Bacillus thermolactis sp. nov., isolated from dairy farms, and emended description of *Bacillus thermoamylovorans*

An Coorevits,^{1,2} Niall A. Logan,³ Anna E. Dinsdale,⁴ Gillian Halket,³ Patsy Scheldeman,⁵ Marc Heyndrickx,⁵ Peter Schumann,⁶ Anita Van Landschoot^{1,2} and Paul De Vos²

Correspondence

An Coorevits

An.Coorevits@HoGent.be

¹Laboratory of Biochemistry and Brewing, Faculty of Applied Engineering Sciences, University College Ghent, Schoonmeerstraat 52, 9000 Ghent, Belgium

²Laboratory of Microbiology (LM-UGent), Department of Biochemistry and Microbiology, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

³Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Cowcaddens Road, Glasgow G4 0BA, UK

⁴Campden BRI, Chipping Campden, Gloucestershire GL55 6LD, UK

⁵Institute for Agricultural and Fisheries Research, Technology and Food Unit, Food Safety, Brusselsesteenweg 370, 9090 Melle, Belgium

⁶DSMZ German Collection of Microorganisms and Cell Cultures, Inhoffenstr. 7B, D-38124 Braunschweig, Germany

A polyphasic taxonomic study was performed on 22 thermotolerant, aerobic, endospore-forming bacteria from dairy environments. Seventeen isolates were retrieved from raw milk, one from a filter cloth and four from grass, straw or milking equipment. These latter four isolates (R-6546, R-7499, R-7764 and R-7440) were identified as *Bacillus thermoamylovorans* based on DNA–DNA hybridizations (values above 70% with *Bacillus thermoamylovorans* LMG 18084^T) but showed discrepancies in characteristics with the original species description, so an emended description of this species is given. According to 16S rRNA gene sequence analysis and DNA–DNA hybridization experiments, the remaining 18 isolates (R-6488^T, R-28193, R-6491, R-6492, R-7336, R-33367, R-6486, R-6770, R-31288, R-28160, R-26358, R-7632, R-26955, R-26950, R-33520, R-6484, R-26954 and R-7165) represented one single species, most closely related to *Bacillus thermoamylovorans* (93.9% 16S rRNA gene sequence similarity), for which the name *Bacillus thermolactis* is proposed. Cells were Gram-stain-positive, facultatively anaerobic, endospore-forming rods that grew optimally at 40–50 °C. The cell wall peptidoglycan type of strain R-6488^T, the proposed type strain, was A1 γ based on *meso*-diaminopimelic acid. Major fatty acids of the strains were C_{16:0} (28.0%), iso-C_{16:0} (12.1%) and iso-C_{15:0} (12.0%). MK-7 was the predominant menaquinone, and major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and some unidentified phospholipids. DNA G + C content was 35.0 mol%. Phenotypic properties allowed discrimination from other thermotolerant species of the genus *Bacillus* and supported the description of the novel species *Bacillus thermolactis*, with strain R-6488^T (=LMG 25569^T =DSM 23332^T) as the proposed type strain.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains R-6484, R-6486, R-6488^T, R-7165, R-6491, R-6492, R-6770, R-7336, R-7632, R-33520, R-28193, R-31288, R-26955, R-26358, R-33367, R-26954, R-26950, R-28160, R-7499, R-6546, R-7764 and R-7440 are FN666256, FN997657, AY397764, FN997658, FN666257, FN997656, FN997655, FN997659, FN997654, AM910339, AM910224, AM910312, AM910187, FN997653, AM910336, AM910186, AM910183, AM910223, AY373320, FN666258, FN666260 and FN666259, respectively.

Five supplementary figures are available with the online version of this paper.

The genus *Bacillus* grew historically as a dumping ground for all Gram-positive, aerobic, spore-forming, rod-shaped bacteria, resulting in a very heterogeneous genus comprising metabolically diverse species. This heterogeneity made it difficult to recognize general characteristics for the genus. Since the use of molecular techniques in the early 1990s, several efforts have been made to elucidate the phylogeny of members within the genus *Bacillus*, resulting in a more reliable taxonomy, including the creation of new genera of spore-forming bacteria such as *Paenibacillus* (Ash *et al.*, 1994), *Brevibacillus*, *Aneurinibacillus* (Shida *et al.*, 1996), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Geobacillus* (Nazina *et al.*, 2001) and *Ureibacillus* (Fortina *et al.*, 2001). Still, at the time of writing, the taxonomy of the genus is constantly evolving, with the accommodation of novel species and the transfer of described species into new or already described genera.

Owing to their endospores, members of the genus *Bacillus* are able to persist in various environments, including foodstuffs, where they often pose contamination and spoilage problems. The dairy industry, for instance, is a field frequently associated with spore-forming contaminants. Bacteria contaminate the milk during the primary production stage (the milking process), and cannot be eliminated during further processing as their spores survive the decontamination steps applied (pasteurization or Ultra Heat Treatment) (Pettersson *et al.*, 1996). These endospore-formers affect milk quality and safety through production of proteolytic and/or lipolytic enzymes that cause off-flavours and structural defects of the milk, and by the potential production of toxins associated with food-borne illnesses (Meer *et al.*, 1991; Crielly *et al.*, 1994; Andersson *et al.*, 1995).

Two previous studies (Scheldeman *et al.*, 2005; Coorevits *et al.*, 2008) focusing on the diversity of (highly) heat resistant endospore-forming bacteria in milk from Belgian dairy farms collected 22 isolates that could not unequivocally be identified to the species level. On the basis of partial 16S rRNA gene sequencing, these isolates appeared to represent *Bacillus thermoamylovorans*, and a novel species of the genus *Bacillus*. *Bacillus thermoamylovorans* was first isolated in 1995 from a palm wine sample in Senegal (Combet-Blanc *et al.*, 1995). It was described as a non-spore-forming, moderately thermophilic, facultatively anaerobic, Gram-positive rod. Following further characterization of the isolates using a polyphasic taxonomic approach as recommended by Logan *et al.* (2009), an emended description of *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995) is given as discrepancies with the original species description were observed, and the species *Bacillus thermolactis* sp. nov. is proposed.

All twenty-two isolates originated from dairy farm environments, more specifically from raw milk samples, straw, grass, milking equipment and a filter cloth, and were retrieved during a summer and a winter isolation campaign at Belgian dairy farms. Samples obtained during the first

study (Scheldeman *et al.*, 2005) were heat-treated (30 min at 100 °C) to select for highly heat-resistant spore-formers specifically, while samples obtained during the later study (Coorevits *et al.*, 2008) were heat treated (10 min at 80 °C) to select for all spore-formers. Samples were incubated at 55 °C for 24 h to select for thermotolerant spore-formers. All isolates were further subcultured on Brain Heart Infusion Agar (BHI, Oxoid), supplemented with vitamin B₁₂ (1 mg ml⁻¹) at 55 °C. The isolates, their sources and isolation conditions are listed in Table 1.

Total genomic DNA for 16S rRNA gene sequencing and DNA fingerprinting was extracted as described by Coorevits *et al.* (2008). The nearly complete 16S rRNA gene sequences of all isolates were generated as described by Heyrman & Swings (2001). Sequencing products were purified with a Big Dye XTerminator Purification kit (Applied Biosystems) according to the manufacturer's instructions using sequential pipetting and a MixMate (Eppendorf) shaking device. Sequences were assembled using the BioNumerics 5.1 software (Applied Maths, Belgium) and the 50 most closely related organisms were appraised using the online FASTA tool of EMBL (<http://www.ebi.ac.uk/Tools/sss/fasta/>). FASTA results indicated that these isolates were members of the genus *Bacillus*. A phylogenetic tree harbouring all species of the genus *Bacillus* with validly described names at the time of writing confirmed the position of strain R-6488^T within this genus, with *Bacillus thermoamylovorans* as its closest relative (Supplementary Figure S1, available in IJSEM online). A detailed view of part of this tree, with all isolates and their closest relatives included, is represented in Fig. 1. Both phylogenetic trees were based on almost complete 16S rRNA gene sequences and were reconstructed by aligning all sequences using CLUSTAL X (Thompson *et al.*, 1997) and trimming the overhangs. The jModelTest 0.1.1 program (Posada, 2008) was then applied to the datasets to determine the best-fit evolutionary model. Maximum-likelihood analyses were performed using PhyML (Guindon & Gascuel, 2003) by applying the parameters determined by jModelTest. Approximate likelihood ratio test values were calculated to assess the reliability of the clusters (Anisimova & Gascuel, 2006). Additional maximum-parsimony and neighbour-joining analyses were performed using MEGA4 (Tamura *et al.*, 2007) for the tree represented in Fig. 1. Resulting phylogenetic trees are shown in Supplementary Figures S2 and S3, respectively, and support the maximum-likelihood analysis. One group of milk isolates (strains R-33520, R-6488^T, R-6770, R-6491, R-7336, R-31288, R-7632, R-7165, R-28193, R-6492, R-6484, R-33367, R-26954, R-26950, R-26955, R-6486, R-26358 and R-28160) showed less than 0.7 % variability in 16S rRNA gene sequences with each other and with two *Bacillus* sp. strains, TAT105 and TAT112, representing two patented micro-organisms. The closest relative to this group of strains was *Bacillus thermoamylovorans* LMG 18084^T, sharing 93.9 % 16S rRNA gene sequence similarity with strain R-6488^T. These values indicated that the

Table 1. Overview of all isolates and their corresponding isolation conditions

For all isolates, the isolation location is Flanders, the isolation medium is BHI + vitamin B₁₂.

Strain	Isolation parameters			Study
	Source	Period	Heat treatment	
<i>Bacillus thermolactis</i> sp. nov.				
R-6484	Filter cloth	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6486	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6488 ^T (=LMG 25569 ^T ; =DSM 23332 ^T)	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-7165	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6491	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6492	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6770	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-7336	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-7632	Raw milk	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-33520	Raw milk	Winter	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-28193	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-31288	Raw milk	Winter	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-26955	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-33378	Raw milk	Winter	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-26358	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-33367	Raw milk	Winter	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-26954	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-26950	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
R-28160	Raw milk	Summer	10 min, 80 °C	Coorevits <i>et al.</i> (2008)
<i>Bacillus thermoamylovorans</i>				
R-7499	Straw	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-6546	Grass	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-7764	Milking equipment	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)
R-7440	Milking equipment	Winter	30 min, 100 °C	Scheldeman <i>et al.</i> (2005)

above-mentioned isolates probably represented a novel species within the genus *Bacillus*. 16S rRNA gene sequences of strains R-7499, R-7440, R-6546 and R-7764 showed at least 99.2 % similarity with each other and with the type strain of *Bacillus thermoamylovorans* (LMG 18084^T), indicating that these isolates probably belonged to this species. Surprisingly, two strains, WSBC20060 and WSBC20059 assigned to *Bacillus circulans*, also clustered in the vicinity of the *Bacillus thermoamylovorans* group. However, no further information could be found about these strains, and it was assumed these strains were probably wrongly identified and most likely should be allocated to *Bacillus thermoamylovorans*.

As prescribed by the minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan *et al.*, 2009), a fingerprint pattern of all isolates was generated using a repetitive sequence-based PCR with a (GTG)₅-primer (Heyrman *et al.*, 2005). This allowed grouping of the isolates into manageable clusters of similar strains as described previously by Heyrman *et al.* (2004). Five unique fingerprint patterns could be distinguished, as shown in Supplementary Figure S4, and were assigned groups A–E (supported by a 90 % cut-off value). For each group, representative strains were chosen for DNA–DNA hybridization experiments, namely strains R-33367, R-7499,

R-7440, R-6488^T and R-6484 (indicated in bold in Supplementary Figure S4). Indeed, it is generally recommended and accepted that strains with a DNA–DNA relatedness value below 70 %, or with 16S rRNA gene sequence dissimilarity above 3 %, are considered as belonging to separate species. Yet, bacterial strains with a difference in 16S rRNA gene sequence of less than 3 % cannot be allocated to the same species without support from DNA–DNA hybridization experiments (Stackebrandt & Ebers, 2006). For this purpose, approximately 1 g biomass from the representative strains, as well as from the type strain LMG 18084^T of *Bacillus thermoamylovorans*, was harvested from tryptone soy agar (TSA; Oxoid) plates, and DNA was purified as described by Logan *et al.* (2000). DNA–DNA hybridization was performed using a modification of the microplate method of Ezaki *et al.* (1989) as described by Willems *et al.* (2001). A hybridization temperature of 32 °C (calculated with correction for the presence of 50 % formamide) was used. DNA–DNA relatedness results confirmed 16S rRNA gene sequence analyses, with strains R-6488^T, R-6484 and R-33367 representing a novel species and strains R-7499 and R-7440 belonging to *Bacillus thermoamylovorans*. An overview of DNA–DNA relatedness values between the strains is given in Table 2. DNA G + C contents of strains R-6488^T and LMG 18084^T were 35.0 mol% and 37.0 mol%,

Whole cell hydrolysates (4 M HCl, 100 °C, 16 h) of strains LMG 18084^T and R-6488^T were subjected to thin layer chromatography on cellulose plates using the solvent system of Rhuland *et al.* (1955). *Meso*-diaminopimelic acid was found as the diagnostic diamino acid. This has only been reported for peptidoglycan type A1γ and for three variations of peptidoglycan type A4γ; however, these variations of peptidoglycan type A4γ have been found so far exclusively in members of the genera *Brachybacterium*, *Dermabacter* and *Devriesia*. It was clear from 16S rRNA gene sequence data that a close relationship with these three genera could be excluded, thus, it was concluded that LMG 18084^T and R-6488^T showed peptidoglycan type A1γ. The presence of *meso*-diaminopimelic acid is a characteristic typical for members of the genus *Bacillus* (Schleifer &

Strain	(GTG) ₅ -group	Hybridization (%) with DNA from:				
		R-7499	R-7440	R-6488 ^T	R-33367	R-6484
R-7499	A	100				
R-7440	B	97.2 ± 12.8	100			
R-6488 ^T	C	18.2 ± 0.4	20.2 ± 0.8	100		
R-33367	D	21.2 ± 1.3	24.3 ± 1.6	100.0 ± 7.7	100	
R-6484	E	20.2 ± 0.7	26.0 ± 4.3	99.5 ± 0.5	98.1 ± 0.3	100
LMG 18084 ^T		98.1 ± 6.5	92.4 ± 7.5	27.7 ± 7.7	20.9 ± 2.9	28.1 ± 1.9

Table 3. Characters for distinguishing between *Bacillus thermolactis* sp. nov., *Bacillus thermoamylovorans* and other thermotolerant species of the genus *Bacillus*

Taxa: 1, *Bacillus thermolactis* sp. nov. R-6488^T; 2, *Bacillus thermoamylovorans* LMG 18084^T; 3, *Bacillus alveayuensis* TM1^T; 4, *Bacillus fumarioli* LMG 17489^T; 5, *Bacillus circulans* LMG 13261^T; 6, *Bacillus coagulans* LMG 6326^T; 7, *Bacillus smithii* LMG 12526^T. ST, Subterminal; T, terminal; P, paracentral; +, >85 % positive; v, variable (26–74 % positive); –, 0–15 % positive; w, weak positive reaction; v (w), variable, and weak when positive; NR, not reported. Data for *Bacillus alveayuensis* are from Bae *et al.* (2005), who obtained biochemical characters using similar methods to those used in the present work; all other data were obtained in the authors' laboratories using the same methods as those described in the present paper.

Characteristic	1	2	3	4	5	6	7
Motility	–	v	+	w	+	+	+
Spore position	ST	ST	T/ST	P/ST	T/ST	ST (P/T)	ST
Sporangia swollen	–	v	+	–	+	Slight	–
Oxidase	+	+	–	–	–	v	w
Starch hydrolysis	w	+	–	–	w	+	w
Casein hydrolysis	+	–	+	–	w	–	–
ONPG production	–	+	NR	–	+	+	–
Voges–Proskauer test	–	v (w)	NR	+	–	w	v
Gelatin hydrolysis	+	+	–	+	v	+	–
Nitrate reduction	+	+	–	–	v	v	–
Acid from:							
Glycerol	–	w	+	v	+	+	+
L-Arabinose	+	+	–	–	+	v	v
Ribose	+	+	–	v	v	v	+
D-Xylose	+	v	–	–	+	v	+
Galactose	–	+	–	v	+	+	–
D-Mannose	–	+	+	+	+	+	v
myo-Inositol	–	–	–	–	+	v	–
Mannitol	w	–	–	+	+	v	+
Methyl D-glucoside	–	+	–	v	+	v	v
N-acetylglucosamine	–	+	–	w	+	+	–
Amygdalin	–	+	–	–	+	v	–
Arbutin	–	+	–	–	+	v	–
Salicin	–	+	–	–	+	v	–
Cellobiose	–	+	–	–	+	v	–
Lactose	–	+	–	v	+	v	–
Melibiose	–	+	–	v	+	+	–
Melezitose	–	v	–	v	+	–	–
Raffinose	–	v	–	v	+	v	–
Starch	w	+	–	–	+	+	–
Glycogen	–	+	–	–	+	–	–
Gentiobiose	–	+	–	–	+	v	–
Turanose	–	–	–	v	+	+	v
2-Keto-D-gluconate	–	–	+	–	v	–	–
Growth at/in:							
30 °C	–	–	–	+	+	+	+
60 °C	+	–	+	–	–	+	+
1 % NaCl	–	+	+	+	+	+	+
2.5 % NaCl	–	+	+	+	+	+	–
Anaerobic growth	+	+	–	–	+	+	+

Kandler, 1972). Menaquinones for type strains LMG 18084^T and R-6488^T were analysed as described by Groth *et al.* (1996). The major menaquinone was MK-7 for both strains, and strain R-6488^T also showed trace amounts of MK-8 (1 %). The type species of the genus *Bacillus*, i.e. *Bacillus subtilis* subsp. *subtilis*, also contains a quinone system with MK-7 predominant (Collins & Jones, 1981),

again supporting the attribution of strain R-6488^T and related isolates to the genus *Bacillus*. Polar lipids were extracted from 100 mg freeze-dried cell material using a chloroform/methanol/0.3 % aqueous NaCl mixture (1:2:0.8, by vol.) (modified after Bligh & Dyer, 1959). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered

into the chloroform phase by adjusting the chloroform/methanol/0.3 % aqueous NaCl mixture to a ratio of 1 : 1 : 0.9 (by vol.). Polar lipids were separated by two dimensional silica gel thin layer chromatography (Machery Nagel art. no. 818135). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.), and the second in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff reagent (alpha-glycols), Dragendorff reagent (quaternary nitrogen) and alpha-naphthol-sulphuric acid (glycolipids). Full details are given in Tindall *et al.* (2007). Polar lipid patterns of strains R-6488^T and LMG 18084^T were quite similar, complex patterns, showing diphosphatidylglycerol, phosphatidylglycerol and four to five unidentified phospholipids as the major components (Supplementary Figure S5). Phosphatidylethanolamine was detected in strain LMG 18084^T but not in strain R-6488^T. These profiles are somewhat similar to the polar lipid profile of *Bacillus subtilis* subsp. *subtilis* DSM 10^T (Kämpfer *et al.*, 2006), with diphosphatidylglycerol and phosphatidylglycerol as the major polar lipids; furthermore, phosphatidylethanolamine (as in strain LMG 18084^T) and three unidentified glycolipids (also observed in strain R-6488^T) were detected. For fatty acid methyl ester (FAME) analysis, strains were pre-cultured, then incubated for exactly 48 h at 52 °C on TSA (BBL, Beclon Dickinson). A loop full of well-grown cells was harvested and fatty acid methyl esters were prepared and extracted according to the standardized protocol of the Microbial Identification System (MIS; Microbial ID). All strains exhibited typical fatty acid profiles for the genus *Bacillus*, with a lot of branched chain components (Kaneda, 1977). Kämpfer (1994) specified fatty acid profiles of members of the genus *Bacillus*, containing large amounts of anteiso-C_{15:0} (26–60 %) and iso-C_{15:0} (13–30 %), and low amounts of unsaturated fatty acids (<3 %). Fatty acid profiles of the dairy strains comply with this profile, and both species can be easily differentiated from one another based on different amounts of these major fatty acids, anteiso-C_{15:0} and iso-C_{15:0}. Strains representing *Bacillus thermoamylovorans* had major amounts of iso-C_{15:0} (mean value 23.2 %), anteiso-C_{15:0} (mean value 23.4 %) and C_{16:0} (mean value 17.7 %); iso-C_{16:0}, iso-C_{17:0}, anteiso-C_{17:0}, iso-C_{14:0} and C_{14:0} were present in moderate amounts (mean values of 7.9 %, 7.1 %, 6.7 %, 5.0 % and 4.9 %, respectively), and trace amounts of C_{16:1ω11c} and C_{18:0} could be observed. Strains representing *Bacillus thermolactis* sp. nov. had major amounts of C_{16:0} (mean value 28.0 %), iso-C_{16:0} (mean value 12.1 %) and iso-C_{15:0} (mean value 12.0 %); C_{17:0}, anteiso-C_{17:0}, anteiso-C_{15:0}, iso-C_{17:0}, C_{16:1ω11c}, C_{14:0} and C_{18:0} were present in moderate amounts (mean values of 10.0 %, 7.6 %, 5.7 %, 5.4 %, 4.9 %, 4.9 % and 4.6 %, respectively), and trace amounts of iso-C_{14:0} and C_{16:1ω7c} were also detected.

Based on cell wall composition, menaquinone analyses and major polar lipids, the dairy farm isolates could be

allocated to the genus *Bacillus*. Furthermore, these data together with other phenotypic and genotypic data described above indicated that strains R-33520, R-6488^T, R-6770, R-6491, R-7336, R-31288, R-7632, R-7165, R-28193, R-6492, R-6484, R-33367, R-26954, R-26950, R-26955, R-6486, R-26358 and R-28160 represented a novel species within the genus *Bacillus*, for which the name *Bacillus thermolactis* sp. nov. is proposed.

The four isolates, R-7499, R-6546, R-7764 and R-7440, identified as *Bacillus thermoamylovorans*, showed some discrepancies with the original species description (Combet-Blanc *et al.*, 1995); therefore, an emended description of this species is given. In contrast to the original data, nitrate reduction was positive and in the API 50 CHB gallery, positive results were obtained for acid production from melibiose and methyl D-glucoside, a weak positive result for glycerol, and negative results for gluconate and rhamnose. Furthermore, endospore formation was observed, and this was not reported in the original description of Combet-Blanc *et al.* (1995). In addition, fatty acid content has been determined, and peptidoglycan, quinone and polar lipid analysis has also been performed.

Description of *Bacillus thermolactis* sp. nov.

Bacillus thermolactis (ther.mo.lac'tis. Gr. adj. *thermos* hot; L. gen. n. *lactis* from milk; N.L. gen. n. *thermolactis* a thermotolerant bacterium isolated from milk).

Facultatively anaerobic, Gram-stain-positive, rod-shaped cells (0.7–0.9 × 4–10 µm) that occur either singly or in short chains of two to four cells, and in filaments. Mainly non-motile but some strains are motile by means of peritrichous flagella. After 24 h of incubation at 50 °C on TSA, colonies are circular and cream-coloured with irregular edges, slightly rough and matt surfaces and glossy centres, and have diameters of approximately 1–4 mm. Endospores are formed within 24 h of incubation at 50 °C on TSA containing 5 mg MnSO₄ l⁻¹; they are ellipsoidal, lie subterminally and do not swell the cells. Growth occurs at pH 7 but not at pH 6 or 8. Growth occurs between 40 and 60 °C, optimally at 50 °C, but does not occur at 30 or 70 °C. Does not tolerate 1 % NaCl (w/v) for growth. Casein is hydrolysed, starch is hydrolysed weakly, and aesculin hydrolysis is variable. Catalase and oxidase production are positive. In the API 20E strip, reactions for gelatin hydrolysis and nitrate reduction are positive. Arginine dihydrolase, citrate utilization, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, *ortho*-nitrophenyl-β-D-galactopyranosidase (ONPG), tryptophan deaminase, urease and the Voges–Proskauer (acetoin production) reactions are negative. Acid but no gas is produced from the following carbohydrates in the API 50 CHB gallery: trehalose (weak), D-fructose, D-glucose, D-xylose, L-arabinose, mannitol (weak), ribose, starch (weak) and sucrose (weak). Acid production is variable between strains for maltose. No acid is produced from: 2-keto-D-gluconate, 5-keto-D-gluconate, adonitol, amygdalin,

arbutin, raffinose, D-arabitol, cellobiose, D-fucose, melezitose, melibiose, turanose, D-arabinose, D-lyxose, D-mannose, D-tagatose, dulcitol, erythritol, galactose, gentiobiose, gluconate, glycerol, glycogen, inulin, L-arabitol, L-fucose, lactose, L-sorbose, L-xylose, *myo*-inositol, methyl D-glucoside, methyl D-mannoside, methyl xyloside, N-acetylglucosamine, rhamnose, salicin, sorbitol or xylitol. Major fatty acids are C_{16:0}, iso-C_{16:0} and iso-C_{15:0}. Major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. MK-7 is the predominant menaquinone and the peptidoglycan type is A1 γ . The mol% G + C of the DNA of the type strain is 35.0 $\pm$ 0.2 mol%.

The type strain is R-6488^T (=LMG 25569^T =DSM 23332^T), isolated from milk and dairy farm environments. In the variable reactions listed above, the type strain is very weakly positive for acid production from maltose, and negative for aesculin hydrolysis.

Emended description of *Bacillus thermoamylovorans*

Bacillus thermoamylovorans (ther.mo.a.my.lo.vo'rans. Gr. adj. *thermos* hot; Gr. n. *amulon* starch; L. part. adj. *vorans* devouring; N.L. part. adj. *thermoamylovorans* utilizing starch at high temperature).

Facultatively anaerobic, Gram-positive, rod-shaped cells (0.45–0.5 $\times$ 3–4 μ m) that occur either singly or in short chains of two to four cells. Usually non-motile but some strains are motile. After 24 h of incubation at 50 °C on TSA, colonies are circular and cream-coloured, with smooth or irregular edges, and have diameters of 0.5–4 mm; colony surfaces range from smooth or glossy to slightly rough or matt. Endospores are formed within 24 h of incubation at 50 °C on TSA containing 5 mg MnSO₄ l⁻¹; they are ellipsoidal, lie subterminally, and occasionally swell the cells. Growth does not occur at pH 5 but can occur between pH 6 and 9, with the optimum for growth being between pH 7 and 9. Growth occurs at 50 °C, but does not occur at 40 or 60 °C. Tolerates 2.5 % NaCl but does not tolerate 5 % NaCl (w/v). Starch and aesculin are hydrolysed, but casein is not. Catalase and oxidase production are positive. In the API 20E strip, reactions for gelatin hydrolysis and nitrate reduction are positive. Reactions for *ortho*-nitrophenyl- β -D-galactopyranosidase (ONPG) production and the Voges–Proskauer (acetoin production) test are variable but the latter, when positive, is weak. Arginine dihydrolase, citrate utilization, hydrogen sulphide, indole, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease reactions are negative. Acid but no gas is produced from the following carbohydrates in the API 50 CHB gallery: amygdalin, arbutin, trehalose, cellobiose, D-fructose, D-glucose, D-mannose, galactose, gentiobiose, glycerol (weak), glycogen, lactose, L-arabinose, maltose, melibiose, methyl D-glucoside, N-acetylglucosamine, ribose, salicin, starch and sucrose. Acid production is variable between strains for raffinose, melezitose, D-xylose and methyl D-mannoside.

Acid is not produced from 2-keto-D-gluconate, 5-keto-D-gluconate, adonitol, D-arabitol, D-fucose, turanose, D-arabinose, D-lyxose, D-tagatose, dulcitol, erythritol, gluconate, inulin, L-arabitol, L-fucose, L-sorbose, L-xylose, mannitol, *myo*-inositol, methyl xyloside, rhamnose, sorbitol or xylitol. Major fatty acids are iso-C_{15:0}, anteiso-C_{15:0} and C_{16:0}. Major polar lipids are diphosphatidylglycerol and phosphatidylglycerol. MK-7 is the predominant menaquinone and the peptidoglycan type is A1 γ . The G + C content of the DNA of the type strain is 37.0 $\pm$ 0.2 mol%. Isolated from Senegalese palm wine, milking equipment, straw and grass.

The type strain is LMG 18084^T. In the variable reactions listed above, the type strain has cream-coloured colonies with slightly rough, matt surfaces and irregular edges. It is positive for acid production from raffinose, melezitose, D-xylose and methyl D-mannoside although the reactions are very weak; and negative for Voges–Proskauer and ONPG.

Acknowledgements

The authors thank the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen and Dr B. J. Tindall (DSMZ, Braunschweig, Germany) for the analysis of polar lipids.

References

- Andersson, A., Rönner, U. & Granum, P. E. (1995). What problems does the food industry have with the spore-forming pathogens *Bacillus cereus* and *Clostridium perfringens*? *Int J Food Microbiol* **28**, 145–155.
- Anisimova, M. & Gascuel, O. (2006). Approximate likelihood-ratio test for branches: a fast, accurate, and powerful alternative. *Syst Biol* **55**, 539–552.
- Ash, C., Priest, F. G. & Collins, M. D. (1994). Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek* **64**, 253–260.
- Bae, S. S., Lee, J.-H. & Kim, S.-J. (2005). *Bacillus alveayuensis* sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu Trough. *Int J Syst Evol Microbiol* **55**, 1211–1215.
- Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
- Collins, M. D. & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbiol Rev* **45**, 316–354.
- Combet-Blanc, Y., Ollivier, B., Streicher, C., Patel, B. K. C., Dwivedi, P. P., Pot, B., Prensier, G. & Garcia, J.-L. (1995). *Bacillus thermoamylovorans* sp. nov., a moderately thermophilic and amylolytic bacterium. *Int J Syst Bacteriol* **45**, 9–16.
- Coorevits, A., De Jonghe, V., Vandroemme, J., Reekmans, R., Heyrman, J., Messens, W., De Vos, P. & Heyndrickx, M. (2008). Comparative analysis of the diversity of aerobic spore-forming bacteria in raw milk from organic and conventional dairy farms. *Syst Appl Microbiol* **31**, 126–140.
- Crielly, E. M., Logan, N. A. & Anderton, A. (1994). Studies on the *Bacillus* flora of milk and milk products. *J Appl Bacteriol* **77**, 256–263.

- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Fortina, M. G., Pukall, R., Schumann, P., Mora, D., Parini, C., Manachini, P. L. & Stackebrandt, E. (2001). *Ureibacillus* gen. nov., a new genus to accommodate *Bacillus thermosphaericus* (Andersson *et al.* 1995), emendation of *Ureibacillus thermosphaericus* and description of *Ureibacillus terrenus* sp. nov. *Int J Syst Evol Microbiol* **51**, 447–455.
- Groth, I., Schumann, P., Weiss, N., Martin, K. & Rainey, F. A. (1996). *Agrococcus jenensis* gen. nov., sp. nov., a new genus of actinomycetes with diaminobutyric acid in the cell wall. *Int J Syst Bacteriol* **46**, 234–239.
- Guindon, S. & Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* **52**, 696–704.
- Heyndrickx, M., Lebbe, L., Kersters, K., De Vos, P., Forsyth, G. & Logan, N. A. (1998). *Virgibacillus*: a new genus to accommodate *Bacillus pantothenicus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenicus*. *Int J Syst Bacteriol* **48**, 99–106.
- Heyrman, J. & Swings, J. (2001). 16S rDNA sequence analysis of bacterial isolates from biodeteriorated mural paintings in the Servilia tomb (necropolis of Carmona, Seville, Spain). *Syst Appl Microbiol* **24**, 417–422.
- Heyrman, J., Vanparys, B., Logan, N. A., Balcaen, A., Rodríguez-Díaz, M., Felske, A. & De Vos, P. (2004). *Bacillus novalis* sp. nov., *Bacillus vireti* sp. nov., *Bacillus soli* sp. nov., *Bacillus bataviensis* sp. nov. and *Bacillus drentensis* sp. nov., from the Drentse A grasslands. *Int J Syst Evol Microbiol* **54**, 47–57.
- Heyrman, J., Verbeeren, J., Schumann, P., Swings, J. & De Vos, P. (2005). Six novel *Arthrobacter* species isolated from deteriorated mural paintings. *Int J Syst Evol Microbiol* **55**, 1457–1464.
- Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86–98.
- Kämpfer, P., Rosselló-Mora, R., Falsen, E., Busse, H.-J. & Tindall, B. J. (2006). *Cohnella thermotolerans* gen. nov., sp. nov., and classification of ‘*Paenibacillus hongkongensis*’ as *Cohnella hongkongensis* sp. nov. *Int J Syst Evol Microbiol* **56**, 781–786.
- Kaneda, T. (1977). Fatty acids of the genus *Bacillus*: an example of branched-chain preference. *Bacteriol Rev* **41**, 391–418.
- Logan, N. A. & De Vos, P. (2009). Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, vol. 3, pp. 21–128. Edited by P. De Vos, G. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer & W. B. Whitman. New York: Springer.
- Logan, N. A., Lebbe, L., Hoste, B., Goris, J., Forsyth, G., Heyndrickx, M., Murray, B. L., Syme, N., Wynn-Williams, D. D. & De Vos, P. (2000). Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of *Bacillus fumarioli* sp. nov. *Int J Syst Evol Microbiol* **50**, 1741–1753.
- Logan, N. A., Berge, O., Bishop, A. H., Busse, H.-J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L. & other authors (2009). Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *Int J Syst Evol Microbiol* **59**, 2114–2121.
- Meer, R. R., Baker, J., Bodyfelt, F. W. & Griffiths, M. W. (1991). Psychrotrophic *Bacillus* spp. in fluid milk products: a review. *J Food Prot* **54**, 969–979.
- Mesbah, M., Premachandran, U. & Whitman, W. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nazina, T. N., Tourova, T. P., Poltarau, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., Lysenko, A. M., Petrunyaka, V. V., Osipov, G. A. & other authors (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol* **51**, 433–446.
- Pettersson, B., Lembke, F., Hammer, P., Stackebrandt, E. & Priest, F. G. (1996). *Bacillus sporothermodurans*, a new species producing highly heat-resistant endospores. *Int J Syst Bacteriol* **46**, 759–764.
- Posada, D. (2008). jModelTest: phylogenetic model averaging. *Mol Biol Evol* **25**, 1253–1256.
- Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behavior of the isomers of α,ϵ -diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844–4846.
- Scheldeman, P., Pil, A., Herman, L., De Vos, P. & Heyndrickx, M. (2005). Incidence and diversity of potentially highly heat-resistant spores isolated at dairy farms. *Appl Environ Microbiol* **71**, 1480–1494.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Shida, O., Takagi, H., Kadowaki, K. & Komagata, K. (1996). Proposal for two new genera, *Brevibacillus* gen. nov. and *Aneurinibacillus* gen. nov. *Int J Syst Bacteriol* **46**, 939–946.
- Stackebrandt, E. & Ebers, J. (2006). Taxonomic parameters revisited: tarnished gold standards. *Microbiol Today* **33**, 152–155.
- Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* **24**, 1596–1599.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Tindall, B. J., Sikorski, J., Smibert, R. M. & Krieg, N. R. (2007). Phenotypic characterization and the principles of comparative systematics. In *Methods for General and Molecular Microbiology*, 3rd edn, pp. 330–393. Edited by C. A. Reddy, T. J. Beveridge, J. A. Breznak, G. Marzluf, T. M. Schmidt & L. R. Snyder. Washington, DC: American Society for Microbiology.
- Willems, A., Doignon-Bourcier, F., Goris, J., Coopman, R., de Lajudie, P., De Vos, P. & Gillis, M. (2001). DNA–DNA hybridization study of *Bradyrhizobium* strains. *Int J Syst Evol Microbiol* **51**, 1315–1322.