NOTE

***Bacillus funiculus* sp. nov., novel filamentous isolates from activated sludge**

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A novel filamentous *Bacillus* strain, NAF001^T, was reported previously that produces endospores and spore-like resting cells; the latter outgrow by budding. Phylogenetic analysis based on 16S rDNA gene sequences reported in the same paper speculated on the proposal of a novel species for this isolate. This communication describes the DNA–DNA relatedness of strain NAF001^T to various members of the genus *Bacillus* and its whole-cell fatty acid and quinone profiles, in order to authenticate the creation of a novel species, for which the name *Bacillus funiculus* sp. nov. is proposed. The type strain is NAF001^T (= JCM 11201^T = CIP 107128^T). Further, features of the binding points between filaments of strain NAF001^T that enable it to form extremely long filaments are captured by electron microscopy.

Keywords: filamentous *Bacillus*, resting cell, binding growth, fatty acid profile, quinone profile

Filamentous bacteria often cause major problems in activated sludge processes, as they are found in a scummy layer formed in treatment tanks and are believed to inhibit the sedimentation of sludge. They are often difficult to cultivate. For this reason, morphological characters alone are taken into consideration for routine analysis. The presence of *Bacillus* strains in activated sludge has been documented, but with little practical significance (Trick *et al.*, 1984; Seviour *et al.*, 1999). In contrast, Iriye (1999) showed the effectiveness of *Bacillus* strains in decreasing the malodour of domestic sewerage facilities. The bacterium described in this paper was isolated from such a facility (Iriye, 1999). Strain NAF001^T is the first phylogenetically characterized filamentous *Bacillus* (Ajithkumar *et al.*, 2001). It formed a long, unbranched, filamentous trichome and developed two kinds of resting cells, namely endospores and unique heat-resistant cells, termed spore-like resting cells, which outgrow by budding. Phylogenetic analysis based on 16S rDNA showed its linkage to the *Bacillus cereus* cluster, though it was very distinct, as shown by the large sequence difference (sequence similarity 95.0%). Strain NAF001^T also showed similar, but slightly higher sequence similarity to *Bacillus megaterium* and

Bacillus cohnii (each 95.2%), suggesting a unique phylogenetic position (see Fig. 5 of Ajithkumar *et al.*, 2001). We further analysed the genetic relatedness of strain NAF001^T to selected members of the *B. cereus* cluster, *B. megaterium*, *B. cohnii* and strain NAF002, an isolate similar to NAF001^T, by DNA–DNA hybridization. Composition of fatty acids, isoprenoid quinones and details of the fine structure of binding points between filaments that result in its strong filamentous growth were analysed, the results of which are presented.

Culture media used to cultivate strain NAF001^T were nutrient broth/glucose (NG broth), nutrient broth/glucose agar (NG agar) and synthetic medium (Ajithkumar *et al.*, 2001). Strain NAF002 was isolated from the stock culture (NG agar) of strain NAF001^T using Columbia agar (Difco) (with 5% sheep blood). Colonies were round, opaque and off-white in NG agar (NAF001^T) and colourless for NAF002 (more notable on Columbia blood agar). Staining was done as described by Murray *et al.* (1994). Strains NAF001^T and NAF002 were filamentous, Gram-variable and peritrichously flagellated. Spores of strain NAF002 were observed on Columbia agar but budding cells were not observed on NG agar, Columbia agar or in synthetic medium (glucose). Carbon utilization tests were carried out using Biolog GP microplates (Biolog Inc.) as well as in minimal medium. Glucose, fructose,

Table 1. Characteristics that differentiate strain NAF001^T from species of the *B. cereus* cluster

Data were taken from Claus & Berkeley (1986) unless otherwise stated. Taxa: 1, strain NAF001^T (data from this study); 2, *Bacillus anthracis*; 3, *B. cereus*; 4, *B. mycoides*; 5, *Bacillus pseudomycoides* (Nakamura, 1998); 6, *Bacillus thuringiensis*; 7, *Bacillus weihenstephanensis* (Lechner *et al.*, 1998). Results are scored as: +, positive; v, variable among strains; –, negative; d, 11–89% strains positive; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Budding cells	+	–	–	–	–	–	–
Anaerobic growth	–	+	+	+	+	+	+
Parasporal crystals	–	–	–	–	–	d	–
Growth at 10 °C	–	–	d	d	–	d	+
Growth with 5% NaCl	–	+	ND	d	+	+	ND
Citrate utilization	–	d	+	d	v	+	ND
Hydrolysis of:							
Casein	–	+	+	+	+	+	+
Gelatin	–	+	+	+	ND	+	+

maltose, sucrose, D-psicose, D-ribose, D-trehalose and glycerol were utilized, while salicin, lactose, L-arabinose, D-arabitol, D-tagatose, xylitol and D-xylose were not. Strain NAF001^T, but not strain NAF002, utilized D-mannitol and D-mannose. Arbutin was utilized by strain NAF002. All biochemical tests for strains NAF001^T (repeated) and NAF002 were carried out as described by Smibert & Krieg (1994). Briefly, catalase, the Voges–Proskauer test, nitrate reduction to nitrite and hydrolysis of starch, aesculin and urea were positive and oxidase, indole, hydrolysis of gelatin, casein and Tween 80 and citrate utilization were negative. Acid was produced from glucose but not from mannitol, xylose or arabinose. Growth was observed with 0.001% lysozyme. Most of the biochemical characteristics thus resemble those of the *Bacillus cereus* group (Claus & Berkeley, 1986). Differential characteristics are presented in Table 1.

In order to analyse the fatty acid profile, cells of strain NAF001^T cultured on NG agar (4 days) were washed with distilled water. Wet cells (200 mg) were refluxed in 10% KOH/65% ethanol (5 ml) for 1 h under a N₂ atmosphere. The reaction mixture was then acidified and extracted with ethyl acetate. Methylated samples were analysed with a GC-MS [JMS-700 with Hewlett Packard 5890 gas chromatograph (column DB-1), temperature 80–220 °C (6 °C min^{–1}), injection temperature 240 °C and flow rate 7.0 ml min^{–1} (He gas)] in EI positive mode. Retention times of each peak were compared with those of standard methylated fatty acids (Supelco). The molecular formula of each peak was determined by high-resolution GC-MS analysis using the JEOL data-processing system. The whole-cell fatty acid profile of NAF001^T was as follows. Straight-chain saturated fatty acids: C_{16:0}, 8.75%; C_{17:0}, 1.24%; C_{18:0}, 1.93%. Monounsaturated fatty

acids: C_{11:1}, 4.25%. Branched saturated fatty acids: iso-C_{15:0}, 18%; anteiso-C_{15:0}, 44.3%; iso-C_{16:0}, 3.84%; and an unidentified fatty acid (hydroxylated, branched C_{15:4}), 13.8%. The fatty acid profile of strain NAF001^T showed an abundance of branched, saturated C₁₅ fatty acids and thus it could fit into the Kaneda group A *Bacillus*, which includes, among others, the *B. cereus* group, *Bacillus subtilis*, *B. megaterium* and *Bacillus pumilus* (Kaneda, 1977).

Isoprenoid quinones were extracted from wet cells (500 mg) with acetone, separated by preparative silica gel TLC using benzene and detected under UV. The major bands due to menaquinones were collected (ubiquinone could be detected as a minor band, but not sufficient for quantification) and analysed with an LC-MS spectrometer (Collins & Jones, 1981; Hiraishi, 1999). The major menaquinone detected in strain NAF001^T was menaquinone-7 (*t_r* 13.91 min, *m/z* 648), which indicates that strain NAF001^T is a member of the genus *Bacillus* (Collins & Jones, 1981).

In order to examine genetic relatedness, DNA was extracted by Marmur's method as described by Johnson (1994). A combination of lysozyme and achromopeptidase was used for the initial lysis and incubated for up to 4–6 h, followed by 4–8 h treatment in an SDS/proteinase K mixture for strains NAF001^T and NAF002. DNA–DNA homology was determined by fluorometric hybridization in microdilution wells (Ezaki *et al.*, 1989) with biotinylated DNA of NAF001^T. Black microplates used were for fluorescence and enhanced binding (Lab Systems). DNA–DNA hybridization was achieved at 36 °C for 2 h in 2 × SSC containing 45% formamide. Fluorescence intensity was measured with an FP3000 Fluorolite microplate reader (Shinseirika) at 360 nm for excitation and 450 nm for emission. The values obtained were as follows: NAF001^T, 100%; NAF002, 92%; *B. cereus* JCM 2152^T, 15%; *B. cohnii* YN-2000 (Yumoto *et al.*, 2000), 10%; *B. megaterium* JCM 2506^T, 12%; *Bacillus mycoides* NCIMB 13305^T, 6%. Such low values clearly indicate the genetic distance of the present isolates from other *Bacillus* species, especially the *B. cereus* group, with which it shares most biochemical characteristics.

It was reported previously that 'cell binding' was a unique character of strain NAF001^T. Germinated cells of strain NAF001^T could bind and then grow into filaments that, in turn, bound to form an extremely long trichome. In order to perform ultrastructural studies of the binding points of strain NAF001^T, cells were cultured in synthetic medium containing glucose as a carbon source for 2 days at 32 °C and pre-fixed in 2.5% glutaraldehyde at 6 °C. Scanning electron micrographs were taken using a scanning electron microscope (Hitachi S-4500). Fig. 1 shows the strong cell binding points that result in the formation of the long filamentous trichome. Two terminal cells could bind tightly during exponential phase (Fig. 1a). This cell–cell binding point was then covered with some unidentified polymeric substance (Fig. 1b). This might be the

Fig. 1. Scanning electron micrographs of strain NAF001^T, highlighting the cell-cell binding point. Strain NAF001^T was incubated in synthetic medium (glucose) for 2 days at 32 °C with shaking at 10 r.p.m. Binding points of strain NAF001^T during early exponential phase (just after binding) (a) and during late exponential phase (b) are shown. Bars, 1 µm.

reason why the filaments are not broken down even during agitated cultivation (120 r.p.m.). The growth process of strain NAF001^T was therefore unique, and a clear understanding of its cell division is a subject of further study.

Based on the results of this and previous studies (Ajithkumar *et al.*, 2001), the name *Bacillus funiculus* sp. nov. is proposed for these isolates and strain NAF001^T is designated as the type strain.

Description of *Bacillus funiculus* sp. nov.

Bacillus funiculus (fu.ni'cu.lus. L. masc. n. *funiculus* string, rope, reflecting the filamentous appearance of cells of this isolate).

Cells (4.0–6.0 × 0.8–2.0 µm) are aerobic and filamentous and produce centrally located ellipsoidal spores. Flagella present (peritrichous). Growth occurs between 20 and 40 °C, with the optimum being around 30 °C. Anaerobic growth, oxidase and indole from tryptophan are negative. Catalase and the Voges–Proskauer test are positive and nitrate is reduced to nitrite. Starch, urea and aesculin are hydrolysed, but casein, gelatin and Tween 80 are not. Glucose, fruc-

tose, maltose, sucrose, D-psicose, D-ribose, D-trehalose and glycerol are utilized but L-arabinose, raffinose, lactose, salicin, D-tagatose, inositol, xylitol, D-xylose, citrate and L-malic acid are not. The major quinone is menaquinone-7. Iso-C_{15:0} (18%) and anteiso-C_{15:0} (44%) are the main fatty acids produced when grown on nutrient broth/glucose agar. The DNA G+C content of the type strain is 37.2 mol% by HPLC.

Isolated from suspended water of a wastewater treatment tank in Fukushima, Japan. The type strain is strain NAF001^T (= JCM 11201^T = CIP 107128^T). Strain NAF002 (= JCM 11493) is a reference strain.

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