Geomicrobium halophilum gen. nov., sp. nov., a moderately halophilic and alkaliphilic bacterium isolated from soil

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Two moderately halophilic and alkaliphilic bacteria, designated strains BH1^T and HN5, were isolated from forest soil and garden soil, respectively, in Japan. Cells of strains BH1^T and HN5 were non-motile, aerobic, bean-shaped, formed irregular clusters with 2–20 cells, Gram-positive and contained A1γ, meso-diaminopimelic acid-type murein. Spore formation was not detected. Growth occurred in 5–25 % (w/v) NaCl (optimum, 10–15 %, w/v), at pH 6.0–10.0 (optimum, pH 8.5–9.0) and at 20–40 °C (optimum, 30 °C). The predominant isoprenoid quinones were menaquinone-7 and menaquinone-6. The phospholipids were diphosphatidylglycerol and phosphatidylglycerol. The major cellular fatty acids were i-C_{15:0}, i-C_{17:0} and i-C_{18:0}. The DNA G + C content of strains BH1^T and HN5 was 45 and 46 mol%, respectively. The 16S rRNA gene sequences of the two strains were 99.9 % similar. DNA–DNA hybridization results indicated high levels of relatedness (88 and 85 % reciprocally). Similarities with recognized species were less than 90.2 %. The phylogenetic and phenotypic characteristics indicated that strains BH1^T and HN5 represent a novel species in a new genus, for which the name *Geomicrobium halophilum* gen. nov., sp. nov. is proposed. The type strain is BH1^T (=JCM 15647^T=DSM 21769^T).

Halophilic micro-organisms require NaCl for growth, while alkaliphilic micro-organisms use Na⁺ for the adjustment of intracellular pH, solute transport and flagella rotation (Krulwich *et al.*, 1982). Thus, both depend on NaCl for growth. A number of haloalkaliphilic species that belong to the genus *Bacillus* and related genera, such as *Alkalibacillus*, *Gracilibacillus* and *Halobacillus*, have been isolated from various saline environments (Fritze, 1996; Jeon *et al.*, 2005; Romano *et al.*, 2005; Spring *et al.*, 1996; Wainø *et al.*, 1999). In recent years, however, some haloalkaliphilic strains such as *Alkalibacillus silvisoli* (Usami *et al.*, 2007), *Bacillus oshimensis* (Yumoto *et al.*, 2005) and *Halalkalibacillus halophilus* (Echigo *et al.*, 2007) have been isolated from ordinary field soils in Japan. In the

present study, we report on the isolation and characterization of moderately haloalkaliphilic strains BH1^T and HN5 from ordinary soils in Japan. We suggest that these strains represent a novel species in a new genus.

Soil samples were taken from a forest in Kawagoe City and a garden in Kawasaki City, Japan. The two sampling sites were about 50 and 5 km from the seashore, respectively. Soil samples were taken from near trees or plants. The pH values of the soil extracts were slightly acidic, pH 5.0–6.0. The isolation medium (JCM medium No. 611, with 20 % NaCl) contained the following (l⁻¹): 5.0 g Casamino acids (Difco), 5.0 g yeast extract (Difco), 1.0 g sodium glutamate.H₂O, 3.0 g trisodium citrate.2H₂O, 2.0 g KCl, 0.2 g MgSO₄.7H₂O, 36 mg FeCl₂.4H₂O, 200.0 g (3.4 M) NaCl and 20.0 g Bacto agar (Difco) (pH 7.2). After autoclaving, the pH was adjusted to 9.5 by adding precalculated amounts of sterile 10 % (w/v) Na₂CO₃ solution. About 0.5 g of the soil samples was placed on agar plates, spread with a spatula, and incubated at 37 °C for 3 weeks in a plastic bag to prevent desiccation. Colonies were picked up, transferred to fresh agar plates, and pure

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains BH1^T and HN5 are AB449106 and AB449107, respectively.

Scanning electron micrographs of strains BH1^T and HN5 and a maximum-likelihood tree showing the relationships of BH1^T, HN5 and other related strains are available with the online version of this paper.

cultures were obtained by plating serial dilutions and repeated transfers on agar plates. After partial sequencing (about 500 bp of the 5'-end) of PCR-amplified 16S rRNA genes of 29 isolates, two strains, BH1^T isolated from a forest soil in Toyo University, Kawagoe City, and strain HN5 isolated from a garden soil in Kawasaki City, were selected for further characterization. The 16S rRNA gene sequence similarity between strains BH1^T and HN5 (1479 bp) was 99.9%. In addition, DNA–DNA hybridization assessed by the fluorometric method of Ezaki *et al.* (1989) showed high levels of relatedness (88 and 85%, reciprocally), suggesting that these two strains should be classified within the same species.

Growth characteristics, production of acid from carbohydrates and sensitivity to antimicrobial agents were determined as described previously (Echigo *et al.*, 2007; Usami *et al.*, 2007). All phenotypic tests were carried out in JCM medium No. 611 with 10% NaCl and incubated at 37 °C. Cell morphology was studied using a phase-contrast microscope (ZEISS; Axiovert 135) and a scanning electron microscope (JEOL; JSM-7400F). Endospore formation by strains BH1^T and HN5 cultivated under various conditions [30 and 37 °C for 1, 2 and 3 days in/on liquid and agar medium (JCM No. 611) with 5, 10, 15, 20 and 25% (w/v) NaCl, pH 7.0, 8.0, 9.0, 9.5 and 10.0] was determined by spore-staining according to the method of Wirtz–Conklin (Murray *et al.*, 1999) or by heating cultures at 70, 80 or 90 °C for 30 min. Alkaliphilic, moderately halophilic bacteria *Alkalibacillus haloalkaliphilus* JCM 12303^T and *Halalkalibacillus halophilus* JCM 14192^T were used as positive control strains. Colonies of strains BH1^T and HN5 formed on agar plates were approximately 1.0–1.5 mm in diameter, brown, opaque and stained the surrounding agar brown. Cells of strain BH1^T and HN5 were non-motile, bean-shaped, approximately 0.5–0.7 × 1.0–1.2 µm and formed irregular clusters with 2–20 cells (Supplementary Fig. S1 in IJSEM Online). Both strains were Gram-positive. Spores were not observed by staining or by phase-contrast microscopy. After the heat treatment, only very few colonies (0–3 out of 2–3 × 10⁴ cells in 20 experiments) were obtained. Growth characteristics were determined under the following conditions with JCM liquid medium No. 611 as basal medium: NaCl concentrations of 0, 1, 2, 3, 4 and 5–30% (w/v) at 5% (w/v) intervals, pH 9.5, 37 °C; pH of 5.0–11.0 at 0.5 intervals, adjusted by 10% (w/v) H₂SO₄ solution or 10% (w/v) KOH solution, 10% (w/v) NaCl, 37 °C; and temperature of 10–70 °C at 10 °C intervals, 10% (w/v) NaCl, pH 9.5. Strains BH1^T and HN5 grew in 5–25% (w/v) NaCl, with optimum growth at 10–15% (w/v) NaCl. No growth was observed in media with 0–4% NaCl. Viable cell numbers did not decrease when cells grown in 10% NaCl medium were suspended in distilled water for 1 h at room temperature. Growth occurred within the pH range 6.0–10.0, with optimum growth at pH 8.5–9.0. The pH values of media adjusted to pH 6.0 and 10.0 were 6.1 and 6.3, and 9.7 and 9.4, at early-exponential and stationary

phases, respectively. The temperature range for growth was 20–40 °C, with optimum growth at 30 °C. Anaerobic growth was not observed in an anaerobic jar after incubation for 7 days at 37 °C. Sole carbon source utilization was tested in modified JCM liquid medium No. 611, without Casamino acids and yeast extract. Strains BH1^T and HN5 showed almost no growth in this medium. They utilized cellobiose, glycerol, lactose, D-mannitol, raffinose, trehalose, D-sorbitol and sucrose as sole carbon sources, but did not use L-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, ribose or D-xylose. Strain BH1^T produced acid from D-glucose, glycerol, D-sorbitol and sucrose, but not from L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, raffinose, ribose, trehalose or D-xylose. Strain HN5 produced acid from D-fructose, D-galactose, D-glucose, glycerol and sucrose, but not from L-arabinose, cellobiose, lactose, maltose, D-mannitol, D-mannose, raffinose, ribose, D-sorbitol, trehalose or D-xylose.

Tests for catalase, oxidase and decarboxylase activities, starch, pullulan, casein, gelatin, Tween 80, tributyrin, DNA, hippurate, aesculin, urea, tyrosine, xanthine and hypoxanthine hydrolysis, indole and H₂S production and reduction of nitrate were performed according to the standard or modified procedures of Smibert & Krieg (1981, 1994), Oren *et al.* (1997) and Schlesner *et al.* (2001). Strains BH1^T and HN5 were catalase-, oxidase- and ornithine decarboxylase-positive, but arginine decarboxylase- and lysine decarboxylase-negative. Casein and gelatin were hydrolysed, but starch, pullulan, Tween 80, tributyrin, DNA, hippurate, aesculin, urea, tyrosine, xanthine and hypoxanthine were not hydrolysed. Indole and H₂S were not produced from tryptophan or thiosulfate. Reduction of nitrate was not detected by using the sulfanilic acid and α-naphthylamine reagent, and formation of gas from nitrate was not detected using Durham tubes under anaerobic conditions.

HPLC analysis of isoprenoid quinones, GC/MS analysis of fatty acid methyl esters and TLC analysis of polar lipids were performed according to the modified procedures of Minnikin *et al.* (1984), Tamaoka (1986) and Komagata & Suzuki (1987). Strains BH1^T and HN5 were cultivated at 30 °C for 3 days in JCM liquid medium No. 611. The isoprenoid quinones of strains BH1^T and HN5 were menaquinone-7 (MK-7) and menaquinone-6 (MK-6) with ratios of 71:20 and 64:18, respectively. No other significant peaks were observed. The phospholipids of strains BH1^T and HN5 were diphosphatidylglycerol and phosphatidylglycerol. The cellular fatty acid profiles of strains BH1^T and HN5 were characterized by saturated branched fatty acids such as i-C_{15:0}, i-C_{17:0} and i-C_{18:0} (42:24:10 and 39:30:8, respectively).

Preparation of peptidoglycan and determination of its structure were performed according to the modified procedures of Schleifer & Kandler (1972), Schleifer (1985) and Schlesner *et al.* (2001) as described previously

(Echigo *et al.*, 2007; Usami *et al.*, 2007). The resulting fingerprints were compared with those from known peptidoglycan structures. Strains BH1^T and HN5 possessed A1 γ , *meso*-diaminopimelic acid-type murein.

Total DNA was extracted by the method of Saito & Miura (1963). The DNA G + C content of strains BH1^T and HN5, determined by the HPLC method of Tamaoka & Komagata (1984), was 45 and 46 mol%, respectively. The 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-AGAGTTTGATCCTGGCTCAG-3' (positions 8–27 according to *Escherichia coli* numbering) and 5'-GGCTACCTTGTACGACTT-3' (positions 1510–1492). The amplified DNA was cloned using the TA Cloning kit (Invitrogen) and sequenced using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing kits (Applied Biosystems) with the primers 5'-GGAAACAGC-TATGACCATG-3' (vector side), 5'-GACTACCAGGGT-ATCTAATC-3' (positions 805–786), 5'-AGGGTTGCG-CTCGTTG-3' (positions 1115–1100) and 5'-GTAAAC-GACGGCCAGT-3' (vector side) on the ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The 16S rRNA gene sequences of strains BH1^T and HN5 (1479 bp) were 99.9% similar, but showed very low similarities with those of any recognized species. The 16S rRNA gene sequence of strain BH1^T was most closely related to that of *Bacillus salarius* [90.2% by the FASTA system (Pearson & Lipman, 1988; Lipman & Pearson, 1985) and 89.9% by the EzTaxon server 2.0 (Chun *et al.*, 2007)], *Bacillus pseudofirmus* (90.0% and 90.3%), *Planomicrobium okeanoikoites* (89.3% and 90.1%), *Bacillus akibai* (89.3% and 89.9%), *Marinococcus halophilus* (88.9% by FASTA) and *Sinobaca qinghaiensis* (88.1% by FASTA). These values are sufficient to permit classification of these strains as a different genus (Ludwig *et al.*, 1998). The sequences of related strains retrieved from the DNA Data Bank of Japan (Miyazaki *et al.*, 2003) were aligned using CLUSTAL_X 2.0.9 (Larkin *et al.*, 2007). The length of the 16S rRNA gene sequences used in the phylogenetic analysis ranged from 1380 bp (*B. salarius*; AY667494) to 1553 bp (*Bacillus subtilis*; X60646). The neighbour-joining tree was reconstructed using the method of Saitou & Nei (1987), and was evaluated by bootstrap sampling methods (Felsenstein, 1985), based on 1000 replicates. Maximum-likelihood analyses were performed with RAxML 7.0.4 using the GTR + Γ model (Stamatakis *et al.*, 2005). Support values for the maximum-likelihood tree were obtained by bootstrapping (100 replicates) using CONSENSE in PHYLIP. The neighbour-joining tree (Fig. 1) and maximum-likelihood tree (Supplementary Fig. S2) showed that strains BH1^T and HN5 were closely related to *M. halophilus*, *B. salarius*, *S. qinghaiensis* and other *Bacillus* species but formed another distinctly separate cluster.

Phenotypic properties of strains BH1^T and HN5 supported the view that the isolates were distinguishable from the most closely related species, *M. halophilus* DSM 20408^T (Hao *et al.*, 1984), *B. salarius* DSM 16461^T (Lim *et al.*, 2006) and *S. qinghaiensis* DSM 17008^T (Li *et al.*, 2006) (Table 1). Cells of strains BH1^T and HN5 were bean-

shaped and formed irregular clusters, whereas *B. salarius* was single rod-shaped and *M. halophilus* and *S. qinghaiensis* were in single spherical form. The predominant isoprenoid quinones of strains BH1^T and HN5 were MK-7 and MK-6, whereas MK-7 was predominant in *B. salarius* and *M. halophilus*, and MK-5 in *S. qinghaiensis*. The major cellular fatty acid of strains BH1^T and HN5 was i-C_{15:0}, whereas the three most closely related species possess ai-C_{15:0}. The other selected characteristics that distinguish strains BH1^T and HN5 from the three most closely related species are shown in Table 1. The above phylogenetic and phenotypic characteristics indicated that strains BH1^T and HN5 represent a novel species in a new genus, for which the name *Geomicrobium halophilum* gen. nov., sp. nov. is proposed.

Description of *Geomicrobium* gen. nov.

Geomicrobium (Ge.o.mi.cro'bi.um. Gr. n. *ge* soil; N.L. neut. n. *microbium* a microbe; N.L. neut. n. *Geomicrobium* a microbe from soil).

Cells are non-motile, Gram-positive, non-spore-forming, bean-shaped, approximately 0.5–0.7 $\times$ 1.0–1.2 μ m and form irregular clusters with 2–20 cells. Cell walls contain A1 γ , *meso*-diaminopimelic acid-type murein. No growth occurs in media without NaCl. Alkaliphilic, mesophilic, aerobic and halophilic. Catalase- and oxidase-positive. The DNA G + C content is 45 mol%. The predominant isoprenoid quinones are MK-7 and MK-6. Major cellular fatty acids are i-C_{15:0}, i-C_{17:0} and i-C_{18:0}. The type species is *Geomicrobium halophilum*.

Description of *Geomicrobium halophilum* sp. nov.

Geomicrobium halophilum (ha.lo.phi'lum. Gr. n. *hals halos* salt; Gr. adj. *philos* loving; N.L. neut. adj. *halophilum* salt-loving).

Exhibits the following characteristics in addition to those given in the genus description. Colonies formed on agar are approximately 1.0–1.5 mm in diameter, brown, opaque and stained the surrounding agar brown. Spore formation is not detected. Growth occurs in 5–25% (w/v) NaCl, with maximum growth at 10–15% (w/v) NaCl. No growth occurs in the absence of NaCl, but cells do not die. Growth occurs at pH 6.0–10.0, with optimum growth at pH 8.5–9.0. Temperature range for growth is 20–40 °C, with optimum growth at 30 °C. Cellobiose, glycerol, lactose, D-mannitol, raffinose, trehalose, D-sorbitol and sucrose can be utilized as sole carbon sources; L-arabinose, D-fructose, D-galactose, D-glucose, maltose, D-mannose, ribose and D-xylose cannot be utilized. Acid is produced from D-glucose, glycerol, D-sorbitol and sucrose, but not from L-arabinose, cellobiose, D-fructose, D-galactose, lactose, maltose, D-mannitol, D-mannose, raffinose, ribose, trehalose or D-xylose. Sensitive to ampicillin (50 μ g per disc), bacitracin (25 μ g per disc), chloramphenicol (25 μ g per disc), erythromycin (25 μ g per disc), gentamicin (50 μ g per

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships of BH1^T, HN5 and other related strains. Bootstrap percentage is based on 1000 replicates; those greater than 60% are shown. *Brevibacillus brevis* JCM 2503^T (D78457) was used as an outgroup. Scale bar, 0.01 changes per nucleotide position.

Table 1. Differential characteristics of strain BH1^T, HN5 and related taxa

Taxa: 1, strains BH1^T/HN5; 2, *Bacillus salarius* DSM 16461^T; 3, *Marinococcus halophilus* DSM 20408^T; 4, *Sinobaca qinghaiensis* DSM 17008^T. Data from Hao *et al.* (1984), Li *et al.* (2006) and Lim *et al.* (2006). +, Positive; –, negative; ND, no data.

Characteristic	1	2	3	4
Colonial pigmentation	Brown	Cream	Yellow–orange	Orange
Cell morphology	Bean-shaped	Short rods	Cocci	Cocci
Spore formation	–	+	–	–
Motility	–	–	+	+
Acid production from:				
L-Arabinose	–	+	ND	ND
Cellobiose	–	ND	–	+
D-Fructose	–/+	+	+	–
Lactose	–	+	ND	ND
Maltose	–	+	–	+
D-Mannitol	–	+	–	+
D-Mannose	–	+	ND	ND
Ribose	–	+	ND	ND
Trehalose	–	+	+	+
D-Xylose	–	+	ND	ND
Oxidase test	+	+	–	–
Hydrolysis of:				
Starch	–	+	–	–
Gelatin	+	ND	+	–
Tween 80	–	+	ND	ND
Aesculin	–	+	+	ND
G + C content (mol%)	45/46	43	46.4	47.0
Isoprenoid quinone	MK-7, MK-6	MK-7	MK-7	MK-5
Major fatty acids	i-C _{15:0} , i-C _{17:0} , i-C _{18:0}	ai-C _{15:0} , ai-C _{17:0} , i-C _{15:0} , i-C _{16:0}	ai-C _{15:0} , ai-C _{17:0} , i-C _{16:0} , C _{16:0}	ai-C _{15:0} , ai-C _{17:0}

disc), novobiocin (25 µg per disc), penicillin G (25 µg per disc), rifampicin (50 µg per disc), streptomycin (100 µg per disc), tetracycline (50 µg per disc) and vancomycin (25 µg per disc); resistant to anisomycin (50 µg per disc), kanamycin (50 µg per disc), neomycin (25 µg per disc) and pravastatin (50 µg per disc).

Ornithine decarboxylase-positive, but arginine decarboxylase- and lysine decarboxylase-negative. Casein and gelatin are hydrolysed; starch, pullulan, Tween 80, tributyrin, DNA, hippurate, aesculin, urea, tyrosine, xanthine and hypoxanthine are not hydrolysed. Indole and H₂S are not produced. Reduction of nitrate and gas formation are not observed. The phospholipids are diphosphatidylglycerol and phosphatidylglycerol.

The type strain is strain BH1^T (=JCM 15647^T=DSM 21769^T), isolated from ordinary soil from a forest in a campus of Toyo University, Kawagoe City, Saitama Prefecture, Japan.

References

- Chun, J., Lee, J.-H., Jung, Y., Kim, M., Kim, S., Kim, B. K. & Lim, Y. W. (2007). EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57**, 2259–2261.
- Echigo, A., Fukushima, T., Mizuki, T., Kamekura, M. & Usami, R. (2007). *Halalkalibacillus halophilus* gen. nov., sp. nov., a new moderately halophilic and alkaliphilic bacterium isolated from a non-saline soil sample in Japan. *Int J Syst Evol Microbiol* **57**, 1081–1085.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fritze, D. (1996). *Bacillus haloalkaliphilus* sp. nov. *Int J Syst Bacteriol* **46**, 98–101.
- Hao, M. V., Kocur, M. & Komagata, K. (1984). *Marinococcus* gen. nov., a new genus for motile cocci with meso-diaminopimelic acid in the cell wall; and *Marinococcus albus* sp. nov. and *Marinococcus halophilus* (Novitsky and Kushner) comb. nov. *J Gen Appl Microbiol* **30**, 449–459.
- Jeon, C. O., Lim, J. M., Lee, J. M., Xu, L. H., Jiang, C. L. & Kim, C. J. (2005). Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **55**, 1891–1896.
- Komagata, K. & Suzuki, K. (1987). Lipid and cell-wall analysis in bacterial systematics. *Methods Microbiol* **19**, 161–207.
- Krulwich, T. A., Guffanti, A. A., Bornstein, R. F. & Hoffstein, J. (1982). A sodium requirement for growth, solute transport, and pH homeostasis in *Bacillus firmus* RAB. *J Biol Chem* **257**, 1885–1889.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A. & other authors (2007). CLUSTAL W and CLUSTAL_X version 2.0. *Bioinformatics* **23**, 2947–2948.
- Li, W. J., Zhang, Y. Q., Schumann, P., Tian, X. P., Zhang, Y. Q., Xu, L. H. & Jiang, C. L. (2006). *Sinococcus qinghaiensis* gen. nov., sp. nov., a novel member of the order Bacillales from a saline soil in China. *Int J Syst Evol Microbiol* **56**, 1189–1192.
- Lim, J. M., Jeon, C. O., Lee, S. M., Lee, J. C., Xu, L. H., Jiang, C. L. & Kim, C. J. (2006). *Bacillus salarius* sp. nov., a halophilic, spore-forming bacterium isolated from a salt lake in China. *Int J Syst Evol Microbiol* **56**, 373–377.
- Lipman, D. J. & Pearson, W. R. (1985). Rapid and sensitive protein similarity searches. *Science* **227**, 1435–1441.
- Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M. & Schleifer, K. H. (1998). Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.
- 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.
- Miyazaki, S., Sugawara, H., Gojobori, T. & Tatenos, Y. (2003). DNA Data Bank of Japan (DDBJ) in XML. *Nucleic Acids Res* **31**, 13–16.
- Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C. & Tenover, R. H. (1999). *Manual of Clinical Microbiology*, 7th edn. Washington DC: American Society for Microbiology.
- Oren, A., Ventosa, A. & Grant, W. D. (1997). Proposed minimal standards for description of new taxa in the order Halobacteriales. *Int J Syst Bacteriol* **47**, 233–238.
- Pearson, W. R. & Lipman, D. J. (1988). Improved tools for biological sequence comparison. *Proc Natl Acad Sci U S A* **85**, 2444–2448.
- Romano, I., Lama, L., Nicolaus, B., Gambacorta, A. & Giordano, A. (2005). *Alkalibacillus filiformis* sp. nov., isolated from a mineral pool in Campania, Italy. *Int J Syst Evol Microbiol* **55**, 2395–2399.
- Saito, H. & Miura, K. (1963). Preparation of transforming deoxyribonucleic acid by phenol treatment. *Biochim Biophys Acta* **72**, 619–629.
- Saitou, N. & Nei, M. (1987). The neighbour-joining methods: 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.
- Schlesner, H., Lawson, P. A., Collins, M. D., Weiss, N., Wehmeyer, U., Völker, H. & Thomm, M. (2001). *Filobacillus milensis* gen. nov., sp. nov., a new halophilic spore-forming bacterium with Orn-D-Glu-type peptidoglycan. *Int J Syst Evol Microbiol* **51**, 425–431.
- 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, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Spring, S., Ludwig, W., Marquez, M. C., Ventosa, A. & Schleifer, K. H. (1996). *Halobacillus* gen. nov., with description of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophila* comb. nov. *Int J Syst Bacteriol* **46**, 492–496.

- Stamatakis, A., Ludwig, T. & Meier, H. (2005).** RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics* **21**, 456–463.
- Tamaoka, J. (1986).** Analysis of bacterial menaquinone mixtures by reverse-phase high-performance liquid chromatography. *Methods Enzymol* **123**, 251–256.
- Tamaoka, J. & Komagata, K. (1984).** Determination of DNA base composition by reversed-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- Usami, R., Echigo, A., Fukushima, T., Mizuki, T., Yoshida, Y. & Kamekura, M. (2007).** *Alkalibacillus silvisoli* sp. nov., an alkaliphilic moderate halophile isolated from non-saline forest soil in Japan. *Int J Syst Evol Microbiol* **57**, 770–774.
- Wainø, M., Tindall, B. J., Schumann, P. & Ingvorsen, K. (1999).** *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., sp. nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int J Syst Bacteriol* **49**, 821–831.
- 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.