Geobacillus debilis sp. nov., a novel obligately thermophilic bacterium isolated from a cool soil environment, and reassignment of Bacillus pallidus to Geobacillus pallidus comb. nov.

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Several aerobic, motile, rod-shaped, thermophilic, spore-forming Geobacillus bacteria predominantly giving a Gram-positive staining reaction were isolated from a cool soil environment in Northern Ireland and taxonomically investigated. Two isolates, F10 and Tt, showed low 16S rRNA gene sequence similarity to recognized members of the genus Geobacillus. Phylogenetic tree investigation using neighbour-joining, maximum-likelihood and parsimony methods indicated that strains F10 and Tt represent a single novel species, for which the name Geobacillus debilis sp. nov. is proposed, with type strain Tt (DSM 16016T = NCIMB 13995T) and which belongs to a subgroup of the genus Geobacillus comprising Geobacillus toebii and Geobacillus caldoxylosilyticus. However, G. debilis showed closest affinities to Bacillus pallidus, which we propose should become Geobacillus pallidus comb. nov.

Over a number of years, the large and diverse grouping of bacteria in the genus Bacillus has been progressively subdivided into the novel genera Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus, Gracilibacillus, Ureibacillus and, most recently, Geobacillus (Nazina et al., 2001). These novel genera are based around separate phylogenetic groups derived from 16S rRNA gene sequence information. The original genus Bacillus contained aerobic and facultatively anaerobic, rod-shaped, Gram-positive (or Gram-variable) endospore-forming bacteria (Claus & Berkeley, 1986).

Many of the organisms previously classified in the genus Bacillus are thermophilic, aerobic, spore-forming organisms, which fall into genetic groups 1 and 5. There have been a number of taxonomic studies of the thermophilic bacilli, which retained these organisms within the genus (White et al., 1993; Rainey et al., 1994). The thermophiles in group 5 have been defined as ‘a phenotypically and phylogenetically coherent group of thermophilic bacilli displaying very high similarity among their 16S rRNA sequences (98-5–99-2 %)’ (Nazina et al., 2001). These observations led this group of workers to erect the novel genus Geobacillus with Geobacillus stearothermophilus as the type species. The two novel species they described (Geobacillus subterraneus and Geobacillus uzenensis) were isolated from subterranean petroleum reservoirs, geothermal locations providing an ideal source of thermophilic microorganisms. In addition, Nazina et al. (2001) transferred some existing Bacillus species into the new genus as G. stearothermophilus, Geobacillus thermocatenulatus, Geobacillus thermodenitrificans, Geobacillus kaustophilus, Geobacillus thermoglucosidasius and Geobacillus thermodenitrificans. Subsequently, other species have been added to the genus, i.e. Geobacillus caldoxylosilyticus, which was originally trivially named ‘Bacillus caldoxylosilyticus’, then transferred to Saccharococcus caldoxylosilyticus (Ahmad et al., 2000), and is now regarded as G. caldoxylosilyticus (Fortina et al., 2001), and lastly Geobacillus toebii (Sung et al., 2002). Not surprisingly, most microbiologists seek to isolate thermophiles from ‘hot’ environments; however, it has recently been shown that extremely thermophilic bacilli are present in cool soil environments at population levels that preclude them being contaminants from other environments (Marchant et al., 2002a, b).

Thermophiles can be categorized as being one of three types: (i) those that are restricted biogeographically and relaxed biogeochimically, (ii) those that are relaxed biogeographically and restricted biogeochimically or (iii) those that are relaxed biogeographically and relaxed biogeochimically. The thermophilic geobacilli fall into the last group because they are widely distributed and not restricted to specialized nutritional environments. Careful examination of the soil environment for thermophilic microorganisms has shown that the diversity is large and that

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there are organisms present that do not fit into the confines of Geobacillus species as recently recognized. In this paper we describe a novel species, Geobacillus debilis sp. nov., isolated from a cool soil environment in Northern Ireland, UK, belonging to a distinct Geobacillus genus subgroup. In addition, following comparison of 16S rRNA gene sequences of several geobacilli with the sequences of strains of Bacillus pallidus we suggest reassigning Bacillus pallidus to Geobacillus pallidus comb. nov.

Soil samples were collected from a site in Northern Ireland within an established mixed wet meadow area, which had been undisturbed for at least 15 years (Irish Grid reference C881 215) and from a similarly undisturbed adjacent site under mixed coniferous and deciduous trees with no ground cover (Irish Grid reference C881 216). The soil type at the sites was basalt till and the samples were taken at a depth of 50 mm below the surface.

One hundred milligrams of soil sample was suspended in 50 ml sterile Ringer’s solution (Oxoid) containing 0·1 % Triton X-100 and placed in a sonicating bath (KS100; Kerry Ultrasonics Ltd) for 10 min. One millilitre of the sample was serially diluted in Ringer’s solution and spread plates were prepared on nutrient broth (Oxoid) at pH 6·5 and incubated at 70 °C for 24 h under aerobic conditions. Cell colonies were isolated either onto specialized Bacillus medium (Leighton & Doi, 1971) or tryptase soya broth solidified with agar or Gelrite Gellan gum (Sigma) and further purified before being stored as stock cultures either at room temperature or at 4 °C.

Morphological and biochemical characterization of the isolates designated F10 (from the meadow site) and TtF (from the wooded site) were carried out using standard methods as described by Marchant et al. (2002b). In order to allow longer incubation times for the biochemical tests, the cultures were incubated at 60 °C rather than at the higher temperatures of 60–70 °C at which the organisms are able to grow.

DNA was extracted from pure cultures of the organisms and the 16S rRNA gene sequences were determined as described by Marchant et al. (2002b). DNA extraction from cultured bacteria was performed using a BIO 101 Inc. Soil DNA extraction kit. PCR was carried out using standard methods with the universal reverse primer 1492R with DNA extraction kit. PCR was carried out using standard methods as described by Marchant et al. (from the wooded site) were carried out using standard methods as described by Marchant et al. (2002b). The sequencing reactions were carried out and analysed using the ABI Prism 3100 Genetic Analyzer system as specified by the manufacturer. The genomic G+C ratio was determined using HPLC by DSMZ, Braunschweig, Germany.

Obligately thermophilic bacilli were easily isolated in large numbers from the cool soil environments examined (Marchant et al., 2002a, b). The strains isolated showed temperature ranges for visible growth of 40–75 °C and had extremely high growth rates with minimum generation times of less than 30 min at 70 °C. Many of the isolates could be readily assigned to existing Geobacillus species (Nazina et al., 2001; Fortina et al., 2001) on the basis of morphology, metabolic characteristics and 16S rRNA gene sequences. Two isolates, F10 and TtF³, showed low 16S rRNA gene sequence similarity with any recognized Geobacillus species, showing 93 % similarity to a sequence for G. stearothermophilus (AY297092) and thus indicating a more distant phylogenetic relationship. It has been proposed that sequence similarity below 95 % indicates a novel species (Fogel et al., 1999), although Stackebrandt & Goebel (1994) set the threshold at 97 %; this suggests that strains F10 and TtF³ represent a novel species of the genus Geobacillus. A higher sequence similarity of 99 % was given for a strain given the name ‘Bacillus thermozeamaize’ (unpublished; accession no. Y288912) isolated from corn steep liquor and that was presumably a thermophile. Construction of phylogenetic trees using neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981, 1988, 1996) and parsimony (Felsenstein, 1996) methods indicated that strains F10 and TtF³ represent a novel species, for which we propose the name Geobacillus debilis sp. nov. However, G. debilis shows close affinities to Bacillus pallidus, which we propose should become Geobacillus pallidus comb. nov. (Fig. 1). G. debilis strains F10 and Tt³ each show 91 % 16S rRNA gene sequence similarity to the sequence of G. pallidus. Comparison of the mean distance analysis tree in Fig. 1 with the tree in Nazina et al. (2001) shows similar relationships. In both cases, G. kaustophilus, G. thermoleovorans, G. thermocatenulatus, G. subterraneus, G. uzenensis and G. thermodenitrificans form a closely related cluster. In addition, G. caldoxylosilyticus and Bacillus (Geobacillus) pallidus occupy similar relative positions in both trees, with subsequently described G. toebii and G. debilis fitted close by in our tree. We have been able to confirm these relationships in a second phylogenetic tree constructed by mean distance analysis (data not shown).

The original description of the genus Geobacillus by Nazina et al. (2001) gave species as rod-shaped cells of varying dimensions but less than 10 μm long. Although there are references to these thermophilic bacilli producing chains of cells, some isolates produce enormously long, unidividually extended at 72 °C for 60 s. PCR products were run on 1·2 or 2 % agarose gels containing ethidium bromide.

DNA sequencing was carried out directly on purified PCR products. Three forward and three reverse sequencing primers were used (Marchant et al., 2002b). The sequencing reactions were carried out and analysed using the ABI Prism 3100 Genetic Analyzer system as specified by the manufacturer. The genomic G+C ratio was determined using HPLC by DSMZ, Braunschweig, Germany.
Flexuous cells (Marchant et al., 2002b). In the case of \textit{G. debilis}, no elongated cells have been observed and the rods were 0.5–1 \mu m wide by 1–14 \mu m long. Spore formation is also a defining character of \textit{Bacillus} and \textit{Geobacillus} and in many strains sporulation is copious and readily initiated. We have only been able to show the production of spores in \textit{G. debilis} following prolonged storage of plate cultures. The spores are produced singly and are not swollen beyond the size of the cells. Cells are motile and produce flat, cream-coloured colonies with smooth margins. Cells stain Gram-negative, but variable Gram-staining reactions are the norm for this group of Gram-positive bacteria.

\textit{G. debilis} shows a temperature range for growth of 50–70 °C, which is a rather narrower range than for other species in the genus. The organism is a strict aerobe and does not use nitrate or sulphate as a terminal electron acceptor.

Detailed characteristics of the two strains of \textit{G. debilis} used in this study are shown in Table 1. Morphological and biochemical characterizations, including alkane utilization (pentane, hexane, heptane, dodecane, hexadecane, octadecane and nonadecane), were carried out using standard methods as described by Marchant et al. (2002b). Characteristics of all recognized \textit{Geobacillus} species are shown in Table 2.

\textbf{Reassignment of \textit{B. pallidus} to \textit{G. pallidus} comb. nov.}

During screening of numerous soil thermophile isolates (Rahman et al., 2004), several showed close 16S rRNA gene sequence similarity to the thermophilic \textit{B. pallidus}, which was originally isolated from thermophilic treated wastes and was described by Scholz et al. (1987). \textit{B. pallidus} has subsequently been employed in studies of thermostable enzymes and biodegradation studies including breakdown of 2-propanol (Bustard et al., 2002). A close examination of the published characteristics of \textit{B. pallidus} (Table 2) together with the morphological description (Scholz et al., 1987) and with the sequence similarity for the 16S rRNA gene available on the European Bioinformatics Institute database and with these of our \textit{Geobacillus} isolates obtained from soil strongly suggests that \textit{B. pallidus} should be transferred and renamed \textit{G. pallidus}. A phylogenetic tree for \textit{Geobacillus} species including \textit{G. pallidus} presented in Fig. 1 indicates the affinity of \textit{G. pallidus} with \textit{G. caldolysolithicus}, \textit{G. toebii} and \textit{G. debilis}.

\textbf{Description of \textit{Geobacillus debilis} sp. nov.}

\textit{Geobacillus debilis} (de’bil.is. L. masc. adj. debilis weak or feeble, referring to the restricted substrate range for this species).

Gram-negative rods, 0.5–1.0 \mu m wide by 1.0–14.0 \mu m long, motile, spores sparsely produced terminally, sporangium not swollen. Colonies flat, cream-coloured with smooth margins. Growth occurs at 50–70 °C, with an optimum above 60 °C. Obligate aerobe. The DNA G+C

\begin{table}[h]
\begin{center}
\textbf{Table 1. Characteristics of \textit{Geobacillus debilis} sp. nov. strains TF\textsuperscript{T} and F10}
\begin{tabular}{|l|l|l|}
\hline
Characteristic & Strain TF\textsuperscript{T} & Strain F10 \\
\hline
Cell size (\mu m) & 0.5 x 1.1–10.9 & 1.1 x 1.1–14.2 \\
Production of acid from: & & \\
Glucose & – & (G) \\
Xylose & – (G) & – \\
Ribose & + & – \\
Trehalose & + & + \\
Sorbitol & + & – (G) \\
Rhamnose & – (G) & – \\
Arabinose & – & + \\
Utilization of: & & \\
Carboxymethyl cellulose & + & – \\
Sodium formate & – & + \\
Casein & – & + \\
Methyl red test & + & + \\
Gelatin liquefaction & + & – \\
Alkane utilization & + & – \\
\hline
\end{tabular}
\end{center}
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Table 2. Characteristics of all recognized Geobacillus species including G. debilis sp. nov. and G. pallidus comb. nov.

<table>
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<tr>
<th>Characteristic</th>
<th>1</th>
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<th>5</th>
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<th>9</th>
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<td>Cell width (μm)</td>
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<td>0.7–1</td>
<td>1.5</td>
<td>0.7–1</td>
<td>0.5–1.2</td>
<td>0.6–1</td>
<td>0.7–1</td>
<td>0.5–1</td>
<td>0.9–1.3</td>
<td>1.1–1.5</td>
<td>0.5–1–1.2</td>
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<td>Cell length (μm)</td>
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<td>3.7</td>
<td>3.5</td>
<td>3–&gt;100</td>
<td>3–7</td>
<td>2–3.5</td>
<td>1.5–3.5</td>
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<td>ND</td>
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<td>DNA G+C content (mol%)</td>
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<td>44</td>
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<td>45–46</td>
<td>52</td>
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<td>48–52</td>
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<td>Cellobiose</td>
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<td>Maltose</td>
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<td>Trehalose</td>
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<td>Xylose</td>
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<td>–</td>
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<td>d</td>
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<td>Arabinose</td>
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<td>+</td>
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<td>d</td>
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<tr>
<td>Gelatin</td>
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<td>d</td>
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<td>Starch</td>
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<td>Alkane utilization</td>
<td>d™</td>
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<td>d™</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
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content is 49·9 mol%. Production of acid from raffinose and trehalose, and also from ribose, sorbitol and arabinose in some strains. No utilization of starch but casein and trehalose, and also from ribose, sorbitol and arabinose in some strains. No utilization of starch but casein and trehalose. No utilization of starch but casein and ribose, sorbitol and arabinose in some strains. No utilization of starch but casein and trehalose. No utilization of starch but casein and ribose, sorbitol and arabinose in some strains. No utilization of starch but casein.

Habitat, soil.

The type strain is Tf™ (= DSM 16016™ = NCIMB 13995™); strains have been isolated from 50 mm undisturbed subsurface soil samples from Northern Ireland.

Description of Geobacillus pallidus (Scholz et al. 1988) Banat, Marchant and Rahman comb. nov.


The description is identical to that given for the genus Geobacillus by Nazina et al. (2001) and the species description given by Scholz et al. (1987). The type strain is H12™ (= ATCC 51176™ = DSM 3670™ = LMG 19006™).

Acknowledgements

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References


