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Bacillus endophyticus sp. nov., isolated from the inner tissues of cotton plants (*Gossypium* sp.)

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Four strains of aerobic, endospore-forming bacteria were isolated from the inner tissues of healthy cotton plants (*Gossypium* sp., Dushanbe, Tajikistan). The organisms had identical randomly amplified polymorphic DNA patterns that distinguished them from other bacilli that are commonly isolated from plant tissues, e.g. *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus subtilis*. PCR amplification of 16S–23S rRNA spacer regions suggested that the four strains could be assigned to two highly related taxa, which correlated with differences in cell morphology. However, the cloned spacer region provided a simple and specific hybridization probe for all four strains. The virtually complete 16S rDNA sequences were prepared for representatives of the two groups (strains 2DT^T and 12DX) and differed by only two bases, thus supporting classification of the four strains in a single taxon at the species level. Phylogenetic analyses indicated that strain 2DT^T belonged to the genus *Bacillus* and was most closely related to *Bacillus sporothermodurans* DSM 10599^T with a sequence similarity of 94·8%. It is concluded that the four strains belong to a novel species of *Bacillus* for which the name *Bacillus endophyticus* sp. nov. is proposed. The type strain is 2DT^T (= UCM B-5715^T = CIP 106778^T).

Keywords: *Bacillus endophyticus*, ribotyping, RAPD, 16S rRNA, 16S–23S rRNA spacer region

INTRODUCTION

Bacillus species are common among the resident microflora of the inner tissues of various species of plants, including cotton (Misaghi & Donndelinger, 1990; McInroy & Kloepper, 1995), grape (Bell *et al*., 1995), peas (Huang *et al*., 1993), spruce (Hallaksela *et al*., 1991; Shishido *et al*., 1999) and sweet corn (McInroy & Kloepper, 1995), where these micro-organisms play an important role in plant protection and growth promotion. It is commonly thought that antagonistic activity is a major factor in their capacity to protect the plant from phytopathogenic fungi and bacteria (Emmert & Handelsman, 1999). Indeed, production of antibiotics and hydrolytic enzymes is a feature of many endophytic bacilli including *Bacillus cereus* (Pleban *et al*., 1997), *Bacillus pumilus* (Pleban & Sørensen, 1996) and *Bacillus subtilis* (Sharga & Lyon, 1998). However, it is becoming apparent that relationships between plants and their endophytic micro-flora are more complex and bacteria can be involved in the production of growth promotion factors and mediators that induce the plant's natural resistance (Strobel & Long, 1998; Wipat & Harwood, 1998).

All endophytic, aerobic, spore-forming bacteria described to date belong to species generally recognized as free-living soil organisms including *B. cereus* (Pleban *et al*., 1997), *Bacillus insolitus* (Bell *et al*., 1995; Sturz *et al*., 1997), *Bacillus megaterium* (McInroy & Kloepper, 1995), *B. pumilus* (McInroy & Kloepper, 1995; Benhamou *et al*., 1998), *B. subtilis* (Misaghi &

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank accession number for the 16S rRNA gene sequence of strain 2DT^T is AF295302.

Donndelinger, 1990) and *Paenibacillus polymyxa* (Shishido *et al*., 1999), although in many instances the bacteria have not been identified beyond genus level (Sturz, 1995; Hallmann *et al*., 1997). To gain more insight into the diversity of endophytic, aerobic, endospore-forming bacteria, a collection of 76 strains isolated from the inner tissues of healthy cotton plants (*Gossypium* sp., Dushanbe, Tajikistan) was characterized phenotypically. The majority were identified as *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *B. megaterium*, *B. pumilus* and *B. subtilis* but four strains could not be assigned to any known species of *Bacillus* or related genera of the aerobic, endospore-forming bacteria. Here, it is shown that these strains are closely related and represent a novel species of the genus *Bacillus*, for which the name *Bacillus endophyticus* sp. nov. is proposed.

METHODS

Isolation of strains and growth conditions. Bacteria were isolated in 1989–1990 from the inner tissues of healthy cotton plants (*Gossypium* sp., Dushanbe, Tajikistan). Parts of stems about 1 cm in diameter were flamed with ethanol to remove surface organisms and the outer layers were excised with a sterile scalpel blade. Slices of the inner stem were placed on nutrient agar plates and incubated at 30 °C for 48 h. Bacterial growth associated with the stem sections was purified by repeated plating on nutrient agar and cultures were maintained as spore suspensions by freezing in 20 % (v/v) glycerol. From this, four unusual strains were isolated: 2DT^T, 10DX, 12DX and 9DT. *B. megaterium* UCM B-5714 and 7DX, *B. pumilus* 7-91D, *B. subtilis* UCM B-5017 and UCM B-5008 (all from the Ukrainian Collection of Microorganisms) and *B. amyloliquefaciens* H, *Bacillus firmus* 3391, *B. licheniformis* DSM 13^T, *B. pumilus* 1005 and *B. subtilis* DSM 10^T (Priest *et al*., 1988) were used as reference strains.

Phenotypic characterization. Cells were examined for morphology using Gram-stained smears and phase-contrast microscopy. A set of 36 physiological characteristics, including acid production from sugars, sodium citrate utilization, urease production, starch and gelatin hydrolysis, arginine dihydrolase, nitrate reduction and the Voges–Proskauer reaction, was carried out using a prototype MICROBACT 36B Bacillus Identification System (Medvet Science) which emulates the standard tests for *Bacillus* described by Gordon *et al*. (1973). Utilization of organic acids was determined using the basal citrate utilization medium of Gordon *et al*. (1973) supplemented with the various carbon sources at 0·2 % of the sodium salt. Tubes were incubated at 28 °C for 3 d and a positive reaction was indicated by development of a red (alkaline) indicator colour. Anaerobic growth was determined in tubes of oxidative and fermentative test medium (Difco) under mineral oil. Casein hydrolysis was detected after incubation of strains for 3 d on nutrient agar supplemented with 2 % skimmed milk. Determination of catalase and oxidase activities, gas production from glucose, phenylalanine deamination and tyrosine degradation were carried out as described by Gordon *et al*. (1973). Growth in the presence of ampicillin (100 μg ml^{-1}), lysozyme (1 mg ml^{-1}) and NaCl (5 and 10 %) was determined in nutrient broth as the basal medium during incubation at 28 °C for 3 d. Growth at different temperatures was determined in tubes of nutrient broth incubated for 3 d in water baths set at 25, 30, 40 and 50 °C.

Molecular methods. Chromosomal DNA was isolated from late exponential phase nutrient broth cultures by phenol extraction as described previously (de Silva *et al*., 1998). Randomly amplified polymorphic DNA (RAPD) analysis was done by PCR amplification using a Perkin-Elmer thermal cycler and primer 0955-03 (5′-CCGGCGGCG-3′) as described by Brousseau *et al*. (1993). PCRs were performed in 100 μl volumes containing 0·5 μg template DNA and 40 pmol primer using Bioline *Taq* DNA polymerase. PCR amplification of *rrn* spacer DNA was performed as described by de Silva *et al*. (1998) in a final volume of 50 μl using 25 ng purified DNA as template. The reaction mixture contained 50 pmol primers 2F (5′-GAACTGGCAGTTG-TACACACCGCCCGTC-3′) and 10R (5′-ACGATGGTA-CCTTTCCCTCACGGTACTG-3′), corresponding to conserved regions of the distal portion of the 16S and proximal region of the 23S rRNA genes, respectively. The reaction comprised an initial denaturation at 95 °C, addition of *Taq* DNA polymerase and 35 cycles of denaturation at 95 °C for 1 min, primer annealing at 42 °C for 1 min and extension at 72 °C for 3 min, with a final extension step at 72 °C for 10 min. Total PCR products were analysed by agarose gel electrophoresis and used for the preparation of a hybridization probe. For the latter, the products from strain 2DT^T were digested with *Sau*3A and ligated into pUC19 that had been digested with *Bam*HI and dephosphorylated with shrimp alkaline phosphatase (Roche). Ligated products were transformed by heat shock into calcium-chloride-treated competent cells of *Escherichia coli* DH5α and white colonies were picked from LB agar plates containing ampicillin (100 μg ml^{-1}), IPTG (0·5 mM) and X-Gal (25 μg ml^{-1}). Plasmid from the white colonies was checked for inserts by agarose gel electrophoresis of minipreps and a recombinant plasmid with an insert of about 750 bp was selected for use as a specific hybridization probe. Labelled probe was prepared by PCR amplification of the insert using primers 1Ins (5′-TGCCTGCAGGTCGACTCTAGAGGATC-3′) and 2Ins (5′-GAATTCGAGCTCGGTACCCGGGGATC-3′), targeted to the flanking regions of the insertion site in pUC19, and incorporating digoxigenin-11-dUTP (Roche) in the reaction mix. PCR was the same as for amplification of the *rrn* spacer DNA except that the annealing temperature was 55 °C. Slot-blot hybridizations were prepared as described previously (de Silva *et al*., 1988).

Sequence and analysis of 16S rRNA genes. Small-subunit rDNA was amplified from genomic DNA (1 ng) from strains 2DT^T and 12DX by PCR. The amplification was performed in a semi-nested fashion using primers and conditions as described recently (de Silva *et al*., 1998). Both strands were sequenced by direct solid-phase 16S rDNA sequencing using the ALF*express* system (Amersham Pharmacia Biotech). Almost complete 16S rDNA sequences were obtained from the sequencing procedure. The gapped BLAST (Altschul *et al*., 1997) option at the home page of National Center for Biotechnology Information was used to screen GenBank for deposited 16S rRNA gene sequences. Sequences obtained from GenBank were combined with aligned data, which were retrieved in aligned format from the Ribosomal Database Project (Maidak *et al*., 1999; Ribosomal Database Project RDP-II, release 7.0, 1999, Michigan State University, East Lansing, MI, USA) using the Genetic Data Environment software alignment tool (Smith, 1992). The total alignment served as a basis for the extraction and analysis of subalignments. The neighbour-joining program

NEIGHBOR (Saitou & Nei, 1987) contained in the phylogenetic program package PHYLIP version 3.573 (Felsenstein, 1993) was used to infer phylogenetic relationships. The tree was calculated from a distance matrix corrected for multiple substitutions at single locations by the method of Jukes & Cantor (1969). The program SEQBOOT was used for statistical testing of the trees by resampling the dataset 500 times.

RESULTS AND DISCUSSION

Morphological and physiological characteristics

During a study of 76 endospore-forming bacteria isolated from the inner tissues of cotton plants, most strains were identified by traditional means as *B. licheniformis* (15 strains), *B. megaterium* (8 strains), *B. pumilus* (6 strains) and *B. subtilis* (43 strains). The recovery of these species is typical of other studies of bacilli from the rhizosphere and plant tissues (Misaghi & Donndelinger, 1990; McInroy & Kloepper, 1995; Benhamou *et al*., 1998). The remaining four strains phenotypically resembled *B. insolitus*, another well-known colonizer of plants (Bell *et al*., 1995; Sturz *et al*., 1997), but differed from this psychrotolerant species in several respects, including spore shape and some physiological features (Table 1). This led us to examine them more fully.

The four strains were Gram-positive rods, 2·5–3·5 × 0·5–1·5 μm and occurred singly and in short chains [strain 12DX (Fig. 1a) and strain 9DT] or in long filamentous chains [strain 2DT^T (Fig. 1b) and strain 10DX]. Spores were ellipsoidal with a terminal/subterminal position in a non-distended sporangium (Fig. 1a). Growth on nutrient agar with 2% (w/v) glucose resulted in vacuole formation in the cytoplasm. On nutrient agar, colonies were slimy or slight fibrous, about 1–3 mm in diameter. Occasionally, all strains produced an intense pink-red pigment that did not diffuse into liquid or the solid medium. Both pigmented and white colonies could sometimes be observed on the same plate, but lines with constant pigmentation could not be isolated and the culture conditions necessary to consistently induce pigmentation could not be defined. However, growth on media with ampicillin and lysozyme usually resulted in red pigmentation, perhaps due to stress induction.

The distinguishing phenotypic characters for these bacteria in the context of other plant-associated bacilli are shown in Table 1. The strains were obligate aerobes that lacked nitrate reductase but could grow weakly under microaerophilic conditions producing acid from a range of sugars. Optimum growth occurred at about 28 °C with a maximum growth temperature between 40 and 50 °C. All strains were resistant to NaCl, even at 10% (w/v), and to ampicillin (100 μg ml^{-1}). They did not hydrolyse casein or starch.

Molecular typing

Since the four unusual strains appeared to be phenotypically similar, they were examined by RAPD analysis to determine their relationships at the genomic level. The strains had similar RAPD patterns that were different from those of reference strains of common plant-associated species such as *B. licheniformis*, *B. megaterium*, *B. pumilus* and *B. subtilis* (Fig. 2). RAPD analyses have similarly proved valuable for defining strains within new species, such as *Bacillus siralis* (Pettersson *et al*., 2000) and *Bacillus weihenstephanensis* (Lechner *et al*., 1998). The levels of similarity revealed here are consistent with the allocation of the four strains to a single distinct taxon and there was no indication of genomic differences correlating to the differences in cellular morphology noted in Fig. 1.

The hypervariable spacer regions between the 16S and 23S rRNA genes have been popular targets for strain typing (Jensen *et al*., 1993; Saruta *et al*., 1997) and strain identification (Barry *et al*., 1991; de Silva *et al*., 1998). Using this approach, two different amplification product profiles were obtained from the four strains (Fig. 3) that were consistent with the phenotypic division and suggested that these four bacteria encompass two lineages within the same taxon. However, a cloned fragment of spacer region DNA of around 750 bp hybridized with DNA from all four strains, supporting their allocation to a single species (Fig. 4). This probe failed to hybridize with DNA from other common, plant-associated bacilli indicating its value for identification of these strains when studying this environment.

Phylogenetic analysis

Sequences of the virtually complete 16S rRNA genes of representative strains 2DT^T and 12DX were determined. The sequences were almost identical, differing at only two sites. This supported assignment of the four strains to a single taxon since strains with such similar rRNA sequences have been classified in different *Bacillus* species by DNA hybridization on only two occasions; *Bacillus globisporus* and *Sporosarcina psychrophila* (Palys *et al*., 2000) and *Bacillus atrophaeus* and *Bacillus mojavensis* (Goto *et al*., 2000). Indeed, strains that share more than 97% rDNA sequence homology generally show >70% DNA reassociation and are classified in the same species (Stackebrandt & Goebel, 1994; Nielsen *et al*., 1995).

The sequence for strain 2DT^T was used to establish the generic position of the four strains. The sequence was inserted into an alignment comprising about 400 published and unpublished *Bacillus*-related 16S rRNA gene sequences. Reference strains and the closest phylogenetic relatives were selected from this dataset for computation of evolutionary relationships. A neighbour-joining tree (Fig. 5) was inferred from an alignment which was corrected for hypervariable sites by applying a 50% nucleotide consistency filter, i.e. only the aligned positions where each position had a nucleotide present in more than 50% of the sequences were used for phylogenetic calculations. *Alicyclobacillus acidocaldarius* DSM 446^T served as outgroup

Table 1. Distinguishing phenotypic characteristics of the cotton plant isolates compared with known plant-associated species

+, >89 % strains positive; D, 11–89 % strains positive; −, <11 % strains positive; numeric values indicate the proportion of the four strains that were positive (strain 12DX was negative in all cases of variable reactions); ND, no data.

Character	*B. insolitus**	*B. licheniformis*†	*B. megaterium*†	*B. pumilus*†	*B. subtilis*†‡	Endophytic isolates
Spore position/ Spore shape	Terminal Round	Central Oval	Central Oval	Central Oval	Central Oval	Terminal Ellipsoidal
Vacuoles in cytoplasm	+	−	+	−	−	+
Cell width >1·0 μm	+	−	+	−	−	+
Motility	D	+	+	+	+	−
Anaerobic growth	−	+	−	−	−	−
Voges–Proskauer	−	+	−	+	+	−
Oxidase	+	+	−	+	+	+
Nitrate reduction	−	+	−	−	+	−
Hydrolysis of:						
Casein	−	+	+	+	+	−
Gelatin	−	+	+	+	+	−
Starch	−	+	+	−	+	−
Urea	−	−	+	−	−	−
Acid from:						
Arabinose	−	+	+	+	−	+
Cellobiose	+	+	+	+	+	−
Galactose	ND	+	+	+	D	−
Glucose	−	+	+	+	+	+
Inositol	ND	+	+	−	+	+
Lactose	−	D	D	D	−	−
Mannitol	−	+	+	−	+	+
Maltose	−	+	+	D	+	+
Mannose	−	+	D	+	+	+
Raffinose	+	+	−	D	+	0·75
Rhamnose	ND	−	−	−	−	+
Sorbitol	ND	−	D	−	+	−
Trehalose	−	+	+	+	+	−
Xylose	−	+	+	+	D	0·75
Use of:						
Acetate	ND	+	+	−	D	−
Citrate	−	+	+	+	+	+
Gluconate	ND	+	+	−	−	+
Propionate	ND	+	−	−	−	−
Arginine dihydrolase	ND	+	−	−	−	−
Phenylalanine deaminase	−	−	+	−	−	−
Growth in:						
5 % NaCl	−	+	+	+	+	+
10 % NaCl	−	−	−	+	D	+
Growth at 50 °C	−	+	−	−	−	−

* Data from Larkin & Stokes (1967) and Rüger *et al*. (2000).

† Data from Priest *et al*. (1988).

‡ Data from Nakamura *et al*. (1999).

(not shown in Fig. 5) and bootstrap percentage values are given only for major clades that showed a stability of at least 80 %. The tree shows a high overall stability and confirms that strain 2DT^T belongs to the genus *Bacillus*. Many nodes in the *Bacillus* clade were only weakly supported despite using differently corrected datasets such as only removing gaps and/or ambiguous positions and/or highly variable regions, etc. Moreover, addition or removal of taxa had very little effect on the node stability (not shown). A deeper analysis of the evolutionary branching pattern within this area of the genus was not considered necessary to establish the generic allocation of this bacterium within the genus *Bacillus*.

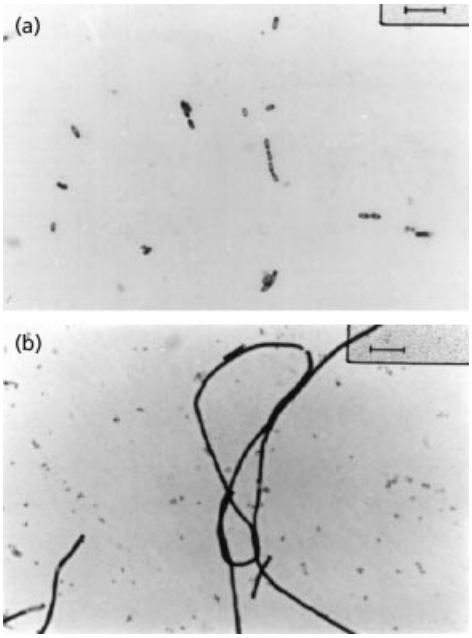

Fig. 1. Gram-stained cells of *Bacillus endophyticus* strain 12DX (a) and strain 2DT^T (b) both grown on nutrient agar for 48 h at 28 °C. Bars, 10 μm.

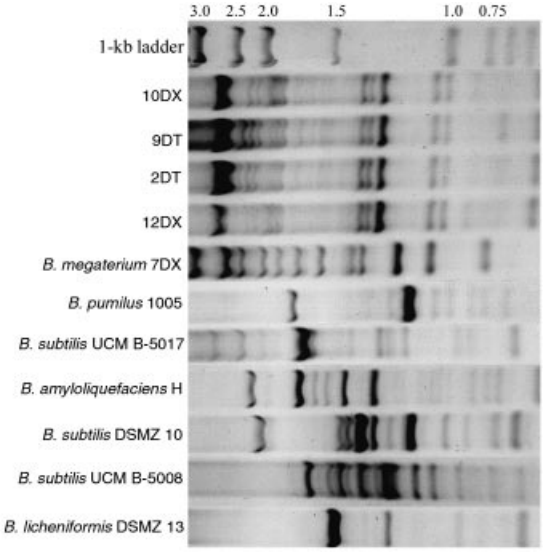

Fig. 2. RAPD patterns of the four isolates from cotton plants and representative strains of other species commonly associated with plants.

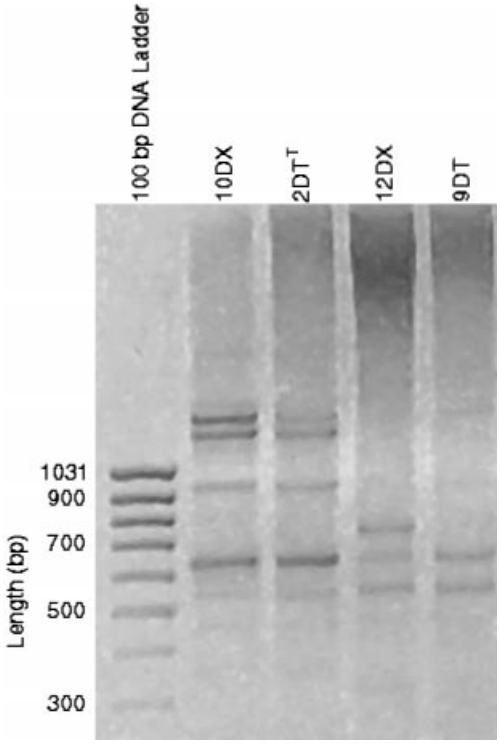

Fig. 3. PCR products from 16S–23S rRNA spacer regions from the four isolates from cotton plants.

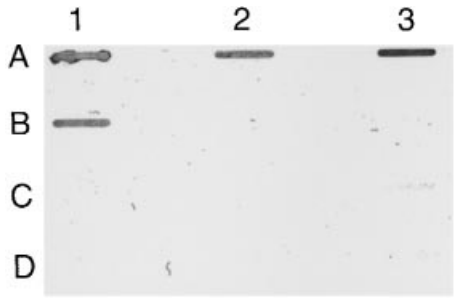

Fig. 4. Slot-blot hybridization between a labelled 16S–23S rDNA fragment from strain 2DT^T and chromosomal DNA from the four isolates from cotton plants and representative strains of other species commonly associated with plants. Slots: A1, strain 10DX; A2, strain 2DT^T; A3, strain 12DX; B1, strain 9DT; B2, *B. megaterium* 7DX; B3, *B. megaterium* UCM B-5714; C1, *B. pumilus* 1005; C2, *B. pumilus* 7-91D, C3, *B. subtilis* UCM B-5008; D1, *B. amyloliquefaciens* H; D2, *B. licheniformis* DSM 13^T; D3, *B. firmus* 3391.

Strain 2DT^T was most closely related to *Bacillus sporothermodurans* DSM 10599^T, *Bacillus circulans* ATCC 4513^T and *Bacillus smithii* DSM 4216^T, with 16S rRNA gene sequence similarity values of 94·8, 94·7 and 94·6 %, respectively. Despite low statistical support, strain 2DT^T grouped repeatedly with *B. smithii* DSM 4216^T in all computed trees and the tree presented in Fig. 5 is thus believed to be representative. Similarity values indicate that strain 2DT^T can be judged to be sufficiently separated from any known species within the genus *Bacillus* to justify its classification into a new species.

To date, strains similar to strain 2DT^T have only been isolated from the inner tissues of cotton plants, although it is likely that they exist outside this habitat.

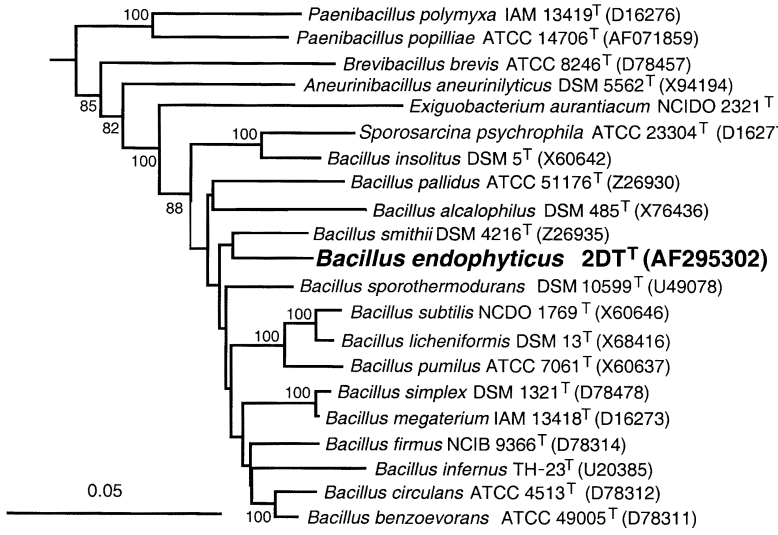

Fig. 5. Phylogenetic tree showing the evolutionary position of strain 2DT^T in the family *Bacillaceae* with special emphasis on the genus *Bacillus*. The tree was constructed with the neighbour-joining algorithm (Saitou & Nei, 1987) from a distance matrix corrected for multiple substitutions by the method of Jukes & Cantor (1969) and *Alicyclobacillus acidocaldarius* DSM 446^T served as outgroup (not shown in the tree). Bar, the number of substitutions at certain nucleotide sites. Bootstrap percentage values from 500 resamplings of the dataset have been included at nodes, but only for those which were statistically supported to 80 % or more.

The microaerophilic metabolism, lack of extracellular protease and amylase, and optimal growth at around 28 °C are consistent with a preferred niche within plants. The name *Bacillus endophyticus* is therefore proposed for this bacterium.

Description of *Bacillus endophyticus* sp. nov.

Bacillus endophyticus (en.do.phy′ti.cus. *endophyticus* adj. derived from Gr. *endo* within and *phyt-* relating to plants; N.L. adj. *endophyticus* within plant, pertaining to the original isolation from plant tissues).

Gram-positive rods, 0·5–1·5 × 2·5–3·5 μm occurring singly and as short or long chains which appear filamentous. Non-motile. Ellipsoidal endospores are located subterminally or terminally in a non-swollen sporangium. Cells grown on media containing 2 % glucose have vacuoles in the cytoplasm. Circular colonies may be red, pink or white, slimy or rough with entire or slightly indented edges, about 1–3 mm diameter. Strictly aerobic. Catalase- and oxidase-positive, urease-negative. Does not produce indole from tryptophan or gas from glucose. Egg-yolk reaction is negative. Voges–Proskauer test and nitrate reduction are negative, ONPG-positive. Does not hydrolyse starch, gelatin or casein. Produces acid from L-arabinose, D-glucose, *meso*-inositol, D-mannitol, D-mannose, melibiose, D-rhamnose, ribose and sucrose, but not from adonitol, galactose, glycerol, glycogen, melezitose, methyl D-glucoside, methyl D-mannoside, lactose or trehalose. Acid production from D-raffinose and D-xylose is variable. Utilizes sodium citrate and gluconate, but not acetate, propionate or tartrate. Arginine dihydrolase and phenylalanine deaminase reactions are negative. Growth occurs at 10–45 °C. Optimal growth temperature is about 28 °C. Growth occurs in the presence of 10 % NaCl, 100 μg ampicillin ml^{-1} and 1 mg lysozyme ml^{-1}. Habitat: associated with plants and isolated from inner tissues of plants. Type strain is 2DT^T, which has been deposited with the Ukrainian Collection of Microorganisms and the Collection de l'Institut Pasteur as UCM B-5715^T and CIP 106778^T, respectively.

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