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Phylogenetic and Taxonomic Analyses of *Rhodopirellula caenicola* Sp. Nov., a New Marine *Planctomycetes* Species Isolated from Iron Sand

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Abstract

A Gram-negative, strictly aerobic, pink-pigmented, non-motile, spherical bacterium designated YM26-125^T was isolated from iron sand. Phylogenetic analyses based on the 16S rRNA gene sequence indicated that the new strain was affiliated with the phylum *Planctomycetes* and shared 96.1% sequence similarity with *Rhodopirellula baltica* SH 1^T. The strain could be also differentiated phenotypically from the *Rhodopirellula baltica* SH 1^T. The major fatty acids of strain YM26-125^T were identified as C16:0 and C18:1 ω9c as defined by the MIDI system. Amino acid analysis of cell wall hydrolysates indicated that the novel isolate did not contain diaminopimelic acid in their cell walls. The DNA G+C content was determined to be 57.5 mol%, the major respiratory quinone was identified as menaquinone 6 (MK-6) and a polar lipid profile was present consisting of a phosphatidylglycerol, a diphosphatidylglycerol and three unidentified phospholipids. From the distinct phylogenetic position and combination of genotypic and phenotypic characteristics, the strain is considered to represent a novel species for which the name *Rhodopirellula caenicola* sp. nov. is proposed. The type strain of *R. caenicola* is YM26-125^T (=KCTC 32995^T=NBRC 110016^T).

Keywords: *Planctomycetes*; *Rhodopirellula caenicola* sp. nov; Iron sand; 16S rRNA gene; Polyphasic taxonomy

Introduction

Members of phylum *Planctomycetes* [1] constitute a significant proportion of diverse range of natural environments including soil, aquatic freshwater, marine macroalgae and invertebrates [2-6]. However, the majority of them are uncultivated and the known ecophysiological types of planctomycetes are quite limited [7-9]. The genus *Rhodopirellula* was first proposed by Schlesner et al. [10]. This genus is affiliated with the family *Planctomycetaceae* within the phylum *Planctomycetes* that is a budding and peptidoglycan-less phylogenetic group [11]. At the time of writing, the genus *Rhodopirellula* (www.bacterio.cict.fr) comprises only one species, *Rhodopirellula baltica* SH 1^T [10] and three not yet been validly published members, '*Rhodopirellula rosea*' LHWP3 [12], '*Rhodopirellula lusitana*' UC17 and '*Rhodopirellula rubra*' LF2 [13]. In 2006, in the course of our study on the diversity of culturable marine bacteria in iron sand samples collected from Murohama Beach, Kamaishi, Iwate, Japan, a bacterium, designated YM26-125^T, was isolated. Phylogenetic analysis based on the 16S rRNA gene sequences revealed that the novel strain was belonged to the family *Planctomycetaceae*, with their closest relative being *Rhodopirellula baltica* SH 1^T that is the only validated species. In this study, we characterised a novel marine planctomycete strain, YM26-125^T, isolated from iron sand by using polyphasic taxonomic methods, including the 16S rRNA gene sequence analysis, physiological, biochemical and chemotaxonomic analyses. Based on the polyphasic taxonomic data, we suggest that the isolate represents a new species of the family *Planctomycetaceae* within the phylum *Planctomycetes*.

Materials and Methods

Isolation of the bacterial strain and culture conditions

Strain YM26-125^T was isolated from iron sand in depth of 30 cm from the surface collected at Murohama Beach, Kamaishi, Iwate, Japan in July 2006. Iron sand (approximately 1 g) was rinsed in 10 mL of sterile artificial seawater and then collected with a magnet. After more than 10 cycles of rinse and collection, a 50 µL of the suspension was spread onto a plate (φ 9 cm) containing medium 'P' [14]. The agar medium was incubated for 4 weeks at room temperature and the pink coloured colonies that grew were purified on a fresh marine agar 2216.

The strain was routinely subcultured on marine agar 2216 at 28°C and maintained in marine broth 2216 (Difco) supplemented with 20% (v/v) glycerol at -70°C.

Morphological, physiological and biochemical analysis

Cell morphology was observed by using transmission electron microscopy (TEM) and motility was measured by phase contrast microscopy (Primo Star; ZEISS). Gliding motility was determined as described by Perry [15]. For TEM, cells were mounted on Formvar-coated copper grids and negatively stained with 1% (w/v) aqueous uranyl acetate. Grids were observed using a Hitachi H-7100 microscope operated at 75 kV at a magnification of 20,000. A flagella staining was carried out according to Blenden and Goldberg [16]. The temperature range (4, 10, 15, 20, 30, 37, 40 and 45°C) and pH range (5.5-9.5) for colony growth were determined by incubating the isolate for 2 weeks on marine agar 2216. The following buffers were used for pH tests: MES (pH 5.5), ACES (pH 6.5 and 7.0), TAPSO (pH 7.6), TAPS (pH 8.5) and CHES (pH 9.0 and 9.5). The NaCl concentration for growth was determined on marine agar 2216 containing 0-10% (w/v) NaCl [17]. Gram-staining was performed using the BD Gram Staining Kit (Becton, Dickinson and Company, USA). Spore formation was tested by staining with malachite green. Anaerobic growth was tested for up to 2 weeks on marine agar 2216 in a jar containing AnaeroPack-Anaero (Mitsubishi Gas Chemical Co, Inc), which works as an O_2 absorber and CO_2 generator. Catalase activity was detected by the observation of the

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formation of bubbles in 3% (v/v) H_2O_2 solution. Oxidase activity test was performed using commercial dropper oxidase (Becton, Dickinson and Co). Degradation of DNA was tested using DNase agar [DNase agar (Scharlau Chemie)] [18] , with DNase activity detected by flooding plates with 1 M HCl. Starch hydrolysis were tested as described by Choi et al. [19]. The ability to hydrolyse casein, Tween 20, Tween 80 and tyrosine were determined according to Hansen and Sørheim [20]. API 20E, API 50CH and API ZYM strips (bioMérieux) were used to determine the physiological and biochemical characteristics. All suspension media for the API test strips were supplemented with 0.85% (w/v) NaCl solution (final concentration). API 20E, API 50CH and API ZYM test strips were read after 72 h incubation at 28°C. Flexirubin-type pigments were investigated by using the bathochromatic shift test with a 20% (w/v) KOH solution [21].

Determination of DNA G+C content, 16S rRNA gene sequencing and phylogenetic analysis

Genomic DNA was prepared according to the method of Marmur [22] from cells grown on marine agar 2216 and the DNA base composition was determined by using the HPLC method of Mesbah et al. [23].

An approximately 1,500 bp long fragment of the 16S rRNA gene was amplified from the extracted DNA by using bacterial universal primers specific to the 16S rRNA gene: 27F and 1,492R (*Escherichia coli* numbering system) [24]. Technical details of phylogenetic and taxonomic analyses were performed according to the method of Liu and Zhao [25]. To ascertain the phylogenetic position of the novel isolate, the 16S rRNA gene sequence of strain YM26-125^T (GenBank/EMBL/DDBJ accession number AB983339) was compared with sequences obtained from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). Multiple alignments of the sequences were performed using CLUSTAL_X (version 1.83) [26]. Alignment gaps and ambiguous bases were not taken into consideration when 1,315 bases of the 16S rRNA gene were compared. Evolutionary distances (distance options according to Kimura's two-parameter model) [27] were calculated and clustering was performed with the neighbour-joining method [28], maximum-parsimony (Fitch) [29] and maximum-likelihood [30] methods using MEGA5 software [31]. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1,000 resamplings [30]. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein [30] with 1,000 replicates.

Chemotaxonomic analysis

Gas chromatography analysis of the cellular fatty acid methyl esters was performed using a culture grown on marine agar 2216 at 28°C for 7 days and fatty acid methyl esters were extracted and prepared according to standard protocols provided by the MIDI/Hewlett Packard Microbial Identification system Sherlock Version 3.10/TSBA 50 [32]. Cell walls were prepared by the methods described by Schleifer and Kandler [33] and the amino acids present in an acid hydrolysate of the cell walls were identified by TLC (Harper and Davis) [34]. Determination of the respiratory quinone system was carried out as described previously (Collins and Jones) [35].

Results and Discussion

Morphological, physiological and biochemical characteristics

Cells of strain YM26-125^T grown on marine agar 2216 were coccoid and mostly 1.0-1.1 µm in diameter, devoid of flagella or cell appendages (Figure 1) and produced a pink pigment. Gliding motility was not observed by a light microscopy. Flexirubin-type pigments were not produced. The strain also showed distinct phenotypic, physiological and biochemical features that discriminated it from the closest described member in the genus *Rhodopirellula* as shown in Table 1.

Molecular phylogenetic analysis

The almost complete 16S rRNA gene sequence was determined for strain YM26-125^T (GenBank/EMBL/DDBJ accession number AB983339). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YM26-125^T belonged to the genus *Rhodopirellula* within the phylum *Planctomycetes* and showed highest sequence similarity (96.1%) to *Rhodopirellula baltica* SH 1^T, which is the only validly described species (Figure 2). Thus, on the basis of phylogenetic data presented, we believe that strain YM26-125^T should be considered as representative of a novel species of the family *Planctomycetaceae* within the phylum *Planctomycetes*.

Chemotaxonomic characteristics

As shown in Table 2, the predominant cellular fatty acids of strain YM26-125^T differentiate it from recognized species of the genus *Rhodopirellula baltica* SH 1^T based on the proportions of C16:0 (23.2%) and C18:1 ω9c (51.8%) as defined by the MIDI system. On the basis of the fatty acid composition, strain YM26-125^T could be differentiated from the phylogenetically closest taxon *Rhodopirellula baltica* SH 1^T as shown in Table 2. Furthermore, strain YM26-125^T could be distinguished from the neighbouring species within the genus *Rhodopirellula* by the presence of C17:0, C16:1 ω11c and iso-C17:0 and the absence of C10:0.

Amino acid analysis of the cell wall hydrolysates indicated the absence of diaminopimelic acid in the cell wall, which suggests that the strain does not contain peptidoglycan in their cell walls. From these results, it is strongly suggested that strain YM26-125^T represents an independent species of the genus *Rhodopirellula* within the phylum *Planctomycetes*.

Polyphasic taxonomic conclusion

From the distinct phylogenetic position and combinations of genotypic and phenotypic characteristics, strain YM26-125^T cannot be assigned to any previously recognized species in the genus *Rhodopirellula* and thus can be described as representing a novel species, *Rhodopirellula caenicola* sp. nov.

Description of *Rhodopirellula caenicola* sp. nov.

Rhodopirellula caenicola (cae.ni'co.la. L. n. *caenum*, mud; L: suff. *-cola*, inhabitant, dweller; N.L. fem. n. *caenicola*, mud inhabitant). Cells are strictly aerobic cocci that are 1.0-1.1 µm in diameter. Cells lack flagella and are non-motile. Neither cellular gliding movement nor swarming growth is observed. Colonies grown on marine agar 2216 are circular and pink pigmented after 7 days of incubation at 28°C. Temperature range for growth is 20-30°C; the optimal temperature is around 28°C but no growth occurs at 4 or 45°C. The pH range for growth is 6-8 (optimum, pH 7), while no growth was observed below 6 or above 8. NaCl is required for growth and can be tolerated at a concentration of up to 5% (w/v) but no growth occurs above 6% (w/v) NaCl. Nitrate and nitrite reduction are negative. Gelatin and urea are hydrolysed but agar, casein, DNA, starch, tyrosine, Tween 20 and Tween 80 are not. The reactions for arginine dihydrolase, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), ornithine decarboxylase, citrate utilization and Voges-Proskauer test are positive but hydrogen sulfide production, indole production and lysine decarboxylase activities are negative (API

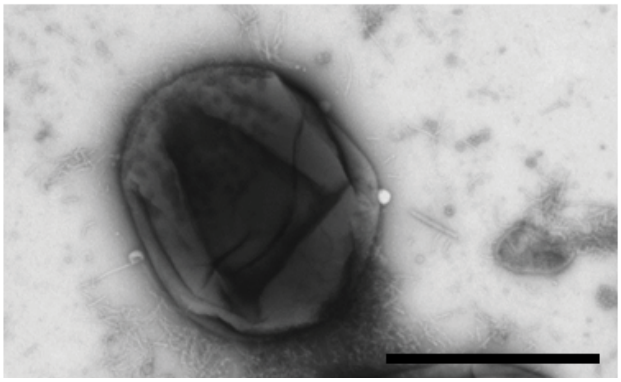

Figure 1: Transmission electron micrograph of a negatively stained cell of strain YM26-125T340. Bar, 1 µm.

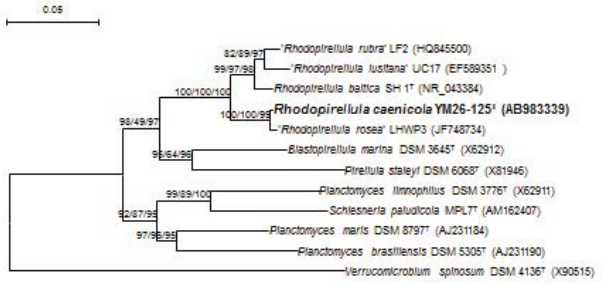

Figure 2: Neighbour-joining tree of 16S rRNA gene sequence similarity, showing the phylogenetic position of strain YM26-125^T and representatives of the phylum Planctomycetes. The sequence of *Verrucomicrobium spinosum* DSM 4136^T (X90515) was used as an out group. The sequence determined in this study is shown in bold. Bootstrap values from neighbour-joining, maximum-parsimony and maximum likelihood analyses are shown (NJ/MP/ML). Bar, 5% sequence divergence.

20E). Acid production tests using API50CH strips give the following reactions: acid is produced from L-xylose, *N*-acetyl-glucosamine, esculin ferric citrate, maltose and melibiose but not from D-arabinose, galactose, glucose, fructose, mannose, arbutin, D-turanose, methyl-β-D-xylopyranoside, salicin, L-arabitol, 5-keto-gluconate, amygdalin, lactose, sucrose, trehalose, starch, glycogen, gentiobiose, L-fucose, ribose, sorbose, rhamnose, sorbitol, methyl-α-D-mannopyranoside, L-arabinose, D-xylose, methyl-α-D-glucopyranoside, cellobiose, melezitose, D-lyxose, D-tagatose, D-fucose, inulin, raffinose, glycerol, erythritol, adonitol, dulcitol, inositol, mannitol, xylitol, D-arabitol, gluconate and 2-keto-gluconate. In the API ZYM strip, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase and α-fucosidase are present but trypsin, acid phosphatase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, esterase lipase (C8), α-galactosidase, lipase (C4), cystine arylamidase, α-chymotrypsin, β-glucuronidase and α-mannosidase are absent. The major fatty acids are C16:0 (23.2%) and C18:1 *ω*9c (51.8%). The major polar lipids are a phosphatidylglycerol, a diphosphatidylglycerol and three unidentified phospholipids. The G+C of the genomic DNA of the type strain is 57.5 mol% (Supplementary Figure 1).

The type strain is YM26-125^T (= KCTC 32995^T=NBRC 110016^T), which was isolated isolated from iron sand collected at Murohama Beach, Kamaishi, Iwate, Japan. The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of strain YM26-125^T is AB983339.

Characteristics	1	2
Pigmentation	Pink	Pink to red
Cell size (µm)	1.0-1.1	1.0-2.5×1.2-2.3
Artificial seawater requirement	–	+
Temperature range for growth (°C)	20-30	5-30
Oxidase	–	+
Hydrolysis of:		
Urea	+	–
Gelatin	+	–
Starch	–	+
Acid production from:		
Cellobiose	–	+
Fructose	–	+
Galactose	–	+
Glycerol	–	+
Lactose	–	+
Enzyme activity of:		
Cystine arylamidase	–	+
α-Fucosidase	+	–
α-Glucosidase	–	+
β-Galactosidase	+	–
β-Glucosidase	–	+
Polar lipids	PG, DPG, 3UPL	PC, PG
DNA G+C content (mol%)	57.5	55
Strains: *1* YM26-125^T (*Rhodopirellula caenicola* sp. nov.; present study), 2 *Rhodopirellula baltica* SH 1^T [10,12]		
Symbols: *PG* phosphatidylglycerol, *DPG* diphosphatidylglycerol, *UPL* unidentified phospholipid, *PC* phosphatidylcholine, + positive, – negative		

Table 1: Differential characteristics of strain YM26 125^T and other closely related taxa.

Fatty acid	1	2
C10:0	–	4.9
C12:0	tr	–
C14:0	tr	–
C16:0	23.2	37.6
C17:0	3.3	–
C18:0	5.8	11.3
C16:1 *ω*11c	2.1	–
C17:1 *ω*8c	4.5	3.9
C18:1 *ω*9c	51.8	28.7
iso-C15:0	tr	–
iso-C17:0	1.3	–
Summed feature 3[a]	1.3	7.3
[a]Summed feature 3 consists of C16:1 *ω7c* and/or iso-C15:0 2-OH.		
Strains: *1* YM26-125^T (*Rhodopirellula caenicola* sp. nov.; present study), 2 *Rhodopirellula baltica* SH 1^T [12]		
Symbol: *tr* trace (less than 1.0%), – not detected. The data were typically obtained by GLC using the MIDI system.		

Table 2: Comparison of cellular fatty acids for strain YM26-125^T and other closely related taxa.

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