Bacillus barbaricus sp. nov., isolated from an experimental wall painting

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In a project concerning bacterial colonization of experimental wall paintings, a large number of isolates have been acquired with high similarities in their whole-cell protein patterns obtained after SDS-PAGE. Of this group, four strains, designated V2-BIII-A2^T, V2-BI-A9, V2-BI-04 and V2-BII-A8, were chosen for further characterization. Banding patterns obtained after ERIC-PCR were barely distinguishable among these four strains, indicating their affiliation within a single species. The isolates also displayed nearly identical biochemical and physiological features. The chemotaxonomic characteristics, including polar lipids, quinone systems, cell-wall diamino acid composition and fatty acid profiles, were in good agreement with those of numerous previously described *Bacillus* species. 16S rDNA analysis of strain V2-BIII-A2^T showed that this bacterium belongs to the genus *Bacillus*, with highest sequence similarities to *Bacillus megaterium* (94.6%), *Bacillus flexus* (94.4%) and the alkaliphilic *Bacillus cohnii* (94.2%). Based on almost identical biochemical, physiological and chemotaxonomic traits, ERIC-PCR-generated genomic fingerprints and comparative 16S rDNA sequence analysis, it is demonstrated that the four isolates represent a novel species of the genus *Bacillus*, for which the name *Bacillus barbaricus* sp. nov. is proposed. The type strain is V2-BIII-A2^T (= CCM 4982^T = DSM 14730^T).

Medieval wall paintings often suffer deterioration caused by microbial biodecay of the paintings and their groundings (Cifferi, 1999; Krumbein *et al.*, 1990; Petushkova *et al.*, 1989; Sorlini *et al.*, 1987). Sampling procedures used for biodecay investigations exacerbate the destruction. In order to achieve a better understanding of bacterial colonization and its contribution to the deterioration of medieval wall paintings, a project was carried out on experimental objects. Two identical wall paintings were produced by the Academy of Fine Arts (Vienna) using materials for the background that were also employed for construction of medieval wall paintings and were painted in a combination of the secco and fresco techniques. They were exposed in two medieval Austrian chapels, the Virgilkapelle, beneath the Stephansplatz in Vienna, and the Katharinenkapelle, in Castle Herberstein in Styria. The Virgilkapelle, which is connected directly to the subway system, is characterized by a relatively stable climate, with temperatures ranging between 18 and 26 °C and relative humidity between 64 and 86 %. The climate in

the Katharinenkapelle is strongly affected by outdoor conditions. The temperature varies between –2 and 20 °C and the relative humidity between 67 and 97 %. Over a period of 2 years, each painting was sampled four times on different areas that were covered with different pigments. Samples were taken by punching out material to a depth of approx. 5 mm using metal tubes (100 mm²). Isolation of bacteria was done as described by Wieser *et al.* (1999). For preliminary clustering of isolates displaying similar colony morphology, their protein patterns were compared after SDS-PAGE. One protein-similarity cluster was found to contain a large number of isolates (results not shown) that had been isolated from both paintings. This observation may indicate that the construction materials were already contaminated with these bacteria or that the paintings became contaminated during construction.

The 16S rDNA sequence of strain V2-BIII-A2^T, selected for reference, was analysed as described previously (Buczolits *et al.*, 2002) and a stretch of 1421 bases was obtained (positions 50–1467, *Escherichia coli* numbering; Brosius *et al.*, 1978). Highest 16S rDNA sequence similarities of V2-BIII-A2^T were obtained to *Bacillus megaterium* DSM 32^T (94.6%), *Bacillus flexus* DSM 1320^T (94.4%) and the obligate alkaliphile *Bacillus cohnii* LMG 16678^T (94.2%).

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of *B. barbaricus* V2-BIII-A2^T is AJ422145.

These values clearly allocate V2-BIII-A2^T to *Bacillus* rRNA group 1 (Ash *et al.*, 1991) and demonstrate that it represents a novel species. From a collection of 35 isolates with highly similar protein patterns, including strain V2-BIII-A2^T, which were isolated in October 1999 from the wall painting exposed in the chapel Virgilkapelle, strains V2-BI-09, V2-BI-A4 and V2-BII-A8 were selected for further taxonomic characterization. Comparison of genomic fingerprints of these four strains obtained after ERIC (enterobacterial repetitive intergenic consensus)-PCR (Wieser & Busse, 2000) supported their affiliation to a single species and clearly distinguished them from selected reference strains (Fig. 1).

In order to screen for isolates of this putative novel *Bacillus* species in subsequent samplings, isolates from both wall paintings were subjected to colony hybridization with an oligonucleotide probe (5'-GCGTTTCTTCTTATTATCCGG-3') specific for strain V2-BIII-A2^T. This probe displayed at least five mismatches with the corresponding sequences of every established *Bacillus* species. The distribution of this assumed species on the experimental wall paintings was studied by employing the probe in colony hybridizations with isolates from the third and fourth samplings. By this means, we were able to show that approx. 20 and 1%, respectively, of the bacterial communities isolated from the wall paintings exposed in the Virgilkapelle and the Katharinenkapelle can be assumed to belong to the same species as V2-BIII-A2^T (results not shown). This assumption was supported by a high degrees of similarity in their protein patterns (results not shown).

Colonies of the four isolates V2-BIII-A2^T, V2-BI-09, V2-BI-A4 and V2-BII-A8 were brownish, opaque and flat and reached a maximum of 7 mm in diameter when grown on

PYES medium (0.3% peptone from casein, 0.3% yeast extract, 0.23% disodium succinate, pH 7.2). Younger colonies had an entire margin, but older colonies sometimes became frayed. Cells were rod-shaped, occurring singly or in filamentous chains. Spores were oval in a swollen sporangium. Cells were Gram-positive, as shown by Gram-staining, KOH test and aminopeptidase test (Moaledj, 1986). Growth at different temperatures was investigated on PYES medium. The four strains grew well in the range 18–37 °C; after 3 weeks of incubation, no growth was observed at 4 or 47 °C. The four strains grew only weakly on PYES medium supplemented with 2% NaCl (w/v) and no growth was observed in the presence of 5 or 10% NaCl (w/v) after 3 weeks of incubation. Good growth on PYES agar was observed for all four isolates under anaerobic conditions as described by Altenburger *et al.* (2002), whereas no growth was detected when the strains were incubated on PYES agar under microaerobic conditions as described by Fox *et al.* (1994). This lack of growth may be due to the 5% H₂ present in the microaerobic atmosphere. In experiments where the pH of PYES medium was adjusted with HCl or NaOH before autoclaving, the strains grew weakly at pH 6.0 and strongly at pH 7.2, 8.0 and 9.5, indicating alkalitolerance of the isolates. The type strain (LMG 16678^T) of the alkaliphilic species *B. cohnii* (Spanka & Fritze, 1993), which was incubated under the same conditions, grew strongly at pH 9.5, moderately at pH 8.0 and not at all at pH 7.2. When the pH was adjusted as described by Nielson *et al.* (1995) using buffered systems, no growth of the four isolates was observed at pH 7.0, 8.0, 9.0, 10.0 or 11.0, whereas *B. cohnii* LMG 16678^T grew best at pH 8–11. Good growth of the four strains was observed on a medium recommended for cultivation of *B. cohnii* LMG 16678^T (0.3% yeast extract, 0.5% peptone, 0.5% NaCl), adjusted to pH 9.5 with Na₂CO₃ after autoclaving.

The physiological and biochemical properties of the four novel isolates, *B. megaterium* DSM 32^T, *B. flexus* DSM 1320^T and *B. cohnii* LMG 16678^T were analysed as described by Kämpfer *et al.* (1991) and are summarized in Table 1. Strains V2-BIII-A2^T, V2-BI-09, V2-BI-A4 and V2-BII-A8 only showed varying results for assimilation of 4-aminobutyrate, fumarate, L-leucine and L-ornithine. The four isolates displayed very similar acid-production profiles from carbohydrates with the API 50 CHB system (see species description).

The chemotaxonomic characteristics of the four isolates were almost identical. Peptidoglycan diamino acids were analysed in cell-wall preparations as described by Bousfield *et al.* (1985). Quinones were extracted and analysed as described previously (Tindall, 1990; Altenburger *et al.*, 1996). Detection of diaminopimelic acid as the characteristic peptidoglycan diamino acid and the predominant menaquinone MK-7 were in good agreement with the characteristics of numerous *Bacillus* species, including *B. megaterium* (Claus & Berkley, 1986) and *B. flexus* (this study). In contrast, Spanka & Fritze (1993) reported that the

Fig. 1. ERIC-PCR-generated genomic fingerprints. Lanes: 1 and 9, 100 bp marker; 2, *B. cohnii* LMG 16678^T; 3, *B. flexus* DSM 1320^T; 4, *B. megaterium* DSM 32^T; 5, V2-BIII-A2^T; 6, V2-BI-04; 7, V2-BII-A8; 8, V2-BI-A9; 10, negative control.

Table 1. Differential physiological and biochemical properties of *B. barbaricus* sp. nov. and related species

Strains: 1, *B. barbaricus* sp. nov. (four strains studied); 2, *B. megaterium* DSM 32^T; 3, *B. flexus* DSM 1320^T; 4, *B. cohnii* LMG 16678^T. +, Positive; –, negative; d, some strains positive; w, weak; ND, not determined; pNP, *p*-nitrophenyl; pNA, *p*-nitroanilide. All seven strains were positive in the catalase test (data for *B. megaterium* from Claus & Berkeley, 1986), for decomposition of starch and casein (data for *B. megaterium* from Priest *et al.*, 1988) and for utilization of acetate and DL-3-hydroxybutyrate. In general, positive reactions for utilization of amino acids were only weak. All seven strains were negative for utilization of L-arabinose, α -D-melibiose, D-xylose, adonitol, D-sorbitol, putrescine, propionate, *trans*-aconitate, adipate, azelate, itaconate, mesaconate, suberate, β -alanine, L-tryptophan and phenylacetate and hydrolysis of pNP β -D-glucuronide, pNP phosphorylcholine, L-alanine pNA and L-proline pNA.

Property	1*	2	3	4
Utilization of:				
<i>N</i> -Acetyl-D-glucosamine	+	+	+	–
<i>p</i> -Arbutin	–	–	+	–
D-Cellobiose	–	–	+	–
D-Fructose	+	+	+	–
D-Galactose	–	–	+	–
Gluconate	+	–†	++†	+
D-Glucose	+	+	+	–
D-Mannose	+	–	–	–
D-Maltose	+	+	+	–
L-Rhamnose	–	+	–	–
D-Ribose	+	–	+	–
Salicin	–	–	+	–
Sucrose	–	–	+	–
D-Trehalose	+	+	+	–
i-Inositol	–	+	+	–
Maltitol	–	–	+	–
D-Mannitol	–	+	–	–
<i>cis</i> -Aconitate	–	–	+	–
4-Aminobutyrate	d (1/4) ^a	–	+	–
Citrate	–	+	+	–
Fumarate	d (1/4) ^a	–	+	+
Glutarate	–	–	+	+
DL-Lactate	–	+	++†	–
L-Malate	+	–	+	+
Oxoglutarate	–	+	+	+
Pyruvate	+	–	+	+
L-Alanine	+	–	+	–
L-Aspartate	–	+	–	–
L-Histidine	+	+	–	–
L-Leucine	d (2/4) ^b	–	–	+
L-Ornithine	d (2/4) ^b	–	–	–
L-Phenylalanine	+	–	–	–
L-Proline	+	–	+	+
L-Serine	+	–	+	+
3-Hydroxybenzoate	–	–	–	+
4-Hydroxybenzoate	–	–	–	+
Hydrolysis of:				
Aesculin	–	+	–	–
pNP β -D-galactopyranoside	–	+	–	–
pNP α -D-glucopyranoside	–	+	–	–
pNP β -D-glucopyranoside	–	+	–	–
bis-pNP phosphate	–	+	–	–
pNP phenylphosphonate	–	+	–	–

Table 1. cont.

Property	1*	2	3	4
2-Deoxythymidine-5'-pNP phosphate	—	+	—	—
L-Glutamate- γ -3-carboxy pNA	—	+	—	—
Decomposition of:				
Hippurate	+	ND	—	+
Urea	—	ND	+	—‡
Tween 20	—	+§	+	+
Tween 80	—	ND	+	+
Growth in the presence of:				
2 % NaCl	w	+§	+	+
5 % NaCl	—	+§	+	+
10 % NaCl	—	—§	w	w
Nitrate reduction	—	—§	—	+
Growth at 47 °C	—	ND	+	+

*Strain-dependent results are scored as: a, V2-BII-A8 negative; b, V2-BII-A8 and V2-BI-A9 negative.

†Data not in line with Priest *et al.* (1988).

‡Data from Spanka & Fritze (1993).

§Data from Priest *et al.* (1988).

||Data not in line with Spanka & Fritze (1993).

alkaliphilic species *B. cohnii* contains a peptidoglycan with ornithine instead of diaminopimelic acid. Analysis of total fatty acids (Kämpfer *et al.*, 1997) showed the predominance of iso- and anteiso-branched fatty acids, typical for members of the genus *Bacillus* (Kämpfer, 1994). Fatty acids with chain lengths of 15 carbons [$C_{15:0}$ anteiso (37–43 %) and $C_{15:0}$ iso (19–22 %)] were quantitatively dominant. The homogeneity in the fatty acid profiles of the four strains supports their allocation into a single species and distinguished them from the relatives *B. megaterium* DSM 32^T, *B. flexus* DSM 1320^T and *B. cohnii* LMG 16678^T (Table 2).

Polar lipids of V2-BIII-A2^T analysed by two-dimensional TLC according to Ventosa *et al.* (1993) are shown in Fig. 2. Phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol were predominant in the polar lipid profile. Furthermore, we detected an unknown aminophospholipid, an unknown phospholipid, an unknown aminolipid and an unknown polar lipid. The unknown phospholipid showed chromatographic behaviour that was almost identical to that of phosphatidylcholine, but it could not be stained with Dragendorff reagent, which is commonly employed for detection of phosphatidylcholine. Glycolipids were not detected. The polar lipid profile was unaffected by the age of the biomass. Only minor quantitative differences in the amounts of certain lipids were found in two other isolates (results not shown). Similar polar lipid profiles, containing the same predominant compounds as well as the unknown phospholipid and the unknown polar lipid, were detected in *B. flexus* DSM 1320^T, *B. cohnii* LMG 16678^T and *B. megaterium* DSM 32^T (results not shown).

Relatively low 16S rDNA sequence similarity values (94.2–94.6 %) to the nearest relatives indicate that

Table 2. Fatty acid compositions of *B. barbaricus* sp. nov. V2-BIII-A2^T and related type strains

Strains: 1, *B. barbaricus* sp. nov. V2-BIII-A2^T (values in parentheses indicate the range of values detected within the three other strains of the species); 2, *B. megaterium* DSM 32^T; 3, *B. flexus* DSM 1320^T; 4, *B. cohnii* LMG 16678^T.

Fatty acid	1	2	3	4
i-C _{14:0}	9.4 (8.8–11.0)	6.7	2.3	2.3
C _{14:0}	0.9 (0.0–1.0)	—	1.6	—
i-C _{15:1} AT	0.7 (0.8–1.0)	—	—	—
i-C _{15:0}	19.0 (19.1–21.8)	13.9	29.6	38.4
ai-C _{15:0}	42.8 (37.5–43.2)	70.3	36.1	17.6
C _{15:0}	0.9 (0.8–1.1)	—	—	—
C _{16:1} ω7c	4.9 (4.6–5.7)	—	2.0	4.0
Summed feature 2*	1.1 (1.2–1.5)	—	—	—
C _{16:0} N	0.8 (0.0–1.1)	—	—	—
i-C _{16:0}	6.0 (6.0–7.1)	—	—	4.9
C _{16:1} ω11c	2.8 (2.3–2.9)	—	8.4	4.0
Summed feature 3*	2.5 (2.2–3.1)	—	—	—
C _{16:0}	2.0 (1.2–2.4)	4.4	2.5	0.6
i-C _{17:1} ω10c	—	—	4.8	—
Summed feature 4*	1.4 (1.1–2.0)	—	5.1	7.0
ai-C _{17:1} A	1.3 (1.3–2.0)	—	—	—
i-C _{17:0}	—	—	2.5	1.8
ai-C _{17:0}	2.6 (1.5–3.0)	4.7	5.0	3.6

*Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 2 contained one or more of C_{12:0} aldehyde or unknown ECL 10.928, i-C_{16:1} I and C_{14:0} 3-OH. Summed feature 3 contained C_{16:1}ω7c and/or C_{15:0} iso 2OH. Summed feature 4 contained i-C_{17:1} I and/or ai-C_{17:1} B.

Fig. 2. Two-dimensional TLC of the polar lipids of *Bacillus barbaricus* V2-BIII-A2^T. Abbreviations: DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown phospholipid; APL, unknown aminophospholipid; AL, unknown aminolipid; L, unknown polar lipid.

V2-BIII-A2^T represents a novel species of the genus *Bacillus*. Almost identical genomic fingerprints (Fig. 1), highly similar whole-cell protein patterns (results not shown) and fatty acid profiles (Table 2) and nearly identical biochemical and physiological properties (Table 1) provide further evidence that the four strains V2-BIII-A2^T, V2-BI-04, V2-BII-A8 and V2-BI-A9 represent a single species of the genus *Bacillus*. It can be distinguished from its close relatives within the genus, *B. megaterium* DSM 32^T, *B. flexus* DSM 1320^T and *B. cohnii* LMG 16678^T, on the basis of genomic fingerprints, biochemical/physiological traits and differences in fatty acid profiles and polar lipid patterns. In conclusion, we describe a novel species, for which we propose the name *Bacillus barbaricus* sp. nov.

Description of *Bacillus barbaricus* sp. nov.

Bacillus barbaricus (bar.ba'ri.cus. L. adj. *barbaricus* strange, foreign, referring to the strange behaviour towards growth at different pH).

Cells are Gram-positive, non-motile rods that produce subterminal, oval endospores in a swollen sporangium. Cells are 0.5 µm wide and 4–5 µm long. Colonies are brownish, opaque, circular, flat and 3–7 mm in diameter when grown on PYES agar. Facultatively anaerobic. Catalase-positive. Oxidase- and urease-negative. Nitrate is not reduced. Indole and H₂S are not produced. Alkalitolerant. Good growth occurs at temperatures ranging from 18 to 37 °C. No growth at 4 or 47 °C or in the presence of 5 or 10 % NaCl, and only weak growth in 2 % NaCl. Acid is produced from D-glucose, N-acetylglucosamine, aesculin, maltose, trehalose, starch

and glycogen. Acid production is variable from D-fructose (V2-BIII-A2^T is weakly positive), galactose, methyl α-D-glucoside, lactose, sucrose and D-turanose (V2-BIII-A2^T is negative). No acid is produced from glycerol, erythritol, D-arabinose, ribose, D-xylose, L-xylose, adonitol, methyl β-xyloside, D-mannose, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, amygdalin, arbutin, salicin, cellobiose, melibiose, inulin, melezitose, D-raffinose, xylitol, β-gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-ketogluconate or 5-ketogluconate. Other physiological and biochemical characteristics are given in Table 1. The cell-wall diamino acid is diaminopimelic acid and MK-7 is the predominant menaquinone. The polar lipid profile is composed of the major compounds phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol and minor amounts of an unknown phospholipid, an unknown aminophospholipid, an unknown aminolipid and one unknown polar lipid. The fatty acid profile consists of the predominant compounds ai-C_{15:0} and i-C_{15:0}; i-C_{14:0} and i-C_{16:0} are present in moderate amounts.

The type strain, V2-BIII-A2^T (=CCM 4982^T=DSM 14730^T), was isolated from an experimental wall painting exposed in the Virgilkapelle in Vienna, Austria.

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