Bacillus foraminis sp. nov., isolated from a non-saline alkaline groundwater

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A low-G + C-content Gram-positive bacterium, designated CV53^T, phylogenetically related to species of the genus *Bacillus*, was isolated from a highly alkaline non-saline groundwater environment (pH 11.4). This organism comprised rod-shaped cells, was aerobic, did not display spore formation, was catalase- and oxidase-negative, had an optimum growth temperature of 40 °C and had an optimum pH of approximately 7.0–8.5. Optimal growth was observed in the absence of NaCl, but growth did occur at NaCl concentrations up to 3.0 %. The strain possessed an A1γ-type peptidoglycan cell wall and the major respiratory quinone was MK-7. The predominant fatty acids were anteiso-C_{15:0}, iso-C_{15:0} and anteiso-C_{17:0}. The G + C content of the DNA was 43.1 mol%. Phylogenetic analyses of the 16S rRNA gene sequence revealed that the novel isolate is closely related to the type strain of *Bacillus jeotgali*, forming a coherent cluster supported by bootstrap analysis at a confidence level of 90 %. The pairwise similarity of the 16S rRNA gene sequences of the two strains is 97.7 %. On the basis of the phylogenetic analyses and the distinct phenotypic characteristics, strain CV53^T represents a novel species within the genus *Bacillus*, for which we propose the name *Bacillus foraminis* sp. nov. The type strain is CV53^T (=LMG 23174^T = CIP 108889^T).

Recently we investigated the bacterial diversity of a groundwater at Cabeço de Vide in southern Portugal. The ophiolite-like geological background of this aquifer and its chemical characteristics strongly suggest serpentinization. This groundwater, which has an outflow at a temperature of 20.5 °C, has a high level of alkalinity (pH 11.4) associated with an extremely low ionic concentration, with Ca²⁺ and OH[−] as the major chemical constituents (Tiago *et al.*, 2004).

The majority of the populations recovered during this survey comprised high-G + C-content Gram-positive bacteria (Tiago *et al.*, 2004). However, several low-G + C-content Gram-positive bacteria were also isolated; one strain, designated CV53^T, was of particular interest and was found to be phylogenetically related to the lineage containing the type strain of the species *Bacillus jeotgali* of the family *Bacillaceae*. In this study, we describe the morphological, physiological, chemotaxonomic and phylogenetic characteristics of strain CV53^T. On the basis of our results we

propose that strain CV53^T represents a novel species of the genus *Bacillus*.

Strain CV53^T was isolated from a non-saline alkaline groundwater environment by using alkaline buffered medium 2 (ABM2), adjusted to pH 8.5, at 37 °C, as described previously (Tiago *et al.*, 2004). The isolate was routinely cultured under the same conditions and maintained at −70 °C in the same medium supplemented with 15 % glycerol. Unless otherwise stated, all morphological examinations and biochemical and tolerance tests were performed on this medium after 6 days incubation, as described previously (Tiago *et al.*, 2005).

The temperature range for growth of strain CV53^T was examined at temperatures between 10 and 50 °C in ABM2 liquid medium buffered at pH 7.0, 8.0 and 9.0. The pH range for growth was determined at 40 °C in the same medium buffered at pH values between 6.0 to 10.0. Growth in the presence of NaCl concentrations up to 5.0 % (w/v) was examined in liquid medium at pH 7.5 and 40 °C, as described previously (Tiago *et al.*, 2005).

Three different agar-based media were used to test for sporulation. Medium A contained (l^{−1}) 5.0 g Bacto peptone (Difco), 3.0 g meat extract (Difco), 5 mg MnSO₄ (Merck) and 16 g agar (Difco) and medium B contained (l^{−1}) 1.0 g

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CV53^T is AJ717382.

A comparison of the fatty acid compositions of strain CV53^T and *B. jeotgali* YKJ-10^T is available as supplementary material in IJSEM Online.

yeast extract (Difco), 1.0 g tryptone (Difco), 10.0 g soluble starch (Merck) and 16 g agar (Difco). The third medium was ABM2 solid medium. All media were adjusted to pH 8.0. Plates were inoculated with 0.3 ml aliquots of an overnight liquid culture and then incubated at 40 °C for up to 15 days to determine the presence of spores. The heat resistance of the cells was determined using cultures in ABM2 medium and in ABM2 medium supplemented with 0.5 % glucose. Aliquots (5 ml) of the cultures were recovered at the exponential (4 h), late-exponential (10 h), stationary (20 h) and late-stationary (48 h, 72 h and 10 days) phases and were heated at 80 °C for 10 min. Aliquots (0.3 ml) of the heated cultures were inoculated onto ABM2 solid medium and incubated for 48 h at 40 °C. In addition, the viability of the cells at each growth stage was checked by subculturing them on the same medium before heating.

The assimilation of single carbon sources was determined using API 50 CH test strips (bioMérieux), using 0.1 M phosphate buffer (pH 7.5) supplemented with 0.3 % (w/v) agar (Difco), 0.05 % NH₄Cl₂ (Merck) and macronutrient and micronutrient solutions described previously (Tiago *et al.*, 2005). Acid production from single carbon sources was determined using API 50 CH test strips (bioMérieux), as recommended by the manufacturer. Results were recorded after incubation at 40 °C for 24 h, 48 h and 5 days. Anaerobic growth was assessed at 40 °C in an anaerobic chamber with a H₂/CO₂ atmosphere (bioMérieux).

Antibiotic susceptibility was determined after 72 h incubation on ABM2 (pH 7.5) at 40 °C, using discs (bioMérieux) containing amoxicillin (25 µg), virginiamycin (15 µg), ceftriaxon (30 µg), cephalothin (30 µg), ceftazidin (30 µg), chloramphenicol (30 µg), colistin (50 µg), doxycycline

(30 µg), gentamicin (10 µg), kanamycin (30 µg), lincomycin (2 µg), nalidixic acid (30 µg), ofloxacin (5 µg), penicillin G (10 U/IE), piperacilin (100 µg), polymyxin B (300 U/IE), streptomycin (10 µg) or tetracycline (30 µg).

Strain CV53^T formed grey-pigmented colonies and comprised Gram-positive, rod-shaped cells (1.0 µm wide and 2.4–3.9 µm long). The isolate had an optimum growth temperature of about 40 °C and did not grow at 10 or 50 °C. The optimum pH for growth of strain CV53^T was between 7.0 and 8.5, but no growth was observed at pH 6.0 or at 10.0. Optimal growth was observed in the absence of NaCl, but poor growth did occur in ABM2 containing up to 3.0 % NaCl. This organism was isolated from an environment with a pH of 11.4, but the optimum pH for growth under laboratory conditions was only around 8.0, and growth did not occur at pH 10.0. However, it is not uncommon for isolates from extreme alkaline environments to grow only at moderate pH values under laboratory conditions (Duckworth *et al.*, 1998; Jones *et al.*, 1998, 1994; Tiago *et al.*, 2004). Furthermore, the optimum pH and temperature for growth and the halotolerance were only slightly different from those of *B. jeotgali*, the most closely related species (which has wider growth limits) (Table 1).

Despite all attempts to find spores, these were not observed; furthermore, the cells did not exhibit heat resistance when subjected to heating at 80 °C for 10 min, indicating that no spores were formed under the conditions tested. In addition, the cells became non-viable after 10 days incubation. To our knowledge, the only previously described *Bacillus* species incapable of spore formation is *Bacillus thermoamylovorans* (Combet-Blanc *et al.*, 1995), but this micro-organism and strain CV53^T clearly belong to distinct phylogenetic lineages (Fig. 1).

Table 1. Phenotypic characteristics that differentiate strain CV53^T from *B. jeotgali* YKJ-10^T

Data for *B. jeotgali* YKJ-10^T were taken from Yoon *et al.* (2001).

Characteristic	Strain CV53 ^T	<i>B. jeotgali</i> YKJ-10 ^T
Source	Alkaline groundwater	Jeotgal (fermented seafood)
Growth temperature (°C)		
Range	15–45	10–45
Optimum	40	30–35
pH for growth		
Range	6.5–9.5	5.0–ND
Optimum	7.0–8.5	7.0–8.0
Tolerance of NaCl (% w/v)	0–4	0–14
Acid production from L-arabinose, ribose, xylose, methyl β-xyloside, galactose, D-mannose, L-rhamnose, mannitol, sorbitol, N-acetylglucosamine, amygdalin, salicin, cellobiose, lactose, melibiose, sucrose, inulin, melezitose, D-raffinose and β-gentiobiose	+	–
Major fatty acid(s)	anteiso-C _{15:0} , iso-C _{15:0} , anteiso-C _{17:0}	iso-C _{15:0}
DNA G + C content (mol%)	43.1	41

Fig. 1. Neighbour-joining phylogenetic dendrogram based on a comparison of the 16S rRNA gene sequences of strain CV53^T and some of its closest phylogenetic relatives. Numbers on the tree indicate bootstrap support (%), derived from 1000 replications. Bar, 1 inferred nucleotide substitution per 100 nt.

Strain CV53^T utilized several sugars and proteinaceous substrates, and acid was produced from several single carbon sources, clearly differentiating strain CV53^T from the type strain of *B. jeotgali* (Table 1).

Cell-wall preparations and peptidoglycan for analysis were obtained as described previously (Schleifer & Kandler, 1972; Schleifer, 1985). Lipoquinones were extracted and identified as described by Tindall (1989). Cultures used for fatty acid analysis were grown on ABM2 media, adjusted to pH 7.5, in sealed plastic bags submerged in a water bath at 40 °C for 24 h; for comparison purposes, cultures grown on TSA medium at 28 °C for 24 h were also analysed. Fatty acid methyl esters were obtained from fresh wet biomass by saponification, methylation and extraction, and the fatty acids were identified and quantified by using the standard MIS Library Generation software (Microbial ID) as described by the manufacturer.

As in many other species of the genus *Bacillus*, the peptidoglycan of strain CV53^T belonged to the A1γ type, which contains *meso*-diaminopimelic acid as the diamino acid. The major respiratory quinone detected was MK-7. The fatty acid composition of the novel organism was made up mainly of anteiso-C_{15:0} (29.7 %), iso-C_{15:0} (29.7 %) and anteiso-C_{17:0} (10.6 %), a profile that clearly differentiates strain CV53^T from *B. jeotgali* (see Supplementary Table S1 available in IJSEM Online).

DNA for the determination of the G+C content was obtained as described by Nielsen *et al.* (1995) and HPLC was performed as described by Mesbah *et al.* (1989). The 16S rRNA gene was sequenced as described by Tiago *et al.* (2004) and phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004). Evolutionary distances were calculated using the Jukes–Cantor method (Jukes & Cantor, 1969), phylogenetic dendrograms were constructed using the neighbour-joining method (Saitou & Nei, 1987) and tree topologies were evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1000 resamplings of the datasets.

The DNA G+C content determined for strain CV53^T was 43.1 mol%. Comparative analyses of 1483 nucleotide positions of the 16S rRNA gene sequence of strain CV53^T with sequences of representatives of the main lines of descent within the domain *Bacteria* indicated that the strain was a member of the family *Bacillaceae*. The novel isolate and the type strain of *B. jeotgali* formed a coherent cluster supported by bootstrap analysis at a confidence level of 90 %, showing the phylogenetic relatedness of the two strains (Fig. 1). The pairwise similarity of the 16S rRNA gene sequences of the two strains was 97.7 %. Although CV53^T was isolated from an alkaline environment, it does not have a close phylogenetic relationship with micro-organisms on the phylogenetic branch that includes some of the most alkaliphilic *Bacillus* species, namely *Bacillus alcalophilus*, *Bacillus pseudoalcalophilus* and *Bacillus krulwichiae* (Fig. 1).

The phylogenetic relationships of strain CV53^T, together with its distinctive phenotypic and chemotaxonomic characteristics, justify the proposal that it represents a novel species of the genus *Bacillus*. Moreover, the novel isolate can be differentiated from the most closely related species, *B. jeotgali*, on the basis of several characteristics (Table 1). Consequently, we propose that strain CV53^T represents a novel species of the genus *Bacillus*, for which we propose the name *Bacillus foraminis* sp. nov.

Description of *Bacillus foraminis* sp. nov.

Bacillus foraminis (fo.ra'mi.nis. L. n. *foramen* -inis a hole; L. gen. n. *foraminis* from a hole).

Forms rod-shaped cells, 1 µm wide and 2.4–3.9 µm long. Gram stain is positive. Spores are not observed under a range of conditions; cells are killed by heating at 80 °C for 8 min. Aerobic and heterotrophic. Nitrate is reduced to nitrite. Colonies are small, smooth, convex and grey. Oxidase- and catalase-positive. The optimum temperature for growth is about 40 °C; no growth occurs at 10 or 50 °C. The optimum pH is between 7.0 and 8.5; no growth occurs at pH 6.0 or 10.0. Does not require NaCl for growth, but

tolerates up to 3.0% NaCl. The diamino acid of the peptidoglycan is *meso*-diaminopimelic acid (A1 γ type). The major respiratory quinone is MK-7. The predominant fatty acids are anteiso-C_{15:0} (29.7%), iso-C_{15:0} (29.7%) and anteiso-C_{17:0} (10.6%). Does not hydrolyse casein or elastin. Hydrolyses aesculin, hippurate, starch, gelatin and arbutin. Urease, β -galactosidase and DNase are detected. Xylanase and arginine dihydrolase are not detected. Sensitive to amoxicillin, cephalothin, chloramphenicol, doxycycline, ofloxacin, penicillin G, streptomycin and tetracycline. Assimilates arabinose, xylose, methyl β -xyloside, galactose, glucose, fructose, mannose, rhamnose, inositol, mannitol, sorbitol, *N*-acetylglucosamine, amygdalin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, melizitose, raffinose, β -gentiobiose, turanose, gluconate and 2-ketogluconate. Acid is produced from glycerol, arabinose, ribose, xylose, methyl β -xyloside, galactose, glucose, fructose, mannose, rhamnose, inositol, mannitol, sorbitol, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melizitose, raffinose, starch, glycogen, β -gentiobiose, turanose, gluconate and 2-ketogluconate. The DNA G + C content of the type strain is 43.1 mol%.

The type strain, CV53^T (=LMG 23174^T=CIP 108889^T), was isolated from groundwater taken from the borehole at Cabeço de Vide in southern Portugal.

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