Alkalibacillus filiformis sp. nov., isolated from a mineral pool in Campania, Italy

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A halo-alkaliphilic, Gram-positive, non-motile bacterium, designated strain 4AG^T, was isolated from a mineral pool located in Malvizza, Campania, southern Italy. On the basis of 16S rRNA gene sequence analysis, strain 4AG^T was shown to belong to the genus *Alkalibacillus* within the phylum *Firmicutes*; its phylogenetic distance from recognized *Alkalibacillus* species was <95.0%. Chemotaxonomic data (MK-7 as the major menaquinone; directly cross-linked meso-diaminopimelic acid in the cell wall; phosphatidylglycerol and diphosphatidylglycerol as major polar lipids; iso-C_{15:0}, anteiso-C_{15:0} and iso-C_{17:0} as major fatty acids; and glycine betaine and glutamate as major compatible solutes) supported the affiliation of the strain to the genus *Alkalibacillus*. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain 4AG^T from the two recognized *Alkalibacillus* species. Strain 4AG^T therefore represents a novel species, for which the name *Alkalibacillus filiformis* sp. nov. is proposed. The type strain is 4AG^T (= DSM 15448^T = ATCC BAA-956^T).

Alkaliphilic micro-organisms, mainly those belonging to the genus *Bacillus*, are of considerable industrial interest, particularly for the production of enzymes (proteases, xylanases, glycosidases, etc.; Fritze, 1996; Nielsen *et al.*, 1995). Alkaliphilic micro-organisms are also often halophilic. There are a large number of recognized species of the genus *Bacillus* and related genera that could be described as moderately halophilic or halotolerant, some of which are also alkaliphilic (Fritze, 1996; Nielsen *et al.*, 1995; Romano *et al.*, 2005; Yumoto *et al.*, 2005). A number of approaches have been used to define this heterogeneous group taxonomically (Fritze, 1996, and references therein). Halophiles, slight halophiles, moderate halophiles, extreme halophiles halotolerants, alkaliphiles, alkalitolerants, etc., are classified in many genera (*Amphibacillus*, *Bacillus*, *Gracilibacillus*, *Halobacillus*, *Marinococcus*, *Filobacillus*, *Halomonas*, *Oceanobacillus* and *Virgibacillus*) (Arahal & Ventosa, 2002, and references therein). Jeon *et al.* (2005) proposed a novel genus, *Alkalibacillus*, based on a reclassification of [*Bacillus*] *haloalkaliphilus* as *Alkalibacillus haloalkaliphilus* and description of the novel species *Alkalibacillus salilacus*.

In this study, a new halo-alkaliphilic strain, designated 4AG^T, is characterized on the basis of a polyphasic approach. The strain was isolated from water samples collected from a mineral pool in Malvizza (Montecalvo Irpino, Avellino Province, Campania Region, southern Italy).

Strain 4AG^T was isolated by the dilution-plating technique, from water (pH 8.0, 18 °C) taken from a small, bubbling pool in the resort of Montecalvo Irpino. The enrichment medium (medium 1) contained the following components (g l⁻¹): Na₂CO₃, 3.0; KCl, 2.0; MgSO₄·7H₂O, 1.0; NaCl, 100; sodium citrate, 3.0; yeast extract, 10.0; MnCl₂·4H₂O, 0.36 × 10⁻³ and FeSO₄, 50 × 10⁻³. Na₂CO₃ and NaCl were autoclaved separately. The pH of medium 1 was 9.0. Strain 4AG^T was grown in medium containing 0–20% (w/v) NaCl. The temperature range for growth was determined by incubating the isolate at 5–55 °C in medium 1. The pH dependence of growth was tested in the pH range 6.5–11.0 in medium 1 without Na₂CO₃. Growth on single carbon sources (medium 2) was tested on liquid media containing (g l⁻¹): K₂HPO₄, 7.0; KH₂PO₄, 2.0; MgSO₄·7H₂O, 0.1; (NH₄)₂SO₄, 1.0; NaCl, 100 and Na₂CO₃, 3.0; plus 500 µl l⁻¹ of a thiamine hydrochloride solution (100 mg l⁻¹) and the tested compounds (10.0 g l⁻¹). Growth under anaerobic conditions at pH 7.5 and 9.0 was carried out in medium 1 in an anaerobic chamber for 5 days. In liquid medium, growth was followed by measuring OD₅₄₀ and was scored as positive at values >0.250. Solid media were prepared by the

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Abbreviations: DPG, diphosphatidylglycerol; meso-Dpm, meso-diaminopimelic acid; PG, phosphatidylglycerol.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 4AG^T is AJ493661.

addition of 1.8% agar (w/v). *A. haloalkaliphilus* was grown according to the method described by Fritze (1996).

Cell morphology and spore formation were determined by using phase-contrast microscopy (Zeiss). Colony morphology was analysed on solid medium by using a Leica M8 stereomicroscope. All growth tests were performed at optimal growth temperatures for 3 days. Sensitivity of the strains to antibiotics was tested on medium 1 supplemented with 1.8% (w/v) agar and Sensi-Discs (6 mm; Oxoid); incubation was for 48 h. The antibiotic tests were carried out according to the method of Romano *et al.* (1993). Casein and gelatin hydrolysis, oxidase, tyrosinase, L-aminopeptidase (Bactident-Merck) and catalase reactivities were tested in medium 1. Nitrate reduction was tested in medium 1 supplemented with 0.1% (w/v) KNO₃ (Jeon *et al.*, 2005). Hydrolysis of hippurate was tested in medium 1 supplemented with 1% (w/v) hippurate (Romano *et al.*, 1996). Gram staining was performed according to the method of Dussault (1955). The KOH test was performed according to the method of Halebian *et al.* (1981). Hydrolysis of *N*'-benzoyl arginine-*p*-nitroaniline stereoisomers was tested by using the method of Oren & Galinski (1994). Extraction of intracellular solutes and their purification and quantification were conducted according to the methods of Romano *et al.* (2001).

Cell mass for quinone and lipid analysis was obtained from culture in medium 1 at 30 °C, pH 9.0 and at optimal NaCl concentration for growth. Lipid analysis was performed as reported by Romano *et al.* (2001). Lipid hydrolysis and identification of the fatty acid methyl esters were performed as reported by Romano *et al.* (2001). Quinones were analysed by liquid chromatography/mass spectroscopy (LC/MS) on a reversed-phase column, by electron ionization/mass spectroscopy (EI/MS) and ¹H NMR spectra. Analyses of peptidoglycan structure and cell-wall sugars were carried out at the DSMZ according to the methods described by Rhuland *et al.* (1955), Schleifer (1985), Schleifer & Kandler (1972) and Stanek & Roberts (1974). Total hydrolysis was carried out by using 4 M HCl at 100 °C for 16 h and partial hydrolysis by using 4 M HCl at 100 °C for 45 min.

The DNA G + C content was determined by the method of Tamaoka & Komagata (1984); DNA was hydrolysed and the resultant nucleotides were analysed by HPLC (Mesbah *et al.*, 1989).

DNA was isolated as described by Romano *et al.* (2003), and DNA–DNA hybridization was performed at the DSMZ with *A. haloalkaliphilus* WN13^T. Hybridization was carried out spectroscopically as described by De Ley *et al.* (1970) with the modifications described by Huss *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program described by Jahnke (1992).

The total 16S rRNA gene sequence was determined by direct sequencing of the PCR-amplified 16S rRNA gene. Genomic DNA extraction, PCR-mediated amplification of the 16S

rRNA gene and purification of the PCR products were carried out as described previously (Romano *et al.*, 2003). Purified PCR products were sequenced by using an ABI Prism Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems). Sequence reactions were electrophoresed by using an Applied Biosystems 373A DNA sequencer. For multiple sequence alignment, the program CLUSTAL W (Chenna *et al.*, 2003) was used. 16S rRNA gene sequences for comparison were obtained from GenBank/EMBL or the RPD database. A phylogenetic tree based on 16S rRNA gene sequence comparisons (Fig. 1) was inferred by using the neighbour-joining method (Saitou & Nei, 1987) contained in the PHYLIP package, version 3.6 (Felsenstein, 2004). Evolutionary-distance matrices for the neighbour-joining method were calculated with the algorithm of Jukes & Cantor (1969) in the program DNADIST. Bootstrap analysis was performed with 1000 resamplings using SEQBOOT. A majority-rule (50%) consensus tree was constructed for the topologies found by using CONSENSE (PHYLIP).

Cells of strain 4AG^T were Gram-positive, non-motile, sporulating rods; although the KOH test indicated that the strain could be a Gram-negative bacterium, the aminopeptidase test and Gram staining demonstrated clearly that the isolate was a Gram-positive bacterium, as also reported for *A. haloalkaliphilus* and for *Filobacillus milosensis* (Fritze, 1996; Schlesner *et al.*, 2001). Colonies of strain 4AG^T on enrichment medium 1 were usually white to transparent. Colonies were smooth, convex and regularly circular.

Fig. 1. Unrooted phylogenetic tree based on 16S rRNA gene sequence comparison, showing the relationship of strain 4AG^T to related taxa. Numbers at nodes indicate bootstrap values (percentages derived from 1000 samples). Bar, 0.01 K_{nuc}.

The isolate required aerobic conditions for growth and grew in medium 1 containing 0–18 % NaCl, optimally at 10 %. Isolate 4AG^T grew over a temperature range of 15–45 °C, optimally at 30 °C. The optimum pH for growth was 9.0 (pH range for growth was 7–10).

Strain 4AG^T accumulated glycine betaine and glutamate under optimal growth conditions. It was able to grow in medium 2 containing glucose. Other carbon sources did not sustain growth. It was sensitive to vancomycin (30 µg), ampicillin (25 µg), novobiocin (30 µg), fusidic acid (10 µg), chloramphenicol (10 µg), erythromycin (5 and 30 µg), penicillin G (2 and 10 iu) and bacitracin (10 µg).

Strain 4AG^T possessed complex lipids based on fatty acids. The total lipid content accounted for 9–11 % of the total dry weight of cells grown at the optimal growth conditions in medium 1 (pH 9.0, 10 % NaCl, 30 °C). Under these conditions, diphosphatidylglycerol (DPG) and phosphatidylglycerol (PG) were the main lipids (about 96 %), as identified by ¹H and ¹³C NMR spectra. Chemical-shift values of signals present in these spectra were identical to those reported by Romano *et al.* (2001), with the exception of the absence of the signals due to double bonds in the fatty acid chains. Assignments were performed by comparison with commercial standards and by DEPT (distortionless enhancement by polarization transfer) experiments. Glycolipids were not detected. The fatty acid composition determined on cells grown under standard growth conditions was characterized by the abundance of branched, saturated acyl chains.

LC/MS and EI/MS analyses of the quinone content of strain 4AG^T (about 0.1 % of the total dry weight of cells) indicated two characteristic major peaks corresponding to MK-7 and demethylmenaquinone with six repeating fully saturated isoprene units (DeMK-6), with a relative ratio of 7:3, respectively. The total hydrolysate of the peptidoglycan preparation contained the amino acids *meso*-diaminopimelic acid (*meso*-Dpm), alanine and glutamic acid. The partial hydrolysate of the peptidoglycan contained the peptides L-Ala–D-Glu and *meso*-Dpm–D-Ala. These data indicate that the peptidoglycan type of strain 4AG^T is A1_γ (*meso*-Dpm directly cross-linked) (DSMZ, 2001). Xylose was detected (trace amounts) as the only cell-wall sugar.

Strain 4AG^T showed a high 16S rRNA gene sequence similarity (98.0 %) to *A. haloalkaliphilus* (Fritze, 1996), and the phylogenetic distance from any other recognized species within the genera *Bacillus*, *Filobacillus*, *Halobacillus*, *Virgibacillus*, *Marinococcus* and *Oceanobacillus* and from *A. salilacus* was <95.0 %.

The G+C content of the DNA of strain 4AG^T was 39.5 mol%. DNA–DNA hybridization between strain 4AG^T and DNA from *A. haloalkaliphilus* DSM 5271^T (WN13^T) indicated levels of DNA–DNA relatedness of 49.2 %. The assignment of strain 4AG^T to a novel species, distinct from *A. haloalkaliphilus*, is clearly supported by both

a low degree (49 %) of DNA–DNA relatedness (Rossellò-Mora & Amman, 2001; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002; Wayne *et al.*, 1987) and by phenotypic differences (Table 1) from the two other recognized *Alkalibacillus* species (Fritze, 1996; Jeon *et al.*, 2005).

Strain 4AG^T can be placed within a coherent group of halophilic or halotolerant taxa of Gram-positive bacteria that do not belong to the genus *Bacillus* (Fig. 1), but comprise the genera *Halobacillus*, *Filobacillus*, *Marinococcus*, *Gracilibacillus*, *Oceanobacillus*, *Virgibacillus*, *Amphibacillus* and *Alkalibacillus* (Arahal *et al.*, 2000; Arahal & Ventosa, 2002; Hao *et al.*, 1984; Heyrman *et al.*, 2003; Jeon *et al.*, 2005; Lu *et al.*, 2001; Schlesner *et al.*, 2001; Spring *et al.*, 1996; Wainø *et al.*, 1999; Yoon *et al.*, 2004; Zhilina *et al.*, 2001, and references therein).

Characteristics differentiating strain 4AG^T from other *Alkalibacillus* species are summarized in Table 1. Cells of strain 4AG^T were non-motile and the strain was less halophilic compared with related *Alkalibacillus* species. Strain 4AG^T and *A. haloalkaliphilus* were able to hydrolyse gelatin, but did not reduce nitrate, in contrast to *A. salilacus*. Strain 4AG^T and *A. haloalkaliphilus* were more alkaliphilic than *A. salilacus*. Strain 4AG^T was the only strain tested that was oxidase-negative.

The quinone content of strain 4AG^T and its closest relatives differed at the level of the minor components. The fatty acid profile of the three species was characterized by a large percentage of branched saturated fatty acids. In *A. haloalkaliphilus*, iso-C_{15:0} was most abundant, whereas in strain 4AG^T iso-C_{17:0} and in *A. salilacus*, anteiso-C_{15:0} was present at a similar level to iso-C_{15:0}.

Strain 4AG^T formed a cluster with other *Alkalibacillus* species and represents a novel species, for which the name *Alkalibacillus filiformis* sp. nov. is proposed.

Description of *Alkalibacillus filiformis* sp. nov.

Alkalibacillus filiformis (fili.for'mis. L. neut. n. *filum* a thread; L. suff. *-formis* -like, of the shape of; N.L. masc. adj. *filiformis* thread-shaped).

Aerobic, alkalitolerant and halotolerant micro-organism of the phylum *Firmicutes*. Cells are Gram-positive, long, sporulating rods, 0.25–0.30 µm in width and 9.0–11.0 µm long. Spores are round-terminal. Colonies are white to transparent. Mesophilic, exhibiting optimum growth at 30 °C, but is able to grow between 15 and 45 °C and at pH 7.0–10.0 (optimal pH, 9.0). Tolerates up to 18 % NaCl [optimum growth at 10 % (w/v)]. Growth occurs in the absence of salt. Able to grow on glucose as sole carbon source. Gelatinase-positive, but negative for oxidase, casein, tyrosine, starch, hippurate and phenylalanine hydrolysis, and nitrate reduction. Catalase reaction is weak. Possesses α-glucosidase activity. Menaquinones found are MK-7 (70 %) and DeMK-6 (30 %) and the predominant polar lipids are PG and DPG. Iso-C_{15:0}, anteiso-C_{15:0}, iso-C_{16:0}, C_{16:0},

Table 1. Differential properties of strain 4AG^T and related *Alkalibacillus* species

Data are taken from Fritze (1996), Jeon *et al.* (2005) and the present study. Abbreviations: ND, no data; +, positive; –, negative; W, weak.

Characteristic	Strain 4AG ^T	<i>A. haloalkaliphilus</i> WN13 ^T (= DSM 5271 ^T)	<i>A. salilacus</i> DSM 16460 ^T
Cell morphology	Long rods	Long rods	Rods
Colony colour	White, transparent	Creamy white/light yellowish, depending on growth conditions	Cream
Motility	–	+	+
Oxidase	–	+	+
Catalase	W	+	+
Hydrolysis of:			
Gelatin	+	+	–
Casein	–	W	–
Starch	–	W	–
Hippurate	–	+	–
Nitrate reduction	–	–	+
NaCl for growth (%):			
Minimum	0	>0	5
Maximum	18	25	20
Optimum	10	5	10–12
pH range	7–10	>7 to >9.7	7.0–9.0
Optimal pH	9.0	9.5	8.0
DNA G+C content (mol%)	39.5	38.0	41
Quinone composition	MK-7 (70 %), DeMK-6 fully saturated (30 %)	MK-7	MK-7
Major fatty acids	Iso-C _{15:0} , anteiso-C _{17:0}	Iso-C _{15:0}	Iso-C _{15:0} , anteiso-C _{15:0}
Osmolytes	Glycine betaine (86 %), glutamate (14 %)*	Different solutes*	ND

*Strain 4AG^T synthesizes mainly ectoine in mineral medium at 10 %. Glycine betaine in the medium was taken up and accumulated.

iso-C_{17:0}, anteiso-C_{17:0} are the main cellular fatty acids. The cell wall is type A1γ (*meso*-Dpm directly cross-linked). Accumulates glycine betaine as a major component and glutamate as minor osmoprotectant. The DNA G+C content is 39.5 mol%. It is related phylogenetically to *Alkalibacillus haloalkaliphilus* on the basis of 16S rRNA gene sequence analysis, but shares only 49.2 % DNA–DNA relatedness with this species. Susceptible to the following antibiotics: chloramphenicol (10 µg), erythromycin (5 and 30 µg), penicillin G (2 and 10 iu), bacitracin (10 µg), vancomycin (30 µg), ampicillin (25 µg), fusidic acid (10 µg) and novobiocin (30 µg), but resistant to streptomycin (25 µg), tetracycline (30 and 50 µg), kanamycin (30 µg), neomycin (30 µg) and gentamicin (30 µg).

The type strain, 4AG^T (= DSM 15448^T = ATCC BAA-956^T), was isolated from water of a small mineral pool with gas bubbles at the Malvizza site (Montecalvo Irpino, Campania Region, Italy).

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References

- Arahal, D. R. & Ventosa, A. (2002). Moderately halophilic and halotolerant species of *Bacillus* and related genera. In *Applications and Systematics of Bacillus and Relatives*, pp. 83–99. Edited by R. C. W. Berkeley, M. Heyndrickx, N. Logan & P. de Vos. Oxford: Blackwell.
- Arahal, D. R., Márquez, M. C., Volcani, B. E., Schleifer, K. H. & Ventosa, A. (2000). Reclassification of *Bacillus marismortui* as *Salicibacillus marismortui* comb. nov. *Int J Syst Evol Microbiol* **50**, 1501–1503.
- Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. & Thompson, J. D. (2003). Multiple sequence alignment with the Clustal series of programs. *Nucleic Acids Res* **31**, 3497–3500.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). The quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- DSMZ (2001). *Catalogue of Strains*, 7th edn. Braunschweig: DSMZ. <http://www.dsmz.de/species/murein.htm>
- Dussault, H. P. (1955). An improved technique for staining red halophilic bacteria. *J Bacteriol* **70**, 484–485.

- Escara, J. F. & Hutton, J. R. (1980). Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. *Biopolymers* **19**, 1315–1327.
- Felsenstein, J. (2004). PHYLIP (Phylogeny Inference Package), version 3.6. Seattle: University of Washington.
- Fritze, D. (1996). *Bacillus haloalkaliphilus* sp. nov. *Int J Syst Bacteriol* **46**, 98–101.
- Haleblian, S., Harris, B., Finegold, S. M. & Rolfe, R. D. (1981). Rapid method that aids in distinguishing gram-positive from gram-negative anaerobic bacteria. *J Clin Microbiol* **13**, 444–448.
- 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.
- Heyrman, J., Logan, N. A., Busse, H.-J., Balcaen, A., Lebbe, L., Rodriguez-Diaz, M., Swings, J. & De Vos, P. (2003). *Virgibacillus carmonensis* sp. nov., *Virgibacillus necropolis* sp. nov. and *Virgibacillus picturae* sp. nov., three novel species isolated from deteriorated mural paintings, transfer of the species of the genus *Salibacillus* to *Virgibacillus*, as *Virgibacillus marismortui* comb. nov. and *Virgibacillus salexigens* comb. nov., and emended description of the genus *Virgibacillus*. *Int J Syst Evol Microbiol* **53**, 501–511.
- Huss, V. A. R., Festl, H. & Schleifer, K.-H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Jahnke, K.-D. (1992). Basic computer program for evaluation of spectroscopic DNA renaturation data from GILFORD system 2600 spectrometer on a PC/XT/AT type personal computer. *J Microbiol Methods* **15**, 61–73.
- 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.
- Jukes, T. H. & Cantor, C. R. (1969). Evolution of protein molecules. In *Mammalian Protein Metabolism*, pp. 21–132. Edited by H. N. Munro. New York: Academic Press.
- Lu, J., Nogi, Y. & Takami, H. (2001). *Oceanobacillus iheyensis* gen. nov. sp. nov., a deep-sea extremely halotolerant and alkaliphilic species isolated from a depth of 1050 m on the Iheya Ridge. *FEMS Microbiol Lett* **205**, 291–297.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Nielsen, P., Fritze, D. & Priest, F. G. (1995). Phenetic diversity of alkaliphilic *Bacillus* strains: proposal for nine new species. *Microbiology* **141**, 1745–1761.
- Oren, A. & Galinski, E. A. (1994). Hydrolysis of N'-benzoyl-arginine-p-nitroanilide stereoisomers as a phenotypic test: a study of Gram-positive halotolerant bacteria. *Syst Appl Microbiol* **17**, 7–10.
- 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.
- Romano, I., Manca, M. C., Lama, L., Nicolaus, B. & Gambacorta, A. (1993). Method for antibiotic assay on *Sulfolobales*. *Biotechnol Tech* **7**, 439–440.
- Romano, I., Nicolaus, B., Lama, L., Manca, M. C. & Gambacorta, A. (1996). Characterization of a haloalkaliphilic strictly aerobic bacterium, isolated from Pantelleria Island. *Syst Appl Microbiol* **19**, 326–333.
- Romano, I., Nicolaus, B., Lama, L., Trabasso, D., Caracciolo, G. & Gambacorta, A. (2001). Accumulation of osmoprotectants and lipid pattern modulation in response to growth conditions by *Halomonas pantelleriense*. *Syst Appl Microbiol* **24**, 342–352.
- Romano, I., Giordano, A., Lama, L., Nicolaus, B. & Gambacorta, A. (2003). *Planococcus rifletensis* sp. nov., isolated from algal mat collected from a sulfurous spring in Campania (Italy). *Syst Appl Microbiol* **26**, 357–366.
- Romano, I., Lama, L., Nicolaus, B., Gambacorta, A. & Giordano, A. (2005). *Bacillus saliphilus* sp. nov., isolated from a mineral pool in Campania, Italy. *Int J Syst Evol Microbiol* **55**, 159–163.
- Rossellò-Mora, R. & Amman, 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.
- Schlesner, H., Lawson, P. A., Collins, M. C., 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.
- 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 halophilus* comb. nov. *Int J Syst Bacteriol* **46**, 492–496.
- 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.
- Stackebrandt, E., Frederiksen, W., Garrity, G. M. & 10 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.
- Staneck, J. L. & Roberts, G. D. (1974). Simplified approach to identification of aerobic actinomycetes by thin-layer chromatography. *Appl Microbiol* **28**, 226–231.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.
- 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 Evol Microbiol* **49**, 821–831.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 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.
- Yoon, H.-J., Oh, T.-K. & Park, Y.-H. (2004). Transfer of *Bacillus halo-denitrificans* Denariáz et al. 1989 to the genus *Virgibacillus* as *Virgibacillus halo-denitrificans* comb. nov. *Int J Syst Evol Microbiol* **54**, 2163–2167.
- 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.
- Zhilina, T. N., Garnova, E. S., Tourova, T. P., Kostrikina, N. A. & Zavarzin, G. A. (2001). *Amphibacillus fermentum* sp. nov. and *Amphibacillus tropicus* sp. nov., new alkaliphilic, facultatively anaerobic, saccharolytic bacilli from Lake Magadi. *Mikrobiologiya* **70**, 825–837 (in Russian).