Bacillus rigiliprofundi sp. nov., an endospore-forming, Mn-oxidizing, moderately halophilic bacterium isolated from deep subseafloor basaltic crust

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A facultatively anaerobic bacterium, designated strain 1MBB1^T, was isolated from basaltic breccia collected from 341 m below the seafloor by seafloor drilling of Rigil Guyot during Integrated Ocean Drilling Program Expedition 330. The cells were straight rods, 0.5 μm wide and 1–3 μm long, that occurred singly and in chains. Strain 1MBB1^T stained Gram-positive. Catalase and oxidase were produced. The isolate grew optimally at 30 °C and pH 7.5, and could grow with up to 12 % (w/v) NaCl. The DNA G+C content was 40.5 mol%. The major cellular fatty acids were $C_{16:1}\omega11c$ (26.5 %), anteiso-$C_{15:0}$ (19.5 %), $C_{16:0}$ (18.7 %) and iso-$C_{15:0}$ (10.4 %), and the cell-wall diamino acid was *meso*-diaminopimelic acid. Endospores of strain 1MBB1^T oxidized Mn(II) to Mn(IV), and siderophore production by vegetative cells was positive. Phylogenetic analysis of the 16S rRNA gene indicated that strain 1MBB1^T was a member of the family *Bacillaceae*, with *Bacillus foraminis* CV53^T and *Bacillus novalis* LMG 21837^T being the closest phylogenetic neighbours (96.5 and 96.2 % similarity, respectively). This is the first novel species described from deep subseafloor basaltic crust. On the basis of our polyphasic analysis, we conclude that strain 1MBB1^T represents a novel species of the genus *Bacillus*, for which we propose the name Bacillus *rigiliprofundi* sp. nov. The type strain is 1MBB1^T (=NCMA B78^T=LMG 28275^T).

There are as many microbial cells in subseafloor sediments as there is in the entire marine water column (Kallmeyer *et al.*, 2012). While few data are currently available, models indicate that a similar microbial biomass also resides in subseafloor basaltic crust (Heberling *et al.*, 2010). Despite the importance of this subsurface biosphere in global biogeochemistry, few isolates from the marine deep subsurface exist. The first isolated deep subsurface bacterium was *Desulfovibrio profundus*, a sulfate-reducing bacterium isolated from 500 m below the seafloor in the Japan Sea (Bale *et al.*, 1997). Since then, several studies have focused on cultivation of microbial isolates from deep sediment (Barnes *et al.*, 1998; Inagaki *et al.*, 2003; Mikucki *et al.*, 2003; Toffin *et al.*, 2004a, b; Biddle *et al.*, 2005; Lee *et al.*, 2005; Batzke *et al.*, 2007; Fichtel *et al.*, 2012), but only one study has focused on cultivation from deep subseafloor basaltic crust (Smith *et al.*, 2011). These authors isolated bacteria from the classes *Gammaproteobacteria*, *Alphaproteobacteria* and *Actinobacteria* by using an osmotic pump sampler that bathed different mineral substrates with fluids from deep subsurface ocean crust and then tested the isolates' ability to oxidize iron and reduce nitrate.

It is expected that sporulation may play an important role in the marine deep subsurface biosphere (Lomstein *et al.*, 2012); therefore, microbial species capable of sporulation are likely to be present in this environment. Species within the genus *Bacillus* are amongst the most common spore-formers isolated from the environment, and they have been isolated from various subsurface environments, including terrestrial subsurface aquifers (Mayhew *et al.*, 2008) and surface marine sediments (Rosson & Nealson, 1982; Francis & Tebo, 2002; Dick *et al.*, 2006).

†Deceased.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 1MBB1^T is KJ630837.

A supplementary figure is available with the online Supplementary Material.

Additionally, endospores made by some of these subsurface isolates from the genus *Bacillus* display Mn-oxidizing activity (Rosson & Nealson, 1982; Francis & Tebo, 2002; Dick *et al.*, 2006; Mayhew *et al.*, 2008), implicating members of the genus *Bacillus* in subsurface Mn biogeochemistry. Interestingly, the two described species of *Bacillus* isolated from terrestrial subsurface aquifers, *Bacillus foraminis* and *B. subterraneus*, do not form endospores, and no novel species of the genus *Bacillus* have been characterized from subseafloor basaltic crust to date. Therefore, it remains unknown whether sporulation may play a role in Mn cycling in this environment.

Here, we present what we believe to be the first characterization of a bacterial isolate from deep subseafloor basaltic crust, isolated from a sample collected during Integrated Ocean Drilling Program Expedition 330. Strain 1MBB1^T was isolated from basaltic breccia collected from 341 m below the seafloor at site U1374A, Rigil Guyot, in the south-west Pacific Ocean (Koppers *et al.*, 2012). The lithology from which the sample was recovered is 71-million-year-old plagioclase–augite–olivine–phyric basalt breccia, with a porosity of 18 %. The sample, 330-U1374A-52R4, was obtained using the rotary core barrel drilling system on the research vessel *JOIDES Resolution*. After recovery on deck, the whole round-core sample was collected in pre-baked (400 °C for 4 h) aluminium foil, rinsed three times with sterile seawater and then split with a flame-sterilized chisel in a specially designed rock sampling box. A slurry was generated from crushed rock pieces and 100 µl was inoculated into 5 ml 1 % marine broth (Difco) diluted with 3.5 % NaCl. The slurry was incubated at 7 °C for 269 days before being transferred onto plates of 10 % marine broth, on which strain 1MBB1^T was isolated through three transfers. A polyphasic characterization was carried out according to the guidelines for description of novel aerobic, endospore-forming species (Logan *et al.*, 2009).

Morphological characteristics were observed under a phase-contrast microscope (Nikon Eclipse 80i) and a scanning electron microscope (JEOL JSM-7001F-LV) using cells from exponentially growing cultures. For electron microscopy, cells were fixed in 10 % formalin in phosphate buffered saline and then prepared for scanning electron microscopy as described previously (Chao & Zhang, 2011). The resultant cell concentrate was dripped onto a 0.2 µm pore size, 13 mm diameter filter and allowed to air dry. The cells affixed to the filter were sequentially dehydrated in 10, 25, 50, 75, 95 and 100 % ethanol, for 15 min each. Cells were critical-point dried and visualized on the scanning electron microscope.

Gram staining was performed using the Neat Stain Gram stain kit (Polysciences, Inc.), as implemented by Microbial ID. Unless stated otherwise, all physiological tests were performed in 7.5 % HM broth (Ventosa *et al.*, 1982; Bagheri *et al.*, 2012), hereafter referred to simply as HM broth. Growth was determined at 4.0, 7.5, 10, 20, 24, 30, 35, 40, 45, 50, 60 and 70 °C, pH 6.0–10.0 (at intervals of 0.5 pH units, with the exception of pH 9.5) and 0, 5, 6.1, 10, 12, 14, 15 and 20 % (w/v) NaCl. For experiments with varying pH and salt concentrations, cultures were maintained at 30 °C. Anaerobic growth was tested on HM agar that was allowed to off-gas O_2 for 3 days before plating, as well as in nitrate reduction broth (per litre, 20 g tryptone, 2 g sodium phosphate dibasic, 1 g glucose and 1 g potassium nitrate) and tryptic soya broth. Resazurin was used in the nitrate reduction broth as a redox indicator. Nitrate reduction broth prepared aerobically (separate from that used to test for anaerobic growth) was used to test for nitrate reduction, with sulfanilamide and naphthylamine as indicators of the presence of nitrite and powdered zinc to confirm or deny reduction of nitrite to N_2. Catalase and oxidase activities were tested using 3 % (w/v) hydrogen peroxide solution and BBL DrySlides (Becton Dickinson), respectively. Hydrolysis of starch was tested using starch agar and noting the presence or absence of clearings around colonies after flooding the plate with iodine (Zimbro *et al.*, 2009). Hydrolysis of casein was tested by looking for clearance around colonies grown on milk agar (Zimbro *et al.*, 2009). GP2 microplates (Biolog) were used according to the manufacturer's specifications to test the capacity for utilization of a variety of carbon sources. API 20E test strips (bioMérieux) were used according to the manufacturer's specifications to test for biochemical characteristics. The growth temperature for both GP2 plates and API 20E test strips was 30 °C. *B. foraminis* LMG 23174^T, *B. novalis* LMG 21837^T and *B. subterraneus* DSM 13966^T were obtained from the Belgian Coordinated Collections of Microorganisms (LMG) or the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and used as reference strains. Temperature tolerance for the reference strains was tested with HM containing 2 % NaCl (*B. foraminis* LMG 23174^T and *B. subterraneus* DSM 13966^T) or tryptic soya broth (Difco) (*B. novalis* LMG 21837^T).

To test for sporulation, cells were inoculated into HM broth, M medium and K medium; the latter two are low- and medium-nutrient broths, respectively, in comparison with the high-nutrient HM broth (Tebo *et al.*, 2007). All three media were supplemented with 5 mg $MnSO_4$ l^{-1}. Cells were collected after 1 week of growth on a rotating table at 30 °C, fixed with formalin and observed under phase-contrast microscopy. Cells were tested for heat tolerance by immersing exponential-phase liquid cultures in 80 °C water baths for 10 and 20 min and then plating 10 µl aliquots on TSA. Aliquots of 10 µl culture were plated on TSA prior to heat treatment to serve as a positive control. Strains were tested for motility by dropping 15 µl exponential-phase liquid cultures on the centre of 0.30 % agar 7.5 % HM plates with 2 % (w/v) NaCl and 10 g tryptone l^{-1}.

Strain 1MBB1^T was Gram-stain-positive, motile and facultatively anaerobic. Cells were rods, ~0.5 µm wide and 1–3 µm long, that occurred singly and in chains (Fig. S1, available in the online Supplementary Material). After

overnight growth on TSA, strain 1MBB1^T formed opaque, cream-coloured colonies that were circular, raised and entire, with a mean diameter of 1 mm. The isolate was moderately halophilic and grew in media containing 0–12 % (w/v) NaCl (optimum 6.1 %), but not at 14 % NaCl. It should be noted that, due to the makeup of HM broth, some KCl and $MgCl_2$ is present in the medium prepared without NaCl; therefore, the salt content in that medium is slightly greater than 0. Strain 1MBB1^T grew at pH 6.5–8.0 (optimum pH 7.5) and at 7.5–45 °C (optimum 30 °C), but not 4.0 or 50 °C. Other phenotypic features are given in Table 1.

Strain 1MBB1^T sporulated completely after growth in M medium at 30 °C for 1 week (Fig. S1) and after growth on K medium after several weeks; some endospores were detected in HM broth at 7.5 °C, but not at 30 °C.

Table 1. Characteristics that distinguish strain 1MBB1^T from the type strains of closely related species of the genus *Bacillus*

Strains: 1, 1MBB1^T; 2, *B. foraminis* LMG 23174^T; 3, *B. subterraneus* DSM 13966^T; 4, *B. novalis* LMG 21837. All data were obtained in this study unless indicated. ND, Not determined; (+), positive in two out of three replicates. Cells of all strains are motile and use maltose.

Characteristic	1	2	3	4
Endospore location/shape*	T/C	No spores	No spores	ST, PC/E
Heat resistance	+	−	−	+
Growth temperature (°C)				
Range	7.5–45	10–45	10–45	7.5–45
Optimum	30	30–35	35–40	35
pH for growth				
Range	6.0–8.5	6.5–9.0	6.5–9.0	5.0–9.5
Optimum	7.5	7.5–8.0	7.0–8.0	7.0–8.0
NaCl concentration for growth (%)				
Range	0–12	0–10	0–9	0–3
Optimum	5–6	0	0	0
Nitrate reduction to N_2	−	+ (nitrite)	+	+ (nitrite)
Anaerobic growth	+	−	+	+
β-Galactosidase (ONPG)	−	+	+	−
Arginine dihydrolase	+	−	+	−
Acetoin production	+	+	−	+
Gelatin hydrolysis	−	+	−	+
Siderophore production	+	(+)†	+	ND
Mn oxidation	+	−	−	−
Substrate utilization				
Tween 40	(+)	+	(+)	−
D-Galactose	−	+	−	−
myo-Inositol	(+)	+	−	−
D-Mannitol	−	+	−	+
D-Mannose	−	+	−	+
Melibiose	−	+	−	−
Salicin	+	+	−	−
Sucrose	+	+	+	(+)
Acetic acid	(+)	+	(+)	−
α-Ketovaleric acid	(+)	(+)	+	−
α-Lactic acid	+	+	+	−
L-Malic acid	−	+	(+)	−
Methyl pyruvate	−	+	(+)	−
Monomethyl succinate	−	+	−	−
Pyruvic acid	+	+	+	(+)
Casein	−	−	−	+
Starch	−	+	+	−
DNA G+C content (mol%)	40.5	43.1[a]‡	43.1[b]	40.5[c]

*PC, Paracentral; ST, subterminal; T, terminal; C, circular; E, ellipsoidal.

†Siderophores present only in stationary phase.

‡Data from: *a*, Tiago *et al.* (2006); *b*, Kanso *et al.* (2002); *c*, Heyrman *et al.* (2006).

Previously, it was suggested that cells of some species of the genus *Bacillus* lyse instead of sporulating in medium that is too rich (Logan *et al.*, 2009); that was likely the case here for HM broth at 30 °C. The sporulating cells observed at 7.5 °C were potentially temperature stressed.

Endospores of some species of the genus *Bacillus* can oxidize reduced manganese (Rosson & Nealson, 1982; Francis & Tebo, 2002), and diverse Mn-oxidizing members of the genus *Bacillus* have been isolated from hydrothermally influenced marine (Dick *et al.*, 2006) and subsurface terrestrial (Mayhew *et al.*, 2008) environments. We tested the ability of strains to oxidize Mn(II) to Mn(IV) by growing them on plates of K medium supplemented with 10 mM HEPES and 100 μM $MnCl_2$ and additionally in liquid M medium (Tebo *et al.*, 2007). Leukoberbelin blue was used to spot test colonies (K medium) or cell pellets (M medium) for the presence of manganese oxides (Krumbein & Altmann, 1973). Strain 1MBB1^T tested positive for manganese oxidation in liquid M medium and on K medium plates.

We tested the ability of strains to produce siderophores, iron-chelating ligands that are important for cellular acquisition of iron in subsurface environments (Butler, 2005). A single colony was inoculated into 40 ml iron-depleted artificial seawater (10 g Casamino acids l^{-1}, 19 mM NH_4Cl, 50 mM $MgSO_4$, 10 mM $CaCl_2$, 300 mM NaCl, 10 mM KCl, 2 mM $NaHCO_3$, 4.6 mM sodium glycerophosphate, 41 mM glycerol, 100 μM HEPES; Martin *et al.*, 2006) supplemented with 1 ml filtered diluted vitamin solution l^{-1} (8.2 μM biotin, 1.6 μM nicotinic acid, 0.33 μM thiamine, 1.46 μM *p*-aminobenzoic acid, 0.21 μM calcium pantothenate, 5 μM pyridoxine hydrochloride, 0.07 μM vitamin B_{12}, 0.5 μM riboflavin, 0.5 μM folic acid) and 41 mM sterile-filtered glucose. The stock solution of vitamins was diluted 10-fold before being added to the samples. After all samples were prepared, they were incubated at 30 °C on a shaking incubator at 200 r.p.m. in the dark. Each day, 2 ml of each culture was subsampled under a sterile laminar flow hood in order to measure optical density and siderophore production via the liquid chrome azurol S (CAS) assay (Schwyn & Neilands, 1987). Samples were measured daily for 1 week for both optical density and siderophore production using a UV spectrophotometer (Beckman Du-50). The peak for the CAS assay was measured at 630 nm. Relative absorbance as a function of days was calculated. If there was a negative slope, siderophores were considered to be present (Schwyn & Neilands, 1987). *B. novalis* LMG 21837^T did not grow in the artificial seawater, and so was not tested for siderophore production. Both strain 1MBB1^T and *B. subterraneus* DSM 13966^T tested positive for siderophore production. *B. foraminis* LMG 23174^T was variably positive, but results were not consistent, and positive results occurred only during stationary phase, unlike strain 1MBB1^T and *B. subterraneus* DSM 13966^T, which produced siderophores during exponential phase.

DNA was extracted from cells from an exponentially growing culture of strain 1MBB1^T using the FastDNA Spin kit for Soil (MP Biomedical) according to the manufacturer's protocols. The 16S rRNA gene was amplified by using the universal bacterial primers 27F and 1492R (Lane, 1991) in PCR, the resultant PCR product was purified with the QIAquick PCR Purification kit (Qiagen) and the purified product was cloned into a plasmid vector using the TOPO TA cloning kit (Life Technologies). Several colonies were picked and screened for the insert by PCR using the primers M13F and M13R. Three PCR products that were the correct size when run on an agarose gel were purified with the QIAquick PCR Purification kit and then sequenced using sequencing primers T7 and T3. Sequence data were assembled and analysed using Geneious version 5.6 (Drummond *et al.*, 2011); all three sequenced 16S rRNA gene clones were identical. Phylogenetic analysis of strain 1MBB1^T and other members of the *Bacillaceae* was conducted using MEGA5 (Tamura *et al.*, 2011); evolutionary distances were calculated from a 1436 bp alignment using the Tamura–Nei model with the G + I rate model. Phylogenetic trees were reconstructed using maximum-likelihood, neighbour-joining and maximum-parsimony algorithms, and tree topologies were evaluated by performing bootstrap analysis of 1000 resamplings of the datasets.

Comparative analyses of the 16S rRNA gene sequence of strain 1MBB1^T with sequences representative of the main lines of descent within the domain *Bacteria* indicated that the strain was a member of the family *Bacillaceae* (Fig. 1). The closest relative of strain 1MBB1^T was *B. foraminis* CV53^T (96.5 % similarity), which was originally isolated from a terrestrial subsurface serpentinizing spring (Tiago *et al.*, 2006), and it was also closely related to *B. subterraneus* C0013B^T (95.7 % similarity), another isolate from a terrestrial subsurface aquifer (Kanso *et al.*, 2002).

Whole-cell fatty acid composition was determined by GC using the Microbial Identification system (Sherlock version 4.5, identification library RTSBA6 6.10; Microbial ID). All strains were grown on TSA for 24 h at 28 °C prior to determination. The major fatty acids of strain 1MBB1^T were $C_{16:0}$, iso-$C_{15:0}$, anteiso-$C_{15:0}$ and $C_{16:1}\omega11c$ (Table 2). These same fatty acids were also dominant in *B. foraminis* LMG 23174^T, but the proportions were different, especially for $C_{16:0}$ and iso-$C_{15:0}$. For both *B. subterraneus* DSM 13966^T and *B. novalis* LMG 21837^T, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ were the only major fatty acids (Table 2). This is in agreement with previous work for *B. novalis* (Heyrman *et al.*, 2004), but no previous data exist for the fatty acid composition of *B. subterraneus*.

DNA for determination of the base composition was isolated from cell pellets by cell disruption and subsequent purification on hydroxyapatite (Cashion *et al.*, 1977), followed by analysis by HPLC (Mesbah *et al.*, 1989). Peptidoglycan structure was determined from cell pellets by disrupting the cells followed by digestion with trypsin, hydrolysis of the enzymic products and analysis with

TLC (Schumann, 2011). Analyses of both DNA base composition and peptidoglycan were conducted at the DSMZ. The DNA G+C content for strain 1MBB1^T was 40.5 mol%. As in many other species of the genus *Bacillus*, the peptidoglycan of strain 1MBB1^T belongs to the A1γ type, which contains *meso*-diaminopimelic acid as the diamino acid.

Strain 1MBB1^T could be distinguished from closely related members of the genus *Bacillus* by a combination of differences in ability to form endospores, NaCl range and optimum for growth, carbohydrate utilization, ability to oxidize Mn and other phenotypic tests, as well as by the genomic DNA G+C content. Therefore, we propose that isolate 1MBB1^T represents a novel species of the

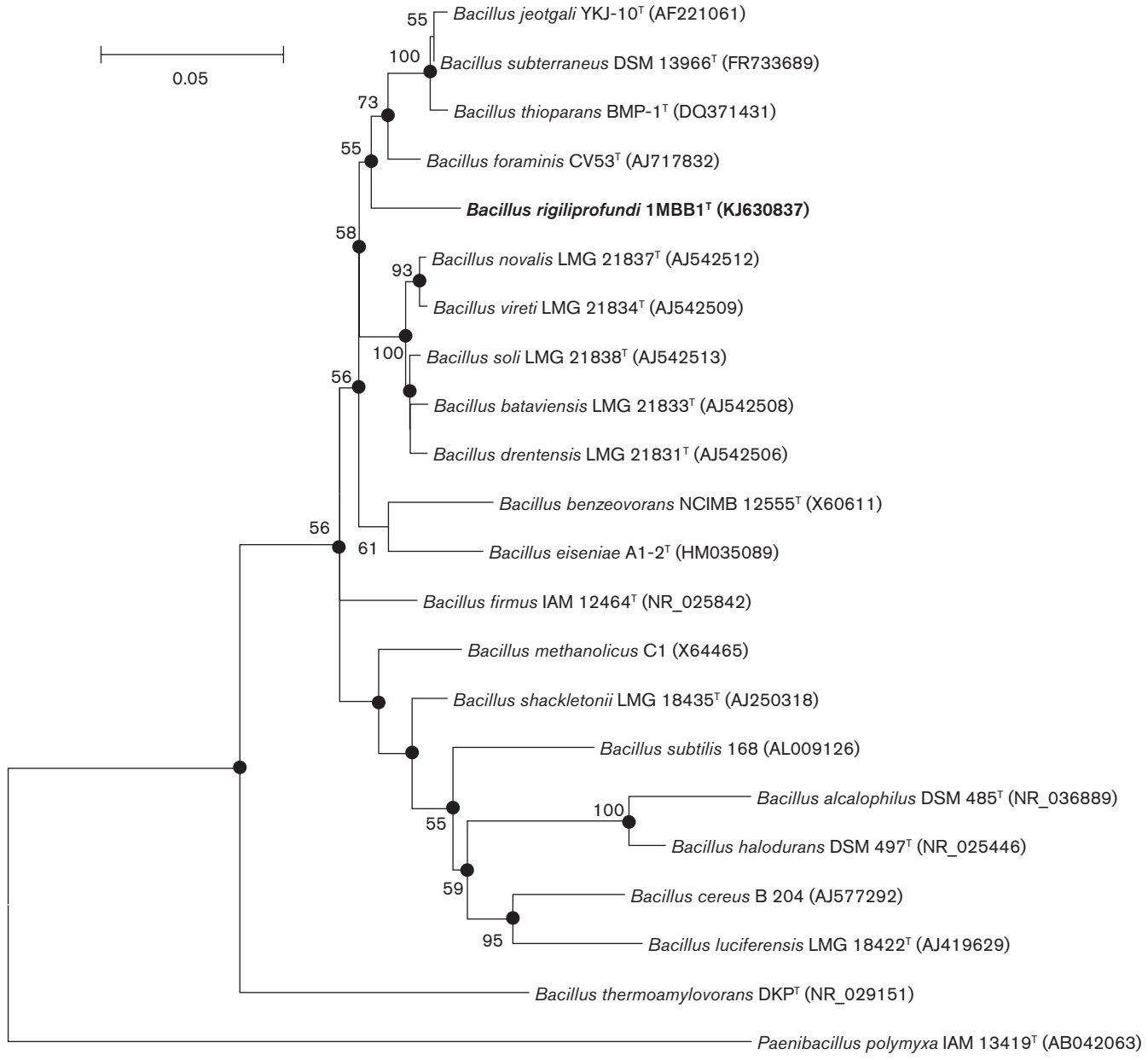

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain 1MBB1^T and closely related species of the genus *Bacillus*. Sequence accession numbers are shown in parentheses. Bootstrap values higher than 50% are indicated at branch points. Filled dots indicate branches in agreement with trees reconstructed using neighbour-joining and maximum-parsimony algorithms. *Paenibacillus polymyxa* IAM 13419^T was used as an outgroup. Bar, 5% sequence divergence.

genus *Bacillus*, for which the name *Bacillus rigiliprofundi* sp. nov. is proposed.

Description of *Bacillus rigiliprofundi* sp. nov.

Bacillus rigiliprofundi (ri.gi′li.pro.fun′di. L. neut. n. *profundum* a deep place; N.L. gen. n. *rigiliprofundi* from deep within Rigil Guyot, from where the type strain was isolated).

Forms rod-shaped cells, 0.5 μm wide and 1–3 μm long, occurring singly, in pairs and in short chains. Gram stain is positive. Endospores are terminal and circular. When grown on TSA, colonies are cream-coloured, circular, raised and entire, with a diameter of 1 mm. Optimal growth occurs at 30 °C; the maximum growth temperature lies between 45 and 50 °C. Growth occurs at 7.5 °C but is not observed at 4 °C after 2 weeks. The minimum pH for growth lies between 5.5 and 6.0, the optimum pH lies between 7.0 and 8.0 and the maximum pH lies between 8.5 and 9.0. Salt is not required for growth, and growth is observed up to 12 % NaCl. Optimal salt concentration is 5–6 %. Oxidase and catalase activities are positive. Positive for the Voges–Proskauer test and arginine dihydrolase activity, but negative for β-galactosidase production, gelatin hydrolysis, indole production, the methyl red test and production of acid from carbohydrates. When in endospore form, positive for oxidation of Mn(II) to Mn(IV). Siderophore production is positive in low-iron medium. Dextran, α-D-glucose, maltose, maltotriose, salicin, sucrose, D-trehalose, L-lactic acid and pyruvic acid are all used consistently as single carbon sources, while utilization of Tween 40, arbutin, *myo*-inositol, acetic acid and α-ketovaleric acid is inconsistent. Hydrolysis of Tween 80, casein and starch is negative. Does not reduce nitrate. The major cellular fatty acids, in decreasing order of abundance, are $C_{16:1}\omega11c$, anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{15:0}$. Minor fatty acids are $C_{14:0}$, iso-$C_{14:0}$, $C_{18:0}$, $C_{18:1}\omega9c$, summed feature 3 ($C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$) and anteiso-$C_{17:0}$. The diagnostic diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid.

The type strain, 1MBB1^T (=NCMA B78^T=LMG 28275^T) was isolated from subsurface ocean crust, 341 m below the seafloor in Rigil Guyot, along the Louisville Seamount Trail. The DNA G+C content of the type strain is 40.5 mol%.

Table 2. Cellular fatty acid compositions of strain 1MBB1^T and type strains of closely related species of the genus *Bacillus*

Strains: 1, 1MBB1^T; 2, *B. foraminis* LMG 23174^T; 3, *B. subterraneus* DSM 13966^T; 4, *B. novalis* LMG 21837. All data were obtained from this study using cells cultivated on TSA for 48 h. Values are percentages of total fatty acids. Fatty acids that made up <1 % of the total in all strains are not shown. −, Not detected.

Fatty acid	1	2	3	4
Straight-chain saturated				
$C_{14:0}$	7.2	4.2	2.5	0.8
$C_{16:0}$	18.7	11.0	3.6	1.6
$C_{18:0}$	3.1	2.1	−	−
Branched saturated				
iso-$C_{14:0}$	4.9	6.4	0.5	0.8
iso-$C_{15:0}$	10.4	24.6	48.9	46.7
anteiso-$C_{15:0}$	19.5	14.3	10.6	39.8
iso-$C_{16:0}$	0.9	4.2	0.5	0.8
iso-$C_{17:0}$	0.4	3.1	2.8	1.2
anteiso-$C_{17:0}$	1.0	3.8	4.2	3.4
Mono-unsaturated				
$C_{16:1}\omega7c$ alcohol	0.5	1.4	1.5	0.6
$C_{16:1}\omega11c$	26.5	18.1	7.7	1.8
iso-$C_{17:1}\omega10c$	0.3	1.1	7.4	0.7
$C_{18:1}\omega9c$	1.8	2.6	0.3	−
Summed features				
Summed feature 3*	1.1	1.2	0.1	−
Summed feature 4*	0.4	0.7	8.3	1.3

*Summed features represent groups of two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of $C_{16:1}\omega7c$ and/or $C_{16:1}\omega6c$; summed feature 4 consisted of anteiso-$C_{17:1}$ B and/or iso-$C_{17:1}$ I.

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