Labeledella gwakjiensis gen. nov., sp. nov., a novel actinomycete of the family *Microbacteriaceae*

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A Gram-positive, non-motile, non-mycelium-forming, rod-shaped actinomycete, designated KSW2-17^T, was isolated from dried seaweed collected from beach sand along the coast of Jeju, Republic of Korea. The organism had ornithine as the diagnostic cell-wall diamino acid, MK-10 and MK-11 as the major menaquinones, and phosphatidylglycerol and diphosphatidylglycerol as polar lipids. The fatty acid profile included predominantly iso- and anteiso-branched acids and a minor amount of tuberculostearic acid (10-methyl C_{18:0}). The DNA G+C content was 68.0 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that the seaweed isolate formed a distinct clade within the radiation of the family *Microbacteriaceae* and had highest sequence similarity (96.1–96.3%) to members of the genera *Cryobacterium*, *Frigoribacterium* and *Rathayibacter*. On the basis of phenotypic and genotypic evidence, strain KSW2-17^T is considered to represent a novel species of a new genus, for which the name *Labeledella gwakjiensis* gen. nov., sp. nov. is proposed. The type strain is KSW2-17^T (=JCM 14008^T=KCTC 19176^T).

The family *Microbacteriaceae* (Park *et al.*, 1993; Stackebrandt *et al.*, 1997) embraces a large group of rod-shaped or rarely coccoid or mycelium-forming actinobacteria that have B-type cell-wall peptidoglycan and unsaturated menaquinones. At the time of writing, the family comprises 20 genera with validly published names, including the recently described genera *Gulosibacter*, *Pseudoclavibacter* (Manai *et al.*, 2004) and *Microcella* (Tiago *et al.*, 2005). The genera of the family can be readily differentiated on the basis of chemotaxonomic characters, such as diagnostic diamino acids of the cell-wall peptidoglycan (ornithine, lysine, 2,4-diaminobutyric acid), peptidoglycan type, major menaquinones and cellular fatty acid composition. Members of the family occur in diverse environments, including seawater and marine mud (Evtushenko & Takeuchi, 2003; Han *et al.*, 2003). In this paper, we describe an actinomycete isolated from seaweed, which represents a novel species of a new genus within the family *Microbacteriaceae*.

Strain KSW2-17^T was isolated from a dried seaweed sample collected at Gwakji Beach in Jeju, Republic of Korea, during a study of exopolysaccharide-producing marine bacteria. A piece of dried seaweed was transferred directly onto a WAT-SW agar plate (Lee, 2006). One colony on the plate, incubated at 30 °C for 14 days, was subcultured onto TSA-SW medium [trypticase soy agar (TSA; Difco) in a mixture of 60% (v/v) natural seawater and 40% (v/v) distilled water]. The pure culture was maintained in 20% glycerol suspension supplemented with 60% natural

seawater at –20 and –80 °C. Strain KSW2-17^T showed good growth on YE-SW medium [ISP 2 medium (Shirling & Gottlieb, 1966) supplemented with 60% (v/v) seawater], TSA and nutrient agar (NA; Difco), but moderate growth on NA-SW (NA plus seawater), marine agar (MA; Difco) and ISP 2 medium.

Cell morphology was observed via phase-contrast and electron microscopy with cells grown on TSA for 6, 15, 24 and 72 h. For scanning electron microscopy, cells were fixed with 1% (w/v) osmium tetroxide for 1 h, dehydrated through a graded ethanol series and substituted with isoamyl acetate. After critical-point drying in CO₂, the samples were coated with gold and observed by using a scanning electron microscope (model S-2460; Hitachi). Cell motility was tested by monitoring the degree of turbidity on motility test medium (0.3% beef extract, 1.0% peptone, 0.5% NaCl and 0.4% Bacto agar) as described by Mac Faddin (1980) and by phase-contrast microscopy. Colony pigmentation and morphology were observed with cultures grown on TSA at 30 °C for 5 days. Oxidase and catalase activities were determined with solutions of 1% (w/v) tetramethyl-*p*-phenylenediamine and 3% (v/v) H₂O₂, respectively. For these tests, cells were grown on TSA at 30 °C for 48 h. β -Galactosidase activity was determined by using the method described by Gosink *et al.* (1998). Gram stain, hydrolysis of aesculin, elastin and starch, reduction of nitrate, and production of H₂S were investigated according to the methods described by Mac Faddin (1980). Growth temperature was determined on TSA at 4–45 °C. NaCl tolerance for growth was tested on TSA supplemented with 1–9% (w/v) NaCl. The pH range

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain KSW2-17^T is DQ533552.

(4.1–10.1) for growth was determined on TSA with pH adjustment at intervals of 1.0 pH unit. Production of acid from carbohydrates and alcohols was determined on Bacto OF basal medium (Difco) supplemented with filter-sterilized carbon source at a final concentration of 1% (w/v). Cells were grown in trypticase soy broth (TSB; Difco) at 30 °C for 2 days and harvested by centrifugation. After washing out with distilled water, the cells were used as inoculum sources for the utilization of carbon sources. The agar plates were incubated at 30 °C for 14 days under aerobic conditions. Urease activity was determined by monitoring a colour change in Bacto urea broth (Difco). DNA hydrolysis was determined by using DNase test agar (Difco) supplemented with methyl green. Other physiological and biochemical properties were tested by using API 20NE and API ZYM strips according to the manufacturer's instructions (bioMérieux), with cells grown on TSA at 30 °C for 2 days. Cells of strain KSW2-17^T were Gram-positive, aerobic, non-spore-forming, non-mycelium-forming, non-motile rods (Fig. 1). The results of phenotypic characterization tests are given in the genus and species descriptions below.

Chromosomal DNA was extracted and purified according to the method of Hopwood *et al.* (1985). The DNA G + C content of strain KSW2-17^T was determined with HPLC as described by Mesbah *et al.* (1989). The base composition of strain KSW2-17^T was 68.0 mol%. The 16S rRNA gene of strain KSW2-17^T was amplified by PCR and directly sequenced as described by Lee (2006). The CLUSTAL_X software (Thompson *et al.*, 1997) was used for multiple alignments of sequences. Phylogenetic analyses were performed using three tree-making algorithms, namely the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A neighbour-joining tree was constructed from evolutionary distances calculated by the

method described by Jukes & Cantor (1969). Tree topology was evaluated by bootstrap analysis (Felsenstein, 1985) of the neighbour-joining data set, based on 1000 replications.

In the neighbour-joining tree (Fig. 2) based on 16S rRNA gene sequences, strain KSW2-17^T formed a distinct clade within the radiation of the family *Microbacteriaceae*, with *Cryobacterium psychrophilum* as its closest neighbour. This relationship was supported by a high bootstrap value (78%) and was also found in trees obtained when other tree-making algorithms were applied. Highest 16S rRNA gene sequence similarity was with the type strains of *Cryobacterium psychrophilum* and *Frigoribacterium faeni* (96.3%), followed by *Rathayibacter tritici* (96.2%) and *Rathayibacter rathayi* (96.1%). Sequence similarities with other members of the family *Microbacteriaceae* were in the range 91.3–95.9%.

Cell biomass for chemical analyses was obtained from cultures grown in TSB for 3 days at 30 °C with shaking. For determining the amino acid composition in the peptidoglycan, cells were ground with aluminium oxide. Purified cell wall was obtained according to the method of Hancock (1994) as follows: cell-wall preparations were mixed in boiling SDS with continuous stirring for 30 min. After harvesting by centrifugation, this treatment was repeated several times until all extractable UV-absorbing material was removed. Finally, sample material was treated with proteinase K (Sigma), recovered by centrifugation and washed four times with water. Hydrolysis of purified cell wall was carried out by using 4 M HCl at 100 °C for 16 h. Quantitative determination of amino acids was performed by reversed-phase HPLC (2690, Waters) of the derivatized amino acid with AccQFluor Reagent (Waters) according to the manufacturer's instructions. The acyl type of the peptidoglycan was analysed according to the method of Uchida & Aida (1984). Polar lipids and menaquinones were extracted by the integrated procedure of Minnikin *et al.* (1984). The purified menaquinones were identified by HPLC as described by Kroppenstedt (1985). The phospholipid composition was determined according to the method of Minnikin *et al.* (1977). Analysis of cellular fatty acids was performed by using GC according to the instructions of the Sherlock Microbial Identification System (version 6; MIDI), with cells grown on TSA for 3 days at 30 °C.

Purified peptidoglycan of strain KSW2-17^T contained ornithine as the diagnostic diamino acid. The molar ratio of alanine/glycine/glutamic acid/ornithine was estimated to be 0.9:1.1:1.0:1.1. Homoserine and other amino acids were not detected. The polar lipid profile contained phosphatidylglycerol and diphosphatidylglycerol. Glycolipids or other phospholipids, namely phosphatidylcholine and ninhydrin-positive phospholipids, were not detected. The major menaquinones were MK-10 (44%) and MK-11 (31%), with lesser amounts of MK-9 and MK-7. The major fatty acids were anteiso-C_{15:0} (49.4%), iso-C_{16:0} (20.5%) and anteiso-C_{17:0} (11.4%). Minor components included C_{16:0} (5.8%),

Fig. 1. Scanning electron micrograph of cells of strain KSW2-17^T grown on TSA for 3 days at 30 °C. Bar, 5 µm.

Fig. 2. Neighbour-joining tree showing the phylogenetic position of strain KSW2-17^T within the family *Microbacteriaceae*. *Micrococcus luteus* DSM 20030^T (AJ536198) was used as outgroup taxon (not shown). Double asterisks indicate the corresponding branches that were also found in both maximum-likelihood and maximum-parsimony trees. Numbers at the branches indicate bootstrap support values, based on 1000 replications (only values above 50% are shown). Bar, 0.01 substitutions per nucleotide position.

C_{18:0} (1.7%), iso-C_{14:0} (2.0%), iso-C_{15:0} (2.0%), C_{18:1}ω₉c (1.8%), 10-methyl C_{18:0} (tuberculostearic acid, 1.8%) and a mixture of iso-C_{15:0} 2-OH and/or C_{16:1}ω₇c (1.6%). The presence of tuberculostearic acid is noteworthy given that this component has not previously been found in representatives of the family *Microbacteriaceae*.

The data obtained show that strain KSW2-17^T is clearly distinguishable phenotypically from its closest phylogenetic relative, *Cryobacterium psychrophilum*, which is psychrophilic (optimum growth temperatures of 9–12 °C; no growth at 18 °C) and has the cell-wall peptidoglycan based upon 2,4-diaminobutyric acid (Suzuki *et al.*, 1997; Inoue and Komagata, 1976). Among the other bacteria sharing relatively high 16S rRNA gene sequence similarity to strain KSW2-17^T, *Frigoribacterium faeni* has a cell-wall peptidoglycan with lysine as the diagnostic diamino acid and MK-9 as the major menaquinone (Kämpfer *et al.*, 2000), while *Rathayibacter* possesses a cell-wall peptidoglycan with 2,4-diaminobutyric acid and does not contain MK-11 as the second major menaquinone (Zgurskaya *et al.*, 1993). The isolate differs from members of the six genera (*Agreia*, *Curtobacterium*, *Gulosibacter*, *Microbacterium*, *Rhodoglobus* and *Salinibacterium*) with ornithine in the cell-wall

peptidoglycan mainly based on the amino acid composition of this polymer and/or the menaquinone system (Table 1). Other characteristics useful for the differentiation of strain KSW2-17^T and related genera are given in Table 1. Members of the remaining genera of the family *Microbacteriaceae* have the cell-wall peptidoglycan based upon 2,4-diaminobutyric acid or lysine.

It is apparent from the genotypic and phenotypic evidence presented that strain KSW2-17^T represents a novel species of a new genus in the family *Microbacteriaceae*, for which the name *Labeledella gwakjiensis* gen. nov., sp. nov. is proposed.

Description of *Labeledella* gen. nov.

Labeledella (La.be.del'la. N.L. fem. n. *Labeledella* named in honour of David P. Labeda, who has made significant contributions to the area of actinomycete taxonomy).

Aerobic, Gram-positive, catalase-positive, oxidase-negative, non-spore-forming, non-motile, rod-shaped cells. Branching or mycelium formation does not occur. V-shaped forms are not found. Mesophilic. Chemoheterotrophic. The predominant menaquinones are MK-10 and MK-11. Peptidoglycan in the cell wall contains ornithine as

Table 1. Differential characteristics between strain KSW2-17^T and related genera in the family *Microbacteriaceae*

Taxa: 1, strain KSW2-17^T; 2, *Agreia*; 3, *Cryobacterium*; 4, *Curtobacterium*; 5, *Frigoribacterium*; 6, *Gulosibacter*; 7, *Microbacterium*; 8, *Rathayibacter*; 9, *Rhodoglobus*; 10, *Salinibacterium*. Data from Evtushenko *et al.* (2001), Suzuki *et al.* (1997), Komagata & Suzuki (1986), Kämpfer *et al.* (2000), Manaia *et al.* (2004), Behrendt *et al.* (2002), Zgurskaya *et al.* (1993), Sheridan *et al.* (2003), Han *et al.* (2003) and this study. +, Positive; –, negative; DAB, 2,4-diaminobutyric acid; Lys, lysine; Orn, D-ornithine; ND, not detected.

Characteristic	1	2	3	4	5	6	7	8	9	10
Diamino acid	Orn	DAB, Orn	DAB	Orn	Lys	Orn	Orn, Lys	DAB	Orn	Lys, Orn
Major menaquinones (MK)	10, 11	10	10	9	9	9	11, 12, 13, 14	10	11, 12	11
DNA G + C content (mol%)	68	67	65	68–75	71.7	65	65–72	63–72	62	61
Motility	–	+	–	+, –	+	–	+, –	–	ND	–
Optimal temperature (°C)	25–30	28	9–12	28	4–10	35–37	28	28	18	25–28

the diagnostic amino acid. The acyl type of murein is acetyl. Polar lipids contain phosphatidylglycerol and diphosphatidylglycerol. Mycolic acids are not present. The cellular fatty acid profile is characterized by the predominance of iso- and anteiso-branched components, together with a small amount of tuberculostearic acid (10-methyl C_{18:0}). Phylogenetically, the genus belongs to the family *Microbacteriaceae*, suborder *Micrococcineae*. The type species is *Labeledella gwakjiensis*.

Description of *Labeledella gwakjiensis* sp. nov.

Labeledella gwakjiensis (gwak.ji.en'sis. N.L. fem. adj. *gwakjiensis* pertaining to Gwakji Beach, Jeju, Republic of Korea, from where the type strain was isolated).

Has the following characteristics in addition to those given for the genus. Cells are 1.0–4.4 µm in length and 0.30–0.38 µm in width. On TSA, colonies are smooth, circular, convex, translucent and yellow-pigmented. Growth occurs between 10 and 37 °C. No growth occurs at 4 or 45 °C. The pH range for growth is pH 5.1–10.1, with optimum growth at pH 7.1. Growth occurs in the presence of up to 5% NaCl. Positive for β-galactosidase but negative for urease. Nitrate is not reduced to nitrite. Hydrolyses aesculin, DNA and starch. Casein and elastin are not hydrolysed. H₂S production is observed. Positive for hydrolysis of gelatin, but negative for indole production, glucose fermentation and arginine dihydrolase (API 20NE). D-Glucose, D-arabinose, D-mannose, D-mannitol and N-acetyl-D-glucosamine are utilized as sole carbon and energy sources but utilization of maltose, gluconate, caproate, adipate, malate, citrate and phenylacetate is not observed (API 20NE). Under aerobic conditions, acid is produced from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, inulin, D-mannose, D-melezitose, methyl α-D-mannoside, D-raffinose, L-rhamnose, salicin, sucrose, D-xylose, glycerol, myo-inositol, D-mannitol and D-sorbitol. Acid is not produced from D-arabinose, melibiose, L-ribose, L-sorbose, adonitol, 2,3-butanediol, dulcitol, myo-erythritol, 1,2-propanediol or D-xylitol. Acid is weakly produced from D-lactose, maltose, methyl α-D-glucoside and trehalose. In the tests with the API ZYM system, positive for leucine

arylamidase, α-galactosidase, α-glucosidase and β-glucosidase. Weakly positive for esterase lipase (C8) but negative for alkaline phosphatase, esterase (C4), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, N-acetyl-α-glucosaminidase, α-mannosidase and α-fucosidase. Major cellular fatty acids are anteiso-C_{15:0} (49.4%), iso-C_{16:0} (20.5%) and anteiso-C_{17:0} (11.4%). Tuberculostearic acid (10-methyl C_{18:0}) is also present as a minor component. Polar lipids contain phosphatidylglycerol and diphosphatidylglycerol. The DNA G + C content is 68.0 mol%.

The type strain, KSW2-17^T (=JCM 14008^T=KCTC 19176^T), was isolated from a dried seaweed sample collected around Gwakji Beach in Jeju, Korea.

Note added in proof

An additional new genus, *Yonghaparkia*, with cell-wall peptidoglycan based on D-glutamate–D-diaminobutyric acid (Yoon *et al.*, 2006), and the species *Microcella alkaliphila* containing D-ornithine in the cell wall (Tiago *et al.*, 2006) have been described since this article was submitted for publication.

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