. Carbon and nitrogen sources were added to a concentration of 0.5 g L^{-1}.

The content of Fe(II) in the water samples was determined on a KFK-3 spectrophotometer (Russia) using α,α-dipyridyl [14].

The concentration of dissolved oxygen was determined with a Hanna HI 9142 oximeter (Finland) at the sampling site; the pH of the water was determined with a portable Hanna Checker 1 pH meter (Finland).

The content of N_2 in the gas phase was determined using a Kristall 5000.1 gas chromatograph (Chromatec, Russia).

The contents of NO_3^-, NO_2^- were determined on a Staier liquid anion exchange chromatograph (Russia).

Biochemical methods. Nitrate and nitrite reductase activities were determined with methyl viologen using the standard techniques [15, 16].

Catalase and oxidase activities in the cell suspension were determined according to the standard procedure [17, 18].

Resistance to antibiotics was determined using the test discs (Difco Laboratories, United States) [19].

Protein content was determined by the Lowry method. The cell suspension obtained on the medium with ferrous iron was pretreated with 1% oxalic acid for 3–5 min in order to dissolve iron oxides.

Analysis of the fatty acid composition of the cells of strain Hf1. The fatty acids were analyzed by gas chromatography and mass spectrometry with a Sherlock gas chromatograph (Microbial Identification System, MIDI Inc., United States) according to the standard procedure [20].

Molecular genetic analysis. DNA extraction. To disintegrate bacterial cells, they were subjected three times to a freezing–thawing procedure (at –70 and 50°C, respectively); the cell suspension was then heated at 80°C for 5 min [21]. DNA was extracted by the phenol method [22].

The DNA G+C base content was determined by the method of Owen et al. [23].

The DNA homology level was determined by the optical reassociation method [24].

PCR amplification of the 16S rRNA gene was carried out with the universal eubacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGYTACCTTGTTACGACTT-3') [25, 26]. The polymerase chain reaction (PCR) was carried out on a GeneAmp PCR System 2700 thermocycler (Applied Biosystems, United States).

Determination of the 16S rRNA gene sequences was carried out on a CEQ 2000 XL automatic sequencer (Beckman Coulter, United States) according to the manufacturer's instructions.

Phylogenetic analysis. For identification of the microorganisms closely related to the obtained strain, the 16S rRNA gene sequences were compared to those of the type strains from the RDBII (Ribosomal Database Project, http://rdp.cme.msu.edu) and NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) databases. The obtained 16S rRNA gene sequences were aligned using the Clustal W software package [27]. The phylogenetic tree was constructed by the methods implemented in the Treecon software package [28]. The GenBank accession number for the 16S rRNA gene sequences of strain $Hf1^T$ is EU670237.

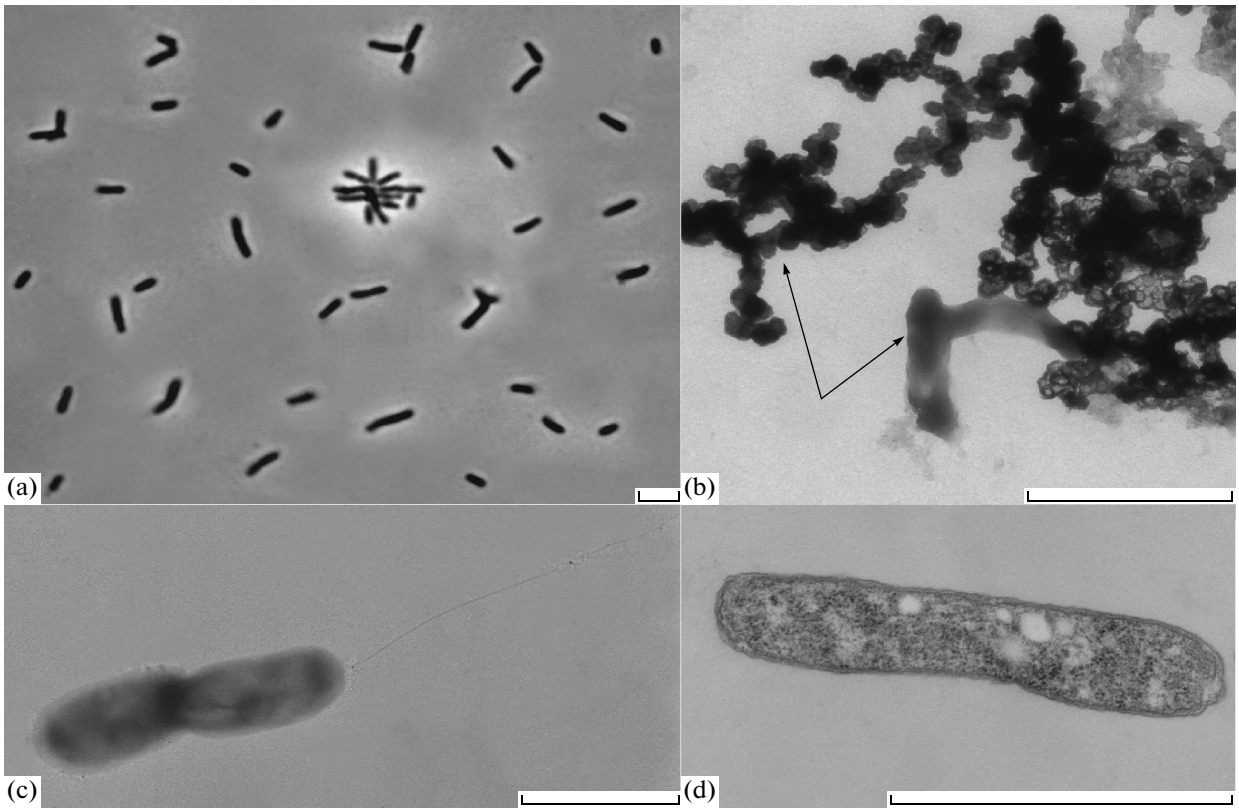

Fig. 1. Morphology and ultrastructure of strain Hf1^T cells: phase contrast microphotographs, Fe(II)-free media (a); iron-encrusted cells (the arrow points to iron oxides on the cell surface and in the medium) (b); cells stained with phosphotungstic acid (c); and cell ultrastructure (d). Electron microphotographs. Scale bar, 1 μm.

RESULTS

Quantitative assessment and isolation of pure cultures. The total number of iron-oxidizing bacteria in the spring water did not exceed 10 cells/mL; in the iron-containing precipitates, it was 10^5–10^7 cells/mL. For the isolation of iron-oxidizing bacteria, samples of fresh ochreous sediments collected near the outlet of the iron-rich spring were used as inoculum.

On the agarized medium, the strain produced small (1–3 mm in diameter), dense spherical colonies, orange-colored due to the formation of iron oxides. In the liquid medium of the same composition, an ochreous precipitate was formed.

Cell morphology and ultrastructure. The cells of strain Hf1 were thin, slightly curved rods (0.4 × 0.9–2.2 μm), motile by means of a single polar flagellum (Figs. 1a, 1c, and 1d). The cells reproduced by binary division. The bacterium was capable of forming rosettes (Fig. 1a). During growth in the medium with ferrous iron, iron oxides were deposited on the cell surface (Fig. 1b). The cell wall is of the gram-negative type.

Cultural properties. The temperature range for the growth of strain Hf1 was 9–38°C, with an optimum at 30°C; the pH range for growth was 6.2–8.5, with an optimum at 7.5. The isolate grew at a NaCl concentration of 0.1–8.5%, with an optimum at 1%.

Physiological and biochemical properties. Oxidation of Fe(II) compounds. During growth of iron-oxidizing bacteria under anaerobic conditions in the mineral medium with NO_3^- as an electron acceptor and $FeSO_4$ as an electron donor, oxidation of Fe(II) occurred primarily via bacterial oxidation; however, up to 20% of Fe(II) was oxidized in the chemical reaction with the newly formed nitrite (Table 1). When NO_3^- was replaced with N_2O, chemical oxidation of Fe(II) was insignificant (Table 2).

Strain Hf1 was capable of both mixotrophic and organotrophic growth under microaerobic or anaerobic conditions; in the latter case, it utilized NO_3^-, N_2O as electron acceptors. During lithotrophic growth, it utilized FeS, $FeSO_4$, and $FeCO_3$ as Fe(II) sources. Under anaerobic conditions, strain Hf1 was not able to utilize NO_2^-, ClO_4^-, S^0, $S_2O_3^{2-}$, and $Fe(OH)_3$ as electron acceptors. During growth in the mineral medium, H_2 was not utilized as an electron donor.

Table 1. Relation between the decrease in the Fe(II) content and the biomass accumulation, strain Hf1, during anaerobic growth in the mineral medium with NO_3^- as an electron acceptor

Time, days	NO_3^-, mM		NO_2^-, mM		Fe(II), mg/L		Cell protein, mg/L
	Control	Experiment	Control	Experiment	Control	Experiment	Experiment
0	7	7	0	0	140	140	4
5	7	5.9	0	0.035	140	130	8.5
10	7	4.7	0	0.08	140	80	11
15	7	2.8	0	0.15	140	55	11

Table 2. Relation between the decrease in the Fe(II) content and the biomass accumulation during anaerobic growth in the mineral media with N_2O as an electron acceptor

Time, days	N_2 in the gas phase, mM	Fe(II), mg/L		Cell protein, mg/L	
		Control	Experiment	Control	Experiment
0	0.047	140	140	0	2
3	0.17	–	120	0	4
6	0.27	130	105	0	6
10	0.33	130	90	0	8
13	0.43	–	75	0	10
17	0.45	130	40	0	11

During organotrophic growth under anaerobic conditions, strain Hf1 reduced NO_3^- only to NO_2^- via the interrupted chain of denitrification.

Accumulation of NO_2^- inhibited growth of the iron-oxidizing bacterium. When NO_3^- was replaced with N_2O, growth was not inhibited and was accompanied by accumulation of N_2 in the gas phase:

$$NO_3^- \longrightarrow NO_2^- \text{—}\vert \longrightarrow N_2O \longrightarrow N_2.$$

Growth was inhibited due to accumulation of NO_2^-, which resulted from low rates of enzymatic activity involved in the chain of reactions causing a reduction of nitrogen compounds: the activity of nitrate reductase was 30 nmol/mL per 1 mg of protein, while the activity of nitrite reductase did not exceed 0.6 nmol/mL per 1 mg of protein.

Carbon and nitrogen sources. During aerobic organotrophic growth, strain Hf1 utilized acetate, succinate, citrate, lactate, malate, fumarate, propionate, pyruvate, butyrate, propanol, glycerol, a wide range of sugars, including ribose, xylose, D,L-arabinose, D,L-glucose, dextrose, fructose, L-sorbose, galactose, D-mannose, 2-deoxyglucose, sucrose, D-lactose, trehalose, D-maltose, cellobiose, melibiose, melizitose, D-raffinose, L-rhamnose, L,CH_3-glucoside, D-glucuronic acid, M-inositol, M-erythritol, L-arabitol, D-glucosamine, N-acetyl-glucosamine, as well as dextrin, glycogen, yeast extract, and peptone, as carbon sources. Weak growth was observed on media with some amino acids, including phenylalanine, tryptophan, and histidine. The strain was not able to utilize oxalate, formate, benzoate, ethanol, buthanol, proline, leucine, alanine, asparagine, glutamine, aspartic and glutamic acids, or casein hydrolysate.

The microorganism utilized ammonium salts, NO_3^-, N_2O, urea, yeast extract, and peptone, and did not utilize NO_2^-, histidine, aspartic and glutamic acids, and casein hydrolysate as nitrogen sources.

The predominant fatty acids were 11-octadecenoic (18:1ω7c; 60.48%), hexadecanoic (16:0; 14.65%), and 11-methyl-octadecenoic (11-Methyl 18:1ω7c; 10.94%).

The strain was sensitive to polymyxin, rifampicin, and tetramycin and resistant to amikacin, bacitracin, kanamycin, mycostatin, and penicillin.

Table 3. Comparative analysis of the properties of strain Hf1^T and the type strains of the described *Hoeflea* species

Differentiating properties	Strain Hf1^T DSM21587^T	*H. phototrophica* DFL-43^T [30]	*H. alexandrii* DFL-43^T [31]	*H. marina* LMG 128^T [32]
Cell size (mm)				
Length	0.9–2.2	0.3–0.5	0.8	0.7–0.9
Width	0.4	0.7–2.0	2.5	1.1–1.4
Motility	+	+	+	+
Growth optima and ranges				
NaCl	1% (0.1–8.5%)	3% (0.5–7%)	0–6.8% (0–11.8%)	3% (0–5%)
Temperature	30°C (9–38°C)	31°C (25–33°C)	30°C (10–42°C)	28°C (4–40°C)
pH	7.5 (6.2–8.5)	7.5 (6–9)	7 (6–9)	7 (6–8)
Anaerobic growth	+	–	–	–
Catalase activity	±	+	+	+
Oxidase activity	+	+	–	+
Utilization of sugars	+	–	+	+
Utilization of Fe(II) as an electron donor	+	ND	ND	–
Electron acceptors				
NO_3^-	+	–	–	–
N_2O	+	ND	ND	ND
NO_2^-	–	ND	ND	ND
Bchl *a*	–	+	–	–
pufL and *pufM* bchl *a* genes	+	+	ND	ND
Main fatty acids:				
16:0	14.65	6.3	7.7	4
18:1ω7c	60.48	62.8	83.1	76
11-Methyl 18:1ω7c	10.94	20.6	3.6	7.5
19:0cyc	3.98	3.4	ND	5.6
DNA G+C base content, mol % (T_m)	57.5	59.3	59.7	53.1

Note: +, detected; –, not detected; ND, not determined.

The results of comparative analysis of the pheno- and genotypic characteristics of strain Hf1, and those of the closely related species [30–32], are listed in Table 3.

Genotypic and phylogenetic analysis. According to the results of phylogenetic analysis based on the comparison of the 16S rRNA gene sequences, strain Hf1 belonged to the cluster of the genus *Hoeflea* (Fig. 2), but formed a separate branch within this genus.

The levels of 16S rRNA similarity between strain Hf1 and *H. phototrophica* DFL-43^T, *H. alexandrii* AM1V30^T, and *H. marina* LMG 128^T were 98.5, 98.2, and 97.8%, respectively.

The level of DNA–DNA homology between strain Hf1 and the type species *H. marina* was 19%. The contents of the G+C base pairs in the DNA of strain Hf1 and the type species were 57.5 and 53.1 mol %, respectively.

DISCUSSION

The isolated strain of neutrophilic facultative anaerobic iron-oxidizing bacteria shared common properties with members of the genus *Hoeflea*. However, comparative analysis of the results of phenotypic and genotypic studies demonstrated that strain Hf1 differed considerably from the type species and the two other described species (Table 3).

Among the main physiological properties of this strain are its ability to utilize Fe(II) compounds as electron donors during lithotrophic growth, and its

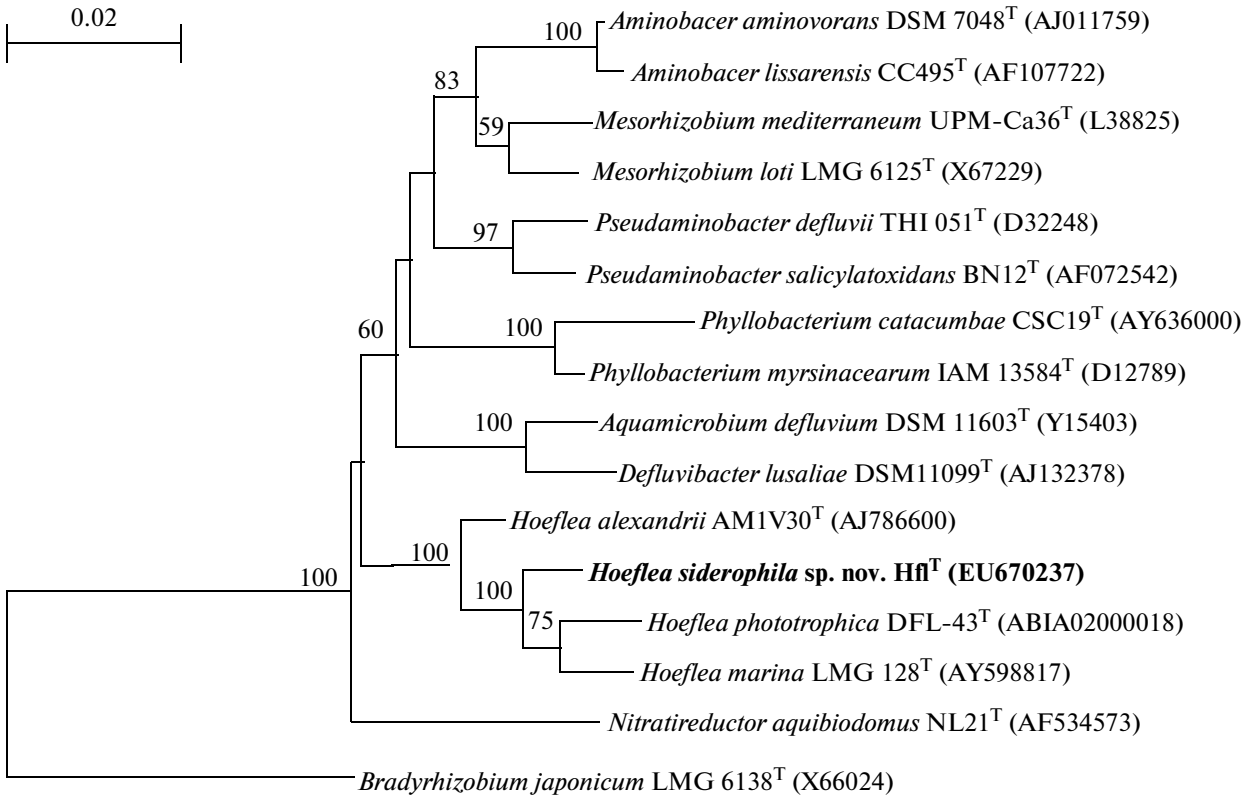

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of *Hoeflea siderophila* sp. nov. Hf1^T within the family *Phyllobacteriaceae*. The numbers at the branching points show the significance of the branching order as determined by bootstrap analysis. Bar, 0.02 substitutions per nucleotide position.The tree was rooted using *Bradyrhizobium japonicum* LMG6138T (X66024) as an outgroup.

ability to utilize NO_3^- as an electron acceptor under anaerobic conditions.

The available data on the levels of DNA–DNA homology between strain Hf1 and the type species *H. marina* suggest similarly low levels of DNA–DNA homology may exist between the studied strain and *H. phototrophica* and *H. alexandrii*. Despite 1.3% difference in their 16S rRNA gene sequences, the level of DNA–DNA homology between the majority of bacterial taxa is lower than 70% [33]. The threshold level of 16S rRNA similarity recommending DNA–DNA hybridization was recently lowered from 97.7 to 97.0% for unclear reasons [34].

The results obtained support classification of strain Hf1^T within the genus *Hoeflea*, family *Phyllobacteriaceae*, order *Rizobiales* of the class *Alphaproteobacteria* as a new species *Hoeflea siderophila* sp. nov.

Taxonomic description of *Hoeflea siderophila* sp. nov. (*Si.de.ro'phi.la.*, *sidero*, iron; *phila*, to like, to require; *siderophila*, iron-requiring).

The cells are thin, slightly curved rods (0.4 × 0.9–2.2 μm), motile by means of a single polar flagellum. The bacterium reproduces by binary division and is capable of producing rosettes. The cell wall structure is of the gram-negative type. The organism is a facultative anaerobe. Optimal growth occurs within a pH range of 6.2–8.5 (with an optimum at 7.5), at 30°C (9–38°C) (with an optimum at 30°C), and in a salinity range of 0.1–8.5% NaCl (with an optimum at 1%). The strain is able to grow under organotrophic and mixotrophic conditions with utilizing Fe(II) as an electron donor via reduction of NO_3^- and N_2O and depositing iron oxides on the cell surface. The microorganism exhibits oxidase activity and low catalase activity. During aerobic organotrophic growth, strain Hf1 utilizes acetate, succinate, citrate, lactate, malate, fumarate, propionate, pyruvate, butyrate, propanol, glycerol, a wide range of sugars, including ribose, xylose, D,L-arabinose, D,L-glucose, dextrose, fructose, L-sorbose, galactose, D-mannose, 2-deoxyglucose, sucrose, D-lactose, trehalose, D-maltose, cellobiose, melibiose, melizitose, D-raffinose, L-rhamnose, L,CH_3-glucoside, D-glucuronic acid, M-inositol, M-erythritol, L-arabitol, D-glucosamine, *N*-acetyl-glucosamine, as well as dextrin, glycogen, yeast extract, and peptone, as carbon sources. Weak growth occurs in media with some amino acids (phenylalanine, tryptophan, and histidine). The strain is unable to utilize oxalate, formate, benzoate, ethanol, buthanol, proline, leucine, alanine, asparagine, glutamine, aspartic and glutamic acids, or casein

hydrolysate. The microorganism utilizes ammonium salts, NO_3^-, N_2O, urea, yeast extract, and peptone as nitrogen sources. The strain is unable to utilize NO_2^-, histidine, aspartic and glutamic acids, or casein hydrolysate. Under anaerobic conditions, strain Hf1 is unable to utilize ClO_4^-, S^0, $S_2O_3^{2-}$, or $Fe(OH)_3$ as electron acceptors. During growth in the mineral medium, H_2 is not utilized as an electron donor. The predominant fatty acids are 11-octadecenoic (18:1ω7c) 60.48%), hexadecanoic (16:0; 14.65%), and 11-methyl-octadecenoic (11-Metyl 18:1ω7c) 10.94%). The strain is sensitive to polymyxin, rifampicin, and tetramycin and resistant to amikacin, bacitracin, kanamycin, mycostatin, and penicillin.

The type strain is Hf1^T (=DSM 21587^T = VKM A7094^T). The GenBank accession number for the 16S rRNA gene sequences of strain 21587^T is EU670237.

ACKNOWLEDGMENTS

The authors thank D.Yu. Sorokin for valuable advice and for help in the experiments, E.N. Detkova for help with DNA/DNA-hybridization, and G.A. Osipov for the analysis of fatty acid composition.

This work was supported by the Russian Foundation for Basic Research, project no. 10-04-01500.

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