Hoeflea anabaenae sp. nov., an epiphytic symbiont that attaches to the heterocysts of a strain of *Anabaena*

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The heterotrophic, epiphytic, symbiotic bacterial strain WH2K^T was previously isolated from a two-member culture in which it was attached to the heterocysts of a strain of *Anabaena* (SSM-00). Analysis of its 16S rRNA gene sequence demonstrated that the symbiont was most closely related to the type strain of *Hoeflea marina* (96.9 % similarity), which belongs to the family *Phyllobacteriaceae* within the order *Rhizobiales* of the class *Alphaproteobacteria*. A polyphasic taxonomic study was performed on strain WH2K^T, which consisted of irregular rods (2–5 μm long, 0.2 μm wide) that appeared to be narrower at one pole. Optimal growth was obtained in complex media with 15 g sea salts l^{-1}, at 18–34 °C (30 °C optimum) and at pH 6.0–8.0 (optimum pH 6.5). Unknown growth requirements were provided by small amounts of yeast extract but not by standard vitamin and trace metal solutions. Of the substrates tested, WH2K^T was able to utilize only acetate, pyruvate, malate and fumarate. Growth was observed only under aerobic and microaerobic conditions, and nitrate was not reduced. No photosynthetic pigments were detected under any of the growth conditions tested. The predominant fatty acids were a summed feature that comprises $C_{18:1}\omega7c$, $C_{18:1}\omega9t$, $C_{18:1}\omega12t$ or any combination of these (64.0 %) and an unidentified fatty acid of equivalent chain length 17.603 (13.5 %). The polar lipid profile consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylcholine, phosphoglycolipid, unknown lipids and an unidentified aminolipid. The only respiratory ubiquinone detected was Q-10. The DNA G+C content of the strain was 58.1 mol%. The organism can form a site-specific attached symbiotic relationship with a species of *Anabaena*. Based on phylogenetic and phenotypic evidence, it is proposed that strain WH2K^T be classified within a novel species of the genus *Hoeflea*, for which the name *Hoeflea anabaenae* sp. nov. is proposed. The type strain is WH2K^T (=CCUG 56626^T =NRRL B-59520^T).

In aquatic environments, synergistic interactions between heterotrophic bacteria and photosynthetic phytoplankton or cyanobacteria are commonplace and are important in global primary production and nutrient cycling (Ashen & Goff, 1998, 2000; Ferrier *et al.*, 2002; Grossart *et al.*, 2006; Paerl & Pinckney, 1996). Some of these associations are believed to be symbiotic and highly specific (Caldwell & Caldwell, 1978a, b; Ferrier *et al.*, 2002; Jasti *et al.*, 2005; Lupton & Marshall, 1981; Paerl, 1978; Paerl & Kellar, 1978). One of the more interesting symbioses occurs between heterotrophic bacteria and filamentous, heterocystous cyanobacteria such as *Anabaena*, *Aphanizomenon* and *Nostoc*, in which the heterotrophic bacteria are attached specifically to the heterocystous cells (Fig. 1a) (Lupton & Marshall, 1981; Paerl, 1977; Paerl & Gallucci, 1985; Stevenson & Waterbury, 2006). In these symbioses, the cyanobacterium provides a ready source of fixed carbon and nitrogen (Behrens *et al.*, 2008; Paerl, 1984), a surface for attachment and a vehicle for spatial localization

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain WH2K^T is DQ364238.

Two supplementary figures and two supplementary tables are available with the online version of this paper.

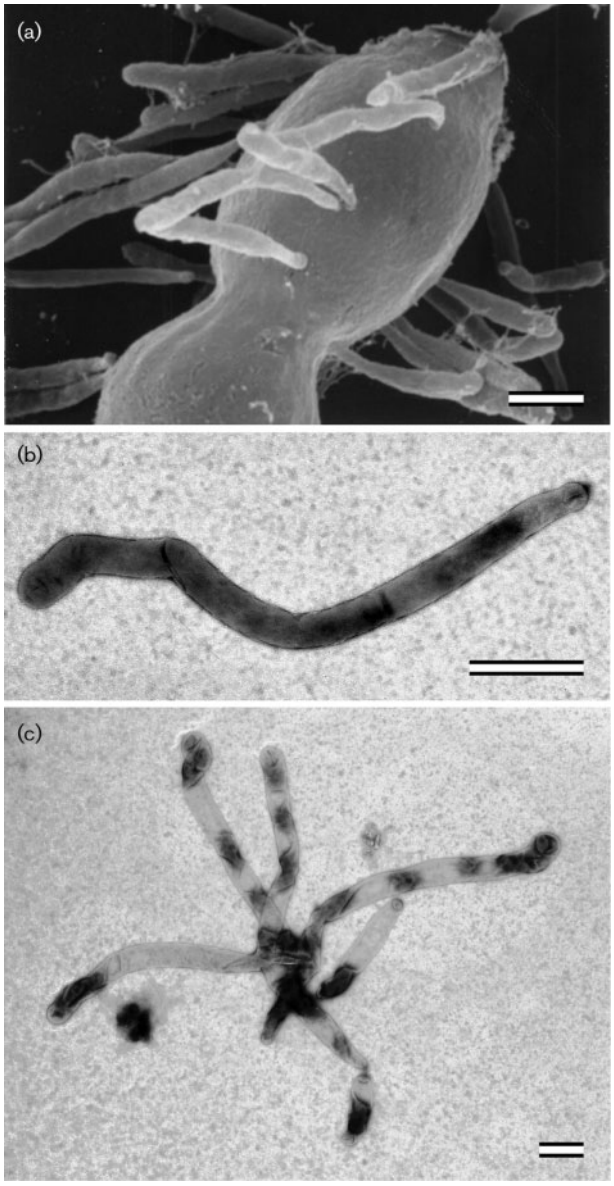

Fig. 1. Scanning and transmission electron micrographs showing the cell morphology of *Hoeflea anabaenae* sp. nov. WH2K^T attached to a heterocystous cell of *Anabaena* sp. SSM-00 (a), as a single cell (b) and as a multicellular rosette in PY medium (c). Bars, 1 μm.

in the environment (Jones & Cannon, 1986). The benefit of this symbiotic relationship, with regard to the *Anabaena* strain, is realized by increased rates of growth and nitrogen fixation in the presence of attached heterotrophic bacteria (Lupton & Marshall, 1981; Paerl, 1977; Schiefer & Caldwell, 1982).

This study describes a novel isolate, strain WH2K^T, that was recovered as the only heterotroph in a two-membered culture, where it was attached to the heterocystous cells of a strain of the filamentous cyanobacterium *Anabaena* (SSM-00) (Stevenson & Waterbury, 2006). In this paper, we characterize the physiology and phylogeny of this strain and compare it with its close relatives. Based on this polyphasic study, incorporating phylogenetic, chemotaxonomic and phenotypic methods, it is proposed that strain WH2K^T represents a novel species of *Hoeflea*.

Strain WH2K^T was isolated under heterotrophic conditions in marine purity (MP) liquid medium from a culture in which it was attached almost exclusively to *Anabaena* heterocysts (Stevenson & Waterbury, 2006). Cells of isolate WH2K^T were irregular, club-shaped rods, 2–5 μm long and 0.2 μm wide (Fig. 1b), and able to form star-shaped aggregates (Fig. 1c) like those of the marine *Agrobacterium* species later reclassified as *Hoeflea marina* (Ahrens, 1968; Peix *et al.*, 2005; Rüger & Höfle, 1992). No obvious motility was observed for WH2K^T, which is in contrast to the rapid motility observed directly for *Hoeflea* species reported previously (Biebl *et al.*, 2006; Palacios *et al.*, 2006; Peix *et al.*, 2005). Transmission electron micrographs of negatively stained cells of strain WH2K^T revealed no visible flagella (Fig. 1b, c). The bacterium was capable of growth in liquid or on solid (15 g agar l^{-1}) MP medium (Rippka *et al.*, 1979), full- and half-strength marine broth (Difco) and marine agar (7.5 g agar l^{-1} added) and liquid or solid PY medium (20 g sea salts, 3 g peptone and 0.5 g yeast extract l^{-1}; Biebl *et al.*, 2005) under aerobic conditions at 30 °C. WH2K^T formed colonies that were small (1–2 mm), non-pigmented and opaque after 7–10 days on all agar media.

PY medium was chosen for most of the comparative studies between WH2K^T and other *Hoeflea* species, as it supported the best growth of all organisms. The permissible (18–34 °C) and optimal (30 °C) growth temperatures were determined sequentially in an incubator, with shaking at 225 r.p.m. with periodic measurement of optical density at 600 nm (OD_{600}). WH2K^T was able to grow at pH 6.0–8.0; maximal OD_{600} was achieved in PY medium at pH 6.5. Sea salts were required at a concentration of at least 5 g l^{-1}, but WH2K^T grew optimally at concentrations between 12.5 and 17.5 g l^{-1} and tolerated only up to 25 g sea salts l^{-1}. No growth was observed when NaCl was substituted for sea salts.

Utilization of carbon sources was determined in experiments modelled after those used for the characterization of *Hoeflea phototrophica*, using a minimal seawater medium containing 20 g sea salts l^{-1} and 0.1 g yeast extract l^{-1} for required growth factors (Biebl *et al.*, 2006). The following carbon sources were tested at 1 g l^{-1} (acids as sodium salts): acetate, pyruvate, fumarate, malate, lactate, glutamate, butyrate, glucose, fructose, sucrose, ethanol, methanol, glycerol and L-arabinose. An organism was considered able to utilize a substrate if it resulted in a significant increase in maximum OD_{600} compared with controls that contained only yeast extract (mean value, $n=4$; Student's t-test, $P<0.05$). Substrate utilization profiles were also determined in this manner for *H. phototrophica* DSM 17068^T, *Hoeflea alexandrii* DSM 16655^T and *H. marina* DSM 16791^T to allow direct comparison (Table 1). WH2K^T was

Table 1. Substrate utilization patterns for strain WH2K^T and type strains of the genus *Hoeflea*

Strains: 1, WH2K^T; 2, *H. phototrophica* DSM 17068^T; 3, *H. alexandrii* DSM 16655^T; 4, *H. marina* DSM 16791^T. Data were obtained in this study. All strains grew on acetate, pyruvate, malate and fumarate.

Growth substrate	1	2	3	4
Glucose	−	+	+	+
Fructose	−	+	+	+
Glycerol	−	−	−	+
Butyrate	−	+	−	−
Lactate	−	+	+	+
L-Arabinose	−	+	+	+
Glutamate	−	+	+	+

able to utilize acetate, pyruvate, malate and fumarate. The other *Hoeflea* type strains were also able to utilize glucose, fructose, sucrose, glutamate and L-arabinose. In addition, *H. phototrophica* DSM 17068^T could use butyrate and *H. marina* DSM 16791^T could use glycerol.

Extracellular enzyme activities were characterized using the API ZYM kit for strain WH2K^T and the other *Hoeflea* type strains, which were sampled from cultures in mid-exponential phase (OD_{600} 0.4–0.5) in 1× PY medium. Strips were incubated at 30 °C for 24 h prior to reading the results, which are compared among all strains in Supplementary Table S1, available in IJSEM Online. Among the *Hoeflea* strains, strain WH2K^T was unique in that it possessed trypsin but lacked esterase lipase.

The production of bacteriochlorophyll *a* and other photopigments by strain WH2K^T was investigated as described by Biebl *et al.* (2006). The absorption spectrum lacked the peaks of bacteriochlorophyll *a* and a carotenoid that are found in *H. phototrophica*. In this regard, strain WH2K^T is like *H. alexandrii* and *H. marina* in that it does not produce photopigments (Palacios *et al.*, 2006).

Long-chain cellular fatty acids, respiratory lipoquinones and polar lipids were extracted from cell biomass of strain WH2K^T, *H. phototrophica* DSM 17068^T, *H. alexandrii* DSM 16655^T and *H. marina* DSM 16791^T harvested from cultures during mid-exponential phase (OD_{600} 0.4–0.5) in PY broth at 30 °C. Long-chain cellular fatty acids were analysed by GC (MIDI Sherlock) at the CCUG as described previously (Kämpfer & Kroppenstedt, 1996). Overall, strain WH2K^T had a fatty acid profile that was similar to those of the other *Hoeflea* type strains, but differences were observed in the presence/absence of several components (Table 2). Fatty acids detected in strain WH2K^T included $C_{18:1}\omega7c/\omega9t/\omega12t$ or any combination of these (64.0 %), ECL 17.603 (13.5 %), ECL 18.846/$C_{19:1}\omega6c$ (7.5 %), $C_{18:1}\omega9c$ (6.6 %), $C_{18:0}$ (4.0 %), 11-methyl $C_{18:1}\omega7c$ (2.6 %) and $C_{16:0}$ (1.8 %). Strain WH2K^T was unique among the *Hoeflea* strains in that it contained the saturated fatty acid $C_{18:0}$ and the unsaturated fatty acid $C_{18:1}\omega9c$, but lacked $C_{16:1}\omega9c$. Polar lipid and respiratory quinone

Table 2. Cellular fatty acid profiles of strain WH2K^T and type strains of the genus *Hoeflea*

Strains: 1, WH2K^T; 2, *H. phototrophica* DSM 17068^T; 3, *H. alexandrii* DSM 16655^T; 4, *H. marina* DSM 16791^T. Data are percentages of total fatty acids and were obtained in this study. Fatty acids that occurred at less than 1.0 % in all four strains are not shown; −, <1.0 %. ECL, Equivalent chain length.

Fatty acid	1	2	3	4
Saturated				
$C_{16:0}$	1.8	9.8	7.0	8.3
$C_{18:0}$	4.0	−	−	−
Unsaturated				
$C_{16:1}\omega11c$	−	1.5	−	−
$C_{16:1}\omega7c$	−	4.7	1.9	2.2
$C_{18:1}\omega9c$	6.6	−	−	−
11-Methyl $C_{18:1}\omega7c$	2.6	3.8	4.1	4.0
Cyclopropane acids				
$C_{19:0}$ cyclo $\omega8c$	−	2.0	−	−
Summed features				
$C_{14:0}$ 3-OH/iso-$C_{16:1}$ I	−	−	−	1.6
$C_{18:1}\omega7c/\omega9t/\omega12t$	64.0	69.4	73.9	72.9
ECL 18.846/$C_{19:1}\omega6c$	7.5	2.4	6.1	5.7
Unidentified				
ECL 17.603	13.5	6.4	7.1	5.4

analyses were carried out by the Identification Service of the DSMZ, Braunschweig, Germany, and Dr B. J. Tindall (DSMZ). Images of TLC polar lipid profiles are shown in Supplementary Fig. S1 and a comparison of profiles is given in Supplementary Table S2. Strain WH2K^T was unique among the *Hoeflea* strains in that it possessed two unidentified phospholipids and did not contain sulfoquinovosyl diacylglycerol. All strains produced ubiquinone 10 (Q-10) as their single respiratory lipoquinone, a feature of the majority of alphaproteobacteria.

The novel isolate WH2K^T forms a unique attached symbiotic relationship with a species of *Anabaena* (Fig. 1a; Stevenson & Waterbury, 2006). The ability to attach to the filamentous heterocystous cyanobacterium *Anabaena* sp. SSM-00 was compared between WH2K^T and the *Hoeflea* type strains in a series of attachment experiments. Cells from WH2K^T and each *Hoeflea* type strain were harvested from mid-exponential phase cultures growing in PY medium. Optical densities of culture aliquots were normalized by dilution with fresh PY medium and cells were harvested by centrifugation. Cells were resuspended in the nitrogen-free, autotrophic seawater medium ½SO (Stevenson & Waterbury, 2006; Waterbury *et al.*, 1986) and added to cultures of *Anabaena* with shaking at 125 r.p.m. under fluorescent light at 25 °C. Attachment was monitored microscopically on days 3, 7, 14, 30 and 49. The level of attachment (if observed) was recorded as the number of attachments per 106 *Anabaena* filaments (mean two heterocysts per filament) and the number of cells attached to each heterocyst. No attachment was observed for

H. marina DSM 16791^T or *H. alexandrii* DSM 16655^T. *H. phototrophica* DSM 17068^T exhibited only rare attachment; six of 106 (5.7 %) filaments were observed to contain attachment of cells of *H. phototrophica* DSM 17068^T at day 49, with only one cell in each case. In contrast, cells of WH2K^T were found attached to 28 % of observed heterocysts at day 7 and 55 % at day 49, with a mean of four cells per heterocyst.

The phylogenetic relationship between WH2K^T and the closest related organisms with validly published names was determined by comparing nearly full-length 16S rRNA gene sequences from prokMSA of the GreenGenes database (DeSantis *et al.*, 2006a, b). Using the phylogenetic software package ARB (Ludwig *et al.*, 2004), sequence alignments were optimized manually based on 16S rRNA secondary structure and filtered to include only homologous nucleotides for analysis. These optimally aligned sequences were used in PAUP* 4b to determine phylogenetic relationships based on neighbour-joining, maximum-parsimony and maximum-likelihood methods (Fig. 2; Swofford, 2003). Bootstrap analyses using distance and maximum-parsimony were based on 1000 resamplings. Nucleotide pairwise sequence similarity of the 16S rRNA gene over 1429 homologous nucleotides was calculated in ARB and using the web-based tool EzTaxon (Chun *et al.*, 2007).

Strain WH2K^T belongs to a cluster of named organisms that includes *H. marina* LMG 128^T (96.9 % similarity), *H. phototrophica* DFL-43^T (96.7 % similarity) and *H. alexandrii* AM1V30^T (96.6 % similarity). However, this cluster also contains many strains isolated from a number of environmental sources that have yet to be described.

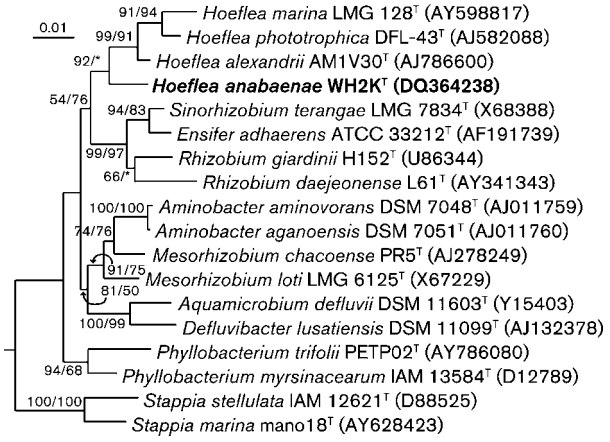

Fig. 2. Phylogenetic tree showing the relationship of *H. anabaenae* sp. nov. WK2K^T to members of related genera in the α-2 subgroup of the *Proteobacteria*, based on 16S rRNA gene sequences using maximum-likelihood analysis. Bootstrap values greater than 50 % (1000 resamplings) for nodes conserved among distance/maximum-parsimony analyses are shown (*, <50 %). Bar, 1 substitution per 100 nucleotide positions.

When these sequences are included in phylogenetic analyses, WH2K^T appears to be more closely related to alphaproteobacteria that have been recovered from marine tunicates (Biebl *et al.*, 2005; Martínez-García *et al.*, 2007), sponges (Sipkema *et al.*, 2009) and coral (Sekar *et al.*, 2008). A phylogenetic tree containing these additional sequences is available as Supplementary Fig. S2. Two of the three described species of the genus *Hoeflea*, *H. phototrophica* and *H. alexandrii*, have been isolated from cultures of the marine dinoflagellates *Alexandrium lusitanicum*, *Prorocentrum lima* and *Alexandrium minutum* (Biebl *et al.*, 2006; Palacios *et al.*, 2006; Peix *et al.*, 2005). Symbiotic relationships may indeed be a life history trait shared between WH2K^T and its close relatives.

Characterization of WH2K^T and comparisons between this novel strain and the type strains of the closest named species, i.e. *H. marina*, *H. phototrophica* and *H. alexandrii*, are detailed in Table 3. Strain WH2K^T has a more limited repertoire of utilizable carbon substrates than the other *Hoeflea* type strains, apparently being able to use only acetate, pyruvate and intermediates of the tricarboxylic acid cycle. This trait may be related to the limited diversity of carbon substrates obtained from its *Anabaena* host during attached symbiosis. The attachment of WH2K^T cells to *Anabaena* sp. SSM-00 is also a distinguishing characteristic. Only *H. phototrophica* DSM 17068^T was observed to attach to the *Anabaena* heterocysts, but at a much lower frequency. Among *Hoeflea* species, only *H. phototrophica* possesses bacteriochlorophyll *a* and carotenoids, which is explained by evidence that this trait was obtained by horizontal gene transfer (Klassen, 2009). The production of photopigments would therefore not be a trait that defines the genus *Hoeflea*.

Description of *Hoeflea anabaenae* sp. nov.

Hoeflea anabaenae (a.na.bae′nae. N.L. fem. gen. n. *anabaenae* of *Anabaena*, a cyanobacterium, from which the type strain was isolated).

Cells are small, irregular club-shaped rods, 0.2 × 2.0–5.0 μm. Colonies grown on PY medium are small (1–2 mm diameter), smooth, flat and opaque with no pigmentation. Cultures require aerobic growth conditions and concentrations of sea salts above 5 g l^{-1} but below 25 g l^{-1} (optimum 15 g l^{-1}), temperatures of 18–34 °C (optimum 30 °C) and pH 6.0–8.0 (optimum pH 6.5). Acetate, pyruvate, fumarate and malate are utilized, but glucose, fructose, sucrose, lactate, glutamate, L-arabinose, butyrate and glycerol are not. Yeast extract is required for growth. Positive for the following enzyme activities (API ZYM): alkaline phosphatase, esterase, leucine aminopeptidase, trypsin, β-galactosidase and β-glucosidase. Negative for the following enzyme activities (API ZYM): esterase lipase, lipase, valine aminopeptidase, cystine aminopeptidase, chymotrypsin, acid phosphatase, phosphohydrolase, α-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Table 3. Characteristics of strain WH2K^T that differentiate it from its closest phylogenetic neighbours

Strains: 1, WH2K^T; 2, *H. phototrophica* DSM 17068^T; 3, *H. alexandrii* DSM 16655^T; 4, *H. marina* DSM 16791^T. Data were obtained in this study unless indicated.

Characteristic	1	2	3	4
Source	Attached symbiont of *Anabaena* sp.	Culture of *Prorocentrum lima*[a]*	Culture of *Alexandrium minutum*[b]	Bulk seawater[a]
Heterocyst attachment	High	Very low	None	None
Salts required for growth	+	+	−	−
Bacteriochlorophyll *a*	−	+[a]	−[b]	−[a]
Substrate utilization				
Glucose	−	+	+	+
Fructose	−	+	+	+
Glycerol	−	−	−	+
Butyrate	−	+	−	−
Lactate	−	+	+	+
L-Arabinose	−	+	+	+
Extracellular enzyme activity				
Esterase lipase (C8)	−	+	+	+
Trypsin	+	−	−	−
Fatty acids (%)†				
$C_{18:0}$	4.0	−	−	−
$C_{16:1}\omega7c$	−	4.7	1.9	2.2
$C_{18:1}\omega9c$	6.6	−	−	−
Polar lipids‡				
PL1	+	−	−	−
PL2	+	−	−	−
SQDG	−	+	+	+
DNA G+C content (mol%)	58.1	59.3[a]	59.7[b]	53.1[a]
16S rRNA gene sequence similarity to strain WH2K^T	(100)	96.7	96.6	96.9

*Data taken from: *a*, Biebl *et al.* (2006); *b*, Palacios *et al.* (2006).
†Values are percentages of total cellular fatty acids.
‡PL1 and PL2, Unknown polar lipids; SQDG, sulfoquinovosyl diacylglycerol.

Does not produce bacteriochlorophyll *a* or photopigments. Fatty acids detected in the type strain include (in decreasing order of abundance) $C_{18:1}\omega7c/\omega9t/\omega12t$ or any combination of these, ECL 17.603, ECL 18.846/$C_{19:1}\omega6c$, $C_{18:1}\omega9c$, $C_{18:0}$, 11-methyl $C_{18:1}\omega7c$ and $C_{16:0}$. Polar lipids include diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidylcholine, an unidentified phosphoglycolipid, two different unidentified phospholipids and an unidentified aminolipid. The only respiratory ubiquinone detected is Q-10. The DNA G+C content of the type strain is 58.1 mol%. Cells are capable of a specific attached symbiotic relationship, only with *Anabaena* sp. SSM-00 and only attached to the surface of its heterocystous cells.

The type strain, WH2K^T (=CCUG 56626^T =NRRL B-59520^T), was isolated from a two-membered culture containing WH2K^T and *Anabaena* sp. SSM-00, which itself was isolated from a brackish marsh in Woods Hole, MA, USA.

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