Phycicola gilvus gen. nov., sp. nov., an actinobacterium isolated from living seaweed

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A Gram-positive, aerobic, non-mycelium-forming actinomycete, designated strain SSWW-21^T, was isolated from a living seaweed sample on the coast of Jeju, Republic of Korea. Cells of the organism were rod-shaped or coccoid depending on culture age. Short rods were observed in young cultures, whereas older cultures predominantly consisted of coccoid cells. Rod-shaped cells were motile by means of flagella, but coccoid cells were non-motile. Budding-like cell division was observed. The temperature and pH for growth were 4–30 °C and pH 6.1–10.1. Growth occurred in the presence of up to 2% NaCl. Phylogenetic analyses showed that the organism formed a distinct clade within the family *Microbacteriaceae*. Comparative 16S rRNA gene sequence analyses showed that strain SSWW-21^T was related most closely to the type strains of *Okibacterium fritillariae* (96.8% similarity), *Leifsonia poae* (96.8%) and *Agreia bicolorata* (96.6%). Levels of 16S rRNA gene sequence similarity between the new isolate and members of other genera within the family *Microbacteriaceae* were in the range 93.1–96.3%. The cell-wall peptidoglycan of strain SSWW-21^T was of the B-type (2,4-diaminobutyric acid as the diagnostic diamino acid). The acyl type of the muramic acid was acetyl. The predominant menaquinone was MK-11. The polar lipid profile contained phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and diphosphatidylglycerol. Mycolic acids were not present. The major cellular fatty acids were saturated branched-chain components (anteiso-C_{15:0} and anteiso-C_{17:0}). The G + C content of the DNA was 69.8 mol%. On the basis of the phenotypic and phylogenetic data presented, strain SSWW-21^T is considered to represent a novel species of a new genus within the family *Microbacteriaceae*, for which the name *Phycicola gilvus* gen. nov., sp. nov. is proposed. The type strain of *Phycicola gilvus* is SSWW-21^T (=KCTC 19185^T =DSM 18319^T).

The family *Microbacteriaceae* Park *et al.* 1993 emend. Rainey, Ward-Rainey and Stackebrandt 1997 (Park *et al.*, 1993; Stackebrandt *et al.*, 1997) as originally described comprised seven actinobacterial genera, namely *Microbacterium* (the type genus), *Agrococcus*, *Agromyces*, *Aureobacterium*, *Clavibacter*, *Curtobacterium* and *Rathibacter*, and was characterized chemotaxonomically by the combination of B-type peptidoglycan and unsaturated menaquinones. Since then, 17 genera, including the recently described genera *Microcella* (Tiago *et al.*, 2005), *Yonghaparkia* (Yoon *et al.*, 2006) and *Labeledella* (Lee, 2007), have been included in the family on the basis of data from polyphasic studies. Among

members of the family, *Salinibacterium amurskyense* (Han *et al.*, 2003) and *Labeledella gwakjiensis* (Lee, 2007) were recently isolated from marine environments, i.e. seawater and dried seaweed, respectively.

Strain SSWW-21^T was isolated from a living seaweed sample collected around Samyang Beach in Jeju, Republic of Korea. Seaweed samples were scraped from rock surfaces of a beach with a spatula and placed directly into sterilized 50-ml Falcon tubes. For bacterial isolation, a seaweed sample (1 g) was placed into a test tube containing 9 ml sterile seawater and mixed in a tube rotator for 30 min. After serial dilution of the sample with sterile seawater, the diluents were transferred onto SC-SW agar plates containing sterilized natural seawater (Lee, 2006). A colony on the plates, incubated at 30 °C for 14 days, was subcultured on YE-SW medium (0.4% yeast extract, 1.0% malt extract,

Abbreviation: DAB, 2,4-diaminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain SSWW-21^T is AM286414.

0.4 % glucose and 1.8 % agar in a mixture of 60 % seawater and 40 % distilled water) and the pure culture was maintained in 20 % (v/v) glycerol supplemented with 60 % sterilized seawater at -20 and -80 °C.

Cell morphology and motility were investigated by using an Olympus light microscope equipped with phase-contrast optics (magnification $\times 400$) and a model JEM-1200EXII transmission electron microscope (JEOL). Cells of strain SSWW-21^T were cultivated in trypticase soy broth (TSB; Difco) at 30 °C for 6, 15, 24, 48 and 72 h. Colony pigmentation and morphology were observed by using 7-day cultures grown at 30 °C on trypticase soy agar (TSA; Difco). Growth was tested at different temperatures (4, 10, 25, 30, 37 and 42 °C) and pH (4.1–10.1). NaCl tolerance for growth was investigated on ISP 2 medium (Shirling & Gottlieb, 1966) supplemented with 1–9 % (w/v) NaCl. Tests for degradation and hydrolysis of several compounds, Gram staining and catalase and oxidase activities were carried out as described by Lee (2006). Urease activity was tested by using Bacto urea broth (Difco). Acid production from carbohydrates and alcohols was determined by using Bacto OF basal medium (Difco) as described by Lee (2007). Tests with API 20E and API ZYM strips (bioMérieux) were performed according to the manufacturer's instructions.

Cells of strain SSWW-21^T were rod-shaped or coccoid depending on culture age (Fig. 1). Rod-shaped cells were observed in young cultures. Older cultures predominantly revealed coccoid cells that were arranged singly or rarely in pairs. V cell arrangements and branching were not found. Both rod and coccoid forms were Gram-positive. Rods were motile by means of flagella, but coccoid cells were

non-motile. Budding-like morphology was also observed (Fig. 1b). Such budding-like morphology has not previously been reported for recognized members of the *Microbacteriaceae*. The results of other cultural, physiological and biochemical tests are given in the genus and species descriptions below.

Extraction of genomic DNA, PCR amplification and direct sequencing of the 16S rRNA gene and phylogenetic analyses were performed as described by Lee (2006). The 16S rRNA gene sequence (1406 nt) of strain SSWW-21^T was aligned with the corresponding sequences retrieved from public databases by using CLUSTAL_X software (Thompson *et al.*, 1997). A final tree was developed by using the neighbour-joining method (Saitou & Nei, 1987) and the model of Jukes & Cantor (1969), on the basis of 1353 unambiguously aligned positions of the sequences of all test strains. Strain SSWW-21^T formed a distinct phylogenetic branch within the radiation of the family *Microbacteriaceae* and was loosely associated with members of the genus *Agrococcus* (Fig. 2). This relationship was supported by a moderate bootstrap value (58 %), but was also found in both the maximum-parsimony and maximum-likelihood trees. Strain SSWW-21^T showed highest 16S rRNA gene sequence similarities to the type strains of *Okibacterium fritillariae* (96.8 %), *Leifsonia poae* (96.8 %) and *Agreia bicolorata* (96.6 %). Strain SSWW-21^T showed levels of 16S rRNA gene sequence similarity of 93.1–96.3 % to representatives of other genera within the family *Microbacteriaceae*.

Purified cell walls were prepared by the method of Schleifer & Kandler (1972). The molar ratios of amino acids were

Fig. 1. Transmission electron micrographs of cells of strain SSWW-21^T cultivated in TSB at 30 °C for 24 (a), 48 (b) and 72 (c) h. Bars, 500 (a), 200 (b) and 100 (c) nm.

Fig. 2. Neighbour-joining tree showing the phylogenetic position of SSWW-21^T within the family *Microbacteriaceae*. Asterisks indicate branches that were also found in both the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Numbers at nodes indicate bootstrap support values (Felsenstein, 1985), based on 1000 replications (only values >40% are indicated). Bar, 0.05 substitutions per nucleotide position.

determined by GC and GC-MS of *N*-heptafluorobutyryl amino acid isobutyl esters, as described by MacKenzie (1987). The amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional TLC on cellulose plates, as described by Schleifer & Kandler (1972). The molar ratio of alanine/glycine/2,4-diaminobutyric acid (DAB)/glutamic acid was estimated to be 0.6:1.2:1.5:1.0. The diagnostic diamino acid in the cell-wall peptidoglycan was DAB. Homoserine was not detected in the peptidoglycan hydrolysates. The partial hydrolysates contained the peptide Gly-Glu typical of B-type peptidoglycans. From these data, the peptidoglycan of strain SSWW-21^T was inferred to be of B2_γ type (or B7; see <http://www.dsmz.de/species/murein.htm>). Analyses of polar lipids (Minnikin *et al.*, 1977), isoprenoid quinones (Kroppenstedt, 1985), mycolic acids (Minnikin *et al.*, 1980) and the acyl type of murein (Uchida & Aida, 1984) were performed as described previously. Cells were cultivated in TSB for 3 days at 30 °C, washed (after harvesting by centrifugation) with distilled water and freeze-dried. The polar lipid profile of strain SSWW-21^T contained phosphatidylcholine,

phosphatidylglycerol, phosphatidylinositol and diphosphatidylglycerol (Fig. 3). The identification of phosphatidylcholine was finally confirmed by TLC of a polar lipid extract supplemented with authentic phosphatidylcholine (Sigma). The presence of phosphatidylcholine is unusual in the family *Microbacteriaceae* and can serve as a key marker for distinguishing the novel isolate from members of genera within the family. The major menaquinone was MK-11 (71%). Minor amounts of MK-10 (15%), MK-12 (9%) and MK-9 (5%) were also detected. No mycolic acids were present. The G+C content of the DNA was determined by HPLC according to the method of Mesbah *et al.* (1989). The DNA G+C content of strain SSWW-21^T was 69.8 mol%.

Preparation and analysis of cellular fatty acid methyl esters were performed by GC according to the standard protocols of the Sherlock Microbial Identification System (version 6; MIDI), by using 3-day cultures grown on TSA at 30 °C. The predominant fatty acids were anteiso-C_{15:0} (49.7%) and anteiso-C_{17:0} (27.3%). The following fatty acids were

Fig. 3. Polar lipid profile of strain SSWW-21^T. DPG, Diphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

present as minor components: iso-C_{16:0} (6.1 %), C_{16:0} (5.1 %), C_{18:0} (5.0 %), iso-C_{15:0} (3.6 %) and iso-C_{17:0} (2.6 %). Other components (<1 %) were C_{9:0} and iso-C_{14:0}.

Differential phenotypic characteristics between strain SSWW-21^T and members of related genera in the family *Microbacteriaceae* are given in Table 1. The new isolate can also be distinguished from all other genera within the family *Microbacteriaceae* based on its polar lipid profile and budding-like cell division. On the basis of the phenotypic and molecular genetic data presented here, strain SSWW-21^T is considered to represent a novel species of a new

genus within the family *Microbacteriaceae*, for which the name *Phycicola gilvus* gen. nov., sp. nov. is proposed.

Description of *Phycicola* gen. nov.

Phycicola [Phy'ci.co.la. L. n. *phycos* -i (from Gr. n. *phukos*) seaweed; L. masc. suffix -cola inhabitant; N.L. masc. n. *Phycicola* inhabitant of seaweed].

Cells are aerobic, Gram-positive, catalase-positive, non-spore-forming, non-mycelium-forming. Cells are rod-shaped or coccoid depending on culture age. Budding-like morphology may be observed. Cell-wall peptidoglycan is of the B-type (DAB as the diagnostic diamino acid). The acyl type of the muramic acid is acetyl. The predominant menaquinone is MK-11. The polar lipid profile contains phosphatidylcholine, phosphatidylglycerol, phosphatidylinositol and diphosphatidylglycerol. Mycolic acids are not present. The major cellular fatty acids are saturated branched-chain components (anteiso-C_{15:0} and anteiso-C_{17:0}). Phylogenetically, the genus belongs to the family *Microbacteriaceae*, suborder *Micrococcineae*. The type species is *Phycicola gilvus*.

Description of *Phycicola gilvus* sp. nov.

Phycicola gilvus (gil'vus. L. masc. adj. *gilvus* pale yellow-coloured).

Has the following characteristics in addition to those given for the genus above. Young cultures contain rod-shaped cells (0.40–0.50 × 0.80–1.20 µm), whereas older cultures contain coccoid cells (0.18–0.33 µm in diameter). Rods are motile by means of flagella, but coccoid cells are non-motile. Colonies are circular, smooth, entire and reach 1–2 mm in diameter after 5 days incubation on TSA. Colony colour is variable depending on culture conditions;

Table 1. Differential phenotypic characteristics between strain SSWW-21^T and members of related genera in the family *Microbacteriaceae*

Taxa: 1, strain SSWW-21^T; 2, *Agreia* (data from Evtushenko *et al.*, 2001; Schumann *et al.*, 2003); 3, *Leifsonia* (Evtushenko *et al.*, 2000; Suzuki *et al.*, 1999); 4, *Okibacterium* (Evtushenko *et al.*, 2002). Orn, D-Ornithine; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PI, phosphatidylinositol; v, variable depending on species. Strain SSWW-21^T can be distinguished from all other genera of the family *Microbacteriaceae* based on its polar lipid profile and budding-like cell division.

Characteristic	1	2	3	4
Cell morphology	Short rods to cocci	Irregular rods	Irregular rods or filaments	Irregular rods or oval to irregular cocci
Budding-like cell division	+	–	–	–
Diphtheroid arrangement	–	+	+	+
Motility	+	v	v	–
Diamino acid(s)	DAB	DAB, Orn	DAB	Lys
Acyl type of murein	Acetyl	Acetyl	Acetyl	Glycolyl
Major menaquinone(s)	11	10 or 10, 11	11, 10 or 11, 12	10, 11
Phospholipids	DPG, PC, PG, PI	DPG, PG	DPG, PG	DPG, PG
DNA G + C content (mol%)	69.8	65–67	66–73	67

colonies are white when cells are incubated in the dark, but yellow when incubated in the light. Oxidase-negative. The temperature range for growth is 4–30 °C, with optimum growth at 25 °C. No growth occurs at ≥ 37 °C. The pH range for growth is pH 6.1–10.1, with optimum growth at pH 7.1–8.1. Growth occurs in the presence of 2 % NaCl but not with 3 % NaCl. Indole and H₂S are not produced. Nitrate is reduced to nitrite. Unable to utilize citrate. Voges–Proskauer test is weakly positive. Negative for arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase. Hydrolyses aesculin but not casein, elastin, starch, gelatin or urea. Decomposition of hypoxanthine, DL-tyrosine and xanthine does not occur. Acid is produced from L-arabinose, cellobiose, dextran, D-galactose, D-glucose, glycerol, maltose, L-rhamnose, D-ribose, salicin and sucrose. Acid production from D-arabinose and D-xylose is weak. Acid is not produced from the following carbon and energy sources: adonitol, 2,3-butanediol, dulcitol, meso-erythritol, D-fructose, myo-inositol, inulin, D-lactose, D-mannitol, D-mannose, melezitose, melibiose, methyl α -D-glucoside, methyl α -D-mannoside, 1,2-propanediol, raffinose, D-sorbitol, L-sorbose, trehalose or D-xylitol. In tests with the API ZYM system, positive for alkaline phosphatase, leucine arylamidase, naphthol-AS-BI phosphohydrolase, acid phosphatase and α -glucosidase. Weakly positive for esterase lipase (C8), trypsin, β -glucosidase and N-acetyl- β -glucosaminidase. The following enzyme activities give negative responses: esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, α -galactosidase, β -galactosidase, α -glucuronidase, α -mannosidase and α -fucosidase. The major fatty acids are anteiso-C_{15:0} (49.7 %) and anteiso-C_{17:0} (27.3 %). The G + C content of the DNA is 69.8 mol%.

The type strain, SSWW-21^T (=KCTC 19185^T =DSM 18319^T), was isolated from a living seaweed sample collected on the coast of Jeju, Republic of Korea.

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