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Ponticoccus gilvus gen. nov., sp. nov., a Novel Member of the Family *Propionibacteriaceae* from Seawater

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A novel actinobacterium, designated strain MSW-19^T, was isolated from a seawater sample in Republic of Korea. Cells were aerobic, Gram-positive, non-endospore-forming, and non-motile cocci. Colonies were circular, convex, opaque, and vivid yellow in colour. A phylogenetic tree based on 16S rRNA gene sequences exhibited that the organism formed a distinct clade within the radius encompassing representatives of the family *Propionibacteriaceae*. The phylogenetic neighbors were the type strains of the genera *Friedmanniella*, *Microlunatus*, *Micropruina*, *Propionicicella*, and *Propionicimonas*. Levels of 16S rRNA gene sequence similarity between the isolate and members of the family were less than 95.3%. The cell wall peptidoglycan of the organism contained LL-diaminopimelic acid as the diagnostic diamino acid. The isolate contained MK-9(H_4) as the predominant menaquinone, ai-$C_{15:0}$ as the major fatty acid and polar lipids including phosphatidylglycerol, phosphatidylethanolamine, and an unknown phospholipid. The G+C content of the DNA was 69.6 mol%. On the basis of the phenotypic and phylogenetic data presented here, the isolate is considered to represent a novel genus and species in the family *Propionibacteriaceae*, for which the name *Ponticoccus gilvus* gen. nov., sp. nov. is proposed. The type strain is strain MSW-19^T (= KCTC 19476^T= DSM 21351^T).

***Keywords*:** *Ponticoccus gilvus* gen. nov., sp. nov., Mara Island, actinomycete

The family *Propionibacteriaceae* Delwiche 1957 emend. Stackebrandt *et al*. (1997) originally included four validly published genera, *Propionibacterium*, *Luteococcus*, *Microlunatus*, and *Propioniferax*. Thereafter, nine genera *Friedmanniella* (Schumann *et al*., 1997), *Tessaracoccus* (Maszenan *et al*., 1999a), *Micropruina* (Shintani *et al*., 2000), *Propionimicrobium* (Stackebrandt *et al*., 2002), *Propionicimonas* (Akasaka *et al*., 2003), *Propionicicella* (Bae *et al*., 2006b), *Brooklawnia* (Bae *et al*., 2006a), *Aestuariimicrobium* (Jung *et al*., 2007), and *Granulicoccus* (Maszenan *et al*., 2007) have been successively described on the basis of their phenotypic features and phylogenetic analyses. Members of the family have cell wall peptidoglycans including LL-diaminopimelic acid (DAP), *meso*-DAP or lysine as the diagnostic diamino acid depending on genus (Pitcher and Collins, 1991; Stackebrandt *et al*., 2002; Akasaka *et al*., 2003). Among these, LL-DAP-containing genera in the peptidoglycan are *Aestuariimicrobium*, *Friedmanniella*, *Granulicoccus, Luteococcus*, *Microlunatus*, *Propionibacterium*, *Propioniferax*, and *Tessaracoccus* and have been mostly isolated from terrestrial environments, activated sludge, and human. However, it was recently reported that other LL-DAP-containing actinomycetes, *Aestuariimicrobium kwangyangensis* (Jung *et al*., 2007) and *Tessaracoccus flavescens* (Lee and Lee, 2008), were isolated from marine environments such as tidal flat and beach sediment. In the previous study of marine bacteria in seashore of Mara Island, Republic of Korea (Lee *et al.*, 2001), a new extracellular-polysaccharide-producing bacterium was reported to be isolated from marine sediment. In the present study, we describe the taxonomic position of a LL-DAP-containing actinomycete isolated from seawater around Mara Island by a polyphasic approach, and propose that it can be assigned as a novel genus and species in the family *Propionibacteriaceae*, *Ponticoccus gilvus* gen. nov., sp. nov. (type strain, MSW-19^T).

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Materials and Methods

Isolation and maintenance of microorganism

Strain MSW-19^T was isolated from a seawater sample in seashore of Mara Island, Jeju, Republic of Korea in June, 2007. A seawater sample (1 L) was filtered with membrane filter (pore size; 0.45 μm). The filter was placed into a sterile falcon tube containing 10 ml distilled water. After mixing for 10 min, aliquots (100 μl) of suspension were directly transferred onto SC-SW agar plate (1% soluble starch, 0.03% casein, 0.2% KNO_3, 0.2% NaCl, 0.002% $CaCO_3$, 1.8% agar, 0.005% $MgSO_4{\cdot}7H_2O$, and 0.001% $FeSO_4{\cdot}7H_2O$ in a mixture of 60% natural seawater and 40% distilled water). In a preliminary study, the number of colony-forming units was higher on media supplemented with 60% (v/v) natural seawater than those with 100% (v/v) or 30% (v/v). The plate was cultured at 30°C for 14 days, and single colony was streaked on yeast extract-malt extract agar (ISP 2 medium; Shirling and Gottlieb, 1966) supplemented with 60% seawater (YE-SW agar). Pure culture was stored on YE-SW agar at 4°C and as 20% glycerol supplemented with

60% seawater at -20°C or -80°C.

Morphological and cultural characterization

Growth on various culture media was tested by using ISP 2 medium (Shirling and Gottlieb, 1966), Trypticase Soy Agar (TSA; Difco, USA), Nutrient Agar (NA; Difco, USA) and Marine Agar 2216 (MA; Difco, USA). The requirement of seawater for growth was tested on above media amended with 60% (v/v) seawater except for MA. Colony morphology and pigmentation were observed and recorded on TSA for 5 days at 30°C. Cell morphology and motility were examined with light and transmission electron microscopes, using cells from exponentially growing cultures. For checking the presence of flagella, cells were negatively stained with 1% phosphotungstic acid, placed on gold-coated grid, and observed with a transmission electron microscope (a model JEM-1200EXII JEOL).

Physiological and biochemical characterization

Growth at various temperatures (4, 10, 20, 30, 37, and 42°C), NaCl concentrations [0~9% (w/v) at interval of 1.0% unit] and pHs (4.1~12.1 at interval of 1.0 unit) was tested on TSA. The results were recorded after incubation for 5 days. Gram stain and degradation were tested as described previously (Lee and Kim, 2007). Oxidase and catalase activities were determined with 3% (v/v) H_2O_2 and 1% (w/v) tetramethyl-*p*-phenylenediamine solutions, respectively. Utilization of carbohydrates was determined using ISP 9 medium as described previously (Lee, 2007). Other physiological and biochemical properties were tested with the API 20NE and API ZYM Kits (bioMérieux, France) according to the manufacturer's directions.

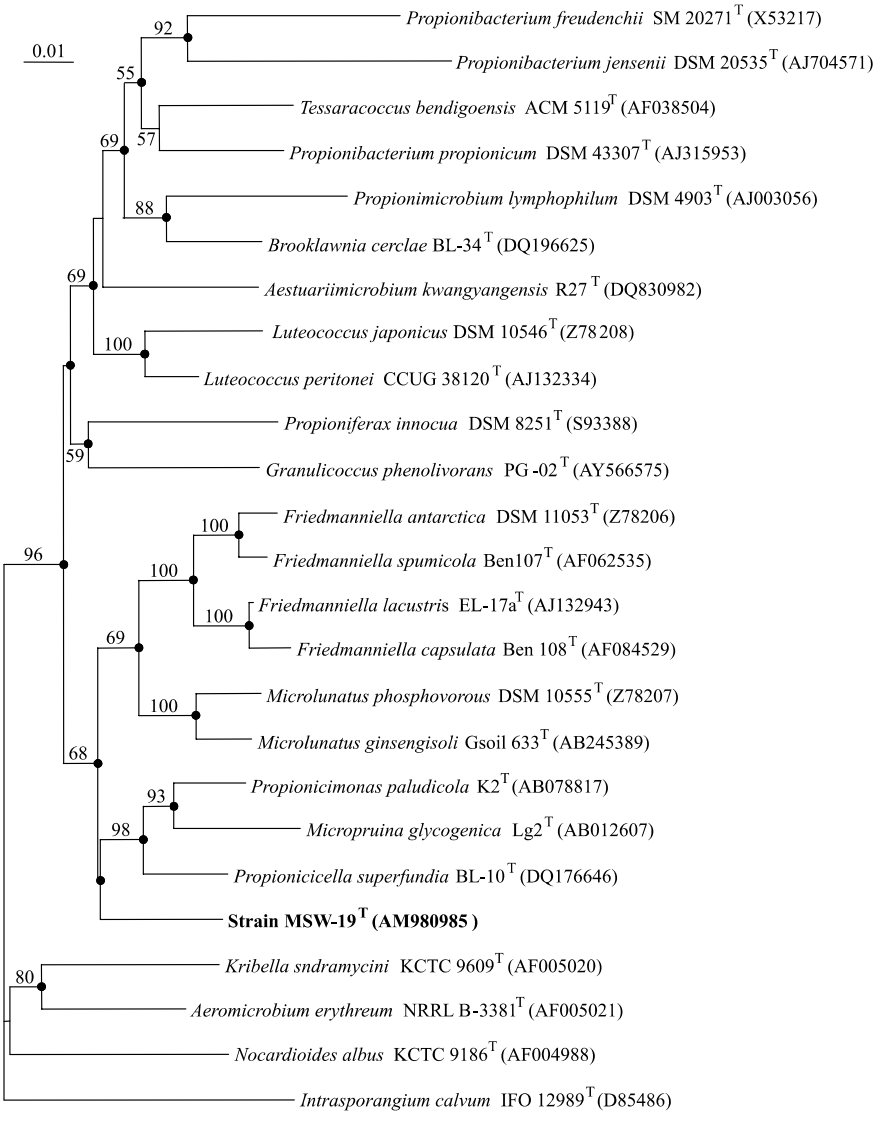

Fig. 1. A neighbor-joining tree showing the phylogenetic relationships between strain MSW-19^T and related species within the suborder *Propionibacterineae*, based on 1209 aligned positions present in 16S rRNA gene sequences of all strains. Filled circles indicated the branches that were also recovered in both maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) trees. Values at nodes are levels of bootstrap support (only values greater than 50% are indicated). Bar, 0.01 substitutions per nucleotide position.

Chemotaxonomic analyses

Cell biomass was obtained from cultures grown in trypticase soy broth (TSB; Difco, USA) for 3 days at 30°C. The isomer of diaminopimelic acid in the cell wall was determined by the method of Staneck and Roberts (1974), using TLC on cellulose plates (10×10 cm). Menaquinones were extracted according to the method of Collins (1985) and identified by HPLC as described previously (Kroppenstedt, 1985). Genomic DNA was extracted and purified by the method of Hopwood *et al*. (1985), and the determination of the G+C content was performed by HPLC (Mesbah *et al*., 1989). Phospholipid composition was determined by the previously described method (Minnikin *et al*., 1977). Fatty acid methyl esters were prepared and analysed by using Sherlock Microbial Identification System (version 6; MIDI) according to the directions of the manufacturer, with cells grown in TSA for 3 days at 30°C.

16S rRNA gene sequence analyses

Extraction of genomic DNA and amplification of 16S rRNA gene by PCR were performed as described by Lee (2007). The amplified PCR product was purified using the Wizard Genomic DNA Purification Kit (Promega, USA). Sequencing of the 16S rRNA gene was performed using an ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an automatic DNA sequencer (model 3730xl; Applied Biosystems, USA). The sequence of strain MSW-19^T was aligned using CLUSTAL X (Thompson *et al.*, 1997) with respect to the corresponding sequences of representatives of the family *Propionibacteriaceae*. Phylogenetic analyses were performed by using NEIGHBOR, DNAPARS, DNAML programs contained in the PHYLIP package (Felsenstein, 1993). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) from evolutionary distances transformed by the model of Jukes and Cantor (1969). The sequence of *Intrasporangium calvum* IFO 12989^T (D85486) was used to root the tree. The topology of the phylogenetic tree was evaluated by bootstrap analysis (Felsenstein, 1985), using 1,000 resampled datasets.

Results and Discussion

A partial 16S rRNA gene sequence (1,436 nt) of strain MSW-19^T was compared with the corresponding sequences of representatives of the family *Propionibacteriaceae* and related taxa. A neighbor-joining tree (Fig. 1) based on 16S rRNA gene sequences revealed that strain MSW-19^T occupied a distinct position within the radius encompassing representatives of the family *Propionibacteriaceae*. The 16S rRNA gene sequence similarity values of strain MSW-19^T to phylogenetic neighbors were found to be with members of the genera *Microlunatus* (94.7~95.3%), *Propionicicella* (94.5%), *Friedmanniella* (92.5~94.3%), and *Micropruina* (93.7%). Levels of 16S rRNA gene sequence similarity of the organism to other representatives of the family were 89.0~94.4%.

Strain MSW-19^T grew well on most of tested media irrespective of supplementation of seawater. Cells were aerobic, Gram-positive, oxidase-negative, catalase-positive, non-motile cocci (0.5~0.7 μm in diameter) that arranged singly, in pairs and short chains (Fig. 2). Colonies of the cells were vivid yellow and approximately 0.2~0.3 mm in diameter after incubation for 5 days. Data for physiological and biochemical tests are given in the species description.

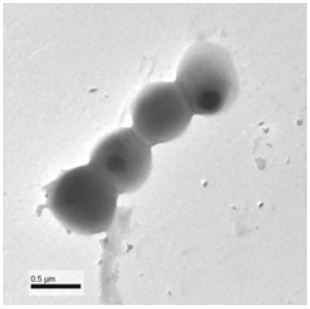

Fig. 2. A transmission electron microscope of cells of strain MSW-19^T grown on TSA at 30°C for 3 days.

The cell wall peptidoglycan of strain MSW-19^T contained LL-DAP as the diagnostic diamino acid. Polar lipid profile consisted of phosphatidylglycerol, phosphatidylethanolamine, and an unknown phospholipid. The major menaquinone was MK-9(H_4). The cellular fatty acids contained anetiso-$C_{15:0}$ (59.1%), iso-$C_{14:0}$ (10.7%), iso-$C_{15:0}$ (5.6%), iso-$C_{16:0}$ (5.3%), iso-$C_{16:1}$ H (4.6%), $C_{16:0}$ (2.1%), iso-$C_{14:0}$ 3-OH (1.8%), $C_{18:0}$ (1.6%), and iso-$C_{17:1}\omega 9c$ (1.5%). The DNA G+C content of strain MSW-19^T was determined as 69.6 mol% by using HPLC.

On the basis of comparative 16S rRNA gene sequence analyses, it is revealed that the phylogenetic neighbors of strain MSW-19^T are the type strains of genera *Friedmanniella*, *Microlunatus*, *Micropruina*, *Propionicicella*, and *Propionicimonas*. Among these, the genera *Micropruina*, *Propionicicella*, and *Propionicimonas* differ from our isolate in that they contained *meso*-DAP in cell wall peptidoglycan. The genera *Friedmanniella* and *Microlunatus* possess the same type of DAP isomer (LL-form), compared with strain MSW-19^T, but they can be readily differentiated from the isolate by temperature and pH ranges for growth, and in the absence of phosphatidylethanolamine in their polar lipid profiles. Other differential characteristics between strain MSW-19^T and its phylogenetic neighbors are given in Table 1.

On the basis of the physiological and chemotaxonomic data as wells as phylogenetic evidence, strain MSW-19^T merits to be assigned a novel genus and species in the family *Propionibacteriaceae*, which it is named *Ponticoccus gilvus* gen. nov., sp. nov..

Description of *Ponticoccus* gen. nov.

Ponticoccus (Pon.ti.coc'cus. L. n. *Pontus-i* sea; N.L. masc. *coccus* from Gr. N. *kokkos* a grain or berry; N.L. masc. n. *Ponticoccus* coccus from sea)

Cells are aerobic, Gram-positive, oxidase-negative, catalase-positive, non-endospore-forming, non-motile cocci that arranged singly, in pairs and in chains. The cell wall peptidoglycan contains LL-DAP as the diagnostic diamino acid. The polar lipid profile includes phosphatidylglycerol, phosphatidylethanolamine, and an unknown phospholipid. The

Table 1. Differential characteristics between strain MSW-19^T and its phylogenetic neighbors

Characteristic	1	2	3	4	5	6
Cell morphology	Cocci	Cocci	Cocci	Cocci	Rods	Irregular rods
Cell size (μm)	0.5∼0.7	0.5∼2.2	0.5∼2.0	0.5∼2.2	0.5×1.7	0.4∼0.5×1.4∼2.2
O_2 requirement	Aerobe	Aerobe	Aerobe	Aerobe	Facultative anaerobe	Facultative anaerobe
Optimum growth temperature (°C)	30	20∼25	25∼30	30	30	35
Growth temperature (°C)	20∼42	9∼37	5∼35 (20∼35)	20∼35	15∼37	10∼40
Optimum growth pH	7.1	6.0∼7.5	7.0	7.0	6.5	6.5
Growth pH range	5.1∼11.1	5.1∼8.7	5.0∼9.0	6.0∼8.0	4.5∼8.5	4.5∼7.5
Oxidase	-	-	+ (-)	+	-	-
Catalase	+	+	+	+	-	-
Urease	-	+	+ (-)	ND	ND	ND
Nitrate reduction	+	-	+ (-)	+	-	-
Isomer of DAP	LL	LL	LL	*meso*	*meso*	*meso*
Major menaquinone(s)	MK-9(H_4)	MK-9(H_4), MK-9(H_2)†, MK-7(H_2)†, and MK-7(H_4)†	MK-9(H_4)	MK-9(H_4)	MK-9	MK-9(H_4), MK-10(H_4)
Polar lipids	PE, PG, PL	PI, PG, DPG, PL GL†	PI, PG, DPG, PL	ND	ND	ND
Major fatty acid (>10%)	ai-$C_{15:0}$	ai-$C_{15:0}$, i-$C_{15:0}$, $C_{18:1}$†	ai-$C_{15:0}$, i-$C_{15:0}$, (i-$C_{16:0}$)	ai-$C_{15:0}$, i-$C_{14:0}$, $C_{16:0}$, i-$C_{16:0}$	ai-$C_{15:0}$, $C_{15:0}$, i-$C_{16:0}$	$C_{15:0}$, ai-$C_{15:0}$, $C_{13:0}$
DNA G+C (mol%)	69.6	69∼74	67.9∼69.8	71	69.9	68.7
Origin of isolation	Seawater	Antarctic sandstone or activated sludge	Sewage treatment plant or soil	Laboratory sequenceing batch reactor	Groundwater	Plant residue in paddy soil

Taxa: 1, Strain MSW-19^T; 2, *Friedmanniella* (Schumann *et al.*, 1997; Maszenan *et al*., 1999b); 3, *Microlunatus* (Nakamura *et al*., 1995; Cui *et al*., 2007); 4, *Micropruina* (Shintani *et al.*, 2000); 5, *Propionicicella* (Bae *et al.*, 2006b); 6, *Propionicimonas* (Akasaka *et al*., 2003). Abbreviation: DAP , diaminopimelic acid; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, unknown phospholipid; GL, unknown glycolipid; ai, anteiso-methyl-branched; i, iso-methyl-branched; ND, not determined. Data in parentheses are taken from the type strain of *Microlunatus ginsengisoil* (Cui *et al*., 2007). †Detected only in the type strain of *Friedmanniella spumicola* (Maszenan *et al*., 1999b).

predominant menaquinone is MK-9(H_4). The major cellular fatty acid is ai-$C_{15:0}$. On the basis of 16S rRNA gene sequence comparisons, the genus belongs to the family *Propionibacteriaceae*. The type species is *Ponticoccus gilvus*.

Description of *Ponticoccus gilvus* sp. nov.

Ponticoccus gilvus (gil'vus. L. masc. adj. *gilvus* pale yellow).

Cells are non-motile cocci (0.5∼0.7 μm in diameter). Colonies are vivid-yellow, circular, convex, entire margin, and approximately 0.2∼0.3 mm in diameter. Chemotaxonomic characteristics are the same as those given in the genus description. Temperature and pH ranges for growth are 20∼42°C and pH 5.1∼11.1, respectively. Grows at 0∼5% (w/v) NaCl. Positive for utilization of D-arabinose, D-maltose, and D-glucose (weakly positive), aesculin degradation, gelatin hydrolysis, and β-galactosidase, but negative for indole production, glucose fermentation, arginine dihydrolase, and utilization of D-mannose, D-mannitol, N-acetyl-D-glucosamine, gluconate, caprate, adipate, malate, citrate, and phenylacetate (API 20NE). Hydrolyzes DNA and elastin, but not casein, cellulose, hypoxanthine, starch, tyrosine, xanthine, and chitin. Positive for esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, α-glucosidase, β-glucosidase, alkaline phosphatase (weakly positive), and esterase (C4) (weakly positive), but negative for lipase (C14), cystein arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase (API ZYM). Utilizes D-arabinose, L-arabinose, D-cellobiose, dextran, D-fructose, D-galactose, D-glucose, inulin, D-lactose, maltose, D-mannose, D-melezitose, melibiose, α-methyl-D-glucoside, α-methyl-D-mannoside, D-raffinose, L-rhamnose, L-ribose, salicin, L-sorbose, sucrose, D-trehalose, D-xylose, adonitol, *meso*-erythritol, glycerol, D-mannitol, D-sorbitol, D-xylitol, citrate, malate, and succinate as sole carbon and energy sources, but not dulcitol, *meso*-inositol, acetate, benzoate, formate, and tartrate. The major cellular fatty acid is ai-$C_{15:0}$ (59.1%). The G+C content of the DNA is 69.6 mol%.

The type strain, MSW-19^T (= KCTC 19476^T= DSM 21351^T), was isolated from a seawater sample in the seashore of Mara Island, Jeju, Republic of Korea.

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