Bacillus herbersteinensis sp. nov.Monika Wieser,¹ Hanna Worliczek,¹ Peter Kämpfer²
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Two bacterial strains, designated D-1,5a^T and D-1,5b, were isolated from a medieval wall painting in the chapel of Castle Herberstein, Styria (Austria). The Gram-positive, heterotrophic, aerobic, spore-forming rods showed nearly identical whole-cell protein patterns, identical genomic fingerprints and identical physiological profiles, demonstrating their relationship at the species level. Both strains contained meso-diaminopimelic acid in their peptidoglycan, possessed a quinone system comprising menaquinone MK-7 and had fatty acid profiles in which C_{15:0} iso and C_{15:0} anteiso were predominant. The 16S rRNA gene sequence of D-1,5a^T showed the highest similarity (99.5 %) to the sequence of *Bacillus* sp. LMG 20243, and *Bacillus flexus* IFO 15715^T was the next most closely related established species (96.5 %). Other type strains, such as *Bacillus fastidiosus* DSM 91^T, *Bacillus indicus* SD/3^T, *Bacillus cibi* JG-30^T, *Bacillus megaterium* IAM 13418^T, *Bacillus cohnii* DSM 6308^T, *Bacillus bataviensis* LMG 21833^T and *Bacillus soli* LMG 21838^T, shared 96.0–96.1 % 16S rRNA gene sequence similarity with D-1,5a^T. The combination of physiological and chemotaxonomic traits distinguishes the two strains from those species sharing the highest sequence similarities (96.0–96.5 %). On the basis of these characteristics and the phylogenetic position of strain D-1,5a^T (= DSM 16534^T = CCM 7228^T), this strain is assigned as the type strain of a novel species of the genus *Bacillus*, for which the name *Bacillus herbersteinensis* sp. nov. is proposed.

From microbiological examinations of a medieval wall painting in the chapel of Castle Herberstein in Styria (Austria), several bacterial strains have been isolated and classified as novel species, including *Agrococcus citreus*, *Micrococcus luteus* (Wieser *et al.*, 1999, 2002), *Brachybacterium fresconis*, *Brachybacterium sacelli*, *Halomonas muralis*, *Bacillus decolorationis*, *Virgibacillus picturae* and *Brevibacterium picturae* (Heyrman *et al.*, 2002a, b, 2003a, b, 2004). During our studies of the microbiological diversity of the wall painting, we isolated, in addition to *A. citreus* and *M. luteus*, other isolates, which were preliminarily identified as members of the *Bacillaceae* (four isolates), the *Microbacteriaceae* (one isolate) and the *Moraxellaceae* (one isolate) (M. Wieser, unpublished results). Here we report the detailed characterization of two strains from this collection, which, in preliminary examinations, differed

by only a single band in the protein patterns after SDS-PAGE (results not shown) performed as described by Altenburger *et al.* (1996).

Strains were isolated and investigated morphologically as described by Wieser *et al.* (1999). NaCl tolerance was tested on PYES agar (0.3 % peptone, 0.3 % yeast extract, 0.23 % disodium succinate, 1.5 % agar, pH 7.2) supplemented with 1, 3, 5, 7 and 10 % NaCl, respectively. Tolerance towards different pH values was examined on PYES agar adjusted with HCl (37 %) or 4 M NaOH to pH 6, 7, 8, 9, 10, 11 and 12, respectively, or on buffered medium as described by Nielsen *et al.* (1995). Hydrolysis of starch was examined on PYES medium as described by Sneath (1986). Other tests were done as described by Kämpfer *et al.* (1991). Optimal growth was obtained on PYES agar or broth, pH 8.0, supplemented with 1 % NaCl (w/v). The characteristics are listed in the species description below and in Table 1.

Identical genomic fingerprints (see Supplementary Fig. S1 available in IJSEM Online) of strains D-1,5a^T and D1,5b obtained after enterobacterial repetitive intergenic consensus (ERIC) sequence PCR (Wieser & Busse, 2000) confirmed indications from protein patterns (results not shown) that these strains were members of a single species.

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Abbreviations: pNA, *p*-nitroanilide; pNP, *p*-nitrophenyl.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of D-1,5a^T is AJ781029.

Genomic fingerprints and polar lipid profiles of strains D-1,5a^T and D-1,5b are available as supplementary figures in IJSEM Online.

Table 1. Characteristics that differentiate *Bacillus herbersteinensis* sp. nov. from *Bacillus* species that share the highest 16S rRNA gene sequence similarity ($\geq 96\%$)

Species: 1, *B. herbersteinensis* sp. nov. (data from this study); 2, *B. bataviensis* (Heyrman *et al.*, 2004); 3, *B. cibi* (Yoon *et al.*, 2005); 4, *B. cohnii* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004); 5, *B. fastidiosus* (Claus & Berkeley, 1986); 6, *B. flexus* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004); 7, *B. indicus* (Suresh *et al.*, 2004); 8, *B. megaterium* (Priest *et al.*, 1988; Täubel *et al.*, 2003; Suresh *et al.*, 2004); 9, *B. soli* (Heyrman *et al.*, 2004). +, Positive; –, negative; W, weakly positive; V, variable; NG, no growth; NA, not analysed.

Characteristic	1	2	3	4	5	6	7	8	9
Sporangium shape	Unswollen	Slightly swollen	Swollen	Swollen	Unswollen	NA	Slightly swollen	Swollen	Slightly swollen
pH tolerance*	7–12	6–9.5 ($>4 < 10$)	5.5–7.5 (>5)	5 (<10)	NA	4.5–9.5	6–7	4.5–9.5	5–9 ($>4 < 9.5$)
NaCl (%) tolerance*	0–5 (<7)	NA	0–12	0–10 (W)	0–5 (<7)	0–10 (W)	0–2	0–5 (<10)	NA
Temperature tolerance (°C)*	4–28 (<37)	<30 –50 (<55)	10–40 ($>4 < 50$)	10–47 (<49)	10–40 ($>5 < 50$)	17–40 ($>5 < 50$)	15–37 (<40)	5 (V)–40 (V)	<30 – >45 (<50)
Nitrate reduction	–	+	–	+	–	–	–	–	+
Starch hydrolysis	+	NA	+	+	–	+	NA	+	NA
Acid production from:									
Salicin	–	W	NA	NA	NA	V	NA	+	–
D-Mannose	–	V	+	–	NA	–	+	V	+
N-Acetylglucosamine	–	+	NA	NA	NA	NA	NA	NA	+
D-Glucose	–	+	+	–	NG	+	–	+	+
Maltose	–	+	+	NA	NA	+	NA	+	+
Trehalose	–	V	+	NA	NA	+	NA	+	+
L-Arabinose	–	–	–	NA	NG	NA	NA	V	–
D-Mannitol	–	+	–	NA	NG	+	–	V	–
D-Xylose	–	–	–	–	NG	–	–	V	–
Assimilation of:									
D-Cellobiose	+	NA	+	–	NA	+	+	–	NA
D-Glucose	+	NA	+	–	NA	+	NA	+	NA
D-Melibiose	+	NA	NA	–	NA	–	+	–	NA
D-Sucrose	+	NA	+	–	NA	+	+	–	NA
D-Trehalose	+	NA	+	NA	NA	+	NA	+	NA
Citrate	+	–	–	–	NA	+	–	+	–
Major fatty acids	ai-C _{15:0} , i-C _{15:0}	i-C _{15:0} , ai-C _{15:0}	i-C _{15:0} , i-C _{14:0}	ai-C _{17:0} , i-C _{15:0} , ai-C _{15:0} or i-C _{15:0} , ai-C _{15:0}	NA	ai-C _{15:0} , i-C _{15:0}	i-C _{15:0} , ai-C _{15:0}	ai-C _{15:0}	i-C _{15:0} , ai-C _{15:0}
Glycolipids (GLs) present	2 GLs	NA	NA	–	NA	–	–	–	NA
Cell-wall diamino acid†	mDpm	NA	NA	Orn	NA	mDpm	L-Orn	mDpm	NA

*Values in parentheses indicate conditions under which no growth was observed.

†mDpm, *meso*-Diaminopimelic acid; Orn, ornithine.

The 16S rRNA gene sequence (1464 bases) of D-1,5a^T was analysed and sequenced according to Wieser *et al.* (1999), using primers 27f, 342f, 519r and 1992r (Lane, 1991). It should be mentioned here that sequencing of the 16S rRNA-encoding gene of D-1,5a^T caused unexpected problems. When primer 27f was used, the sequence could be determined only up to position 192. Ambiguous results were obtained for subsequent bases. A similar observation was made at the same position in the sequence when primer 519r was employed, and the base at position 193 (A or G) could not be determined in either of the two sequencing

directions. This observation might be explained by the presence of two rRNA-encoding genes in D-1,5a^T, one of which contains a gap or insertion at this position. Sequence comparisons (Pearson & Lipman, 1988) revealed the highest scores with the type strains of established species *Bacillus flexus* IFO 15715^T (96.5%), *Bacillus fastidiosus* DSM 91^T (96.1%), *Bacillus indicus* SD/3^T (96.1%), *Bacillus cibi* JG-30^T (96.0%), *Bacillus megaterium* IAM 13418^T (96.0%), *Bacillus cohnii* DSM 6308^T (96.0%), *Bacillus bataviensis* LMG 21833^T (96.0%), *Bacillus soli* LMG 21838^T (96.0%), *Bacillus asahii* MA001^T (95.8%), *Bacillus muralis* LMG

20238^T (95.8 %), *Bacillus novalis* LMG 21837^T (95.7 %), *Bacillus niacini* IFO 15566^T (95.7 %) and *Bacillus firmus* IAM 12464^T (95.6 %), indicating no relatedness between D-1,5a^T and any of them at the species level. Interestingly, the 16S rRNA gene sequence of D-1,5a^T shared the highest similarity (99.5 %) with the sequence of *Bacillus* sp. LMG 20243, which was recently isolated from a mural painting in St. Martins church in Greene-Kreiensen (Lower Saxony, Germany; Gorbushina *et al.*, 2004) and shared 97.1 % similarity with *Bacillus* sp. LMG 19415, which, like D-1,5a^T, had been isolated from the wall painting in the chapel of Castle Herberstein (Gurtner *et al.*, 2000). The phylogenetic position of strain D-1,5a^T was calculated using the PHYLIP software package (Felsenstein, 1993) after alignment of the 16S rRNA gene sequences using CLUSTAL X (Thompson *et al.*, 1997) and manual editing of sequences for ambiguous nucleotides and gaps using BioEdit (Hall, 1999). In all algorithms, the phylogenetically next most closely related species to strain D-1,5a^T were (i) the type strain of the type species of the genus *Bacillus*, *Bacillus subtilis* DSM 10^T (Fig. 1), although the two strains share only 95.3 % 16S rRNA gene sequence similarity, and (ii) *Bacillus fastidiosus* DSM 91^T. This branching was statistically supported by a moderate bootstrap value (81 %). However, these results indicate that strain D-1,5a^T may be considered as a species of the genus *Bacillus sensu stricto*. For G+C content

determination, DNA was extracted and purified as described by Auling *et al.* (1985). The DNA G+C contents of strains D-1,5a^T and D1,5b were determined by HPLC (Kaneko *et al.*, 1986) as 36.2 and 36.9 mol%, respectively. These values differ only slightly from those reported for *Bacillus fastidiosus* (34.3–35.1 mol%; Claus & Berkeley, 1986), *Bacillus flexus* (37.0–37.9 mol%; Priest *et al.*, 1988), *Bacillus megaterium* (37.3 mol%; Claus & Berkeley, 1986) and *Bacillus niacini* (37.0–39.0 mol%; Nagel & Andreesen, 1991).

Isoprenoid quinones and polar lipids were extracted and analysed as described previously (Tindall, 1990; Ventosa *et al.*, 1993; Altenburger *et al.*, 1996). Fatty acid methyl esters were extracted from biomass grown on nutrient agar (1⁻¹; 5 g peptone from casein, 3 g meat extract, 3 g yeast extract, 15 g agar) and was analysed as described by Kämpfer (1994). The diagnostic cell-wall diamino acid was determined as described by Schleifer (1985).

The major respiratory menaquinone in the two strains was found to be MK-7 and the diagnostic diamino acid in their cell walls was *meso*-diaminopimelic acid. These characteristics are in agreement with those of numerous species of the genus *Bacillus*, including the type species, *Bacillus subtilis* (Claus & Berkeley, 1986). The polar lipid profiles of D-1,5a^T (see Supplementary Fig. S2 in IJSEM Online) and D1,5b (results not shown) were identical. Major to moderate amounts of diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids, which might correspond to monoglucosyl diacylglycerol and diglucosyldiacyl glycerol (as reported to be present in a strain of *Geobacillus stearothermophilus*; Minnikin *et al.*, 1974), and moderate to minor amounts of two unknown phospholipids and two unknown polar lipids were detected. The presence of two glycolipids and the absence of phosphatidylethanolamine clearly distinguish the two strains from *Bacillus flexus* DSM 1320^T, *Bacillus cohnii* LMG 16678^T and *Bacillus megaterium* DSM 32^T (Täubel *et al.*, 2003). On the other hand, the presence of the two glycolipids might confirm the closer phylogenetic relatedness to *Bacillus subtilis* (Fig. 1), which has been reported to contain glycolipids (Brundish *et al.*, 1965; Bishop *et al.*, 1967). The fatty acid profiles of strains D-1,5a^T and D1,5b contained the major compounds C_{15:0} iso, C_{15:0} anteiso, C_{14:0} iso, C_{16:0} iso, C_{16:0} and C_{17:0} iso, which are characteristic of numerous taxa within the bacilli (Kämpfer, 1994). The relative fatty acid concentrations are listed below in the species description.

All of the characteristics determined for strain D-1,5a^T are in accordance with those of the genus *Bacillus*. On the basis of phylogenetic distance from established *Bacillus* species, also indicated by relatively low 16S rRNA gene sequence similarities (<97 %) and the combination of unique phenotypic characteristics, it is demonstrable that D-1,5a^T is not affiliated with any species of this genus. In conclusion, we describe D-1,5a^T as the type strain of a novel species, for which we propose the name *Bacillus herbersteinensis* sp. nov.

Fig. 1. Maximum-likelihood phylogenetic tree showing the position of strain D-1,5a^T among other *Bacillus* species on the basis of 16S rRNA gene sequences. Bootstrap values (expressed as percentages of 100 replications) greater than 70 % are shown at branching points. Bar, 10 % sequence divergence. The tree was rooted using *Paenibacillus polymyxa* DSM 36^T as an outgroup.

Description of *Bacillus herbersteinensis* sp. nov.

Bacillus herbersteinensis (her.ber.stein'en.sis. N.L. masc. adj. *herbersteinensis* pertaining to Castle Herberstein in Styria, in which the chapel with the medieval wall painting is located from which the type strain was isolated).

Cells are motile, rod-shaped, Gram-positive in the KOH and aminopeptidase tests, rod-shaped and produce oval spores in terminal, unswollen sporangia. Colonies on PYES agar are 2–3 mm in diameter, slightly raised, irregular and cream-coloured to beige. Older colonies are more translucent. Catalase- and oxidase-positive. Growth occurs at 4 and 28 °C, in the presence of 0, 1, 3 and 5 % NaCl (w/v) but not at 7 or 10 % NaCl (w/v). On buffered medium, growth is observed at pH 7 (weakly), 8 and 9. On unbuffered medium, growth is observed at pH 7 (weakly), 8, 9, 10, 11 and 12 but not at pH 6. Negative for nitrate reduction and haemolysis. Starch, *p*-nitrophenyl (pNP) β -D-galactopyranoside, pNP α -D-glucopyranoside, pNP β -D-glucopyranoside and bis-pNP phosphate are hydrolysed. pNP β -D-xyloside, pNP phenylphosphonate, pNP phosphorylcholine, 2-deoxythymidine-5'-pNP phosphate, L-alanine pNA (pNA, *p*-nitroanilide), L-glutamate γ -3-carboxy-pNA, L-proline pNA and pNP β -D-glucuronide are not hydrolysed. Acid is not produced from adonitol, L-arabinose, D-arabitol, cellobiose, dulcitol, erythritol, glucose, inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, methyl D-glucoside, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or D-xylose. *N*-Acetyl-D-glucosamine, L-arabinose (weakly), *p*-arbutin (weakly), D-cellobiose, D-fructose, D-galactose, gluconic acid, D-glucose, D-maltose, D-mannitol, D-mannose, α -D-melibiose, L-rhamnose, D-ribose, salicin, D-sorbitol, D-sucrose, D-trehalose, D-xylose, acetate (weakly), *cis*-aconitate (weakly), L-aspartate, citrate, fumarate, DL-3-hydroxybutyrate, DL-lactate, L-malate, L-ornithine (weakly), 2-oxoglutarate (weakly), pyruvate and L-proline are assimilated. Adonitol, *i*-inositol, maltitol, putrescine, azelate, glutarate, itaconate, *trans*-aconitate, adipate, propionate, 4-aminobutyric acid, 4-hydroxybenzoic acid, mesaconate, suberate, L-alanine, β -alanine, L-serine, L-histidine, L-leucine, L-phenylalanine, L-tryptophan, 3-hydroxybenzoic acid and phenylacetate are not assimilated. The fatty acid profile consists of C_{14:0} iso (8.7–14.2 %), C_{14:0} (0–0.5 %), C_{15:0} iso (17.7–27.4 %), C_{15:0} anteiso (17.0–23.4 %), C_{15:0} (1.9–3.2 %), C_{16:1} ω 7c (2.9–5.1 %), C_{16:1} ω 11c (1.9–2.4 %), C_{16:0} iso (8.4–15.8 %), C_{16:0} (4.8–5.9 %), C_{17:1} ω 10c iso (2.4–2.5 %), C_{17:1} ω 5c iso (1.0 %), C_{17:0} iso (3.7–5.1 %), C_{17:0} anteiso (1.1–1.2 %) and C_{17:0} (1.1–1.6 %). The diamino acid in the cell wall is *meso*-diaminopimelic acid. The predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. Additionally, moderate to minor amounts of a second unknown glycolipid, two phospholipids and four polar lipids are present. The major respiratory quinone is MK-7. The DNA G + C content is 36.2–36.9 mol% (HPLC).

The type strain is strain D-1,5a^T (= DSM 16534^T = CCM 7228^T). Strains D-1,5a^T and D-1,5b were both isolated from

a damaged wall painting in the chapel of Castle Herberstein, Styria (Austria).

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