Prostheco bacter fluviatilis sp. nov., which lacks the bacterial tubulin *btubA* and *btubB* genes

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Leptothrix cholodnii is a sheathed bacterium often found in metal-rich and oligotrophic aquatic environments. A bacterial strain that is able to degrade the NaOH-treated sheath of *L. cholodnii* was isolated. The isolate was a Gram-negative, aerobic and prosthecate bacterium. The optimum growth temperature and pH were 30 °C and pH 7.0, respectively. The DNA G + C content was 62.9 mol%. The major respiratory quinone was MK-6. A phylogenetic analysis based on the 16S rRNA gene indicated that the isolate is a member of the genus *Prostheco bacter*. The nearest relative was the type strain of *Prostheco bacter vanneervanii*, with a similarity of 97.1 %. However, the isolate does not possess the bacterial tubulin genes, *btubA* and *btubB*, unique to known species of the genus *Prostheco bacter*. It is proposed that the isolate represents a novel species, *Prostheco bacter fluviatilis* sp. nov. The type strain is HAQ-1^T (=JCM 14805^T =KACC 12649^T =KCTC 22182^T).

Members of the *Sphaerotilus–Leptothrix* group are aquatic filamentous bacteria that are able to oxidize metal ions (Eikelboom, 1975; van Veen *et al.*, 1978) and play a role in biomineralization (Ghiorse & Ehrlich, 1992). Their filamentous growth is accomplished by the formation of a sheath, a tube-like extracellular structure in which cells are enclosed, forming a filament. Insoluble metal oxides generated by this group of bacteria are not accumulated directly on the cell surface, but are deposited on the sheath (van Veen *et al.*, 1978), possibly allowing steady cell division. The sheaths of the *Sphaerotilus–Leptothrix* group are formed by association of mucopolysaccharide modified with a cysteine-rich peptide and probably comprise a novel category of structural glycoconjugates (Emerson & Ghiorse, 1993; Makita *et al.*, 2006; Takeda *et al.*, 1998, 2003, 2005). To investigate the chemical structure of a complicated structural macromolecule, an enzyme is desired that degrades the macromolecule specifically, such as lysozyme for peptidoglycans. A degrading enzyme specific for the sheath of *Sphaerotilus natans* is already available from *Paenibacillus koleovorans* (Takeda *et al.*, 2002, 2003). In contrast, no microbe that degrades the sheath of *Leptothrix cholodnii* has so far been isolated.

Therefore, we attempted to isolate microbes from soil and river water capable of growth on the sheath of *L. cholodnii*. Although we could not find any microbes with this desirable property, a bacterial strain that can utilize the polysaccharide prepared from the sheath was obtained. Based on a taxonomic characterization, the isolate was found to be a novel strain related to *Prostheco bacter vanneervanii*. Interestingly, two bacterial tubulin genes (*btubA* and *btubB*) unique to the genus *Prostheco bacter* were not detected in the isolate.

The sheath of *L. cholodnii* ATCC 51168^T was obtained by a previously described method (Takeda *et al.*, 2005) and used to prepare a screening medium of the following composition (l⁻¹ distilled water): 0.25 g sheath material, 0.2 g (NH₄)₂SO₄, 0.3 g NaNO₃, 0.2 g CaCl₂, 0.06 g MgSO₄·7H₂O, 0.1 g KH₂PO₄, 0.15 g Na₂HPO₄·2H₂O and 2.8 mg FeSO₄·7H₂O. Screening was carried out aerobically at 25 °C, but no sheath-degrading microbes were detected. We then tried to find microbes capable of growing on *N*-acetylated mucopolysaccharide that was prepared from the sheath by the method described below. Sheath material (50 mg) was soaked in 50 ml 3 M NaOH followed by incubation at 30 °C for 72 h to remove the peptide moiety and to release the mucopolysaccharide. The solution was neutralized with phosphoric acid, passed through a glass filter and dialysed against a 0.1 M NaHCO₃ solution. To the dialysate, a saturating amount of NaHCO₃ was added and then acetic anhydride was slowly added until the pH of the solution reached about 6. The solution

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HAQ-1^T is AB305640.

A graph showing growth of strain HAQ-1^T on mucopolysaccharide and figures showing the positions of PCR primers specific for the *btubA* and *btubB* genes are available as supplementary material with the online version of this paper.

was passed through a column (2.5 × 22 cm) packed with AG50W-X8 resin (H⁺ form; Bio-Rad) and the eluate was dialysed against distilled water. The dialysate was finally lyophilized to recover the *N*-acetylated mucopolysaccharide (25–30 mg). The composition of the modified screening medium for isolation of microbes that utilize the *N*-acetylated mucopolysaccharide was the same as that of the original screening medium described above except that the sheath material was replaced with the *N*-acetylated mucopolysaccharide. A culture supplemented with river water collected from the Yamada River (Munakata, Fukuoka, Japan) became turbid at 25 °C within 2 months. After subculturing three times with the modified screening medium, colony isolation was carried out with plates of the same medium (1.5 % agar) at 25 °C. Each colony formed on the plates was transferred to liquid medium and incubated at 25 °C. One of the cultures became turbid within 1 month and the isolate was designated strain HAQ-1^T. More rapid growth was observed, and degradation of the *N*-acetylated mucopolysaccharide was confirmed, in a medium supplemented with soytone peptone as the nitrogen source (Supplementary Fig. S1, available in IJSEM Online). However, strain HAQ-1^T could not grow on the original screening medium.

When strain HAQ-1^T was cultured aerobically in a complex medium (MMB medium; Staley & Mandel, 1973) at 30 °C for 3 days, straight, fusiform cells with prosthecae at one pole were observed (Fig. 1a). Cell division occurred at the non-prosthecae pole (Fig. 1a). Cells were 0.3–0.7 µm wide and 1.8–8 µm long (Fig. 1). The cells lacked flagella and were non-motile. Spores were not formed. These features are typical for the genus *Prostheco bacter* (Staley *et al.*, 1976). Cells of strain HAQ-1^T tended to attach to each other and to form cell clumps (Fig. 1b), different from known *Prostheco bacter* strains (Staley *et al.*, 1976). It is likely that the expanded fibrous material becomes interlaced to bind the adjoining cells (Fig. 1a). Gram-staining was negative using Favour-G reagents (Nussui Pharmaceutical). Anaerobic growth in MMB medium at 30 °C was tested using a BBL GasPack pouch (Becton Dickinson), and no growth was observed within 2 months. Colonies formed on solidified MMB medium were pale yellow, convex and opaque.

For determination of the effect of pH on growth, the bacterium was cultured statically in MMB medium at various pHs (by addition of HCl or NaOH) at 30 °C for 4 days, and the optical density at 660 nm was then measured. Growth of strain HAQ-1^T occurred at pH 4.5–8.1, with an optimum at pH 6.5–7.0. The effect of temperature was tested by static cultivation for 1 month in MMB medium at various temperatures. Growth was assessed by an increase in the optical density at 1–3 day intervals. The growth temperature range of strain HAQ-1^T was 10–38 °C with an optimum at 30 °C. The doubling time at 30 °C was estimated to be 4.6 h by measuring the optical density at intervals of 4 h during shaking cultivation in MMB medium. These phenotypic properties

Fig. 1. Negatively stained electron micrograph (top; bar, 1 µm) and phase-contrast micrograph (bottom; bar 10 µm) of strain HAQ-1^T. Strain HAQ-1^T was cultured statically in MMB medium for 2 days and then subjected to electron microscopy (JEM-2000 FX II; JEOL) and phase-contrast microscopy (Axioskop; Zeiss).

are similar to those of other members of the genus *Prostheco bacter*. Cells grown on MMB agar plates at 30 °C for 3 days formed a few bubbles in 3 % (v/v) H₂O₂ solution, thus exhibiting a weak catalase activity. Oxidase activity was positive, because the cells oxidized *p*-phenylenediamine. The capacity to utilize various sugars was tested using defined DM medium (van Ert & Staley, 1971) as a basal medium according to the method of Staley *et al.* (1976). Utilization was judged by an increase in the turbidity of the cultures, with results given in the species description and in Table 1. The carbohydrate utilization pattern of strain HAQ-1^T is useful in differentiating it from type strains of the genus *Prostheco bacter*.

Total DNA of strain HAQ-1^T grown on MMB medium was prepared using a Genomic-Prep blood DNA isolation kit

Table 1. Carbohydrate utilization of strain HAQ-1^T and type strains of *Prostheco bacter* species

Strains: 1, *P. de joneii* FC1^T; 2 *P. vanneervanii* FC2^T; 3, *P. debontii* FC3^T; 4, *P. fusiformis* FC4^T; 5, strain HAQ-1^T. Reference data are from Hedlund *et al.* (1997). All strains were positive for utilization of D-glucose, D-galactose, D-mannose, D-xylose, sucrose, maltose and cellobiose.

Carbohydrate	1	2	3	4	5
L-Rhamnose	+	+	+	+	–
Trehalose	+	+	+	+	–
D-Ribose	+	+	+	–	+
L-Arabinose	–	–	+	+	–
D-Fructose	–	+	+	–	–
Raffinose	–	+	–	–	–
Glycogen	+	+	–	–	–
D-Glucosamine	–	+	–	–	–
N-Acetyl-D-glucosamine	+	+	+	–	+
Lactose	+	+	+	+	–
Melibiose	+	+	+	+	–

(GE healthcare). The 16S rRNA gene (1488 bp) was amplified by PCR with the primers 8F (Hallbeck *et al.*, 1993) and 1492R (Lane, 1991). PCR products were cloned into the vector pCR2.1 using a TOPO-TA cloning kit (Invitrogen) and plasmid DNA was prepared by a Plasmid Miniprep kit I.E.Z.N.A. (Omega Bio-tech). Phylogenetic analysis was done according to previously described methods (Takeda *et al.*, 2002). Strain HAQ-1^T could be placed into the cluster of the genus *Prostheco bacter* (Fig. 2), indicating that the strain is a member of this genus. The closest relative of strain HAQ-1^T was the type strain of *P. vanneervanii*. Sequence similarity values with the type strains of the genus *Prostheco bacter* ranged from 93.1 % (*Prostheco bacter debontii*) to 97.1 % (*P. vanneervanii*).

The G+C content of the DNA of strain HAQ-1^T was estimated to be 62.9 mol% by the HPLC method (Takeda *et al.*

Fig. 2. Phylogenetic position of strain HAQ-1^T among the species of the genus *Prostheco bacter*. The tree was constructed from an evolutionary distance matrix based on the neighbour-joining method (Saitou & Nei, 1987). Bootstrap probabilities (percentages of 1000 replications) are indicated at branch points. Accession numbers are shown in parentheses. The sequence of *Verrucomicrobium spinosum* DSM 4136^T was used to root the tree. Bar, 0.02 changes per nucleotide position.

al., 2002). This value is slightly higher than the range (54–61 mol%) determined for other members of the genus *Prostheco bacter* (Staley *et al.*, 1976) and the family *Verrucomicrobiaceae* (Hedlund *et al.*, 1997). Both ubiquinone and menaquinone were detected by TLC according to a previously described method (Takeda *et al.*, 2004). The spot of ubiquinone on the TLC plate was faint. Menaquinone was recovered from the plate and was further purified by HPLC under the following conditions: column, COSMOSIL 5C₁₈-MS-II (4.6 × 150 mm; Nacalai Tesque); temperature, ambient; eluent, methanol/isopropyl ether (75:25); flow rate, 1 ml min^{–1}; detection, A₂₇₀. The predominant menaquinone was MK-6(H₂), based on its retention time. *Prostheco bacter* strains are reported to contain both ubiquinone (not identified) and menaquinone [MK-6(H₂)] as major quinones (Hedlund *et al.*, 1996; Staley *et al.*, 1976), supporting the assumption that strain HAQ-1^T should be classified into the genus *Prostheco bacter*. Except for the G+C content, the major chemotaxonomic properties of strain HAQ-1^T were similar to those of the genus *Prostheco bacter* (Staley *et al.*, 1976). In addition, the cellular fatty acids were identified by GC-MS according to previously described methods (Takeda *et al.*, 2004). The major cellular fatty acids of strain HAQ-1^T were C_{14:0} (42.0%), C_{16:1} (17.7%), anteiso-C_{15:0} (9.7%), and iso-C_{14:0} (5.7%).

The genes *btubA* and *btubB*, which share high sequence identity with eukaryotic α - and β -tubulin genes, have been found previously in the genus *Prostheco bacter* and their expression has been confirmed in *Prostheco bacter de joneii* (Jenkins *et al.*, 2002). These genes are assumed to have been transferred from a eukaryotic cell by horizontal gene transfer (Schlieper *et al.*, 2005). *BtubA* and *BtubB* potentially form filaments in a similar arrangement to $\alpha\beta$ -tubulin (Schlieper *et al.*, 2005; Sontag *et al.*, 2005), and there is the possibility that these proteins contribute to the maintenance of the prothecate cell shape of *Prostheco bacter* strains (Sontag *et al.*, 2005). However, the roles of these genes in *Prostheco bacter* strains have not yet been confirmed (Pillhofer *et al.*, 2007). To elucidate whether strain HAQ-1^T has *btubA* and *btubB*, PCR amplification of these genes was attempted using the primers and temperature program reported by Jenkins *et al.* (2002). Despite repeated trials, no products were obtained. For confirmation, other PCR primer sets (BTUBA-SF/BTUBA-SR for *btubA*; BTUBB-SF/BTUBB-SR for *btubB*) specific for *btubA* and *btubB* were newly designed on the basis of nucleotide sequences available from GenBank (Supplementary Figs S2 and S3). *P. vanneervanii* ATCC 700199^T was grown in MMB medium at 25 °C for 3 days and its genomic DNA was prepared as described above as a positive control for the PCR assay. The desired PCR products were amplified from DNA of *P. vanneervanii* ATCC 700199^T (Fig. 3). In contrast, no products were obtained from DNA of strain HAQ-1^T (Fig. 3), indicating that strain HAQ-1^T does not harbour *btubA* and *btubB*. These genes have probably been lost in some species during their evolution and are not essential for growth of

Fig. 3. Detection of the *btubB* (lanes 1 and 2) and *btubA* (lanes 3 and 4) genes by PCR. The PCR consisted of 30 cycles of 94 °C (30 s), 55 °C (30 s) and 72 °C (30 s), followed by extension at 72 °C for 7 min. PCR products were separated on a 1% (w/v) agarose gel. Lanes 1 and 3 were loaded with products from DNA of *P. vanneervanii* ATCC 700199^T, while lanes 2 and 4 were loaded with DNA of strain HAQ-1^T. Lane 5, 1 kb DNA ladder (Promega).

*Prostheco*bacter strains. Since other *Prostheco*bacter strains have *btubA* and *btubB*, the absence of these genes is a significant feature of strain HAQ-1^T. The genetic and phenotypic features of strain HAQ-1^T suggest that it represents a novel species of the genus *Prostheco*bacter. The name *Prostheco*bacter *fluviatilis* sp. nov. is now proposed. In order to include strain HAQ-1^T in the genus *Prostheco*bacter, we also propose emended descriptions of the genus *Prostheco*bacter and the family *Verrucomicrobiaceae*.

Emended description of the family *Verrucomicrobiaceae* Ward-Rainey *et al.* 1996

The formal description remains as given by Hedlund *et al.* (1997) with the following modification. The G + C content of the DNA ranges from 54 to 63 mol%.

Emended description of the genus *Prostheco*bacter (ex Staley *et al.* 1976) Staley *et al.* 1980

The formal description remains as given by Staley *et al.* (1976) with the following modification. The G + C content of the DNA ranges from 54 to 63 mol%.

Description of *Prostheco*bacter *fluviatilis* sp. nov.

*Prostheco*bacter *fluviatilis* (flu.vi.a.til'is. L. masc. adj. *fluviatilis* of or belonging to a river).

Cells are fusiform (0.3–0.7 × 1.8–8 µm) and straight. Growth occurs under aerobic conditions. Cells grown on

MMB medium occasionally form clumps. Colonies on MMB agar are pale yellow, convex and opaque. Growth occurs in the temperature range 10–38 °C, with optimum growth at 30 °C. Optimum growth occurs at pH 6.5–7.0. Ammonium salts are utilized as a nitrogen source. Vitamins are not required. The following carbon sources are utilized: D-glucose, D-galactose, D-mannose, sucrose, maltose, D-xylose, cellobiose, D-ribose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. The following carbon sources are not utilized: lactose, melibiose, L-rhamnose, trehalose, L-arabinose, D-fructose, raffinose, glycogen, D-glucosamine, D-galactosamine and galacturonic acid. The G + C content of the DNA of the type strain is 62.9 mol%. The type strain does not appear to possess the bacterial tubulin genes *btubA* and *btubB* based on PCR data.

The type strain is HAQ-1^T (=JCM 14805^T =KACC 12649^T =KCTC 22182^T), isolated from river water.

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