

A report on 14 unrecorded bacterial species isolated from the Nakdong River, South Korea

Ja Young Cho^{1,†}, Kiwoon Baek^{2,†}, Eui-Jin Kim¹, Ji-Hye Han¹, Seoni Hwang¹ and Ahyoung Choi^{3,*}

¹Microbial Research Department, Nakdonggang National Institute of Biological Resources (NNIBR), Sangju 37242, Republic of Korea

²Bioresources Collection & Bioinformation Department, Nakdonggang National Institute of Biological Resources (NNIBR), Sangju 37242, Republic of Korea

³Strategic Planning Department, Nakdonggang National Institute of Biological Resources (NNIBR), Sangju 37242, Republic of Korea

*Correspondent: aychoi@nnibr.re.kr

†These authors contributed equally to this work.

As a part of the research project “Survey of freshwater organisms and specimen collection,” freshwater samples were collected from the Nakdong River. Among the bacterial isolates, we selected strains that showed higher than 98.7% 16S rRNA gene sequence similarity with confirmed bacterial species previously unreported in South Korea. The 14 new records to South Korea were phylogenetically diverse and belonged to four phyla, six classes, 11 orders, and 14 genera. At the genus level, these species were found to be affiliated with *Reyranella*, *Ferrovibrio*, *Brevundimonas*, and *Aquidulcibacter* of the class *Alphaproteobacteria*; *Pseudomonas*, *Cellvibrio*, and *Photobacterium* of the class *Gammaproteobacteria*; *Paenibacillus* and *Bacillus* of the phylum *Firmicutes*; *Chryseobacterium*, *Flavobacterium*, *Pedobacter* of the phylum *Bacteroidetes*; and *Actinomadura* and *Leifsonia* of the phylum *Actinobacteria*. These species were further characterized by examining their Gram reaction, colony and cell morphologies, biochemical properties, and phylogenetic positions. The detailed descriptions of these 14 previously unreported species are provided.

Keywords: Freshwater, Nakdong River, unreported bacterial species

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INTRODUCTION

The microbial community in a river water ecosystem is different from that in any other ecosystems, such as soil and marine. These freshwater bacteria are the key players of global environmental processes and carbon cycling (Falkowski *et al.*, 2008; Williamson *et al.*, 2018). The bacterial community in freshwater is predominantly composed of *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Cytophaga-Flavobacterium-Bacteroidetes*, and *Verrucomicrobia* (Newton *et al.*, 2011). Advances in in-depth sequencing technology have quickly improved our knowledge of bacterial diversity and the key processes of microbial ecology, which led to the understanding of the relationship between the functioning of aquatic ecosystems and the formation of bacterial communities on ecology (Edwards *et al.*, 2006; Huse *et al.*, 2008). However, the cultivation of a predominant group of microorganisms

is essential for understanding the bacteria and their ecological roles in freshwater environments.

Our research site, the Nakdong River is the longest river in South Korea, and its total watershed is 23,384 square kilometers wide. The Nakdong River and its tributaries also serve as a major source of drinking water for the inhabitants of the river basin and nearby locations. However, environmental changes have occurred, as untreated nitrogen and phosphorus nutrients flow into the river. The acceleration of eutrophication caused by the inflow of contaminated waters has led to an increase in phytoplankton abundance and changed bacterial species composition in rivers (Sin *et al.*, 2005; Chun *et al.*, 2019). Thus, there has been a significant increase in academic interest in the relationship between river ecosystems and microorganisms that act as decomposers (Miettinen *et al.*, 1997; Zhou *et al.*, 2016).

As a part of the research program conducted and sup-

ported by the Nakdonggang Institute of Biological Resources (NNIBR), freshwater samples were collected from the Nakdong River, and almost 300 bacterial strains were isolated. Through phylogenetic analyses based on 16S rRNA gene sequences, 14 bacterial species were recognized as bacterial species previously unrecorded in South Korea. Here, we report the phylogenetic and phenotypic characterization of these bacterial species.

MATERIALS AND METHODS

A total of 14 bacterial strains were isolated from the Nakdong River (36°27'15.91"N, 128°15'30.65"E and 35°18'8.73"N, 128°58'45.8"E) by the standard dilution plating method on marine agar 2211 (MA) (Becton Dickinson), 1/10-diluted MA, nutrient agar (NA), tryptic soy agar (TSA), Reasoner's 2A (R2A), 1/10-diluted R2A media, R2A with sea water (M-R2A) and subsequently incubated at 20–25°C for 3–7 days. All the strains were purified as single colonies after serial dilution spreading, and the pure cultures were stored as 20% glycerol suspension at –80°C. The strain IDs, sources of isolation, culture media, and incubation conditions are summarized in Table 1.

The colony morphologies of the bacterial strains were observed on agar plates with a magnifying glass after the cells were cultivated until the stationary phase. Cellular morphology and size were examined by transmission electron microscopy. Gram staining was performed using a Gram-staining kit (bioMérieux). Biochemical characteristics were assessed using API 20NE galleries (bioMérieux) according to the manufacturer's instructions.

For 16S rRNA sequence analysis, the genomic DNA of isolates were extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions. The 16S rRNA gene of strains were amplified by PCR with universal bacterial primers 27F and 1492R and sequenced by Sanger sequencing (Weisburg *et al.*, 1991). The resultant almost full-length 16S rRNA gene sequences were identified using the "16S based ID" service in EzBioCloud (Yoon *et al.*, 2017). Sequence similarity of 98.7% was set as the cutoff value; bacterial strains $\geq 98.7\%$ sequence similarity with known bacterial species previously unreported in South Korea were selected as unreported bacterial species. For phylogenetic analyses, multiple sequence alignments between the 16S rRNA gene sequences of the isolates and those of the reference type strains were carried out using the ClustalW program and manually checked with EzEditor (Jeon *et al.*, 2014). Based on the sequences aligned, phylogenetic trees were generated using a neighbor-joining method (Saitou and Nei, 1987) with the Kimura 2-parameter model (Kimura,

1980) implemented in MEGA 7.0 software (Kumar *et al.*, 2016). The robustness of the inferred phylogenetic trees was evaluated by bootstrap analyses based on 1,000 random re-samplings (Felsenstein, 1985).

RESULTS AND DISCUSSION

The 14 strains represent 14 species previously unrecorded in South Korea and belong to four phyla, six classes, 11 orders, and 14 genera. The taxonomic composition and identification results of these species are summarized in Table 1. At the genus level, the unreported species were found to belong to *Reyranella*, *Ferrovibrio*, *Brevundimonas*, and *Aquidulcibacter* of the class *Alphaproteobacteria*; *Pseudomonas*, *Cellvibrio*, and *Photobacterium* of the class *Gammaproteobacteria*; *Paenibacillus* and *Bacillus* of the phylum *Firmicutes*; *Chryseobacterium*, *Flavobacterium*, and *Pedobacter* of the phylum *Bacteroidetes*; and *Actinomadura* and *Leifsonia* of the phylum *Actinobacteria*. The phylogenetic tree of the bacterial strains assigned to the classes *Alphaproteobacteria* and *Gammaproteobacteria* is shown in Fig. 1 and the tree of the phyla *Firmicutes*, *Bacteroidetes*, and *Actinobacteria* is shown in Fig. 2. The transmission electron microscopy images of the isolates are provided in Fig. 3.

In summary, in this study, 14 bacterial species were identified as bacterial species previously unreported in South Korea freshwater ecosystems, and here, we presented and described the characteristics of these unreported bacterial species.

Description of *Reyranella massiliensis* 5SWB3-2

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are circular, convex and ivory colored after three days of incubation at 25°C on TSA. Positive for nitrate reduction and urease activities in API 20NE, but negative for indole production, glucose fermentation, arginine dihydrolase, esculin hydrolysis, gelatin hydrolysis, and β -galactosidase. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain 5SWB3-2 displays the highest 16S rRNA gene sequence similarity with *Reyranella massiliensis* 521^T (99.0%). Strain 5SWB3-2 (=NNIBR2017BA16) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MG818310.

Description of *Ferrovibrio denitrificans* 6SW1-49

Cells are Gram-staining-negative, flagellated, and rod-shaped. Colonies are circular, convex, and white colored

Table 1. Summary of unrecorded freshwater bacterial strains isolated from the Nakdong River and their taxonomic affiliations.

Isolate	Order	Family	Strain ID	NNIBR ID	Accession number	Most closely related species	Similarity (%)	Medium	Incubation conditions	
Nakdong River (Sangju)	Rhodospirillales	Rhodospirillaceae	5SWB3-2	NNIBR2017301BA16	MG818310	<i>Reyranella massiliensis</i>	99.0	TSA	25°C, 3d	
	Sneathiellales	<i>Ferrovibrio_f</i>	6SW1-49	NNIBR2017301BA17	MG818311	<i>Ferrovibrio denitrificans</i>	98.9	R2A	25°C, 3d	
	Caulobacterales	Caulobacteraceae	19SA03-R-5	NNIBR2019643BA6	MN540834	<i>Brevundimonas viscosa</i>	99.5	R2A	25°C, 4d	
	Pseudomonadales	Pseudomonadaceae	5SWB1-35	NNIBR2017301BA15	MG818309	<i>Pseudomonas anguilliseptica</i>	99.8	R2A	25°C, 3d	
	Cellvibrionales	Cellvibrionaceae	19SA03-N-12	NNIBR2019643BA2	MN540796	<i>Cellvibrio fibrivorans</i>	99.1	NA	25°C, 3d	
	Bacillales	Paenibacillaceae	BK-49	NNIBR2015296BA21	KU360712	<i>Paenibacillus barcinonensis</i>	99.0	NA	30°C, 3d	
		Bacillaceae	19SA03-T-11	NNIBR2019643BA5	MN540832	<i>Bacillus nealsonii</i>	99.4	TSA	25°C, 3d	
	Flavobacteriales	Flavobacteriaceae	SWB1-24	NNIBR2017301BA13	MG818307	<i>Chryseobacterium limigenitum</i>	99.9	TSA	25°C, 3d	
	Sphingobacteriales	Sphingobacteriaceae	19SA03-R-9	NNIBR2019643BA9	MN540836	<i>Pedobacter ruber</i>	99.3	R2A	25°C, 3d	
	Streptosporangiales	Thermomonosporaceae	SWB3-6	NNIBR2017301BA14	MG818308	<i>Actinoadura coerulea</i>	98.8	TSA	25°C, 3d	
	Micrococcales	Microbacteriaceae	19SA03-T-4	NNIBR2019643BA4	MN540803	<i>Leifsonia kapiensis</i>	98.8	TSA	25°C, 3d	
	Nakdong River (Mulgeum)	Caulobacterales	Caulobacterales	19MK03-0.1R-21	NNIBR2019643BA7	MN540833	<i>Aquidulcibacter paucihalophilus</i>	99.8	R2A	25°C, 4d
		Vibrionales	Vibrionales	19MK03-MR-19	NNIBR2019643BA10	MN540837	<i>Photobacterium indicum</i>	99.4	M-R2A	25°C, 10d
		Flavobacteriales	Flavobacteriales	19MK03-0.1M-18	NNIBR2019643BA8	MN540835	<i>Flavobacterium buctense</i>	98.8	R2A	25°C, 3d

after three days of incubation at 25°C on R2A. Positive for reduction of indole production and urease activities in API 20NE, but negative for reduction of nitrate reduction, glucose fermentation, arginine dihydrolase, esculin hydrolysis, gelatin hydrolysis, and β -galactosidase. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain 6SW1-49 displays the highest 16S rRNA gene sequence similarity with *Ferrovibrio denitrificans* S3^T (98.9%). Strain 6SW1-49 (= NNIBR 2017BA17) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MG818311.

Description of *Brevundimonas viscosa* 19SA03-R-5

Cells are Gram-staining-negative, flagellated, and rod-shaped. Colonies are circular, smooth, slightly convex and white colored after four days of incubation at 25°C on R2A. Positive for nitrate reduction, esculin hydrolysis, and β -galactosidase in API 20NE, but negative for indole production, glucose fermentation, arginine dihydrolase, urease, and gelatin hydrolysis. D-Glucose and D-maltose are utilized. Does not utilize L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain 19SA03-R-5 displays the highest 16S rRNA gene sequence similarity with *Brevundimonas viscosa* F3^T (99.5%). Strain 19SA03-R-5 (= NNIBR2019643BA6) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540834.

Description of *Aquidulcibacter paucihalophilus* 19MK03-0.1R-21

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are convex, smooth and yellow colored after 4 days of incubation at 25°C on R2A. Positive for nitrate reduction, esculin hydrolysis, and β -galactosidase in API 20NE, but negative for indole production, glucose fermentation, arginine dihydrolase, urease, and gelatin hydrolysis. D-Glucose is utilized. Does not utilize L-arabinose, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. Strain 19MK03-0.1R-21 displays the highest 16S rRNA gene sequence similarity with *Aquidulcibacter paucihalophilus* TH1-2^T (99.8%). Strain 19MK03-0.1R-21 (= NNIBR2019643BA7) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540833.

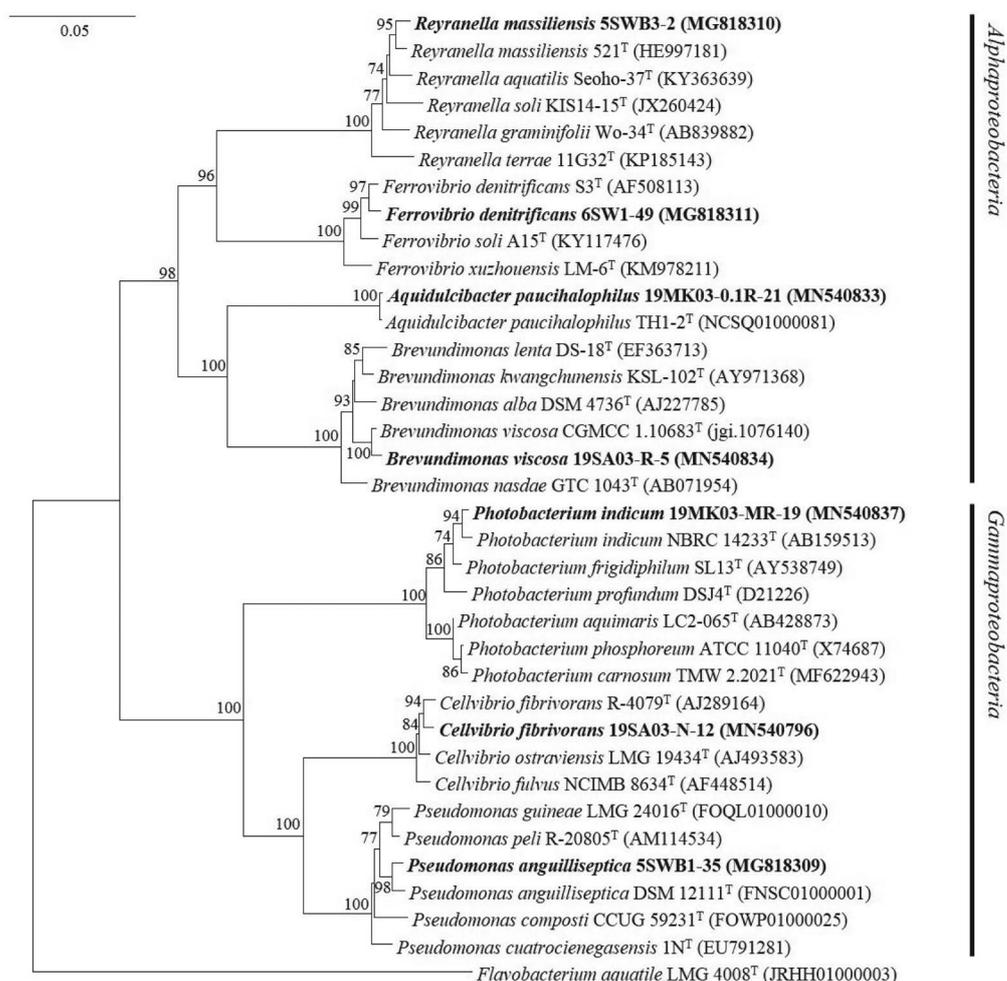


Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their relatives in the classes *Alphaproteobacteria* and *Gammaproteobacteria*. Bootstrap values (>70%) are shown at nodes. Bar, 0.05 substitutions per nucleotide position.

Description of *Pseudomonas anguilliseptica* 5SWB1-35

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are circular, convex, and ivory colored after three days of incubation at 25°C on R2A. Positive for nitrate reduction and arginine dihydrolase in API 20NE, but negative for reduction of indole production, glucose fermentation, urease, esculin hydrolysis, gelatin hydrolysis, and β -galactosidase. Malic acid is utilized. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate, and phenylacetic acid. Strain 5SWB1-35 displays the highest 16S rRNA gene sequence similarity with *Pseudomonas anguilliseptica* DSM 12111^T (99.1%). Strain 5SWB1-35 (=NNIBR2017BA15) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MG818309.

Description of *Cellvibrio fibrivorans* 19SA03-N-12

Cells are Gram-staining-negative, non-flagellated, and short rod-shaped. Colonies are low-convex, circular, and pale-yellow colored after three days of incubation at 25°C on NA. Positive for nitrate reduction, esculin hydrolysis, gelatin hydrolysis, and β -galactosidase in API 20NE, but negative for indole production, glucose fermentation, arginine dihydrolase, and urease activities. D-Glucose, D-mannose, D-mannitol, D-maltose, potassium gluconate, malic acid, and trisodium citrate are utilized. Does not utilize L-arabinose, *N*-acetyl-glucosamine, capric acid, adipic acid, and phenylacetic acid. Strain 19SA03-N-12 displays the highest 16S rRNA gene sequence similarity with *Cellvibrio fibrivorans* R-4079^T (99.0%). Strain 19SA03-N-12 (=NNIBR2019643BA2) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540796.

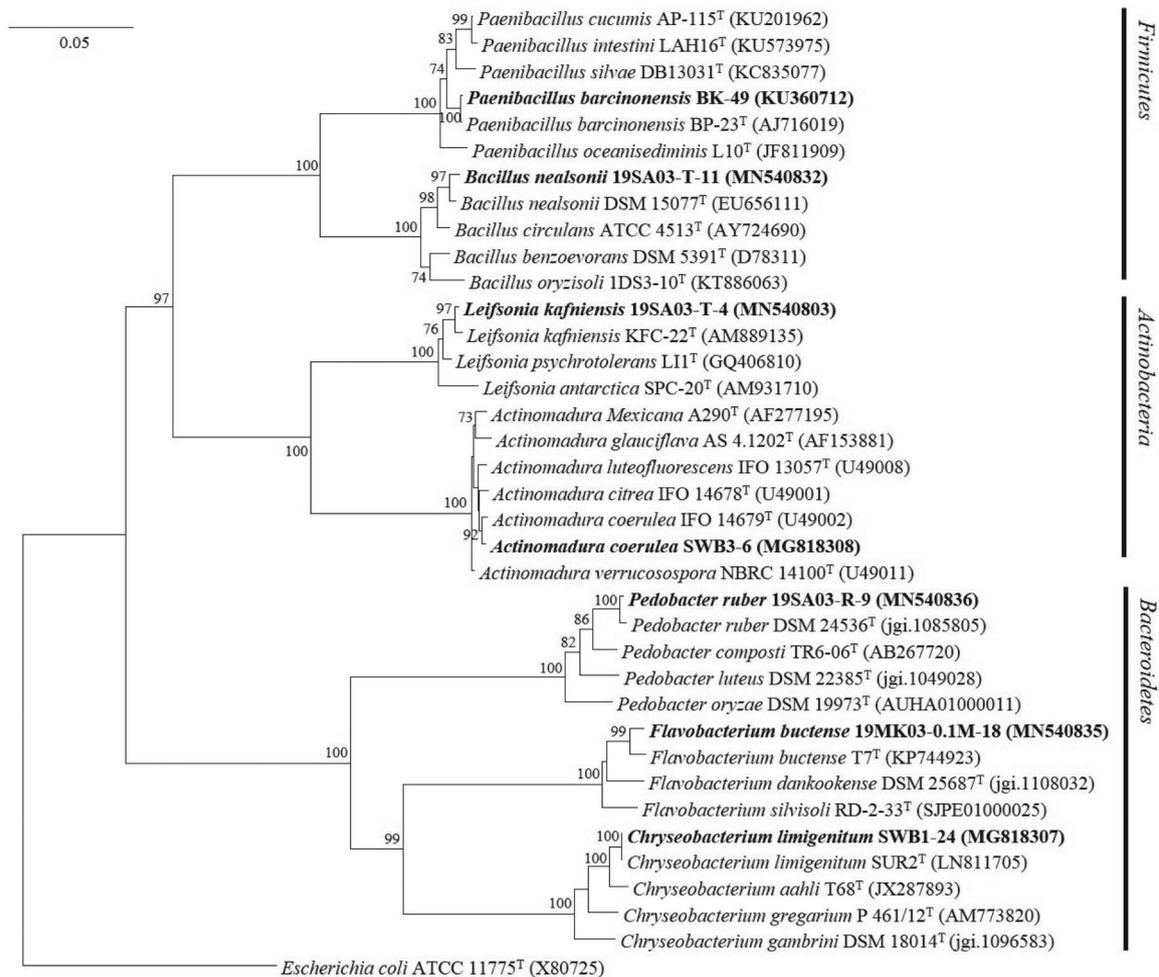


Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their relatives in the phyla *Firmicutes*, *Bacteroidetes* and *Actinobacteria*. Bootstrap values (>70%) are shown at nodes. Bar, 0.05 substitutions per nucleotide position.

Description of *Photobacterium indicum* 19MK03-MR-19

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are circular, convex and ivory colored after 10 days of incubation at 25°C on M-R2A. Positive for nitrate reduction, indole production, arginine dihydrolase, and urease activities in API 20NE, but negative for glucose fermentation, esculin hydrolysis, gelatin hydrolysis, and β -galactosidase. D-Glucose and D-mannitol are utilized. Does not utilize L-arabinose, D-mannose, *N*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain 19MK03-MR-19 displays the highest 16S rRNA gene sequence similarity with *Photobacterium indicum* ATCC 19614^T (99.4%). Strain 19MK03-MR-19 (= NNIBR2019643BA10) was isolated from a freshwater sample of Nakdong River. The GenBank accession num-

ber of 16S rRNA gene sequence is MN540837.

Description of *Paenibacillus barcinonensis* BK-49

Cells are Gram-staining-positive, non-flagellated, and rod-shaped. Colonies are circular, slightly irregular, and pale yellow colored after three days of incubation at 30°C on NA. Positive for esculin hydrolysis and β -galactosidase in API 20NE, but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, and gelatin hydrolysis. L-Arabinose, D-mannose, D-maltose, and potassium gluconate are utilized. Does not utilize D-glucose, D-mannitol, *N*-acetyl-glucosamine, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain BK-49 displays the highest 16S rRNA gene sequence similarity with *Paenibacillus barcinonensis* BP-23^T (99.9%). Strain BK-49 (= NNIBR2015BA21) was isolated from a freshwater

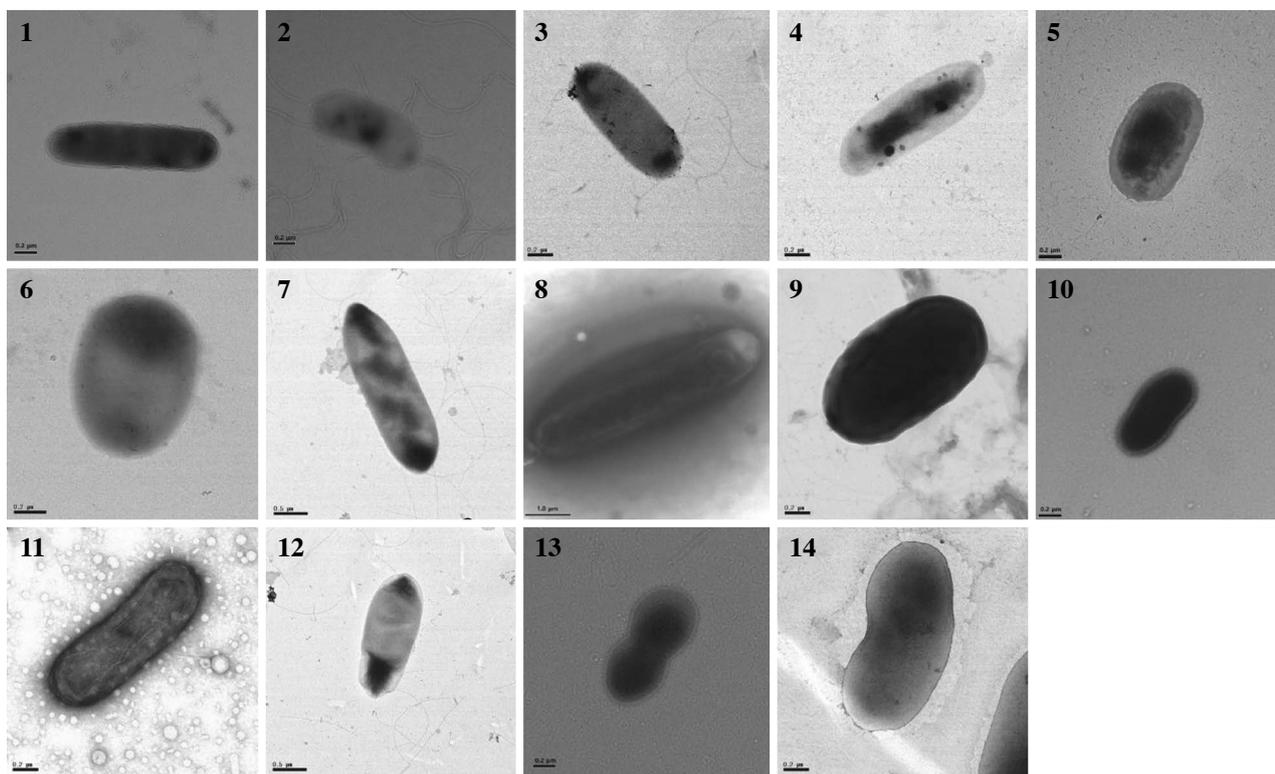


Fig. 3. Transmission electron micrographs of cells of the strains isolated in this study. Strains: 1, 5SWB3-2; 2, 6SW1-49; 3, 19SA03-R-5; 4, 19MK03-0.1R-21; 5, 5SWB1-35; 6, 19SA03-N-12; 7, 19MK03-MR-19; 8, BK-49; 9, 19SA03-T-11; 10, SWB1-24; 11, 19MK03-0.1M-18; 12, 19SA03-R-9; 13, SWB3-6; 14, 19SA03-T-4.

sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is KU360712.

Description of *Bacillus nealsonii* 19SA03-T-11

Cells are Gram-staining-positive, non-flagellated, and rod-shaped. Colonies are irregular, smooth, and beige colored after three days of incubation at 25°C on TSA. Positive for nitrate reduction, glucose fermentation, esculin hydrolysis, and β -galactosidase in API 20NE, but negative for indole production, arginine dihydrolase, urease, and gelatin hydrolysis. D-Glucose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, and malic acid are utilized. Does not utilize L-arabinose, capric acid, adipic acid, trisodium citrate, and phenylacetic acid. Strain 19SA03-T-11 displays the highest 16S rRNA gene sequence similarity with *Bacillus nealsonii* FO-92^T (99.3%). Strain 19SA03-T-11 (=NNIBR2019643BA5) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540832.

Description of *Chryseobacterium limigenitum* SWB1-24

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are circular, smooth, and yellow

colored after three days of incubation at 25°C on TSA. Positive for reduction of esculin hydrolysis, gelatin hydrolysis, and β -galactosidase in API 20NE, but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, and urease activities. D-Glucose and D-mannose are utilized. Does not utilize L-arabinose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain SWB1-24 displays the highest 16S rRNA gene sequence similarity with *Chryseobacterium limigenitum* SUR2^T (98.8%). Strain SWB1-24 (=NNIBR2017BA13) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MG818307.

Description of *Flavobacterium buctense* 19MK03-0.1M-18

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are circular, convex, smooth, and yellow colored after three days of incubation at 25°C on R2A. Positive for arginine dihydrolase, urease, esculin hydrolysis, and β -galactosidase in API 20NE, but negative for nitrate reduction, indole production, glucose

fermentation, and gelatin hydrolysis. D-Glucose, D-maltose, and phenylacetic acid are utilized. Does not utilize L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, and trisodium citrate. Strain 19MK03-0.1M-18 displays the highest 16S rRNA gene sequence similarity with *Flavobacterium buctense* T7^T (98.8%). Strain (= NNIBR2019643BA8) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540835.

Description of *Pedobacter ruber* 19SA03-R-9

Cells are Gram-staining-negative, non-flagellated, and rod-shaped. Colonies are smooth, round, convex, and red colored after seven days of incubation at 25°C on R2A. Positive for esculin hydrolysis and β -galactosidase in API 20NE, but negative for nitrate reduction, indole production, glucose fermentation, arginine dihydrolase, urease, and gelatin hydrolysis. L-Arbinose, D-mannose, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, and phenylacetic acid are utilized. Does not utilize D-glucose and trisodium citrate. Strain 19SA03-R-9 displays the highest 16S rRNA gene sequence similarity with *Pedobacter ruber* W1^T (99.6%). Strain 19SA03-R-9 (= NNIBR2019643BA9) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540836.

Description of *Actinomadura coerulea* SWB3-6

Cells are Gram-staining-positive, non-flagellated, and coccoid-shaped. Colonies are crateriform, convex, and pink colored after three days of incubation at 25°C on TSA. Positive for reduction of indole production, urease, esculin hydrolysis, and gelatin hydrolysis in API 20NE, but negative for nitrate reduction, glucose fermentation, arginine dihydrolase, and β -galactosidase. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain SWB3-6 displays the highest 16S rRNA gene sequence similarity with *Actinomadura coerulea* IFO 14679^T (99.5%). Strain SWB3-6 (= NNIBR2017BA14) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MG818308.

Description of *Leifsonia kafniensis* 19SA03-T-4

Cells are Gram-staining-positive, non-flagellated, and curved rod-shaped. Colonies are circular, convex, and pale-yellow colored after three days of incubation at 25°C on TSA. Positive for nitrate reduction, glucose fermentation, esculin hydrolysis, gelatinase, and β -galactosidase

in API 20NE, but negative for indole production, arginine dihydrolase and urease activities. D-Glucose, L-arabinose, D-mannose, *N*-acetyl-glucosamine, and trisodium citrate are utilized. Does not utilize D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, and phenylacetic acid. Strain 19SA03-T-4 displays the highest 16S rRNA gene sequence similarity with *Leifsonia kafniensis* KFC-22^T (99.6%). Strain 19SA03-T-4 (= NNIBR2019643BA4) was isolated from a freshwater sample of Nakdong River. The GenBank accession number of 16S rRNA gene sequence is MN540803.

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