Bacillus plakortidis sp. nov. and *Bacillus murimartini* sp. nov., novel alkalitolerant members of rRNA group 6

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The Gram-positive, alkali- and salt-tolerant marine bacterium strain P203^T is described together with its closest phylogenetic neighbour, terrestrial isolate LMG 21005^T. Strain P203^T was isolated from material from the sponge *Plakortis simplex* that was obtained from the Sula-Ridge, Norwegian Sea. Strain LMG 21005^T was an undescribed strain that was isolated from a church wall mural in Germany. Strains P203^T and LMG 21005^T were identified as novel alkalitolerant members of the *Bacillus* rRNA group 6 with a 16S rRNA gene sequence similarity of 99.5 %. The closest described neighbour, *Bacillus gibsonii* DSM 8722^T, showed 99.0 % gene sequence similarity with P203^T and 98.8 % similarity with strain LMG 21005^T. Despite the high 16S rRNA gene sequence similarity, DNA–DNA cross-hybridization revealed only 25.8–34.1 % similarity amongst the three strains. The DNA G + C contents were 41.1 mol% for strain P203^T and 39.6 mol% for strain LMG 21005^T. Both strains grew well between pH 7 and pH 11. Strain P203^T showed growth at moderate temperatures (from 4 to 30 °C) and in the presence of up to 12 % (w/v) NaCl at pH 9.7, whereas strain LMG 21005^T was not salt tolerant (up to 4 % NaCl) and no growth was observed at 4 °C. The major fatty acids of strains P203^T, LMG 21005^T and the type strain of *B. gibsonii* were the saturated terminally methyl-branched compounds iso-C_{15:0} (19.8, 15.6 and 28.0 %, respectively) and anteiso-C_{15:0} (57.1, 48.6 and 45.2 %, respectively). Physiological and biochemical tests allowed genotypic and phenotypic differentiation of strains P203^T and LMG 21005^T from the six related *Bacillus* species with validly published names and supported the proposal of two novel species, *Bacillus plakortidis* [type strain P203^T (=DSM 19153^T=NCIMB 14288^T)] and *Bacillus murimartini* [type strain LMG 21005^T (=NCIMB 14102^T)].

Abbreviations: AZCL, azurine cross-linked; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains *Bacillus plakortidis* sp. nov. DSM 19153^T and *Bacillus murimartini* sp. nov. LMG 21005^T are AJ880003 and AJ316316, respectively.

A table detailing the fatty acid content of strains DSM 19153^T and LMG 21005^T and electron micrographs showing the morphology of cells of the two novel strains are available with the online version of this paper.

Marine sponges are a recognized source of a variety of bioactive metabolites of pharmaceutical and biotechnological importance (Proksch *et al.*, 2002). In many cases, such compounds originate from the bacteria associated with the sponge rather than the sponge itself (Haygood *et al.*, 1999; Pabel *et al.*, 2003). Marine species of the genus *Bacillus* are common in the seawater column (Siefert *et al.*, 2000), but are also found in association with sponges (Hentschel *et al.*, 2003). Recently, *Bacillus pseudofirmus* strain 01-855 was isolated from the sponge *Aplysina aerophoba* (Karpushova *et al.*, 2005). The sponge *Plakortis simplex* (class

Demospongiae), used as the source for bacterial isolates in this study, was obtained from the Sula-Ridge region of the Norwegian Sea at a depth of about 290 m (64.06° N 08.09° E, water temperature about 7.6 °C) in 1999 (Pape, 2004). Strain P203^T was isolated from a homogenized sample of *Plakortis simplex* on CSA plates (1⁻¹: 1 g casein, 5 g MgSO₄·7H₂O, 0.5 g K₂HPO₄·3H₂O, 10 g soluble starch and 30 g sea salt) at 8 °C.

Strain P203^T was identified as a member of the genus *Bacillus* by partial 16S rRNA gene amplification using the oligonucleotides 16Sf (5'-GAGTTTGATCCTGGCTCAG-3') and 16Sr (5'-AGAAAGGAGGTGATCCAGCC-3') for the PCR product and standard primers for direct sequencing (Brosius *et al.*, 1978). The 16S rRNA gene sequence of strain P203^T was a continuous stretch of 1433 bp and was compared with the EMBL nucleotide database by basic local alignment. Multiple alignments of the sequence were performed and the nucleotide substitution rate was calculated. Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004) and PHYLIP (Felsenstein, 1989) after multiple alignment of data by CLUSTAL W (v. 1.83; Thompson *et al.*, 1994). Distances (distance options according to the Kimura two-parameter model) and clustering with the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1000 replications.

The phylogenetic tree constructed suggested that strain P203^T is a member of rRNA group 6 (Fig. 1). This group comprises alkaliphilic species of the genus *Bacillus*; *B. agaradhaerens*, *B. clausii*, *B. clarkii*, *B. gibsonii*, *B. halmaphilus*, *B. halodurans*, *B. horikoshii*, *B. pseudocaliphilus* and *B. pseudofirmus* (Nielsen *et al.*, 1995).

Subsequently *B. horti* (Yumoto *et al.*, 1998), *B. okuhidensis* (Li *et al.*, 2002), *B. krulwichiae* (Yumoto *et al.*, 2003), *B. decolorationis* (Heyrman *et al.*, 2003), *B. patagoniensis* (Olivera *et al.*, 2005) and *B. okhensis* (Nowlan *et al.*, 2006) have been added to *Bacillus* rRNA group 6.

The 16S rRNA gene sequence of strain P203^T showed 99.0 % similarity to the sequence of *B. gibsonii* DSM 8722^T. However, the undescribed *Bacillus* strain LMG 21005^T shared 99.5 % 16S rRNA gene sequence similarity to strain P203^T and we therefore decided to characterize strain LMG 21005^T as well. Strain LMG 21005^T was isolated from a wall painting in St. Martin's church, Greene-Kreienzen, Germany (Gorbushina *et al.*, 2004). *B. gibsonii* DSM 8722^T was found to be the closest recognized neighbour of strain LMG 21005^T (98.8 % gene sequence similarity).

DNA–DNA hybridization reactions and DNA G+C content determination were performed at the DSMZ (Braunschweig, Germany). DNA G+C content was analysed by HPLC according to the methods of Mesbah *et al.* (1989) and Tamaoka & Komagata (1984). Genomic DNA samples from *B. gibsonii* DSM 8722^T, strain P203^T and strain LMG 21005^T were cross-hybridized. DNA of strain P203^T showed relatedness values of 31.0 and 34.1 % with strain LMG 21005^T (reciprocal values are given) and 25.8 and 29.5 % with *B. gibsonii* DSM 8722^T. The DNA–DNA relatedness of strain LMG 21005^T and *B. gibsonii* DSM 8722^T was equally low (23.9 and 29.9 %). These values were well below the recommended 70 % threshold for the assignment of novel species (Stackebrandt & Goebel, 1994). The DNA G+C content of strains P203^T and LMG 21005^T and *B. gibsonii* DSM 8722^T were 41.1, 39.6 and 41.7 mol%, respectively.

Fig. 1. Neighbour-joining phylogenetic tree indicating the position of strains P203^T and LMG 21005^T among related species of the genus *Bacillus*. The previously established *Bacillus* rRNA group 6 (Nielsen *et al.*, 1995) was extended to include the new isolates discovered thereafter. *Staphylococcus aureus* subsp. *aureus* was used as an outgroup. The values at branch points indicate the percentages of occurrence in 1000 bootstrapped trees. Bar, 0.02 substitutions per nucleotide position.

Growth, substrate utilization and enzymic hydrolysis reactions were performed in triplicate and observed for 48–72 h at 20 °C. Growth at NaCl concentrations of 10 % (w/v) or higher and growth below 10 °C were recorded for up to 15 days. Medium DSM 31 (5 g peptone, 3 g meat extract in 1 l 0.1 M NaHCO₃/Na₂CO₃ buffer, pH 9.7) was supplemented with NaCl (0–20 % in 2 % intervals) for salt tolerance tests. The growth range from pH 6 to pH 12 (in 0.5 pH intervals) was tested in LB medium (deionized water 1 l⁻¹; 5 g yeast extract, 10 g tryptone and 10 g NaCl) adjusted to pH 6–8.5 with 0.1 M sodium phosphate buffer, to pH 9–10 with 0.1 M NaHCO₃ and to pH 10.5–12 with 0.1 M sodium sesquicarbonate/NaOH buffer.

The marine strain P203^T was identified as a novel halo- and alkalitolerant species of the genus *Bacillus* by means of growth in NaCl concentrations ranging from 0 % to 12 % (at pH 9.7) and at pH values ranging from pH 7 to pH 10 (at 1 % NaCl). A large percentage (40 %) of the previously defined members of the *Bacillus* rRNA group 6 (Nielsen *et al.*, 1995) exhibit a salt tolerance of up to 10 % NaCl. However, the minimum growth temperature of strain P203^T was lower (4 °C) compared with all other strains of the members of rRNA group 6 and to other sponge-associated *Bacillus* strains that have been isolated from the Gulf of Mexico (15 °C; Siefert *et al.*, 2000). *B. gibsonii* DSM 8722^T failed to grow at 40 °C, whereas strains P203^T and LMG 21005^T could not grow at or above 37 °C. Strain LMG 21005^T was not salt-tolerant (up to 4 % NaCl at pH 9.7) and no growth at 4 °C was observed. Strains of *B. gibsonii* grow best at around pH 8 (Nielsen *et al.*, 1995) and strains P203^T and LMG 21005^T showed the same behaviour. No growth was detected for strains P203^T and LMG 21005^T under anaerobic conditions as tested in DSMZ medium 31 by substituting air with N₂ gas. The results are summarized and compared with related species of the genus *Bacillus* in Table 1.

The cellular fatty acid contents of strains P203^T, LMG 21005^T and *B. gibsonii* DSM 8722^T were analysed from overnight cultures grown in LB medium (100 ml) as described previously (Thiel *et al.*, 2002). Structural elucidations of mono-unsaturated fatty acids were performed on their dimethyldisulfide derivatives (Buser *et al.*, 1983) and on their saturated structural analogues after hydrogenation. The fatty acids of strains P203^T, LMG 21005^T and *B. gibsonii* DSM 8722^T differed particularly with regard to the saturated terminally methyl-branched compounds iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{17:0} which represented the major fatty acids (see Supplementary Table S1 in IJSEM Online).

Overnight cultures of strains LMG 21005^T and P203^T were resuspended in 0.1 M phosphate buffer and used for electron microscopy. The suspension was dropped on a carbon-coated copper grid, covered with 4 % OsO₄ for 30 s and negatively stained for 5 min with 2 % uranyl acetate for examination. Shadow casting was performed using chromium chip sublimation at a 30° angle. Immediately

after staining and shadow casting, cells were observed with a transmission electron microscope (Jem 1010; JEOL) at 80 kV electron acceleration. The cell sizes (approx. 2 µm in length and 0.5 µm in width) and shapes of strains P203^T and LMG 21005^T appeared similar by TEM (see Supplementary Fig. S1 in IJSEM Online).

The results of the chemotaxonomic analysis are given in the species descriptions and in Table 1 and show the differences between strains P203^T and LMG 21005^T and six related strains of the genus *Bacillus*. Enzymic activities were tested by inoculating a 3 day-old fluid culture onto solidified marine agar broth (Difco) supplemented with 0.1 % dye-bound (azurine cross-linked, AZCL) substrates (Megazyme), 1 % skimmed milk powder from a local drug store or 1 % Tweens 20, 60 and 80. Halo or blue colour formation after 2 days at 20 °C indicated hydrolytic activity. Halo diameter was measured and noted on a scale from 1 to 5. Further enzymic hydrolysis and physiological characterization was performed with API ZYM, API 20E and API 50CH colorimetric tests as recommended by the manufacturer (bioMérieux). A 5 ml sample of a 3 day-old culture was washed with PBS buffer and resuspended in the same volume of an appropriate medium (0.85 % NaCl or API CHB media). Strips were read visually after 1 and 3 days and the colour change was noted on a scale from 1 to 5. All physiological experiments were performed in triplicate.

Strains P203^T and LMG 21005^T could utilize the following substrates: glycerol, glucose, D-fructose, D-mannose, D-mannitol, arbutin, aesculin, salicin, maltose, trehalose and hydrolysis of ONPG was observed. Strains P203^T and LMG 21005^T could be distinguished from related species by the following characteristics: different flagella types, growth at 4 °C and lack of growth at 40 °C, very different salt tolerances, inability to utilize starch or pullulan, and the ability to utilize D-galactose, D-ribose, D-cellobiose, D-lactose, D-melibiose, sucrose, citrate and gelatin. Comparison of enzyme patterns of strains P203^T, LMG 21005^T and *B. gibsonii* DSM 8722^T was performed with the API ZYM test and analysis of AZCL-substrate hydrolysis (Table 2). Strain P203^T could degrade AZCL-arabinan and AZCL-galactan, while *B. gibsonii* DSM 8722^T could not utilize AZCL-arabinan and strain LMG 21005^T could not utilize AZCL-galactan. All three strains could utilize skimmed milk, AZCL-casein and glyceryl tributyrat but not the substrates AZCL-xyloglucan, AZCL-pullulan, AZCL-arabinoxylan, AZCL-dextran, AZCL-cellulose, AZCL-β-glucan, AZCL-xylan, red amylose, red pullulan and red starch under the tested conditions. Only strain LMG 21005^T utilized AZCL-galactomannan and *B. gibsonii* DSM 8722^T could not degrade azocasein. Indole production (using Kovács reagent) was negative; the Voges–Proskauer reaction (acetoin production) was positive for strain P203^T, strain LMG 21005^T and *B. gibsonii* DSM 8722^T.

In summary, the carbohydrate utilization of strains P203^T and LMG 21005^T was distinct from that of *B. gibsonii* DSM 8722^T. Based on the phenotypic characterization and the

Table 1. Phenotypic characteristics of strains P203^T and LMG 21005^T and six recognized alkalitolerant *Bacillus* species

Taxa: 1, strain P203^T; 2, strain LMG 21005^T; 3, *B. gibsonii* (five strains); 4, *B. decolorationis* (ten strains; data from Heyrman *et al.*, 2003); 5, *B. clausii* (six strains, Nielsen *et al.*, 1995); 6, *B. patagoniensis* (four strains, Olivera *et al.*, 2005); 7, *B. okuhidensis* (two strains, Li *et al.*, 2002); 8, *B. hwajinpoensis* (Yoon *et al.*, 2004). All strains were able to hydrolyse casein but not Tween 80, were positive in catalase and oxidase tests and gave negative results for the utilization of erythritol, L-xylose, adonitol, sorbose, dulcitol, L-arabitol, gluconate and 2-ketogluconate. +, Positive; –, negative; ++, optimal conditions; v, variable; w, weak; C, central; ST, subterminal; PC, para-central; T, terminal; E, ellipsoid; SW, swollen; >, growth over 40 °C; ND, not determined; ONPG, 2-nitrophenyl β-D-galactopyranoside; VP, Voges–Proskauer reagent.

Characteristic	1	2	3	4	5	6	7	8
Spore	C to ST	C to ST	ST	C to ST	ST to PC, E	ST	T, E	ST, SW
DNA G+C content (mol%)	41.1	39.6	40.6–41.7	39.8–0.0	42.8–5.5	38.4–9.7	41.0–1.1	40.9
Nitrate reduction	–	–	v	+	+	–	+	+
Flagella/motility	Peritrichous	Polar	Polar	+	+	Peritrichous	Bipolar	None/–
Isolation source	Sponge	Wall	Soil	Wall	Soil	Soil	Hot spring	Seawater
Growth at (°C):								
5	+	–	–*	+	–	+	–	–
30	+	+	+	++	+	+	+	++
7	–	–	v	++	+	+	+	+
40	–	–	–	+	>	>	+	+
Growth at pH:								
6	w	–	w	v	–	–	+	++
7	+	+	+	++	+	+	+	++
8	++	++	++	–	++	++	+	+
10	+	+	+	–	–	+	+	+(9.5)
11	+	+	+	–	–	–	+	–
Growth in NaCl (% w/v):								
8	+	–	+	+	+	+	+	+
12	+	–	v	–	–	+	–	+
13	–	–	–	–	–	+	–	+
15	–	–	–	–	–	+	–	<20 %
Hydrolysis of:								
Starch	–	–	–	ND	+	+	+	+
Tween 20	–	–	–	ND	–	+	–	+
Tween 40	ND	ND	v	ND	–	+	–	+
Utilization of:								
Acetoin production (VP)	+	–	+	–	ND	ND	ND	ND
Amygdalin	–	–	v	–	+	–	ND	ND
D-Cellobiose	w	–	+	w	+	+	ND	+
Citrate	+	–	–*	–	–	ND	ND	ND
D-Galactose	–	w	v	v	v	–	+	+
β-Galactoside (ONPG)	+	+	+	v	ND	ND	ND	ND
Gelatin	+	–	+	w	+	ND	ND	+
Gentiobiose	–	–	+	w	v	+	+	ND
D-Lactose	+	–	+	v	v	–	+	–
D-Melezitose	–	–	+	–	+	–	–	–
D-Melibiose	+	–	+	–	+	–	+	+
D-Raffinose	–	–	+	–	+	+	–	+
L-Rhamnose	–	–	v	–	+	+	+	–
D-Ribose	–	w	+	w	+	+	ND	+
Sucrose	+	–	+	w	+	+	ND	ND
Trehalose	+	+	+	w	+	+	ND	ND
L-Tryptophan deaminase	w	–	+	–	ND	ND	ND	ND
Turanose	–	–	+	–	+	+	+	ND
D-Xylose	–	–	v	–	+	–	+	–

*Result for *B. gibsonii* DSM 8722^T obtained in this study.

Table 2. API ZYM characterization of strain P203^T, strain LMG 21005^T and *B. gibsonii* DSM 8722^T

All strains showed positive reactions for the enzymes alkaline phosphatase, C4 and C8 esterase/lipase and β -galactosidase, but gave negative results for C14 lipase, α -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. All strains degraded skimmed milk, AZCL-casein and glyceryl tributyrates. None of the strains could degrade the following AZCL substrates: xyloglucan, pullulan, arabinoxylin, dextran, cellulose, β -glucan, collagen, xylan or red amylose, red pullulan or starch. +, Positive; –, negative; w, weak reaction in API ZYM test or halo formation on AZCL-substrates or azocasein.

Enzyme/substrate	Strain P203 ^T	Strain LMG 21005 ^T	<i>B. gibsonii</i> DSM 8722 ^T
Acid phosphatase	–	+	w
Azocasein	+	–	+
α -Chymotrypsin	–	w	–
Cysteine arylamidase	–	–	+
Esterase C4	w	+	+
β -Glucosidase	w	–	+
β -Glucuronidase	+	–	–
Leucine arylamidase	–	w	+
Naphthol-AS-BI-phosphohydrolase	–	+	w
Trypsin	–	–	+
Valine arylamidase	–	–	+
AZCL carbohydrates			
Arabinan	+	+	–
Galactan	+	–	+
Galactomannan	–	+	–

wide difference in the enzyme fingerprint, we propose that the marine strain P203^T and strain LMG 21005^T represent two novel species of the genus *Bacillus*, for which the names *Bacillus plakortidis* sp. nov. and *Bacillus murimartini* sp. nov. are proposed.

Description of *Bacillus plakortidis* sp. nov.

Bacillus plakortidis [pla.kor'ti.dis. N.L. (Gr.-derived) gen. n. of *Plakortis* a genus of sponges].

Cells are rod-shaped with a length of approx. 2–3 μ m and a width of 0.5 μ m. Cells are motile by means of peritrichous flagella, especially at high pH. Spores are located central to paracentral and are seldom observed. Colonies are circular, convex, smooth and yellowish. Cells stain slightly Gram-positive in the exponential phase. The temperature range for growth is 4–30 °C, with optimal growth at 15–20 °C. The pH range for growth is from pH 6.5 to pH 10, the optimal pH is 8.5. Cells grow in the absence or presence of NaCl up to a concentration of 12 % (w/v). Terminally methyl-branched saturated fatty acids iso-C_{15:0} (19.8 %) and anteiso-C_{15:0} (57.1 %) represent the major fatty acids. Catalase and oxidase tests are positive. Nitrate is reduced to nitrite. Casein, gelatin, arabinan and galactan are hydrolysed, but not starch or galactomannan. Tween 80 is not hydrolysed but glyceryl tributyrates is hydrolysed. Utilizes *N*-acetylglucosamine, sorbitol, D-lactose, D-melibiose, sucrose and citrate. The DNA G+C content is 41.1 mol%.

The type strain, strain P203^T (=DSM 19153^T=NCIMB 14288^T), was isolated from the marine sponge *Plakortis simplex* from the Norwegian Sea.

Description of *Bacillus murimartini* sp. nov.

Bacillus murimartini [m.uri.mar.ti'ni. L. n. *murus* wall; N.L. gen. n. *martini* of Martin (masc. name of a saint); N.L. gen. n. *murimartini* from the wall of the (St.) Martin church in Greene-Kreienzen, Germany].

Cells are rod-shaped with a length of approx. 2–3 μ m and a width of 0.5 μ m. Cells are motile by means of peritrichous flagella but are less motile than *B. plakortidis* DSM 19153^T and *B. gibsonii* DSM 8722^T. Spore formation is rarely observed. Spores are ellipsoidal in ageing cells and are located paracentrally. Colonies are circular, convex, smooth and yellowish. Cells stain slightly Gram-positive in the exponential phase. The temperature range for growth is 10–30 °C, with optimal growth between 15 and 20 °C. The pH range for growth is from pH 7 to pH 10; the optimal pH is 8.5. Cells grow in the absence or presence of NaCl up to a concentration of 4 % (w/v). Terminally methyl-branched saturated fatty acids iso-C_{15:0} (28.6 %) and anteiso-C_{15:0} (45.2 %) represent the major cellular fatty acids. Catalase, oxidase and acid phosphatase tests are positive. Nitrate is reduced to nitrite. Casein and galactomannan are hydrolysed, but not starch, gelatin or galactan. Tween 20 and 80 are not hydrolysed but glyceryl tributyrates is hydrolysed. Utilizes *N*-acetylglucosamine and sorbitol, but not D-lactose, D-melibiose, sucrose or citrate. The G+C content of the DNA is 39.6 mol%.

The type strain, LMG 21005^T (=NCIMB 14102^T), was isolated from a mural painting from a church in Germany.

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