***Bacillus siralis* sp. nov., a novel species from silage with a higher order structural attribute in the 16S rRNA genes**

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A novel bacterial strain (171544^T) was recently isolated from silage and was classified in the genus *Bacillus* by 16S rDNA sequence analysis. Additional silage samples have been investigated in the present study and four organisms resembling strain 171544^T were isolated. Phenotypic and genotypic characterization of these bacteria showed that they constitute a new species of the genus *Bacillus*. This taxon was positioned in the family *Bacillaceae* on the basis of evolutionary distance trees using 16S rDNA sequences. *Bacillus circulans*, *Bacillus firmus* and *Bacillus benzoovorans* were the most closely related species with 16S rDNA similarities of 97.2, 96.3 and 95.9%, respectively. All five silage isolates shared a higher order structural feature in the 3' region of the 16S rRNA gene comprising an extension to helix 49 of 24 bp and highly similar random amplified polymorphic DNA patterns that distinguished them from the type strains of *B. circulans* and *B. firmus*. Moreover, they possessed a unique pattern of phenotypic features including subterminally or terminally located endospores which distinctly swelled the sporangium, strictly aerobic metabolism but with the ability to utilize nitrate as a terminal electron acceptor under anaerobic conditions, and hydrolysis of casein but not starch. The name *Bacillus siralis* is therefore proposed for this new taxon. The type strain of *B. siralis* is strain 171544^T (= NCIMB 13601^T = CIP 106295^T).

Keywords: *Bacillus siralis*, taxonomy, phylogeny, 16S rRNA, rRNA extension

INTRODUCTION

Previous studies on bacterial biodiversity in grassland soils using molecular biology techniques have revealed that most of the 16S rDNA sequences obtained originated from bacterial taxa that had either not been cultured *in vitro* or had not been characterized at the 16S rDNA level (Borneman *et al.*, 1996; Felske *et al.*, 1998). In these studies, many of the clones were phylogenetically related to members of the genus *Bacillus*. Recently, several novel *Bacillus* strains have been isolated from grass silage which appear to be phylogenetically similar to the grassland soil clones (De Silva *et al.*, 1998). Sequence analysis of the 16S rRNA genes of some of these organisms revealed that

several possible new species of the genus *Bacillus* were present in the silage (De Silva *et al.*, 1998).

One of the silage isolates (strain 171544^T) showed an unusually long extension to the 16S rRNA gene located in the penultimate loop of the 16S rRNA molecule. This type of macro-heterogeneity has been rarely reported in Gram-positive bacteria, for example in *Clostridium paradoxum* (Rainey *et al.*, 1996) and *Desulfotomaculum australicum* (Patel *et al.*, 1992). However, insertions in both the 16S and 23S rRNA genes have been noted in several Gram-negative bacteria including *Campylobacter helveticus* (Linton *et al.*, 1994a), *Campylobacter sputorum* (Van Camp *et al.*, 1993), *Helicobacter canis* (Linton *et al.*, 1994b), *Leptospira* (Ralph & McClelland, 1994), salmonellae (Burgin *et al.*, 1990; Mattatall & Sanderson, 1998) and *Yersinia enterocolitica* (Skurnik & Toivanen, 1991).

In this study, four more isolates resembling strain

Abbreviation: RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA sequence of *Bacillus siralis* strain 171544^T is AF071856.

171544^T have been isolated from other silage samples and subjected to biochemical, morphological and genotypic characterizations. All five strains were genotypically and phenotypically homogeneous, shared the same unique element in the 16S rRNA genes and were distinct from all other species of the genus *Bacillus*.

METHODS

Strains and culture conditions. *Bacillus* strain 171544^T, an isolate from silage, has been described previously (De Silva *et al.*, 1998). Similar strains were isolated from freeze-dried silage samples (kindly provided by Fritz Lembke, TetraPak GmbH, Stuttgart, Germany). Lyophilized silage (1 g) was suspended in 1 ml sterile water, heated at 100 °C for 90 min and diluted to 10⁻¹ and 10⁻² in sterile water. Aliquots (50 µl) were spread on NYSM agar (100 ml nutrient agar containing 0.5 g yeast extract l⁻¹ and 1 ml of a solution containing 5 × 10⁻⁵ M MnCl₂, 7 × 10⁻⁴ M CaCl₂, 1 × 10⁻³ M MgCl₂) and brain heart infusion (BHI; Oxoid) agar plates and incubated for 24–48 h at 37 °C. Colonies from both media resembling *Bacillus* strain 171544^T were purified on NYSM agar and examined microscopically after Gram-staining. Isolates appearing as slender rods with a subterminal, oval endospore in a swollen sporangium were retained. Strains on NYSM agar were stored at room temperature and subcultured every 2 weeks. Stock cultures were kept at -70 °C in BHI broth containing 20% glycerol (v/v) and in freeze-dried form at room temperature. Reference strains from our culture collection included: *Bacillus circulans* DSM 11^T, *Bacillus firmus* DSM 12^T, *Bacillus pumilus* 5HR (isolated

from silage), *Bacillus sporothermodurans* M215^T and *Bacillus subtilis* DSM 10^T.

Morphological and physiological characterization. Cellular morphology was determined from Gram-stained cultures grown for 24 h on NYSM agar at 37 °C. Spore morphology was determined after a further 24-h incubation using phase-contrast microscopy. The physiological tests shown in Table 1 were conducted according to recognized methods (Gordon *et al.*, 1973), with the exceptions that aesculin and gelatin hydrolysis were detected according to Priest *et al.* (1988) and a prototype identification kit for *Bacillus* species (Microbact 36B; Medvet Science) was used for the detection of arginine dihydrolase and acid production from carbohydrates. The latter is based on indicator (bromocresol purple) change due to acid production from sugar fermentation during a 3-d incubation period (similar to the standard methods for *Bacillus*; Gordon *et al.*, 1973). The correlation of this method with acid production in ammonium salt sugar medium (Gordon *et al.*, 1973) has been evaluated and confirmed by examination of reference strains in the Microbact 36B system (data not shown). Strains were examined for resistance to antibiotics using impregnated discs (Mast Groups) placed on NYSM agar plates seeded with a light lawn of bacteria and incubated at 37 °C for 24 h. A distinct inhibition zone indicated susceptibility to the antibiotic.

Molecular methods. Chromosomal DNA was purified as described previously (Aquino de Muro *et al.*, 1992). PCR reactions were typically performed in a final volume of 100 µl using 80 pmol of each primer, 50 ng of template and 1 mM of each dNTP. *Taq* DNA polymerase (Bioline) was added after the initial denaturation at 95 °C for 10 min.

Table 1. Distinctive phenotypic properties of *B. soralis* and phylogenetically related species

+, > 90% strains positive; -, < 10% strains positive; v, 11–89% strains positive; NT, not tested. Data taken from the following references: *B. benzoevorans*, Pichinoty *et al.* (1984); *B. circulans* and *B. firmus*, Claus & Berkeley (1986). Data for 10% NaCl and growth at 50 °C from Priest *et al.* (1988), data for arginine dihydrolase, this study.

Characteristic	<i>B. benzoevorans</i>	<i>B. circulans</i>	<i>B. firmus</i>	<i>B. soralis</i>
Cell shape	Filamentous	Rods	Rods	Rods
Spore shape	Oval	Oval	Oval	Oval
Swollen sporangia	—	+	—	+
Anaerobic growth	—	+	—	—
Nitrate reduction	+	v	+	+
Arginine dihydrolase	NT	—	—	v
Acid from:				
Arabinose	—	+	—	—
Glucose	—	+	+	—
Mannitol	—	+	+	—
Xylose	—	+	—	—
Acetylmethylcarbinol production	—	—	—	—
Hydrolysis of:				
Aesculin	NT	+	—	+
Casein	NT	—	+	+
Gelatin	—	+	+	+
Starch	—	+	+	—
Use of citrate	v	—	—	—
Growth in:				
5% NaCl	—	+	+	+
10% NaCl	—	—	+	—
Growth at 50 °C	—	—	—	+

Reactions comprised 25 cycles of 94 °C for 1 min, annealing at the relevant temperature for 1.5 min and extension at 72 °C for 1.5 min, with a final extension at 72 °C for 5 min before the reduction of the reaction temperature to 4 °C. Products were electrophoresed in 2% agarose gels at 60 V for 2 h using Tris/acetate buffer. Primers (MWG Biotech) were prepared to amplify from the 5'-end of the 16S rRNA gene (8F; 5'-AGA GTT TGA TCM TGG CTC AG-3') into the insertion sequence in helix 49 (1452R; 5'-GTG GTT TCA CTG AAT TTC CGA C-3') with an annealing temperature of 58 °C and to amplify the internal section of helix 49 [primers 1372F (5'-CAC ACC GCC CGT CAC ACC-3') and 1504R (5'-GAC TTC ACC CCA ATC ATC-3')] with an annealing temperature of 55 °C. Randomly amplified polymorphic DNA (RAPD) patterns were prepared using the primer 5'-CCG AGT CCA-3' and 50 ng chromosomal DNA as template. Denaturation at 95 °C for 10 min was followed by 30 cycles of 95 °C for 45 s, 30 °C for 10 s ramped at 1 °C per 2 s to 72 °C for 2 min and a final hold at 72 °C for 7 min. Products were analysed in 1.5% agarose gels using Tris/acetate buffer.

Phylogenetic analysis. The virtually complete sequence of the 16S rRNA genes of strain 171544^T was determined by a solid-phase DNA sequencing method as described previously (De Silva *et al.*, 1998). The sequences were aligned manually by using the Genetic Data Environment software (GDE; Smith, 1992). The alignment comprised previously described 16S rRNA gene sequences from *Bacillus* species retrieved from GenBank invoking the gapped-BLAST option (Altschul *et al.*, 1997) at the NCBI home page and those available in an alignment format from RDP, the Ribosomal Database Project (Maidak *et al.*, 1999; RDP-II, 1999). Phylogenetic calculations were performed by using algorithms implemented in the phylogenetic program package PHYLIP version 3.573 (Felsenstein, 1993). The tree was derived by using the neighbour-joining program (Saitou & Nei, 1987) from a distance matrix corrected for multiple substitutions at single locations by the one-parameter model of Jukes & Cantor (1969).

Nucleotide accession numbers. The GenBank accession numbers for the 16S rRNA sequences of the reference strains used for phylogenetic calculations are given in Fig. 2.

RESULTS

Isolation of strains similar to *Bacillus* 171544

Strain 171544^T was originally isolated after prolonged heat treatment of silage samples (De Silva *et al.*, 1998). The same enrichment procedure was used here (90 min at 100 °C) and colonies of aerobic, endospore-forming bacteria were selected after incubation on BHI and NYSM agars for 2 d. Four isolates with cellular and colonial morphology similar to that of strain 171544^T were purified on NYSM agar, retained and labelled 1HR, 2HR, 3HR and 4HR. These bacteria were obtained from two separate silage samples. Presumptive identification as members of the same taxon as strain 171544^T was obtained by PCR amplification using a universal forward primer, which recognized the 5'-end of the 16S rRNA gene and a reverse primer targeted to a significantly protruding structural feature in the 16S rRNA gene. This insertion is positioned near the 3'-end of the 16S rRNA gene of strain 171544^T (De Silva *et al.*, 1998). An amplification product of

Fig. 1. Agarose gel electrophoresis of PCR products from 16S rRNA genes of strain 171544^T and related bacteria using (a) a forward primer to the proximal end of the gene and a reverse primer in the extension to helix 49 and (b) forward and reverse primers flanking the extension to helix 49 (see text for details). (a) Lanes: 1, 1 kbp ladder; 2, *B. pumilus* 5HR; 3, strain 171544^T; 4, 1HR; 5, 2HR; 6, 3HR; 7, 4HR. (b) Lanes: 1, 100-bp ladder; 2, strain 171544^T; 3, 1HR; 4, 2HR; 5, *B. pumilus* 5HR; 6, *B. subtilis* DSM 10^T; 7, *B. sporothermodurans* M215^T.

about 1.5 kb was produced when DNAs from strains 171544^T, 1HR, 2HR, 3HR and 4HR were used as template but not with DNA from typical species of *Bacillus* including *B. pumilus* 5HR (Fig. 1a), *B. sporothermodurans* M215^T or *B. subtilis* DSM 10^T (data not shown). This was strong evidence for the presence of the insertion in the four new silage isolates. Similarly, the insertion could be recognized using primers that flanked this protrusion, thus resulting in amplification products from strain 171544^T and similar isolates being larger (about 150 bp) than those from other *Bacillus* species (about 100 bp; Fig. 1b). Subsequent sequencing of these products confirmed that insertions in strains 1HR, 2HR, 3HR and 4HR were identical to that found in strain 171544^T.

Molecular phylogeny

The virtually complete 16S rDNA sequence of strain 171544^T was compared with sequences of related organisms. Phylogenetic trees were constructed using different data sets as obtained from a starting alignment comprising about 400 published and unpublished *Bacillus*-related 16S rRNA gene sequences. Several subsets were selected for phylogenetic calculations and each was compensated by removing gaps, ambiguously aligned positions and positions for which a certain nucleotide composition could not be observed in more than half of the sequences. Despite slight shifts in branching order, strain 171544^T consistently clustered

Fig. 2. An evolutionary distance tree showing the phylogenetic placement of *Bacillus siralis* strain 171544^T and its relatives among the members of the genus *Bacillus* and closely grouping 16S rDNA clone sequences as obtained from soil and aquatic niches. The tree was constructed from a distance matrix corrected by the method of Jukes & Cantor (1969) by using the neighbour-joining program of Saitou & Nei (1987). The scale bar denotes the number of substitutions per nucleotide position. *Brevibacillus brevis* ATCC 8246^T and *Brevibacillus thermoruber* DSM 7064^T served as outgroups. Accession numbers are given in parentheses.

with *Bacillus benzoovorans*, *B. circulans* and *B. firmus* in all trees, confirming the relatedness of these taxa. A representative phylogenetic tree inferred by the neighbour-joining method (Saitou & Nei, 1987) from a distance matrix corrected for multiple nucleotide substitutions at single locations by the one-parameter model of Jukes & Cantor (1969) is shown in Fig. 2. Bootstrap analysis indicated that the region of the tree containing *B. firmus* and relatives has weak statistical support for the different branching orders and bootstrap values were, therefore, not included at the individual nodes of the dendrogram.

From 16S rRNA gene sequence similarities, strain 171544^T was most closely related to *B. circulans* (similarity value of 97.2%) and *B. firmus* (96.3%) and less so to the filamentous bacterium *B. benzoovorans* (95.9%). Besides the clustering to known *Bacillus* species, strain 171544^T showed relatively close relationships to a set of 16S rDNA clones enriched from grassland soil (Felske *et al.*, 1998), in particular, soil clones DA036 and DA066, and the sediment clone BPC094 showed 96.1, 95.5 and 95.7% similarity to

strain 171544^T, respectively. Because the closest phylogenetic relatives of strain 171544^T lay on the borderline of 97% rRNA similarity, which is generally recognized as the upper limit for separate speciation, the silage isolates were compared with the type strains of *B. circulans* and *B. firmus* by RAPD analysis (Fig. 3). This revealed that the silage isolates had almost identical chromosomal structures that were distinct from those of *B. circulans* and *B. firmus*. In summary, strain 171544^T-like isolates constitute a separate taxon within *Bacillus*, however the precise phylogenetic position of these bacteria could not be solved with certainty.

Helix 49, an idiosyncrasy for the 171544^T-like strains

Previously, it has been reported that strain 171544^T has an unusually long insertion sequence in the penultimate stem-loop structure of the 16S rRNA gene (De Silva *et al.*, 1998) and, in this study, strains 1HR, 2HR, 3HR and 4HR were found to have identical structures. These extensions are positioned in helix 49 using the nomenclature of Van de Peer *et al.* (1996) from positions 1449 to 1454 according to

Fig. 3. RAPD patterns generated from chromosomal DNA of the silage isolates and reference strains of *B. circulans* and *B. firmus*. Lanes: 1, 1-kb DNA ladder; 2, strain 171544^T; 3, 1HR; 4, 2HR; 5, 3HR; 6, 4HR; 7, *B. circulans* DSM 11^T; 8, *B. firmus* DSM 12^T; 9, 1-kb ladder.

Fig. 4. Sequence of the 49-nt insertion in helix 49 of the 16S rRNA genes of *Bacillus siralis* strain 171544^T and relatives showing a most likely base-pairing structure. The dashed line denotes the origin of the extension as compared to *E. coli* and most other prokaryotes. The actual nucleotide positions according to *E. coli* are 1449 and 1454.

Escherichia coli numbering (Brosius *et al.*, 1978). The total length of the extension was found to be 49 nt and a plausible secondary structure, deciphered by applying comparative analysis, is shown in Fig. 4. In this model, all but one of the inserted bases, a purine forming a unilaterally bulged residue, can be defined as constituents of a stem structure terminated by a tetraloop. Almost all of the base pairs (83%) are of the canonical Watson–Crick type. Searching GenBank using this insertion sequence resulted in no significant

Fig. 5. Phase-contrast photomicrograph of *Bacillus siralis* strain 171544^T after growth on NYSM agar for 36 h. Bar, 10 μm.

similarity to any deposited sequence from the *Bacteria*, *Archaea* or *Eucarya*. Only 13 small subunit rRNA sequences showed this type of attribute, and the insertion present in the phylogenetically closely related sediment clone BPC094 (Fig. 2) showed the highest sequence similarity to that of strain 171544^T, namely 49%.

Phenotypic characteristics of *Bacillus* strain 171544^T and relatives

Bacillus strains 171544^T, 1HR, 2HR, 3HR and 4HR were morphologically and physiologically homogeneous. Cells were rod-shaped being 2.0–3.0 μm in length by about 0.5–0.8 μm in diameter and stained Gram-positive. After incubation for 24 h on BHI agar, large colonies (3–5 mm in diameter) were formed which were brown to light brown in colour. The colony surface was granular but glistening and shiny. On NYSM agar, the colonies were 1–3 mm in diameter and lighter in colour. Spores first appeared on NYSM agar after 24 h and were oval, located subterminally to terminally in a swollen sporangium (Fig. 5).

Strain 171544^T and the related silage isolates were examined for a range of biochemical and physiological characters (Table 1). They were strict aerobes that did not produce acid from sugars or sugar alcohols (see species description). They were negative for the Voges–Proskauer reaction but they could use nitrate as a terminal electron acceptor. All of the strains hydrolysed aesculin, casein, gelatin and tributyrin, but not starch. Growth was observed in 7% NaCl, but not 10%. The bacteria did not grow at temperatures above 50 °C. All strains were resistant to discs containing 5 μg erythromycin and 1 U penicillin G, but were generally sensitive to a range of other antibiotics including chloramphenicol (25 μg), gentamicin (10 μg), novobiocin (5 μg) streptomycin (10 μg) and

tetracycline (25 µg). These features distinguished the silage isolates from phylogenetically related species such as *B. benzoovorans*, *B. circulans* and *B. firmus* (Table 1).

DISCUSSION

Endospore-forming bacteria predominate in the upper layers of silage bins, but are less common in the bulk anaerobic regions (Dutkiewicz *et al.*, 1989). Although several studies have enumerated total aerobic endospore-forming bacteria, the diversity of species has received scant attention. In a recent study, *B. pumilus*, *Bacillus cereus*, *B. circulans*, *Bacillus licheniformis*, *Bacillus sphaericus*, *Brevibacillus choshinensis* and *Paenibacillus validus* were associated with aerobic spoilage of maize silage (Inglis *et al.*, 1999). However, our previous study (De Silva *et al.*, 1998) and the results presented here show that silage is a rich source of novel endospore-forming bacteria.

Phylogenetic analysis revealed that *Bacillus* strain 171544^T and relatives were members of the genus *Bacillus* (Fig. 2). The high representation of soil clones in the phylogenetic neighbourhood of strain 171544^T suggests that these bacteria are part of a larger community of endospore-forming bacteria that inhabit grassland soils from which they gain access to silage. The closest relative to strain 171544^T was *B. circulans*, which shared 97.2% 16S rDNA sequence similarity. This is on the 97% upper limit for species delineation, as suggested by Stackebrandt & Goebel (1994), and supports the classification of these bacteria in distinct species. Further evidence for the separation of these bacteria from *B. circulans* and *B. firmus* was provided by their distinctive RAPD patterns.

The isolates were characterized at the molecular level by an insertion in the 16S rRNA genes. This insertion is almost certainly present in all copies of the *rrs* gene because there were no frameshifts in the sequencing electropherograms such as those caused by the heterogeneities in 16S rRNA genes found in *B. sporothermodurans* (Pettersson *et al.*, 1996). The actual extension was found to result in an almost doubling of the stem length of helix 49 (Fig. 4) and, since it was identical in all strains, the oligonucleotide primer 1452R was found to be reliable for the detection of 171544^T-like strains (Fig. 1a).

Very few other rDNA sequences shared a similar structure, e.g. the sediment clone BPC094, some *Desulfotomaculum* and *Thermoanaerobacter* species and some mitochondrial rDNAs. Since strain 171544^T and the sediment clone BPC094 are close phylogenetic relatives (Fig. 2), it is tempting to speculate in a shared origin of this insertion in these two 16S rRNA genes. Reasons for this would be that they are of identical length, they form stable base pairing (BPC094 data not shown) and they both protrude from the same nucleotide positions. However, while the overall 16S rRNA gene sequence similarity between *Bacillus* strain 171544^T and clone BPC094 is 95.7%, the nucleotide

similarity of the insertion sequences in helix 49 is only 49% indicating that if they do have a common origin, these insertions are subject to rapid evolutionary change. It has not been demonstrated whether the insertion is present in the 16S rRNA molecule or removed by processing, as it is for example in *Clostridium paradoxum* (Rainey *et al.*, 1996) and *Salmonella typhimurium* (Mattatall & Sanderson, 1996), because of difficulties obtaining reverse transcriptase products from purified rRNA (data not shown).

Bacillus strain 171544^T and the four silage isolates form a homogeneous taxon as defined by the 16S rRNA insertion, genomically (by RAPD analysis) and physiologically. Morphologically, the cells resemble those of *B. circulans*, but they can be readily differentiated from the latter by virtue of their strict aerobic metabolism. Moreover, the organisms can be readily distinguished from other phylogenetically related species using traditional phenotypic tests (Table 1). It is therefore proposed that these bacteria be given species status within the genus *Bacillus* as *Bacillus siralis*.

Description of *Bacillus siralis* sp. nov.

Bacillus siralis (si.ra'lis. L. masc. n. *sirus* grain pit, silo; L. adj. *siralis* belonging to the silo).

Cells are rod-shaped, 2.0–3.0 µm in length by about 0.5–0.8 µm in diameter and occur singly. Gram-positive. Spores are oval, located subterminally to terminally in a swollen sporangium. Colonies on BHI agar after incubation for 24 h are 3–5 mm diameter, brown to light brown in colour. The colony surface is granular, but glistening and shiny, circular, entire. On nutrient agar, the colonies are smaller (1–3 mm in diameter), pale and opaque. Aerobic, catalase- and oxidase-positive. Nitrate in broth is reduced to nitrite, but not nitrogen gas. Arginine is not hydrolysed (strain 1HR is an exception) and citrate is not used as carbon source. Resistant to erythromycin (5 µg ml⁻¹). Butanediol (Voges–Proskauer test) is not produced and acids are not produced from a range of sugars and sugar alcohols including arabinose, cellobiose, fructose, galactose, glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose and xylose. Aesculin, casein and gelatin, but not starch, are hydrolysed. Grows in 7% NaCl, but not 10%, and at 50 °C, but not 55 °C. Contains characteristic inserts of 49 bases in the distal region of the 16S rRNA genes. The full sequence of the 16S rRNA gene has database accession number AF071856. Source: silage. The type strain (171544^T) has been deposited in the National Collection of Industrial and Marine Bacteria as NCIMB 13601^T and in the Collection de l'Institut Pasteur as CIP 106295^T.

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