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Published in:
International Journal of Systematic and Evolutionary Microbiology

DOI:
10.1099/ijs.0.026740-0

Publication date:
2012

Document Version
Peer reviewed version

IJSEM Papers in Press. Published March 11, 2011 as doi:10.1099/ijs.0.026740-0

Emended descriptions of *Bacillus sporothermodurans* and *Bacillus oleronius* with the inclusion of dairy farm isolates of both species

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Subject category: Taxonomic note

Running title: Emended descriptions of *B. sporothermodurans* and *B. oleronius*

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Bacillus sporothermodurans is an industrially important micro-organism because of its ability to produce endospores which resist ultra high temperature (UHT) and industrial sterilization processes. It was described by Pettersson *et al.* (1996) based on seven genetically homogeneous isolates all from UHT-milk. *Bacillus oleronius*, the closest phylogenetic neighbor of *B. sporothermodurans*, was described by Kuhnigk *et al.* (1995), based on a single strain, isolated from the hindgut of the termite *Reticulitermes santonensis*. A polyphasic study of a heterogeneous collection of *B. sporothermodurans* and *B. oleronius* strains isolated from various sources and geographic origins led to an emended description of both species. Additional data presented are the results of fatty acids, quinones and/or cell wall analysis (polar lipids). DNA-DNA hybridizations confirmed 3 subgroups of strains obtained after SDS-PAGE analysis of cellular proteins as *B. sporothermodurans*. One named *B. sporothermodurans* strain (R-7489) was reclassified as a *Bacillus fordii* strain. The phenotypic profiles of both species were rather heterogeneous, sometimes different from the original descriptions and did not differ in a large number of characters, although *B. oleronius* generally gave stronger reactions in its positive tests than did *B. sporothermodurans*; the variable and weak reactions for both organisms with some substrates blurred the distinction between both. However, differences in polar lipid, SDS-PAGE and menaquinone profiles clearly allow distinction between the two species.

Bacillus sporothermodurans was described by Pettersson *et al.* (1996) as a species producing highly heat-resistant endospores that may survive ultra high temperature (UHT) treatment of milk. The spore resistance to UHT-conditions (typically 140°C for a few seconds) was proven both with an isolate from UHT-milk (Huemer *et al.*, 1998) as with spores naturally occurring in contaminated UHT-milk (Scheldeman *et al.*, 2006). Isolates of this species were also found in sterilized milk and milk products such as UHT-cream, chocolate milk, evaporated milk and reconstituted milk (Herman *et al.*, 2000). As a consequence, surviving spores may germinate and grow in the consumer milk or milk product causing the technological problem of non-sterility. This problem has been encountered by different dairy companies worldwide with the

remarkable observation that one clone (HRS-clone) was concerned in the majority of the cases; only a few German isolates were found to be different from this HRS-clone by a non-hierarchical three-dimensional scaling of molecular typing data (Guillaume-Gentil *et al.* 2002). In the same study, farm isolates of *B. sporothermodurans* isolated from raw milk, feed concentrate and silage were shown to be genetically very heterogeneous and different from the HRS-clone. These farm isolates were identified as *B. sporothermodurans* by a specific PCR and for an isolate from raw milk and from feed concentrate also by DNA-DNA hybridization (Scheldeman *et al.*, 2002). In the same study as well as in Vaerewijck *et al.* (2001), *Bacillus oleronius* was isolated from raw milk and feed concentrate. Hitherto, this species had only been isolated from the hindgut of a termite and described on the basis of one isolate from this source (Kuhningk *et al.*, 1995).

Because of the observed genetic heterogeneity, the original description of *B. sporothermodurans*, which was based on only a few genetically homogeneous UHT isolates belonging to the HRS-clone (Petterson *et al.*, 1996), may no longer be adequate. One example is the upper growth limit reported by Petterson *et al.* (1996) to be 50 °C for the type strain but shown to be as high as 55 °C for farm isolates (Scheldeman *et al.*, 2002). This prompted us to prepare an emended description of this industrially important organism as well as of its phylogenetic neighbour, *B. oleronius*, with the inclusion of genetically heterogeneous farm isolates for both species.

The *B. sporothermodurans* and *B. oleronius* strains used are listed in Table 1. They were grown on Brain Heart Infusion (BHI) (Oxoid) supplemented with Bacteriological agar no. 1 (15 g l^{-1}) (Oxoid) and filter sterilized vitamin B_{12} (1 mg l^{-1}) at 37 °C for 48 h for all analyses (unless otherwise indicated). The *B. sporothermodurans* specific PCR (BSPO-PCR) described by Scheldeman *et al.* (2002) was carried out on all strains listed above as well as on some additional *Bacillus fordii* strains described by Scheldeman *et al.* (2004). For determination of the G + C content of some selected strains, approximately 1 gram of biomass was harvested from agar plates and DNA was purified as in Logan *et al.* (2000) with the modifications as

outlined by Heyndrickx *et al.* (2004). The G + C content of DNA was determined by HPLC using further specifications given by Logan *et al.* (2000). DNA-DNA hybridizations were performed for strains *B. sporothermodurans* LMG 17668^T, LMG 19637 and R-6778, for *Bacillus shackletonii* LMG 18435^T and for *Bacillus acidicola* DSM 14745^T using a modification (Willems *et al.*, 2001) of the microplate method described by Ezaki *et al.* (1989) with a reassociation temperature of 37 °C. All strains (together with the additional *B. fordii* strains mentioned above), grown on supplemented BHI as outlined above, were subjected to SDS-PAGE analysis of whole cell proteins according to Pot *et al.* (1994). The SDS-PAGE data were collected and interpreted as described by Vauterin & Vauterin (1992). For gas chromatographic analysis of methylated cellular fatty acids (FAME), cells of the same strains were grown for 24h on supplemented BHI. Further analysis was performed as described by Dawyndt *et al.* (2006). All *B. sporothermodurans* and *B. oleronius* strains together with type (and reference) strains of unreactive or weakly reactive species were phenotypically characterized by the methods of Logan & Berkeley (1984): thirteen routine biochemical tests, and 48 tests for acid production from a range of carbohydrates were made using the API 20E and 50CHB kits (bioMérieux), following cultivation of *B. sporothermodurans* and *B. oleronius* strains on BHI agar supplemented with vitamin B_{12} at 37 ºC for 24 hours. Other phenotypic characters were determined as described by Heyrman *et al.* (2004), but using BHI or BHI agar supplemented with vitamin B_{12} as the basal media. Phenotypic data for other species were obtained on Tryptic Soy Agar (TSA) (Oxoid). For peptidoglycan analysis, whole cell hydrolysates (4N HCl, 100°C, 16 hours) of *B. sporothermodurans* LMG 17668^T and *B. oleronius* LMG 17952^T were subjected to thin layer chromatography on cellulose plates using the solvent system of Rhuland *et al.* (1955). Quinones for both strains were determined as described by Groth *et al.* (1996). For polar lipid determination, cells of both type strains were grown for 24h at 37 °C in BHI broth (1liter flasks) supplemented with vitamin B_{12} and analyses were carried out by the Identification Service of the DSMZ (Braunschweig,

Germany) by two-dimensional silica gel thin-layer chromatography and detection with appropriate reagents (Tindall *et al.*, 1990a & b).

Numerical analysis of SDS-PAGE patterns of whole cell proteins (supplementary Fig.) revealed three groups that all contain strains sharing at least 80 % similarity between their SDS-PAGE profiles. Groups 1 and 2 are subdivided at about 75 % similarity and group 3 branches at about 65 %. The first group is quite heterogeneous and contains the *B. sporothermodurans* isolates. This group can be further subdivided in three SDS-PAGE subgroups (I to III). The first subgroup I contains sixteen strains, including the type strain, the UHT-milk strains and strains from various other sources (sterilized milk, raw milk, soy and feed concentrate). The second subgroup II contains three Belgian strains (R-6777, R-6778 and R-6779) from different sources. The third subgroup III contains five Belgian strains isolated from feed concentrate. The second main SDS-PAGE group contains different strains previously described as *B. fordii* (Scheldeman *et al.*, 2004), as well as one strain (R-7489) labelled as *B. sporothermodurans*. The attribution of R-7489 to *B. sporothermodurans* is based on a positive reaction in the *B. sporothermodurans* specific PCR (BSPO-PCR) that was thought to be specific for *B. sporothermodurans* (Scheldeman *et al.*, 2002). However, the type strain of *B. fordii* (not described at the time) also gives a weak amplicon in the BSPO-PCR. On the basis of the performed analysis, R-7489 should be attributed to *B. fordii*. The third SDS-PAGE group in the SDS-PAGE clustering contains named *B. oleronius* strains and five strains putatively identified as *B. oleronius* by fatty acid analysis (Scheldeman *et al.*, 2004). SDS-PAGE thus confirmed the attribution of the latter strains to *B. oleronius*. The *B. oleronius* strains can be distinguished on the basis of their SDS-PAGE profile by a strong band in the region of 85 kDalton.

B. sporothermodurans M215^T (= LMG 17668^T) (U49078) shares 98.2% 16S rRNA gene sequence similarity with *B. oleronius* DSM 9356^T (= LMG 17952^T) (X82492), 97.6% with *B. acidicola* 105-2^T (= DSM 14745^T) (AF547209) and 97.2% with *B. shackletonii* LMG 18435^T (AJ250318). *B.*

oleronius DSM 9356^T (= LMG 17952^T) (X82942) shares 98% 16S rRNA gene sequence similarity with *B. acidicola* 105-2^T (= DSM 14745^T) and 96.6% with *B. shackletonii* LMG 18435^T (AJ250318). The species status of both *B. sporothermodurans* and *B. oleronius*, showing >97% 16S rRNA gene sequence similarity with the above mentioned closely related species, was confirmed by low DNA-DNA hybridisation values (< 70%) as previously determined by Scheldeman *et al.* (2002) and Albert *et al.* (2005), and as determined in this study (Table 2). DNA-DNA hybridisation values determined by Scheldeman *et al.* (2002) and during this study for *B. sporothemodurans* strains LMG 17833 and LMG 19620 (both belonging to SDS-PAGE subgroup I), R-6778 (SDS-PAGE subgroup II) and LMG 19637 (SDS-PAGE subgroup III) with the *B. sporothermodurans* type strain LMG 17668^T confirmed the allocation of all three observed SDS-PAGE subgroups to the species *B. sporothermodurans* (hybridisation values > 70% (Table 2).

Fatty acid analysis did not allow a clear distinction between *B. sporothermodurans* and *B. oleronius* (Table 3). The *B. sporothermodurans* strains showed some more heterogeneity than the *B. oleronius* strains (as can be derived from the greater standard deviation values), but this heterogeneity could not be linked to the heterogeneity observed in SDS-PAGE analysis. Both species could clearly be differentiated from *B. fordii* by lower amounts of the dominant fatty acid iso-$C_{15:0}$, and higher amounts of anteiso-$C_{17:0}$.

B. sporothermodurans strains often grew poorly and benefited from the addition of vitamin B_{12} to the medium. They also showed weak reactions in the phenotypic tests, and these reactions were not appreciably enhanced by the addition of vitamin B_{12} to the test media. The characters were not only weak but also variable, so that the phenotypic data do not give a clearly distinctive profile that is characteristic for the species. *B. sporothermodurans* UHT milk isolates not only grew poorly but also sporulated poorly, yet their spores showed very high heat resistances (Scheldeman *et al.*, 2006). This property of resistance to high temperatures decreased with subculture in the laboratory. Sporulation was enhanced by the addition of soil extract to the BHI medium supplemented with

$MnSO_4$ and vitamin B_{12}, but it is not known whether such spores have enhanced heat resistance. Isolates from farm environments grew more readily than UHT milk isolates and their spores did not show very high heat resistances (Scheldeman *et al.*, 2006). Strains of *B. oleronius* did not require the growth medium to be supplemented with vitamin B_{12}, and all strains sporulated well. The phenotypic profiles of the two species did not differ in a large number of characters, although *B. oleronius* generally gave stronger reactions in its positive tests than did *B. sporothermodurans*; the variable and weak reactions for both organisms with some other substrates blurred the distinction between the two species (Table 4). The differentiation table includes the phylogenetic closest relatives of both species, namely *B. acidicola* and *B. shackletonii*, as well as phenotypically related species.

The type strains of *B. sporothermodurans* and *B. oleronius* contained both *meso*-diaminopimelic acid as diagnostic diamino acid of the cell wall peptidoglycan. This means that both species show the peptidoglycan type A1γ (this can be concluded from the fact that *meso*-diaminopimelic acid has been reported up to now only for the peptidoglycan type A1γ and for three variatons of the type A4 γ; these variations of the type A4 γ have been found so far exclusively in the genera *Brachybacterium* and *Dermabacter*, with which *B. sporothermodurans* and *B. oleronius* have no relationship). The major polar lipids of the type strains of *B. sporothermodurans* and *B. oleronius*, LMG 17668^T and LMG 17952^T were diphosphatidylglycerol (DPG), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE), typical for the genus *Bacillus*. Minor amounts of an aminophospholipid (PN) and an unidentified phospholipid could also be detected. However, both species can be differentiated from each other based on the presence of three other unidentified phospholipids (PL's) in *B. oleronius*.

The type strain of *B. sporothermodurans* contained the menaquinones MK-7, MK-8 and MK-6 (in the ratio 51 : 45 : 1), while the type strain of *B. oleronius* contained MK-7, MK-8 and MK-9 (ratio 52.6 : 46.9 : 0.4).

Overall, it has been shown here that strains of *B. sporothermodurans* and *B. oleronius* are rather heterogeneous in phenotypic characteristics but belong to these respective valid species. Both species are difficult to differentiate from each other on the basis of biochemical and physiological characteristics. The identification of members of these species requires the use of a polyphasic approach based on well defined phenotypic, chemotaxonomic and/or genetic taxonomic properties as described above. An emended description of both species is warranted as given below, including new data on fatty acid analysis, diagnostic diamino acid, peptidoglycan type, polar lipids and menaquinone profile for *B. sporothermodurans,* and polar lipids and menaquinone profile for *B. oleronius*. Furthermore, some additions and modifications were made on cell morphology and spore properties and on biochemical characteristics based on API 50CHB and API 20E test results as contradictions were observed with the original species descriptions. Specifically for *B. sporothermodurans*, more of these tests (e.g. API 50 CHB) appeared positive in our hands.

Emended description of *Bacillus sporothermodurans*.

Bacillus sporothermodurans (spo.ro.ther.mo.du'rans, Gr. n. *spora*, seed, and in biology a spore; Gr. adj. *thermos*, warm, hot; L. part. adj. *durans*, resisting. N. L. part adj. *sporothermodurans*, with heat-resisting spores).

Aerobic, Gram-positive cells that usually occur as motile, thin rods in chains. Strains require vitamin B_{12} for satisfactory growth. After 2d on Brain Heart Infusion (BHI) agar supplemented with 5 mgl^{-1} $MnSO_4$ and with 1 mg l^{-1} vitamin B_{12}, colonies are 1-2mm diameter, flat, circular, entire, beige or cream and smooth or glossy in appearance. They bear spherical to ellipsoidal endospores which lie in paracentral and subterminal, sometimes terminal, positions within slightly swollen and unswollen sporangia. Sporulation is infrequent but can be enhanced by using BHI-soil extract agar supplemented with vitamin B_{12} and $MnSO_4$. Spores of strains isolated from UHT milk grow poorly and sporulate poorly, but their spores show very high heat resistance and have the ability to survive

ultra high temperature treatment (UHT). This very high heat resistance may decrease upon subculture. Isolates from farm environments may grow more readily than UHT milk isolates but be less heat resistant. Oxidase and catalase positive. Casein and starch are not hydrolysed. In the API 20E strip: nitrate is reduced to nitrite, the Voges-Proskauer reaction is variable, citrate utilisation is variable, hydrogen sulphide and indole are not produced, and the ONPG reaction is negative. Gelatin and aesculin are hydrolysed, urea is not. Growth may occur between 20 and 55 ^{o}C, with an optimum of about 37 ^{o}C. Growth occurs between pH 5 and 9, and NaCl is tolerated up to 5% (w/v). In the API 50CHB gallery, acid without gas is produced from *N*-acetyl-glucosamine, D-glucose, D-fructose, maltose, and from sucrose and D-trehalose by most strains, but reactions may be weak. Acid production from the following carbohydrates is variable: amygdalin, arbutin, D-cellobiose, gentiobiose, glycerol, mannitol, D-mannose, D-melezitose, methyl-D-glucoside, salicin, starch (weak), D-tagatose, D-turanose and xylitol (weak). Acid is not produced from the following carbohydrates: adonitol, D-arabinose, L-arabinose, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, L-fucose, galactose, gentiobiose, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, glycogen, inulin, lactose, D-lyxose, D-melibiose, *meso*-inositol, methyl-D-mannoside, methyl-xyloside, D-raffinose, rhamnose, ribose, sorbitol, L-sorbose and D-xylose.

The major cellular fatty acids (mean percentage ± standard deviation of total fatty acids) after 24 h growth on BHI supplemented with vitamin B_{12} at 37 °C are: iso-$C_{15:0}$ (33.28 ± 4.42), anteiso-$C_{15:0}$ (24.87 ± 2.32) and anteiso-$C_{17:0}$ (19.58 ± 3.45). The following fatty acids are present in smaller amounts (mean percentage ± standard deviation of total fatty acids): $C_{16:0}$ (6.69 ± 2.32), iso-$C_{16:0}$ (6.40 ± 1.61), iso-$C_{17:0}$ (5.29 ± 1.22), iso-$C_{14:0}$ (1.77 ± 0.70) and $C_{14:0}$ (1.51 ± 0.81). The type strain has *meso*-diaminopimelic acid as the cell wall diagnostic diamino acid and contains the polar lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminophospholipid and a phospholipid. Major menaquinones are MK7 and MK8. The G+C content is 36.7 mol% for the type strain LMG 17668^{T} (=LMG 17894^{T} (contains colony types t_1 and t_2), = DSM 10599^{T}, = M215^{T}, = MB 581^{T}) and 36.2 mol% and 36.5 mol% for strains LMG 19620 and LMG 17883, respectively;

the 16S rRNA gene sequence of the type strain is deposited at EMBL/GenBank under accession numbers U49078 (type I), U49079 (type II) and U49080 (type III). In the variable characters listed above, the type strain gives the following reactions: the Voges-Proskauer reaction is positive, citrate utilisation is negative, and acid without gas is produced from arbutin, D-cellobiose, glycerol, mannitol, D-melezitose, salicin, D-tagatose, D-turanose and xylitol, but not from amygdalin, gentiobiose, D-mannose, methyl-D-glucoside and starch. Spores, though scanty, are ellipsoidal, terminal, and do not swell the sporangia.

Emended description of *Bacillus oleronius*.

Bacillus oleronius (o.le.ro'ni.us, N. L. masc. adj. *oleronius*, pertaining to the island îsle d'Oléron (France), the place where the organism was first isolated from the hindgut of a termite).
Cells are non-motile, Gram-negative, medium-sized rods, that occur singly and in pairs, and sometimes form short chains of 3-4 cells. They bear ellipsoidal endospores that lie in subterminal and paracentral positions within swollen sporangia. Spores do not show very high heat resistance. After 2 d on TSA colonies are approximately 1-2 mm diameter, circular, entire, shiny, beige or cream and butyrous with slightly translucent edges. Organisms are strictly aerobic and catalase positive. Growth may occur between 30 and 50 ^{o}C, with an optimum of 37 ^{o}C. Growth occurs at pH 5.7 and at 6.8. NaCl is tolerated up to 7% (w/v). Casein is not hydrolysed and starch is sometimes hydrolysed weakly. In the API 20E strip: nitrate is reduced to nitrite, the Voges-Proskauer reaction is variable, citrate is not utilized, hydrogen sulphide and indole are not produced, and the ONPG reaction is negative. Aesculin is hydrolysed, gelatin is weakly hydrolysed and urea is not hydrolysed. In the API 50CHB gallery: acid without gas is produced from *N*-acetyl-glucosamine, D-cellobiose, D-fructose, D-glucose, mannitol and D-tagatose. Acid production from the following carbohydrates is variable, and when positive it is weak: galactose, glycerol, maltose, D-mannose, ribose, salicin, starch and D-trehalose. Acid is not produced from the following carbohydrates: adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, dulcitol, erythritol, D- and L-

fucose, gentiobiose, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, methyl-D-glucoside, glycogen, *meso*-inositol, inulin, lactose, D- and L-lyxose, methyl-D-mannoside, D-melibiose, D-melezitose, rhamnose, D-raffinose, sorbitol, L-sorbose, sucrose, D-turanose, xylitol, D- and L-xylose and methyl-xyloside.

The major cellular fatty acids (mean percentage $\pm$ standard deviation of total fatty acids) after 24 h growth on BHI supplemented with vitamin B_{12} at 37 °C are: iso-$C_{15:0}$ (39.24 ± 1.38), anteiso-$C_{15:0}$ (22.89 ± 2.22) and anteiso-$C_{17:0}$ (20.78 ± 0.85). The following fatty acids are present in smaller amounts (mean percentage $\pm$ standard deviation of total fatty acids): iso-$C_{17:0}$ (6.76 ± 1.25), iso-$C_{16:0}$ (5.37 ± 0.50), $C_{16:0}$ (3.35 ± 0.29) and iso-$C_{14:0}$ (1.09 ± 0.15). The type strain has *meso*-diaminopimelic acid as the cell wall diagnostic diamino acid and contains the polar lipids diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, aminophospholipid and four different phospholipids. Major menaquinones are MK7 and MK8. The G+C content is 35.2 mol% for the type strain LMG 17952^T (= DSM 9356^T, Rt 10^T) and 34.7 mol% for LMG 17886; the 16S rRNA gene sequence of the type strain is deposited at EMBL/GenBank under accession number X82492. In the variable characters listed above the type strain is positive for the Voges-Proskauer reaction, and acid without gas is produced from: glycerol, maltose, ribose, starch (weak) and D-trehalose, but not from galactose, D-mannose or salicin.

ACKNOWLEDGEMENTS

We are very grateful to Elly Engels for excellent technical assistance. We are most grateful to bioMérieux sa for providing API materials and for supporting G. F., M. R.-D. and A.D.; P. D. V. is indebted to the Fund for Scientific Research - Flanders for personnel and research grant G.0156.02. J. H. is most grateful to FWO-Vlaanderen for a personal grant. We thank J. P. Euzéby for revision of the etymology.

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Table 1: *B. sporothermodurans* and *B. oleronius* strains used and their origin.

Original species identification	Eventual new species identification	LMG number	R-number*	Other designations†	Source
B. sporothermodurans		17668^T	1948	LMG 17894^T (t_1 and t_2)‡, M215^T, MB 581^T, DSM 10599^T	UHT-milk, Italy
B. sporothermodurans		17897	1951	MB 372	UHT-milk, Germany
B. sporothermodurans		18461		MB 512	UHT-milk, Belgium
B. sporothermodurans		18465		MB 373	UHT-milk, Germany
B. sporothermodurans			17269	MB 1320	sterilized milk, Dominican republic
B. sporothermodurans		17883	1937	MB 385	raw milk, Belgium
B. sporothermodurans		19637	3247	MB 1668	feed concentrate, Belgium
B. sporothermodurans			6645	MB 1493	feed concentrate, Belgium
B. sporothermodurans			6662	MB 1494	soy, Belgium
B. sporothermodurans			6665	MB 1495	soy, Belgium
B. sporothermodurans			6689	MB 1496	feed concentrate, Belgium
B. sporothermodurans			6701	MB 1497	feed concentrate, Belgium
B. sporothermodurans			6710	MB 1498	feed concentrate, Belgium
B. sporothermodurans			6713	MB 1499	feed concentrate, Belgium
B. sporothermodurans			6723	MB 1500	feed concentrate, Belgium
B. sporothermodurans			6725	MB 1501	feed concentrate, Belgium
B. sporothermodurans			6744	MB 1502	feed concentrate, Belgium
B. sporothermodurans			6777	MB 1503	soy, Belgium
B. sporothermodurans			6778	MB 1504	pulp, Belgium
B. sporothermodurans			6779	MB 1505	silage, Belgium
B. sporothermodurans			6786	MB 1506	feed concentrate, Belgium
B. sporothermodurans			7342	MB 1508	feed concentrate, Belgium
B. sporothermodurans	*B. fordii*		7489	MB 1509	feed concentrate, Belgium
B. sporothermodurans		19617	3227	MB 1316	feed concentrate, Belgium
B. sporothermodurans		19620	3299	MB 1317	feed concentrate, Belgium
B. oleronius		17882	1936	MB 382	raw milk, Belgium
B. oleronius		17884	1938	MB 386	raw milk, Belgium

B. oleronius		17886	1940	MB 397	raw milk, Belgium
B. oleronius		17887	1941	MB 398	raw milk, Belgium
B. oleronius		17952^{T}		DSM 9356^{T}	termite
B. oleronius		19619	3297	MB 1318	feed concentrate, Belgium
B. oleronius.			6450		milk installation, Belgium
B. oleronius			6691		feed concentrate, Belgium
B. oleronius			7770	MB 2102	milk installation, Belgium
Bacillus sp. [§]	*B. oleronius*	6724			feed concentrate, Belgium
Bacillus sp. [§]	*B. oleronius*	6464			filter cloth, Belgium
Bacillus sp.[§]	*B. oleronius*	6955		MB 2101	milk installation, Belgium
Bacillus sp. [§]	*B. oleronius*	6463			filter cloth, Belgium
Bacillus sp. [§]	*B. oleronius*	6504			milk installation, Belgium
B. fordii		22080^{T}	7190^{T}	DSM 16014^{T}	raw milk, Belgium
				MB 1507^{T}	

*: R, Research collection of the LMG Bacteria Culture Collection; †: MB, collection of the molecular bacteriology lab of the Institute of Agricultural & Fisheries Reseach (ILVO), Technology and Food Science Unit, Melle, Belgium; ‡: colony types; §: strains putatively identified as *B. oleronius* by FAME (Scheldeman *et al.*, 2004).

398 Table 2: DNA-DNA hybridisation values for *Bacillus sporothermodurans* strains and closest
399 relatives.

			LMG 17668^T	LMG 18435^T	DSM 14745^T	LMG 17952^T
Bacillus sporothermodurans	SDS-PAGE subgroup I	LMG 17668^T	100%			
		LMG 17833	81%*			
		LMG 19620	88%*			
	SDS-PAGE subgroup II	R-6778	83%			
	SDS-PAGE subgroup III	LMG 19637	76%			
Bacillus shackletonii		LMG 18435^T	42%	100%		
Bacillus acidicola		DSM 14745^T	35%†	25%	100%	
Bacillus oleronius		LMG 17952^T	16%*	ND	32%†	100%

400 *: data from Scheldeman *et al.* (2002); †: data from Albert *et al.* (2005)

401 **Table 3**: Cellular fatty acid methyl ester profiles of *B. sporothermodurans*, *B. oleronius* and *B.*
402 *fordii*. Presented values are averages for the number of strains analysed (mentioned between
403 brackets) with the corresponding standard deviation.

Fatty acids	*B. sporothermodurans* (n = 24)	*B. oleronius* (n = 14)	*B. fordii* (n = 2)
Saturated fatty acids			
$C_{14:0}$	1.51 ± 0.81	< 1.00	< 1.00
$C_{16:0}$	6.69 ± 2.32	3.35 ± 0.29	2.65 ± 0.10
Branched fatty acids			
iso-$C_{14:0}$	1.77 ± 0.70	1.09 ± 0.15	< 1.00
iso-$C_{15:0}$	33.28 ± 4.42	39.24 ± 1.38	47.19 ± 0.86
anteiso-$C_{15:0}$	24.87 ± 2.32	22.89 ± 2.22	25.55 ± 5.49
iso-$C_{16:0}$	6.40 ± 1.61	5.37 ± 0.50	2.10 ± 1.44
iso-$C_{17:0}$	5.29 ± 1.22	6.76 ± 1.25	7.65 ± 4.19
anteiso-$C_{17:0}$	19.58 ± 3.45	20.78 ± 0.85	12.65 ± 2.12

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Table 4. Some characters for distinguishing between *B. sporothermodurans, B. oleronius, B. fordii, B. acidicola, B. shackletonii* and some other weakly reacting species. All data were obtained in the course of this study or have been taken from other studies in the authors' laboratories. All characters were determined using tests in API 20E and API50CHB systems with the exception of microscope observations, starch and casein hydrolysis.

+, > 85% positive; (+), 75-84% positive; v, variable (26-74% positive); -, 0-15% positive; w, weak positive reaction; +/w, positive or weak positive reaction; v/w, variable and when positive the reaction is weak.

	B. sporothermodurans	*B. oleronius*	*B. ruris*	*B. circulans* T	*B. lentus* T	*B. firmus*	*B. fordii*	*B. acidicola*
Motility	v	-	+	+	+	+	+	+
Spores observed	rarely	+	+	+	+	+	+	+
Spores spherical	v	-	-	-	-	-	-	-
Sporangia swollen	v	+	+	+	-	-	v	+
Anaerobic growth	-	-	+	+	-	+	-	-
Gram reaction	+	-	+	+	+	+	-	+
Starch hydrolysis	-	v/w	+	+	+	+	-	-
Casein hydrolysis	-	-	-	w	-	+	-	
API 20E tests								
ONPG reaction	-	-	+	+	+	-	-	-
Citrate utilisation (Simmons')	v	-	-	-	-	-	-	+
Urease production	-	-	-	-	+	-	-	-
Voges-Proskauer test	v	v/w	-	w	-	-	-	-
Gelatin hydrolysis	+	+/w	-	-	-	+	-	-
Nitrate reduction	+	+	+	+	+	v	-	+
API 50 CHB tests								
Glycerol	v	v/w	-	+	-	+	-	-
L-arabinose	-	-	+	+	w	-	-	-
Ribose	-	v/w	+	w	w	w	-	+
D-xylose	-	-	+	+	w	v	-	+
Methyl-xyloside	-	-	-	+	-	-	-	-
Galactose	-	v/w	v	+	w	-	-	+
D-mannose	v	v/w	+	+	+	-	-	w
Meso-inositol	-	-	-	+	w	-	-	-

mannitol	v	+	v	+	-	+	-	w
sorbitol	-	-	-	+	w	-	-	-
Methyl-D-glucoside	v	-	v	+	+	-	-	+
Amygdalin	v	-	-	+	w	-	-	+
Arbutin	v	-	-	+	w	-	-	+
Salicin	v	v/w	v	+	w	-	-	+
D-cellobiose	v	+	v	+	w	-	-	+
Maltose	+	v/w	v	+	w	+	-	+
Lactose	-	-	+	+	+	-	-	w
D-melibiose	-	-	+	+	w	-	-	+
Sucrose	(+)	-	+	+	+	+	-	+
D-trehalose	(+)	v/w	+	+	+	+	-	+
Inulin	-	-	v	+	-	-	-	+
D-melezitose	v	-	+	+	w	-	-	w
D-raffinose	-	-	v	+	w	-	-	+
Glycogen	-	-	v	+	-	+	-	+
Xylitol	v/w	-	-	+	-	-	-	w
Gentiobiose	v	-	-	+	w	-	-	+
D-turanose	v	-	-	+	w	-	-	-

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Legend Supplementary figure. Normalized computer profiles from SDS-PAGE analyses of whole cell proteins of isolates belonging to the species *Bacillus sporothermodurans, B. oleronius* and *B. fordii*..The dendrogram is based on UPGMA clustering of the correlation coefficient (*r*) of the total protein profiles. The zone used for clustering is indicated in gray on the kDalton bar above the profiles.

Supplementary figure