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

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A novel filamentous Bacillus strain, NAF001T, 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 NAF001T 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 NAF001T ( = JCM 11201T = CIP 107128T ). Further, features of the binding points between filaments of strain NAF001T that enable it to form extremely long filaments are captured by electron microscopy.

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Table 1. Characteristics that differentiate strain NAF001T from species of the B. cereus cluster

Data were taken from Claus & Berkeley (1986) unless otherwise stated. Taxa: 1, strain NAF001T (data from this study); 2, Bacillus anthracis; 3, B. cereus; 4, B. mycoides; 5, Bacillus pseudomycoidei (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.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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</thead>
<tbody>
<tr>
<td>Budding cells</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Anaerobic growth</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Parasporal crystals</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>d</td>
<td>−</td>
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<tr>
<td>Growth at 10°C</td>
<td>−</td>
<td>d</td>
<td>d</td>
<td>−</td>
<td>d</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Growth with 5% NaCl</td>
<td>−</td>
<td>+</td>
<td>ND</td>
<td>d</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Citrate utilization</td>
<td>−</td>
<td>d</td>
<td>+</td>
<td>d</td>
<td>V</td>
<td>+</td>
<td>ND</td>
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<td>Hydrolysis of:</td>
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<tr>
<td>Casein</td>
<td>−</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>Gelatin</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>+</td>
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</tbody>
</table>

maltose, sucrose, d-psicose, d-ribose, d-trehalose and glycerol were utilized, while salcin, lactose, l-arabinose, d-arabitol, d-tagatose, xylitol and d-xylene were not. Strain NAF001T, but not strain NAF002, utilized d-mannitol and d-mannose. Arbutin was utilized by strain NAF002. All biochemical tests for strains NAF001T (repeated) and NAF002 were carried out as described by Smibert & Krieg (1994). Briefly, catalase, the Voges–Proskauer test, nitrate reduction to nitrite and hydroylsis of starch, aesculin and urea were positive and oxidase, indole, hydrolsis 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 NAF001T 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 70 ml min⁻¹ (He gas) in EI positive mode. Retention times of each peak were analysed with a GC-MS [JMS-700 with Hewlett Packard 5890 gas chromatograph (column DB-1)].

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 NAF001T was menaquinone-7 (t1, 13±91 min, m/z 648), which indicates that strain NAF001T 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 (1971). 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/protease K mixture for strains NAF001T and NAF002. DNA–DNA homology was determined by fluorometric hybridization in microdilution wells (Ezaki et al., 1989) with biotinylated DNA of NAF001T. 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: NAF001T, 100%; NAF002, 92%; B. cereus JCM 2152T, 15%; B. cohnii NY-2000 (Yumoto et al., 2000), 10%; B. megaterium JCM 2506T, 12%; Bacillus mycoides NCIMB 13305T, 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 NAF001T. Germinated cells of strain NAF001T 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 NAF001T, 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...
Based on the results of this and previous studies, 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 NAF001T 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, oxidad 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, fructose, maltose, sucrose, d-psicose, d-ribose, d-trehalose and glycerol are utilized but t-arabinose, raffinose, lactose, salicin, d-tagatose, inositol, xylitol, d-xylene, citrate and t-malic acid are not. The major quinone is menaquinone-7. Iso-C15:0 (18 %) and anteiso-C15:0 (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 NAF001T (= JCM 11201T = CIP 107128T). Strain NAF002 (= JCM 11493) is a reference strain.

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