Ferriphaselus amnicola gen. nov., sp. nov., a neutrophilic, stalk-forming, iron-oxidizing bacterium isolated from an iron-rich groundwater seep

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A neutrophilic, stalk-forming, iron-oxidizing bacterium, strain OYT1-T, which was isolated from a groundwater seep in Ohyato Park, Tokyo, Japan, was subjected to taxonomic analysis. OYT1-T was a motile, bean-shaped, Gram-negative bacterium that was able to grow at 8–30 °C (optimally at 25–30 °C) and at pH 5.6–7.3 (optimally at pH 6.1–6.5). The strain grew microaerobically and autotrophically. Major cellular fatty acids detected were C16:1ω7c/C16:1ω6c and C16:0. The total DNA G+C content was 57.6 mol%. 16S rRNA gene sequence analysis revealed that strain OYT1-T was affiliated with the class Betaproteobacteria and clustered with iron-oxidizing bacteria isolated from groundwater seeps and wetlands and with uncultured clones detected in freshwater iron-rich environments. Based on the phenotypic and phylogenetic characteristics of strain OYT1-T, we propose that the strain represents a novel species in a new genus, for which the name Ferriphaselus amnicola gen. nov., sp. nov. is proposed; the type strain of Ferriphaselus amnicola is OYT1-T (=JCM 18545-T=DSM 26810-T).

Neutrophilic, microaerophilic, iron-oxidizing bacteria are widely distributed in iron-rich freshwater and marine environments, as well as in deep-sea hydrothermal vents (Emerson et al., 2010). Many of these microbes are chemolithoautotrophs that play a role in iron and carbon cycling in these environments. One of the first described iron-oxidizing bacteria, Gallionella ferruginea, is a neutrophilic, microaerophilic micro-organism belonging to the class Betaproteobacteria (Hallbeck et al., 1993). This species was first reported in the 1830s (Ehrenberg, 1836), and is known as a twisted-ribbon-like stalk-forming bacterium (Vatter & Wolfe, 1956); however, the cultured strain of G. ferruginea is not available from any culture collections. In addition, some iron-oxidizing bacteria, such as Gallionella capsiferriformans ES-2, Sideroxydans lithothrophicus strains ES-1 and LD-1, Sideroxydans paludicola BrT and Sideroxydans sp. strain CL21, have been isolated since the discovery of G. ferruginea, although no evidence of stalk production by these bacteria has been reported (Emerson & Moyer, 1997; Lüdecke et al., 2010; Weiss et al., 2007). A stalk-forming, iron-oxidizing bacterium, Mariprofundus ferrooxydans, was isolated from an iron-rich microbial mat at a deep-sea hydrothermal vent (Emerson & Moyer, 2002) and affiliated with the candidate class ‘Candidatus Zetaproteobacteria’ (Emerson et al., 2007). More recently, a stalk-forming, iron-oxidizing betaproteobacterium, strain R-1, was isolated from a groundwater seep (Krepski et al., 2012). Twisted stalks are often observed in iron-rich freshwater and marine environments by microscopy (e.g. Chan et al., 2009; Emerson & Moyer, 2002; Emerson & Revsbech, 1994; Kato et al., 2009) and can be recognized as an indicator of the presence of iron-oxidizing bacteria. The stalks contain carboxyl-rich polysaccharides and may prevent cell encrustation with iron oxides (Chan et al., 2009, 2011). We isolated another stalk-forming, iron-oxidizing betaproteobacterium, strain OYT1-T, from an iron-rich groundwater seep and briefly determined its physiological and phylogenetic characteristics (Kato et al., 2013). Here, we describe the detailed physiological and phylogenetic characteristics of OYT1-T and propose that this isolate represents a novel species in a new genus.

Strain OYT1-T was isolated from an iron-rich floc sample collected from a groundwater seep in Ohyato Park, Tokyo, Japan, by a serial dilution-to-extinction method using 96-well plates (Kato et al., 2013). OYT1-T was maintained using an agarose-stabilized gradient culture technique as described previously (Emerson & Merrill Floyd, 2005). In this method, opposing concentration gradients of oxygen and Fe(II) were established in culture tubes. The basal...
medium, modified Wolfe’s mineral medium (MWMM), was composed of 1 g NH₄Cl, 0.2 g MgSO₄·7H₂O, 0.1 g CaCl₂·2H₂O, 0.05 g K₂HPO₄, 0.42 g NaHCO₃, 10 ml trace element solution (JCM medium no. 151) and 10 ml vitamin solution (JCM medium no. 197) per litre distilled water. This medium was buffered with MES (10 mM) to pH 6.2. The top layer, consisting of high-melting-point (HM) agarose- [0.15% (v/v) agarose type II medium EEO; Sigma-Aldrich] stabilized MWMM, and the bottom layer, consisting of HM-agarose-stabilized (1% v/v) FeS as Fe(II) source, were prepared separately. A gas mixture of N₂/CO₂/O₂ (79:20:1, 0.1 MPa) was used as a headspace. Unless otherwise indicated, growth tests were carried out with the above medium at pH 6.2 and 25°C.

The temperature and pH ranges and optima for growth, requirement for energy sources and growth rate have been reported previously (Kato et al., 2013). In brief, OYT1T grew at 8–30°C and pH 5.6–7.3. Optimum growth was observed at 25–30°C and pH 6.1–6.5. No growth was observed at 4 or 35°C. OYT1T grew on iron-containing media with FeS or FeCl₂ as the iron source but not on iron-free media with the other inorganic and organic substrates [thiosulfate, sulfide, nitrite, MnCl₂, pyruvate, glucose and acetate]. The strain could grow on zero-valent iron (Fe⁰). OYT1T grew with 0–0.8% (w/v) NaCl and did not grow anaerobically with nitrate as an electron acceptor. The doubling time during exponential growth under optimal conditions was 10.9 h.

As reported previously, cells of strain OYT1T produced stalks in an HM-agarose-stabilized medium at pH 7.1–7.3, but not at pH 6.5 or below (Kato et al., 2013). Instead, they produced extracellular particulate oxides under lower pH (Fig. S1, available in the Supplementary Material). Strain R-1, 'G. capsiferriformans', 'S. lithotrophicus' and 'S. paludicola' also produce such particulate oxides (Emerson & Moyer, 1997; Krepski et al., 2012; Weiss et al., 2007). It should be noted that OYT1T produced stalks in a low-melting-point (LM) agarose [0.15% (w/v) certified low-melt agarose; Bio-Rad] -stabilized or agarose-free liquid medium at pH 6.2 (Fig. 1). The stalks consisted of thin filaments (20–200 nm wide), determined by transmission electron microscopy (Fig. 1; Kato et al., 2013), which resemble those produced by G. ferruginea (Vatter & Wolfe, 1956), strain R-1 (Krepškienė et al., 2012) and M. ferrooxydans (Chan et al., 2011). All stalks represented a left-handed (S-type) helical structure. As with stalks produced by strain R-1 (Krepškienė et al., 2012) and M. ferrooxydans (Chan et al., 2011), stalks of strain OYT1T contained polysaccharides (Fig. S2), as determined by lectin-staining with concanavalin A, as described previously (Chan et al., 2011).

Cells of OYT1T were Gram-negative, gently curved, short rods (0.8–1.9 × 0.7–0.9 µm). No spore formation was observed under any of the growth conditions examined. Cells were motile by a polar flagellum (Fig. S3), as observed in the agarose-free liquid medium. Colonies appeared in LM-agarose (0.15%, w/v) -stabilized or agarose-free liquid cultures at early stages (approx. 12 h) at pH 6.2, similar to cultures of G. ferruginea (Kucera & Wolfe, 1957); however, colonies did not appear in HM-agarose-stabilized cultures. A white band appeared in LM-agarose-stabilized and HM-agarose-stabilized cultures after 1 or 2 days, like cultures of ‘G. capsiferriformans’, ‘S. lithotrophicus’ and ‘S. paludicola’ (Emerson & Moyer, 1997; Weiss et al., 2007). The colony and band consisted of stalks and contained particulate oxides. The colour of the colony and band changed to orange or ochre (rust colour) after 2–3 days.

Fatty acid methyl esters were identified using the procedure recommended for use with the Microbial Identification System (MIDI; Sherlock Microbial Identification System version 4.0). Cells were grown in gradient plates (Emerson & Merrill Floyd, 2005), which consisted of agarose-free liquid MMW (350 ml total) and FeS/agarose plug in plates. These plates were incubated in an anaerobic jar with the gas mixture of N₂/CO₂/O₂ (79:20:1). After 3 days (late exponential growth phase), total cells and iron oxides were harvested from the plates and the oxides were dissolved with a solution of dithionite (50 g l⁻¹) in 0.2 M citrate and 0.35 M acetic acid (Kostka & Luther, 1994). Cells were centrifuged, rinsed three times in sterile buffered MMW and submitted to MIDI Laboratories (Newark, DE, USA) for fatty acid analysis. The cellular fatty acid profile of OYT1T contained C₁₀:0-3-OH (3.4%), C₁₂:0 (2.2%), C₁₄:0 (1.6%), C₁₅:0 (1.2%), C₁₆:0 N-alcohol (0.9%), C₁₆:1ω7c and/or C₁₆:1ω6c (59.9%), C₁₆:1ω5c (1.2%), C₁₆:0 (22.2%), C₁₇:1ω6c (0.7%), C₁₈:3ω6c (6.9,12) (0.8%), C₁₈:1ω9c (1.4%), C₁₈:1ω7c (2.2%) and C₁₈:0 (2.3%).
For DNA extraction, cells were grown in gradient plates as described above. Total DNA was extracted from cells by using a FastDNA spin kit for soil and the FastPrep instrument (MP Biomedicals). DNA base composition was determined by the HPLC method as described by Tamaoka (1994). The G+C content of the total DNA was 57.6 ± 0.4 mol% (two determinations).

OYT\textsuperscript{T} was affiliated with the Gallionella-related cluster within the class Betaproteobacteria, as shown by phylogenetic analysis using the 16S rRNA gene sequence of OYT\textsuperscript{T} (Kato et al., 2013). In addition, we checked the 16S rRNA gene sequence by direct sequencing of PCR products amplified from the extracted DNA. These results suggest that the strain contains one copy of the rRNA operon or a set of identical rRNA operons. Based on the BLAST search result, the nearest cultivated neighbour of OYT\textsuperscript{T} was strain R-1 (97.8 % similarity). The 16S rRNA gene sequence of OYT\textsuperscript{T} showed high similarity to two environmental clone sequences, oytB002 (99.9 % similarity; GenBank accession no. AB722190), recovered from an iron-rich floc in a freshwater creek in Big Basin Redwoods State Park, CA, USA (Duckworth et al., 2009). The 16S rRNA gene sequences of OYT\textsuperscript{T} and close relatives deposited in public databases (GenBank/EMBL/DDBJ) were aligned using MUSCLE (Edgar, 2004), and gap positions were removed from the alignment dataset using Gblock (Castresana, 2000). A maximum-likelihood tree was reconstructed with the alignment dataset (1266 bases) using PhyML (Guindon et al., 2010) with the general-time-reversible model of nucleotide substitution incorporating invariable sites and discrete gamma distribution (GTR + I + G). Bootstrap values were estimated from 1000 replicates. OYT\textsuperscript{T} was distant from members of the genera Gallionella and ‘Sideroxydans’ (92.9–94.0 % similarity) and formed a distinct lineage with strain R-1 and environmental clones, as strongly supported by a high bootstrap value (Fig. 2).

Our previous report (Kato et al., 2013) and the present study show that strain OYT\textsuperscript{T} is a neutrophilic, microaerophilic, stalk-forming, chemolithoautotrophic, iron-oxidizing bacterium. The Calvin–Benson–Bassham cycle is probably used for autotrophic growth, because the strain possesses a ribulose-1,5-bisphosphate carboxylase/oxygenase gene, \textit{cbbM}, encoding a key enzyme of the cycle (Kato et al., 2013). Based on phylogenetic analysis of the 16S rRNA gene sequence, OYT\textsuperscript{T} should not be affiliated with the genera Gallionella, ‘Sideroxydans’ or ‘Nitrotoga’ (Fig. 2). The phenotypic characteristics and overall fatty acid profile of OYT\textsuperscript{T} can be distinguished from those of \textit{G. ferruginea} and related isolates (Tables 1 and S1). Although OYT\textsuperscript{T} produces stalks like those of \textit{G. ferruginea} and strain R-1,
the growth temperature and pH distinguish OYT1T from these two micro-organisms. The phenotypic characteristics of the other iron-oxidizing isolates are slightly different from those of OYT1T, and these isolates do not produce stalks. On the basis of the phenotypic and phylogenetic properties, strain OYT1T represents a novel species in a new genus. Here, the name Ferriphaselus amnicola gen. nov., sp. nov. is proposed to accommodate strain OYT1T. Strain R-1 may represent another species of this genus, because of the lower 16S rRNA gene sequence similarity (i.e. 97.8 %) to OYT1T. However, more detailed phenotypic analyses are required to distinguish strain R-1 from OYT1T.

**Description of Ferriphaselus amnicola sp. nov.**

Ferriphaselus amnicola [am.ni’co.la. L. masc. n. amnis a stream, a small river; L. suff. -cola (from L. n. incola) a dweller, an inhabitant; N.L. masc. n. amnicola an inhabitant of a stream].

Morphological, cultural properties and phylogenetic features are as described for the genus. Grows at 8–30 °C (optimally at 25–30 °C) and pH 5.6–7.3 (optimally at pH 6.1–6.5). Utilizes ferrous iron as an energy source for lithotrophic growth. Does not utilize thiosulfate, sulfide, nitrite, Mn(II), pyruvate, glucose or acetate as an energy source. The doubling time under optimal conditions is 10.9 h.

The type strain is OYT1T (=JCM 18545T=DSM 26810T), isolated from an iron-rich floc in a groundwater seep in Ohyato Park, Tokyo, Japan. The total DNA G+C content of the type strain is 57.6 mol%.

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**References**


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