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 sacelli*, *Halomonas muralis*, *Bacillus decolorationis*, *Virgibacillus picturae* and *Brevibacterium picturae* (Heyrman et al., 2002a, b, 2003). 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*Bacillaceae* (four isolates), the *Microbacteriaceae* (one isolate) and the *Moraxellaceae* (one isolate) (M. Wieser, unpublished results).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, 0·2 % 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-1,5aT 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.
The 16S rRNA gene sequence (1464 bases) of D-1,5aT 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-1,5aT 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-1,5aT, 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 DSM 15715T (96·5%), Bacillus fastidiosus DSM 91T (96·1%), Bacillus indicus SD/3T (96·1%), Bacillus cibi JG-30T (96·0%), Bacillus megaterium IAM 13418T (96·0%), Bacillus cohnii DSM 6308T (96·0%), Bacillus bataviensis LMG 21833T (96·0%), Bacillus soli LMG 21838T (96·0%), Bacillus asahii MA001T (95·8%), Bacillus muralis LMG
20238\textsuperscript{T} (95.8\%), Bacillus novalis LMG 21837\textsuperscript{T} (95.7\%), Bacillus niacini IFO 15566\textsuperscript{T} (95.7\%) and Bacillus firmus IAM 12464\textsuperscript{T} (95.6\%), indicating no relatedness between D-1,5a\textsuperscript{T} and any of them at the species level. Interestingly, the moderate bootstrap value (81\%). However, these results

DSM 91T. This branching was statistically supported by a rRNA gene sequence similarity, and (ii)

rRNA gene sequences usingCLUSTAL X (Thompson

software package (Felsenstein, 1993) after alignment of the

Fig. 1, although the two strains share only 95\% of 16S rRNA gene sequence similarity, and (ii) Bacillus fastidiosus DSM 91\textsuperscript{T}. This branching was statistically supported by a moderate bootstrap value (81\%). However, these results indicate that strain D-1,5a\textsuperscript{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\textsuperscript{T} and D1,5b were determined by HPLC (Kaneko et al., 1988) 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 & Arendees, 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\% peptone from casein, 3\% yeast extract, 1\% 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 wall was \textit{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\textsuperscript{T} (see Supplementary Fig. S2 in IJSEM Online) and D1,5b (results not shown) were identical. Major to moderate amounts of diphasphatidylglycerol, phosphatidylglycerol, two unknown glycolipids, which might correspond to monoglucosyl diacylglycerol and diglucosylglycerol were detected. The presence of two glycolipids and the absence of phosphatidylethanolamine clearly distinguish the two strains from Bacillus flexus DSM 1320\textsuperscript{T}, Bacillus subtilis DSM 32\textsuperscript{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\textsuperscript{T} and D1,5b contained the major compounds C\textsubscript{15:0} iso, C\textsubscript{15:0} anteiso, C\textsubscript{14:0} iso, C\textsubscript{16:0} iso, C\textsubscript{16:0} and C\textsubscript{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\textsuperscript{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\textsuperscript{T} is not affiliated with any species of this genus. In conclusion, we describe D-1,5a\textsuperscript{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\textsuperscript{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\textsuperscript{T} as an outgroup.

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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. Growth is observed at pH 7 (weakly), 8 and 9. On unbuffered medium, growth occurs 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 phosphonylcholine, 2-deoxythymidine-5'-pNP phosphate, L-alanine pNA (pNA, p-nitroanilide), L-glutamate γ-3-carboxy-pNA, L-proline pNA and pNP β-D-glucoronic acid are not hydrolysed. Acid is not produced from adonitol, L-arabinose, D-arabitol, cellulbiose, dulcitol, erythritol, glucose, inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, methyl D-glucoside, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose or D-xylose. The type strain is strain D-15aT (DSM 16534T = CCM 7228T). Strains D-1,5aT and D-1,5b were both isolated from a damaged wall painting in the chapel of Castle Herberstein, Styria (Austria).

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References


Bacillus herbersteinensis sp. nov.


