***Marinospirillum* gen. nov., with descriptions of *Marinospirillum megaterium* sp. nov., isolated from kusaya gravy, and transfer of *Oceanospirillum minutulum* to *Marinospirillum minutulum* comb. nov.**

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Two strains of helical, halophilic, Gram-negative, heterotrophic bacteria were isolated from kusaya gravy which is a traditional Japanese fermented brine. These strains were motile by means of a single polar or bipolar tuft flagellum. They had a large cell size, were helical, formed coccoid bodies, were microaerophilic and had quinone type Q-8. The DNA G+C content of the strains was 44–45 mol%. A detailed investigation of the phenotypic, chemotaxonomic and phylogenetic characteristics of the strains revealed that they represent a new species of halophilic helical bacteria. The sequence of the 16S rRNA gene of strain H7^T, designated the type strain of the new isolates, and all of the *Oceanospirillum* species except for *Oceanospirillum linum* were determined. Phylogenetic analysis indicated that these strains were closely related to *Oceanospirillum minutulum*, with enough distance to separate the *O. minutulum*/new isolate H7^T cluster from *Oceanospirillum sensu stricto* on the genus level. It is proposed that a new genus, *Marinospirillum*, be created; this genus should include *Marinospirillum minutulum* ATCC 19193^T (formerly *Oceanospirillum minutulum*) as the type species, as well as *Marinospirillum megaterium* JCM 10129^T (=H7^T).

Keywords: *Marinospirillum*, *Oceanospirillum*, kusaya gravy, halophilic helical bacteria

INTRODUCTION

Members of the genus *Oceanospirillum* are widely distributed in the ocean. Previous studies have isolated several kinds of *Oceanospirillum* species from putrid marine shellfish (Terasaki, 1972, 1973, 1975, 1979; Watanabe, 1959) and coastal sea water (Baumann *et al.*, 1972; Hylemon *et al.*, 1973; Linn & Krieg, 1978; Williams & Rittenberg, 1957). In previous studies (Fujii, 1978; Fujii *et al.*, 1990), *Oceanospirillum*-like bacteria were isolated from kusaya gravy, which is used for producing Japanese traditional dried fish. The

main chemical characteristics of kusaya gravy are as follows: low salinity (NaCl concentration of approximately 3%) (Fujii, 1977), low dissolved oxygen concentration (Satomi *et al.*, 1997b), and large amounts of volatile basic nitrogen compounds (Fujii, 1977; Satomi *et al.*, 1997b). Microbiologically, the predominant bacteria in kusaya are *Corynebacterium*-like bacteria (Fujii, 1978; Shimidu *et al.*, 1967, 1969), *Peptococcaceae* (Fujii *et al.*, 1985, 1993), helical bacteria (Fujii, 1978; Fujii *et al.*, 1990; Kosuge *et al.*, 1978), and bacteria that are unculturable on agar surfaces including the new isolates (Fujii *et al.*, 1990; Satomi *et al.*, 1997b). According to *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994), all helical, halophilic, chemo-organotrophic and aerobic bacteria belong to the genus *Oceanospirillum*. Recently, however, interspecies diversity of this group of bacteria was pointed out by several workers, based on rRNA–DNA hybridization experiments (Pot *et al.*, 1989),

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Abbreviations: TEM, transmission electron microscopy; PHB, poly- β -hydroxybutyrate.

The DDBJ accession numbers for the 16S sequences reported in this paper are AB006760–AB006771.

fatty acid analysis (Sakane & Yokota, 1994), isoprenoid quinone analysis (Sakane & Yokota, 1994) and polyamine composition (Hamana *et al.*, 1994). Thus, the precise identification of halophilic helical bacteria is difficult based on phenotypic characteristics alone. Phenotypically, the new isolates were quite different from other previously described species of this genus in that they had a large cell size and were unable to form colonies on agar plates aerobically. In this study, additional phenotypic and chemotaxonomic characteristics, as well as 16S rRNA gene (16S rDNA) sequencing data were obtained. The latter are useful for determining phylogenetic placement and species affiliation. In addition, DNA–DNA hybridization was performed. On the basis of these results, it is proposed that the isolates reported here and *Oceanospirillum minutulum* should be placed in a new genus, *Marinospirillum*, and that the new isolates should be placed in a new species, *Marinospirillum megaterium*.

METHODS

Bacterial strains and growth conditions. Two strains (H7^T and SP5) were isolated from kusaya gravy. Both strains were isolated from gravy produced by the same manufacturer but

at different times; H7^T was isolated in September 1988 and SP5 was isolated in June 1993. Isolation and purification procedures were as described previously (Fujii *et al.*, 1990). Briefly, a loopful of gravy was used to inoculate the inlet tube of a horizontal glass tube in which the inlet and outlet ends were bent upwards. After an appropriate time, the isolates predominantly accumulated in the outlet because of their excellent motility. By repeating this selective accumulation and purification process alone, the isolates could almost be purified (microscopic observation). Isolation was done by streaking on agar plates. The ability of the new isolates to form colonies on the agar surface was low and was lost after several generations. The isolates (H7^T and SP5) have been deposited in the Japan Collection of Microorganisms (JCM) as *Marinospirillum megaterium* JCM 10129^T (=H7^T) and JCM 10130 (=SP5). These strains were maintained on stab cultures in 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. The pH was adjusted to 8.0 with 1.0 M NaOH. The cultures were incubated for 1 week at 25 °C. To obtain large amounts of cells, cells were incubated in TSSY broth at 90 r.p.m. on a rotary shaker. Cells could not grow in liquid media with vigorous shaking or under static conditions.

Oceanospirillum species were used for comparison; these

Table 1. Designations and/or accession numbers of the genus *Marinospirillum* and related bacteria analysed in this study

ATCC, American Type Culture Collection, Rockville, MD, USA; IFO, Institute for Fermentation, Osaka, Japan; and JCM, Japan Collection of Microorganisms, Saitama, Japan.

Species	Strain	Sequence accession no.
<i>Marinospirillum minutulum</i>	ATCC 19193 ^T (=IFO 15450 ^T)	AB006769*
<i>Marinospirillum megaterium</i>	H7 ^T (=JCM 10129 ^T)	AB006770*
<i>Marinospirillum megaterium</i>	SP5 (=JCM 10130)	
<i>Oceanospirillum linum</i>	IFO 15448 ^T (=ATCC 11336 ^T)	M22365
<i>Oceanospirillum maris</i> subsp. <i>maris</i>	ATCC 27509 ^T	AB006771*
<i>Oceanospirillum maris</i> subsp. <i>hiroshimense</i>	IFO 13616 ^T	AB006762*
<i>Oceanospirillum maris</i> subsp. <i>williamsae</i>	IFO 15468 ^T (=ATCC 29547 ^T)	AB006763*
<i>Oceanospirillum beijerinckii</i> subsp. <i>beijerinckii</i>	IFO 15445 ^T (=ATCC 12754 ^T)	AB006760*
<i>Oceanospirillum beijerinckii</i> subsp. <i>pelagicum</i>	IFO 13612 ^T (=ATCC 33337 ^T)	AB006761*
<i>Oceanospirillum multiglobuliferum</i>	IFO 13614 ^T (=ATCC 33336 ^T)	AB006764*
<i>Oceanospirillum japonicum</i>	ATCC 19191 ^T (=IFO 15446 ^T)	AB006766*
<i>Oceanospirillum kriegii</i>	IFO 15467 ^T (=ATCC 27133 ^T)	AB006767*
<i>Oceanospirillum jannaschii</i>	IFO 15466 ^T (=ATCC 27135 ^T)	AB006765*
<i>Oceanospirillum pusillum</i>	IFO 13613 ^T (=ATCC 33338 ^T)	AB006768*
<i>Deleya salina</i>	ATCC 49509 ^T	X87217
<i>Halomonas elongata</i>	ATCC 33173 ^T	X67023
<i>Marinomonas vaga</i>	ATCC 27119 ^T	X67025
<i>Marinobacter hydrocarbonoclasticus</i>	ATCC 49840 ^T	X67022
<i>Marinobacterium georgiense</i>	ATCC 700074 ^T	U58339
<i>Volcaniella eurihalina</i>	ATCC 49336 ^T	X87218
<i>Vibrio parahaemolyticus</i>	ATCC 17802 ^T	X56580
<i>Escherichia coli</i> †		J01859
<i>Proteus vulgaris</i>	IFAM 1731	X07652

* Sequenced in this study.

† Consensus sequence of multiple strains.

Fig. 1. Scanning electron micrographs of strain H7^T, vegetative cells (a) and coccoid bodies (b). Negatively stained cells (c) observed by TEM. (a, b) Bar, 1 µm; (c) bar, 2 µm.

strains were obtained from the Institute for Fermentation, Osaka (IFO), the American Type Culture Collection (ATCC), and Y. Terasaki (formerly Suzugamine Women's College). The recommended growth media were used to grow these organisms.

Morphology. The cellular morphology of the new isolates,

which contained both vegetative and coccoid body cells, and flagella formation were observed by electron microscopy. To obtain the photographs for scanning electron microscopy, cells were fixed for 2 h in 2% glutaraldehyde and dehydrated by increasing the concentration of ethanol and isoamyl acetate. Isoamyl acetate was evaporated by critical lyophilization and cells were sputter-coated with gold. Flagella formation was observed by transmission electron microscopy (TEM); cells were stained with uranium acetate. Helix type and motility were observed by phase-contrast microscopy. Gram staining was performed by the modified Hucker procedure (Conn *et al.*, 1957). Accumulation of poly-β-hydroxybutyrate (PHB) was determined by the Sudan black staining method (Burdon, 1946).

Physiological and biochemical characteristics. Optimum growth temperature, pH and NaCl concentration were determined using TSSY semi-solid agar medium. Effects of different oxygen concentrations (0, 3, 6, 9, 12 and 15%) on growth of the H7^T isolate were studied on TSSY semi-solid agar medium using a mixed atmosphere consisting of nitrogen and oxygen. Other physiological and biochemical tests were performed in accordance with standard protocols (Krieg, 1984; Pot *et al.*, 1992). Unless indicated otherwise, the inocula used for these tests were prepared from exponential growth stage cells in TSSY semi-solid agar. In the physiological and biochemical tests, *Marinospirillum minutum* was used as a reference strain.

Isoprenoid quinone composition. The isoprenoid quinone type and isoprenoid length were analysed using the methods described by Yamada & Kuraishi (1982). Total acetone soluble extracts of whole cells were separated by one-dimensional chromatography on silica gel thin layers (Merck) with benzene for eluent. Isoprenoid length was analysed by HPLC using a reverse-phase column (Cosmosil C18 Econopak column; Nakarai Tesque).

DNA preparation. Cells were suspended in TE buffer (pH 8.0) and treated with SDS (0.5% final concentration) for lysis. Chromosomal DNA was purified by standard methods (Sambrook *et al.*, 1989) except that the final steps of the procedure were modified as described by Satomi *et al.* (1997a).

DNA base composition and DNA–DNA homology. The DNA G+C content was determined by the HPLC methods of Tamaoka & Komagata (1984). DNA–DNA homology was studied by microplate hybridization methods (Ezaki *et al.*, 1989) with photobiotin labelling and colorimetric detection as described previously (Satomi *et al.*, 1997a).

16S rDNA sequencing and phylogenetic analysis. 16S rDNA was amplified by PCR. For amplification of 16S rDNA, a universal primer set, corresponding to positions 8–27 (forward primer) and 1492–1510 (reverse primer) of the *Escherichia coli* numbering system, was used (Weisburg *et al.*, 1991). The PCR operating conditions were as described by Suzuki & Yamasato (1994). The PCR products were visualized by electrophoresis with 1.5% agarose gel (Nippon Gene) stained with ethidium bromide. Direct sequencing of the amplified DNA fragments was performed as described previously (Satomi *et al.*, 1997a). Similarity of 16S rDNA sequence between strain H7^T and other organisms was compared with all sequence data in the GenBank, EMBL and DDBJ databases using the BLAST algorithm (Altschul *et al.*, 1990). Data analysis was performed with CLUSTAL W software (Thompson *et al.*, 1994) as described previously (Satomi *et al.*, 1997a). Nucleotide substitution rates (K_{unc} values) were calculated by the methods of Kimura (1980).

and a phylogenetic tree was constructed using the neighbour-joining method of Saitou & Nei (1987). The accession numbers of the new isolates and newly determined reference strains used in the phylogenetic analysis are shown in Table 1.

RESULTS

Morphology and culture characteristics

The new strains isolated from kusaya gravy were Gram-negative, rigidly helical (Fig. 1a), and motile. Coccoid body formation was positive after 7 d culture (Fig. 1b), but no spores were produced. Cell size in optimum conditions was 0.8–1.2 µm in diameter by 5–15 µm in length and the thin-wall coccoid body was 2.0–2.5 µm in diameter. Motility was by means of large bipolar tufts of flagella (Fig. 1c).

New isolates required NaCl for growth; growth was observed at media NaCl concentrations of 0.5–9.0% (w/v). Optimum growth occurred in 3% NaCl. These strains grew at temperatures of 4–25 °C, with the optimum temperature for growth at 20–25 °C. The pH range for growth was 7.5–9.0, with the optimum at pH 8.0. In TSSY semi-solid medium, the optimum oxygen concentration was 6% and growth was not observed at concentrations of 0–3%; cells were thus regarded as microaerophilic. The effects of H₂ and CO₂ concentrations on growth were not studied. Cells could not grow in liquid media in static or aerobic conditions with vigorous shaking. Moreover, they could not grow on agar plates under all oxygen concentrations studied.

Physiology and biochemical characteristics

All of the new isolates were oxidase-positive and did not catabolize carbohydrates. Intracellular PHB was formed. Nitrate reduction and denitrifying activity were negative. The catalase reaction was negative or weakly positive. The DNA G + C contents of the two new isolates, H7^T and SP5, were 44.4 and 44.8 mol %, respectively. Biochemical and physiological characteristics useful for identifying and differentiating the new isolates and *O. minutulum* are shown in Table 2. The characteristics of both species given in Table 2 were studied under the same conditions.

Isoprenoid quinone analysis

The isoprenoid quinone composition of strain H7^T was determined. It contained ubiquinone, which consisted of mainly Q-8. In this new isolate, a small amount of menaquinone was detected, but too little for precise analysis.

16S rRNA gene sequence comparisons

To investigate the phylogenetic relationships of the new isolates, the 16S rDNA sequences of the representative strain H7^T and of all of the *Oceanospirillum* species, except for *Oceanospirillum linum*, already deposited in the database were determined. The sequences obtained in this study were aligned together with reference sequences from the DDBJ database. Sequence comparisons and phylogenetic analysis are

Table 2. Characteristics which differentiate the new helical bacteria isolated from kusaya gravy and *M. minutulum*

Both species were Gram-negative spirals that formed coccoid bodies but did not form spores. They were motile by means of a polar tuft flagellum, accumulated PHB and had a positive oxidase reaction; the cell shape helix type was anticlockwise and the major isoprenoid quinone type was Q-8. All strains were negative for acid production from sugars, production of pigment, indole, and H₂S, and hydrolysis of casein, aesculin, gelatin, hippurate and starch. None of the strains produced DNase, RNase, phosphatase or urease.

Characteristic	New isolates (H7 ^T and SP5)	<i>M. minutulum</i> ATCC 19193 ^T
Cell length (µm)	5–15	2–2.8
Cell diameter (µm)	0.8–1.2	0.3–0.4
Oxygen requirement*	Mi	Ae
Catalase	– or W†	+
Reduction of nitrate	–	+
Temperature range for growth (°C)	4–25	4–30
Range of NaCl (%) for growth	0.5–9.0	0.2–10.0
Range of pH for growth	7.5–9.0	7.0–10.5
Growth in the presence of:		
1% Glycine	–	+
1% Ox gall	–	+
DNA G + C (mol %)	44–45	42.5‡

* Mi, Microaerophilic; Ae, aerophilic.

† W, Weak reaction, i.e. several bubbles were observed in the catalase test performed on centrifuged cells or on cell debris sonically disrupted in neutralization buffer.

‡ Data from Sakane & Yokota (1994).

Fig. 2. Phylogenetic tree constructed by the neighbour-joining method based on 16S rDNA sequences of new isolate H7^T and related bacteria. Bar, 0.02 K_{nuc} .

shown in Fig. 2. The 16S rDNA sequence of H7^T was most similar (level of sequence similarity was 97.5%) to the sequence of *O. minutulum*; other reference strains exhibited only low levels of similarity (less than 90%). On the phylogenetic tree of 16S rDNA, *Oceanospirillum kriegii*, *Oceanospirillum jannaschii*, *Oceanospirillum japonicum* and *O. minutulum* had separate positions among the γ -subclass *Proteobacteria* (Stackebrandt *et al.*, 1988); *Oceanospirillum*

pusillum belongs to the α -subclass *Proteobacteria* (Woese *et al.*, 1985).

DNA-DNA hybridization

Table 3 shows the levels of DNA-DNA relatedness among the strains studied. DNA-DNA hybridization experiments performed with labelled DNAs from strains H7^T, *O. minutulum* and *O. linum* revealed that the new isolates were closely related to each other but exhibited relatively low levels of hybridization (about 30%) with *O. minutulum*. No meaningful DNA-DNA hybridization was recognized between the new isolates/*O. minutulum* group and *O. linum*.

DISCUSSION

The results of these phenotypic, chemotaxonomic and phylogenetic analyses clearly indicate that the new strains (H7^T and SP5), isolated from kusaya gravy in Japan, form a homogeneous group and represent a new species of halophilic helical bacteria. These bacteria are Gram-negative, rigidly helical, non-spore-producing, coccoid body-forming, motile, halophilic, microaerobic and PHB-accumulating. They are also oxidase-positive, do not catabolize carbohydrates or reduce nitrate, and are unable to grow with 1% ox gall and glycine. The catalase reaction is negative or weakly positive. Usually, they cannot grow on agar surfaces. They have genomic DNA G+C contents of 44–45 mol%. The isoprenoid quinone type of strain H7^T is Q-8. Their genotypic and chemotaxonomic characteristics indicate that the new isolates are similar to members of the genus *Oceanospirillum*. However, their taxonomic place could not be determined exactly, since phenotypically they were quite different from other species in this genus in their large cell size and inability to grow on agar surfaces. Based on rRNA-DNA hybridization experiments, Pot *et al.* (1989) reported that the taxonomic relationships in this genus

Table 3. Levels of DNA-DNA reassociation between new isolates and *Oceanospirillum* species

Source of unlabelled DNA	Relatedness (%) to labelled DNA from:		
	H7 ^T	ATCC 19193 ^T	IFO 15448 ^T
<i>M. megaterium</i> H7 ^T (=JCM 10129 ^T)	100	32	7
<i>M. megaterium</i> SP5 (=JCM 10130)	100	28	7
<i>M. minutulum</i> ATCC 19193 ^T	28	100	5
<i>O. linum</i> IFO 15448 ^T	5	8	100
<i>O. maris</i> subsp. <i>hiroshimense</i> IFO 13616 ^T	12	7	37
<i>O. beijerinckii</i> subsp. <i>beijerinckii</i> IFO 15445 ^T	4	6	21
<i>O. multiglobuliferum</i> IFO 13614 ^T	8	8	17
<i>O. japonicum</i> ATCC 19191 ^T	12	8	7
<i>O. jannaschii</i> IFO 15466 ^T	2	6	9
<i>O. kriegii</i> IFO 15467 ^T	7	8	10
<i>O. pusillum</i> IFO 13613 ^T	2	2	2

were heterogeneous. This suggestion was confirmed by the studies of fatty acid, quinone and polyamine composition (Hamana *et al.*, 1994; Sakane & Yokota, 1994). 16S rDNA sequence analysis, which is a reliable method for determining species affiliations, was carried out in this study. A comparative sequence analysis of 16S rDNA revealed that strain H7^T and *O. minutulum* are closely related (similarity of 97.5%), but they could be distinguished as different species by their phenotypic features and phylogenetic distance. The low levels of 16S rDNA sequence similarity between strain H7^T/*O. minutulum* group and other halophilic helical bacteria indicated that these bacteria are phylogenetically different enough to be different genera. This notion is strongly supported by the DNA–DNA hybridization results, which satisfy the recommendation of the Ad Hoc Committee on reconciliation of approaches to bacterial systematics (Wayne *et al.*, 1987). The phylogenetic tree of 16S rDNA (Fig. 2) indicates that the genus *Oceanospirillum* is clearly a heterogeneous group and their phylogenetic relationships are supported by the results of rRNA–DNA hybridization reported by Pot *et al.* (1989) except in the case of *O. japonicum*. These previous results and those presented here suggest that the genus *Oceanospirillum* consists of *O. linum*, which is the type species of this genus, *Oceanospirillum maris*, *Oceanospirillum beijerinckii* and *Oceanospirillum multiglobuliferum*. Other species, *O. jannaschii*, *O. japonicum*, *O. kriegii*, *O. minutulum* and *O. pusillum*, should be eliminated from this genus and transferred to another genus. Determination of their precise taxonomic positions needs further study to detect other, possibly more closely related organisms to these species. In this report, *O. japonicum* is not included in *Oceanospirillum* species, because it could not form a coccoid body (Hylemon *et al.*, 1973; Krieg, 1984; Watanabe, 1959) and did not cluster with *Oceanospirillum sensu stricto* at levels suitable for the same genus based on 16S rDNA sequences. The exclusion of *O. japonicum* from the genus is supported by the insignificant degree of reassociation between its DNA and DNA from *O. linum*.

On the basis of phenotypic, chemotaxonomic and phylogenetic studies, it is concluded that the new isolates should be classified as new species belonging to the same genus as *O. minutulum*. Therefore, it is proposed that a new genus, *Marinospirillum*, should be created for the new isolates and *O. minutulum*.

Several phenotypic features allowed differentiation between *O. minutulum* and the new isolates (Table 2), and the low levels of DNA similarity on both organisms (similarity levels based on DNA–DNA hybridization of about 30%) justify placing them in different species. Thus, the name *Marinospirillum megaterium* sp. nov. is proposed; strain H7^T is the type strain of this species. *Oceanospirillum minutulum* should be transferred to the genus *Marinospirillum* as *Marinospirillum minutulum*.

Description of *Marinospirillum* gen. nov.

Marinospirillum (Ma.ri.no.spi.ril'lum. L. adj. *marinus* of the sea; Gr. n. *spira* a spiral; M.L. dim. neut. n. *spirillum* a small spiral; *Marinospirillum* a small spiral from the sea).

Gram-negative, rigidly helical, non-spore-producing, coccoid body-forming, halophilic, aerobic or micro-aerobic, chemo-heterotrophic and PHB-accumulating bacteria. Motile by means of flagella. Oxidase-positive. Catalase reaction is negative or positive. Carbohydrates are not catabolized. Genomic DNA G+C content of 42–45 mol% (as determined by HPLC). The isoprenoid quinone type is Q-8. The type species is *Marinospirillum minutulum*.

Description of *Marinospirillum megaterium* sp. nov.

Marinospirillum megaterium (me.ga.te'ri.um. Gr. adj. *mega* large; Gr. n. *teras*, *teratis* monster, beast; M.L. n. *megaterium* big beast).

In addition to those given above in the description of the genus, *M. megaterium* has the following characteristics. Rigid helical shaped cells are 0.8–1.2 µm in diameter by 5–15 µm in length and thin-wall coccoid bodies are 2.0–2.5 µm in diameter. NaCl is required for growth; growth occurs at NaCl concentrations of 0.5–9.0% (w/v) and is optimal at 3% NaCl. The temperature range for growth is 4–25 °C, with an optimum of 20–25 °C. The pH range for growth is 7.5–9.0, with an optimum of pH 8.0. Cells were microaerophilic. Cells cannot grow in liquid media in static or aerobic conditions with vigorous shaking or on plate surfaces. Catalase production is weakly positive. Gelatin, hippurate and starch are not hydrolysed. Nitrate is not reduced. DNase, RNase, urease and phosphatase are not produced. The DNA G+C content is 44–45 mol%. The type strain is strain H7^T (=JCM 10129^T), which was isolated from kusaya gravy.

Transfer of *Oceanospirillum minutulum* to the genus *Marinospirillum* as *Marinospirillum minutulum* comb. nov.

Marinospirillum minutulum (mi.nu'tu.lum. L. dim. neut. adj. *minutulum* very little).

Physiological, chemotaxonomic and phylogenetic data indicate that *O. minutulum* is more closely related to *M. megaterium* than to *O. linum*, the type species of the genus *Oceanospirillum*. The original description of the species given by Watanabe (1959) is unchanged.

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REFERENCES

- Altschul, S. A., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Baumann, L., Baumann, P., Mandel, M. & Allen, R. D. (1972). Taxonomy of aerobic marine eubacteria. *J Bacteriol* **110**, 402–429.
- Burdon, K. L. (1946). Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *J Bacteriol* **52**, 665–678.
- Conn, H. J., Bartholomew, J. W. & Jennison, M. W. (1957). Staining methods. In *Manual of Microbiological Methods*, pp. 10–36. Edited by Society of American Bacteriologists. New York: McGraw-Hill.
- 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 Niiijima and Oshima islands. *Nippon Suisan Gakkaishi* **43**, 517–521.
- Fujii, T. (1978). Microbial flora of 'Kusaya' brine of Niiijima 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.
- 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, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. & Williams, S. T. (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 Biol* **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. 71–95. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Linn, D. M. & Krieg, N. R. (1978). Occurrence of two organisms in cultures of the type strain of *Spirillum lunatum*: proposal for rejection of the name *Spirillum lunatum* and characterization of *Oceanospirillum maris* subsp. *williamsae* and an unclassified vibrioid bacterium. *Int J Syst Bacteriol* **28**, 132–138.
- 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.
- Pot, B., Gillis, M. & De Ley, J. (1992). The genus *Oceanospirillum*. In *The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications*, 2nd edn, vol. 4, pp. 3230–3236. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sakane, T. & Yokota, 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, 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., Satou, 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.
- Shimidu, A., Mochizuki, A., Shimizu, U. & Aiso, K. (1967). Studies of kusaya-I. Chemical composition and preserving effect of the curing brine for kusaya. *Nippon Suisan Gakkaishi* **33**, 1143–1146.
- Shimidu, U., Aiso, K., Shimizu, W. & Mochizuki, A. (1969). Studies of kusaya-II. Microbiological examination of a kusaya brine. *Nippon Suisan Gakkaishi* **35**, 109–115.
- Stackebrandt, E., Murray, R. G. E. & Trüper, H. G. (1988). *Proteobacteria* classis nov., a name for the phylogenetic taxon that includes the "Purple bacteria and their relatives". *Int J Syst Bacteriol* **38**, 321–325.
- Suzuki, T. & Yamasato, K. (1994). Phylogeny of spore-forming lactic acid bacteria based on 16S rRNA gene sequences. *FEMS Microbiol Lett* **115**, 13–18.
- Tamaoka, J. & Komagata, K. (1984). Determination of DNA base composition by reversed-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.
- Terasaki, Y. (1973). Studies on the genus *Spirillum* Ehrenberg. II. Comments on type and reference strains of *Spirillum* and description of new species and new subspecies. *Bull Suzugamine Women's Coll Nat Sci* **17**, 1–71.
- Terasaki, Y. (1975). Freeze-dried cultures of water spirilla made on experimental basis. *Bull Suzugamine Women's Coll Nat Sci* **19**, 1–10.
- Terasaki, Y. (1979). Transfer of five species and two subspecies of *Spirillum* to other genera (*Aquaspirillum* and *Oceanospirillum*), with emended descriptions of the species and subspecies. *Int J Syst Bacteriol* **29**, 130–144.
- 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.

- Watanabe, N. (1959).** On four new halophilic species of *Spirillum*. *Bot Mag Tokyo* **72**, 77–86.
- 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.
- Williams, M. A. & Rittenberg, S. C. (1957).** A taxonomic study of the genus *Spirillum* Ehrenberg. *Int Bull Bacteriol Nomencl Taxon* **7**, 49–111.
- Woese, C. R., Weisburg, W. G., Hahn, C. M., Paster, B. J., Zablen, L. B., Lewis, B. J., Macke, T. J., Ludwig, W. & Stackebrandt, E. (1985).** The phylogeny of purple bacteria: the gamma subdivision. *Syst Appl Microbiol* **6**, 25–33.
- Yamada, Y. & Kuraishi, I. (1982).** Ubiquinone and Menaquinone. In *Biseibutsu no Kagakubunrui Jikkenhou*, pp. 143–155. Edited by K. Komagata. Tokyo: Gakkai Shuppan Center (in Japanese).