Bacillus lehensis sp. nov., an alkalitolerant bacterium isolated from soil

A. Ghosh,[1] M. Bhardwaj,[2] T. Satyanarayana,[2] M. Khurana,[1] S. Mayilraj[1] and R. K. Jain[1]

[1]Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Sector-39A, Chandigarh 160036, India

[2]Microbiology, University of Delhi, South Campus, Benito Juarez Road, New Delhi 110021, India

Correspondence
R. K. Jain
rkj@imtech.res.in

A Gram-positive, endospore-forming, alkalitolerant bacterial strain, designated MLB2^T, was isolated from soil from Leh, India, and was subjected to a polyphasic taxonomic study. The strain exhibited phenotypic properties that included chemotaxonomic characteristics consistent with its classification in the genus *Bacillus*. Growth was observed at pH 7.0–11.0, but not at pH 6.0. The DNA G+C content was 41.4 mol%. The highest level of 16S rRNA gene sequence similarity was with *Bacillus oshimensis* JCM 12663^T (98.8 %). However, DNA–DNA hybridization experiments indicated low levels of genomic relatedness with the type strains of *B. oshimensis* (62 %), *Bacillus patagoniensis* (55 %), *Bacillus clausii* (51 %) and *Bacillus gibsonii* (34 %), the species with which strain MLB2^T formed a coherent cluster (based on the results of the phylogenetic analysis). On the basis of the phenotypic characteristics and genotypic distinctiveness of strain MLB2^T, it should be classified within a novel species of *Bacillus*, for which the name *Bacillus lehensis* sp. nov. is proposed. The type strain is MLB2^T (=MTCC 7633^T=JCM 13820^T).

In recent years there has been increasing interest in alkaliphilic and alkalitolerant micro-organisms, attributable to their ability to grow under extreme conditions as well as to the use of their enzymes in biotechnological applications. Naturally occurring alkaline environments, which include soda lakes, deserts and arid soils, harbour a wide range of alkaliphilic and alkalitolerant micro-organisms (Ulukanli & Diurak, 2002). The genus *Bacillus* currently includes 19 species characterized as alkaliphilic and alkalitolerant (http://www.bacterio.cict.fr/b/bacillus.html), and many of them have been studied with a view to finding industrial applications (Horikoshi, 1991; Nielsen *et al.*, 1995; Yumoto *et al.*, 1998). Alkaliphilic enzymes (such as alkaline cellulases and alkaline proteases) produced by *Bacillus* species are used in the detergent industries (Horikoshi, 1999). In this work, we describe an alkalitolerant, endospore-forming bacterium, designated MLB2^T, isolated from pristine soil samples from Leh, India (at 3500 m above sea level and at temperatures ranging from −20 to 30 °C). Characterization of the protease produced by the strain indicated optimal activity at pH 10.0.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MLB2^T is AY793550.

A table showing the fatty acid compositions of strain MLB2^T and its close relatives is available as supplementary material in IJSEM Online.

Strain MLB2^T was isolated on tryptone soya agar (TSA, pH 7.3; HiMedia) at 25 °C by means of the dilution plating technique. Subculturing was performed on TSA at 25 °C for 24 h and the bacterial isolate was maintained as glycerol stock at −70 °C. Reference strains used were *Bacillus oshimensis* JCM 12663^T (=MTCC 7915^T), obtained from the Japan Collection of Microorganisms (Saitama, Japan), and *Bacillus patagoniensis* DSM 16117^T (=MTCC 7916^T), *Bacillus clausii* DSM 8716^T (=MTCC 7914^T), *Bacillus gibsonii* DSM 8722^T (=MTCC 7917^T) and *Bacillus alcalophilus* DSM 485^T (=MTCC 7913^T), from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany).

Colony morphology was examined by studying the growth of the strain on TSA at 25 °C for 24 h. Cell morphology was investigated by means of light microscopy (Zeiss) at ×1000 and scanning electron microscopy (Stereoscan 260; Leica). Motility was checked using the method described by Skerman (1967). The Gram reaction was determined using the HiMedia Gram-staining kit according to the manufacturer's instructions. Growth at different temperatures and NaCl concentrations was studied as described by Cowan & Steel (1965). Growth at different pH values was tested using tryptone soya broth (HiMedia) as growth medium and the pH was adjusted using appropriate biological buffers (Nakajima *et al.*, 2005). All biochemical

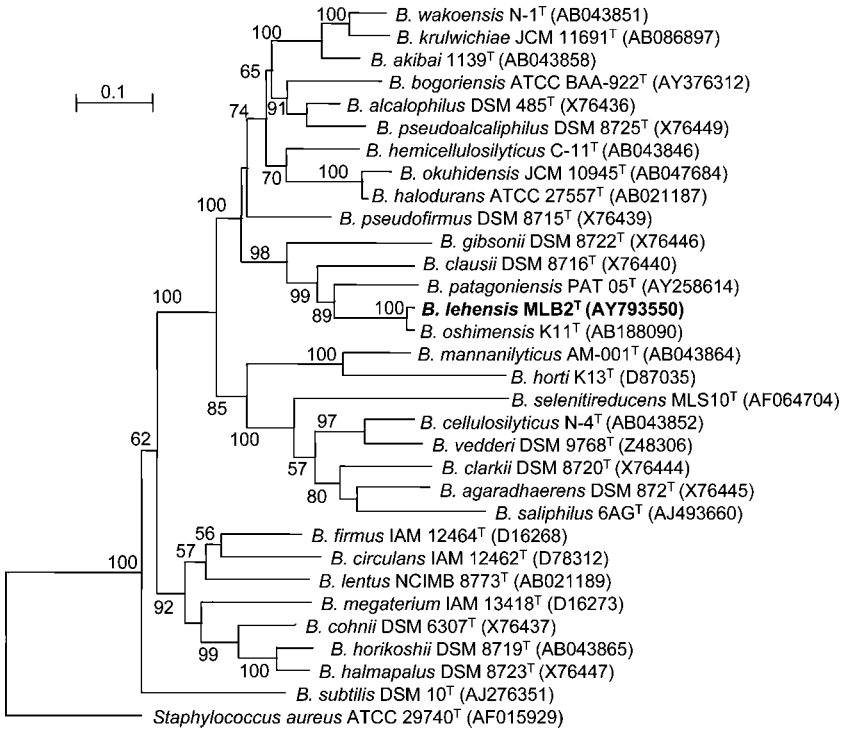

Fig. 1. Neighbour-joining phylogenetic tree indicating the position of strain MLB2^T among related species of the genus *Bacillus*. Bootstrap values (expressed as percentages of 1000 replications) greater than 50 % are shown at the branch points. *Staphylococcus aureus* ATCC 29740^T was used as an outgroup. Bar, 0.1 substitutions per site.

and physiological studies were carried out at pH 8.0. The following characteristics were determined as described by Cowan & Steel (1965): hydrolysis of gelatin, casein, starch, hippurate, ONPG and 4-methylumbelliferone glucuronide; Voges–Proskauer, methyl red, oxidation–fermentation tests; catalase and oxidase (oxidation of tetramethyl-*p*-phenylenediamine dihydrochloride; Sigma) activities; growth on Simmons' citrate and MacConkey agar; production of H_2S and indole; reduction of nitrate; tyrosinase activity; arginine hydrolysis; and deamination of phenylalanine. The assimilation of various substrates for growth was determined by using a Biolog GP2 MicroPlate as described by Mayilraj *et al.* (2006). Acid production from various carbohydrates was tested as described by Claus & Berkeley (1986). The sensitivity of the strain to various antibiotics was tested using antibiotic-susceptibility discs (HiMedia).

Freeze-dried cells used for chemotaxonomic analyses (with the exception of the fatty acid study) were prepared after growth of the strain in tryptone soya broth for 24 h at 25 °C. Polar lipids were analysed as described by Suresh *et al.* (2004). The diagnostic amino acids and whole-cell sugars were determined using TLC, as described by Staneck & Roberts (1974). Isoprenoid quinones were extracted (Minnikin *et al.*, 1984) and separated by reversed-phase HPLC (Kroppenstedt, 1982). For cellular fatty acid analysis, the strains were grown on TSA and a fatty acid methyl ester analysis was then performed with the Sherlock Microbial Identification System (MIDI), as described previously (Pandey *et al.*, 2002). The G+C content of genomic DNA was determined spectrophotometrically (Lambda 35; Perkin Elmer) using the thermal denaturation method (Mandel & Marmur, 1968).

The genomic DNA was isolated using a genomic DNA-isolation kit (Qiagen). PCR amplification, cloning and sequencing of the 16S rRNA gene were performed as described previously (Ghosh *et al.*, 2006). The 16S rRNA gene sequences of closely related taxa with validly published names were retrieved from the GenBank database using BLASTN (Altschul *et al.*, 1997) and aligned using the CLUSTAL_X program (Thompson *et al.*, 1997); the alignment was edited manually. For the neighbour-joining analysis (Saitou & Nei, 1987), the distances between the sequences were calculated using the method of Jukes & Cantor (1969). A bootstrap analysis of 1000 replications was performed to assess the confidence limits of the branching (Felsenstein, 1985). DNA–DNA hybridization was performed each time with freshly isolated genomic DNA and was repeated three times using the membrane filter method (Tourova & Antonov, 1987).

The phenotypic properties are shown in detail in Table 1 and also in the species description. Growth was observed at pH 7.0–11.0, but not at pH 6.0, and the optimum pH for growth (pH 8.0) confirmed strain MLB2^T to be an alkalitolerant bacterium. The cells of strain MLB2^T were sensitive to the following antibiotics (μg per disc): ampicillin (10), chloramphenicol (30), ciprofloxacin (5), erythromycin (10), furazolidone (50), gentamicin (10), nalidixic acid (30), neomycin (30), oleandomycin (15), rifampicin (5), spectinomycin (100), streptomycin (10), sulphadiazine (300), sulphamethizole (300) and tetracycline (30). The cells were resistant to the following antibiotics (μg per disc): cephaloridine (30), cloxacillin (5), colistin (10), novobiocin (30), penicillin (1.5), sulphafurazole (100), tobramycin (10) and trimethoprim (25).

Table 1. Characteristics that differentiate strain MLB2^T from phylogenetically related type strains

Strains: 1, MLB2^T; 2, *B. oshimensis* JCM 12663^T; 3, *B. patagoniensis* DSM 16117^T; 4, *B. clausii* DSM 8716^T; 5, *B. gibsonii* DSM 8722^T; 6, *B. alcalophilus* DSM 485^T. All strains were found to produce acid from glycerol, 2-ketogluconate and D-maltose but not from inositol or D-melezitose. All strains were positive for the assimilation of acetic acid, D-fructose, D-maltose and D-mannose, but negative for the assimilation of adenosine, D-cellobiose, 2-deoxyadenosine, D-fructose 6-phosphate, L-fucose, D-glucose 6-phosphate, D-gluconic acid, DL-α-glycerol phosphate, β-hydroxybutyric acid, inosine, α-ketoglutaric acid, methyl α-D-galactoside, methyl α-D-glucoside, stachyose and succinic acid as growth substrates (Biolog). All data were obtained from this study unless otherwise indicated. +, Positive; W, weakly positive; −, negative.

Characteristic	1	2	3	4	5	6
Motility	+	−	+	+	+	+
Growth at pH 7.0	+	+	+	+	+	−
Reduction of nitrate	+	−	−	+	W	−
Hydrolysis of:						
Hippurate	+	−	−	−	+	−
4-Methylumbelliferone glucuronide	+	+	−	−	+	−
Tween 20	−	+	+	−	−	−
Tween 40	+	+	+	−	−	+
Tween 60	W	+	+	−	−	+
Tween 80	−	−	W	−	−	W
Acid production (aerobically) from:						
D-Arabinose	−	−	−	+	−	+
Glycogen	+	−	W	−	−	+
D-Lactose	+	−	−	−	+	+
D-Mannose	−	+	W	+	+	−
Assimilation of:*						
N-Acetyl-D-glucosamine	−	−	+	+	−	W
Amygdalin	−	−	+	+	+	−
D-Arabitol	+	−	−	−	−	−
Arbutin	+	−	+	−	+	−
2,3-Butanediol	−	−	+	+	−	−
β-Cyclodextrin	+	−	−	+	W	−
Dextrin	+	−	−	−	+	W
D-Galactose	−	+	−	+	W	−
Gentiobiose	−	−	+	−	+	−
Glycerol	−	+	+	−	W	−
2-Ketogluconate	W	−	+	+	−	−
α-Ketovaleric acid	+	−	+	−	−	+
Maltotriose	+	−	−	+	+	W
D-Mannitol	+	−	+	W	+	−
D-Melezitose	+	−	−	−	W	−
Melibiose	−	−	W	−	+	+
Methyl β-D-glucoside	−	+	+	+	+	W
Methyl D-glucose	+	−	W	−	+	−
Palatinose	+	−	−	W	+	−
Propionic acid	+	−	W	+	−	−
D-Psicose	+	−	−	−	+	W
Pyruvic acid	+	−	−	−	+	+
Pyruvic acid methyl ester	−	+	+	−	−	−
D-Raffinose	−	−	+	−	+	−
L-Rhamnose	−	−	+	−	−	+
Salicin	−	−	+	+	+	+
D-Sorbitol	+	−	+	+	W	−
Sucrose	+	−	+	+	+	+
D-Tagatose	−	+	−	+	−	W
Thymidine	−	−	+	−	−	−
D-Trehalose	+	−	+	+	+	−

Table 1. cont.

Characteristic	1	2	3	4	5	6
Turanose	+	−	+	+	+	+
D-Xylose	+	+	−	−	+	−
DNA G+C content (mol%)†	41.4	40.8[a]	39.7[b]	42.8–45.5[c]	40.6–41.7[c]	36.2–38.4[c]

*Using Biolog GP2 microplates.
†Data from other studies are indicated as follows: *a*, Yumoto *et al.* (2005); *b*, Olivera *et al.* (2005); *c*, Nielsen *et al.* (1995). Ranges indicate data from more than one strain.

Most of the chemotaxonomic properties (presented in the species description) were typical of members of the genus *Bacillus*. The isoprenoid quinones present in strain MLB2^T were MK-7 and MK-6. The fatty acid methyl ester profile of the novel strain matched qualitatively those of phylogenetically related species (see Supplementary Table S1 available in IJSEM Online). The DNA G+C content was calculated as 41.4 mol% (mean of three replications), which falls within the defined range (32–69 mol%) accepted for *Bacillus* species (Fritze *et al.*, 1990).

The complete sequence (1541 bases) of the 16S rRNA gene of strain MLB2^T was determined and was compared with those of closely related taxa retrieved from the GenBank database. The phylogenetic tree constructed using the neighbour-joining method suggested that this strain is a member of group 6 (Nielsen *et al.*, 1994) of the genus *Bacillus*; MLB2^T formed a clade with *B. oshimensis* K11^T with a bootstrap value of 100 % (Fig. 1). Pairwise sequence analysis revealed that the highest sequence similarity was with *B. oshimensis* K11^T (98.8 %), followed by *B. patagoniensis* PAT 05^T (98.5 %) and *B. clausii* DSM 8716^T (97.3 %); the remaining species with validly published names showed less than 97 % similarity.

In the DNA–DNA hybridization analysis, strain MLB2^T exhibited 62±2 % similarity to *B. oshimensis* JCM 12663^T, 55±3 % similarity to *B. patagoniensis* DSM 16117^T, 51±3 % similarity to *B. clausii* DSM 8716^T, 34±3 % similarity to *B. gibsonii* DSM 8722^T and 21±2 % similarity to *B. alcalophilus* DSM 485^T. These values for hybridization were lower than the recommended threshold value accepted for defining a novel species (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), hence supporting the distinct position of strain MLB2^T within the genus *Bacillus*. On the basis of the differential phenotypic (Table 1) and genotypic properties, strain MLB2^T should be assigned to a novel species of the genus *Bacillus*, for which the name *Bacillus lehensis* sp. nov. is proposed.

Description of *Bacillus lehensis* sp. nov.

Bacillus lehensis (le.hen′sis. N.L. masc. adj. *lehensis* pertaining to Leh, in India, where the type strain was isolated).

Cells are aerobic, Gram-positive, motile rods (0.5–0.8 × 1.0–3.8 μm). Oval spores develop subterminally in the cells and sporangia are not swollen. Colonies are circular, convex, smooth and pigmented creamish-yellow. Catalase and oxidase are produced. Negative for H_2S, indole and urease production, in the methyl red and Voges–Proskauer tests, for ONPG hydrolysis and in the oxidation–fermentation test. No growth occurs on MacConkey agar or Simmons' citrate agar. Growth occurs at temperatures in the range 10–37 °C (optimum temperature, 25 °C), pH 7.0–11.0 (optimum, pH 8.0) and at NaCl concentrations up to 12 %. Nitrate is reduced to nitrite. Assimilation of substrates for growth and production of acid from carbohydrates under aerobic conditions are shown in Table 1. Hydrolyses casein, gelatin, hippurate and starch. Negative for arginine dihydrolase, phenylalanine deaminase and tyrosinase activity; positive for 4-methylumbelliferone glucuronide activity. Cell wall contains *meso*-diaminopimelic acid as the diagnostic diamino acid; D-glucose, D-galactose and D-xylose are major cell-wall sugars. The predominant polar lipids are diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol, along with two unknown phospholipids (PL1 and PL2). The major isoprenoid quinone is MK-7. The major cellular fatty acids are iso-$C_{15:0}$ (57.0 %), anteiso-$C_{15:0}$ (17.5 %) and iso-$C_{17:0}$ (8.2 %). The DNA G+C content is 41.4 mol%.

The type strain, MLB2^T (=MTCC 7633^T=JCM 13820^T), was isolated from soil collected from Leh, India.

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References

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872, 174^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 2,

pp. 1105–1139. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.

Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Fritze, D., Flossdorf, J. & Claus, D. (1990). Taxonomy of alkaliphilic *Bacillus* strains. *Int J Syst Bacteriol* **40**, 92–97.

Ghosh, A., Paul, D., Prakash, D., Mayilraj, S. & Jain, R. K. (2006). *Rhodococcus imtechensis* sp. nov., a nitrophenol-degrading actinomycete. *Int J Syst Evol Microbiol* **56**, 1965–1969.

Horikoshi, K. (1991). *Microorganisms in Alkaline Environments*. Weinheim: VCH.

Horikoshi, K. (1999). Alkaliphiles: some applications of their products for biotechnology. *Microbiol Mol Biol Rev* **63**, 735–750.

Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, vol. 3, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.

Kroppenstedt, R. M. (1982). Separation of bacterial menaquinones by HPLC using reverse phase RP 18 and a silver loaded ion exchanger as stationary phases. *J Liq Chromatogr* **5**, 2359–2387.

Mandel, M. & Marmur, J. (1968). Use of ultraviolet absorbance-temperature profile for determining the guanine plus cytosine content of DNA. *Methods Enzymol* **12B**, 195–206.

Mayilraj, S., Saha, P., Suresh, K. & Saini, H. S. (2006). *Ornithinimicrobium kibberense* sp. nov., isolated from the Himalayas, India. *Int J Syst Evol Microbiol* **56**, 1657–1661.

Minnikin, D. E., O'Donnell, A. G., Goodfellow, M., Alderson, G., Athalye, M., Schaal, A. & Parlett, J. H. (1984). An integrated procedure for the extraction of bacterial isoprenoid quinones and polar lipids. *J Microbiol Methods* **2**, 233–241.

Nakajima, K., Hirota, K., Nodasaka, Y. & Yumoto, I. (2005). *Alkalibacterium iburiense* sp. nov., an obligate alkaliphile that reduces an indigo dye. *Int J Syst Evol Microbiol* **55**, 1525–1530.

Nielsen, P., Rainey, F. A., Outtrup, H., Priest, F. G. & Fritze, D. (1994). Comparative 16S rDNA sequence analysis of some alkaliphilic bacilli and the establishment of a sixth rRNA group within the genus *Bacillus*. *FEMS Microbiol Lett* **117**, 61–66.

Nielsen, P., Fritze, D. & Priest, F. G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745–1761.

Olivera, N., Sineriz, F. & Breccia, J. D. (2005). *Bacillus patagoniensis* sp. nov., a novel alkalitolerant bacterium from the rhizosphere of *Atriplex lampa* in Patagonia, Argentina. *Int J Syst Evol Microbiol* **55**, 443–447.

Pandey, K. K., Mayilraj, S. & Chakraborti, T. (2002). *Pseudomonas indica* sp. nov., a novel butane-utilizing species. *Int J Syst Evol Microbiol* **52**, 1559–1567.

Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Skerman, V. B. D. (1967). *A Guide to the Identification of the Genera of Bacteria*, 2nd edn. Baltimore: Williams & Wilkins.

Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin layer chromatography. *Appl Microbiol* **28**, 226–231.

Suresh, K., Prabagaran, S. R., Sengupta, S. & Shivaji, S. (2004). *Bacillus indicus* sp. nov., an arsenic-resistant bacterium isolated from an aquifer in West Bengal, India. *Int J Syst Evol Microbiol* **54**, 1369–1375.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Tourova, T. P. & Antonov, A. S. (1987). Identification of microorganisms by rapid DNA-DNA hybridization. *Methods Microbiol* **19**, 333–355.

Ulukanli, Z. & Diurak, M. (2002). Alkaliphilic microorganisms and habitats. *Turk J Biol* **26**, 181–191.

Wayne, L. G., Brenner, D. J., Colwell, R. R., Grimont, P. A. D., Kandler, O., Krichevsky, M. I., Moore, L. H., Moore, W. E. C., Murray, R. G. E. & other authors (1987). International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.

Yumoto, I., Yamazaki, K., Sawabe, T., Nakano, K., Kawasaki, K., Ezura, Y. & Shinano, H. (1998). *Bacillus horti* sp. nov., a new Gram-negative alkaliphilic bacillus. *Int J Syst Bacteriol* **48**, 565–571.

Yumoto, I., Hirota, K., Goto, T., Nodasaka, Y. & Nakajima, K. (2005). *Bacillus oshimensis* sp. nov., a moderately halophilic, non-motile alkaliphile. *Int J Syst Evol Microbiol* **55**, 907–911.