Marinospirillum insulare sp. nov., a novel halophilic helical bacterium isolated from kusaya gravy

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A novel species that belongs to the genus *Marinospirillum* is described on the basis of phenotypic characteristics, phylogenetic analysis of 16S rRNA and *gyrB* gene sequences and DNA–DNA hybridization. Four strains of helical, halophilic, Gram-negative, heterotrophic bacteria were isolated from kusaya gravy, which is fermented brine that is used for the production of traditional dried fish in the Izu Islands of Japan. All of the new isolates were motile by means of bipolar tuft flagella, of small cell size, coccoid-body-forming and aerophilic; it was concluded that they belong to the same bacterial species, based on DNA–DNA hybridization values (> 70 % DNA relatedness). DNA G + C contents of the new strains were 42–43 mol% and they had isoprenoid quinone Q-8 as the major component. Phylogenetic analysis of 16S rRNA gene sequences indicated that the new isolates were members of the genus *Marinospirillum*; sequence similarity of the new isolates to *Marinospirillum minutulum*, *Marinospirillum megaterium* and *Marinospirillum alkaliphilum* was 98.5, 98.2 and 95.2 %, respectively. Phylogenetic analysis based on the *gyrB* gene indicated that the new isolates had enough phylogenetic distance from *M. minutulum* and *M. megaterium* to be regarded as different species, with 84.7 and 78.7 % sequence similarity, respectively. DNA–DNA hybridization showed that the new isolates had < 36 % DNA relatedness to *M. minutulum* and *M. megaterium*, supporting the phylogenetic conclusion. Thus, a novel species is proposed: *Marinospirillum insulare* sp. nov. (type strain, K^T = LMG 21802^T = NBRC 100033^T).

Members of the genus *Marinospirillum*, which was established in 1998 (Satomi *et al.*, 1998), have been isolated from putrid shellfish (Terasaki, 1972), kusaya gravy (Satomi *et al.*, 1998) and an alkaline soda lake in China (Zhang *et al.*, 2002); three species have so far been reported in this genus (*Marinospirillum minutulum*, *Marinospirillum megaterium* and *Marinospirillum alkaliphilum*). All species that belong to this genus are helical, halophilic, Gram-negative, heterotrophic and motile by means of a single polar flagellum or bipolar flagellar tuft and belong to the γ -subclass of the *Proteobacteria*. Almost all of their characteristics are similar to those of the genus *Oceanospirillum* (Hylemon *et al.*, 1973; Krieg, 1984; Holt *et al.*, 1994; Satomi *et al.*, 2002), indicating

that it is difficult to distinguish between these genera solely on the basis of phenotype.

In previous investigations of the functions and diversity of micro-organisms in a traditional fermented brine called kusaya gravy (Fujii, 1977, 1978; Fujii *et al.*, 1985, 1993; Satomi *et al.*, 1997b), which is used for producing Japanese traditional dried fish, halophilic and large helical bacteria have been isolated (Satomi *et al.*, 1998). Also, small helical bacteria that were similar to *M. minutulum* were isolated occasionally by using agar media (Fujii, 1978; Kosuge *et al.*, 1978), but their biological and taxonomic features have not been investigated. In order to determine their taxonomic position, phenotypic, chemotaxonomic and phylogenetic analyses were performed.

Bacterial strains and growth conditions

Bacterial strains tested in this study are shown in Table 1. Four new strains (K^T, P2, O5 and NB11) were isolated from kusaya gravy that was manufactured on different islands or at different times. Strain K^T was isolated from kusaya gravy that was used on Niiijima Island in 1988, strains P2 and O5 were isolated from kusaya gravy of Oshima Island in 1976 and strain NB11 was isolated from kusaya gravy of Shikine Island in 1980. Isolation and purification procedures were described previously (Fujii *et al.*, 1990); they were

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Abbreviation: PHB, poly- β -hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences generated in this study are AB098510–AB098513 and that for the *gyrB* sequence of strain K^T is AB098514.

Tables showing differential characteristics and DNA–DNA hybridization values of *Marinospirillum* species and a phylogenetic tree based on the *gyrB* gene are available as supplementary material in IJSEM Online.

Table 1. Strains investigated in this study and their isolation source

ATCC, American Type Culture Collection, Manassas, VA, USA; JCM, Japan Collection of Micro-organisms, Saitama, Japan; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium; NBRC, National Institute of Technology and Evaluation, Biological Resource Center, Japan.

Species and strain	Source, place and year of isolation
<i>Marinospirillum insulare</i> :	
K ^T = LMG 21802 ^T = NBRC 100033 ^T	Kusaya gravy, Niijima Island, 1988
P2 = LMG 21803 = NBRC 100036	Kusaya gravy, Oshima Island, 1976
O5 = LMG 21804 = NBRC 100035	Kusaya gravy, Oshima Island, 1976
NB11 = LMG 21805 = NBRC 100034	Kusaya gravy, Shikine Island, 1980
<i>Marinospirillum minutulum</i> :	
ATCC 19193 ^T = NBRC 15450 ^T	Marine shellfish, Hiroshima, 1959
NB10	Kusaya gravy, Shikine Island, 1980
<i>Marinospirillum megaterium</i> JCM 10129 ^T	Kusaya gravy, Niijima Island, 1988

performed by using TSSY semi-solid agar, which contained the following ingredients: 1.7 % trypticase peptone (BBL), 0.3 % phytone peptone (BBL), 0.1 % yeast nitrogen base (Difco), 3.0 % NaCl and 0.2 % agar. All strains have been deposited in the BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium (LMG) and the National Institute of Technology and Evaluation, Biological Resource Center, Japan (NBRC) as *Marinospirillum insulare* (K^T = LMG 21802^T = NBRC 100033^T, P2 = LMG 21803 = NBRC 100036, O5 = LMG 21804 = NBRC 100035 and NB11 = LMG 21805 = NBRC 100034). They were maintained on a stab culture in semi-solid marine broth (MB; Difco) that contained 0.2 % agar. The pH was adjusted to 8.0 with 1.0 M NaOH. The culture was incubated for 3 days at 25 °C in semi-solid MB. To obtain a large amount of cells, cells were cultured in TSSY broth or MB at 100 r.p.m. on a rotary shaker.

In taxonomic tests, *M. minutulum* ATCC 19193^T, *M. minutulum* NB10 and *M. megaterium* JCM 10129^T were used as reference strains.

Morphology and growth characteristics

Cellular morphology and motility of the new isolates were observed by using phase-contrast microscopy. Their helix type was determined according to *Bergey's Manual of Systematic Bacteriology* (Krieg, 1984). Gram-staining was performed by using the modified Hucker method (Smibert & Krieg, 1994). Accumulation of poly-β-hydroxybutyrate (PHB) was determined by the Sudan black staining method (Smibert & Krieg, 1994). The new isolates were Gram-negative, rigidly helical, PHB-accumulating and motile by means of bipolar tuft flagella. Thin-wall coccoid body formation was positive after 7 days culture in TSSY, but no spores were produced. Cell size under optimum conditions was 0.1–0.2 µm in diameter and 2.5–7.5 µm in length and the thin-wall coccoid body was 2.0–2.5 µm in diameter. Colonies were entire, smooth, opaque, ivory, non-luminescent

and about 1 mm in diameter on TSSY agar plates or marine agar 2216 (MA; Difco) plates that were incubated at 25 °C for 6 days. The concentration range of NaCl that supported growth was determined in non-salt TSSY medium supplemented with 0, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 7.5 or 10.0 % (w/v) NaCl for 3 weeks. All new isolates were able to grow at 0.5–10 % NaCl, but not at 0%; optimal concentration for growth was 2–3 %. Temperature tolerance was tested at 0, 4, 10, 15, 20, 25, 30, 37 and 40 °C in TSSY medium for 3 weeks. All strains were able to grow at 4–37 °C; optimum growth occurred at 25–30 °C. Ability to grow at pH 6.0–11.0 was tested at 0.5-unit increments in TSSY medium with the pH adjusted with 0.1 M NaOH. All cultures grew in a pH range of 6.5–10.0, with optimum growth at pH 7.5–8.0. Oxygen requirement for growth was determined by comparison of growth on TSSY plates in an anaerobic jar and under aerobic conditions. All strains tested required aerobic conditions for growth.

Phenotypic characteristics

Routine biochemical tests were carried out by using API kits (API 20NE, API ZYM and API 50CH; bioMérieux) that were prepared according to the instruction manual, except that cells were suspended in saline solution with 2.5 % NaCl as the final concentration. Further tests for utilization as the sole carbon source (mainly amino acids) were performed by adding energy sources (0.1 % final concentration) to the basal medium (Baumann *et al.*, 1972), which was solidified with noble agar (Difco). Observation of utilization tests was continued for 3 weeks. Haemolytic activity was determined on trypticase soy agar (BBL) supplemented with 2.5 % NaCl and 5 % defibrinated sheep blood. Casein hydrolysis, production of DNase, RNase, lipase (hydrolysis of Tweens 40 or 80) and phenylalanine deaminase, hippurate hydrolysis and O/129 antibacterial susceptibility were tested as described by Smibert & Krieg (1994). Chitin and alginate hydrolysis was tested as described by West & Colwell (1984).

All new isolates showed key morphological and physiological characteristics of the genus *Marinospirillum*, i.e. they were Gram-negative, halophilic and helical, formed coccoid bodies, were motile by means of bipolar tuft flagella, accumulated PHB, showed a positive reaction for oxidase and did not produce acid from glucose. This indicated that all new isolates were members of the genus *Marinospirillum*. The new isolates were able to grow at $>30^{\circ}\text{C}$ and in growth media supplemented with 10% NaCl, reduced nitrate and had a small cell size, suggesting that they were similar to *M. minutulum*. The other two species of this genus have significantly different phenotypic characteristics: *M. alkaliphilum* is able to grow at 49°C , is positive for urease and fails to grow in $>5\%$ NaCl, whilst *M. megaterium* has a large cell size, is microaerophilic, is not able to grow at $>30^{\circ}\text{C}$ and does not reduce nitrate. Details of the phenotypic characteristics for the new isolates are given in Supplementary Table A in IJSEM Online.

Isoprenoid quinone and fatty acid compositions

Whole-cell lipids were extracted by using the method of Bligh & Dyer (1959). Isoprenoid quinone type and isoprenoid length were analysed as described by Akagawa-Matsushita *et al.* (1992). Total acetone-soluble extracts of whole-cell lipids were separated by one-dimensional chromatography on cellulose thin layers (Merck), with benzene as the eluent. Isoprenoid length was analysed by HPLC, using a reverse-phase column (Cosmosil C18; Nacalai Tesque). All new isolates contained ubiquinone that consisted mainly of Q-8. Menaquinone and methyl-menaquinone were not detected. Other *Marinospirillum* species also have Q-8 as the major isoprenoid quinone (Satomi *et al.*, 1998; Zhang *et al.*, 2002). For analysis of fatty acid composition, whole-cell lipids were converted to fatty acid methyl esters by the methods given by the American Oil Chemist's Society (1990). The fatty acid methyl esters were analysed by GLC with a model GC15A chromatograph (Shimadzu) equipped with a polar capillary column (Hi-cap CBP20; Shimadzu). GLC operating conditions were described previously (Satomi *et al.*, 1997a). The major fatty acids of all new isolates were $\text{C}_{16:0}$, $\text{C}_{16:1}$ and $\text{C}_{18:1}(n-7)$; this is a similar composition to that of *M. minutulum*.

DNA preparation and DNA base composition

Cells were collected by centrifugation from TSSY broth, suspended in TE (Tris/EDTA) buffer (pH 8.0) and treated with SDS (final concentration, 0.5%) for lysis. Chromosomal DNA was purified by standard methods (Sambrook *et al.*, 1989). DNA base composition (G+C content) was determined by the HPLC method of Tamaoka & Komagata (1984). The G+C contents of new isolates K^T, P2, NB11 and O5 were 42.1, 42.4, 42.1 and 42.5 mol%, respectively.

Phylogenetic analysis

16S rDNA and *gyrB* were amplified via PCR with universal primer sets that were described by Weisburg *et al.* (1991) and Yamamoto & Harayama (1995), respectively, and sequenced as described previously (Satomi *et al.*, 1997a). The GenBank/DDBJ accession numbers of the 16S rRNA gene sequences generated in this study are AB098510–AB098513 and the *gyrB* gene sequence of strain K^T is AB098514. The 1.4 kbp nucleotide sequences of the 16S rRNA genes [covering base positions 66–1448 (*Escherichia coli* numbering)] and 1.1 kbp nucleotide sequence of the *gyrB* gene [covering base positions 316–1472 (*E. coli* numbering)] were used for phylogenetic analysis. The 16S rDNA sequences of the new isolates were compared with all sequence data maintained in GenBank/EMBL/DDBJ by using the BLAST algorithm (Altschul *et al.*, 1990). Multiple alignment, calculation of nucleotide substitution rates (K_{nuc} values) as described by Kimura (1980) and construction of phylogenetic trees by the neighbour-joining method (Saitou & Nei, 1987) were performed by using the CLUSTAL W program (Thompson *et al.*, 1994). Alignment gaps, primer regions for PCR amplification and unidentified base positions were not taken into consideration for the calculations. The robustness of the topology of the phylogenetic trees was evaluated by bootstrap analysis over 1000 replications. 16S rRNA gene sequence similarity among the new isolates was $>99.8\%$ (2 bases difference) and three of the strains, K^T, P2 and NB11, had identical sequences. The phylogenetic tree that was based on the 16S rRNA gene (Fig. 1) indicated that the new isolates clustered with members of the genus *Marinospirillum*; the closest species to the new isolates were *M. minutulum* and *M. megaterium*, with 98.0 and 98.2% sequence similarity, respectively. 16S rRNA gene sequence similarity between the new isolate and *M. alkaliphilum*, which has recently been reported as a novel species of this genus, was 95.3%. Generally, the threshold of sequence similarity to separate species based on the 16S rRNA gene is 97% (Stackebrandt & Goebel, 1994), suggesting that the small phylogenetic distances of the new isolates from *M. minutulum* and *M. megaterium* do not warrant their separation as different species. On the other hand, phylogenetic analysis based on the *gyrB* gene showed clearly that they were separated as three different species with significant sequence diversity, which was 16.3 and 22.3% between the new isolates and *M. minutulum* and *M. megaterium*, respectively (see Supplementary Figure in IJSEM Online). However, threshold values to separate species based on *gyrB* sequence diversity are known to vary, depending on the genus. For instance, the threshold value of the genus *Oceanospirillum*, based on *gyrB* sequence diversity, was concluded to be about 20% (Satomi *et al.*, 2002), whereas the value of the genus *Shewanella* was defined as 10% (Venkateswaran *et al.*, 1999; Satomi *et al.*, 2003). Therefore, it is necessary to confirm that the new isolates are a novel species by using genetic analysis.

Fig. 1. Phylogenetic tree of the genus *Marinospirillum* based on nucleotide sequences of the 16S rRNA gene. The tree was constructed by using the neighbour-joining method and genetic distances were computed by using Kimura's model. Numbers at nodes indicate percentages of occurrence in 1000 bootstrapped trees. *Terasakiella pusilla* was the outgroup. GenBank accession numbers are given in parentheses; the sequence determined in this study is shown in bold type. Bar, 0.01 genetic distance.

DNA–DNA hybridization

In order to confirm the phylogenetic conclusion, DNA–DNA homology was studied by using microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection, as described previously (Satomi *et al.*, 1997a). A DNA–DNA hybridization experiment revealed that the four new isolates were closely related amongst themselves (71–82 % DNA relatedness), suggesting that they belong to the same species, but they exhibited relatively low levels of hybridization with *M. minutulum* (31 %) and *M. megaterium* (36 %) (see Supplementary Table B in IJSEM Online). This indicated strongly that the new isolates are a novel species of the genus *Marinospirillum*, according to the criteria for differentiation of bacterial species (Wayne *et al.*, 1987). Genetic analysis clearly divided them as different species, although the new isolates had similar characteristics to *M. minutulum*.

On the basis of polyphasic studies, the four new strains from kusaya gravy were classified as a novel bacterial species of the genus *Marinospirillum*, *Marinospirillum insulare* sp. nov.

Description of *Marinospirillum insulare* sp. nov.

Marinospirillum insulare (in.su.la're. L. neut. adj. *insulare* of or belonging to an island, insular).

Gram-negative, rigidly helical, halophilic, non-spore-producing, coccoid-body-forming, aerobic, chemoheterotrophic and PHB-accumulating bacteria. Motile by means of bipolar tufts of flagella. Cells are 0.1–0.2 µm in diameter and 2.5–7.5 µm in length. NaCl is required for growth; growth occurs at NaCl concentrations of 0.5–10 % (w/v) and is optimal at 2–3 % NaCl. Temperature range for growth is 4–37 °C; optimal temperature is 25–30 °C. pH range for growth is 6.5–10.0; optimal pH is 8.0. Colonies are entire, smooth, opaque, ivory, non-luminescent and about 1 mm in diameter on marine agar 2216 (Difco) plates when incubated at 25 °C for 6 days. Oxidase, catalase, alkaline

phosphatase and esterase are positive. Nitrate is reduced. DNase, RNase, acid phosphatase, urease, *N*-acetylglucosaminase and β-galactosidase are not produced. Carbohydrates are not catabolized. Gelatin, hippurate and starch are not hydrolysed. Tolerance of 1 % glycine and Oxgall is positive. PHB, valerate and D-alanine are utilized as energy sources. Isoprenoid quinone type is Q-8. Whole-cell fatty acids consist mainly of C_{16:0}, C_{16:1} and C_{18:1}(*n*-7). DNA G + C content is 42–43 mol%.

The type strain is K^T=LMG 21802^T=NBRC 100033^T; isolated from kusaya gravy in Japan.

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References

- Akagawa-Matsushita, M., Ito, T., Katayama, Y., Kuraishi, H. & Yamasato, K. (1992). Isoprenoid quinone composition of some marine *Alteromonas*, *Marinomonas*, *Deleya*, *Pseudomonas* and *Shewanella* species. *J Gen Microbiol* **138**, 2275–2281.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- American Oil Chemist's Society (1990). *Official Methods and Recommended Practices*, 4th edn. Chicago: American Oil Chemist's Society.
- Baumann, L., Baumann, P., Mandel, M. & Allen, R. D. (1972). Taxonomy of aerobic marine eubacteria. *J Bacteriol* **110**, 402–429.
- Bligh, E. G. & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**, 911–917.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in microdilution 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.

Fujii, T. (1977). Studies on 'Kusaya'-I. Comparison of composition of 'Kusaya' brine at Nijima and Oshima islands. *Nippon Suisan Gakkaishi* **43**, 517–521.

Fujii, T. (1978). Microbial flora of 'Kusaya' brine of Nijima and Oshima islands. *Nippon Suisan Gakkaishi* **44**, 45–48.

Fujii, T., Sugita, H. & Deguchi, Y. (1985). Obligately anaerobic bacteria isolated from 'Kusaya' gravy. *Nippon Suisan Gakkaishi* **51**, 473–478.

Fujii, T., Hayashi, M. & Okuzumi, M. (1990). New device for isolation of spiral bacteria from 'Kusaya' gravy. *Nippon Suisan Gakkaishi* **56**, 1661.

Fujii, T., Kuda, T. & Okuzumi, M. (1993). Anaerobic microflora of 'Kusaya' gravy. *Nippon Suisan Gakkaishi* **59**, 185.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (editors) (1994). *Bergey's Manual of Determinative Bacteriology*, 9th edn. Baltimore: Williams & Wilkins.

Hylemon, P. B., Wells, J. S., Jr, Krieg, N. R. & Jannasch, H. W. (1973). The genus *Spirillum*: a taxonomic study. *Int J Syst Bacteriol* **23**, 340–380.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* **16**, 111–120.

Kosuge, T., Yokota, M., Ohba, H. & Nagano, H. (1978). Study on kusaya-I. Antiseptic effects of kusaya brine. *Nippon Suisan Gakkaishi* **44**, 1141–1146.

Krieg, N. R. (1984). Aerobic/microaerophilic, motile, helical/vibrioid Gram-negative bacteria. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 71–93. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

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

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Satomi, M., Kimura, B., Mizoi, M., Sato, T. & Fujii, T. (1997a). *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. *Int J Syst Bacteriol* **47**, 832–836.

Satomi, M., Kimura, B., Takahashi, G. & Fujii, T. (1997b). Microbial diversity of kusaya gravy. *Fish Sci (Tokyo)* **63**, 1019–1023.

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.

Satomi, M., Kimura, B., Hamada, T., Harayama, S. & Fujii, T. (2002). Phylogenetic study of the genus *Oceanospirillum* based on 16S rRNA

and *gyrB* genes: emended description of the genus *Oceanospirillum*, description of *Pseudospirillum* gen. nov., *Oceanobacter* gen. nov. and *Terasakiella* gen. nov. and transfer of *Oceanospirillum jannaschii* and *Pseudomonas stanieri* to *Marinobacterium* as *Marinobacterium jannaschii* comb. nov. and *Marinobacterium stanieri* comb. nov. *Int J Syst Evol Microbiol* **52**, 739–747.

Satomi, M., Oikawa, H. & Yano, Y. (2003). *Shewanella marinintestina* sp. nov., *Shewanella schlegeliana* sp. nov. and *Shewanella sairae* sp. nov., novel eicosapentaenoic-acid-producing marine bacteria isolated from sea-animal intestines. *Int J Syst Evol Microbiol* **53**, 491–499.

Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–655. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.

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.

Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reverse-phase high-performance liquid chromatography. *FEMS Microbiol Lett* **25**, 125–128.

Terasaki, Y. (1972). Studies on the genus *Spirillum* Ehrenberg. I. Morphological, physiological, and biochemical characteristics of water spirilla. *Bull Suzugamine Women's Coll Nat Sci* **16**, 1–46.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Venkateswaran, K., Moser, D. P., Dollhopf, M. E. & 10 other authors (1999). Polyphasic taxonomy of the genus *Shewanella* and description of *Shewanella oneidensis* sp. nov. *Int J Syst Bacteriol* **49**, 705–724.

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.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.

West, P. A. & Colwell, R. R. (1984). Identification and classification of *Vibrionaceae* – an overview. In *Vibrios in the Environment*, pp. 285–363. Edited by R. R. Colwell. New York: Wiley.

Yamamoto, S. & Harayama, S. (1995). PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. *Appl Environ Microbiol* **61**, 1104–1109.

Zhang, W., Xue, Y., Ma, Y., Grant, W. D., Ventosa, A. & Zhou, P. (2002). *Marinospirillum alkaliphilum* sp. nov., a new alkaliphilic helical bacterium from Haoji soda lake in Inner Mongolia Autonomous Region of China. *Extremophiles* **6**, 33–37.