Proteocatella sphenisci gen. nov., sp. nov., a psychrotolerant, spore-forming anaerobe isolated from penguin guano

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A novel, obligately anaerobic, psychrotolerant bacterium, designated strain PPP2^T, was isolated from guano of the Magellanic penguin (*Spheniscus magellanicus*) in Chilean Patagonia. Cells were Gram-stain-positive, spore-forming, straight rods (0.7–0.8×3.0–5.0 µm) that were motile by means of peritrichous flagella. Growth was observed at pH 6.7–9.7 (optimum pH 8.3) and 2–37 °C (optimum 29 °C). Growth was observed between 0 and 4 % (w/v) NaCl with optimum growth at 0.5 % (w/v). Strain PPP2^T was a catalase-negative chemo-organoheterotroph that was capable of fermentative metabolism. Peptone, bacto-tryptone, Casamino acids, oxalate, starch, chitin and yeast extract were utilized as substrates. The major metabolic products were acetate, butyrate and ethanol. Strain PPP2^T was resistant to ampicillin, but sensitive to tetracycline, chloramphenicol, rifampicin, kanamycin, vancomycin and gentamicin. The DNA G + C content of strain PPP2^T was 39.5 mol%. Phylogenetic analysis revealed that strain PPP2^T was related most closely to *Clostridium sticklandii* SR (~90 % 16S rRNA gene sequence similarity). On the basis of phylogenetic analysis and phenotypic characteristics, strain PPP2^T is considered to represent a novel species of a new genus, for which the name *Proteocatella sphenisci* gen. nov., sp. nov. is proposed. The type strain of *Proteocatella sphenisci* is PPP2^T (=ATCC BAA-755^T =JCM 12175^T =CIP 108034^T).

Microbiological investigations of penguin guano collected in Chilean Patagonia led to the isolation of two anaerobic bacterial strains capable of growth at low temperatures (Hoover *et al.*, 2002). One of these strains, PmagG1^T, was identified as a psychrotolerant, saccharolytic bacterium that was capable of growth at –5 °C, and was subsequently described as representing a novel species, *Trichococcus patagoniensis* (Pikuta *et al.*, 2006). The second strain, designated PPP2^T, was a Gram-positive, spore-forming proteolytic anaerobe that did not show an ability to grow at subzero temperatures (Pikuta *et al.*, 2003; Pikuta & Hoover, 2003, 2004). Phylogenetic analysis showed that

strain PPP2^T was only very distantly related to any recognized species (Pikuta *et al.*, 2003). Its closest neighbour on the phylogenetic tree, *Clostridium sticklandii* SR, shared only 91.3 % 16S rRNA gene sequence similarity. Phenotypically, the novel isolate was mesophilic and psychrotolerant, did not require NaCl for growth, was neutrophilic and alkalitolerant, and showed obligately anaerobic metabolism. It was a fermentative proteolytic bacterium that was able to use various products of proteolysis as substrates, but not sugars. The present article provides a detailed taxonomic description of strain PPP2^T, and shows that it represents a novel species of a new genus within the family *Clostridiaceae*.

Strain PPP2^T was isolated from a sample of guano of the Magellanic penguin (*Spheniscus magellanicus*) in Chilean Patagonia. Detailed descriptions of the sampling procedure and transportation to the laboratory are given in Pikuta *et al.* (2006).

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain PPP2^T is AF450134.

A figure showing the temperature range and optimum for growth of strain PPP2^T and a table giving the fatty acid composition of strain PPP2^T are available as supplementary material with the online version of this paper.

An enrichment culture was obtained anaerobically on medium comprising (per litre): 30.0 g NaCl, 0.3 g KCl, 0.3 g KH_2PO_4 , 0.1 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.0 g NH_4Cl , 0.0125 g $\text{CaSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g NaHCO_3 , 0.4 g $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.001 g resazurin, 0.2 g yeast extract, 3.0 g peptone, 2 ml vitamin solution (Wolin *et al.*, 1963) and 1 ml trace mineral solution [containing (per 500 ml): 392 mg $(\text{NH}_4)_2\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O}$, 119 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 65.16 mg NiCl_2 , 12.1 mg Na_2MoO_4 , 16.5 mg $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, 71.8 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 101 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 43.7 mg Na_2SeO_4 , 3.1 mg H_3BO_3 , 49.5 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and 5 ml HCl]. Final pH of the medium was 7.1–7.2. High-purity nitrogen was used as the gas phase. A homogeneous sample (0.5 g) was injected into a Hungate tube, containing an anaerobic medium, and incubated for 7–10 days at 2 °C. A pure culture was obtained by serial dilution in Hungate tubes and was supported on the medium described above except that the NaCl concentration was reduced to 0.5 % (w/v). The eighth dilution of a morphologically monotypic culture exhibiting growth at 2 °C on peptone-containing medium was chosen for inoculation on agar medium for growth of colonies.

Isolation of colonies was performed by the roll-tube method on 3 % (w/v) agar medium. After 7–10 days cultivation on agar medium at 2 °C, colonies of strain PPP2^T were creamy yellow and rounded lens-shaped with a diameter of 1–2 mm. Young colonies were white. Colonies had a rough surface and central conical protuberance. Edges of the colonies were thin and smooth, and the centre of the colonies had a denser consistency and darker colour. Purification of strain PPP2^T was performed at 2 °C. Experiments with the pure culture were performed at 6–22 °C. Purity of the culture was determined based on negative growth on D-glucose medium at room temperature and by microscope observation.

The morphology of the new isolate was examined under a phase-contrast microscope (Fisher Micromaster) and FEI Tecnai 20 transmission electron microscope (FEI Co.) at 200 kV with attached digital camera. Cells of strain PPP2^T were rod-shaped, with a diameter of 0.7–0.8 µm and length of 3–5 µm (Fig. 1a). Cells were motile by means of peritrichous flagella (Fig. 1b). For transmission electron microscopy, cells were concentrated by centrifugation, resuspended in 50 mM cacodylate buffer, 50 mM NaCl, pH 8.3, and then fixed with 2 % glutaraldehyde for 20 min at room temperature and negatively stained with 2 % uranyl acetate for 10–60 s.

Gram staining of cell membranes revealed a blue colour, typical of Gram-positive bacteria. Large, circular spores with a diameter twice the cell diameter were located at the end of cells (terminal position). Cells occurred singly, in pairs or in short, irregularly curved chains. Cell division occurred by fission, sometimes unequally with the formation of terminally rounded mini-cells.

Growth was determined based on cell counts with the phase-contrast microscope or by measuring the optical density at 595 nm (Genesis 5; Spectronic Instruments).

Fig. 1. (a) Phase-contrast micrograph of live culture showing the morphology of cells of strain PPP2^T and a spore within the sporangium. Bar, 10 µm. (b) Transmission electron micrograph of a cell of strain PPP2^T with peritrichous flagella. Bar, 500 nm.

Catalase activity was determined based on the reaction with hydrogen peroxide (Gerhardt *et al.*, 1994). All test substrates (3 g l^{-1}) were added to medium containing 0.1 g yeast extract. End products of peptone fermentation in the liquid phase were determined by HPLC. Separation was done on an Aminex HPX-87H (Bio-Rad) column with 5 mM H_2SO_4 as the mobile phase. Gases were measured via a Varian 3700 gas chromatograph equipped with a Porapak Q column and thermal conductivity detector. Nitrogen was used as the carrier gas.

Strain PPP2^T grew only under strictly anaerobic conditions. It was catalase-negative. Growth was observed at 0–4 % (w/v) NaCl, with optimum growth at 0.5 %; no growth occurred at 5 % NaCl. The pH range for growth was 6.7–9.7 with optimum growth at pH 8.3. The new isolate was a psychrotolerant mesophile, growing between 2 and 37 °C with optimum growth at 29 °C (Supplementary Fig. S1, available in IJSEM Online).

Strain PPP2^T possessed a chemo-organoheterotrophic metabolism and was able to grow on the following substrates: peptone, bacto-tryptone, Casamino acids, yeast extract, starch, chitin and oxalate. Growth on starch and chitin was weak (three or four cells per field) on all three transfers for each substrate. No growth was observed on

formate, acetate, lactate, pyruvate, propionate, butyrate, citrate, methanol, ethanol, glycerol, acetone, D-mannitol, D-glucose, D-fructose, D-ribose, trehalose, D-arabinose, maltose, D-mannose, lactose, sucrose, cellobiose, pectin, N-acetylglucosamine, urea, trimethylamine, triethylamine or betaine. Separate amino acids on mineral medium supplemented with yeast extract (0.1 g l^{-1}) did not support growth of strain PPP2^T. No growth occurred on L-cysteine, L-cystine, L- or D-methionine, L- or D-proline, L- or D-lysine, L- or D-serine, L- or D-arginine, L-tyrosine, glycine, *trans*-4-hydroxy-L-proline, L- or D-histidine hydrochloride, L-glutamine, L- or D-aspartic acid, L- or D-leucine, L- or D-tryptophan, L- or D-valine, L- or D-alanine, L-glutamic acid, L-phenylalanine, L-isoleucine, L- or D-threonine or L-asparagine.

Interestingly, strain PPP2^T grew on sodium oxalate only in medium supplemented with selenium as a trace element. Cell morphology of strain PPP2^T on this substrate was atypical, with a tendency for the cells to appear swollen and with a hexagonal crystalline shape (data not shown).

The Stickland reaction was negative for the following combinations of amino acids: L-proline + L-leucine, L-proline + L-isoleucine, L-proline + L-valine, L-proline + L-alanine, glycine + L-leucine, glycine + L-isoleucine, glycine + L-valine, glycine + L-alanine and L-tryptophan + L-valine.

Two gaseous metabolic products of the culture grown on peptone-containing medium were CO₂ and H₂ (both in trace quantities, <1%). In the liquid phase, acetate (21 mM), ethanol (4.3 mM) and butyrate (5 mM) were found.

Antibiotic resistance was tested by using the following: ampicillin, kanamycin, gentamicin, vancomycin, tetracycline, rifampicin ($250 \mu\text{g ml}^{-1}$) and chloramphenicol ($125 \mu\text{g ml}^{-1}$). Strain PPP2^T was sensitive to kanamycin, gentamicin, tetracycline, rifampicin, vancomycin and chloramphenicol, but was resistant to ampicillin.

Fatty acid methyl esters (FAMES) were extracted from fresh biomass incubated for 4 days at 22 °C and were identified following the procedure recommended by the Microbial Identification System (MIDI Inc.) anaerobic Moore Library. The extraction procedure included sonication, methylation, organic extraction (transfer of FAMES from an aqueous phase to an organic phase) and a base wash; cell extracts were then analysed by GC (Sherlock Microbial Identification System version 4.0; MIDI Inc.). The predominant FAMES of strain PPP2^T were C_{14:0} (15.11% of the total), C_{16:0} (13.97%) and dimethyl aldehyde C_{18:1} *cis*11 (12.74%) (Supplementary Table S1).

The G + C content of the genomic DNA of strain PPP^T was measured by HPLC as described by Mesbah *et al.* (1989) except that an Alltima C18 column (250 mm × 4.6 mm, 5- μm particle size; Alltech) and 8% (v/v) methanol were used. The results reported are means $\pm$ SD of two determinations for each of two DNA degradations. The G + C content of the genomic DNA of strain PPP^T was $39.5 \pm 0.2 \text{ mol\%}$ ($n=4$).

Isolation of genomic DNA, amplification of the 16S rRNA gene and sequence determination were performed as described by Hoover *et al.* (2003). The consensus sequence was assembled by using sequence data from four different clones. The 16S rRNA gene sequence obtained was aligned with 21 similar sequences selected from a BLAST search (Altschul *et al.*, 1990) via the GenBank database. Alignment was performed by using the CLUSTAL W program (Thompson *et al.*, 1994). Pairwise distances were computed with MEGA version 4 (Tamura *et al.*, 2007) by using the Jukes–Cantor model (Jukes & Cantor, 1969). An unrooted phylogenetic tree was constructed with the same program according to the neighbour-joining method (Saitou & Nei, 1987). Sites with gaps in any sequence of the alignment were excluded from all analyses.

A sequence covering 1450 nt of the 16S rRNA gene of strain PPP2^T was obtained, corresponding to positions 27–1492 of the *Escherichia coli* 16S rRNA gene sequence. The G + C content of this gene sequence was 53.59 mol%. A comparison with all sequences available in the GenBank database revealed over 80% similarity with sequences from the genera *Clostridium*, *Sporacetigenium*, *Eubacterium*, *Peptostreptococcus*, *Filifactor*, *Alkaliphilus*, *Geosporobacter*, *Acidaminobacter* and *Fusibacter*. Although the phylogenetic dendrogram, based on 1265 common nucleotide positions (Fig. 2), showed strain PPP2^T to be related most closely to members of the genus *Filifactor*, highest 16S rRNA gene sequence similarity was observed with *Clostridium sticklandii* SR (91.3% within the 1265 sites used for the tree and 89.4% over a 1450-nt global alignment).

Strain PPP2^T was isolated from an alkaline sample of Magellanic penguin guano. The fact that Magellanic penguins are endemic to the southern tip of South America, a region with a very cold climate, is reflected in the physiological characteristics of strain PPP2^T: the strain showed tolerance to low temperature (down to 2 °C), high pH and marine concentrations of NaCl. All these environmental factors have had an influence on the phenotype of strain PPP2^T, probably as a result of the adaptive flexibility of its genome. The ability of the new isolate to use exclusively products of proteolysis and oxalate (but not sugars) is probably due to the restricted diet of these penguins: they feed on marine fish and crustaceans.

It is of note that strain PPP2^T is resistant to ampicillin, a characteristic that is extremely rare in environmental bacterial strains. The closest phylogenetic neighbour to strain PPP2^T based on 16S rRNA gene sequences was *C. sticklandii* SR, but with ~10% sequence divergence, and therefore phenotypic comparisons with other recognized species were unnecessary. Strain PPP2^T showed phenotypic features typical of the family *Clostridiales*: strictly anaerobic growth, spore formation, Gram-positive cell walls, fermentative metabolism and acetogenesis. Table 1 shows distinguishing features between strain PPP2^T and recognized mesophilic, anaerobic, acetogenic genera of the family *Clostridiales*.

Fig. 2. Unrooted neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain PPP2^T among its closest neighbours. Numbers at nodes are bootstrap percentages based on 2000 replications. Accession numbers are given in parentheses. Bar, 1 substitution per 100 nucleotide positions.

Based on its phenotypic and genotypic characteristics (Gram-positive cell wall, spore formation, obligately anaerobic and fermentative metabolism, mesophilic/psychrotolerant and neutrophilic/alkalitolerant physiology, no NaCl requirement for growth, fatty acid profile and 16S rRNA gene sequence), strain PPP2^T is considered to represent a novel species of a new genus, for which the name *Proteocatella sphenisci* gen. nov., sp. nov. is proposed.

Description of *Proteocatella* gen. nov.

Proteocatella (Pro'te.o.ca.tel'la. N.L. n. *proteinum* protein; N.L. pref. *proteo-* prefix referring to protein used in compound words; L. fem. n. *catella* small chain; N.L. fem. n. *Proteocatella* a small chain using proteins).

Mesophilic, spore-forming, fermentative rods with Gram-positive cell-wall structure. Obligate anaerobes. Alkalitolerant.

Table 1. Differential characteristics between strain PPP2^T and related anaerobic, mesophilic, acetogenic genera within the family Clostridiaceae

Taxa: 1, strain PPP2^T; 2, *Clostridium* (data from Hippe *et al.*, 1992); 3, *Sporacetigenium* (Chen *et al.*, 2006); 4, *Eubacterium* (Andreesen, 1992); 5, *Filifactor* (Collins *et al.*, 1994; Love *et al.*, 1979); 6, *Alkaliphilus* (Takai *et al.*, 2001; Cao *et al.*, 2003); 7, *Geosporobacter* (Klouche *et al.*, 2007); 8, *Fusibacter* (Ravot *et al.*, 1999); 9, *Acidaminobacter* (Stams & Hansen, 1984); 10, *Tissierella* (Collins & Shah, 1986; Farrow *et al.*, 1995; Bae *et al.*, 2004); 11, *Natronincola* (Zhilina *et al.*, 1998); 12, *Tindallia* (Kevbrin *et al.*, 1998). ND, No data; +, positive; −, negative; +/−, variable.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Cell motility	+	+	+	+	−	+	−	+	−	+/−	+	+
Gram reaction	+	+/−	+	+/−	+/−	+	+	+	−	+/−	+	+
Spore formation	+	+	+	−	+	+	+	−	−	−	+/−†	+/−†
Relation to O ₂ *	OAN	AN (ATL)	OAN	OAN	OAN	OAN	OAN	OAN	OAN	OAN	OAN	OAN
Catalase	−	−	−	ND	−	ND	ND	ND	−	ND	ND	−
NaCl requirement	−	+/−	−	−	−	−	−	−	−	+	+	+
Carbonate requirement	−	−	−	−	−	−‡	−	−	−	+	+	−
Substrate utilization												
Sugars	−	+/−	+	+/−	−	+	+	+	−	−	−	−
Proteolysis products	+	+/−	+	+/−	+	+	+	−	+	+	+	+
Amino acids	−	+/−	ND	+/−	ND	−	+	−	+	+	+	−
Stickland reaction	−	+	ND	+/−	ND	ND	+	−	−	+/−	ND	+
Acetogenesis	+	+	+	+	+	ND	+	+	+	+	+	+
DNA G + C content (mol%)	39.5	21–54	53.9	30–55	ND	30–36	42.2	43	48.1	28–32	31.9	26.9–40

*AN (ATL), Aerotolerant anaerobe; OAN, obligate anaerobe.

†Oligospore-forming.

‡K. Takai, personal communication.

Do not require carbonate ions or NaCl for growth. Psychrotolerant mesophiles. Acetogenic (producing acetate as major end product). The type species is *Proteocatella sphenisci*.

Description of *Proteocatella sphenisci* sp. nov.

Proteocatella sphenisci (sphe.nis'ci. N.L. gen. n. *sphenisci* of *Spheniscus*, zoological name of a genus of penguin, referring to the isolation of the type strain from guano of *Spheniscus magellanicus*, the Magellanic penguin).

Has the following characteristics in addition to those given for the genus above. Cells are flexible, motile rods, 0.7–0.8 × 3.0–5.0 µm, that tend to form long chains. Multiplies by fission, sometimes unequally with the formation of terminally round mini-cells. Motile by means of flagella. Spores are spherical. Sporangium is not swollen. Grows at 2–37 °C (optimum 29 °C) and at pH 6.7–9.7 (at 22 °C) (optimum pH 8.3). NaCl range for growth is 0–4 % (w/v); optimal growth at 0.5 % (w/v) NaCl. Catalase-negative. Heterotrophic growth with peptone, bacto-tryptone, Casamino acids, oxalate, starch, chitin and yeast extract. End products of peptone fermentation are acetate, butyrate and ethanol and, in the gas phase, minor products are hydrogen and carbon dioxide. Resistant to ampicillin, but sensitive to kanamycin, gentamicin, tetracycline, rifampicin, vancomycin and chloramphenicol. The G + C content of the genomic DNA of the type strain is 39.5 mol% (HPLC).

The type strain, PPP2^T (=ATCC BAA-755^T =JCM 12175^T =CIP 108034^T), was isolated from guano of Magellanic penguins (*Spheniscus magellanicus*) inhabiting the coast of Chilean Patagonia.

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