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Marinospirillum alkaliphilum sp. nov., a new alkaliphilic helical bacterium from Haoji soda lake in Inner Mongolia Autonomous Region of China

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Abstract A new helical, alkaliphilic, gram-negative, chemoorganotrophic bacterium designated strain Z4^T was isolated from Haoji soda lake in Inner Mongolia Autonomous Region, China. The isolate grows at salinities between 0.2% and 5.0% (w/v) NaCl and pH range 7.0–11.0, with an optimum at 2.0% (w/v) NaCl and pH 9.5. Its growth temperature ranges from 8° to 49°C with an optimum at 37°C. The G+C content of the DNA is 46.8 mol%. The major isoprenoid quinone is ubiquinone 8 (Q-8). Phylogenetic analyses based on 16S rDNA sequence comparison indicates that strain Z4^T is a member of the genus *Marinospirillum*. Phenotypic features and DNA–DNA homology of less than 20% with the described species of *Marinospirillum* support the view that strain Z4^T represents a new species of the genus *Marinospirillum*. Strain Z4^T (= AS 1.2746) is proposed as the type strain of a new species, named *Marinospirillum alkaliphilum* sp. nov.

Key words *Marinospirillum alkaliphilum* sp. nov. · Helical bacteria · DNA–DNA hybridization · Phylogeny · Soda lake · Alkaliphile

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Introduction

Helical, moderately halophilic chemoorganotrophic and aerobic bacteria are widely distributed in the ocean. Several species of this group of bacteria have been isolated from decaying seaweed and coastal seawater (Holt et al. 1994). Previously, these spirilla were assigned to the genus *Oceanospirillum* on the basis of phenotypic characteristics (Krieg 1984). However, considerable interspecies diversity of the genus *Oceanospirillum* was also indicated, based on rRNA–DNA hybridization experiments (Pot et al. 1989), polyamine composition (Hamana et al. 1994), fatty acid analysis, and isoprenoid quinone analysis (Sakane and Yokota 1994). Recently, 16S rDNA sequence comparisons have been carried out on representatives of the genus (Satomi et al. 1998). Phylogenetic analysis indicated that the genus *Oceanospirillum* was clearly a heterogeneous group, and a new genus, *Marinospirillum*, was proposed to accommodate *Oceanospirillum minutulum* from the marine environment and the other two isolates from Kusaya gravy, a traditional Japanese fermented brine (Satomi et al. 1998). Two validly described species are currently named in the genus *Marinospirillum*: *Marinospirillum minutulum* and *Marinospirillum megaterium*, including strains SP5 and H7^T.

Soda lakes are among the most productive naturally occurring aquatic environments on earth and harbor dense populations of chemoorganotrophic bacteria (Jones et al. 1994; Duckworth et al. 1996). In our investigations of microbial biodiversity in Haoji soda lake in Inner Mongolia Autonomous Region of China, a large number of aerobic chemoorganotrophic bacteria were isolated, including rods, cocci, and helical bacteria (Zhang et al. 2001). The majority of the isolates had a requirement for high pH levels. Phylogenetic analysis of about 20 of the isolates based on 16S rDNA sequence comparison indicated that gram-negative alkaliphiles from Haoji soda lake were associated with members of the genera *Pseudomonas*, *Halomonas*, *Cyclobacterium*, and *Marinospirillum*. In this article, we report in detail the properties of one of the soda lake isolates and show that it represents a new species, for which we propose the name *Marinospirillum alkaliphilum* sp. nov.

Materials and methods

Bacterial strains and culture conditions

Strain Z4^T was isolated from a water sample (pH 9.5) of Haoji soda lake (48°23′ N and 118°28′ E) located in Inner Mongolia Autonomous Region, China. Samples were originally collected in sterile vials and brought back to the laboratory. The isolate is deposited in the China General Microbiological Culture Collection Center as AS1.2746. *Marinospirillum minutulum* ATCC 19193^T and *Marinospirillum megaterium* JCM 10129^T were used as reference strains in this study.

Enrichment and isolation procedures were performed according to Horikoshi (1971). Strain Z4^T was grown aerobically at 37°C in a complex medium having the following composition (in g l^{-1}): glucose, 10; polypeptone, 5; yeast extract, 5; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2CO_3, 10. When required, the medium was solidified with 20 g agar l^{-1}.

Phenotypic characteristics

Cellular morphology was examined by phase-contrast microscopy without fixation and by electron microscopy. To obtain the photographs for scanning electron microscopy and transmission electron microscopy, cells were fixed with acetic acid as described by Dussault (1955). The Gram reaction was determined by the KOH lysis method of Gregersen (1978).

The methods used for physiological studies were described previously (Gerhardt et al. 1981; Ventosa et al. 1982; Quesada et al. 1984). Hydrolysis of urea was tested according to Spanka and Fritze (1993). Accumulation of poly-β hydroxybutyrate (PHB) was determined by the Sudan black staining method (Burdon 1946). Unless otherwise indicated, the tests were carried out in media with 1.0% Na_2CO_3 at pH 9.6 and incubated at 37°C. Growth was monitored by turbidity at OD_{600}.

Isoprenoid quinone composition

Isoprenoid quinones were extracted and purified from freeze-dried cells using the method of Stackebrandt et al. (1995), and examined by high performance liquid chromatography (HPLC) methods with apparatus (model 510; Waters, Milford, MA, USA) equipped with a Zorbax ODS HPLC column (HP, Palo Alto, CA, USA). Dry cells were extracted in acetone at 20°C for 16 h. Acetone-soluble extracts of whole cells were separated by one-dimensional thin-layer chromatography (TLC) on silica gel plates (Kieselgel $60F_{254}$; Merck, Darmstadt, Germany) and analyzed by reverse-phase HPLC.

DNA studies

Chromosomal DNA was extracted and purified by standard methods (Sambrook et al. 1989). Cells were suspended in TE buffer (pH 8.0) and treated with sodium dodecyl sulfate (SDS) (1.0% final concentration) for lysis.

The G+C content of the DNA was determined by the thermal denaturation method (Marmur and Doty 1962). DNA–DNA hybridization was carried out as described by Tindall et al. (1984) with a minor modification: DNA fragments were labeled with [α-^{32}P] dATP using the nick translation kit (Boehringer, Mannheim, Germany).

16S rDNA sequence determination and phylogenetic analysis

Polymerase chain reaction (PCR) amplification of the 16S rDNA and subsequent direct sequencing of the amplified PCR fragments were performed as described previously (Duckworth et al. 1996) with 27F and 1541R (5′-AAGG AGGTGATCCAGGCCGCA) for eubacteria.

The raw sequence dataset including the nearly complete sequence (1,437 bases) was aligned using Clustal W version 1.8 (Thompson et al. 1994). The phylogenetic analysis for multiple sequence alignments was performed with the Treecon W version 1.2 (Van de Peer and de Wachter 1994). The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) with the Kimura two-parameter calculation model in Treecon W version 1.2. The 16S rRNA gene sequence of strain Z4^T has been deposited in the GenBank database under accession number AF275713. The designations and 16S rRNA sequence accession numbers of the reference strains used in the phylogenetic analysis are shown in Fig. 2.

Results and discussion

Phenotypic characteristics

The new strain designated strain Z4^T was isolated from Haoji soda lake in Inner Mongolia Autonomous Region, China. It is strictly aerobic, gram negative, helical, and motile. The helix type was counterclockwise. Motility was by means of large bipolar tufts of flagella, but bipolar monoflagella and twin flagella were also observed by transmission electron microscopy (Fig. 1). Endospores were not detected. Coccoid bodies were formed after 6 days culture on agar plate. Under optimum conditions, cells were 0.2–0.3 μm in diameter and 2.7–4.0 μm in length. Circular, smooth, opalescent colonies 0.5 mm in diameter were formed after overnight culture at 37°C.

The new isolate required Na^+ for growth. No growth occurred without added NaCl in the medium when Na_2CO_3 was substituted by KOH. Growth occurred in media with NaCl concentrations of 0.2%–5.0%, with optimum growth at 2.0% NaCl. The strain grew at temperatures of 8.0°–49°C, with the optimum temperature at 37°C. The pH range for growth is 7.0–11.0, with the optimum at pH 9.5.

Strain Z4^T was positive for the tests of oxidase, catalase, urease, and nitrate reduction, but negative for methyl red

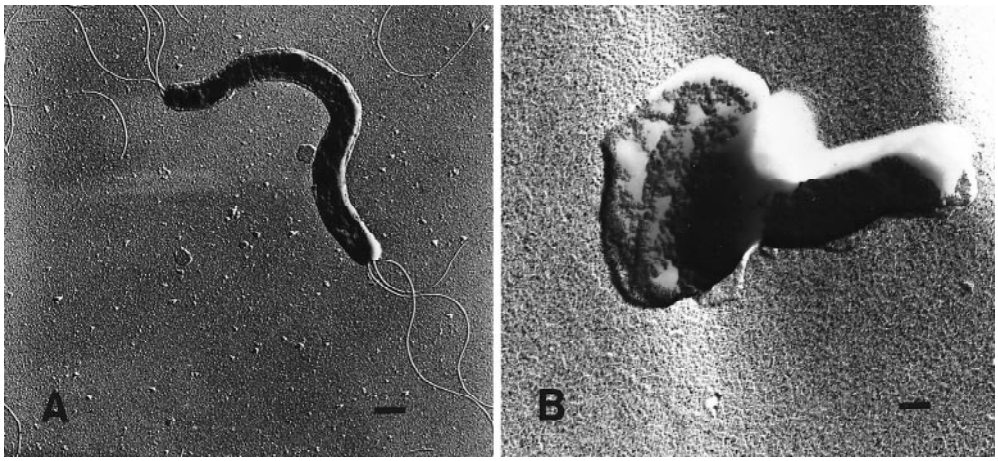

Fig. 1A,B. Transmission electron photomicrographs of strain Z4^T. **A** Vegetative cell with bipolar tufts of flagella. *Bar* 0.3 μm. **B** Coccoid body after 6 days of culture. *Bar* 0.05 μm

Table 1. Comparison of characteristics of members of the genus *Marinospirillum*

Characteristic	Strain Z4^T	*Marinospirillum minutulum* ATCC 19193^T	*Marinospirillum megaterium* JCM 10129^T
Cell length (μm)	2.7–4.0	2–2.8	5–15
Cell diameter (μm)	0.2–0.3	0.3–0.4	0.8–1.2
Colony forming	+	+	–
Oxygen requirement	Aerobic	Aerobic	Microaerophilic
Catalase	+	+	– or W
Reduction of nitrate	+	+	–
Temperature range for growth (°C)	8.0–49	11–37	4–25
Optimum temperature (°C)	37	30	20–25
Range of NaCl for growth (%)	0.2–5.0	0.2–8.0	0.9–9.0
Range of pH for growth	7.0–11.0	7.0–10.5	7.5–9.0
Optimum pH	9.5	9.0	8.0
Urease	+	–	–
Isoprenoid quinone	Q8 and MK-6	Q8	Q-8 and MK
DNA G+C (mol%)	46.8	42.5	44–45

All species were gram-negative spirals that and did not form spores. They were motile by means of a polar flagellum. All strains accumulated poly-β hydroxybutyrate (PHB) and were oxidase positive. The cell shape (helix type) was counterclockwise and the major isoprenoid quinone type was Q-8. None of the strains produced acid from carbohydrates (including sugars glucose, fructose, sucrose, galactose, maltose, trehalose, cellobiose, D-mannitol, arabinose, rhamnose, xylose, lactose, melibiose, raffinose, adonitol, sorbitol, inositol). Pigment, indole, and H_2S were not produced. Casein, gelatin, starch, or Tween 20, 60, and 80 were not hydrolyzed
MK-6, a small amount of MK-6; MK, below the level of detection; W, weak reaction; –, no reaction

test, Voges–Proskauer (V-P; acetyl methyl methane) test, and esterase activity. Intracellular PHB was formed. Carbohydrates were not catabolized. Pigment, indole, and H_2S were not produced. Casein, gelatin, starch, and Tween 20, 60, and 80 were not hydrolyzed.

Biochemical and physiological characteristics useful for identifying and differentiating strain Z4^T, *M. minutulum* ATCC 19193^T, and *M. megaterium* JCM 10129^T are shown in Table 1.

Chemotaxonomic and genotypic characteristics

The isoprenoid quinone composition of strain Z4^T was determined. The major quinone in strain Z4^T was Q-8, which is characteristic of the genus *Marinospirillum*. In addition, a detectable amount of menaquinone with six isoprene units (MK-6) was also present in strain Z4^T. The DNA G+C content of strain Z4^T was 46.8 mol%. These data were compatible with the assignment of strain Z4^T to the genus *Marinospirillum*.

Phylogenetic analysis and DNA–DNA homology

The almost complete 16S rDNA sequence (1,437 bp) of strain Z4^T was determined. The alignments of this sequence with sequences available from the Gene Bank, EMBL, and DDBJ databases showed that the closest relatives to strain Z4^T were *M. minutulum* ATCC 19193^T and *M. megaterium* JCM 10129^T, with sequence similarities of 96% and 95%, respectively. The level of 16S rDNA sequence similarities between strain Z4^T and other gram-negative spirilla was lower than 90%. The phylogenetic tree (Fig. 2) clearly indicates that strain Z4^T and the other known species of genus *Marinospirillum* are grouped into the same lineage, and this

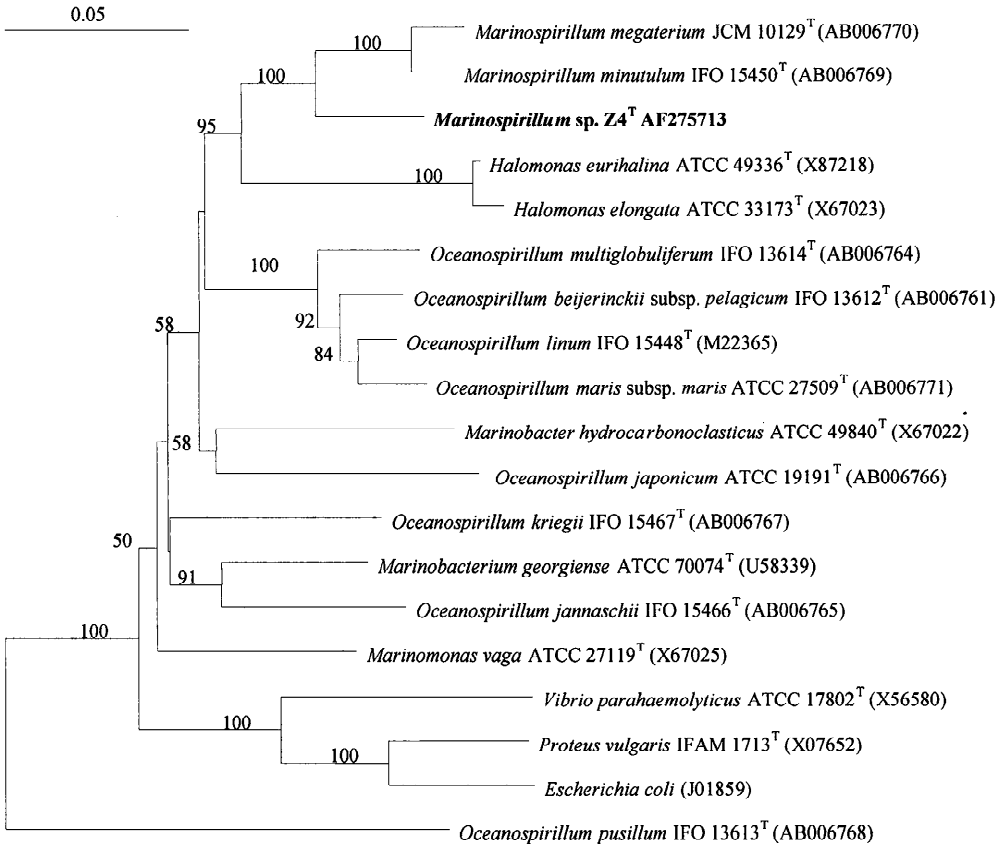

Fig. 2. Phylogenetic tree of strain Z4T and other related species based on their 16S rDNA sequences. Evolutionary distances were calculated by the neighbor-joining method and Kimura two-parameter calculation model. The *numbers* represent the confidence level from 100 replicate bootstrap samplings. *Bar* 0.1 substitutions per nucleotide

branch received 100% bootstrap support. Based on both sequence dissimilarity values (>4%) and phylogenetic relationships with known species, the isolate does not belong to any other previously described species and likely represents a new *Marinospirillum* species (Collins et al. 1999).

DNA–DNA homology was studied to confirm the species status of strain Z4T. The level of DNA–DNA homology was 12.1% between strain Z4T and *M. minutulum* ATCC 19193T and 16.7% between strain Z4T and *M. megaterium* JCM 10129T. According to Wayne et al. (1987), less than 70% DNA–DNA homology is considered to be the threshold value for the delineation of genospecies, and therefore the values were sufficiently low to separate strain Z4T from the two other species of *Marinospirillum*.

According to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994), all helical, moderately halophilic, chemoorganotrophic, and aerobic bacteria belong to the genus *Oceanospirillum*. Based on rRNA–DNA hybridization experiments, Pot et al. (1989) reported that the taxonomic relationships of species of this genus were heterogeneous. The genus *Marinospirillum* was subsequently proposed as a new genus based on the phylogenetic analysis of 16S rRNA and other characteristics (Satomi et al. 1998). To date, the genus *Marinospirillum* consists of two valid species: *M. minutulum* ATCC 19193T and *M. megaterium* JCM 10129T (Satomi et al. 1998). Our phylogenetic analysis demonstrates that strain Z4T is most closely related to members of the genus *Marinospirillum*. Furthermore, strain Z4T shares several phenotypic properties with the species of the genus *Marinospirillum*: e.g., gram-negative, rigidly helical, non-spore-forming, motile, slightly halophilic, aerobic, oxidase-positive, PHB-accumulating, unable to catabolize carbohydrates, genomic DNA G+C content of 46.8 mol%, and isoprenoid quinone type Q-8. These genotypic and phenotypic characteristics indicate that the new isolate is a member of the genus *Marinospirillum*.

Strain Z4T can be differentiated from any currently validly published species of this genus on the basis of several phenotypic characteristics (see Table 1). The low DNA–DNA homology value between strain Z4T and other *Marinospirillum* species also confirmed the new species status of strain Z4T. Therefore, we propose strain Z4T as a new species, *Marinospirillum alkaliphilum*.

Although *Marinospirillum* species have been isolated previously from the ocean environment and artificial brines (Satomi et al. 1998), strain Z4T is the first isolate of the genus *Marinospirillum* from a soda lake.

Description of *Marinospirillum alkaliphilum* sp. nov.

Marinospirillum alkaliphilum (Arabic n. *al-qây*, saltwort; French n. alcali, alkali; M.L. n. alcali, alkali; Gr. adj. philus, loving; M.L. masc. adj. alkaliphilum, liking alkaline [media]).

Gram-negative, rigidly helical, non-spore-producing, aerobic, chemoorganotrophic and PHB-accumulating.

Cells are 0.2–0.3 μm in diameter and 2.7–4.0 μm in length. Motility by bipolar flagella. Coccoid bodies are observed after 6 days of culture. NaCl is required for growth, and growth occurs at NaCl concentrations of 0.2%–5.0% (w/v), with an optimum of 2.0% NaCl. The temperature range for growth is 8.0°–49.2°C, with an optimum at 37.9°C. The pH range for growth is 7.0–11.0, with an optimum at pH 9.5. Oxidase and catalase tests are positive. Carbohydrates are not catabolized. Starch, gelatin, and Tween 20, 60, and 80 are not hydrolyzed. Nitrate is reduced. The G+C content of the DNA is 46.8 mol% (determined by the thermal denaturation method). The predominant isoprenoid quinone type is Q-8 and a small amount of MK-6 is also detected. Isolated from Haoji soda lake of China. The type strain is Z4^T, deposited as AS 1.2746 in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

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References

Burdon KL (1946) Fatty material in bacteria and fungi revealed by staining dried fixed slide preparations. J Bacteriol 52:665–678

Collins MD, Bernard KA, Hutson RA, Sjoden B, Nyberg A, Falsen E (1999) *Corynebacterium sundsvallense* sp. nov., from human clinical specimens. Int J Syst Bacteriol 49:361–366

Duckworth AW, Grant WD, Jones BE, Steenbergen RV (1996) Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol Ecol 19:181–191

Dussault RR (1955) An improved technique for staining red halophilic bacteria. J Bacteriol 70:484–485

Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Philips GB (1981) Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC

Gregersen T (1978) Rapid method for distinction of gram-negative from gram-positive bacteria. Eur J Appl Microbiol Biotechnol 5:123–127

Hamana K, Sakane T, Yokota A (1994) Polyamine analysis of the genera *Aquaspirillum*, *Magnetospirillum*, *Oceanospirillum*, and *Spirillum*. J Gen Appl Microbiol 40:75–82

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. Williams and Wilkins, Baltimore

Horikoshi K (1971) Production of alkaline enzymes by alkalophilic microorganisms. Part 1. Alkaline protease produced by *Bacillus* no. 221. Agric Biol Chem 36:1407–1414

Jones BE, Grant WD, Collins NC, Mwatha WE (1994) Alkaliphiles: diversity and identification. In: Priest FG, Ramos-Cormenzana A, Tindall BJ (eds) Bacterial diversity and systematics. Plenum, New York, pp 195–229

Krieg NR (1984) Genus *Oceanospirillum*. In: Krieg NR, Holt JG (eds) Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore, pp 104–110

Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118

Pot B, Gillis M, Hoste B, Van De Velde A, Bekaert F, Kersters K, De Ley J (1989) Intra- and intergenetic relationships of the genus *Oceanospirillum*. Int J Syst Bacteriol 39:23–34

Quesada E, Ventosa A, Ruiz-Berraquero F, Ramos-Cormenzana A (1984) *Deleya halophila*, a new species of moderately halophilic bacteria. Int J Syst Bacteriol 50:1297–1303

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

Sakane T, Yokata A (1994) Chemotaxonomic investigation of heterotrophic, aerobic and microaerophilic spirilla, the genera *Aquaspirillum*, *Magnetospirillum*, and *Oceanospirillum*. Syst Appl Microbiol 17:128–134

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor, New York

Satomi M, Kimura B, Hayashi M, Shouzen Y, Okuzumi M, Fujii T (1998) *Marinospirillum* gen. nov., with descriptions of *Marinospirillum megaterium* sp. nov., isolated from kusaya gravy, and transfer of *Oceanospirillum minutulum* to *Marinospirillum minutulum* comb. nov. Int J Syst Bacteriol 48:1341–1348

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, Koch C, Schumann P (1995) Taxonomic dissection of the genus *Micrococcus.* Int J Syst Bacteriol 45:682–692

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Tindall BJ, Ross HNM, Grant WD (1984) *Natronobacterium* gen. nov. and *Natronococcus* gen. nov., two new genera of haloalkaliphilic archaebacteria. Syst Appl Microbiol 5:41–57

Van de Peer Y, de Wachter R (1994) TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Appl Biosci 10:569–570

Ventosa A, Quesada E, Rodriguez-Valera F, Ruiz-Berraquero F, Ramos-Cormenzana A (1982) Numerical taxonomy of moderately halophilic gram-negative rods. J Gen Microbiol 128:1959–1968

Wayne LG, Brenner DJ, Colwell RR, Grimont PAD, Kandler O, Krichevsky MI, Moore LH, Moore WEC, Murray RGE, Stackebrandt E, Starr MP, Truper HG (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

Zhang W, Mao W, Xue Y, Ma Y, Zhou P (2001) Biodiversity of alkaliphilic bacteria in Hailaer soda lakes, Inner Mongolia Autonomous Region of China. Biodivers Sci 9:44–50