***Bacillus thermodenitrificans* sp. nov., nom. rev.**

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A polyphasic study was performed on 10 soil isolates of thermophilic denitrifying *Bacillus* strains from different geographical areas. The presence of two main characteristic bands following amplification of the internal transcribed spacer (ITS) region of *rrn* operons suggests a close relatedness to '*Bacillus thermodenitrificans*'. The isolates cluster around two strains of '*B. thermodenitrificans*' in riboprint and fatty acid analyses, though differences occur at the strain level. Subsequent DNA–DNA reassociation studies including the 10 isolates, '*B. thermodenitrificans*' DSM 465^T and DSM 466, and *Bacillus stearothermophilus* ATCC 12980^T and *Bacillus thermoleovorans* ATCC 43513^T revealed such a high level of genomic relatedness between the isolates and the DSM strains (> 73% similarity) that they must be considered strains of the same taxon. The degree of DNA–DNA similarity between the 12 strains of '*B. thermodenitrificans*' and the type strains of the other two phylogenetically neighbouring *Bacillus* species was significantly lower (21–43% similarity). Based upon phylogenetic, chemotaxonomic and phenotypic evidence, the designation of *B. thermodenitrificans* sp. nov., nom. rev. is proposed. The type strain of *B. thermodenitrificans* is DSM 465^T.

Keywords: *Bacillus thermodenitrificans*, DNA–DNA hybridization, chemotaxonomy, riboprinting, ITS analysis

INTRODUCTION

Following extensive phylogenetic analyses, the genus *Bacillus* has undergone major taxonomic revisions, leading to the establishment of novel genera (Wisotzkey *et al.*, 1992; Shida *et al.*, 1996; Heyndrickx *et al.*, 1998) and the recognition of new affiliations at the level of genera (Ash *et al.*, 1991; Rheims *et al.*, 1999) and species (Sunna *et al.*, 1997), while at the same time the list of species, including thermophilic representatives, has been extended (Manachini *et al.*, 1985; Demharter & Hensel, 1989; Andersson *et al.*, 1995; Combet-Blanc *et al.*, 1995; Meier-Stauffer *et al.*, 1996; Pettersson *et al.*, 1996). Despite the progress in rapidly recognizing close degrees of strain relatedness through analysis of 16S rDNA similarities, species delineation requires additional evidence. A DNA–DNA similarity of less than about 70% is the most convincing parameter, but relevant data are missing for strains of '*Bacillus thermodenitrificans*' and their

closest phylogenetic neighbours, e.g. the type strains of *Bacillus stearothermophilus* and *Bacillus thermoleovorans* (Rainey *et al.*, 1994). The latter species have recently been shown in a polyphasic study to embrace *Bacillus kaustophilus*, *Bacillus thermocatenulatus*, '*Bacillus caldolyticus*', '*Bacillus caldovelox*' and '*Bacillus caldotenax*' (Sunna *et al.*, 1997).

Although classified in group II of Klaushofer & Hollaus (1970) and in group Ia of Walker & Wolf (1971), '*B. thermodenitrificans*' has remained an unvalidated species. However, the result of recent phylogenetic (Rainey *et al.*, 1994), chemotaxonomic (White *et al.*, 1993) and numerical phenetic (Martinson *et al.*, 1996) studies suggested that this taxon merits species status.

In a systematic study of 85 aerobic, thermophilic and spore-forming isolates from soils of different geographical areas (Mora *et al.*, 1998), 34 strains (40%) were shown to be related to '*B. thermodenitrificans*'. This paper provides genomic, chemotaxonomic and physiological evidence for the validation of the species '*B. thermodenitrificans*'.

Abbreviation: ITS, internal transcribed spacer.

METHODS

Bacterial strains and growth conditions. The strains used in this study have been previously isolated from soil samples of different geographical areas (Mora *et al.*, 1998). The designation and origin of the isolates as well as that of the reference strains used are compiled in Table 1. Strains were routinely maintained at 4 °C after growth at 55 °C for 6–12 h on nutrient agar. For long-term maintenance, cell suspensions were stored at –80 °C in broth cultures supplemented with 15% (w/v) glycerol.

Phenotypic characterization. All assays were performed in triplicate and repeated when inconsistent results were obtained. Unless otherwise stated, all cultures were incubated at 55 °C for 1–7 d.

The following characteristics were determined by the methods described by Smith *et al.* (1952): anaerobic growth in glucose broth, anaerobic production of gas from nitrate, citrate utilization, starch and casein hydrolysis, indole, acetoin and catalase production. Reduction of nitrate was examined using the method of Lanyi (1987). The urease test followed the method as described by Atlas (1993). Utilization of compounds as sole carbon source was tested on agar (in plates) containing (g l⁻¹): (NH₄)₂HPO₄, 1; KCl, 0.2; MgSO₄, 0.2; yeast extract, 0.2; bromocresol purple, 0.004; agar, 15; pH 7, supplemented with 0.5% (w/v) of each carbon source sterilized separately. The temperature range for growth was determined in nutrient agar plates incubated at 50, 55, 60, 65, 70 and 75 °C. Growth was recorded daily for up to 4 d. For pH studies, nutrient broth was used with pH adjusted from 5 to 11. Phenol tolerance levels were determined in LB broth supplemented with a phenol concentration ranging from 1 to 25 mM.

Determination of G + C content of DNA. DNA base composition was determined by the thermal denaturation method described by Marmur & Doty (1962), using the equation of Owen & Hill (1979). DNA from *Escherichia coli* strain B (Sigma) was used as internal standard.

DNA–DNA hybridization method. DNA was isolated and purified as described previously (Manachini *et al.*, 1985). DNA–DNA homology was determined by the optical

renaturation rates method (Kurtzman *et al.*, 1979) with a model Response spectrophotometer equipped with Advance Kinetics Graphic Version 1.3 thermoprogrammer (Gilford System, Ciba Corning Diagnostics). For all samples tested the melting temperature (T_m) was calculated in 5 × SSC, the same salt concentration used for the determination of DNA–DNA similarities. The temperature of hybridization was 25 °C below the T_m calculated previously. The equation of Seidler & Mandel (1971) was used to calculate the extent of DNA–DNA reassociation.

Plasmid detection. Detection of plasmid DNA followed the alkaline extraction procedure described by Sambrook *et al.* (1989).

Ribotyping. Ribotyping was performed on *B. thermoleovorans* DSM 5366^T, *B. kaustophilus* DSM 7263, *B. stearothermophilus* DSM 22^T, '*B. thermodenitrificans*' DSM 465^T and 466, and 10 soil isolates as described by Allerberger & Fritschel (1999). Analysis was done with the RiboPrinter Microbial Characterization System (Qualicon, DuPont) (Bruce, 1996). Each sample lane was normalized to a standard marker set, characterized and identified using similarity measurements to previously run strains and reference patterns. Binary similarity values of riboprint patterns were used to generate an average linkage clustering dendrogram (Sneath & Sokal, 1973). The reproducibility of the analysis was tested by comparing the riboprint pattern obtained from freshly grown strains and from frozen cultures of the same strains after different times. The patterns obtained were comparable both in the distribution (number and position of bands) and in the intensity of fragments generated.

Internal transcribed spacer (ITS) fingerprints. These experiments were performed as previously described (Mora *et al.*, 1998). Primer set C (forward primer 5'-GTCGTA-ACAAGGTAGCCGTA-3' and reverse primer 5'-CAAG-GCATCCACCGT-3') was used to amplify the region between the 16S and 23S rDNA genes. The reproducibility of the methods was tested by comparing the amplified pattern of several amplification reactions and also using template DNA as purified for the DNA–DNA reassociation experiments.

Table 1. Strains of '*B. thermodenitrificans*' isolated from soil and reference strains used in this study

Strain	Origin
TH6A	New isolate from People's Republic of China
TH8A	New isolate from People's Republic of China
TH4B	New isolate from People's Republic of China
TH45A	New isolate from Czech Republic
TH33A	New isolate from Sardinia, Italy (= DSM 13147)
TH35A	New isolate from Bali, Indonesia (= DSM 13148)
TH51A	New isolate from Harvey Bay, Australia
TH61A	New isolate from Java, Indonesia
BI5A	New isolate from Saudi Arabia (= DSM 13149)
TU6F3	New isolate from Turkey
' <i>B. thermodenitrificans</i> '	DSM 465 ^T
' <i>B. thermodenitrificans</i> '	DSM 466
<i>B. stearothermophilus</i>	ATCC 12980 ^T = DSM 22 ^T
<i>B. thermoleovorans</i>	ATCC 43513 ^T = DSM 5366 ^T

Cellular fatty acid composition. Analyses of fatty acid composition followed the methods described by Reischl *et al.* (1998).

Detection of endonuclease activity. The strains were screened for endonuclease activity as described by Zahner & Priest (1997). The endonuclease assay consisted of testing different aliquots of extract with 2 µg substrate (λ DNA and pBR322) in a total volume of 50 µl. Different buffers (low-, medium-, high-salt) and different incubation temperatures (37, 45, 55 °C) were assayed.

RESULTS AND DISCUSSION

Ten representative strains were selected from the 34 strains previously shown on the basis of *rrn* spacer polymorphism to cluster with '*B. thermodenitrificans*' DSM 465^T and DSM 466 (Klaushofer & Hollaus, 1970), apart from the type strains of *B. stearothersophilus* and *B. thermoleovorans*. Although it has already been suggested by Rainey *et al.* (1994) on the basis of 16S rDNA similarity values that '*B. thermodenitrificans*' constitutes an individual phylogenetic lineage, no attempts have been made to validate this species. The isolation of strains which genomically resemble '*B. thermodenitrificans*' initiated this study which leads to the validation of the species.

Rainey *et al.* (1994) demonstrated that '*B. thermodenitrificans*' could be considered a separate taxon, with *B. stearothersophilus* and *B. thermoleovorans* showing less than 98.7% 16S rDNA similarity with strain DSM 465^T. Our data, obtained by comparison of the published 16S rDNA sequences of '*B. thermodenitrificans*' DSM 465^T with those contained in other databases, using the WU-BLASTN program, also confirmed *B. stearothersophilus* and *B. thermoleovorans* to be the neighbouring species, with identities ranging from 97 to 98%. For this reason, in this study '*B. thermodenitrificans*' strains were only compared with the two most closely related thermophilic bacilli.

As reported previously (Mora *et al.*, 1998), '*B. thermodenitrificans*' can be differentiated from other thermo-

Fig. 1. ITS patterns of '*B. thermodenitrificans*' strains studied. Lanes: 1–11, DSM 465^T, TH8A, TH51A, TH6A, TH35A, TH33A, TH61A, TH45A, TU6F3, B15A, TH4B, respectively; M, molecular mass marker VI (2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220, 154 bp; Boehringer Mannheim).

Fig. 2. Diversity of normalized ribotype patterns found within isolates related to strains of '*B. thermodenitrificans*' and ribotype patterns of the type strains of *B. stearothersophilus* and *B. thermoleovorans*. Lanes 1–15, '*B. thermodenitrificans*' DSM 466, TH61A, DSM 465^T, TH6A, TH51A, TH4B, TH33A, B15A, TH45A, TH35A, TU6F3, TH8A, *B. kaustophilus* DSM 7263, *B. thermoleovorans* DSM 5366^T, *B. stearothersophilus* DSM 22^T, respectively.

philic *Bacillus* species by a distinctive pattern of PCR fragments, generated with 16S–23S rDNA spacer-specific primers. The banding patterns of the isolates (Fig. 1) were characterized by the presence of two main amplification products of about 625 and 450 bp that should be considered specific and distinctive for the species. Additional fragments ranging from 200 to 500 bp and greater than 1750 bp were present, allowing the characterization of the isolates at the strain level. However, the observed polymorphism did not reflect the geographical origin and did not appear consistent with the phenotypic traits tested.

Analysis of *rrn* operons was extended by ribotyping, which included the hybridization of separated *Eco*RI-generated DNA fragments with a labelled *rrn* operon probe and visualization of the labelled fragments by a fully automated procedure. The riboprint patterns generated (Fig. 2) were used by the RiboPrint system to calculate binary similarity values which in turn were used to construct a dendrogram of relatedness (Fig. 3). Also, ribotyping provided a powerful means of typing these bacteria at the species and strain levels. Indeed, all isolates cluster around the two strains of '*B. thermodenitrificans*', while the reference strains of *B. stearothersophilus* and *B. thermoleovorans* branch separately. The depth of the '*B. thermodenitrificans*' cluster is comparable to that of *B. thermoleovorans*, indicating that these two taxonomic units exhibit a significant degree of difference in the genomic organization and/or in the sequence of their *rrn* operons. Moreover, within the species, the strains displayed some differences in their ribopattern that could be useful in strain characterization.

Fig. 3. Dendrogram of relationship based upon similarity of average linkage clustering of ribotype pattern (Fig. 2).

Table 2. Percentage cellular fatty acid composition of some '*B. thermodenitrificans*' isolates and the reference strains

Fatty acid	' <i>B. thermodenitrificans</i> ' DSM 465 ^T	' <i>B. thermodenitrificans</i> ' DSM 466	TH33A	TH35A	B15A	TU6F3	<i>B. stearothermophilus</i> ATCC 12980 ^T	<i>B. thermoleovorans</i> ATCC 43513 ^T
iso-14:0	0.38	0.37	0.46	0.37	0.48	-	-	0.45
14:0	0.41	0.67	0.53	0.48	0.88	0.72	1.32	1.15
iso-15:0	35.64	37.83	37.68	36.02	36.88	37.60	33.46	38.28
anteiso-15:0	1.28	1.39	1.55	1.42	1.60	1.55	4.14	2.14
15:0	1.79	1.34	1.88	1.63	1.81	2.25	-	0.56
iso-16:0	8.07	8.26	8.57	8.38	8.53	7.05	6.43	7.51
16:0	6.77	8.50	7.53	7.66	10.79	10.43	17.35	8.45
iso-17:1 ω 5c	0.98	1.26	0.70	0.79	0.32	-	-	2.39
iso-17:0	34.23	30.00	30.99	32.58	29.48	30.98	21.52	26.11
anteiso-17:0	6.40	5.83	6.61	6.69	5.97	7.08	12.65	6.94
17:0	1.61	1.27	1.35	1.52	1.17	1.74	-	0.35
iso-18:0	0.34	0.37	0.29	0.35	0.30	0.61	1.75	-
18:0	0.55	0.68	0.48	0.66	0.65	-	-	0.64
iso-19:0	0.74	0.85	0.67	0.69	0.67	-	-	-
Others	0.81	1.38	0.71	0.76	0.47	-	1.38	5.03

Fig. 4. Dendrogram of fatty acid similarities (Euclidian distance) based on data of strains shown in Table 2.

A similar picture is seen from the analysis of cellular fatty acids (Table 2 and Fig. 4). The principal fatty acids are iso-15:0 and iso-17:0, accounting for 66–69% of total fatty acids, followed by iso-16:0, 16:0 and anteiso-17:0, accounting for about 8.0, 7.5 and 6.5%, respectively. Differences in the levels of

Table 3. G + C content of the new '*B. thermodenitrificans*' isolates and the reference strains tested and DNA–DNA homology with '*B. thermodenitrificans*' DSM 465^T

Strain	G + C (mol%)	Percentage DNA homology with strain DSM 465 ^T
' <i>B. thermodenitrificans</i> ' DSM 465 ^T	50.3	(100)
' <i>B. thermodenitrificans</i> ' DSM 466	51.2	98
TH6A	48.6	94
TH8A	48.4	91
TH4B	50.5	74
TH45A	52.3	93
TH33A	48.6	88
TH35A	51.3	91
TH51A	52.3	73
TH61A	50.3	79
BI5A	49.2	87
TU6F3	48.2	87
<i>B. stearrowthermophilus</i> ATCC 12980 ^T	50.7	43
<i>B. thermoleovorans</i> ATCC 43513 ^T	52.5	21

Table 4. Phenotypic characteristics of the new isolates and reference strains used in this study

Symbols: +, positive; –, negative; w, weak reaction. All isolates and reference strains were positive for the following tests: Gram reaction, catalase, production of gas from nitrate, nitrate reduction, utilization of glucose, fructose, maltose, trehalose, mannose. They were all negative in the following reactions: indole and urease production; Voges–Proskauer.

Characteristic	' <i>B. thermodenitrificans</i> ' DSM 465 ^T	' <i>B. thermodenitrificans</i> ' DSM 466	TH6A	TH8A	TH4B	TH45A	TH33A	TH35A	TH51A	TH61A	BI5A	TU6F3	<i>B. stearrowthermophilus</i> ATCC 12980 ^T	<i>B. thermoleovorans</i> ATCC 43513 ^T
Growth at 45 °C	+	+	w	w	+	w	+	w	w	–	–	w	w	–
Growth at 70 °C	+	+	+	+	–	+	–	+	+	+	+	+	–	+
Growth at pH 9	–	+	–	–	–	–	–	+	–	–	–	–	–	–
NaCl (3%)	+	+	+	+	+	+	+	+	+	+	+	+	+	–
Denitrification	w	+	+	+	+	+	+	+	+	+	+	+	–	–
Anaerobic growth	+	+	w	+	w	+	+	–	w	–	+	w	w	w
Hydrolysis of casein	w	–	–	–	–	–	w	–	w	–	+	–	+	w
Hydrolysis of starch	+	w	w	w	w	+	+	+	+	+	w	–	+	+
Utilization of:														
Rhamnose	–	–	–	–	+	w	–	w	w	–	w	w	–	–
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Cellobiose	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Arabinose	+	+	+	+	w	+	+	+	+	w	+	+	–	–
Citrate	–	+	w	w	–	+	w	w	+	–	+	w	–	w
Phenol resistance (mM)	20	15	20	10	15	20	10	15	20	20	20	15	20	15

16:0, iso-17:0, anteiso-15:0 and anteiso-17:0, as well as the presence of minor amounts of 15:0 and 17:0 differentiate strains of the '*B. thermodenitrificans*' taxon from the type strain of *B. stearrowthermophilus*. The differences between strains of '*B. thermodenitrificans*' and the type strain of *B. thermoleovorans* are less substantial. A dendrogram of fatty acid relationships shows four soil isolates clustering with strains of '*B. thermodenitrificans*' and this group is separate from the reference strains of the two thermophilic *Bacillus* species (Fig. 4).

Analysis of the base composition of DNA (Table 3) revealed that all soil isolates and the two strains of '*B.*

thermodenitrificans' exhibit similar values ranging from 48.4 to 52.5 mol% G + C. Both DSM strains of '*B. thermodenitrificans*' have very similar values (50.3 and 51.2 mol%) in accordance with their phylogenetic and chemotaxonomic closeness. This contradicts the finding of Pichinoty *et al.* (1978) who reported a difference of 11 mol% in the G + C content of these two strains.

DNA–DNA reassociation studies were performed to determine whether the thermophilic soil isolates were members of the taxon '*B. thermodenitrificans*' and whether this taxon was genomically distinct enough to warrant its description as a new species. Using DNA of

strain DSM 465^T as a reference, similarity values of soil isolates and DSM 466 ranged between 73 and 94 %, while only low relatedness was found with the type strains of *B. stearothersophilus* (ATCC 12980^T = DSM 22^T) and *B. thermoleovorans* (ATCC 43513^T = DSM 5366^T) (43 and 21 % similarity, respectively) (Table 3). These data show that the taxon '*B. thermodenitrificans*' is genetically rather homogeneous and well differentiated from the other two species.

The main phenotypic characteristics are reported in Table 4. All properties listed in the literature for '*B. thermodenitrificans*' were confirmed. The strains were positive for the following tests: Gram reaction, catalase, growth at 65 °C, growth in presence of 3 % NaCl, nitrate and nitrite reduction to gas, anaerobic production of gas from nitrate, and utilization of glucose, fructose, maltose, trehalose, mannose, lactose, cellobiose, galactose, xylose, ribose and arabinose. The strains were resistant to phenol concentrations of 10–20 mM. They were negative for the following reactions: indole and urease production, and the Voges–Proskauer test (acetylmethylcarbinol production). No strain produced endonuclease activity. Extra-chromosomal elements were not found. Strains varied in the following tests: growth at pH 9, anaerobic growth in glucose broth, hydrolysis of casein and starch, and utilization of rhamnose and citrate. In addition to the denitrification property, sufficient phenotypic differences existed between the '*B. thermodenitrificans*' strains and the type strains of *B. stearothersophilus* and *B. thermoleovorans* to support the proposal of a new species. Even if the ability to reduce nitrate to gaseous compounds occurs among some strains of *B. stearothersophilus* and *B. thermoleovorans* (data not shown), '*B. thermodenitrificans*' differs from *B. stearothersophilus* in the ability to utilize a larger number of carbon sources, and from *B. thermoleovorans* in the utilization of lactose and arabinose and growth in presence of 3 % NaCl.

The results of this polyphasic study support the proposal of a new *Bacillus* species for which the invalid epithet *thermodenitrificans* (Klaushofer & Hollaus, 1970) is maintained.

Three new isolates have been deposited in the DSMZ with the following accession numbers: strain TH33A = DSM 13147; strain TH35A = DSM 13148; strain BI5A = DSM 13149.

Description of *Bacillus thermodenitrificans* (ex Klaushofer and Hollaus 1970) sp. nov., nom. rev.

Bacillus thermodenitrificans (ther.mo.de.ni.tri'fi.cans. Gr. n. *therme* heat; M.L. part. adj. *denitrificans* denitrifying; *thermodenitrificans* thermophilic denitrifying, referring to two of the main characteristics).

Gram-positive, straight rods 0.5–1 × 1.5–2.5 µm; endospores are oval, subterminal or terminal, not distending the sporangium. Colonies are flat with

lobate margins and off-white to beige. Growth occurs at 50–65 °C and some strains, including the type strain, are capable of growth at 45 and 70 °C. The optimum pH values for growth are from 6 to 8. Positive for catalase, growth in presence of 3 % NaCl, nitrate and nitrite reduction to gas and anaerobic production of gas from nitrate. Most strains are able to hydrolyse starch and a few degrade casein weakly. Negative for indole and urease production and Voges–Proskauer reaction. Glucose, fructose, maltose, trehalose, mannose, lactose, cellobiose, galactose, xylose, ribose and arabinose are utilized as sole carbon sources. The strains are resistant to phenol concentrations of 10–20 mM. The main fatty acids are iso-15:0 and iso-17:0; minor acids are 16:0, iso-16:0 and anteiso-17:0. The G + C content is 48.2–52.3 mol %. The natural habitat is soil. The type strain is DSM 465^T.

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