Bacillus patagoniensis sp. nov., a novel alkalitolerant bacterium from the rhizosphere of *Atriplex lampa* in Patagonia, Argentina

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A Gram-positive, rod-shaped, spore-forming bacterium (PAT 05^T) was isolated from the rhizosphere of the perennial shrub *Atriplex lampa* in north-eastern Patagonia, Argentina. Its overall biochemical and physiological characteristics indicated that this strain should be placed in the alkaliphilic *Bacillus* group. Strain PAT 05^T grew at pH 7–10 (optimum pH 8), but not at pH 6. Its DNA G+C content was 39·7 mol%. Sequence analysis of the 16S rRNA gene of PAT 05^T revealed the closest match (99·6 % similarity) with *Bacillus* sp. DSM 8714. The highest level of DNA–DNA relatedness (88·6 %) was also found with this strain. On the basis of 16S rRNA gene sequence similarity and phylogenetic analysis, G+C content and DNA–DNA hybridization data, strain PAT 05^T is related at the species level to *Bacillus* sp. DSM 8714, a member of a group referred as phenon 4a by Nielsen *et al*. [Nielsen, P., Fritze, D. & Priest, F. G. (1995). *Microbiology* **141**, 1745–1761], which still lacks taxonomic standing. These results support the proposal of strain PAT 05^T (=DSM 16117^T=ATCC BAA-965^T) as the type strain of *Bacillus patagoniensis* sp. nov.

The classification of alkaliphilic *Bacillus* species has been subject to revisions that have involved their phylogenetic and phenotypic characteristics (Fritze *et al.*, 1990; Nielsen *et al.*, 1994, 1995). As a result of these studies, nine novel species were described, *Bacillus agaradhaerens*, *Bacillus clarkii*, *Bacillus clausii*, *Bacillus gibsonii*, *Bacillus halmapalus*, *Bacillus halodurans*, *Bacillus horikoshii*, *Bacillus pseudalkaliphilus* and *Bacillus pseudofirmus*, in addition to the previously known species *Bacillus cohnii* and *Bacillus alcalophilus*. Since then, the classification of novel alkalitolerant and alkaliphilic strains has led to the proposal of species such as *Bacillus vedderi* (Agnew *et al.*, 1995), *Bacillus haloalkaliphilus* (Fritze, 1996), *Bacillus horti* (Yumoto *et al.*, 1998), *Bacillus arseniciselenatis* and *Bacillus selenitireducens* (Switzer Blum *et al.*, 1998), *Bacillus okuhidensis* (Li *et al.*, 2002) and *Bacillus krulwichiae* (Yumoto *et al.*, 2003).

Naturally occurring alkaline environments harbour a wide range of alkaliphilic micro-organisms. Desert soils, such as the arid soils in north-eastern Patagonia (Argentina), are exposed to wind and water erosion, as well as salinization and alkalinization processes associated with non-irrigated lands. The physical processes that cause losses of fine material, organic matter and nutrients from the topsoil lead to the concentration of soil resources underneath remnant plant patches (Mazzarino *et al.*, 1996). There is very limited knowledge about the microbial diversity of Patagonian arid soils, especially from vegetated soil microsites characterized by alkaline and saline conditions.

During the characterization of proteolytic micro-organisms from such soils, the strain PAT 05^T was isolated from the rhizosphere of *Atriplex lampa*, a perennial shrub that is able to colonize alkaline and saline areas. PAT 05^T is a producer of alkaline proteases that, considering their characteristics such as high optimum pH, high stability and residual activity in the presence of denaturing and chelating agents, could be a promising system enzyme for a detergent formulation (Olivera *et al.*, 2003). This study

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain PAT 05^T is AY258614.

An electron photomicrograph of a negatively stained cell of strain PAT 05^T is available as supplementary material in IJSEM Online.

focuses on phenotypic, phylogenetic and DNA–DNA relatedness analyses performed in order to establish the taxonomic position of strain PAT 05^T.

Strain PAT 05^T was originally isolated using an agar medium composed of 1 % (w/v) skimmed milk, 0·1 % (w/v) yeast extract, 5 % (w/v) NaCl and 0·1 M Na_2CO_3 (separately autoclaved) to provide pH 10 (Olivera *et al.*, 2003). For routine growth, isolate PAT 05^T was cultured in LB medium supplemented with 5 % (w/v) NaCl and 0·1 M Na_2CO_3.

Phenotypic tests were based on the methods described by Claus & Berkeley (1986) with media adjusted to approximately pH 10 according to Fritze *et al.* (1990). The API 50 CH gallery (bioMérieux) was used for carbohydrate utilization tests according to the procedure described by Nielsen *et al.* (1995). Acid production from carbohydrates was determined by the method of Hugh & Leifson (1953) using thymol blue instead of bromothymol blue at pH 10 (Yumoto *et al.*, 2003). Doubling times at pH 6, 7, 8, 9 and 10 were evaluated in LB broth with 5 % NaCl (w/v); triplicate cultures were incubated at 200 r.p.m. and 25 °C and quantified by optical density at 600 nm. Tolerance to salt was investigated by using different NaCl concentrations in LB broth, 0·1 M Na_2CO_3. The effect of temperature on growth was determined in the same medium with 5 % NaCl and 0·1 M Na_2CO_3. Cellular morphology and size and endospores were examined by phase-contrast microscopy (Carl Zeiss Photomicroscope III). Flagellation was examined using transmission electron microscopy (JEOL CX 100) of negatively stained cells (Tesche & Schmiady, 1985).

The 16S rRNA gene sequence (corresponding to positions 27–1492 in the *Escherichia coli* gene) was amplified by PCR as described by DeLong (1992), using a GeneAmp model 2700 thermal cycler (Applied Biosystems). Sequencing of both strands of PCR-amplified fragments was performed using the dideoxy chain-termination method by the commercial services of GATC Biotech AG. 16S rRNA gene sequence similarity searches against the NCBI database were carried out using BLAST (Altschul *et al.*, 1990). Sequences showing a relevant degree of similarity were imported into the CLUSTAL W program (Thompson *et al.*, 1994), aligned and corrected manually. The percentage of similarity was calculated in the BioEdit program version 5.0.9 (Hall, 1999). Phylogenetic analyses were performed using the branch and bound parsimony algorithm with PAUP program version 4.0b10 (Swofford, 2001). Sites involving gaps were treated as missing characters. The results were evaluated with 1000-replication jackknife analysis and the length and the consistency (CI) and retention (RI) indices of the trees were calculated.

The DNA G+C content was determined by reverse-phase HPLC by the commercial services of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). DNA–DNA hybridization analyses were also performed by the DSMZ. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Hybridization was carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980), using a model 2600 spectrophotometer equipped with a model 2527-R thermoprogrammer and plotter (Gilford Instrument Laboratories). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

The overall biochemical and physiological characteristics (see species description) indicate that strain PAT 05^T should be placed in the alkaliphilic *Bacillus* group. It grew as creamy white-coloured colonies and the cells were rod-shaped with peritrichous flagella (an electron photomicrograph is available as supplementary material in IJSEM Online). Subterminal oval endospores were observed in slightly swollen sporangia (Fig. 1). Strain PAT 05^T did not grow under anaerobic conditions. Most of its phenotypic properties are shown in Table 1 and they are compared with those of related alkalitolerant *Bacillus* strains. PAT 05^T grew at pH 7–10, while growth was undetectable at pH 6. Optimal growth (doubling time, t_d 86 min) was obtained at pH 8, although it was able to grow at pH 7 (t_d 99 min), pH 9 (t_d 88 min) and pH 10 (t_d 106 min). The range of temperature for growth was 5–40 °C. These results indicate that strain PAT 05^T is an alkalitolerant/moderately alkaliphilic micro-organism, and its capacity to grow at low temperatures and high salinity revealed that it is also psychrotolerant and halotolerant (Table 1).

The G+C content of genomic DNA of strain PAT 05^T was 39·7 mol%, a value comparable to those found in a group of alkaliphilic *Bacillus* strains referred as phenon 4 by Nielsen *et al.* (1995). Analysis of 1422 bases of the 16S rRNA gene of PAT 05^T confirmed that the closest match (99·6 % similarity) was to the sequence from the alkaliphilic *Bacillus* strain DSM 8714, which belongs to phenon 4 group a (Nielsen *et al.*, 1994, 1995), and which still lacks taxonomic standing. The next highest similarity (98·0 %)

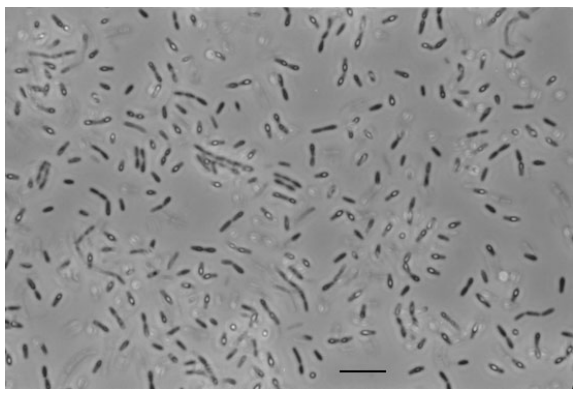

Fig. 1. Phase-contrast micrograph showing endospores of strain PAT 05^T. Bar, 10 μm.

Table 1. Phenotypic characteristics of *Bacillus patagoniensis* sp. nov. and other alkalitolerant bacilli

Taxa: 1, *B. patagoniensis* sp. nov.; 2, *Bacillus* sp. phenon 4a; 3, *Bacillus* sp. phenon 4b; 4, *B. clausii*; 5, *B. gibsonii*; 6, *B. halmapalus*; 7, *B. horikoshii*. +, Positive; −, negative; V, variable; W, weakly positive. Data for reference taxa are from Nielsen *et al.* (1995).

Character	1	2	3	4	5	6	7
Growth at:							
10 °C	+	+	+	−	V	+	+
40 °C	W	−	+	+	−	+	+
50 °C	−	−	−	+	−	−	−
Growth in NaCl:							
5 %	+	+	+	+	+	−	+
10 %	+	+	+	V	V	−	−
15 %	+	+	+	−	−	−	−
Hydrolysis of:							
Starch	+	+	+	+	−	+	+
Tween 20	+	−	−	−	−	−	−
Tween 40	+	−	−	−	V	−	V
Tween 60	+	−	−	−	V	−	V
MUG*	−	−	−	−	+	−	−
Nitrate reduction	−	−	−	+	V	−	−
Utilization of:							
L-Arabinose	−	−	V	+	+	−	−
D-Ribose	+	−	V	+	+	−	V
D-Xylose	−	−	V	+	V	V	V
D-Mannose	+	V	V	+	+	+	V
L-Rhamnose	+	−	−	+	V	−	−
Dulcitol	−	−	V	+	−	−	−
D-Sorbitol	+	−	−	+	−	−	−
Methyl α-D-glucoside	−	V	V	+	−	−	V
N-Acetylglucosamine	+	V	V	+	−	+	+
Amygdalin	−	V	V	+	V	+	V
Salicin	+	V	V	+	+	V	−
D-Lactose	−	−	−	V	+	−	−
D-Melibiose	−	V	V	+	+	−	−
D-Melezitose	−	V	V	+	+	−	−
D-Raffinose	+	V	V	+	+	−	−
Starch	−	−	V	+	−	+	+
Glycogen	−	−	−	+	−	+	+
Xylitol	−	−	V	+	−	−	−
Gentiobiose	+	−	V	V	+	−	V
D-Turanose	+	−	V	+	+	+	V
D-Lyxose	−	−	V	+	−	−	−

*4-Methylumbelliferyl β-D-glucuronide.

was to *Bacillus* sp. WW3-SN6 (Ntougias & Russell, 2000). Similarity values in the range 95·3–89·5 % were obtained when comparing the 16S rRNA gene sequence of PAT 05^T to those of type strains of alkaliphilic *Bacillus* species described by Nielsen *et al.* (1995). The highest similarity values (96·2 and 95·3 %) with strains characterized to the species level were to *B. clausii* SIN84 Enterogermina (Senesi *et al.*, 2001) and the type strain of *B. clausii*, DSM 8716^T, respectively. Levels of similarity between 16S rRNA gene sequences below 97 % suggest that the strains do not correspond to the same species (Stackebrandt & Goebel, 1994).

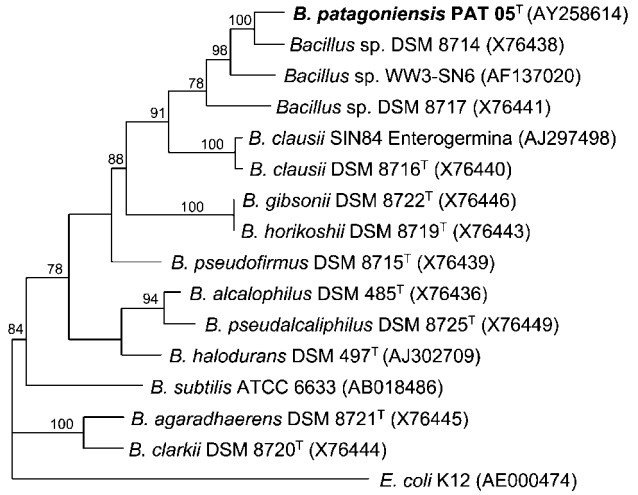

Fig. 2. Most-parsimonious phylogenetic tree of strain PAT 05^T derived from 16S rRNA gene sequence data. Numbers at internal nodes are jackknife support values (%). The 16S rRNA gene sequence of *E. coli* was chosen arbitrarily as the outgroup sequence.

To characterize strain PAT 05^T further, a phylogenetic tree based on its 16S rRNA gene sequence was constructed (Fig. 2). From the total of 1422 bp, 183 were parsimony informative. A single most-parsimonious tree was obtained; its length was 750 steps and the CI and RI were respectively 0·7120 and 0·6516. The phylogenetic tree revealed that PAT 05^T forms a distinct clade in the alkaliphilic *Bacillus* tree together with *Bacillus* spp. DSM 8714, WW3-SN6 and DSM 8717. The taxonomic integrity of this clade was supported by the 78 % jackknife value obtained. The cladogram also showed that this clade is the sister group of the clade containing *B. clausii* strains, with 91 % recovery in jackknife analysis. These results confirmed that PAT 05^T is closely related to taxa referred to as phenon 4 groups a and b, whose reference strains are *Bacillus* spp. DSM 8714 and DSM 8717, respectively (Nielsen *et al.*, 1995). As was the case for PAT 05^T, soil was the source of isolation of group 4a strains, while group 4b strains were isolated from animal manures.

The DNA–DNA hybridization results support the conclusion that strains PAT 05^T and *Bacillus* sp. DSM 8714 are related at the species level, with 88·6 % DNA–DNA relatedness (in 2 × SSC at 64 °C), while PAT 05^{T}and *Bacillus* sp. DSM 8717 are not (20·5 % DNA–DNA relatedness). The recommendations of the ad hoc committee (Wayne *et al.*, 1987) considered a threshold value of 70 % DNA–DNA relatedness for definition of bacterial species.

All these results confirmed that strain PAT 05^T should be classified in a novel species together with strains belonging to phenon 4a (Nielsen *et al.*, 1995). We propose the name *Bacillus patagoniensis* sp. nov., the type strain being PAT 05^T (=DSM 16117^T=ATCC BAA-965^T).

Description of *Bacillus patagoniensis* sp. nov.

Bacillus patagoniensis (pa.ta.go′ni.en.sis. N.L. masc. adj. *patagoniensis* pertaining to Patagonia, in Argentina, where the type strain was isolated).

Cells are aerobic rods (2·4–3·2 × 0·8–1·1 μm) with peritrichous flagella and they occur singly, in pairs or in chains. Endospores are observed as subterminal oval spores. Colonies are cream–white. Gram, oxidase and catalase reactions are positive. Growth occurs at pH 7–10, with an optimum at about pH 8. There is growth between 5 and 40 °C and with 15 % NaCl. Nitrate is not reduced to nitrite. Hydrolysis of casein, gelatin, starch and Tweens 20, 40 and 60 is observed, but Tween 80 and 4-methylumbelliferyl β-D-glucuronide are not hydrolysed. Phenylalanine is not deaminated. Utilizes glycerol, D-ribose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol, *N*-acetylglucosamine, salicin, D-cellobiose, D-maltose, sucrose, D-trehalose, D-raffinose, gentiobiose, D-turanose and potassium 2-ketogluconate but not D-arabinose, L-arabinose, D-xylose, D-galactose, L-sorbose, inositol, starch, xylitol, D-lyxose, D-arabitol or gluconate. Acid, but no gas, is produced from glycerol, D-glucose, D-mannitol, D-sorbitol, D-maltose, D-ribose, D-raffinose and D-cellobiose. The DNA G+C content of the type strain is 39·7 mol% as determined by HPLC.

The type strain is PAT 05^T (=DSM 16117^T=ATCC BAA-965^T), isolated from the rhizosphere of the perennial shrub *Atriplex lampa* in north-eastern Patagonia, Argentina.

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