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 Tf^T, 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 Tf^T represent a single novel species, for which the name *Geobacillus debilis* sp. nov. is proposed, with type strain Tf^T (= DSM 16016^T = NCIMB 13995^T) 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 micro-organisms. In addition, Nazina *et al.* (2001) transferred some existing *Bacillus* species into the new genus as *G. stearothermophilus*, *Geobacillus thermocatenulatus*, *Geobacillus thermoleovorans*, *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 caldoxylolyticus*', 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 biogeochemically, (ii) those that are relaxed biogeographically and restricted biogeochemically or (iii) those that are relaxed biogeographically and relaxed biogeochemically. 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 micro-organisms has shown that the diversity is large and that

The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequences of F10 and Tf^T are AJ564608 and AJ564616, respectively.

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 6.8–7.2 solidified with 0.8 % Gellan Gelrite gum (Sigma) and incubated at 70 °C for 24 h under aerobic conditions. Resulting colonies were isolated either onto specialized *Bacillus* medium (Leighton & Doi, 1971) or trypticase 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 Tf^T (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 *Bacteria*-specific primer 27F (Bond *et al.*, 2000) for the 16S rRNA gene. PCR amplification was carried out in reaction mixtures containing 0.2 mM each dNTP, 1.5 mM MgCl₂, 25 µM each primer, 1 µl template DNA and 2.5 U *Taq* polymerase in a final volume of 100 µl PCR buffer. Thermocycling was carried out in a Whatman Biometra T personal thermocycler under the following conditions: initial denaturation at 95 °C for 120 s, followed by 20 cycles of denaturation at 95 °C for 60 s, primer annealing at 45 °C for 45 s, elongation at 72 °C for 90 s with a final

extension at 72 °C for 600 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.

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 Tf^T, 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 Tf^T 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 Tf^T 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 Tf^T 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. caldioxysilyticus* 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, undivided

Fig. 1. Phylogenetic analysis of thermophilic bacterial isolates F10 and TfT obtained from Northern Ireland soil. The tree topology is based on neighbour-joining analysis of 16S rRNA gene sequences. Database sequence accession numbers are given in parentheses. *Bacillus subtilis* was used as an outgroup. Only bootstrap values >80% are shown at nodes (based on 1000 bootstrap resamplings). Bar, 0.05 substitutions per nucleotide position.

flexuous cells (Marchant *et al.*, 2002b). In the case of *G. debilis*, no elongated cells have been observed and the rods were 0.5–1 µm wide by 1–14 µm long. Spore formation is also a defining character of *Bacillus* and *Geobacillus* and in many strains sporulation is copious and readily initiated. We have only been able to show the production of spores in *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.

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 *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 *Geobacillus* species are shown in Table 2.

Reassignment of *B. pallidus* to *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 *B. pallidus*, which was originally isolated from thermophilic treated wastes

Table 1. Characteristics of *Geobacillus debilis* sp. nov. strains TfT and F10

+, Positive; –, negative; +^w, weakly positive; G, growth but no acid production. No acid production by either strain from mannose, fructose, maltose, lactose, sucrose, galactose, glycerol or cellobiose. Both strains stained Gram-negative and gave negative results for Voges–Proskauer, indole production, urea hydrolysis and utilization of amylopectin, cellulose, citrate, sodium acetate, starch, glycogen and Tween 80. Both strains grew at 50–70 °C and gave positive results for sporulation, motility, production of acid from raffinose, utilization of *myo*-inositol and the oxidase and catalase tests.

Characteristic	Strain TfT	Strain F10
Cell size (µm)	0.5 × 1.1–10.9	1.1 × 1.1–14.2
Production of acid from:		
Glucose	–	– (G)
Xylose	– (G)	–
Ribose	+ ^w	–
Trehalose	+ ^w	+
Sorbitol	+ ^w	– (G)
Rhamnose	– (G)	–
Arabinose	–	+ ^w
Utilization of:		
Carboxymethyl cellulose	+	–
Sodium formate	–	+ ^w
Casein	–	+
Methyl red test	+ ^w	+ ^w
Gelatin liquefaction	+	–
Alkane utilization	+ ^w	–

and was described by Scholz *et al.* (1987). *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 *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 *Geobacillus* isolates obtained from soil strongly suggests that *B. pallidus* should be transferred and renamed *G. pallidus*. A phylogenetic tree for *Geobacillus* species including *G. pallidus* presented in Fig. 1 indicates the affinity of *G. pallidus* with *G. caldoxylosilyticus*, *G. toebii* and *G. debilis*.

Description of *Geobacillus debilis* sp. nov.

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 µm wide by 1.0–14.0 µm 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

Table 2. Characteristics of all recognized *Geobacillus* species including *G. debilis* sp. nov. and *G. pallidus* comb. nov.

Strains: 1, *G. debilis* sp. nov. DSM 16016^T (data from this study); 2, *G. pallidus* comb. nov. DSM 3670^T (data from Scholz *et al.*, 1987); 3, *G. toebii* DSM 14590^T (Sung *et al.*, 2002); 4, *G. kaustophilus* NCIMB 8547^T (Sunna *et al.*, 1997); 5, *G. caldxylosilyticus* ATCC 700356^T (Ahmad *et al.*, 2000); 6, *G. thermoglucosidasius* ATCC 43742^T (Suzuki *et al.*, 1983); 7, *G. stearothermophilus* NCDO 1768^T; 8, *G. thermo-leovorans* ATCC 43513^T; 9, *G. thermodenitrificans* DSM 465^T; 10, *G. uzenensis* DSM 13551^T; 11, *G. subterraneus* DSM 13552^T; 12, *G. thermocatenulatus* DSM 730^T. Unless indicated otherwise, data are from Nazina *et al.* (2001) and White *et al.* (1993). +, Positive; –, negative; +^w, weakly positive; d, variable between strains; d^w, weak variable reaction between strains; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Cell width (µm)	0.5–1	0.8–0.9	0.7–1	1.5	0.7–1	0.5–1.2	0.6–1	0.7–1	0.5–1	0.9–1.3	1–1.5	0.5–1.2
Cell length (µm)	1.0–14.2	2–5	3–7	3.5	3–>100	3–7	2–3.5	1.5–3.5	1.5–2.5	4.7–8	4.7–7	3–7
Motility	+	+	+	–	–	+	+	–	ND	+	+	+
Temperature range for growth (°C)	50–70	30–70	44–80	40–75	43–80	42–69	37–65	40–80	45–70	45–65	45–65	42–69
DNA G + C content (mol%)	50	39–41	44	54	44	45–46	52	52–58	48–52	50–52	50–52	55
Acid produced from:												
Cellobiose	–	–	–	+	+	+	–	+	+	+	+	+
Maltose	–	d	–	+	+	+	+	+	+	+	ND	+
Mannose	–	–	+	+	+	+	+	d	+	+	+	+
Sucrose	–	ND	+	+	d	+	+	d	d	ND	ND	ND
Trehalose	+	d	–	+	+	+	d	+	+	+	ND	ND
Xylose	–	–	–	+	+	+	–	d	+	–	–	+
Arabinose	d ^w	–	–	–	+	–	–	d	+	+	–	–
Ribose	d ^w	–	–	–	–	–	ND	d	+	+	+	ND
Citrate used	–	–	–	+	–	–	d	–	ND	–	–	d
Hydrolysis of:												
Casein	d	–	–	+	+	+	d	+	–	–	–	+
Gelatin	d	–	–	+	–	–	d	–	ND	+	–	–
Starch	–	+ ^w	–	+	+	+	+	+	+	+	+	+
Alkane utilization	d ^w	ND	d ^w	+	+	ND	+	+	ND	+	+	+

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 gelatin used in some strains; very limited use of alkanes. Habitat, soil.

The type strain is Tf^T (=DSM 16016^T=NCIMB 13995^T); 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.

Basonym: *Bacillus pallidus* Scholz *et al.* 1988.

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^T (=ATCC 51176^T=DSM 3670^T=LMG 19006^T).

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