

## *Bacillus purgationiresistans* sp. nov., isolated from a drinking-water treatment plant

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A Gram-positive, aerobic, non-motile, endospore-forming rod, designated DS22<sup>T</sup>, was isolated from a drinking-water treatment plant. Cells were catalase- and oxidase-positive. Growth occurred at 15–37 °C, at pH 7–10 and with <8 % (w/v) NaCl (optimum growth: 30 °C, pH 7–8 and 1–3 % NaCl). The major respiratory quinone was menaquinone 7, the G + C content of the genomic DNA was 36.5 mol% and the cell wall contained meso-diaminopimelic acid. On the basis of 16S rRNA gene sequence analysis, strain DS22<sup>T</sup> was a member of the genus *Bacillus*. Its closest phylogenetic neighbours were *Bacillus horneckiae* NRRL B-59162<sup>T</sup> (98.5 % 16S rRNA gene sequence similarity), *Bacillus oceanisediminis* H2<sup>T</sup> (97.9 %), *Bacillus infantis* SMC 4352-1<sup>T</sup> (97.4 %), *Bacillus firmus* IAM 12464<sup>T</sup> (96.8 %) and *Bacillus muralis* LMG 20238<sup>T</sup> (96.8 %). DNA–DNA hybridization, and biochemical and physiological characterization allowed the differentiation of strain DS22<sup>T</sup> from its closest phylogenetic neighbours. The data supports the proposal of a novel species, *Bacillus purgationiresistans* sp. nov.; the type strain is DS22<sup>T</sup> (=DSM 23494<sup>T</sup>=NRRL B-59432<sup>T</sup>=LMG 25783<sup>T</sup>).

Gram-positive endospore-forming bacteria of the genus *Bacillus* are widespread in nature and can be found in a large variety of environments, such as terrestrial and aquatic habitats, clinical samples and even spacecraft-assembly facilities. Such ubiquity is also reflected in the phenotypic and phylogenetic diversity of this genus, which currently includes more than 150 species (Logan *et al.*, 2009).

In many world regions, drinking-water treatment involves the disinfection processes of ozonation and chlorination. Such treatments are known to impose dramatic changes in the water's bacterial population, markedly by a shift to Gram-positive bacteria, namely endospore formers of the genus *Bacillus* (Norton & LeChevallier, 2000). A single strain, designated DS22<sup>T</sup>, was isolated from a drinking-water treatment plant located in northern Portugal. In this

plant, the water is collected from a river basin and treated by initial filtration, ozonation and treatment with activated carbon followed by a final disinfection with chlorine. According to our data (unpublished), this treatment reduces the number of total cells by 99 % and the number of cultivable bacterial counts by about 98 %. After membrane filtration of 1 l water from the final reservoir, strain DS22<sup>T</sup> was isolated on mannitol salt agar (MSA; Pronadisa). The isolate was purified by subcultivation on plate count agar (PCA; Pronadisa), which contains (l<sup>-1</sup>): 5 g tryptone, 2.5 g yeast extract, 1 g glucose, 15 g agar. Cultures were incubated at 30 °C. Strain DS22<sup>T</sup> was preserved at –80 °C in nutrient broth with 15 % (v/v) glycerol.

Colony and cell morphology, Gram-staining, cytochrome *c* oxidase and catalase tests, endospore production, motility and casein hydrolysis were analysed according to the methodologies of Murray *et al.* (1994) and Smibert & Krieg (1994). Additional phenotypic characterization was based on methods described previously (Vaz-Moreira *et al.*, 2007a, 2010). Conditions for growth were tested at

Abbreviation: FAMES, fatty acid methyl esters.

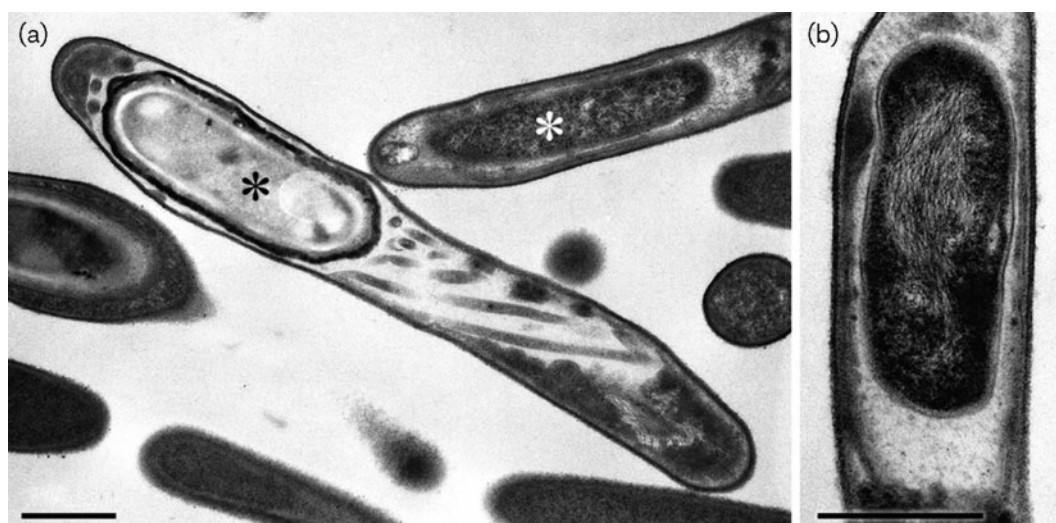
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DS22<sup>T</sup> is FR666703.

6–40 °C, with 0.1–10.0 % (w/v) NaCl and at pH 5.0–10.5. Biochemical and nutritional tests were performed using the API 20NE, API ZYM and API 50CH systems inoculated with API 50 CHB/E medium (bioMérieux), according to the manufacturer's instructions. Additionally, the assimilation of L-alanine, L-histidine, lactic acid, L-proline, propionic acid and L-serine was tested in mineral medium B supplemented with 4 mM  $(\text{NH}_4)_2\text{SO}_4$  and 40 µg vitamins and nitrogenated bases  $\text{l}^{-1}$  and 5 mg amino acids  $\text{l}^{-1}$  (Vaz-Moreira *et al.*, 2007b). The Voges–Proskauer test was assayed in methyl red and Voges–Proskauer media (Oxoid) at 30 °C for 48 h. Hydrolysis of Tween 80 and starch was tested as described by Tiago *et al.* (2004). The ability to grow on MSA and *Bacillus cereus* agar (Bio-Rad) at 30 °C was tested after 4 days of incubation. Lecithinase activity was observed on *B. cereus* agar supplemented with egg yolk (Merck) by the formation of a translucent halo. Production of gas from glucose was tested in glucose broth (Pronadisa). Phenotypic tests, for which results may vary between different laboratories, were assayed in parallel with the reference strains *Bacillus oceanisediminis* H2<sup>T</sup>, *Bacillus horneckiae* NRRL B-59162<sup>T</sup>, *Bacillus muralis* DSM 16288<sup>T</sup>, *Bacillus firmus* DSM 12<sup>T</sup> and *Bacillus infantis* DSM 19098<sup>T</sup>.

Endospores were observed by transmission electron microscopy. Bacteria were fixed for 4 h at 4 °C in 2.5 % glutaraldehyde and 4 % formaldehyde (obtained from hydrolysis of *para*-formaldehyde) diluted with 0.1 M cacodylate buffer (pH 7.4). After washing in the same buffer, bacteria were post-fixed overnight in 2 %  $\text{OsO}_4$  buffered with cacodylate, washed in buffer, treated with 1 % uranyl acetate for 1 h, dehydrated in increasing concentrations of ethanol and embedded in Epon. Ultra-thin sections were stained with uranyl acetate and lead citrate and observed in a JEOL 100CXII transmission electron microscope (60 kV). The G+C content of the

genomic DNA and the respiratory quinones were analysed as described previously (Vaz-Moreira *et al.*, 2007a) using the methods of Mesbah *et al.* (1989) and Tindall (1989), respectively. The polar lipid composition was determined as described previously (Manaia *et al.*, 2004). Fatty acid methyl esters (FAMES) were analysed using cells prepared for 24 h on tryptic casein soy agar (TSA; Pronadisa) at 28 °C. All six strains (the isolate and the five reference strains) grew as expected and provided sufficient cells of comparable physiological age from the third streak on TSA. Cell harvesting and FAME preparation were performed as described by Kuykendall *et al.* (1988). The separation, identification and quantification of the individual FAMES were done using the Sherlock Microbial Identification System version 4.6 (MIDI). FAMES were extracted and analysed twice. Diaminopimelic acid isomers in whole-cell hydrolysates (4 M HCl, 100 °C, 16 h) were analysed by TLC on cellulose plates using described solvent systems (Rhuland *et al.*, 1955; Schleifer & Kandler, 1972).

The nucleotide sequence of the 16S rRNA gene was determined after PCR amplification of total DNA extracts as described elsewhere (Ferreira da Silva *et al.*, 2007). The 16S rRNA gene sequence was compared with others available in the public databases using the FASTA package from EMBL-EBI (<http://www.ebi.ac.uk>). Phylogenetic analysis was conducted using MEGA version 4.0.2 (Tamura *et al.*, 2007). Sequence relatedness was estimated using the model of Jukes & Cantor (1969) and dendrograms were created using the neighbour-joining method. Tree stability was assessed by also constructing trees with the maximum-parsimony and maximum-likelihood methods. Non-homologous and ambiguous nucleotide positions were excluded from the calculations and a total of 1149 nt positions were included in the analysis. For spectroscopic DNA–DNA hybridization, cells were disrupted using a French pressure



**Fig. 1.** Transmission electron micrographs of cells of strain DS22<sup>T</sup>. (a) Cells after growth for 2 days at 30 °C on nutrient agar, showing cell morphology and endospore positions (asterisks). (b) Detail of an endospore. Bars, 0.5 µm.

cell (Thermo Spectronic) and DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multi-cell changer and a temperature controller with an *in situ* temperature probe (Varian).

On TSA after 48 h at 30 °C, strain DS22<sup>T</sup> formed white, slightly convex colonies with irregular margins (approximately 2 mm in diameter). Growth on other media such as PCA, R2A agar (Difco) and *B. cereus* agar was slightly slower. Even though strain DS22<sup>T</sup> was isolated on MSA, growth was not observed on this medium. This could have been because of different culture conditions, as this study isolated the strain with the membrane-filtration method, which avoids direct contact between cells and the medium,

**Table 1.** Distinctive characteristics of strain DS22<sup>T</sup> and its closest phylogenetic neighbours

Strains: 1, *Bacillus purgationiresistans* sp. nov. DS22<sup>T</sup>; 2, *B. horneckiae* NRRL B-59162<sup>T</sup>; 3, *B. oceanisediminis* H2<sup>T</sup>; 4, *B. muralis* DSM 16288<sup>T</sup>; 5, *B. firmus* DSM 12<sup>T</sup>; 6, *B. infantis* DSM 19098<sup>T</sup>. Data are from this study unless otherwise indicated. +, Positive; w, weakly positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6
Colony pigmentation	White	White	White	Pink	White	Pink
Growth on/at:						
MSA	–	+	+	–	–	+
40 °C	–	+	+	+	+	+
9 % NaCl	–	+	+	–	–	+
Nitrate reduction	–	+	+	–*	+	–
Cytochrome <i>c</i> oxidase	+	–	+	+	–	–
Hydrolysis of:						
Starch	–	–	+	–*	–*	+
Aesculin	–	–	–	+	–	w
Assimilation of:						
D-Glucose	–	–	+	+	+	+
L-Arabinose	–	–	–	+	–	–
D-Mannose	–	–	–	+	–	–
D-Mannitol	–	–	–	+	+	+
N-Acetylglucosamine	–	–	+	+	+	w
Maltose	–	+	+	+	+	+
Potassium gluconate	–	+	+	+	–	+
Adipate	–	+	–	–	–	–
Malate	–	+	+	–	w	+
Citrate	–	+	+	+	–	–
L-Alanine	–	+	+	+	–	–
L-Histidine	–	+	+	+	–	–
Lactic acid	–	–	–*	+	+	+
L-Proline	–	+	–	+	+	–
Propionic acid	–	–	–	+	–	–
L-Serine	–	+	+	+	+	–
Enzymes						
Alkaline phosphatase	–	+	+	+	–	–
Leucine arylamidase	+	+	+	+	+	–
Trypsin	–	–	–	–	–	+
α-Chymotrypsin	+	+	+	+	–	w
Acid phosphatase	–	+	–	–	–	–
β-Galactosidase	–	–	–*	+	–	+
α-Glucosidase	–	–	+	+	+	+
DNA G + C content (mol%)†‡	36.5	35.6 <sup>a</sup>	44.8 <sup>b</sup>	ND	46.1–47.4 <sup>d</sup>	40.8 <sup>e</sup>
Isolation source‡	Treated water	Clean room <sup>a</sup>	Sediment <sup>b</sup>	Mural painting <sup>c</sup>	Soil <sup>d</sup>	Sepsis <sup>e</sup>

\*Differs from the original description.

†DNA G + C values for columns 1 and 5 were determined by HPLC; values for columns 2, 3 and 6 were determined by thermal denaturation.

‡Data were taken from: *a*, Vaishampayan *et al.* (2010); *b*, Zhang *et al.* (2010); *c*, Heyrman *et al.* (2005); *d*, Sneath (1986); *e*, Ko *et al.* (2006).

or because the NaCl concentration in MSA (7.5 %) is close to the upper limit for growth of strain DS22<sup>T</sup>. Strain DS22<sup>T</sup> formed subterminal endospores in a non-swollen sporangium (Fig. 1).

The G + C content of the genomic DNA of strain DS22<sup>T</sup> was determined to be  $36.5 \pm 0.12$  mol% (Table 1). Strain DS22<sup>T</sup> had the respiratory quinone menaquinone 7 (MK-7) and the cell wall contained *meso*-diaminopimelic acid. The major cellular fatty acids were iso-C<sub>15:0</sub>, C<sub>16:1</sub>ω7*c* alcohol, anteiso-C<sub>15:0</sub> and iso-C<sub>14:0</sub> (Table 2). The polar lipid analysis showed the predominance of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol (Fig. 2), which are characteristic of the genus *Bacillus* (Kämpfer *et al.*, 2006; Vaishampayan *et al.*, 2010; Zhang *et al.*, 2010). The unidentified phospholipids found in strain DS22<sup>T</sup> are also present in *B. horneckiae* NRRL B-59162<sup>T</sup> (Vaishampayan *et al.*, 2010) and included three phospholipids and two aminophospholipids. One of the aminophospholipids has also been found in *B. subtilis* DSM 10<sup>T</sup> (Kämpfer *et al.*, 2006). However, the glycolipid β-gentiobiosyldiacylglycerol, which is present in *B. subtilis*

DSM 10<sup>T</sup> (Kämpfer *et al.*, 2006), was not detected in strain DS22<sup>T</sup>.

The chemotaxonomic characterization of strain DS22<sup>T</sup> was confirmed by the results of the 16S rRNA gene sequence analysis. In the neighbour-joining tree, strain DS22<sup>T</sup> was placed in a cluster within the genus *Bacillus* (Fig. 3). Strain DS22<sup>T</sup> was most closely related to *B. horneckiae* NRRL B-59162<sup>T</sup> (98.5 % 16S rRNA gene sequence similarity), *B. oceanisediminis* H2<sup>T</sup> (97.9 %), *B. infantis* SMC 4352-1<sup>T</sup> (97.4 %), *B. firmus* IAM 12464<sup>T</sup> (96.8 %) and *B. muralis* LMG 20238<sup>T</sup> (96.8 %).

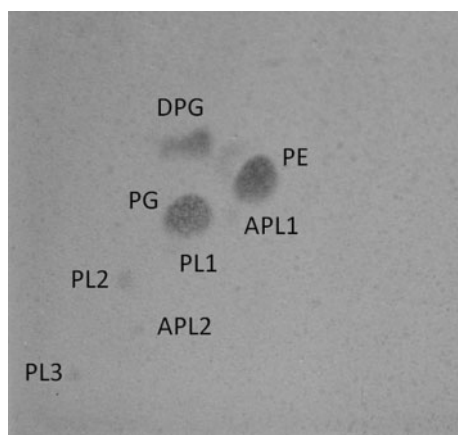
Strain DS22<sup>T</sup> was compared with the reference strains with respect to phenotypic properties (Table 1) and cellular fatty acid composition (Table 2), which allowed the observation of distinctive features. Strain DS22<sup>T</sup> could be distinguished phenotypically from all of the reference strains by its inability to grow at 40 °C and assimilate any of the tested carbon sources, from *B. muralis* DSM 16288<sup>T</sup> and *B. infantis* DSM 19098<sup>T</sup> by colony colour, from *B. horneckiae* NRRL B-59162<sup>T</sup>, *B. oceanisediminis* H2<sup>T</sup> and *B. firmus* DSM 12<sup>T</sup> by its inability to reduce nitrate, and from *B.*

**Table 2.** Cellular fatty acid compositions of strain DS22<sup>T</sup> and its closest phylogenetic neighbours

Strains 1, *Bacillus purgationiresistans* sp. nov. DS22<sup>T</sup>; 2, *B. horneckiae* NRRL B-59162<sup>T</sup>; 3, *B. oceanisediminis* H2<sup>T</sup>; 4, *B. muralis* DSM 16288<sup>T</sup>; 5, *B. firmus* DSM 12<sup>T</sup>; 6, *B. infantis* DSM 19098<sup>T</sup>. All data were taken from this study. Cells were cultivated on TSA at 28 °C for 24 h. —, Not detected.

Fatty acid (%)	1	2	3	4	5	6
Saturated straight-chain						
C <sub>14:0</sub>	0.9	0.7	6.4	3.9	13.4	1.7
C <sub>15:0</sub>	—	—	—	—	—	—
C <sub>16:0</sub>	0.5	0.8	13.5	4.5	15.7	0.9
C <sub>16:0</sub> N alcohol	—	—	—	—	0.3	—
Unsaturated straight-chain						
C <sub>16:1</sub> ω5 <i>c</i>	—	—	1.4	—	3.7	—
C <sub>16:1</sub> ω11 <i>c</i>	2.5	2.1	13.1	9.4	14.6	1.5
C <sub>16:1</sub> ω7 <i>c</i> alcohol	15.2	9.8	3.2	1.9	1.2	1.5
Summed feature 3*	—	—	1.4	—	1.2	—
Saturated branched-chain						
iso-C <sub>13:0</sub>	0.2	—	—	0.3	—	0.2
iso-C <sub>14:0</sub>	9.2	4.1	3.9	3.8	2.3	1.3
iso-C <sub>15:0</sub>	41.9	51.1	27.7	27.7	23.9	49.2
iso-C <sub>16:0</sub>	6.7	5.9	4.1	1.1	1.7	1.3
iso-C <sub>17:0</sub>	1.1	3.2	1.6	1.2	0.9	1.4
anteiso-C <sub>13:0</sub>	—	—	—	0.3	—	0.1
anteiso-C <sub>15:0</sub>	14.2	13.5	15.8	42.1	16.5	29.9
anteiso-C <sub>17:0</sub>	2.3	3.3	3.4	1.0	2.5	5.4
Unsaturated branched-chain						
iso-C <sub>15:1</sub> ω9 <i>c</i>	—	—	—	0.5	—	0.5
iso-C <sub>17:1</sub> ω10 <i>c</i>	1.8	2.7	0.8	1.5	0.4	1.3
Summed feature 4*	3.5	2.9	2.0	0.9	1.5	3.7

\*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C<sub>16:1</sub>ω7*c* and/or C<sub>16:1</sub>ω6*c*. Summed feature 4 consisted of iso-C<sub>17:1</sub> and/or anteiso-C<sub>17:1</sub>.

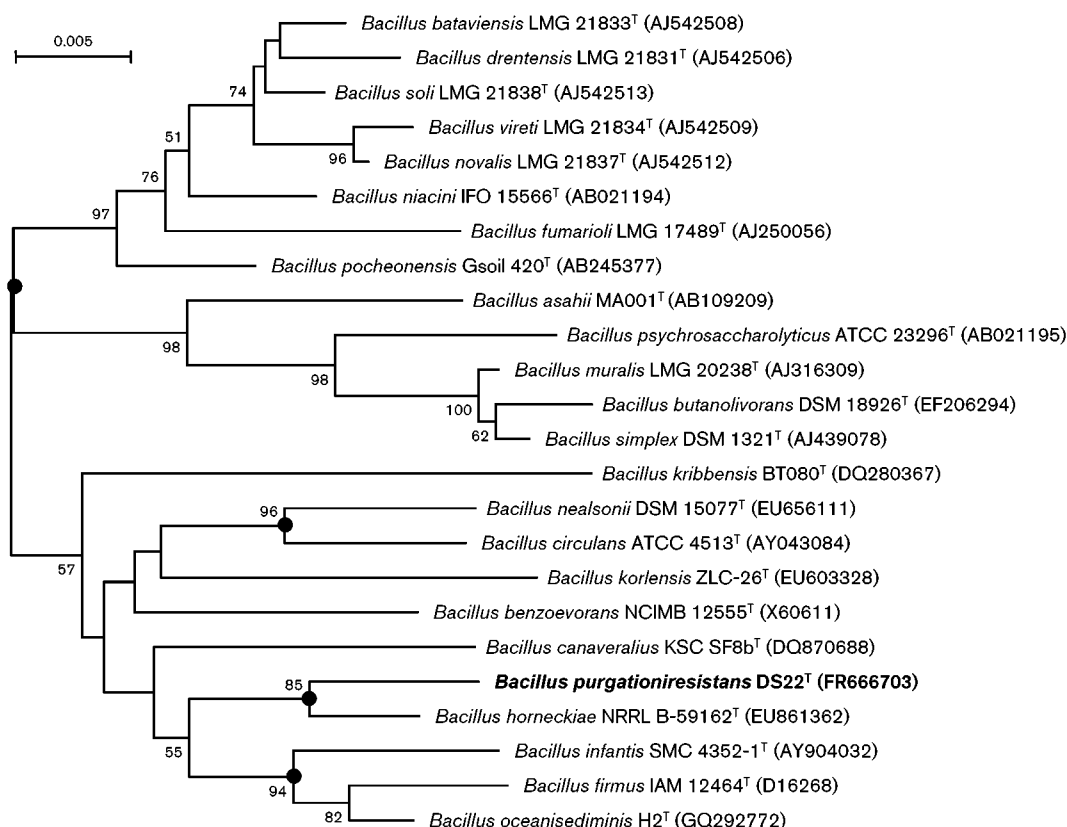


**Fig. 2.** Polar lipid profile of strain DS22<sup>T</sup> after separation by two-dimensional TLC, spraying with 50 % (v/v) aqueous sulfuric acid and charring at 160 °C for 25 min. APL, Unknown aminophospholipid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown phospholipid.

*horneckiae* NRRL B-59162<sup>T</sup> by its inability to produce acid from API 50CH carbon sources. Strain DS22<sup>T</sup> could also be distinguished from the reference strains on the basis of differences in the fatty acid composition, mainly because of the proportions of C<sub>16:1ω7c</sub> alcohol, iso-C<sub>14:0</sub> and iso-C<sub>15:0</sub>. The phenotypic and fatty acid data suggested that strain DS22<sup>T</sup> represented a distinct novel species.

Low DNA–DNA relatedness was observed between strain DS22<sup>T</sup> and its closest neighbours: 13.6 and 7.6 % with *B. oceanisediminis* H2<sup>T</sup>, 32.3 and 30.0 % with *B. horneckiae* NRRL B-59162<sup>T</sup>, 17.5 and 12.9 % with *B. muralis* DSM 16288<sup>T</sup>, 13.8 and 13.7 % with *B. infantis* DSM 19098<sup>T</sup>, and 16.6 and 8.1 % with *B. firmus* DSM 12<sup>T</sup>. These values were clearly below the threshold of 70 % DNA–DNA relatedness recommended for the definition of bacterial species (Wayne *et al.*, 1987).

On the basis of the differentiation of strain DS22<sup>T</sup> from its closest phylogenetic neighbours by 16S rRNA gene sequence analysis, DNA–DNA relatedness, cellular fatty acids and physiological characters, strain DS22<sup>T</sup> is proposed to represent a novel species, *Bacillus purgationiresistans* sp. nov.



**Fig. 3.** Neighbour-joining phylogenetic tree derived from 16S rRNA gene sequences, showing the relationships of strain DS22<sup>T</sup> with members of the genus *Bacillus*. Bootstrap values (≥ 50 %) based on 1000 resamplings are shown at branch nodes. Filled circles indicate that the corresponding nodes were recovered in trees generated with the maximum-parsimony and maximum-likelihood methods. Bar, 1 substitution per 200 nt.

## Description of *Bacillus purgationiresistans* sp. nov.

*Bacillus purgationiresistans* (pur.ga.ti.o.ni.re.sis'tans. L. n. *purgatio* -onis a cleansing, purification; L. part. adj. *resistans* resisting; N.L. part. adj. *purgationiresistans* resisting cleansing, purification).

Colonies are white and slightly convex (~2 mm diameter) with irregular edges on TSA after 48 h at 30 °C. Forms pink colonies on *B. cereus* agar. Rods (3.5 µm long and 0.5 µm wide in very young cultures) are non-motile, aerobic and Gram-positive with subterminal endospores in a non-swollen sporangium. Catalase- and cytochrome *c* oxidase-positive. Grows at 15–37 °C, at pH 7–10 and with <8 % NaCl (optimum growth at about 30 °C, pH 7–8 and 1–3 % NaCl). Does not grow on MSA. Nitrate is not reduced. Citrate is not used. H<sub>2</sub>S, indole and acetoin are not produced. No fermentation or gas production from D-glucose. Gelatin, Tween 80 and casein are hydrolysed, but starch and aesculin are not. Esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase and lecithinase are produced, but arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, acid phosphatase, alkaline phosphatase, lipase (C14), cystine arylamidase, trypsin, α- and β-galactosidases, β-glucuronidase, α- and β-glucosidases, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not produced. Acid is not produced from any of the carbon sources in the API 50CH system. None of the carbon sources in the API 20E and API 20NE systems are oxidized or assimilated; also, L-alanine, L-histidine, lactic acid, L-proline, propionic acid and L-serine tested in mineral medium are not assimilated. The predominant cellular fatty acids are iso-C<sub>15:0</sub>, C<sub>16:1</sub>ω7c alcohol, anteiso-C<sub>15:0</sub> and iso-C<sub>14:0</sub> and the major respiratory quinone is MK-7 (100 %). The predominant polar lipids are phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The peptidoglycan contains meso-diaminopimelic acid.

The type strain is DS22<sup>T</sup> (=DSM 23494<sup>T</sup>=NRRL B-59432<sup>T</sup>=LMG 25783<sup>T</sup>), isolated from water of the final reservoir of a drinking-water treatment plant. The DNA G + C content of the type strain is 36.5 mol%.

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