Bacillus alkalitelluris sp. nov., an alkaliphilic bacterium isolated from sandy soil

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A Gram-positive, alkaliphilic bacterium, designated strain BA288^T, was isolated from sandy soil. Cells were facultatively anaerobic, endospore-forming rods that were motile by means of peritrichous flagella. The strain grew at 15–40 °C and pH 7.0–11.0 (optimally at 30 °C and pH 9.0–9.5) and at salinities of 0–4 % (w/v) NaCl. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain BA288^T belonged to the genus *Bacillus* and that *Bacillus herbersteinensis* D-1,5a^T, *Bacillus humi* LMG 22167^T, *Bacillus cohnii* DSM 6307^T and *Bacillus litoralis* SW-211^T were the closest neighbours (96.2, 96.0, 96.0 and 95.9 % sequence similarity, respectively). The genomic DNA G + C content was 37.9 mol% and the predominant menaquinone was MK-7. The major cellular fatty acids were anteiso-C_{15:0}, iso-C_{15:0}, C_{16:0} and iso-C_{14:0}. The peptidoglycan type was A1γ (meso-diaminopimelic acid). Therefore, on the basis of phylogenetic, phenotypic and chemotaxonomic properties, strain BA288^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus alkalitelluris* sp. nov. is proposed. The type strain is BA288^T (=KCTC 3947^T =DSM 16976^T).

Many alkaliphilic and alkalitolerant *Bacillus* species have been isolated from a wide range of alkaline habitats, such as soda lakes, deserts and arid soils (Li *et al.*, 2002; Ulukanli & Diurak, 2002; Täubel *et al.*, 2003; Vargas *et al.*, 2005; Yumoto *et al.*, 2003, 2005). At least 19 alkaliphilic and alkalitolerant *Bacillus* species have been identified to date (Vedder, 1934; Spanka & Fritze, 1993; Nielsen *et al.*, 1995; Agnew *et al.*, 1995; Fritze, 1996; Switzer Blum *et al.*, 1998; Yumoto *et al.*, 1998, 2003; Olivera *et al.*, 2005; Ghosh *et al.*, 2007). These bacteria constitute the sixth rRNA group in the genus *Bacillus* (Nielsen *et al.*, 1994) and they have attracted much attention with regard to industrial applications, basic research and biotechnological exploration (Duckworth *et al.*, 1996; Krulwich & Guffanti, 1989; Martins *et al.*, 2001; Nielsen *et al.*, 1995; Nogi *et al.*, 2005). During screening for alkaliphilic bacteria, we isolated an alkaliphilic bacterium, designated strain BA288^T, belonging to the genus *Bacillus*. In this study, we describe the results of a polyphasic study designed to characterize this organism, which was isolated from sandy soil from the Keumsan region of Korea.

During screening for alkaliphilic bacteria, strain BA288^T was isolated from sandy soil near an abandoned mine at Keumsan in Korea. For isolation, soil samples were diluted serially with a 1 % (w/v) saline solution, spread on R2A agar (Difco) adjusted to pH 9.2 by the addition of Na₂CO₃/NaHCO₃ and incubated for 2 days at 30 °C. The isolate was routinely cultured aerobically on R2A for 2 days at 30 °C. Growth was tested at different temperatures (4–55 °C) and at different pH values (5.0–11.0) in R2A. Media of different pH were prepared using the appropriate biological buffers: Na₂HPO₄/NaH₂PO₄ for below pH 8.0, Na₂CO₃/NaHCO₃ for pH 8.0–10.0 and Na₂HPO₄/NaOH for pH 11.0 (Bates & Bower, 1956; Gomori, 1955). Requirements for, and tolerance of, NaCl were determined in R2A broth supplemented with NaCl. Cell growth was monitored by measuring the optical density at 600 nm. Cell biomass of strain BA288^T was obtained from cultures grown on R2A at 30 °C. Cell morphology was studied using light microscopy and transmission electron microscopy. Motility was observed after 12 and 36 h in wet mounts by using a light microscope (E600; Nikon). The flagellum type was examined with transmission electron microscopy, using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids and negatively stained with 1 % potassium phosphotungstate (pH 7.0) and the grids were examined in a Phillips 201 transmission electron microscope operated at 80 kV. Endospores were stained according to the method of

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BA288^T is AY829448.

Maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences for strain BA288^T and related taxa are available as supplementary material with the online version of this paper.

Schaeffer–Fulton (Smibert & Krieg, 1981). Gram staining was determined using the bioMérieux Gram stain kit according to the manufacturer's instructions. Catalase activity was determined by testing for bubble production in a 3% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was determined by testing for oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine by using a Bactident oxidase strip (Merck). Nitrate reduction and hydrolysis of compounds were determined on R2A according to the methods described by Cowan & Steel (1965), Lányi (1987) and Smibert & Krieg (1994). Carbon-source utilization tests and acid-production tests were performed using API 50 CH and API 50 CHB galleries according to the instructions of the manufacturer (bioMérieux). Two drops of cell suspension (pH 9.0) were inoculated into the ampoule. Growth under anaerobic conditions was determined in an anaerobic chamber ($\text{H}_2/\text{CO}_2/\text{N}_2$, 5:10:85 by vol.; Mart Microbiology) after 5 days incubation at 30 °C on R2A.

On R2A medium, strain BA288^T formed cream-coloured, smooth, circular colonies after incubation at 30 °C for 2 days. The strain grew at salt concentrations in the range 0–4% (w/v) NaCl, with optimum growth occurring at 0–1% (w/v) NaCl. The strain did not grow in the presence of $\geq 5\%$ (w/v) NaCl. Growth was observed at initial pH of between pH 7.0 and 11.0 in R2A; optimal growth occurred at pH 9.0–9.5, and no growth was observed at pH 6.4. Growth was observed at temperatures between 15 and 40 °C, the optimum temperature being 30 °C. Strain BA288^T comprised Gram-positive, facultatively anaerobic rods, 0.4–0.5 μm wide and 2.4–3.0 μm long. Terminal, ellipsoidal endospores were formed and cells were motile by means of peritrichous flagella. The strain was oxidase- and catalase-positive and did not reduce nitrate to nitrite. In contrast to its closest neighbours, strain BA288^T showed anaerobic growth. The phenotypic characteristics that serve to distinguish strain BA288^T from the type strains of closely related *Bacillus* species are shown in Table 1.

For quantitative analysis of whole-cell fatty acids, strain BA288^T was cultivated on R2A adjusted to pH 9.0 for 2 days at 30 °C. Fatty acid methyl esters were analysed by GC/MS according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987), using an HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; GROM). Methanol/2-propanol (2:1, v/v) was used as the mobile phase and quinone was detected at 270 nm. The peptidoglycan structure was elucidated by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972), using paper chromatography (Rhuland *et al.*, 1955). Quantitative analysis of amino acids in the total hydrolysate was performed using GC and GC/MS as

described by MacKenzie (1987) and Hasegawa *et al.* (1983). The N-terminal residue of the interpeptide bridge was determined by means of dinitrophenylation as described by Schleifer (1985). The G+C content (mol%) was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984).

The predominant isoprenoid quinone of this strain was MK-7. The cell-wall peptidoglycan was of the A1 γ type, containing *meso*-diaminopimelic acid as the diagnostic diamino acid (as is the case in the great majority of the members of the genus *Bacillus*; Claus & Berkeley, 1986). The cellular fatty acids comprised anteiso-C_{15:0} (30.9%), iso-C_{15:0} (16.7%), C_{16:0} (12.3%), iso-C_{14:0} (8.2%), iso-C_{16:0} (7.3%), iso-C_{17:0} (5.8%), anteiso-C_{17:0} (4.7%), C_{16:1 ω 11c} (3.9%), C_{15:0} (1.4%), C_{17:0} (1.3%), C_{16:1 ω 7c} alcohol (0.9%), C_{18:0} (0.5%), C_{16:0} N alcohol (0.5%), C_{17:1 ω 5c} (0.5%) and C_{17:1 ω 10c} (0.4%). The presence of branched, saturated fatty acids as the major fatty acids corresponded with the profiles for the type strains of the genus *Bacillus* (Kämpfer, 1994). Fatty acids anteiso-C_{15:0} and iso-C_{15:0} were major components in strain BA288^T; the most closely related type strains, *Bacillus herbersteiniensis* D-1,5a^T, *Bacillus humi* LMG 22167^T and *Bacillus litoralis* SW-211^T, showed similar components. Comparative 16S rRNA gene sequence analysis showed that the isolate was most closely related to *B. herbersteiniensis* D-1,5a^T, *B. humi* LMG 22167^T, *B. cohnii* DSM 6307^T and *B. litoralis* SW-211^T, with sequence similarities of 96.2, 96.0, 96.0 and 95.9%, respectively. These similarities are sufficiently low (<97%) to justify the definition of a novel species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). The DNA G+C content of strain BA288^T was 37.9 mol%. The major fatty acid profile, the major isoprenoid quinone and the DNA G+C content are typical of the group classically defined as the genus *Bacillus* (Arahal *et al.*, 1999; Fritze, 1996; Nielsen *et al.*, 1994, 1995; Priest *et al.*, 1988).

The 16S rRNA gene was amplified using a PCR with the Eubac 27F and 1492R primers (DeLong, 1992) and the PCR products were purified using the QIAquick PCR purification kit (Qiagen). Sequencing of the purified 16S rRNA gene was performed using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems), as recommended by the manufacturer, and five primers (337F, 785F, 1225F, 518R, 1100R). The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. The 16S rRNA gene sequence of strain BA288^T was aligned with those of *Bacillus* species by using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix, version 1.1 (Ribosomal Database Project II; <http://rdp.cme.msu.edu/>; Cole *et al.*, 2007). Gaps at the 5' and 3' ends of the alignment were included for further analysis. Phylogenetic trees were inferred using three tree-making algorithms, i.e. neighbour joining (Saitou & Nei, 1987), maximum likelihood (Felsenstein, 1981) and maximum parsimony (Kluge & Farris, 1969),

Table 1. Characteristics that serve to differentiate strain BA288^T from related *Bacillus* species

Taxa: 1, strain BA288^T; 2, *B. litoralis* (data from Yoon & Oh, 2005); 3, *B. herbersteinensis* (Wieser *et al.*, 2005); 4, *B. humi* (Heyrman *et al.*, 2005); 5, *B. endophyticus* (Reva *et al.*, 2002); 6, *B. asahii* (Yumoto *et al.*, 2004); 7, *B. funiculus* (Ajithkumar *et al.*, 2002); 8, *B. cibi* (Yoon *et al.*, 2005); 9, *B. indicus* (Suresh *et al.*, 2004); 10, *B. horikoshii* (Nielsen *et al.*, 1995; Li *et al.*, 2002; Logan *et al.*, 2002); 11, *B. halmopalus* (Nielsen *et al.*, 1995; Li *et al.*, 2002; Logan *et al.*, 2002); 12, *B. cohnii* (Spanka & Fritze, 1993; Suresh *et al.*, 2004); 13, *B. flexus* (Claus & Berkeley, 1986; Suresh *et al.*, 2004); 14, *B. megaterium* (Claus & Berkeley, 1986; Reva *et al.*, 2002; Täubel *et al.*, 2003); 15, *B. simplex* (Priest *et al.*, 1988; De Clerck *et al.*, 2004). In all cases, cells are catalase-positive rods. +, Positive; –, negative; w, weakly positive; v, variable; ND, no data available. Data in parentheses are for the respective type strains.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Spore shape*	E	E	E	E or S	E	E	E	E	ND	E	E	E	ND	E	E or C
Spore position†	T	C, S or T	T	S or T	S or T	C	C	C or S	S	S	C or S	T	ND	C	C, S or T
Oxidase	+	+	+	+	+	+	–	+	ND	ND	ND	+	ND	–	–
Anaerobic growth	+	–	–	W	–	–	–	–	–	–	–	ND	ND	–	–
Growth at/in:															
5 % (w/v) NaCl	–	+	+	ND	+	–	–	+	–	+	–	+	+	+	+
10 % (w/v) NaCl	–	+	–	ND	+	–	–	+	–	–	–	–	ND	–	–
10 °C	–	+	+	–	+	–	–	+	–	+	+	+	ND	+	ND
45 °C	–	+	–	–	+	+	–	+	–	–	–	+	ND	ND	ND
Nitrate reduction	–	–	–	+	–	W	+	–	–	–	–	+	–	–	+
Hydrolysis of:															
Aesculin	+	+	ND	+	ND	ND	+	+	+	+	+	(–)	–	+	–
Casein	–	+	ND	ND	–	+	–	+	ND	+	+	v (+)	ND	+	+
Gelatin	–	+	ND	–	–	–	–	–	+	+	+	+	+	+	+
Starch	+	+	+	–	–	W	+	+	+	+	+	+	–	+	+
Tween 80	–	+	ND	ND	ND	ND	–	+	ND	–	–	v (+)	ND	ND	V
Growth on:															
D-Galactose	–	+	+	–	ND	ND	ND	+	ND	–	–	ND	ND	(–)	ND
Cellobiose	–	+	+	–	ND	ND	ND	+	+	ND	ND	(–)	+	(–)	V
D-Mannose	–	–	+	–	ND	ND	ND	+	+	V	+	(–)	–	(–)	–
Sucrose	–	+	+	–	ND	ND	+	+	+	ND	ND	(–)	+	(–)	ND
Maltose	–	+	+	–	ND	ND	+	+	+	ND	ND	(–)	+	(+)	–
Pyruvate	–	+	+	–	ND	ND	ND	+	–	ND	ND	(+)	–	(–)	ND
Acid production from:															
D-Glucose	–	+	–	–	+	–	+	+	–	+	+	(–)	+	+	+
Lactose	–	+	–	+	–	–	ND	–	–	–	–	(–)	–	V	–
D-Mannitol	–	–	–	–	+	–	–	–	–	+	–	(–)	–	+	V
D-Mannose	–	–	–	–	+	–	ND	+	+	V	+	(–)	–	V	V
D-Xylose	–	+	–	–	V	–	–	–	–	V	V	(–)	–	+	–
DNA G + C content (mol%)	37.9	35.2	36.2–36.9	37.5	ND	39.4	37.2	45	41.2	41.1–42.0	38.6	33.9–35.0	38.4	36.5–45.0	40–41

*C, Cylindrical; E, ellipsoidal or oval; S, spherical.

†C, Central or paracentral; S, subterminal; T, terminal.

available in the PHYLIP software package (version 3.6) (Felsenstein, 2002). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of Kimura's two-parameter model (Kimura, 1980) with the DNADIST program. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (with 1000 replications) was performed with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package.

The almost-complete 16S rRNA gene sequence (1515 nt) of strain BA288^T was obtained and used for an initial BLAST

search in GenBank and for phylogenetic analysis. A phylogenetic analysis based on 16S rRNA gene sequences showed that strain BA288^T formed a distinct line within the genus *Bacillus* and joined a clade with the type strain of *B. litoralis* in the neighbour-joining analysis (Fig. 1). Phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms also supported the positioning of strain BA288^T in a clade with the type strain of *B. litoralis* (see Supplementary Figs S1 and S2, available in IJSEM

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships between strain BA288^T and related taxa. Numbers at branching nodes are bootstrap percentages (based on 1000 replications); only values greater than 50 % are indicated. Bar, 0.01 substitutions per nucleotide position.

Online). Comparative 16S rRNA gene sequence analysis showed that the isolate was most closely related to *B. herbersteinensis* D-1,5a^T, *B. humi* LMG 22167^T, *B. cohnii* DSM 6307^T and *B. litoralis* SW-211^T, with sequence similarities of 96.2, 96.0, 96.0 and 95.9 %, respectively; these levels of similarity are sufficiently low (<97 %) to justify defining the isolate as a representative of a novel species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002).

Therefore, on the basis of physiological, biochemical and phylogenetic properties, strain BA288^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus alkalitelluris* sp. nov. is proposed.

Description of *Bacillus alkalitelluris* sp. nov.

Bacillus alkalitelluris (al.ka.li.tel.lu'ris. N.L. n. *alkali* alkali; L. gen. n. *telluris* of the soil or earth; N.L. gen. n. *alkalitelluris* of alkaline soil).

Cells are facultatively anaerobic, Gram-positive, endospore-forming (ellipsoidal, terminal) rods, 0.4–0.5 × 2.4–3.0 µm. Colonies on R2A are creamy, smooth and circular. Cells are motile rods with peritrichous flagella. Grows at salinities of 0–4 % (w/v) NaCl. Optimal growth occurs at 0–1 % (w/v) NaCl. No growth occurs in the presence of ≥5 % (w/v) NaCl. Grows between 15 and 40 °C (optimally at 30 °C) and from pH 7.0 to 11.0 (optimally at pH 9.0–9.5) in R2A broth. Growth occurs under anaerobic

conditions on R2A agar. Oxidase- and catalase-positive. Nitrate is not reduced to nitrite. H₂S and indole are not produced. Aesculin and starch are hydrolysed. Casein, gelatin, Tweens 80 and 20, xanthine and hypoxanthine are not hydrolysed. Acids are produced from potassium 5-ketogluconate and aesculin, but not from L-arabinose, D-arabitol, D-fructose, D-galactose, gentiobiose, glycogen, D-glucose, inositol, D-lactose, D-mannitol, D-mannose, melezitose, raffinose, L-rhamnose, D-sorbitol, salicin, D-tagatose, turanose, xylitol or D-xylose. The following substrates are not utilized for growth: L-arabinose, cellobiose, D-fructose, D-galactose, D-glucose, lactose, D-mannose, maltose, sucrose and pyruvate. Acetate, benzoate, citrate, formate, L-glutamate, L-malate, succinate and D-xylose are utilized. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid (A1_γ type). The predominant isoprenoid quinone is MK-7. The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and C_{16:0}. The DNA G + C content of the type strain is 37.9 mol%.

The type strain, BA288^T (=KCTC 3947^T =DSM 16976^T), was isolated from sandy soil near an abandoned mine in the Keumsan region of Korea.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Centre Program of the Korean Ministry of Science and Technology and by the KRIBB Research Initiative Program, Republic of Korea.

References

- Agnew, M. D., Koval, S. F. & Jarrell, K. F. (1995). Isolation and characterization of novel alkaliphiles from bauxite-processing waste and description of *Bacillus vedderi* sp. nov., a new obligate alkaliphile. *Syst Appl Microbiol* **18**, 221–230.
- Ajithkumar, V. P., Ajithkumar, B., Lriye, R. & Sakai, T. (2002). *Bacillus funiculus* sp. nov., filamentous isolates from activated sludge. *Int J Syst Evol Microbiol* **52**, 1141–1144.
- Arahal, D. R., Marquez, M. C., Volcani, B. E., Schleifer, K. H. & Ventosa, A. (1999). *Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea. *Int J Syst Bacteriol* **49**, 521–530.
- Bates, R. G. & Bower, V. E. (1956). Alkaline solutions for pH control. *Anal Chem* **28**, 1322–1324.
- Claus, D. & Berkeley, R. C. W. (1986). Genus *Bacillus* Cohn 1872. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1105–1140. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam-Syed-Mohideen, A. S., McGarrell, D. M., Bandela, A. M., Cardenas, E., Garrity, G. M. & Tiedje, J. M. (2007). The ribosomal database project (RDP-II): introducing *myRDP* space and quality controlled public data. *Nucleic Acids Res* **35**, D169–D172.
- Cowan, S. T. & Steel, K. J. (1965). *Manual for the Identification of Medical Bacteria*. London: Cambridge University Press.
- De Clerck, E., Rodríguez-Díaz, M., Vanhoutte, T., Heyman, J., Logan, N. A. & De Vos, P. (2004). *Anoxybacillus contaminans* sp. nov. and *Bacillus gelatini* sp. nov., isolated from contaminated gelatin batches. *Int J Syst Evol Microbiol* **54**, 941–946.
- DeLong, E. F. (1992). Archaea in coastal marine environments. *Proc Natl Acad Sci U S A* **89**, 5685–5689.
- Duckworth, A. W., Grant, W. D., Jones, B. E. & Steenbergen, R. V. (1996). Phylogenetic diversity of soda lake alkaliphiles. *FEMS Microbiol Ecol* **19**, 181–191.
- Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J Mol Evol* **17**, 368–376.
- Felsenstein, J. (2002). PHYLIP (phylogeny inference package), version 3.6a. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Fritze, D. (1996). *Bacillus haloalkaliphilus* sp. nov. *Int J Syst Bacteriol* **46**, 98–101.
- Ghosh, A., Bhardwaj, M., Satyanarayana, T., Khurana, M., Mayilraj, S. & Jain, R. K. (2007). *Bacillus lehensis* sp. nov., an alkalitolerant bacterium isolated from soil. *Int J Syst Evol Microbiol* **57**, 238–242.
- Gomori, G. (1955). Preparation of buffers for use in enzyme studies. *Methods Enzymol* **1**, 138–146.
- Hasegawa, T., Takizawa, M. & Tanida, S. (1983). A rapid analysis for chemical grouping of aerobic actinomycetes. *J Gen Appl Microbiol* **29**, 319–322.
- Heyman, J., Rodríguez-Díaz, M., Devos, J., Felske, A., Logan, N. A. & De Vos, P. (2005). *Bacillus arenosi* sp. nov., *Bacillus arvi* sp. nov. and *Bacillus humi* sp. nov., isolated from soil. *Int J Syst Evol Microbiol* **55**, 111–117.
- Kämpfer, P. (1994). Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *Syst Appl Microbiol* **17**, 86–98.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.
- Kluge, A. G. & Farris, F. S. (1969). Quantitative phyletics and the evolution of anurans. *Syst Zool* **18**, 1–32.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Krulwich, T. A. & Guffanti, A. A. (1989). Alkalophilic bacteria. *Annu Rev Microbiol* **43**, 435–463.
- Lányi, B. (1987). Classical and rapid identification methods for medically important bacteria. *Methods Microbiol* **19**, 1–67.
- Li, Z., Kawamura, Y., Shida, O., Yamagata, S., Deguchi, T. & Ezaki, T. (2002). *Bacillus okuhidensis* sp. nov., isolated from the Okuhida spa area of Japan. *Int J Syst Evol Microbiol* **52**, 1205–1209.
- Logan, N. A., Lebbe, L., Verhelst, A., Goris, J., Forsyth, G., Rodríguez-Díaz, M., Heyndrickx, M. & De Vos, P. (2002). *Bacillus luciferensis* sp. nov., from volcanic soil on Candlemas Island, South Sandwich archipelago. *Int J Syst Evol Microbiol* **52**, 1985–1989.
- MacKenzie, S. L. (1987). Gas chromatographic analysis of amino acids as the N-heptafluorobutyl isobutyl esters. *J Assoc Off Anal Chem* **70**, 151–160.
- Martins, R. F., Davids, W., Abu Al-Soud, W., Levander, F., Rådström, P. & Hatti-Kaul, R. (2001). Starch-hydrolyzing bacteria from Ethiopian soda lakes. *Extremophiles* **5**, 135–144.
- 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**, 16–65.
- Nielsen, P., Fritze, D. & Priest, F. G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745–1761.
- Nogi, Y., Takami, H. & Horikoshi, K. (2005). Characterization of alkaliphilic *Bacillus* strains used in industry: proposal of five novel species. *Int J Syst Evol Microbiol* **55**, 2309–2315.
- Olivera, N., Siñeriz, 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.
- Priest, F. G., Goodfellow, M. & Todd, C. (1988). A numerical classification of the genus *Bacillus*. *J Gen Microbiol* **134**, 1847–1882.
- Reva, O. N., Smirnov, V. V., Pettersson, B. & Priest, F. G. (2002). *Bacillus endophyticus* sp. nov., isolated from the inner tissues of cotton plants (*Gossypium* sp.). *Int J Syst Evol Microbiol* **52**, 101–107.
- Rhuland, L. E., Work, E., Denman, R. F. & Hoare, D. S. (1955). The behavior of the isomers of α,ϵ -diaminopimelic acid on paper chromatograms. *J Am Chem Soc* **77**, 4844–4846.
- Rosselló-Mora, R. & Amann, R. (2001). The species concept for prokaryotes. *FEMS Microbiol Rev* **25**, 39–67.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Schleifer, K. H. (1985). Analysis of the chemical composition and primary structure of murein. *Methods Microbiol* **18**, 123–156.
- Schleifer, K. H. & Kandler, O. (1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol Rev* **36**, 407–477.
- Smibert, R. M. & Krieg, N. R. (1981). General characterization. In *Manual of Methods for General Microbiology*, pp. 409–443. Edited by P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg & G. B. Phillips. Washington, DC: American Society for Microbiology.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–654. Edited by P. Gerhardt. Washington, DC: American Society for Microbiology.

- Spanka, R. & Fritze, D. (1993).** *Bacillus cohnii* sp. nov., a new, obligately alkaliphilic, oval-spore-forming *Bacillus* species with ornithine and aspartic acid instead of diaminopimelic acid in the cell wall. *Int J Syst Bacteriol* **43**, 150–156.
- Stackebrandt, E., Frederikson, W., Garrity, G. M., Grimont, P. A. D., Kämpfer, P., Maiden, M. C. J., Nesme, X., Rosselló-Mora, R., Swings, J. & other authors (2002).** Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology. *Int J Syst Evol Microbiol* **52**, 1043–1047.
- Suresh, K., Prabakaran, 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.
- Switzer Blum, J., Burns Bindi, A., Buzzelli, J., Stolz, J. F. & Oremland, R. S. (1998).** *Bacillus arsenicoselenatis* sp. nov., and *Bacillus selenitireducens* sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Arch Microbiol* **171**, 19–30.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Täubel, M., Kämpfer, P., Buczolits, S., Lubitz, W. & Busse, H.-J. (2003).** *Bacillus barbaricus* sp. nov., isolated from an experimental wall painting. *Int J Syst Evol Microbiol* **53**, 725–730.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Ulukanli, Z. & Diurak, M. (2002).** Alkaliphilic microorganisms and habitats. *Turk J Biol* **26**, 181–191.
- Vargas, V. A., Delgado, O. D., Hatti-Kaul, R. & Mattiasson, B. (2005).** *Bacillus bogoriensis* sp. nov., a novel alkaliphilic, halotolerant bacterium isolated from a Kenyan soda lake. *Int J Syst Evol Microbiol* **55**, 899–902.
- Vedder, A. (1934).** *Bacillus alcalophilus* n. sp.; benevens enkele ervaringen met sterk alcalische voedingbodems. *Antonie van Leeuwenhoek* **1**, 141–147 (in Dutch).
- Wieser, M., Worliczek, H., Kämpfer, P. & Busse, H.-J. (2005).** *Bacillus herbersteinensis* sp. nov. *Int J Syst Evol Microbiol* **55**, 2119–2123.
- Yoon, J.-H. & Oh, T.-K. (2005).** *Bacillus litoralis* sp. nov., isolated from a tidal flat of the Yellow Sea in Korea. *Int J Syst Evol Microbiol* **55**, 1945–1948.
- Yoon, J.-H., Lee, C.-H. & Oh, T.-K. (2005).** *Bacillus cibi* sp. nov., isolated from jeotgal, a traditional Korean fermented seafood. *Int J Syst Evol Microbiol* **55**, 733–736.
- 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., Yamaga, S., Sogabe, Y., Nodasaka, Y., Matsuyama, H., Nakajima, K. & Suemori, A. (2003).** *Bacillus krulwichiae* sp. nov., a halotolerant obligate alkaliphile that utilizes benzoate and *m*-hydroxybenzoate. *Int J Syst Evol Microbiol* **53**, 1531–1536.
- Yumoto, I., Hirota, K., Yamaga, S., Nodasaka, Y., Kawasaki, T., Matsuyama, H. & Nakajima, K. (2004).** *Bacillus asahii* sp. nov., a novel bacterium isolated from soil with the ability to deodorize the bad smell generated from short-chain fatty acids. *Int J Syst Evol Microbiol* **54**, 1997–2001.
- 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.