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

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A Gram-positive, alkaliphilic bacterium, designated strain BA288⁷, 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 BA288⁷ belonged to the genus *Bacillus* and that *Bacillus herbersteinensis* D-1,5a⁷, *Bacillus humi* LMG 22167⁷, *Bacillus cohui DSM 6307⁷ and *Bacillus litoralis* SW-211⁷ 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-C₁₅ : ₀, iso-C₁₅ : ₀, C₁₆ : ₀ and iso-C₁₄ : ₀. The peptidoglycan type was A₁,₁ (meso-diaminopimelic acid). Therefore, on the basis of phylogenetic, phenotypic and chemotaxonomic properties, strain BA288⁷ represents a novel species of the genus *Bacillus*, for which the name *Bacillus alkalitelluris* sp. nov. is proposed. The type strain is BA288⁷ (=KCTC 3947⁷ =DSM 16976⁷).

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; Ulukənli & Diurak, 2002; Täuβel *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 & Fritzke, 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; Kruwlitch & 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 BA288⁷, 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 BA288⁷ 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: Na₃HPO₄/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 BA288⁷ 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 BA288⁷ is AY829448.

Maximum-likelihood and maximum-parsimony phylogenetic trees based on 16S rRNA gene sequences for strain BA288⁷ and related taxa are available as supplementary material with the online version of this paper.

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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 in a 1% (w/v) tetramethyl-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 (H2/CO2/N2, 5:10:85 by vol.; Mart Microbiology) after 5 days incubation at 30 °C on R2A.

On R2A medium, strain BA288T 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 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 BA288T 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 BA288T showed anaerobic growth. The phenotypic characteristics that serve to distinguish strain BA288T from the type strains of closely related Bacillus species are shown in Table 1.

For quantitative analysis of whole-cell fatty acids, strain BA288T 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 A1γ type, containing meso-diaminopimelamic 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-C15:0 (30.9%), iso-C15:0 (16.7%), C16:0 (12.3%), iso-C14:0 (8.2%), iso-C16:0 (7.3%), iso-C17:0 (5.8%), anteiso-C17:0 (4.7%), C16:1ω11c (3.9%), C15:0 (1.4%), C17:0 (1.3%), C16:1ω7c alcohol (0.9%), C18:0 (0.5%), C16:0 N-alkanol (0.5%), C17:1ω5c (0.5%) and C17:1ω10c (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-C15:0 and iso-C15:0 were major components in strain BA288T; the most closely related type strains, Bacillus herbersteinensis D-1,5aT, Bacillus humi LMG 22167T and Bacillus litoralis SW-211T, showed similar components. Comparative 16S rRNA gene sequence analysis showed that the isolate was most closely related to B. herbersteinensis D-1,5aT, B. humi LMG 22167T, B. cohnni DSM 6307T and B. litoralis SW-211T, with sequence similarities of 96.2, 96.0, 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 BA288T 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 (Arahel 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, 1518F, 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 BA288T 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 (Klug & Farris, 1969),
Table 1. Characteristics that serve to differentiate strain BA288<sup>T</sup> from related Bacillus species

| 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                          | ND                         | E                          | E or C                     | T                           |
| Spore position†               | T, C, S or T               | T, S or T                  | S or T                     | C, C or S                  | C                          | C                          | C                          | C                          | S                          | S                          | C                          | C                          | C or S                     | T                           |
| Oxidase                       | +                          | +                          | +                          | +                          | +                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | +                          |
| Anaerobic growth              | +                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | +                          | ND                         | ND                         | ND                         | +                          | +                          | +                          |
| Growth at/in:                 | 5% (w/v) NaCl              | –                          | –                          | +                          | +                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | 10% (w/v) NaCl             | –                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | 10 °C                      | –                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | 45 °C                      | –                          | –                          | –                          | +                          | +                          | –                          | –                          | ND                         | +                          | ND                         | ND                         | ND                         | ND                         | ND                         |
| Nitrate reduction             | –                          | –                          | –                          | +                          | +                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
| Hydrolysis of:                |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |                           |
|                               | Aesculin                   | +                          | +                          | ND                         | ND                         | ND                         | +                          | +                          | +                          | +                          | (–)                        | (–)                        | (–)                        | (–)                        | (–)                        |
|                               | Cascin                     | –                          | –                          | ND                         | ND                         | ND                         | –                          | +                          | –                          | (–)                        | (–)                        | (–)                        | (–)                        | (–)                        | (–)                        |
|                               | Gelatin                    | –                          | –                          | ND                         | ND                         | ND                         | –                          | –                          | –                          | –                          | (–)                        | (–)                        | (–)                        | (–)                        | (–)                        |
|                               | Starch                     | +                          | +                          | –                          | –                          | W                          | –                          | –                          | –                          | +                          | +                          | +                          | +                          | +                          | +                          |
|                               | Tween 80                   | +                          | +                          | +                          | –                          | –                          | –                          | –                          | –                          | +                          | –                          | +                          | +                          | +                          | +                          |
| Growth on:                    | d-Galactose                | –                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          | +                          |
|                               | Cellobiose                 | –                          | +                          | +                          | +                          | +                          | +                          | ND                         | +                          | ND                         | (–)                        | +                          | (–)                        | (–)                        | (–)                        |
|                               | d-Mannose                  | –                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | Sucrose                    | –                          | –                          | +                          | –                          | ND                         | +                          | +                          | +                          | ND                         | (–)                        | +                          | (–)                        | (–)                        | (–)                        |
|                               | Maltose                    | –                          | –                          | +                          | –                          | ND                         | +                          | +                          | +                          | ND                         | (–)                        | +                          | (–)                        | (–)                        | (–)                        |
|                               | Pyruvate                   | –                          | –                          | +                          | –                          | –                          | –                          | –                          | –                          | ND                         | (–)                        | +                          | (–)                        | (–)                        | (–)                        |
| Acid production from:         | d-Glucose                  | –                          | –                          | –                          | –                          | –                          | +                          | –                          | +                          | +                          | (–)                        | +                          | +                          | +                          | +                          |
|                               | Lactose                    | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | d-Mannitol                 | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | d-Mannose                  | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
|                               | d-Xylose                   | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          | –                          |
| 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                      |

*– Cylindrical; e, elliptoidal 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> in a clad with the type strain of B. litoralis (see Supplementary Figs S1 and S2, available in IJSEM.)
Online). Comparative 16S rRNA gene sequence analysis showed that the isolate was most closely related to *B. herbersteinensis* D-1,5aT, *B. humi* LMG 22167T, *B. cohnii* DSM 6307T and *B. litoralis* SW-211T, 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 (Rossello-Mora & Amann, 2001; Stackebrandt et al., 2002).

Therefore, on the basis of physiological, biochemical and phylogenetic properties, strain BA288T 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-fructose, D-galactose, gentiobiose, glycogen, D-glucose, inositol, D-lactose, D-mannitol, D-mannose, melezitose, raffinose, D-sorbitol, salicin, D-tagatose, turanose, xylitol or D-xylene. 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-xylene are utilized. The cell-wall peptidoglycan contains meso-diaminopimelic acid (A1γ type). The predominant isoprenoid quinone is MK-7. The major fatty acids are anteiso-C15 : 0, iso-C15 : 0 and C16 : 0. The DNA G+C content of the type strain is 37.9 mol%.

The type strain, BA288T (=KCTC 3947T =DSM 16976T), was isolated from sandy soil near an abandoned mine in the Keumsan region of Korea.

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Bacillus alkalitelluris sp. nov.

References


