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Full Paper

Phycisphaera mikurensis gen. nov., sp. nov., isolated from a marine alga, and proposal of *Phycisphaeraceae* fam. nov., *Phycisphaerales* ord. nov. and *Phycisphaerae* classis nov. in the phylum *Planctomycetes*

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Three strains, FYK2301M01^T, FYK2301M18 and FYK2301M52, all being Gram-negative, spherical, motile and facultatively anaerobic, were isolated from a marine alga (*Porphyra* sp.) collected on Mikura Island, Japan. Colonies of the strains were circular and pink-pigmented on Marine Agar 2216 (Difco) at 25°C. Cells of the strains reproduced by binary fission. The G+C content of the DNA was 73 mol%. The major isoprenoid quinone was MK-6. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strains are the members of the WPS-1 group (Nogales et al., 2001) comprising no validly described taxa within the phylum *Planctomycetes*. The highest similarity value of the 16S rRNA gene sequences of the strains to those in the established bacterial taxa was only 78.7% to *Planctomyces brasiliensis* DSM 5305^T. From the taxonomic data obtained in this study, it is proposed that the new marine isolates be placed in a novel genus and species named *Phycisphaera mikurensis* gen. nov., sp. nov. within a new family, order and class *Phycisphaeraceae* fam. nov., *Phycisphaerales* ord. nov. and *Phycisphaerae* classis nov. in the phylum *Planctomycetes*. The type strain of *Phycisphaera mikurensis* is FYK2301M01^T (= NBRC 102666^T = KCTC 22515^T).

Key Words——binary fission; marine alga; new class; *Phycisphaera mikurensis*; *Planctomycetes*

Introduction

We have reported that marine organisms are interesting sources for the discovery of novel lineages of bacteria (Fukunaga et al., 2006, 2008; Kurahashi and Yokota, 2002, 2004, 2007a, b). During this series of studies, three strains isolated from a marine alga (*Porphyra* sp.) were classified as members of the WPS-1 group in the phylum *Planctomycetes*.

Planctomycetes is one of the phyla in the PVC (*Planctomycetes*, *Verrucomicrobia* and *Chlamydiae*) superphylum (Wagner and Horn, 2006), which is a

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The DDBJ/GenBank/EMBL accession numbers for the 16S rRNA gene sequences of *Phycisphaera mikurensis* strain FYK2301M01^T (= NBRC 102666^T = KCTC 22515^T), FYK2301M18 (=NBRC 102667) and FYK2301M52 (=NBRC 102668) are AB447464, AB474363 and AB474364, respectively.

synonym of "*Planctobacteria*" proposed by Cavalier-Smith (2002). All the members of the phylum *Planctomycetes* are Gram-negative, reproduce by budding and lack peptideglycan in their cell envelope. Some members of the phylum possess membrane-bounded intracellular compartments (Fuerst, 2005). *Planctomycetes* are ubiquitously found in diverse aquatic and terrestrial environments (Janssen, 2006; Kohler et al., 2008; Santelli et al., 2008; Wang et al., 2002); however the majority of them are uncultured (Chouari et al., 2003; Derakshani et al., 2001; Elshahed et al., 2007).

Currently, the phylum *Planctomycetes* is divided into three putative classes (Elshahed et al., 2007; Janssen, 2006): (i) the cultured planctomycete group corresponding to the class *Planctomycetacia* including all previously described genera, (ii) the uncultured planctomycete group which was referred to as WPS-1 (Nogales et al., 2001), and (iii) the deeply branching planctomycete group including anaerobic ammonium-oxidizing bacteria (anammox), which have not yet been isolated in pure cultures.

We isolated three strains, FYK2301M01^T, FYK2301 M18 and FYK2301M52, from a marine alga (*Porphyra* sp.). These strains were classified as members of the WPS-1 group in the phylum *Planctomycetes*. In this paper, polyphasic characterizations of these novel isolates are reported.

Materials and Methods

Bacterial strains and media. Strains FYK2301M01^T, FYK2301M18 and FYK2301M52 were isolated by the following method. A marine alga (*Porphyra* sp.) collected on Mikura Island, a Japanese island in the Pacific Ocean, was washed with sterile artificial seawater (ASW; Naigai Chemical Products) and homogenized with a hand-held homogenizer. Subsequently, the algal homogenate was serially diluted 10-fold in sterile ASW and suitable dilutions were plated onto 1/5-strength Marine Agar 2216 (Difco). The plates were incubated at 20°C for 30 days and colonies that grew were purified on the same medium.

Phenotypic characteristics. The morphology of the cells was observed under a light microscope, while the ultrastructure of negatively-stained cells with 1% (w/v) phosphotungstic acid was observed under a transmission electron microscope (Hitachi, H7600). The Gram-staining procedure was described by Gerhardt et al. (1994). For the preparation of ultrathin sections, cells of FYK2301M01^T were processed by rapid freezing in liquid propane cooled with liquid nitrogen, cryosubstituted with 2% (w/v) OsO_4 in acetone for 3 days, and embedded in epoxy resin. The ultrathin sections were double-stained with the three-times diluted Pt-blue staining solution (Inaga et al., 2007) for 10 min followed by the lead-stain solution (Reynolds, 1963) for 1 min. Physiological and biochemical tests were performed by API 20NE, API 50 CH, API ZYM (bioMérieux) and Biolog GN2 microtiter plates. The manufacturer's instructions were followed except that inocula for API 20NE were prepared in ASW while those for API 50 CH in a 50 : 50 (v : v) solution of filter-sterilized CHB medium (bioMérieux) and ASW. API strips were incubated at 25°C for 3 days after inoculation. Oxidative utilization of 95 carbon sources was tested using Biolog GN2 (Rüger and Krambeck, 1994), while agar hydrolysis was determined by cultivating the strains on Marine Agar. Degradation of starch, casein, chitin, DNA and gelatin was tested according to the protocol of Cowan and Steel (1993). Presence of catalase was revealed with 3% (v/v) hydrogen peroxide. The oxidation or fermentation of D-xylose (O/F test; Hugh and Leifson (1953)) was checked by using O/F basal medium (Eiken Kizai) prepared with the ASW. Absorption spectra (260–700 nm) of methanol extracts from the cells were examined by spectroscopy to test the presence of carotenoid-type pigments. Growth at different temperatures (4, 10, 15, 20, 30, 37, 40, 45 and 55°C) was assessed on agar plates. The salt tolerance was tested with R2A agar (Difco) suspended either in ASW (0–350%, v/v) and or in NaCl solution (0–20%, v/v), and the plates were incubated at 25°C. The test for anaerobic growth was conducted on Marine Agar at 25°C in a GasPak anaerobic jar (Becton Dickinson).

Chemotaxonomy. The DNA G+C contents were determined by HPLC analysis (Mesbah et al., 1989). Major respiratory quinone was revealed using the protocol of Fukunaga et al. (2008). The extraction of cellular fatty acids from cells grown for 5 days at 25°C on Marine Agar and the determination of fatty acid content by gas chromatography were carried out by using the Microbial Identification (MIDI) System (MIDI Labs) according to the manufacturer's instructions. Fatty acids not identified by MIDI System were further analyzed using GC-MS (Agillent). An acid hydrolysate of the cells of FYK2301M01^T was prepared by the methods described by König et al. (1984), and its amino acids were analyzed by HPLC (Tamura et al., 1994). Suscep-

tibility to antibiotics was determined by a micro agar dilution method using Marine Agar for FYK2301M01^T, FYK2301M18 and FYK2301M52 to give final concentrations of ampicillin, penicillin, kanamycin and polymyxin B, from 0 to 1,000 μg/ml. Minimal inhibitory concentrations (MICs) of the drugs were determined after cultivation at 25°C for 9 days.

Phylogenetic analysis. The 16S rRNA gene was analyzed as described by Fukunaga et al. (2006). The random amplified polymorphic DNA (RAPD)-PCR was performed according to Nakagawa et al. (1998). DNA-DNA hybridizations were performed using photobiotin-labelled DNA and microplates (Ezaki et al., 1989). The sequences was aligned against an ARB dataset using the ARB program package (Ludwig et al., 2004). 16S rRNA gene sequences related to that of the isolates thus selected were downloaded from DDBJ and aligned by using the Clustal X program (Thompson et al., 1997). Putative chimeric sequences were identified by the program Mallard (Ashelford et al., 2006). Phylogenetic trees were inferred by using neighbor-joining (NJ; Saitou and Nei (1987)), maximum-parsimony (MP; Swofford (2000)) and maximum-likelihood (ML; Adachi and Hasegawa (1996)) algorithms. The topology of the trees was tested by bootstrap resampling analyses (Felsenstein, 1985) of 1,000 replicates for NJ and MP, and by local bootstrap probabilities (Hasegawa and Kishino, 1994) of 100 replicates for ML.

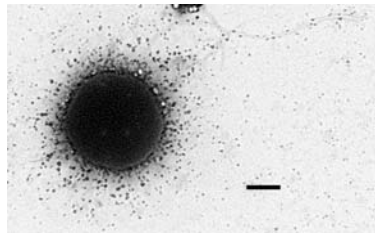
Results and Discussion

Morphological characteristics

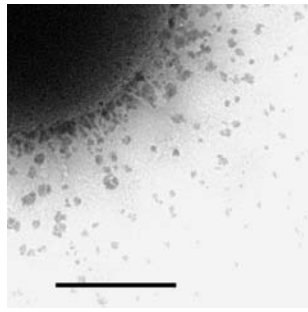
FYK2301M01^T, FYK2301M18 and FYK2301M52 formed pink to red colonies on Marine Agar plates 2216 (Difco), after 5−7 days of incubation at 25°C. The sizes of colonies of these strains were slightly different: FYK2301M01^T formed the largest colonies followed by FYK2301M18 and FYK2301M52. Cells were stained Gram-negatively, motile and coccoid (0.5−1.3 μm in diameter), and had a single flagellum (Fig.1 (a)) and pili (Fig. 1 (b)). Stalk-like structures and crateriform structures were not observed. Examination of an ultrathin section showed that cells reproduced by binary fission (Fig. 1 (c)). A circular intracytoplasmic membrane-like structure was observed (Fig. 1 (d)).

Phenotypic characteristics

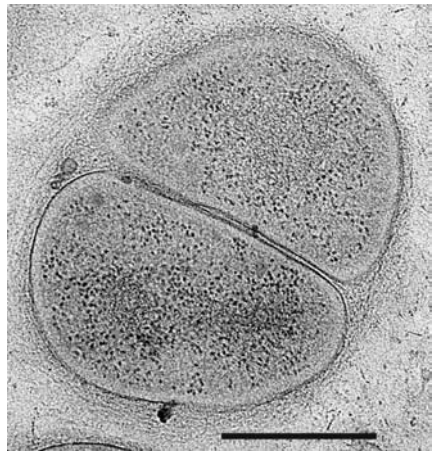
FYK2301M01^T, FYK2301M18 and FYK2301M52 required seawater for growth. No growth was detected



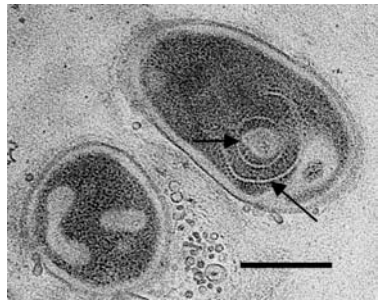
(a)



(b)



(c)



(d)

Fig. 1. Electron micrographs of negatively stained cells of strain FYK2301M01^T grown on Marine Agar, demonstrating (a) flagellation and (b) pili formation.

Morphology of ultarthin sectioned cells of FYK2301M01^T was observed under transmission electron microscope demonstrating that (c) cells reproduce by binary fission, and (d) an intracytoplasmic membrane-like structure (indicated by arrows) exists. Bar, 0.5 μm.

on R2A agar supplemented only with NaCl. The three strains grew in 30–200% ASW (optimally in 70–100% ASW). The temperature range for growth was 10–30°C (optimally 25–30°C). Carotenoid-type pigment was observed with a maximum absorption at 496 nm and two shoulders at 467 and 596 nm in methanol. Agar hydrolysis was observed on Marine Agar plates. Starch, casein, chitin, DNA and gelatin were not degraded. Catalase activity was detected, but oxidase activity was not detected in the three strains. D-Xylose was fermented by the three isolates under anaerobic conditions. Anaerobic growth on Marine Agar was observed. Acid production, enzymatic activities and utilization of carbon sources are summarized in the genus and species descriptions.

Chemotaxonomic characteristics

The DNA G+C contents of strains FYK2301M01^T, FYK2301M18 and FYK2301M52 were determined by HPLC analysis (Mesbah et al., 1989) to be 73.2±0.4, 73.2±0.6 and 73.3±0.5 mol%, respectively. The DNA-DNA hybridization values among strains FYK2301M01^T, FYK2301M18 and FYK2301M52 were 78.2–99.9%. The major respiratory quinone was menaquinone-6. The cellular fatty acid profiles of strains FYK2301M01^T, FYK2301M18 and FYK2301M52 are shown in Table 1. The major fatty acids in the isolates were $C_{16:0}$ (34.0–36.5% of the total fatty acids), iso-$C_{16:1}$ (24.7–27.6%) and iso-$C_{16:0}$ (14.2–15.4%). The hydroxy fatty acid in the isolates was iso-$C_{14:0}$ 3-OH (2.5–3.2%). Muramic acid and diaminopimelic acid were not detected. Strains FYK2301M01^T, FYK2301M18 and FYK2301M52 were resistant to ampicillin and penicillin (MICs > 1,000 μg/ml) but sensitive to kanamycin (MICs being 3, 6.5, 6.5 μg/ml for FYK2301M01^T, FYK2301M18 and FYK2301M52, respectively) and polymyxin B (MICs being 0.78, 0.78 and 1.56 μg/ml). The results of an amino acid analysis of the whole cell hydrolysate and the resistance to ampicillin/penicillin which inhibit cell wall synthesis indicated that the cells lack peptidoglycan.

Phylogenetic analyses

The 16S rRNA gene sequences of FYK2301M01^T, FYK2301M18 and FYK2301M52 were 100% identical. However, the RAPD-PCR profiles of the three isolates were different from each other indicating that these strains were not clonal. A phylogenetic tree inferred by the NJ algorithm based on 1,134-bp-long rRNA gene sequences is shown in Fig. 2. The novel strains were included within the WPS-1 group in the phylum *Planctomycetes*. The node for the WPS-1 group was strongly supported by high bootstrap values (100% in the NJ, MP and ML trees), and clearly separated from the nearest clades, namely the class *Planctomycetacia* and the deep-branching "anammox-planctomycetes" group. Although the successful cultivation of two strains belonging to the WPS-1 group has been reported by Davis et al. (2005), no description of these strains has, to our knowledge, appeared in the literature since then. Among species with validly published names, the highest similarity value was 78.7% with *Planctomyces brasiliensis* DSM 5305^T. Since no validly described taxa has yet been reported in the WPS-1 group, the strains FYK2301M01^T, FYK2301M18 and FYK2301M52 should represent the WPS-1 group. All 16S rRNA gene sequences mostly from the environmental clones belonging to the WPS-1 group shared only 70.1–79.8% similarity to the members of adjacent clades, including the member of the class "*Planctomy-*

Table 1. Cellular fatty acid composition (%) of *Phycisphaera mikurensis* (FYK2301M01^T, FYK2301M18 and FYK2301M52).

Fatty acid	
Saturated straight-chain	
14:0	tr
15:0	2.1–2.5
16:0	34.0–36.5
17:0	2.1–3.2
18:0	1.2–2.4
Unsaturated straight-chain	
12:1	4.5–6.3
13:1	tr
15:1 ω8*c*	tr
Branched	
iso-14:0	tr
anteiso-15:0	tr
iso-16:0	14.2–15.4
iso-16:1	24.7–27.6
anteiso-17:0	2.6–3.1
Hydroxy substituted	
iso 14:0 3-OH	2.5–3.2
18:0 3-OH	tr
Unidentified	
ECL 14.95	1.5–1.6
Summed feature 4[a]	1.2–1.3

[a]Summed feature 4 contained 17:1 anteiso and/or 17:1 iso.
Values shown are percentages of total fatty acid. ECL, equivalent chain length; tr, trace amount (< 1.0%).

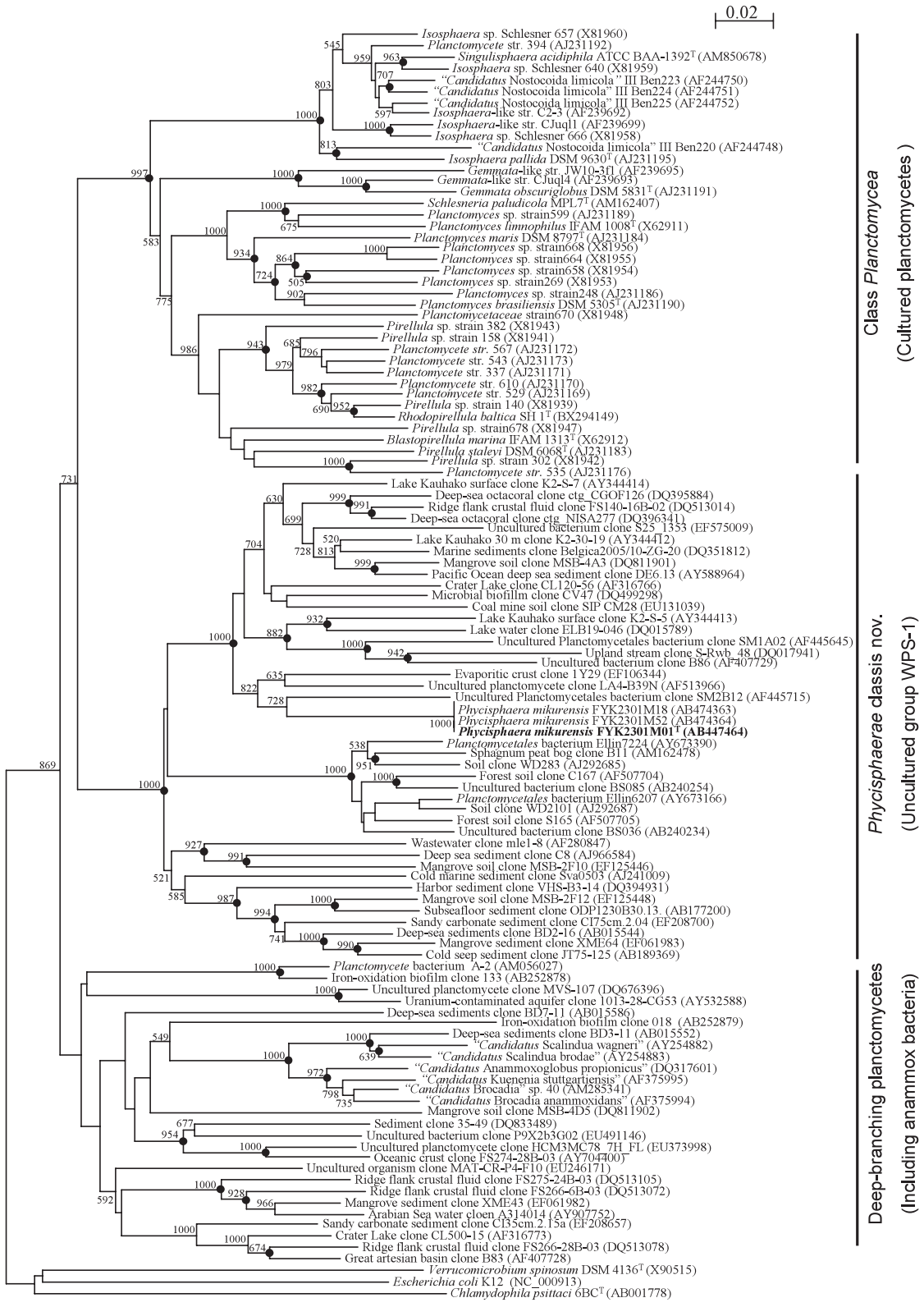


Fig. 2. Neighbor-joining tree of the phylum *Planctomycetes* based on 1,134-base-long 16S rRNA gene sequences.

Bootstrap values above 50% are shown. The closed circle at each node indicates that the node is supported by both the maximum-likelihood and maximum-parsimony analyses with bootstrap values higher than 70%. Bar 0.02 K_{nuc}.

cetacia" and the deep-branching planctomycete group. This low 16S rRNA gene sequence similarity suggests that the WPS-1 group represented by FYK 2301M01^T, FYK2301M18 and FYK2301M52 should be ranked as a novel class.

Taxonomic remarks on the strains

In addition to phylogenetic differentiation of the novel strains from other members of the class *Planctomycetacia*, phenotypic characteristics clearly differentiated these strains from those in the genera of the class *Planctomycetacia*, namely *Blastopirellula*, *Gemmata*, *Isosphaera*, *Pirellula*, *Planctomyces*, *Rhodopirellula*, *Schlesneria* and *Singulisphaera* (Table 2). The G+C contents of the novel strains were 13–20% higher than those of the genera in the class *Planctomycetacia* showing distinct genetic difference between the FYK 2301M01^T, FYK2301M18 and FYK2301M52 and the class *Planctomycetacia*. The oxidase activity of the novel strains was different from that of the members of *Planctomycetacia*. Based on these phenotypic and genetic differences from the described genera, it is proposed that FYK2301M01^T, FYK2301M18 and FYK 2301M52 represent a novel species in a new genus, *Phycisphaera mikurensis* gen. nov., sp.

Based on the phylogenetic placements of the putative class WPS-1, we formally propose the name of *Phycisphaerae* classis nov., with a new order *Phycisphaerales* ord. nov. and a new family *Phycisphaerales* fam. nov. The notable phenotypic property that clearly distinguishes the members of *Phycisphaerae* from *Planctomycetacia* is cell reproduction: members of the class *Phycisphaerae* reproduce by binary fission, while members of *Planctomycetacia* reproduce by budding.

Description of *Phycisphaerae* classis nov.

Phycisphaerae (Phy.ci.spha'e.ra. e. N.L. fem. n. *Phycisphaera,* type genus of the class; -ae ending to denote a class; N.L. fem. pl. n. *Phycisphaerae,* the *Phycisphaera* class).

Equivalent to WPS-1 (Elshahed et al., 2007; Nogales et al., 2001) of the phylum *Planctomycetes* and is defined by phylogenetic analyses based on 16S rRNA gene sequences obtained from one cultured representative and a wide range of uncultured bacteria retrieved mainly from marine and soil habitats. Gram-negative. The class comprises the genus *Phycisphaera*.

Description of *Phycisphaerales* ord. nov.

(Phy.ci.spha'e.ra'les. N.L. fem. n. *Phycisphaerales,* type genus of the order; -ales ending to denote a order; N.L. fem. pl. n. *Phycisphaerales,* the order of the genus *Phycisphaera*).

The description is the same as that for the genus *Phycisphaera*. The order contains the family *Phycisphaeraceae*. The type genus is *Phycisphaera*.

Table 2. Differential characteristics of *Phycisphaera* gen. nov. and related genera.

Characteristic	1	2	3	4	5	6	7	8	9
Cell shape	Spherical	Ovoid, ellipsoidal or pear-shaped	Coccoid	Spherical	Pear or teardrop	Ovoid to spherical	Ovoid, ellipsoidal or pear-shaped	Ellipsoi-dal	Spherical
Motility	+	+	+	+	+	+[a]	+	+	−
Budding	−	+	+	+	+	+	+	+	+
Flagella type	Single	Single, subpolar	Polar bundle	−	Single, polar	Single, polar	Single, subpolar	Double, subpolar	−
Stalk	−	ND	−	−	−	+	ND	+	−
Oxidase	−	+	+	ND	+	+[b]	+	+	+
DNA G+C content (mol%)	73.2±0.6	57.4±0.3	64.4±1.0	62.2	54–57	50.5–57.7	57.4	54.5–56.5	57.8–59.9

[a], *P. maris* (Bauld & Staley, 1976); [b], *P. brasiliensis* (Schlesner, 1989).

Strains: 1, *Phycisphaera*; 2, *Blastopirellula* (Schlesner, 1986; Schlesner et al., 2004); 3, *Gemmata* (Franzmann and Skerman, 1984); 4, *Isosphaera* (Giovannoni et al., 1987); 5, *Pirellula* (Schlesner and Hirsch, 1984; Schlesner et al., 2004); 6, *Planctomyces* (Bauld and Staley, 1976; Hirsch and Mueller, 1985; Schlesner, 1989; Starr and Schmidt, 1984); 7, *Rhodopirellula* (Schlesner et al., 2004); 8, *Schlesneria* (Kulichevskaya et al., 2007); 9, *Singulisphaera* (Kulichevskaya et al., 2008).

ND, no data available; −, negative; +, positive.

Description of *Phycisphaeraceae* fam. nov.

(Phy.ci.spha'e.ra.ce.ae. N.L. fem. n. *Phycisphaera,* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Phycisphaeraceae,* the family of the genus *Phycisphaera*).

The description is the same as that for the genus *Phycisphaera*. The family contains the type genus *Phycisphaera*.

Description of *Phycisphaera* gen. nov.

Phycisphaera (Phy.ci.spha'e.ra. L. n. *phycos*, seaweed; L. fem. n. *sphaera*, a globe, sphere; N.L. fem. n. *Phycisphaera*, a spherical bacterium isolated from a sea-weed.)

Cells are Gram-negative, motile, spherical and facultatively anaerobic. MK-6 is the major menaquinone. Reproduce by binary fission. Usual components of bacterial cell walls such as muramic acid and diaminopimelic acid are not detected. Resistant to ampicillin and penicillin. The G+C content of the genomic DNA of strain FYK2301M01^T, the type strain of the type species, *Phycosphaera mikuraensis*, is 73 mol%.

Description of *Phycisphaera mikurensis* sp. nov.

Phycisphaera mikurensis (mi.ku.ren'sis. N.L. fem. adj. *mikurensis*, pertaining to Mikura Island, Japan, from where the type strain was isolated).

In addition to the characteristics given for the genus description, this species displays the following characteristics. Cells are spherical (0.5−1.3 μm in diameter), covered with pili and motile with a single flagellum. Stalk-like structures are not observed. Colonies on Marine Agar are circular, smooth and pink to red in color. A carotenoid-type pigment (maximum absorption at 496 nm and two shoulders at 467 and 596 nm in methanol) is detected. Grows on Marine Agar at temperatures between 10 and 30°C (optimally at 25−30°C). Growth is observed on R2A agar supplemented with 30−200% ASW (optimally with 70−100%). Catalase activity is positive, oxidase activity is negative. Agar is degraded on Marine Agar. Degradation of starch, casein, chitin, DNA and gelatin is not detected. Growth on D-xylose under anaerobic conditions is observed. Reduction of nitrate to nitrite is observed, acid from glucose and indole from tryptophan is not produced. β-Glucosidase and β-galactosidase are positive. The activities for arginine dihydrolase, urease and protease are not detected. Activities in the API ZYM strip are detected for alkaline phosphatase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Activities of esterase C4, esterase lipase C8, lipase C4, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are not observed. Acids are produced from esculin and 5-keto-gluconate, and weakly from D-arabinose, L-arabinose, ribose, D-xylose, L-xylose, methyl-β-D-xylopyranoside, D-fructose, rhamnose and glycogen on the API 50 CH strips. The following substrates are utilized (Biolog): L-arabinose, D-fructose and α-D-glucose, and some strains utilized: α-cyclodextrin, glycogen, D-cellobiose, α-D-lactose, maltose, D-mannose, β-methyl-D-glucoside, L-rhamnose, L-glutamic acid, inosine, phenyethylamine, α-D-glucose-1-phosphate and D-glucose-6-phosphate. The major cellular fatty acids are $C_{16:0}$, iso-$C_{16:1}$ and iso-$C_{16:0}$ while major hydroxy fatty acid is iso-$C_{14:0}$ 3-OH. The DNA G+C content is 73 mol%. The type strain is FYK2301M01^T (=NBRC 102666^T=KCTC 22515^T) isolated from a marine alga collected from Mikurashima island, Japan. Strains FYK2301M18 (=NBRC 102667) and FYK2301M52 (=NBRC 102668) are also included in the species.

Acknowledgments

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References

Adachi, J. and Hasegawa, M. (1996) MOLPHY Version 2.3-Programs for Molecular Phylogenetics Based on Maximum Likelihood. Computer Science Monograph No. 28, Institute of Statistical Mathematics, Tokyo.

Ashelford, K. E., Chuzhanova, N. A., Fry, J. C., Jones, A. J., and Weightman, A. J. (2006) New screening software shows that most recent large 16S rRNA gene clone libraries contain chimeras. *Appl. Environ. Microbiol.,* **72**, 5734−5741.

Bauld, J. and Staley, J. T. (1976) *Planctomyces maris* sp. nov.: A marine isolate of the *Planctomyces-Blastocaulis* group of

budding bacteria. *J. Gen. Microbiol.,* **97**, 45–55.

Cavalier-Smith, T. (2002) The neomuran origin of archaebacteria, the negibacterial root of the universal tree and bacterial megaclassification. *Int. J. Syst. Evol. Microbiol.,* **52**, 7–76.

Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., and Sghir, A. (2003) Molecular evidence for novel planctomycete diversity in a municipal wastewater treatment plant. *Appl. Environ. Microbiol.,* **69**, 7354–7363.

Cowan, S. T. and Steel, L. J. (1993) Manual for the Identification of Medical Bacteria, 3rd ed., Cambridge University Press, London, pp. 226–236.

Davis, K. E., Joseph, S. J., and Janssen, P. H. (2005) Effects of growth medium, inoculum size, and incubation time on culturability and isolation of soil bacteria. *Appl. Environ. Microbiol.,* **71**, 826–834.

Derakshani, M., Lukow, T., and Liesack, W. (2001) Novel bacterial lineages at the (sub)division level as detected by signature nucleotide-targeted recovery of 16S rRNA genes from bulk soil and rice roots of flooded rice microcosms. *Appl. Environ. Microbiol.,* **67**, 623–631.

Elshahed, M. S., Youssef, N. H., Luo, Q., Najar, F. Z., Roe, B. A., Sisk, T. M., Buhring, S. I., Hinrichs, K. U., and Krumholz, L. R. (2007) Phylogenetic and metabolic diversity of *Planctomycetes* from anaerobic, sulfide- and sulfur-rich Zodletone Spring, Oklahoma. *Appl. Environ. Microbiol.,* **73**, 4707–4716.

Ezaki, T., Hashimoto, Y., and 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.

Felsenstein, J. (1985) Confidence limits on phylogenies: An approach using the bootstrap. *Evolution,* **39**, 783–791.

Franzmann, P. D. and Skerman, V. B. (1984) *Gemmata obscuriglobus*, a new genus and species of the budding bacteria. *Antonie van Leeuwenhoek,* **50**, 261–268.

Fuerst, J. A. (2005) Intracellular compartmentation in planctomycetes. *Annu. Rev. Microbiol.,* **59**, 299–328.

Fukunaga, Y., Kurahashi, M., Tanaka, K., Yanagi, K., Yokota, A., and Harayama, S. (2006) *Pseudovibrio ascidiaceicola* sp. nov., isolated from ascidians (sea squirts). *Int. J. Syst. Evol. Microbiol.,* **56**, 343–347.

Fukunaga, Y., Kurahashi, M., Yanagi, K., Yokota, A., and Harayama, S. (2008) *Acanthopleuribacter pedis* gen. nov., sp. nov., a marine bacterium isolated from a chiton, and description of *Acanthopleuribacteraceae* fam. nov., *Acanthopleuribacterales* ord. nov., *Holophagaceae* fam. nov., *Holophagales* ord. nov. and *Holophagae* classis nov. in the phylum '*Acidobacteria.*' *Int. J. Syst. Evol. Microbiol.,* **58**, 2597–2601.

Gerhardt, P., Murray, R. G. E., Wood, W. A., and Krieg, N. R. (1994) Methods for General and Molecular Bacterioloy, American Society for Microbiology, Washington, DC.

Giovannoni, S. J., Schabtach, E., and Castenholz, R. W. (1987) *Isosphaera pallida*, gen. and comb. nov., a gliding, budding eubacterium from hot springs. *Arch. Microbiol.,* **147**, 276–284.

Hasegawa, M. and Kishino, H. (1994) Accuracies of the simple methods for estimating the bootstrap probability of a maximum-likelihood tree. *Mol. Biol. Evol.,* **11**, 142–145.

Hirsch, P. and Mueller, M. (1985) *Planctomyces limnophilus* sp. nov., a stalked and budding bacterium from freshwater. *Syst. Appl. Microbiol.,* **6**, 276–280.

Hugh, R. and Leifson, E. (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *J. Bacteriol.,* **66**, 24–26.

Inaga, S., Katsumoto, T., Tanaka, K., Kameie, T., Nakane, H., and Naguro, T. (2007) Platinum blue as an alternative to uranyl acetate for staining in transmission electron microscopy. *Arch. Histol. Cytol.,* **70**, 43–49.

Janssen, P. H. (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl. Environ. Microbiol.,* **72**, 1719–1728.

Kohler, T., Stingl, U., Meuser, K., and Brune, A. (2008) Novel lineages of *Planctomycetes* densely colonize the alkaline gut of soil-feeding termites (*Cubitermes* spp.). *Environ. Microbiol.,* **10**, 1260–1270.

König, E., Schlesner, H., and Hirsch, P. (1984) Cell wall studies on budding bacteria of the *Planctomyces*/*Pasteuria* group and on a *Prosthecomicrobium* sp. *Arch. Microbiol.,* **138**, 200–205.

Kulichevskaya, I. S., Ivanova, A. O., Baulina, O. I., Bodelier, P. L., Damste, J. S., and Dedysh, S. N. (2008) *Singulisphaera acidiphila* gen. nov., sp. nov., a non-filamentous, *Isosphaera*-like planctomycete from acidic northern wetlands. *Int. J. Syst. Evol. Microbiol.,* **58**, 1186–1193.

Kulichevskaya, I. S., Ivanova, A. O., Belova, S. E., Baulina, O. I., Bodelier, P. L., Rijpstra, W. I., Sinninghe Damste, J. S., Zavarzin, G. A., and Dedysh, S. N. (2007) *Schlesneria paludicola* gen. nov., sp. nov., the first acidophilic member of the order *Planctomycetales*, from Sphagnum-dominated boreal wetlands. *Int. J. Syst. Evol. Microbiol.,* **57**, 2680–2687.

Kurahashi, M. and Yokota, A. (2002) A preliminary report of phylogenetic diversity of bacterial strains isolated from marine creatures. *J. Gen. Appl. Microbiol.,* **48**, 251–259.

Kurahashi, M. and Yokota, A. (2004) *Agarivorans albus* gen. nov., sp. nov., a gamma-proteobacterium isolated from marine animals. *Int. J. Syst. Evol. Microbiol.,* **54**, 693–697.

Kurahashi, M. and Yokota, A. (2007a) *Endozoicomonas elysicola* gen. nov., sp. nov., a gamma-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst. Appl. Microbiol.,* **30**, 202–206.

Kurahashi, M. and Yokota, A. (2007b) *Tateyamaria omphalii* gen. nov., sp. nov., an alpha-proteobacterium isolated from a top shell *Omphalius pfeifferi pfeifferi*. *Syst. Appl. Microbiol.,* **30**, 371–375.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K. H. (2004) ARB: A software environment for sequence data. *Nucleic Acids Res.,* **32**, 1363–1371.

Mesbah, M., Premachandran, U., and Whitman, W. (1989) Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int. J. Syst. Bacteriol.,* **39**, 159–167.

Nakagawa, Y., Kawasaki, H., Nishii, T., Sakane, T., Yokota, A., and Hatano, K. (1998) Utilization of RAPD for quality control of bacterial cultures preserved in culture collections. *Microbiol. Cult. Coll.,* **14**, 15–21.

Nogales, B., Moore, E. R., Llobet-Brossa, E., Rossello-Mora, R., Amann, R., and Timmis, K. N. (2001) Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl. Environ. Microbiol.,* **67**, 1874–1884.

Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol.,* **17**, 208–212.

Rüger, H.-J. and Krambeck, H.-J. (1994) Evaluation of the BIOLOG substrate metabolism system for classification of marine bacteria. *Syst. Appl. Microbiol.,* **17**, 281–288.

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

Santelli, C. M., Orcutt, B. N., Banning, E., Bach, W., Moyer, C. L., Sogin, M. L., Staudigel, H., and Edwards, K. J. (2008) Abundance and diversity of microbial life in ocean crust. *Nature,* **453**, 653–656.

Schlesner, H. (1986) *Pirella marina* sp. nov., a budding, peptidoglycan-less bacterium from brackish water. *Syst. Appl. Microbiol.,* **8**, 177–180.

Schlesner, H. (1989) *Planctomyces brasiliensis* sp. nov., a halotolerant bacterium from a salt pit. *Syst. Appl. Microbiol.,* **12**, 159–161.

Schlesner, H. and Hirsch, P. (1984) Assignment of ATCC 27377 to *Pirella* gen. nov. as *Pirella staleyi* comb. nov. *Int. J. Syst. Bacteriol.,* **34**, 492–495.

Schlesner, H., Rensmann, C., Tindall, B. J., Gade, D., Rabus, R., Pfeiffer, S., and Hirsch, P. (2004) Taxonomic heterogeneity within the *Planctomycetales* as derived by DNA-DNA hybridization, description of *Rhodopirellula baltica* gen. nov., sp. nov., transfer of *Pirellula marina* to the genus *Blastopirellula* gen. nov. as *Blastopirellula marina* comb. nov. and emended description of the genus *Pirellula*. *Int. J. Syst. Evol. Microbiol.,* **54**, 1567–1580.

Starr, M. P. and Schmidt, J. M. (1984) *Planctomyces stranskae* (ex Wawrik 1952) sp.nov., nom.rev. and *Planctomyces guttaeformis* (ex Hortobyi 1965) sp.nov., nom.rev. *Int. J. Syst. Bacteriol.,* **34**, 470–477.

Swofford, D. L. (2000) PAUP*: Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4, Sinauer Associates, Sunderland, MA.

Tamura, T., Nakagaito, Y., Nishii, T., Hasegawa, T., Stackebrandt, E., and Yokota, A. (1994) A new genus of the order *Actinomycetales*, *Couchioplanes* gen. nov., with descriptions of *Couchioplanes caeruleus* (Horan and Brodsky 1986) comb. nov. and *Couchioplanes caeruleus* subsp. *azureus* subsp. nov. *Int. J. Syst. Bacteriol.,* **44**, 193–203.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.,* **25**, 4876–4882.

Wagner, M. and Horn, M. (2006) The *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance. *Curr. Opin. Biotechnol.,* **17**, 241–249.

Wang, J., Jenkins, C., Webb, R. I., and Fuerst, J. A. (2002) Isolation of *Gemmata*-like and *Isosphaera*-like planctomycete bacteria from soil and freshwater. *Appl. Environ. Microbiol.,* **68**, 417–422.