

# Aerobic endospore-forming bacteria from geothermal environments in northern Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago, with the proposal of *Bacillus fumarioli* sp. nov.

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**Aerobic endospore-forming bacteria were isolated from soils taken from active fumaroles on Mount Rittmann and Mount Melbourne in northern Victoria Land, Antarctica, and from active and inactive fumaroles on Candlemas Island, South Sandwich archipelago. The Mt Rittmann and Mt Melbourne soils yielded a dominant, moderately thermophilic and acidophilic, aerobic endospore-former growing at pH 5.5 and 50 °C, and further strains of the same organism were isolated from a cold, dead fumarole at Clinker Gulch, Candlemas Island. Amplified rDNA restriction analysis, SDS-PAGE and routine phenotypic tests show that the Candlemas Island isolates are not distinguishable from the Mt Rittmann strains, although the two sites are 5600 km apart, and 16S rDNA sequence comparisons and DNA relatedness data support the proposal of a new species, *Bacillus fumarioli*, the type strain of which is LMG 17489<sup>T</sup>.**

**Keywords:** *Bacillus*, *Bacillus fumarioli*, Antarctica, geothermal soils, thermoacidophile

## INTRODUCTION

Although Antarctica is largely an ice-bound continent that relies upon solar heating during the summer to support a sparse growth of terrestrial life, there exists a small number of sites where volcanic activity warms the soil and steam emissions from fumaroles condense to maintain relatively steady water supplies. The unique selective pressures of such sites make the organisms that live there of special biological interest (Broady, 1993). The Cenozoic period has seen constant volcanic activity in Antarctica, and steaming ground is

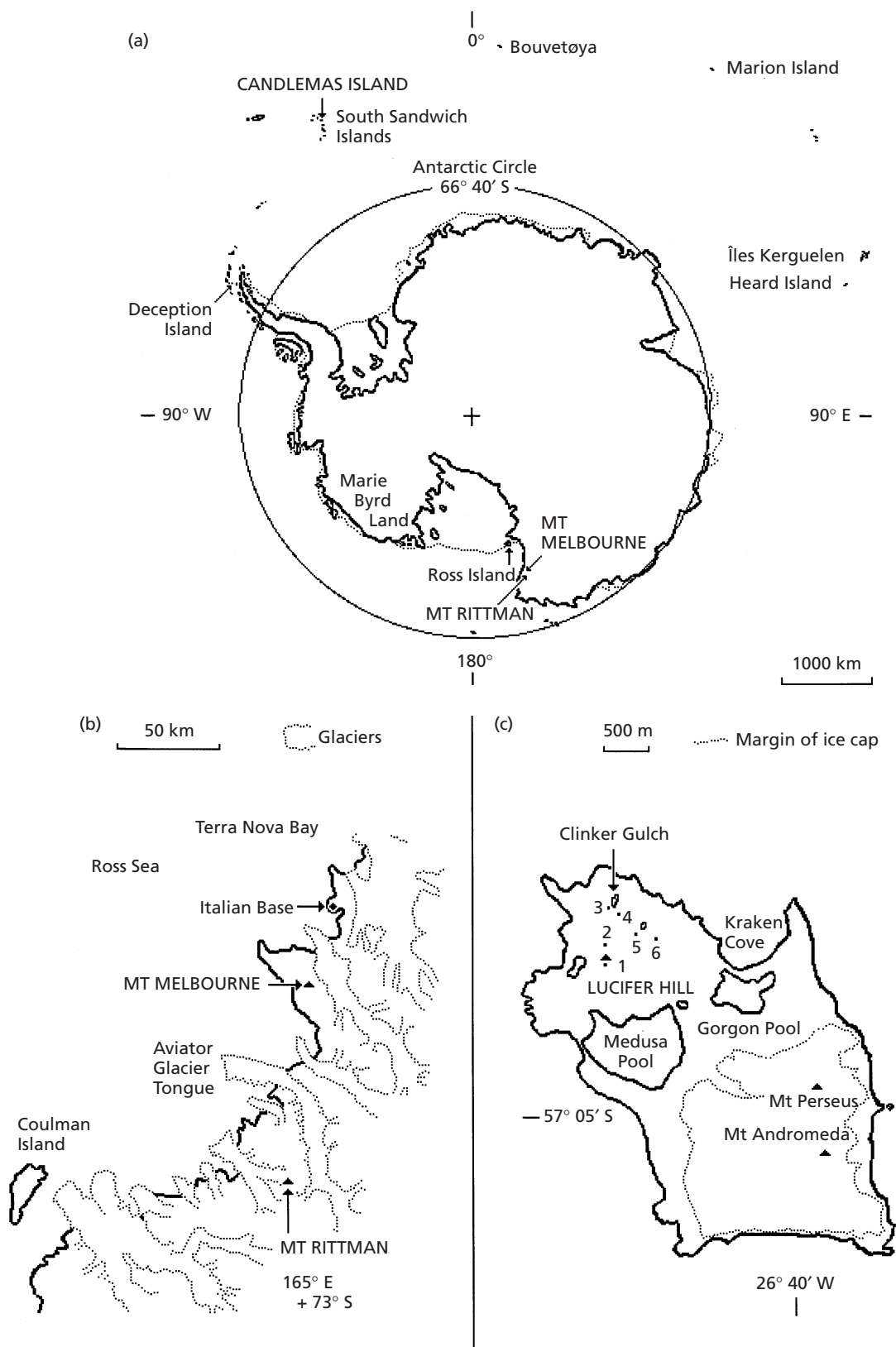
to be found in a number of circumpolar islands, including Deception Island, the South Sandwich Islands, Bouvetøya, Marion Island and Îles Kerguelen, and on continental Antarctica at Mount Erebus on Ross Island, Mount Melbourne and Mount Rittmann in northern Victoria Land, and in Marie Byrd Land (Fig. 1a).

Mounts Erebus and Melbourne represent two of the four provinces of the McMurdo Volcanic Group, which is one of the most extensive alkali volcanic provinces in the world (Harrington, 1958). In 1988–1989, the 4th Italian Antarctic Expedition discovered a new volcano, Mt Rittmann (Armenti & Tripodo, 1991) (Fig. 1b), in the Melbourne Province, and in 1990–1991 the 6th Italian Expedition found fumaroles in a minor calderic structure of Mt Rittmann (73° 28' S, 165° 36' E; Bonaccorso *et al.*, 1991). This geothermal site, and others at the summits of Mt Melbourne and Mt Erebus, harbour unique vegetation communities which appear to have formed following colonization by propagules from circumpolar continents (Linskens *et al.*, 1993). The geothermally heated biosystem at Mt

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**Abbreviations:** ARDRA, amplified rDNA restriction analysis; FAME, fatty acid methyl ester.

The EMBL accession numbers for the 16S rRNA gene sequence of *B. fumarioli* strain LMG 17489<sup>T</sup> is AJ250056. The accession numbers for the other 16S rRNA gene sequences are: LMG 17492, AJ250057; LMG 18409, AJ250058; LMG 18418, AJ250059; LMG 18419, AJ250317; LMG 18435, AJ250318; LMG 18437, AJ250319.



**Fig. 1.** (a) Map of Antarctica and the subantarctic islands, with geothermal sites named, and the sites sampled indicated by names in capital letters; (b) map of northern Victoria Land showing the locations of Mt Melbourne and Mt Rittmann; (c) map of Candlemas Island, with sampling sites 1–6 indicated.

Rittmann has been described by Bargagli *et al.* (1996). It is a rough slope of gravelly sand which lies at an altitude of about 2600 m, and which has small fumaroles whose internal temperatures (at 10 cm depth) range between 50 and 63 °C; patches of moss grow on the warm soil which has a relatively high moisture content (by Antarctic standards) and a pH of around 5.4.

Lucifer Hill on Candlemas Island (57° 05' S, 26° 40' W; Fig. 1c), in the South Sandwich Islands, reaches an altitude of 232 m and also bears patches of moss around active fumaroles high on the hill, around inactive fumaroles at Clinker Gulch, and on lava soils at the base of the volcanic cone, with temperatures ranging from 85 °C down to 0 °C.

Soil samples were collected from Mt Melbourne and the Mt Rittmann geothermal site by British Antarctic Survey (BAS) members of the international BIOTEX 1 expedition during the 1995–1996 austral summer, and from Lucifer Hill, Candlemas Island, by BAS personnel during the 1996–1997 season, with a view to investigating the aerobic endospore-forming bacterial floras of these sites. The present paper describes the isolation and characterization of heterotrophic strains isolated from soils whose temperatures ranged from 0 to 60 °C on Candlemas Island, and from fumarole soil at 50 °C from Mt Rittmann. A moderately thermophilic and acidophilic, aerobic endospore-forming organism appeared to dominate the heterotrophic population of the Mt Rittmann fumarole soil samples, and was also found in soil taken from a cold, dead fumarole at Clinker Gulch, Candlemas Island; further strains were isolated from soils of Mt Rittmann and Cryptogam Ridge, Mt Melbourne (74° 22' S, 164° 40' E; Broady *et al.*, 1987) during the 1998–1999 austral summer. We propose this organism as a new species with the name *Bacillus fumarioli*.

## METHODS

**Isolation and maintenance of strains.** Duplicate soil samples of roughly 500 g were collected aseptically from (i) a fumarole on Mt Rittmann (Fig. 1b; altitude 2600 m; internal temperature of fumarole 50 °C at 10 cm depth), (ii) geothermally heated soil on the north-west ridge of Mt Melbourne (Fig. 1b; altitude 2500 m) during the 1995–1996 austral summer, and (iii) six sites on Lucifer Hill, Candlemas Island (Fig. 1c): two active fumaroles (sites 1 and 2, temperature range 30–60 °C) at the top of the cone (altitude 232 m), two cold, dead fumaroles (sites 3 and 4; temperature range 10–20 °C and 0–15 °C respectively; altitude 20 m) in Clinker Gulch, and two other areas of unheated ground (sites 5 and 6; temperature range 0–15 °C; altitude 30 m) at the foot of the hill, during the 1996–1997 austral summer. One of the sample duplicates from each site was stored chilled (about 4 °C) and the other was deep-frozen (about –20 °C) between collection in the Antarctic and study in Glasgow. Ten samples of mossy and moss-free soils from Mt Rittmann (soil temperature range 8.3–58.5 °C, and 62.5 °C at 5 cm depth within a fumarole), and eight samples of mossy and moss-free soils from Cryptogam Ridge (altitude 2700 m, soil temperatures 3.2–42.0 °C and 47.0 °C at 5 cm

depth within a fumarole) and two samples of moss-free soil from the NW ridge of Mt Melbourne (soil temperatures 28.0 and 36.7 °C) collected during the 1998–1999 austral summer were used fresh for isolations and counts of moderately thermoacidophilic bacteria when examined at the Terra Nova Bay base laboratory as soon as possible after collection, and then stored frozen for transit to Glasgow. Soil samples (temperatures ranging from –1.0 °C to +16.5 °C) were also collected from 25 non-geothermal sites within a 50 km radius of Mt Melbourne during the 1998–1999 season. Aerobic endospore-forming bacteria were sought in the 1995–1996 and 1996–1997 samples by adding 1 g quantities of soil to 9 ml Trypticase Soy Broth (TSB; Oxoid) in duplicate at pH 5.5 and 7.0 for Mt Rittmann and Mt Melbourne samples, and at pH 4.5, 5.5 and 6.5 for Candlemas Island samples, and heat-treating one of each pair at 80 °C for 10 min to kill vegetative cells. Spread plates inoculated with 0.1 ml soil suspension on Trypticase Soy Agar (Oxoid) at the appropriate pH and with 5 mg l<sup>-1</sup> MnSO<sub>4</sub> to enhance sporulation (TSA MnSO<sub>4</sub>) were incubated at 15, 30, 50 and 65 °C for Mt Rittmann samples and at 20, 30, 40, 50 and 60 °C for Candlemas Island samples, and the suspensions remaining were incubated at the same range of temperatures in waterbaths. The 1998–1999 samples from Mt Rittmann and Mt Melbourne and from 25 other sites were cultivated at 50 °C in a modification of the medium B described by Nicolaus *et al.* (1998), here called *Bacillus fumarioli* broth (BFB), and plated on a solid medium called *Bacillus fumarioli* agar (BFA). BFB contained 4 g yeast extract, 2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 3 g KH<sub>2</sub>PO<sub>4</sub> and 4 ml each of solutions A and B l<sup>-1</sup> [A, 125 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 g MgSO<sub>4</sub>·7H<sub>2</sub>O l<sup>-1</sup>; B, 62.5 g CaCl<sub>2</sub>·2H<sub>2</sub>O l<sup>-1</sup>], adjusted to pH 5.5. BFA was prepared by adding 5 mg MnSO<sub>4</sub>·4H<sub>2</sub>O and 18 g agar l<sup>-1</sup> to BFB prior to autoclaving. TSB and BFB cultures which became turbid were subcultured by streaking onto TSA MnSO<sub>4</sub> or BFA at the appropriate pH, and incubating at the appropriate temperature. Colonies appearing on spread plates and streak plates were screened for vegetative and sporangial morphologies by phase-contrast microscopy, and endospore-forming rods were streak purified and then transferred to slopes of the same medium for storage at 4 °C after incubation and confirmation of sporulation by microscopy. Proportions of spores and vegetative cells of the moderately thermoacidophilic endospore-former in five 1998–1999 Mt Rittmann soils, taken from one area of loose, moss-free soil about 2 m in diameter and showing a range of temperatures (8.3, 14.5, 28, 42.2 and 58.5 °C), were determined by BFA plate counts of duplicate soil suspensions of 1 g soil crumbs in 9 ml quarter-strength Ringer's solution, one of which was heated at 80 °C for 10 min to destroy vegetative cells prior to plating and incubation at 50 °C.

**DNA preparation.** For amplified rDNA restriction analysis (ARDRA), total genomic DNA was purified using a slight modification of the method of Pitcher *et al.* (1989), as described previously (Heyndrickx *et al.*, 1995, 1996). For DNA–DNA binding experiments and DNA base composition studies, DNA was prepared using a combination of the methods of Marmur (1961) and Pitcher *et al.* (1989), as described by Heyndrickx *et al.* (1995). A compiled description follows: (all buffers and aqueous solutions were prepared with autoclaved MilliQ-water) exponentially growing cells (0.75–1.5 g) were harvested from cultures on solid media in 14 cm diameter Petri dishes and suspended in 15 ml RS buffer (Resuspension buffer contained 0.15 M NaCl, and 0.01 M EDTA at pH 8.0). After centrifugation,

the cell pellet was resuspended in 3 ml lysozyme solution (50 mg lysozyme ml<sup>-1</sup>) and 60 µl RNase solution (10 mg RNase ml<sup>-1</sup>) and incubated for 1 h at 37 °C. Cells were lysed by adding 15 ml GES (10 g guanidium isothiocyanate/0.5 M EDTA at pH 8.0/1 g Sarkosyl, made up to 100 ml) reagent after gentle shaking. Cooling in ice water for 15 min was followed by addition of 7.5 ml ammonium acetate (578.1 g ammonium acetate l<sup>-1</sup>; filter-sterilized), gentle shaking and another 15 min in ice. The crude DNA extract was mixed with 15 ml of a cold chloroform-isoamyl alcohol mixture (24:1 ratio). The solution was then vigorously shaken and centrifuged (20 min at 15000 g). The supernatant was carefully transferred to a plastic vial and the DNA was precipitated by adding 0.54 vols 2-propanol. The precipitated DNA was twisted on a glass rod and washed in an ethanol gradient (70, 85 and 90%, v/v), air-dried, and dissolved in 15 ml TE (Tris/HCl/EDTA) buffer. After an additional RNase (75 µl of 10 mg RNase ml<sup>-1</sup>) treatment of 1 h at 37 °C, 625 µl acetate EDTA (3 M ammonium acetate, 1 mM EDTA, pH 7.5) was added. Addition of 5 ml chloroform and a 20 min centrifugation at 10000 g separated the protein fraction from the water phase containing the DNA. To the latter, 2 vols 95% (v/v) denatured ethanol were added in a separate vial to precipitate the high-molecular-mass DNA which was twisted on a glass rod, air-dried, washed in an ethanol gradient (70, 80 and 90%, v/v, ethanol in water) and dissolved in 5 ml of 0.1 × SSC. DNA solutions were stored at -80 °C.

**ARDRA.** Enzymically amplified 16S rDNA was obtained by PCR and analysed by restriction digestion with five restriction enzymes (*HaeIII*, *DpnII*, *RsaI*, *BfaI* and *Tru9I*) as described previously (Heyndrickx *et al.*, 1996). Identification of the Antarctic isolates was attempted by comparing ARDRA profiles with a database of over 1000 authentic strains of aerobic endospore-formers.

**DNA base composition.** DNA was enzymically degraded into nucleosides as described by Mesbah *et al.* (1989). The nucleoside mixture obtained was then separated by HPLC using a Waters Symmetry Shield C8 column thermostabilized at 37 °C. The solvent was 0.02 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda phage DNA (Sigma) was used as the calibration reference.

**DNA-DNA hybridizations.** DNA-DNA hybridizations were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using a HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements (excitation filter of 360 nm, emission filter 465 nm). The optimal renaturation temperature was determined according to the equation of De Ley *et al.* (1970).

**16S rDNA sequencing.** The following conserved sequences (primers) were used to amplify fragments of the 16S rRNA gene: A GTT TGA TCC TGG CTC AG (MH1); C TGG CTC AGG AC/TG AAC GCT G (ARI C/T); AGA GTT TGA TCC TGG CTC AG (pA); TAC CTT GTT ACG ACT TCA CCC CA (MH2) and AAG GAG GTG ATC CAG CCG CA (pH). The primer combinations used for the sequenced strains are shown in parentheses: LMG 17489 (MH1, MH2), LMG 17492 (ARI C/T, pH), LMG 18409 (pA, pH), LMG 18418 (pA, pH), LMG 18419 (ARI C/T, pH), LMG 18435 (pA, pH) and LMG 18437 (ARI C/T, pH). The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing was performed using an Applied Biosystems 377 DNA Sequencer and the protocols of the manufacturer (Perkin-Elmer Biosystems) using the ABI

Prism Dye and/or Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The sequencing primers used were as in Coenye *et al.* (1999). The sequences of strains belonging to the same or different phylogenetic groups were retrieved from the EMBL database and aligned with the consensus sequence using GeneBase software version 1.0 (Applied Maths, Kortrijk, Belgium). A 16S rDNA tree was constructed using the GeneBase software. Type strains of a number of *Bacillus* species and relatives were selected based on FASTA searches (Pearson & Lipman, 1988). If more than one sequence of the type strain of a species was available in the database, the most complete sequence (the lowest number of ambiguities and/or longest part of the 16S RNA gene sequenced) was chosen.

**SDS-PAGE of whole-cell proteins.** Cells were grown on TSA at 52 °C and pH 5.5, the SDS protein extracts prepared and electrophoresed according to Pot *et al.* (1994) and the data collected and interpreted as described by Vauterin & Vauterin (1992).

**Gas chromatographic analysis of methylated fatty acids (FAMES).** Cells were grown on TSA at pH 5.5 and 52 °C. Details of harvesting and analysis can be found in Heyndrickx *et al.* (1998) and Vauterin *et al.* (1991). The standardized MIS database could not be used for identification because the growth conditions of the strains did not conform to the standard conditions prescribed by the manufacturer.

**Phenotypic characterization and numerical analysis.** At the beginning of this study all strains were grown and maintained on TSA MnSO<sub>4</sub> at the pH of isolation, with incubation at the temperature of isolation. Subsequently, the isolates growing at pH 5.5 at 50 °C were found to give improved growth when cultivated in BFB and better growth and sporulation on BFA, and these media were used throughout the remainder of the study. Isolates growing at pH 5.5 and 50 °C were grown for 24–72 h at 50 °C on BFA and vegetative cells and sporangia were observed by phase-contrast microscopy for presence of motile cells, chains of cells, curved rods, rods with tapered ends, vacuoles, spores, swollen sporangia, parasporal crystals, parasporal bodies, shape of spores ellipsoidal, cylindrical or spherical, position of spores terminal, subterminal or central/paracentral; these strains were also examined for haemolysis on 5% horse blood using Columbia Blood Agar base (Oxoid), and for casein and starch hydrolysis, egg yolk reaction, and NaCl sensitivity using the methods of Gordon *et al.* (1973) but with media adjusted to pH 5.5, inoculating with overnight cultures in TSB, and incubating at 50 °C. Maximum and minimum growth temperatures were determined by incubating 10 ml BFB cultures in waterbaths set to 30, 37, 40, 45, 50, 55, 60 and 65 °C, and pH ranges for growth were determined using 10 ml BFB cultures adjusted to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0; both series were examined for turbidity at 24 h intervals. Anaerobic growth was tested for by incubating cultures on BFA plates in a GasPak jar, with aerobically incubated plates as controls. Antibiotic sensitivity tests using Mastring discs (MAST Diagnostics) containing 25 µg ampicillin, 50 µg chloramphenicol, 100 µg colistin sulphate, 30 µg kanamycin, 30 µg nalidixic acid, 50 µg nitrofurantoin, 25 µg streptomycin, and 100 µg tetracycline were carried out on plates of Drug Sensitivity Test Agar (Oxoid) adjusted to pH 5.5 and incubated at 50 °C. Strains were characterized using 49 carbohydrate tests in the API 50CH gallery. As the 50CHB suspension medium was unsuitable for these acidophilic strains, a medium was

adapted from the one described by Deinhard *et al.* (1987); it was adjusted to pH 6 and used 0.033 % bromocresol purple as indicator. These phenotypic data were subjected to numerical analysis as described by Heyndrickx *et al.* (1998), but reference strains growing at pH 5.5–6.0 were not available.

Strains isolated at pH 6.0 or 7.0 were characterized using API 20E and 50 CHB kits (bioMérieux) following the methods of Logan & Berkeley (1984), but with modifications of incubation time according to the organisms' optimum growth temperatures. Strains growing at 20, 40, 50 and 60 °C were incubated for 36–72 h, 18–36 h and 12–24 h and 12–24 h respectively. Strains giving weak reactions in the API 20E and 50 CHB kits were also characterized using the API Biotype 100 kit (Heyndrickx *et al.*, 1997). Analysis of the data along with characters for two reference strains of each established species of *Aneurinibacillus*, *Bacillus*, *Brevibacillus*, *Paenibacillus* and *Virgibacillus* was performed in order to identify the strains, using the numerical method described by Heyndrickx *et al.* (1998).

## RESULTS

### Isolation of strains

Plates and broths inoculated from the 1995–1996 Mt Rittmann samples yielded aerobic endospore-formers at both pH 5.5 and 7.0, and representative colonies were subcultured for further study. However, isolates obtained at pH 7.0, whose colonial and microscopic morphologies resembled those of isolates obtained at pH 5.5, did not survive subculture. No aerobic endospore-formers were isolated at either pH from the samples from the NW ridge of Mt Melbourne. No growth occurred at 65 °C and no spore-formers were isolated from broths or plates at 15 and 30 °C. Large numbers of colonies of aerobic endospore-formers grew at 50 °C on pH 5.5 plates, and 40 isolates of moderate thermoacidophiles representative of four colony types, designated L (large), S (small), Sm (smooth) and Ir (iridescent) were selected for further study. As polyphasic taxonomic analysis of these strains (see below) revealed a homogeneous group representing a new species, they are hereinafter referred to as *B. fumarioli* (Table 1). Plates and broths inoculated from Candlemas Island samples yielded a variety of aerobic endospore-formers, and the representatives selected for further study are also listed in Table 1, with identification results for strains isolated at pH 6.0 or 7.0 shown in Table 2. Further moderately thermoacidophilic strains, which have not yet been fully characterized, but whose growth conditions, colonies and microscopic morphologies identified them as *B. fumarioli*, were isolated from soils of Mt Rittmann and Cryptogam Ridge, Mt Melbourne, during the 1998–1999 austral summer, but not from the soils of the NW ridge of Mt Melbourne (two other kinds of aerobic endospore-former were isolated from this site; they appeared to be present in small numbers and to be difficult to cultivate, and their identities have not yet been investigated), or from the 25 soil samples collected from non-geothermal sites within a 50 km radius of Mt Melbourne. These further isolates of *B. fumarioli* were found in soils whose temperatures ranged from 3.4 to

62.5 °C, where they existed as both spores and vegetative cells, the proportions of sporulated cells tending to be higher at the temperature extremes (9 % at 8.3 °C; 29 % at 58.5 °C), and lower (3 % at 42.5 °C) at temperatures approaching the optimum growth temperature (50 °C) of the organisms. The counts of vegetative cells of *B. fumarioli* were not confused by the appearance of many colonies of other organisms.

### ARDRA

The 29 strains of *B. fumarioli* from the 1995–1996 Mt Rittmann samples and from Candlemas Island were separated from all the other organisms studied in the numerical analysis of their combined ARDRA patterns (Fig. 2), and merge to form one large cluster at 85 % S. Six of the strains isolated from Candlemas Island (LMG 18467–18472) show 100 % similarity and might be regarded as clonal; Mt Rittmann isolate LMG 18407 also shows 100 % similarity with this group. These seven strains merge with seven Mt Rittmann isolates (LMG 18408–18411, 18413, 18415, 18416) at 97 % similarity, so that the Mt Rittmann and Candlemas Island isolates do not appear to represent distinct lineages, yet there might be some phylogenetic diversity within the two populations.

### 16S rDNA sequencing

The 16S rDNA sequences for strains LMG 17489, LMG 17492, LMG 18409 and LMG 18418 showed high similarity (Fig. 3). The lowest sequence similarity in rDNA sequence, of 99.4 %, was found between *B. fumarioli* strains LMG 17489 from Mt Rittmann and LMG 17418 from Candlemas Island. The similarities between the 16S rDNA sequences of *B. fumarioli* LMG 17489<sup>T</sup> (EMBL accession no. AJ250056), *B. fumarioli* LMG 17492 (AJ250057), *B. fumarioli* LMG 18409 (AJ250058) and *B. fumarioli* LMG 18418 (AJ250059) were at least 99.8 %. A FASTA search indicated that the highest similarity between these four strains and sequences in the EMBL database was no more than 96.5 % (with '*Bacillus pseudomegaterium*'; EMBL accession number X77791). This observation supports a separate species rank for these Antarctic isolates.

The two Candlemas Island isolates LMG 18419 (AJ250317) and LMG 18437 (AJ250319) showed very high 16S rDNA sequence similarity (above 99 %) and seem to belong to a distinct species at the border of *Paenibacillus* (Fig. 3; similarity in 16S rDNA sequence with established *Paenibacillus* species is 96 % at most, according to a FASTA search in the EMBL database). Classification of these strains must wait until more strains of this putative new taxon become available. Strain LMG 18435 (AJ250318) also appeared to represent a new species (highest similarities were between 96.8 % and 97.3 % with *Bacillus sporothermodurans* entries in the EMBL database; accession nos U49078, U49079 and U49080), but classification must again await further strains.

**Table 1.** Antarctic isolates studied and the methods used for characterization

Abbreviations: LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie Gent, Universiteit Gent, Belgium; Ir, iridescent colonies; L, large colonies; S, small colonies; Sm, smooth colonies; R, Mount Rittmann; c, chilled sample; f, frozen sample; p, heat-treated (pasteurized) sample; u, unpasteurized sample; SSI, South Sandwich Islands sample; numbers 1–6 in parentheses for SSI strains refer to sampling sites shown in Fig. 1(c).

Identity	LMG no.	Other designation	Source and isolation conditions*	Methods applied†		
				API	PAGE	FAME
<i>B. fumarioli</i>	17487	Rcp L1	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C	+	+	
<i>B. fumarioli</i>	17488	Rfu L10	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	17489 <sup>‡</sup>	Rcp Sm1	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	17490	Rfu Sm10	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	17491	Rcu Ir1	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	17492 <sup>‡</sup>	Rcp S1	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	17493	Rcu Ir3	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	17494	Rfu S10	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18407	Rcp L2	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	
<i>B. fumarioli</i>	18408	Rcu L4	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C			
<i>B. fumarioli</i>	18409 <sup>‡</sup>	Rcu L5	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18410	Rcu L6	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18411	Rfu L12	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C			
<i>B. fumarioli</i>	18412	Rcu Ir2	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18413	Rcu Sm4	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18414	Rcu Sm5	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C			+
<i>B. fumarioli</i>	18415	Rcu S3	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18416	Rfu S7	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18417	Rfu S9	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18430	Rcu Sm3	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18431	Rcu S4	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	
<i>B. fumarioli</i>	18432	Rcu S5	Soil from active fumarole, Mt Rittmann, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18418 <sup>‡</sup>	SSI009	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18467	SSI094	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C		+	
<i>B. fumarioli</i>	18468	SSI095	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	18469	SSI096	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C	+	+	+
<i>B. fumarioli</i>	18470	SSI097	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C		+	+
<i>B. fumarioli</i>	18471	SSI098	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C	+	+	
<i>B. fumarioli</i>	18472	SSI010	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 50 °C	+	+	
<i>Bacillus</i> sp.	18419 <sup>‡</sup>	SSI018	Soil from active fumarole (2), Lucifer Hill, pH 6, 50 °C	+	+	+
<i>Bacillus</i> sp.	18420	SSI056	Soil from active fumarole (2), Lucifer Hill, pH 5.5, 30 °C	+		
<i>Bacillus</i> sp.	18421	SSI073	Soil from lava area (6), Lucifer Hill, pH 5.5, 30 °C	+	+	+
<i>Bacillus</i> sp.	18422	SSI061	Soil from active fumarole (1), Lucifer Hill, pH 6, 30 °C	+		
<i>Bacillus</i> sp.	18423	SSI064	Soil from active fumarole (1), Lucifer Hill, pH 5.5, 30 °C	+		
<i>Bacillus</i> sp.	18424	SSI086	Soil from active fumarole (1), Lucifer Hill, pH 6, 20 °C	+		
<i>Bacillus</i> sp.	18425	SSI092	Soil from dead fumarole (3), Clinker Gulch, pH 6, 20 °C	+		
<i>Bacillus</i> sp.	18433	SSI001	Soil from active fumarole (1), Lucifer Hill, pH 6, 60 °C	+	+	
<i>Bacillus</i> sp.	18434	SSI004	Soil from active fumarole (2), Lucifer Hill, pH 6, 60 °C	+		
<i>Bacillus</i> sp.	18435 <sup>‡</sup>	SSI024	Soil from lava area (6), Lucifer Hill, pH 5.5, 40 °C	+	+	
<i>Bacillus</i> sp.	18436	SSI038	Soil from dead fumarole (3), Clinker Gulch, pH 5.5, 40 °C	+	+	+
<i>Bacillus</i> sp.	18437 <sup>‡</sup>	SSI048	Soil from active fumarole (2), Lucifer Hill, pH 5.5, 40 °C	+	+	+
<i>Bacillus</i> sp.	18438	SSI027	Soil from active fumarole (2), Lucifer Hill, pH 6, 40 °C	+		
<i>Bacillus</i> sp.	18439	SSI068	Soil from dead fumarole (4), Lucifer Hill, pH 6, 30 °C	+	+	+
<i>Bacillus</i> sp.	18440	SSI074	Soil from lava area (5), Lucifer Hill, pH 6, 20 °C	+		
<i>Bacillus</i> sp.	18442	SSI082	Soil from lava area (5), Lucifer Hill, pH 6, 20 °C	+		

\* The pH and temperature given indicate isolation conditions and not the conditions of the site at the time of sampling.

† ARDRA was applied to all strains. Abbreviations: API, tests in API System and other routine biochemical tests and morphological observations; FAMES, fatty acid methyl esters; PAGE, PAGE of whole-cell proteins.

‡ Strain subjected to 16S rDNA sequencing.

**Table 2.** Identifications by ARDRA and API tests of strains of aerobic endospore-forming bacteria isolated at pH 6.0 or 7.0 from soils of Candlemas Island

LMG no.	ARDRA	%S	API	%S	Identity
18419	<i>B. circulans</i>	85.7	<i>P. thiaminolyticus</i>	72.5	Unidentified
18420	<i>B. cereus</i> †	100	<i>B. cereus</i>	90.0	<i>B. cereus</i>
18421	<i>A. aneurinilyticus</i>	96.0	<i>Brevibacillus</i> sp.*	82.5	<i>A. aneurinilyticus</i>
18422	<i>B. horikoshii</i>	89.8	<i>B. stearothermophilus</i>	77.5	Unidentified
18423	<i>B. fusiformis</i>	97.7	<i>B. pasteurii</i> / <i>Sporosarcina ureae</i> *	82.5	<i>B. sphaericus</i> / <i>B. fusiformis</i> group
18424	<i>B. sphaericus</i>	100	<i>B. globisporus</i> *	87.5	<i>B. sphaericus</i> group
18425	<i>B. cereus</i>	100	<i>B. cereus</i>	90.0	<i>B. cereus</i>
18433	' <i>B. thermodenitrificans</i> '	95.5	<i>B. stearothermophilus</i> / ' <i>B. thermodenitrificans</i> '	82.5	' <i>B. thermodenitrificans</i> '
18434	' <i>B. thermodenitrificans</i> '	95.5	<i>B. stearothermophilus</i> / ' <i>B. thermodenitrificans</i> '	82.5	' <i>B. thermodenitrificans</i> '
18435	<i>P. thiaminolyticus</i>	93.6	<i>B. horikoshii</i>	90.0	Unidentified
18436	<i>B. lentus</i>	93.3	No reactions		<i>B. lentus</i>
18437	<i>B. circulans</i>	87.5	<i>B. circulans</i>	80.0	Atypical <i>B. circulans</i>
18438	<i>B. cereus</i>	100	<i>B. cereus</i>	90.0	<i>B. cereus</i>
18439	Unidentified	No score	<i>B. circulans</i>	80.0	Unidentified
18440	<i>B.adius</i>	86.4	No reactions		Unidentified
18442	' <i>B. carotarum</i> '	100	<i>Aneurinibacillus</i> sp.	85.0	Unidentified

\* Strains characterized using API Biotype 100 kit; all other strains were characterized using the API 20E/50 CHB kits.

† Not shown in Fig. 2.

## PAGE

In the numerical analysis of SDS-PAGE patterns of whole-cell proteins for 26 *B. fumarioli* strains (Fig. 4), all patterns showed similarities of 87% or greater. Four strains from Candlemas Island (LMG 18467, LMG 18468, LMG 18470 and LMG 18471) clustered at 96.5% similarity and this group merged with all the other *B. fumarioli* strains at 94% similarity or higher. The 26 *B. fumarioli* isolates merged with the other Candlemas Island strains (those isolated at pH 6.0 or 7.0) at about 77% similarity or lower. The reproducibility of the method is such that the observed subgrouping of the *B. fumarioli* strains is not significant above 93% similarity, and so, as with the ARDRA results, the patterns represent members of a distinct and homogeneous taxon.

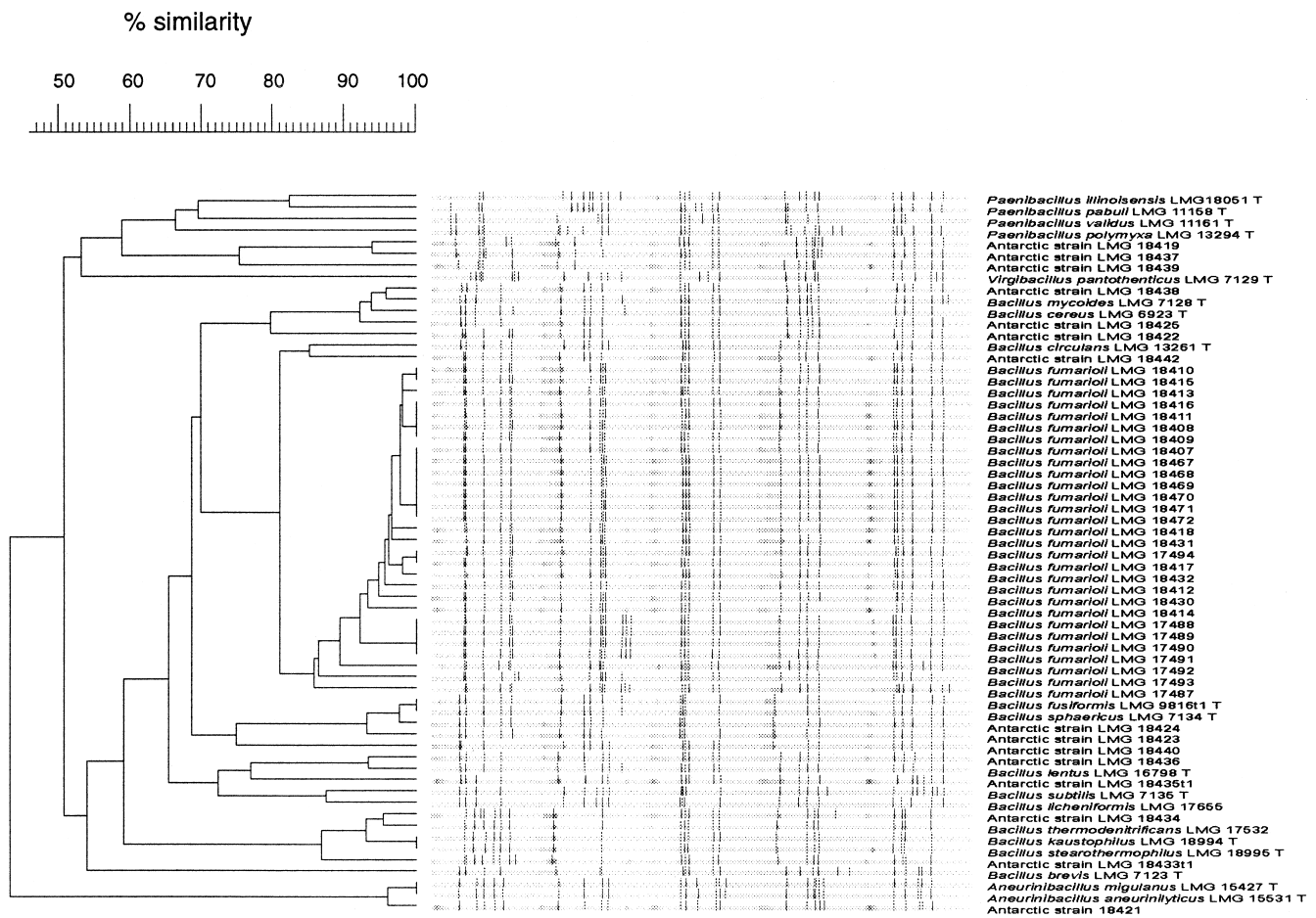
## FAME

The fatty acid profiles of 20 *B. fumarioli* strains were very homogeneous and were used to calculate a mean fatty acid pattern. Fatty acid profiles of other strains of the same group were not used to calculate the mean because they showed low overall peak areas, which might lead to uncertain interpretation. Visual inspection of the analyses however did not show different

patterns overall. The mean calculated FAME profile for *B. fumarioli* is (where fatty acid contents are given as percentage of total, fatty acids representing less than 1% of the total amount are omitted, and the standard deviations are shown in parentheses): iso-C<sub>15:0</sub>, 51.34 (2.98); anteiso-C<sub>15:0</sub>, 6.30 (0.88); iso-C<sub>16:0</sub>, 5.98 (0.98); anteiso-C<sub>16:0</sub>, 3.57 (0.69); iso-C<sub>17:0:ω10e</sub>, 1.98 (1.53); iso-C<sub>17:0</sub>, 15.70 (2.61); anteiso-C<sub>17:0</sub>, 14.01 (2.02). All these fatty acids are present in all strains except for iso-C<sub>17:0:ω10e</sub> which was absent from four out of the 20 strains used to calculate the mean value.

## DNA

Strain LMG 17489<sup>T</sup> showed 96% DNA–DNA relatedness with LMG 18409 and 96.8% relatedness with LMG 17492; LMG 17492 and LMG 18409 showed 98% relatedness. Strain LMG 18418 from Candlemas Island showed only 60% DNA relatedness with each of the three Mt Rittmann isolates LMG 17489<sup>T</sup>, LMG 17492 and LMG 18409, but for the present we propose to regard all four strains as belonging to a single species, as there are no outstanding phenotypic characters that might warrant the recognition of two closely related species. The G + C values were LMG 17489, 40.7%; LMG 18409, 40.7%; LMG 17492, 40.5%; LMG 18418, 40.6 mol%.



**Fig. 2.** Dendrogram based on UPGMA clustering of correlation coefficients of normalized 16S rDNA restriction profiles (ARDRA) of Antarctic isolates of aerobic endospore-forming bacteria and a selection of *Bacillus* (*sensu lato*) strains belonging to different rRNA groups.

### Phenotypic tests

The nine strains of *B. fumarioli* which were subjected to phenotypic tests (Table 1) showed similar characters as summarized in Table 3 and in the description of *B. fumarioli* given below. Numerical analysis of these data (not shown) revealed a single cluster of these strains merging at 85%  $S_G$ , implying appreciable phenotypic diversity within the group but no sub-clustering suggestive of, for example, subspecific types associated with the two geographically distinct sources of the strains.

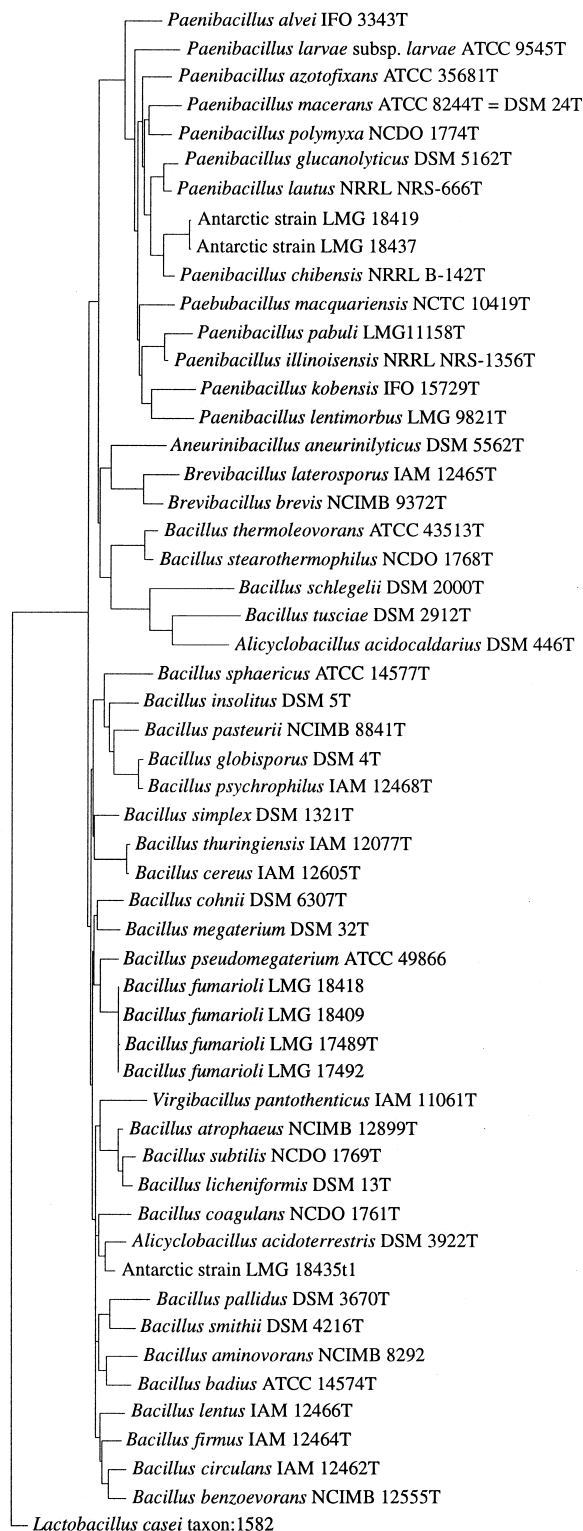
### Candlemas Island isolates growing at pH 6.0 or 7.0

As shown by Table 2, three strains (LMG 18420, LMG 18425 and LMG 18438) could confidently be identified as *Bacillus cereus*, and two strains (LMG 18433 and LMG 18434) were identified as '*Bacillus thermodenitrificans*', which is a thermophile and a close relative of *Bacillus stearothermophilus*. Strain LMG 18437 was identified as *Bacillus circulans* by both methods, but it does not represent a typical strain of this species as similarities were low. Of the remaining ten strains, six

(LMG 18419, LMG 18422, LMG 18435, LMG 18439, LMG 18440, LMG 18442) gave different identifications by ARDRA and API tests and, as their similarities to reference strains were low in one or both of the analyses, they remain unidentified. A search of the EMBL sequence database also did not achieve clear-cut identification of strains LMG 18419, LMG 18435 and LMG 18437 on the basis of their 16S rDNA sequences. Strain LMG 18440 gave no reactions in API tests, and *Bacillus badius* (the identification given by ARDRA) is indeed an unreactive organism in phenotypic tests, but the similarity to *B. badius* in ARDRA is too low for confidence. Strain LMG 18436 also gave no reactions in API tests, but this is consistent with the ARDRA identification of *Bacillus lentus* (often a slow performer in phenotypic tests) at a high enough similarity to the reference strain for confidence that it might belong to this species. Strains LMG 18421, LMG 18423 and LMG 18424 showed high similarities to reference strains of *Aneurinibacillus aneurinilyticus*, *Bacillus fusiformis* and *Bacillus sphaericus*, respectively, in ARDRA; the identifications by API tests do not match the ARDRA results, but as



10 % Dissimilarity



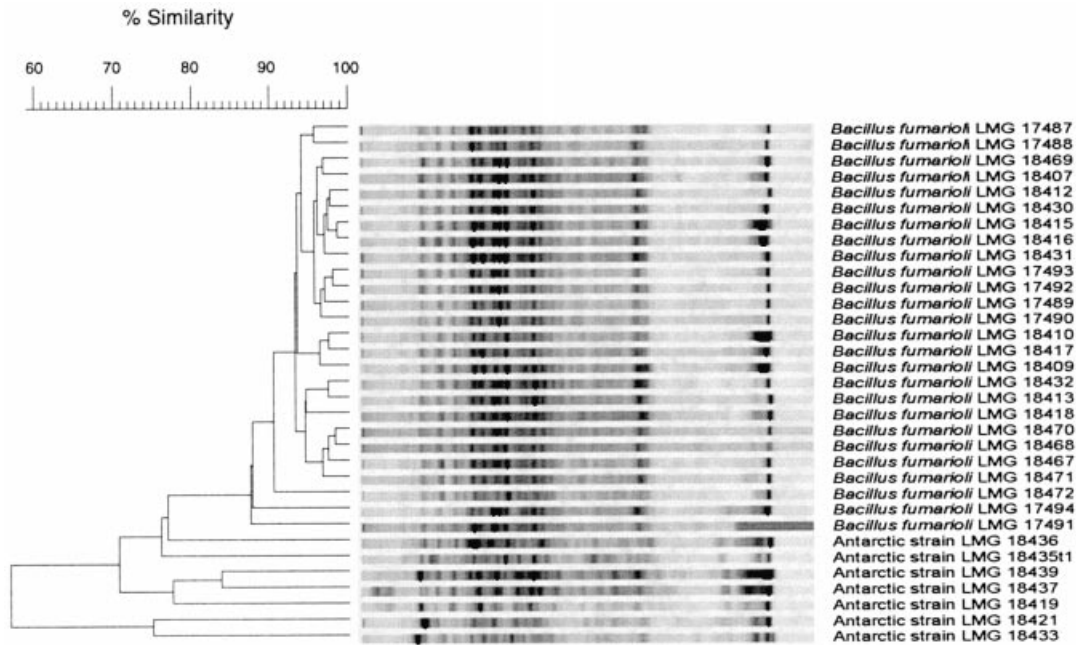
**Fig. 3.** Neighbour-joining clustering of 16S rDNA sequences (rooted with *Lactobacillus casei* as reference) based on a selection of 16S rDNA sequences from the EMBL database and *B. fumarioli* LMG 17489<sup>T</sup>, *B. fumarioli* LMG 17492, *B. fumarioli* LMG 18409, *B. fumarioli* LMG 18418 and unnamed strains LMG 18435, LMG 18419 and LMG 18437.

these organisms gave few positive reactions in the API kits, and were identified as close relatives of the species identified by ARDRA, the identifications by ARDRA may be accepted with confidence.

**DISCUSSION**

The *B. fumarioli* strains from Mt Rittmann and Candlemas Island showed similar profiles in ARDRA, PAGE, FAME and phenotypic analyses and are clearly members of a single species. It is of particular interest that the isolates from the two quite different environments which lie about 5600 km apart (that on Mt Rittmann being an active fumarole at an altitude of 2600 m and 40 km inland on the Antarctic continent, and that on Candlemas Island being a cold, dead fumarole lying close to sea level and being influenced by the Southern Ocean only about 300 m away), show similar phenotypic behaviour and substantial genotypic similarity (in 16S rDNA sequence and overall DNA relatedness). This finding suggests that colonization of these two sites has been from a common source, and this is most likely to have occurred from the air as free spores or spores attached to plant propagules; birds are not known to visit Mt Rittmann and human visits to this site have only occurred since 1988/1989 and not in conjunction with visits to Candlemas Island. Humans have only occasionally visited the latter site since its discovery by Captain James Cook in 1775, and a first survey by a BAS team in 1964, but its aerobic endospore-forming flora could owe something to contamination by humans.

The *B. fumarioli* strains we analysed were isolated from the 1995–1996 season’s soil samples from Mt Rittmann and the 1996–1997 samples from Candlemas Island. The organism was then reisolated from all ten samples collected from Mt Rittmann, and from all eight samples collected from Cryptogam Ridge, Mt Melbourne, during the 1998–1999 season, but not from the 1995–1996 or 1998–1999 samples from the NW ridge of Mt Melbourne or from local, unheated soils. It is of special interest that the NW ridge of Mt Melbourne failed to yield the organism from samples taken on two occasions. Why this particular geothermal site, lying a short distance from Cryptogam Ridge (and apparently having a similar soil), should not yield *B. fumarioli* is not understood, and the near-absence of moss from this site (Broady *et al.*, 1987) is also unexplained, yet *B. fumarioli* was isolated from both mossy and moss-free areas of Cryptogam Ridge and Mt Rittmann. Broady *et al.* (1987) remarked on the low diversity of Victoria Land warm ground bryophytes compared with Deception Island and the South Sandwich Islands in maritime Antarctica. They noted that the geothermal areas of Mt Erebus and Mt Melbourne are, in comparison with maritime Antarctica, much further from the rich propagule sources of more temperate lands to the north and west; nonetheless, the northern Victoria Land volcanoes do lie in the tracks of cyclonic depressions spiralling southwards over the Southern Ocean and so might readily

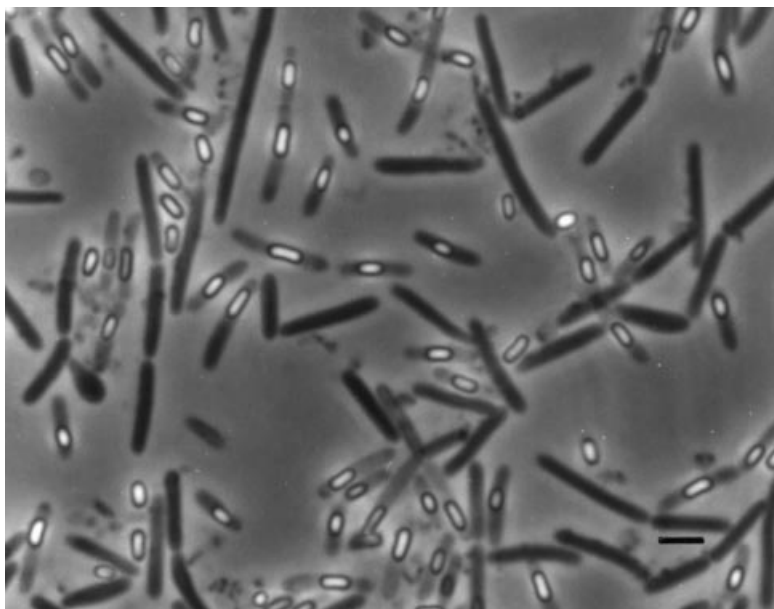


**Fig. 4.** Normalized computer profiles from SDS-PAGE analyses of whole-cell proteins of Antarctic isolates. The dendrogram is based on UPGMA clustering of the correlation coefficient (*r*) of the total protein profiles. The zone used for clustering was between point 30 and point 330 (a complete lane contains 400 points). The subculture of strain LMG 18335 contained two types (t1 and t2) with slightly different patterns (two bands different). Only t1 is included in the figure.

**Table 3.** Characters for differentiating some thermotolerant and thermophilic *Alicyclobacillus* and *Bacillus* species

Data from Bonjour & Aragno (1984), Claus & Berkeley (1986), Gordon *et al.* (1973), Nicolaus *et al.* (1996, 1998), Schenk & Aragno (1979), Suzuki *et al.* (1983), White *et al.* (1993), Zarilla & Perry (1987). Species: 1, *B. fumarioli*; 2, '*A. acidocaldarius* subsp. *rittmannii*'; 3, '*B. thermoantarcticus*'; 3, *B. tusciae*; 4, *B. schlegelii*; 5, *B. coagulans*; 6, *B. stearothermophilus*; 7, '*B. thermodenitrificans*'; 9, *B. thermoglucosidasius*; 10, *B. thermoleovorans*. +, > 85% positive; -, 0-15% positive; W, weak positive reaction; V, varies within strains; d, varies between strains; NR, not reported; for spore shape: C, cylindrical, E, ellipsoidal; S, spherical; for spore position: C, central or paracentral; ST, subterminal; T, terminal.

Character	Species									
	1	2	3	4	5	6	7	8	9	10
Growth temperature (°C)										
Maximum	55	70	65	< 65	< 80	55-60	65-75	70	69	70
Optimum	50	63	63	55	70	36-43	68	65-68	61-63	55-65
Minimum	25-30	45	37	47	> 37	15-25	30-45	< 65	42	45
pH range	4.0-6.5	2.5-5.0	5.5-9.0	3.5-6.0	NR	4.0-> 6.8	> 5.7-> 6.8	5.5-NR	< 6.5-8.7	6.2-7.5
Optimum pH	5.5	4.0	6.0	4.5	6.0-7.0	6.0	6.0-6.5	6.0	6.5	6.2-7.5
Motility	+	-	+	+	+	+	+	-	d	-
Spore shape	E/C	E	E	E	S	E	E	E	E	E
Spore position	C/ST	C/T	T	ST	T	ST/T	ST/T	T/ST	T	T
Sporangia swollen	-	-	NR	+	+	V	+	+	+	+
Anaerobic growth	-	+	-	-	-	+	-	+	+	-
Nitrate reduction	-	W	-	+	+	d	d	+	+	+
Gelatin hydrolysis	+	-	+	NR	-	-	+	d	-	NR
Casein hydrolysis	-	-	-	NR	-	d	d	d	+	NR
G + C mol% of type strain	40.7	64.3	53.7	57.7	64.4	47.1	51.9	52.2	45	58



**Fig. 5.** Photomicrograph of sporangia and vegetative cells of the type strain of *B. fumarioli* viewed by phase-contrast microscopy; ellipsoidal and cylindrical spores lie paracentrally and subterminally in unswollen sporangia. Bar, 2  $\mu$ m.

receive exotic propagules from temperate regions (Wynn-Williams, 1991). Other possible reasons for the observed low diversity are that both of the Victoria Land volcanoes have been vigorously active within the past few hundred years (Nathan & Schulte, 1967; Bonaccorso *et al.*, 1991), that propagules would experience harsher conditions in transit to these sites (in comparison with maritime Antarctic sites), and that long periods of darkness on warm, moist soils may be inimical to some potential colonists. Broady *et al.* (1987) also reported that none of the local cold-ground bryophytes of Victoria Land had colonized the volcanoes and inferred that the soil chemistry of the fumarole environment might be unsuitable, as dispersal problems would not seem to be great. It is remarkable, therefore, to find *B. fumarioli* in soils of both Victoria Land and maritime Antarctica (Candlemas Island), and noteworthy that it was not isolated from 25 cold soils local to Mt Melbourne, especially as the Candlemas Island soil yielding the organism was not geothermally active at the time of sampling. Llarch *et al.* (1997) did not find *B. fumarioli* in their geothermal soils from Deception Island, but they did not cultivate at pH 5.5 from their samples.

It is notable for the Candlemas Island strains growing at pH 6 or 7 that only eight of the 16 isolates examined received similar identifications by ARDRA and phenotypic analysis, and that six remained unidentifiable. Although the unidentified isolates may represent new taxa or atypical members of established taxa, as Llarch *et al.* (1997) found on Deception Island, further isolates would need to be isolated and examined before any taxonomic conclusions could be drawn.

In conclusion, our polyphasic data show that the moderately thermoacidophilic, aerobic endospore-forming isolates from Mt Rittmann and Candlemas Island represent a new taxon, which is distinct from '*Bacillus thermoantarcticus*' isolated from Mt Melbourne by Nicolaus *et al.* (1996) and '*Alicyclobacillus acidocaldarius* subsp. *rittmannii*' isolated from Mt Rittmann (Nicolaus *et al.*, 1998). The description of the new species follows, and Table 3 shows characters that distinguish it from some other thermophilic, aerobic endospore-forming bacteria.

#### Description of *Bacillus fumarioli*

*Bacillus fumarioli* (fum.a.rio'li. M. L. adj. *fumarioli* from L. masc. n. *fumariolum* a smoke vent; whence fumarole, a hole emitting gases in a volcanic area, referring to the habitat from which the organisms were isolated).

Isolated from geothermal soils and active and inactive fumaroles in north Victoria Land, Antarctica, and Candlemas Island, South Sandwich archipelago. Cells are feebly motile, Gram-positive, round-ended rods (0.5–0.8 by 4–8  $\mu$ m) occurring singly and in pairs and chains on BFA medium at pH 5.5. On TSA MnSO<sub>4</sub> the rods tend to become pleomorphic and then ghost cells after 48–72 h incubation. Endospores may be formed in small numbers on TSA MnSO<sub>4</sub> and in large numbers on BFA medium at pH 5.5; they are ellipsoidal to cylindrical, lie paracentrally and subterminally, and do not swell the sporangia (Fig. 5). After 2–3 d on TSA colonies are 5–10 mm in diameter, low convex, circular and slightly irregular, butyrous, brownish-cream and opaque with a smooth, glossy and sometimes slightly

iridescent appearance; on BFA medium colonies are 3–8 mm in diameter, low convex, circular and slightly irregular, with some trailing along streak lines, butyrous, creamy-brown (sometimes darker with age in their centres), and opaque with a smooth, glossy appearance. Minimum temperature for growth lies between 25 and 30 °C, the optimum temperature for growth is 50 °C, and the maximum growth temperature is 55 °C. Minimum pH for growth lies between 4.0 and 5.0, the optimum pH for growth is 5.5, and the maximum pH for growth lies between 6.0 and 6.5. Organisms are strictly aerobic and catalase-positive. In the API 20E strip, Voges–Proskauer reaction is positive, and gelatin is hydrolysed; reactions for ONPG hydrolysis, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulphide production, urease, tryptophan deaminase, indole production and nitrate reduction are negative. Hydrolysis of aesculin and of casein negative. Acid without gas is produced from the following carbohydrates: D-fructose, D-glucose, mannitol, D-mannose, N-acetylglucosamine (weak), sucrose, trehalose (weak). Acid production from the following carbohydrates is variable: galactose, glycerol, lactose, maltose, D-melibiose, D-melezitose, methyl  $\alpha$ -D-glucoside, D-raffinose, ribose and D-turanose. Hydrolysis of aesculin is positive. Acid is not produced from the following carbohydrates: adonitol, amygdalin, D- and L-arabinose, D- and L-arabitol, arbutin, cellobiose, dulcitol, erythritol, D- and L-fucose,  $\beta$ -gentiobiose, gluconate, glycogen, meso-inositol, inulin, 2-keto-D-gluconate, 5-keto-D-gluconate, D-lyxose, methyl  $\alpha$ -D-mannoside, methyl-xyloside, rhamnose, salicin, sorbitol, L-sorbose, starch, D-tagatose, xylitol, and D- and L-xylose. Sensitive to disks of the following antibiotics: ampicillin (25  $\mu$ g), chloramphenicol (50  $\mu$ g), colistin sulphate (100  $\mu$ g), kanamycin (30  $\mu$ g), nitrofurantoin (50  $\mu$ g), streptomycin (25  $\mu$ g) and tetracycline (100  $\mu$ g); resistant to nalidixic acid (30  $\mu$ g).

The major cellular fatty acid is iso-C<sub>15:0</sub>, present at a level of about 50% of the total fatty acids, while iso-C<sub>17:0</sub> and anteiso-C<sub>17:0</sub> appear in roughly equal amounts and account for about 30% of the total fatty acids.

The G+C content is 40.7 mol% for the type strain, strain LMG 17489 (Rcp Sm1); the 16S rRNA sequence of this strain is deposited at EMBL under accession number AJ250056. In the variable characters listed above, the type strain produces acid without gas from glycerol, maltose, D-melezitose, D-raffinose, D-turanose; and not from galactose, lactose, D-melibiose, methyl  $\alpha$ -D-glucoside and ribose.

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