Paenibacillus cineris sp. nov. and *Paenibacillus cookii* sp. nov., from Antarctic volcanic soils and a gelatin-processing plant

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Seven strains of aerobic, endospore-forming bacteria were found in soil taken from an active fumarole on Lucifer Hill, Candlemas Island, South Sandwich archipelago, Antarctica, and four strains were from soil of an inactive fumarole at the foot of the hill. Amplified rDNA restriction analysis, 16S rDNA sequence comparisons, SDS-PAGE and routine phenotypic tests support the proposal of two novel species of *Paenibacillus*, *Paenibacillus cineris* sp. nov. and *Paenibacillus cookii* sp. nov., the type strains of which are LMG 18439^T (= CIP 108109^T) and LMG 18419^T (= CIP 108110^T), respectively. A further strain, isolated from a gelatin-production process, showed more than 99% 16S rDNA sequence similarity to the proposed *P. cookii* type strain and, although the gelatin isolate was atypical when compared with the fumarole isolates by repeated element primed-PCR, SDS-PAGE and phenotypic analyses, it was shown by DNA–DNA reassociation studies to belong to the same species. Strains of *P. cookii* produce spreading growth with motile microcolonies. Both species produce swollen sporangia that are typical for the genus, they both show 97.6% 16S rDNA sequence similarity to *Paenibacillus azoreducens*, they have 51.5–51.6 mol% G + C in their DNA and their major fatty acid is anteiso-C_{15:0}; however, fatty acids C_{16:0} and anteiso-C_{17:0} represent, respectively, 18 and 10% of the total in *P. cineris*, but 11 and 20% in *P. cookii*.

Logan *et al.* (2000) reported the isolation of aerobic, endospore-forming bacteria from volcanic soils of Candlemas Island in the South Sandwich archipelago in Antarctica and found that two of their strains (LMG 18419 and LMG 18437) found in warm soil taken from a mossy area on an active fumarole at the summit of Lucifer Hill on

Candlemas Island and one strain (LMG 18439) isolated from the soil of a cold, inactive fumarole at the foot of the hill represented two unidentifiable species of *Paenibacillus*. The present paper describes the characterization of seven further *Paenibacillus* strains isolated from the same soils and one isolate from a Belgian gelatin-production plant and proposes the novel species *Paenibacillus cineris* sp. nov. and *Paenibacillus cookii* sp. nov., with LMG 18439^T and LMG 18419^T as the respective type strains. Two other strains not identified by Logan *et al.* (2000), LMG 18422^T and LMG 18435^T, have since been proposed as the type strains of *Bacillus luciferensis* (Logan *et al.*, 2002) and *Bacillus shackletonii* (Logan *et al.*, 2004), respectively.

Antarctic strains were isolated and maintained on trypticase soy agar (TSA; Oxoid) containing 5 mg MnSO₄ l⁻¹ (TSA MnSO₄) to enhance sporulation, as described by Logan *et al.* (2000). Strains LMG 18419^T and LMG 18437 and the additional strains R-11662 (= B1719), R-11663 (= B1735), R-11664 (= B1749), R-11665 (= B1750) and R-11666 (= B1751) were isolated from a sample of soil by moss on

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Abbreviations: ARDRA, amplified rDNA restriction analysis; FAMES, fatty acid methyl esters.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *P. cineris* strains LMG 18439^T (= CIP 108109^T) and LMG 21976 and *P. cookii* strains LMG 18419^T (= CIP 108110^T), 18437 and R-11600 determined in this work are AJ575658, AJ575659, AJ250317, AJ250319 and AJ438302, respectively.

A photomicrograph of colonies, an extended phylogenetic tree and detailed fatty acid compositions are available as supplementary material in IJSEM Online.

a warm, active fumarole (temperature range 30–60 °C; altitude 232 m; site 2 in Logan *et al.*, 2000; Plate I in Tomblin, 1979) near the northern crater of Lucifer Hill, at the top of the scoria cone. Strain LMG 18439^T and the further strains LMG 21976 (= B1770), R-11672 (= B1769) and R-11674 (= B1771) were isolated from a sample of ashy soil near limited moss growth on a cold, inactive fumarole (temperature range 0–15 °C; altitude 20 m; site 4 in Logan *et al.*, 2000) at the southern end of Clinker Gulch (Plate IVb in Tomblin, 1979; Fig. 2 in Longton & Holdgate, 1979), a gully that appears to represent a former shoreline and which lies at the northern foot of Lucifer Hill. A description of Candlemas Island is given by Logan *et al.* (2002). Strain R-11600 (= B2765), isolated from a gelatin extract of bovine bones (De Clerck & De Vos, 2002), was included in the study because of its high similarity in 16S rDNA sequence to LMG 18419^T, the type strain of *P. cookii*. This gelatin strain was isolated on a medium the composition of which was based on the gelatin extract that was collected at the point of sampling. The gelatin extract was sterilized and used as a liquid medium for enrichment and isolates were then obtained in pure culture by streak dilution from the enrichment medium onto plates prepared by adding 2% agar (LAB M) to fresh enrichment medium. Strains were characterized by amplified rDNA restriction analysis (ARDRA), SDS-PAGE analysis, gas chromatographic analysis of fatty acid methyl esters (FAMES) and phenotypic tests, with numerical analysis of data, as described by Logan *et al.* (2000, 2002). For observations on sporangia, cells were grown on TSA MnSO₄ and on *Bacillus fumarioli* agar (BFA) at pH 6.5 (Logan *et al.*, 2000). Antibiotic sensitivities were measured using Mastrings (MAST Diagnostics) on Iso-Sensitest agar (Oxoid). 16S rDNA sequences were obtained for strains LMG 18419^T, LMG 18437, LMG 18439^T, LMG 21976 and R-11600 and aligned with sequences in the EMBL database; cluster analysis was performed using CLUSTAL W (Thompson *et al.*, 1994) and trees were constructed using TREECON software (Van de Peer & De Wachter, 1994). DNA base compositions of these strains were also determined, as described by Logan *et al.* (2000).

The strains were found to be Gram-variable or Gram-negative, facultatively anaerobic, motile rods that formed ellipsoidal spores that lay paracentrally or subterminally in swollen or slightly swollen sporangia (Fig. 1). On TSA plates, the strains from site 4 (proposed as *P. cineris*) formed discrete colonies, while those from site 2 (proposed as *P. cookii*) showed yellowish, spreading growth with motile colonies (Supplementary Fig. A in IJSEM Online), which was reminiscent of some strains that were previously named *Bacillus circulans* (illustrated in Logan & Turnbull, 2003), but which are now allocated to *Paenibacillus glucanolyticus*, *Paenibacillus lautus* and some unidentified *Paenibacillus* spp. (Alexander & Priest, 1989; N. A. Logan, unpublished observations), and also reminiscent of colonies of *Paenibacillus alvei* (illustrated in Logan & Turnbull, 2003), but they did not have the odour associated with cultures of *P. alvei*. As well as showing different colonial morphologies,

Fig. 1. Phase-contrast photomicrographs of sporangia and vegetative cells of the type strains of *Paenibacillus cineris* sp. nov. (top), showing ellipsoidal endospores that are located paracentrally and subterminally and swell the sporangia, and *Paenibacillus cookii* sp. nov. (bottom), showing ellipsoidal endospores that lie subterminally and swell the sporangia slightly. Bars, 2 µm.

the two groups of strains from the separate Candlemas Island sites showed two distinct patterns of results in the phenotypic characters; however, each group of strains showed sufficient within-group phenotypic variation to suggest that they are not repeated isolations of the same strain. The seven strains from site 2 clustered together at 92.5% similarity in a UPGMA cluster analysis (not shown) based upon these characters; the gelatin isolate joined them at 85% similarity, and this cluster merged with strains of *Paenibacillus polymyxa* and other *Paenibacillus* species at 80% similarity. In the same analysis, the four strains from site 4 clustered together at 90% similarity and this cluster merged with other strains of *Paenibacillus* and strains from site 2 at only 77.5% similarity. These results indicate that the two groups are phenotypically distinct from each other and from established species of *Paenibacillus*.

It is of interest that, although the temperature of the soil of the active fumarole at site 2 ranged from 30 °C and that of the dead fumarole at site 4 ranged from 5 °C, all of our isolates showed similar temperature ranges for growth, with minima at 0 °C and maxima at 50 °C. Perhaps these temperature ranges reflect a versatility necessary to organisms living in such volcanic soils where geothermal activity at a given site is impermanent, as the organisms do not appear to have evolved growth ranges restricted to the measured temperatures of their soils at the times of sampling. The pH ranges of the strains were also broadly similar, but showed appreciable variation within the groups, with the strains from site 2 showing minima of 5.0–5.5 and maxima of 7.0–10.0 and those from site 4 having minima of 5.0–6.5 and maxima of 7.5–11.0.

The two groups of strains could also be distinguished by their fatty acid profiles; although anteiso-C_{15:0} was the dominant component in all of the strains, as it was for the other *Paenibacillus* strains studied (Table 1), the strains from site 4 had more of this fatty acid and more of C_{16:0} than did the strains from site 2, while the strains from site 2 had more of anteiso-C_{17:0} than did strains from site 4.

Comparison of the ARDRA patterns of strains LMG 18419^T and LMG 18437 with a database of over 1000 authentic strains of species of aerobic, endospore-forming bacteria did not yield an identification; the highest similarity found for LMG 18419^T and 18437 was 77 % with strains of *Paenibacillus amylolyticus* and *Paenibacillus glucanolyticus*. The highest similarity found for LMG 18439^T was 73 % with LMG 18419^T and LMG 18437 and strains of *P. amylolyticus* and *P. glucanolyticus*. The results of SDS-PAGE analysis showed a close grouping within isolates from each site (Fig. 2). The gelatin isolate R-11600 clustered with the isolates from site 2 but showed a somewhat different profile. In 16S rDNA sequence comparisons with entries in the EMBL database, the closest matches achieved for LMG 18419^T and LMG 18437 (EMBL accession nos AJ250317 and AJ250319) were 97.6 % to *Paenibacillus azoreducens* (Fig. 3); the closest matches achieved for LMG 18439^T and LMG 21976 (EMBL accession nos AJ575658 and AJ575659) were also 97.6 % to *P. azoreducens* (Fig. 3). The gelatin isolate R-11600 (AJ438302) showed 99.7 % similarity to *P. cookii* LMG 18419^T. A fuller 16S rDNA sequence comparison tree is available as Supplementary Fig. B in IJSEM Online.

The six other strains from site 2 were further compared with the gelatin isolate by means of rep-PCR with the (GTG)₅ and BOX A1R primers (Fig. 4). As with PAGE, this technique also showed the gelatin isolate to be an outlier of the cluster containing the fumarole isolates from site 2. The gelatin isolate also produced spreading, motile colonies on agar media and differed from the Candlemas isolates of *P. cookii* in only two phenotypic characters: it produced acid from adonitol and it did not produce acid from D-fructose. A DNA–DNA reassociation of 78 % was found between

Table 1. Some characters for distinguishing between *P. cineris* and *P. cookii* and closely related *Paenibacillus* species

Taxa: 1, *P. cineris* sp. nov.; 2, *P. cookii* sp. nov.; 3, *P. azoreducens*; 4, *P. amylolyticus*; 5, *P. polymyxa*; 6, *P. glucanolyticus*; 7, *P. borealis*. +, >85 % positive; (+), 75–84 % positive; v, variable (26–74 % positive); (–), 16–25 % positive; –, 0–15 % positive; w, weak positive reaction; +/w, positive or weak positive reaction; v(w), variable and, when positive, the reaction is weak. Fatty acid data for *P. azoreducens* are from Meehan *et al.* (2001) and those for *P. borealis* are from Elo *et al.* (2001). With the exception of microscopic observations, growth at 50 °C and casein hydrolysis, all characters were determined using tests in API 20E and 50 CHB systems. All species produce ellipsoidal spores that swell the sporangia.

Character	1	2	3	4	5	6	7
Spore position*	CS	S	S	ST	S	T	ST
Spreading growth	–	+	–	–	–	v	–
Growth at 50 °C	+	+	+	–	–	–	–
Gelatin hydrolysis	–	v	w	+	+	+	–
Casein hydrolysis	w	w	–	w	+	+	–
Production of H ₂ S	–	–	+	–	–	–	+
Voges–Proskauer	v(w)	+	w	–	v	–	–
Gas production from glucose	–	–	–	–	–	+	–†
Acid from:							
N-Acetylglucosamine	+	(–)	+	+	–	+	+
D-Arabinose	v(w)	–	+	–	–	–	+
L-Fucose	–	+/w	–	–	–	v	w
Glycerol	–	w	w	–	+	+	–†
Glycogen	v	+	+	+	+	+	+
meso-Inositol	+	–	–	–	–	–	–
5-Keto-D-gluconate	–	v(w)	–	w	–	–	w
Mannitol	+	–	+	+	+	+	+
D-Melezitose	+	+	–	–	v	+	+
Sorbitol	v(w)	–	–	–	–	–	+
Oxidase	+	+	–	–	–	+	–
Growth in 5 % NaCl	–	+	–	–	–	+	–
Fatty acid content:‡							
iso-C _{15:0}	6	6.5	6	6	10.5	7	12.5
anteiso-C _{15:0}	46	36	40	42.5	52	58.5	43
C _{16:0}	18	11	22	20	12	7	12
iso-C _{16:0}	9	13	8.5	10.5	7	5	10.5
anteiso-C _{17:0}	10	20	20	8.5	4.5	5.5	2.5

*C, Central/paracentral; s, subterminal; T, terminal.

†Our results for the type strain differ from those given in the published description (Elo *et al.*, 2001).

‡Major components are expressed as percentages of the total and rounded to nearest 0.5 %.

R-11600 and LMG 18419^T, demonstrating that these strains belong to the same species.

It may seem remarkable that *P. cookii* has been isolated from two such disparate and geographically separated environments, but Logan *et al.* (2000) found *Bacillus fumarioli* in

Fig. 2. Normalized computer profiles from SDS-PAGE analyses of whole-cell proteins of *P. cineris* and *P. cookii* strains. The dendrogram is based on UPGMA clustering of the correlation coefficient (*r*) of the total protein profiles.

Fig. 3. Phylogenetic positions of *P. cineris* and *P. cookii* among the type strains of a selection of other *Paenibacillus* species, based on neighbour-joining clustering of a 1367 bp portion of the 16S rRNA gene. Bootstrap values (expressed as percentages of 1000 replications) $\geq 60\%$ are shown at branch points. Accession numbers are given in parentheses. The scale bar indicates 0.02 nucleotide substitutions per nucleotide position. Tree rooted using *B. subtilis* DSM 10^T (AJ276351) as an outgroup.

Fig. 4. Cluster analysis based on linearly combined BOX- and (GTG)₅-PCR profiles of *P. cookii* isolates. The dendrogram was constructed using UPGMA with correlation levels expressed as percentages of Pearson's correlation coefficient.

both northern Victoria Land and Candlemas Island, which are geographically isolated from each other, presumably distributed by the action of wind, and this species has subsequently been isolated from European and American gelatin-production plants. Our failure to identify the Candlemas Island strains and the gelatin isolate by the genotypic and phenotypic methods applied, the similarities of the Antarctic strains to each other in the phenotypic analyses and the DNA relatedness of the gelatin isolate to one of the groups of Antarctic strains support the proposal of two novel species, the descriptions of which follow.

Description of *Paenibacillus cineris* sp. nov.

Paenibacillus cineris (ci'ne.ris. L. gen. masc. n. *cineris* of ash, referring to the volcanic, ash-based soil from which the type strain was isolated).

Cells are motile, round-ended rods (0.7–0.9 × 2.5–4.0 µm) occurring singly and in pairs. Gram-negative or Gram-variable. Endospores are formed within 2–3 days incubation on BFA at 30 °C; they are ellipsoidal, lie paracentrally and subterminally and swell the sporangia (Fig. 1, top). After 2 days at 30 °C, colonies are 1–5 mm in diameter, low convex and circular with slightly irregular edges; they are opaque and glossy and light beige to greyish in colour with paler margins. Grows on nutrient agar. Facultatively anaerobic, catalase- and oxidase-positive. Minimum temperature for growth lies between 8 and 15 °C and the maximum growth temperature is 50 °C. Minimum pH for growth lies between 5.0 and 6.5, the optimum pH for growth is 7.0 and the maximum pH for growth varies between strains and lies between 7.5 and 11.0. Growth occurs in the presence of 3 % NaCl, but is inhibited by 5 % NaCl. Hydrolysis of casein is weakly positive. In the API 20E strip, reactions for ONPG hydrolysis and nitrate reduction are positive. Voges–Proskauer reaction is variable and positive reactions are weak. Gelatin is not hydrolysed and reactions for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulphide production, urease, tryptophan deaminase and indole production are negative. Hydrolysis of aesculin is positive. Acid is produced without gas from the following carbohydrates in the API 50 CH gallery using the CHB suspension medium: *N*-acetylglucosamine, amygdalin, L-arabinose, arbutin, D-cellobiose, D-fructose, galactose, gentiobiose, D-glucose, *meso*-inositol, lactose, maltose, mannitol, D-melezitose, D-melibiose, methyl D-glucoside, methyl D-mannoside (weak), methyl xyloside, D-raffinose, ribose, salicin, starch, sucrose, D-trehalose, D-turanose and D-xylose. Production of acid is variable for D-arabinose (positives are weak), glycogen, inulin, D-mannose and sorbitol (positives are weak). Acid is not produced from the following carbohydrates: adonitol, D- and L-arabitol, dulcitol, erythritol, D- and L-fucose, glycerol, gluconate, 2-keto-D-gluconate, 5-keto-D-gluconate, D-lyxose, rhamnose, L-sorbose, D-tagatose, L-xylose and xylitol. Strains are resistant to disks of the antibiotics kanamycin (30 µg) and streptomycin (25 µg) and moderately resistant to

tetracycline (100 µg); sensitive to ampicillin (25 µg), chloramphenicol (50 µg), colistin sulphate (100 µg), nalidixic acid (30 µg) and nitrofurantoin (50 µg). The major cellular fatty acids are anteiso-C_{15:0}, C_{16:0}, anteiso-C_{17:0} and iso-C_{16:0} (respectively representing about 46, 18, 10 and 9 % of total fatty acids). The following fatty acids are present in smaller, decreasing amounts (between about 6 and 1 %): iso-C_{15:0}, C_{14:0}, iso-C_{17:0}, C_{15:0} and iso-C_{14:0}. (Supplementary Table in IJSEM Online).

The G + C content is 51.5 mol% for the type strain, strain LMG 18439^T (=CIP 108109^T=Logan collection no. B1768^T=isolate SSI068); the 16S rDNA sequence of this strain is deposited at EMBL under accession number AJ575658. In the variable reactions listed above, the type strain grows in the temperature range 15–50 °C and in the pH range 5.0–10.5; it is negative for the Voges–Proskauer reaction, production of acid is weak for D-arabinose, inulin and sorbitol and negative for glycogen and D-mannose. Reference strains are LMG 21976, R-11672 and R-11674. Strains were isolated from the ashy soil of a cold, dead fumarole at the foot of Lucifer Hill, a volcano on Candlemas Island, South Sandwich archipelago, Antarctica.

Description of *Paenibacillus cookii* sp. nov.

Paenibacillus cookii [cook'i.i. N.L. gen. n. *cookii* of Cook, referring to Captain James Cook, of HMS *Resolution*, who discovered Candlemas Island on Candlemas Day (2 February), 1775].

Cells are motile, round-ended rods (0.6–0.8 × 3.0–3.5 µm) occurring singly and in pairs. Gram-negative or Gram-variable. Endospores are formed within 2–3 days incubation on BFA incubated at 30 °C; they are ellipsoidal, lie subterminally and swell the sporangia slightly (Fig. 1, bottom). After 2 days at 30 °C, colonies are 1–4 mm in diameter, convex, yellowish and transparent with opaque centres; motile microcolonies are formed and spread across the surface of the agar, rotating clockwise and anticlockwise (Supplementary Fig. A). Grows on nutrient agar. Facultatively anaerobic. Catalase-positive or weakly positive and oxidase-positive. Minimum temperature for growth lies between 15 and 20 °C and the maximum growth temperature is 50 °C. Minimum pH for growth lies between 5 and 5.5, the optimum pH for growth is 7.0 and the maximum pH for growth lies between 7.5 and 10. Growth occurs in the presence of 5 % NaCl, but is inhibited by 7 % NaCl. Casein hydrolysis is positive but weak. In the API 20E strip, ONPG hydrolysis, Voges–Proskauer reaction and nitrate reduction are positive. Hydrolysis of gelatin is variable while reactions for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, hydrogen sulphide production, urease, tryptophan deaminase and indole production are negative. In the API 50CH gallery using the CHB suspension medium, hydrolysis of aesculin is positive and acid is produced without gas from the following carbohydrates: amygdalin, L-arabinose, arbutin, D-cellobiose, galactose, gentiobiose, D-glucose, glycerol, glycogen, lactose,

maltose, D-melezitose, D-melibiose, methyl D-glucoside, methyl xyloside, D-raffinose, ribose, salicin, starch, sucrose, D-trehalose, D-turanose, D-xylose. Production of acid without gas is variable for the following carbohydrates: *N*-acetylglucosamine, D-fructose (weak), L-fucose, gluconate (weak), 5-keto-D-gluconate (weak), D-mannose and methyl D-mannoside. Acid is not produced from the following carbohydrates: adonitol, D-arabinose, D- and L-arabitol, dulcitol, erythritol, D-fucose, *meso*-inositol, inulin, 2-keto-D-gluconate, D-lyxose, mannitol, rhamnose, sorbitol, L-sorbose, D-tagatose, L-xylose and xylitol. Sensitive to disks of the antibiotics ampicillin (25 µg), chloramphenicol (50 µg), colistin sulphate (100 µg), kanamycin (30 µg), nalidixic acid (30 µg), nitrofurantoin (50 µg), streptomycin (25 µg) and tetracycline (100 µg). The major cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0}, C_{16:0} and iso-C_{15:0} (respectively representing about 36, 20, 13, 11 and 6.5 % of total fatty acids). The following fatty acids are present in smaller, decreasing amounts (between about 5 and 1 %): iso-C_{17:0}, C_{14:0}, iso-C_{14:0}, C_{12:0} and C_{15:0} (Supplementary Table).

The G + C content is 51.6 mol% for the type strain, strain LMG 18419^T (=CIP 108110^T=Logan collection no. B1718^T=isolate SSI018); the 16S rDNA sequence of this strain is deposited at EMBL under accession number AJ250317. In the variable reactions listed above, the type strain has a minimum growth temperature of 20 °C and grows in the pH range 5.0–9.5; it does not hydrolyse gelatin; acid production is positive for L-fucose, weak for gluconate and D-mannose and negative for *N*-acetylglucosamine, D-fructose, 5-keto-D-gluconate and methyl D-mannoside. Reference strains are LMG 18437, R-11600, R-11662, R-11664, R-11665 and R-11666. Isolated from geothermal soil taken from an active fumarole on Lucifer Hill, a volcano on Candlemas Island, South Sandwich archipelago, Antarctica, and from a gelatin extract of bovine bones (strain R-11600).

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