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Twelve previously unrecorded bacterial species, isolated from the Nakdong River, South Korea

Hyangmi Kim* and Ji-Hye Han

Bacteria Research Team, Nakdonggang National Institute of Biological Resources (NNIBR), Sangju 37242, Republic of Korea

**Correspondent: hmkim@nnibr.re.kr*

During a survey of indigenous prokaryotic species diversity of the upstream Nakdong River, South Korea, 12 bacterial strains were isolated for further analysis. These bacterial strains were identified showing at least 98.7% 16S rRNA gene sequence similarity with known bacterial species that were previously unreported in South Korea. The 12 bacterial strains were phylogenetically diverse and assigned to four classes, eight orders, nine families, and ten different genera. The isolates were identified as *Leucobacter holotrichiae* (99.1%), *Leucobacter tardus* (99.9%), *Rhodococcus rhodochrous* (99.9%), *Tessaracoccus oleiagri* (100%), and *Paeniglutamicibacter cryotolerans* (99.3%), of the class *Actinobacteria*; *Bacillus coagulans* (99.7%) and *Bacillus wudalianchiensis* (99.1%) of the class *Bacilli*; *Ochrobactrum pseudogrignonense* (99.2%) and *Paracoccus thiocyanatus* (100%) of the class *Alphaproteobacteria*; and *Ideonella azotifigens* (99.0%), *Polaromonas glacialis* (99.3%), and *Herbaspirillum seropedicae* (99.5%) of the class *Betaproteobacteria*. The cellular and colonial morphology, biochemical properties, and phylogenetic position of these isolates were examined, and species descriptions are provided.

Keywords: Freshwater, Nakdong River, unrecorded bacterial species

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Introduction

The Nakdong River is the longest river in South Korea and is used as a major source of drinking water for residents. However, the river passes through major cities with several industrial parks including chemical, electrical, and petrochemical complexes, and various organic chemicals are discharged into the river (Oh *et al*., 2009). These discharges affect eutrophication, intrinsic ecological functions, and microbial diversity (Tekile *et al*., 2015; Kim *et al*., 2018) in the river, and studies on water quality, pollution sources, and bacterial communities in the Nacdong River have been reported frequently (Lee *et al*., 2017; Kim *et al*., 2018; Kim *et al*., 2020).

Next-generation sequencing (NGS) technologies are becoming widely used for microbial diversity studies and data on the freshwater microbial community structure is accumulating. Studies based on the 16S rRNA gene sequence have confirmed that soil, ocean, and freshwater habitats exhibit distinct differences in their microbial community structure (Newton *et al*., 2011). For example, *Actinobacteria* is often the numerically dominant phylum in lakes; they account for more than 50% of the bacteria in the surface waters, but have proven difficult to cultivate (Glöckner *et al*., 2000; Newton *et al*., 2011). Cultivation of isolates is essential to understand the physiological and ecological role of bacteria and to use them industrially.

During the indigenous bacterial diversity survey performed by the Nakdonggang Institute of Biological Resources (NNIBR), freshwater samples were collected from the upstream Nakdong River. Through phylogenetic analyses based on 16S rRNA gene sequences, 12 bacterial species were identified as being previously unrecorded in South Korea. Here, we report the phylogenetic and phenotypic characterization of these bacterial species.

Materials and Methods

Freshwater environmental samples were collected from the river surface and sediment in the Sangjubo (36°27′22.92″N, 128°15′34.27″E), the sediment in a branch of the Sangjubo (36°28′15.16″N, 128°9′14.34″E), and the river surface and sediment in a branch of the Nakdong

River, Bonghwa region, South Korea (37°37′51.06″N, 129°2′36.13″E). Samples were suspended in distilled water and serially diluted. Reasoner's Agar (R2A; BD), Tryptic Soy Agar (TSA; BD), and Luria-Bertani Agar (LB; BD) were inoculated and the plates were incubated at 25°C for 3 days (Table 1). All strains were purified as single colonies and stored as a 20% (w/v) glycerol suspension at −80°C.

Colony morphology and cell size of the strains were observed by using a transmission electron microscope (H-7650, Hitachi). Transmission electron micrographs of the strains are shown in Figure 2. The Gram reaction was performed using a Gram-staining kit (bioMérieux). Biochemical characteristics were tested using API 20NE (bioMérieux) according to the manufacturer's instructions. Oxidase activities were measured using oxidase reagent (bioMérieux).

For 16S rRNA gene sequence analysis, the genomic DNA of isolates was extracted using the DNeasy Blood and Tissue kit (Qiagen). Amplification and sequencing of the 16S rRNA gene were performed by Macrogen (South Korea) using 27F and 1492R universal bacterial primers (Weisburg *et al*., 1991). 16S rRNA gene sequences were compared with sequences retrieved from the EzBioCloud server (Yoon *et al*., 2017). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981), and maximum-parsimony (Fitch, 1971) algorithms in MEGA7 (Kumar *et al*., 2016) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

Results and Discussion

The 12 strains isolated from upstream sections of the Nakdong River were confirmed as previously unrecorded bacterial species by base on the 16S rRNA gene sequence similarity (≥98.7%). The taxonomic identification of the isolates is summarized in Table 1. The phylogenetic tree showed the closest relationship between the isolates and the type strains of validly published species in Figure 1. At the genus level, the unrecorded species were assigned to *Leucobacter* (two strains), *Rhodococcus*, *Tessaracoccus*, and *Paeniglutamicibacter* of the class *Actinobacteria*; *Bacillus* (two strains) of the class *Bacilli*; *Ochrobactrum* and *Paracoccus* of the class *Alphaproteobacteria*; and *Ideonella*, *Polaromonas* and *Herbaspirillum* of the class *Betaproteobacteria*. Transmission electron micrographs of the isolates are shown in Figure 2. Detailed morphological and physiological characteristics of the isolates are given in the strain descriptions. Based on the results from this study, the 12 bacterial isolates were classified as members of *Leucobacter holotrichiae*, *Leucobacter tardus*, *Rhodococcus rhodochrous*, *Tessaracoccus*

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

Class	Order	Family	Strain ID	NNIBR ID	Accession number	Most closely related species		Similarity (%)	Isolation source	Medium
						Closest type strain	Accession number			
Actinobacteria	*Micrococcales*	*Microbacteriaceae*	TS8-160	NNIBR2020641BA456	MT871980	*Leucobacter holotrichiae*	KJ461711	99.1	Sediment	TSA
	Micrococcales	*Microbacteriaceae*	LB1-15	NNIBR2020641BA457	MT871979	*Leucobacter tardus*	AM940158	99.9	Sediment	LB agar
	Mycobacteriales	*Nocardiaceae*	L-23	NNIBR2020641BA459	MT871977	*Rhodococcus rhodochrous*	BBXP01000056	99.9	Sediment	LB agar
	Propionibacteriales	*Propionibacteriaceae*	L-80	NNIBR2020641BA455	MT871981	*Tessaracoccus oleiagri*	jgi.1058080	100.0	Sediment	R2A
	Micrococcales	*Micrococcaceae*	DB04	NNIBR2019641BA9	MN602462	*Paeniglutamicibacter cryotolerans*	GQ406812	99.3	Sediment	R2A
Bacilli	*Bacillales*	*Bacillaceae*	CB2-2	NNIBR2020641BA460	MT871976	*Bacillus coagulans*	CP009709	99.7	Water	TSA
	Bacillales	*Bacillaceae*	RE3-5	NNIBR2020641BA461	MT871975	*Bacillus wudalianchiensis*	MAYT01000030	99.1	Water	R2A
Alphaproteobacteria	*Rhizobiales*	*Brucellaceae*	RB1-8	NNIBR2020641BA462	MT871974	*Ochrobactrum pseudogrignonense*	NNRM01000012	99.2	Water	R2A
	Rhodobacterales	*Rhodobacteraceae*	C-2	NNIBR2020641BA458	MT871978	*Paracoccus thiocyanatus*	jgi.1095766	100.0	Sediment	TSA
Betaproteobacteria	*Burkholderiales*	*Comamonadaceae*	DA05	NNIBR2019641BA6	MN602457	*Ideonella azotifigens*	EU542576	99.0	Water	R2A
	Burkholderiales	*Comamonadaceae*	SS10	NNIBR2019641BA7	MN602465	*Polaromonas glacialis*	HM583568	99.3	Sediment	R2A
	Burkholderiales	*Oxalobacteraceae*	GS32	NNIBR2019641BA8	MN602463	*Herbaspirillum seropedicae*	CP011930	99.5	Sediment	R2A

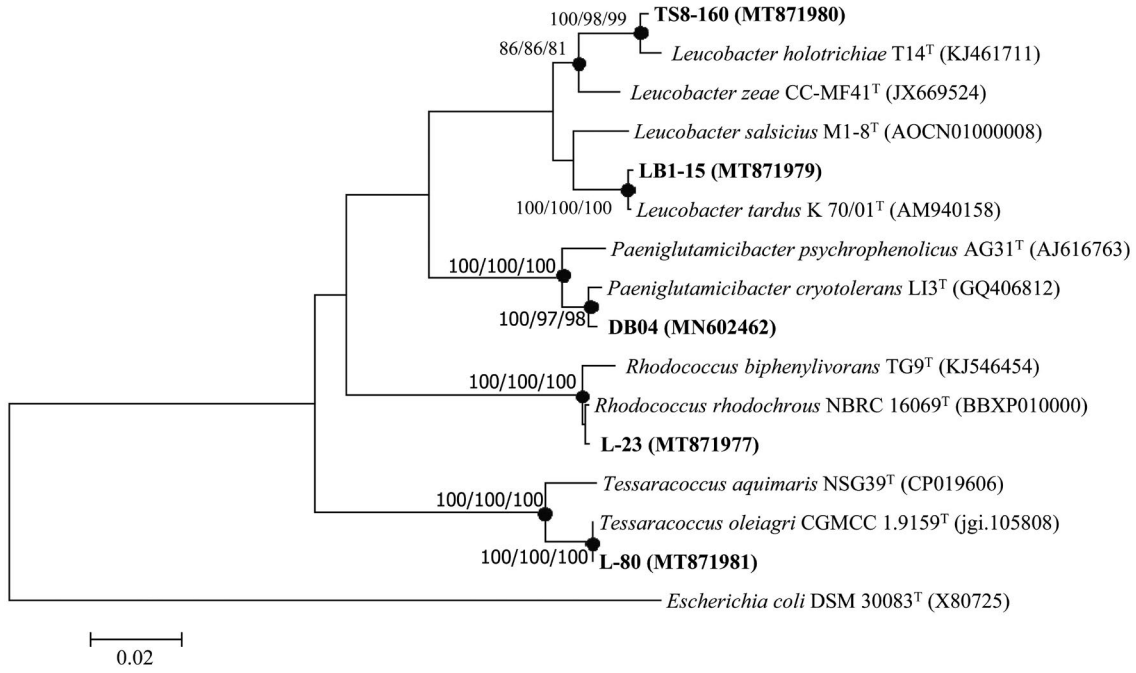

Fig. 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their closest relatives of the class *Actinobacteria*. The tree was reconstructed using neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) algorithms. Filled circles indicate corresponding branches present in the phylogenetic tree generated using the three different tree construction methods. Bootstrap values (expressed as percentages of 1000 replications) of above 70% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

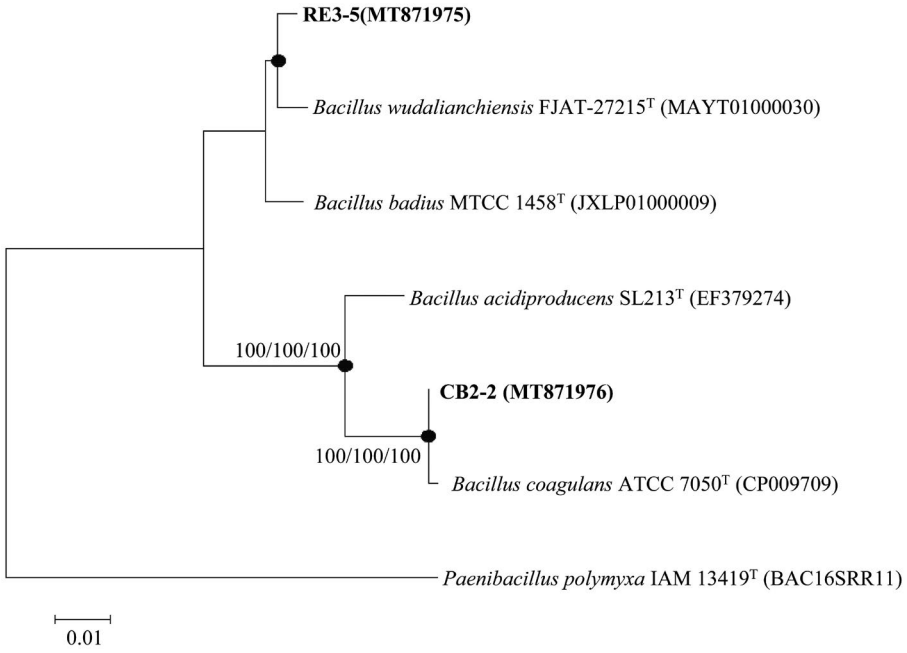

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their closest relatives of the class *Bacilli*. The tree was reconstructed using neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) algorithms. Filled circles indicate corresponding branches present in the phylogenetic tree generated using the three different tree construction methods. Bootstrap values (expressed as percentages of 1000 replications) of above 70% are shown at branch points. Bar, 0.01 substitutions per nucleotide position.

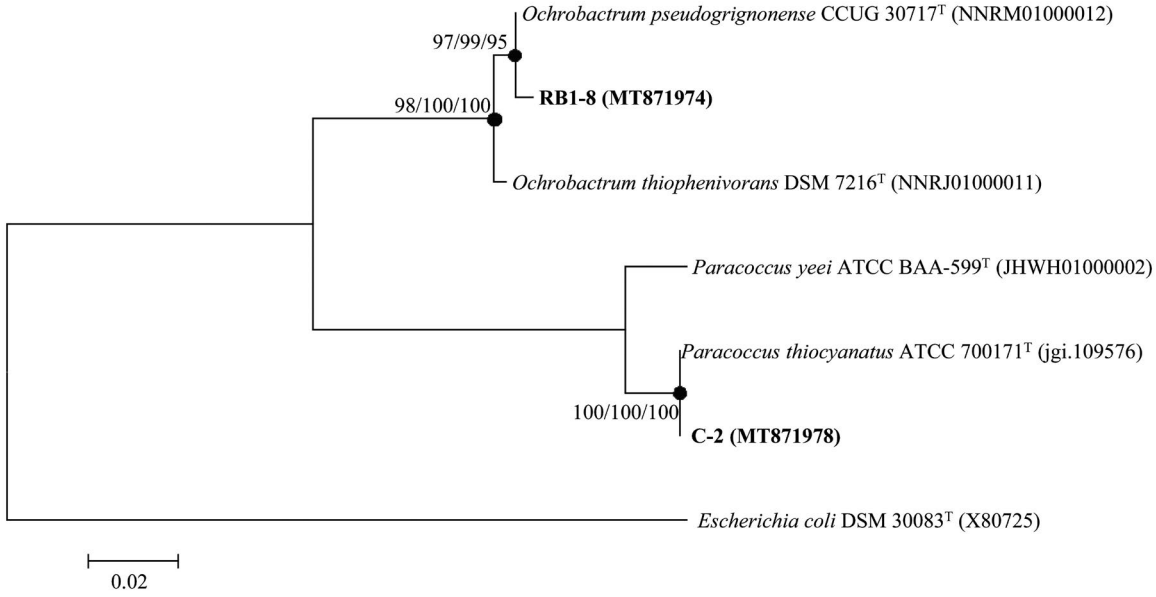

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their closest relatives of the class *Alphaproteobacteria*. The tree was reconstructed using neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) algorithms. Filled circles indicate corresponding branches present in the phylogenetic tree generated using the three different tree construction methods. Bootstrap values (expressed as percentages of 1000 replications) of above 70% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

oleiagri, *Paeniglutamicibacter cryotolerans*, *Bacillus coagulans*, *Bacillus wudalianchiensis*, *Ochrobactrum pseudogrignonense*, *Paracoccus thiocyanatus*, *Ideonella azotifigens*, *Polaromonas glacialis*, and *Herbaspirillum seropedicae*, which have not been officially reported in Korea to date. Thus, we describe the characteristics of these unreported bacterial species.

Description of *Leucobacter holotrichiae* TS8-160

Cells are Gram-staining-positive, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, and cream colored after 3 days on TSA at 25°C. Negative for oxidase. Does not hydrolyze esculin and gelatin. Does not reduce nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase and urease. Assimilates glucose, mannose (weakly), *N*-acetyl-glucosamine (weakly), potassium gluconate (weakly), capric acid (weakly) and trisodium citrate (weakly), but not arabinose, mannitol, maltose, adipic acid, malic acid, and phenylacetic acid. Strain TS8-160 (=NNIBR2020641BA 456) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871980.

Description of *Leucobacter tardus* LB1-15

Cells are Gram-staining-positive, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, and cream yellow colored after 3 days on LB at 25°C. Negative for oxidase. Does not hydrolyze esculin and gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase, and urease. Assimilates glucose (weakly), mannitol, potassium gluconate (weakly), malic acid, and trisodium citrate (weakly), but not arabinose, mannose, *N*-acetyl-glucosamine, maltose, capric acid, adipic acid, and phenylacetic acid. Strain LB1-15 (=NNIBR2020641BA457) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871979.

Description of *Rhodococcus rhodochrous* L-23

Cells are Gram-staining-positive, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, and cream orange colored after 3 days on LB at 25°C. Negative for oxidase. Does not hydrolyze esculin and gelatin. Reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase, and urease. Assimilates glucose (weakly), mannose (weakly), mannitol (weakly), adipic acid, malic acid, and trisodium citrate, but not arabinose, *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, and phenylacetic acid. Strain L-23 (=NNIBR2020641BA459) was isolated from sediment of the Nakdong River. The GenBank accession

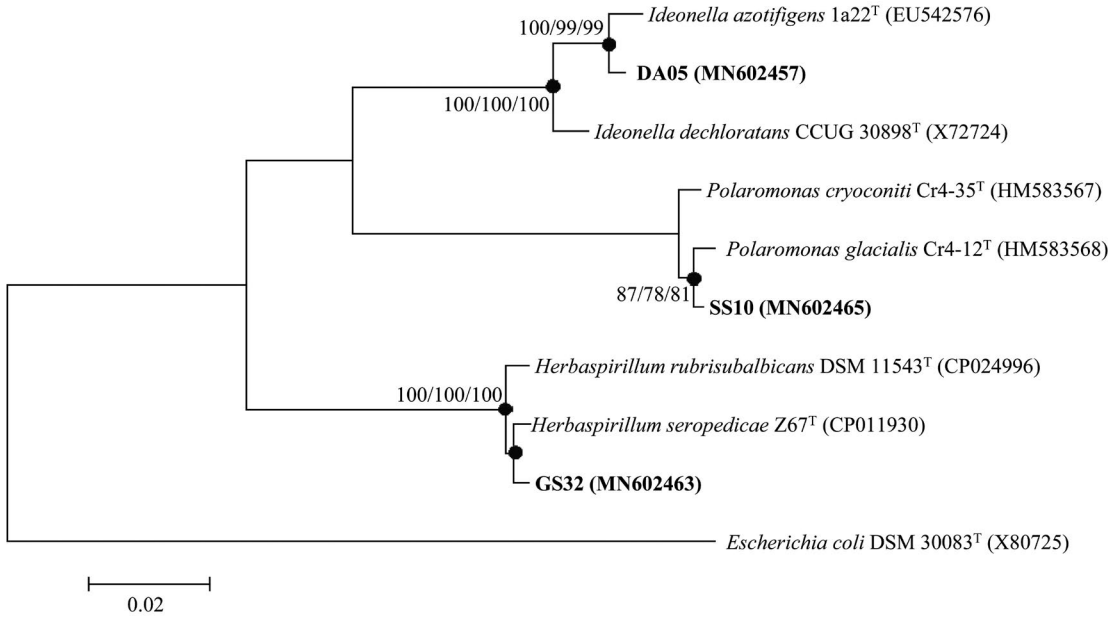

Fig. 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between the strains isolated in this study and their closest relatives of the class *Betaproteobacteria*. The tree was reconstructed using neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) algorithms. Filled circles indicate corresponding branches present in the phylogenetic tree generated using the three different tree construction methods. Bootstrap values (expressed as percentages of 1000 replications) of above 70% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.

number for the 16S rRNA gene sequence is MT871977.

Description of *Tessaracoccus oleiagri* L-80

Cells are Gram-staining-positive, non-flagellated, rod-shaped, and aerobic. Colonies are circular, convex, smooth, and cream colored after 3 days on LB at 25°C. Negative for oxidase. Hydrolyzes esculin, but not gelatin. Reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of β-galactosidase, but not arginine dihydrolase and urease. Assimilates mannitol, maltose and potassium gluconate, but not glucose, arabinose, mannose, *N*-acetyl-glucosamine, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain L-80 (=NNIBR2020641BA455) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871981.

Description of *Paeniglutamicibacter cryotolerans* DB04

Cells are Gram-staining-negative, non-flagellated, rod-shaped, and aerobic. Colonies are opaque, round, convex, and yellow colored after 3 days on R2A at 25°C. Positive for oxidase. Hydrolyzes gelatin, but not esculin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of urease and β-galactosidase, but not arginine dihydrolase. Assimilates maltose, potassium gluconate, malic acid, trisodium citrate, and phenylacetic acid (weakly), but not glucose, arabinose, mannose, mannitol, *N*-acetyl-glucosamine, capric acid, and adipic acid. Strain DB04 (=NNIBR 2019641BA9) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MN602462.

Description of *Bacillus coagulans* CB2-2

Cells are Gram-staining-positive, flagellated, rod-shaped, and aerobic. Colonies are round, convex, and cream yellow colored after 3 days on TSA at 25°C. Positive for oxidase. Hydrolyze esculin, but not gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of β-galactosidase, but not arginine dihydrolase and urease. Assimilates glucose, mannose, *N*-acetyl-glucosamine, but not arabinose, mannitol, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. Strain CB2-2 (=NNIBR2020641BA 460) was isolated from water of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871976.

Description of *Bacillus wudalianchiensis* RE3-5

Cells are Gram-staining-positive, flagellated, rod-

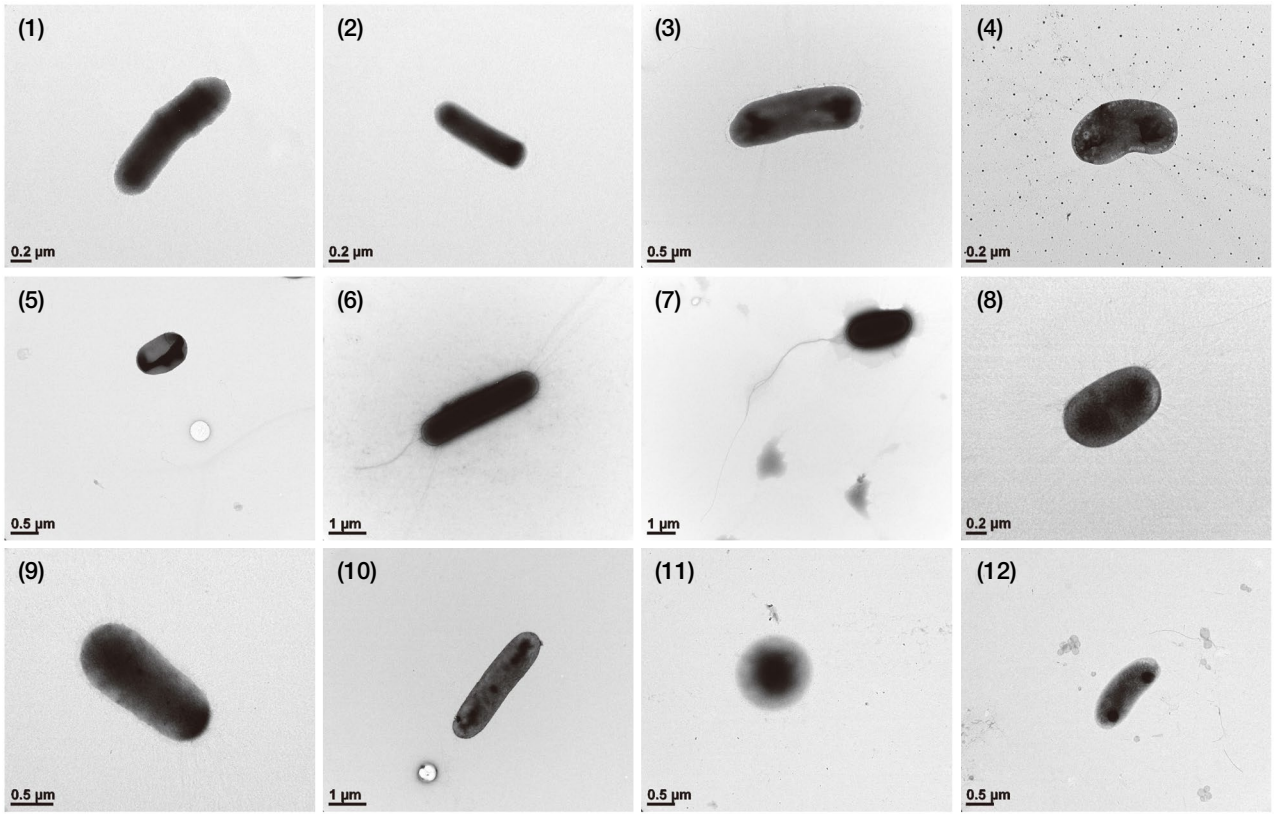

Fig. 5. Transmission electron micrographs of cells of the strains isolated in the study. Stains: 1, TS8-160; 2, LB1-15; 3, L-23; 4, L-80; 5, DB04; 6, CB2-2; 7, RE3-5; 8, RB1-8; 9, C-2; 10, DA05; 11, SS10; 12, GS32.

shaped, and aerobic. Colonies are round, convex, and cream colored after 3 days on R2A at 25°C. Positive for oxidase. Does not hydrolyze esculin and gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of urease, but not arginine dihydrolase and β-galactosidase. Assimilates arabinose, mannose, *N*-acetyl-glucosamine, maltose, potassium gluconate, adipic acid, and phenylacetic acid, but not glucose, mannitol, capric acid, malic acid, and trisodium citrate. Strain RE3-5 (=NNIBR2020641BA461) was isolated from water of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871975.

Description of *Ochrobactrum pseudogrignonense* RB1-8

Cells are Gram-staining-negative, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, and white colored after 3 days on R2A at 25°C. Positive for oxidase. Does not hydrolyze esculin and gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of urease, but not arginine dihydrolase and β-galactosidase. Assimilates glucose, arabinose, mannose, mannitol, and malic acid, but not *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Strain RB1-8 (=NNIBR2020641BA462) was isolated from water of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871974.

Description of *Paracoccus thiocyanatus* C-2

Cells are Gram-staining-negative, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, and cream colored after 3 days on TSA at 25°C. Positive for oxidase. Does not hydrolyze esculin and gelatin. Reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase and urease. Assimilates glucose, arabinose, mannitol, and phenylacetic acid, but not mannose, *N*-acetyl-glucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid and trisodium citrate. Strain C-2 (=NNIBR2020641BA458) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MT871978.

Description of *Ideonella azotifigens* DA05

Cells are Gram-staining-negative, non-flagellated, rod-shaped, and aerobic. Colonies are round, convex, shiny, and cream white colored after 3 days on R2A at 25°C. Positive for oxidase. Hydrolyzes gelatin (weakly), but not esculin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase, and urease. Assimilates mannose, maltose, potassium gluconate, and malic acid, but not glucose, arabinose, mannitol, *N*-acetyl-glucosamine, capric acid, adipic acid, trisodium citrate, and phenylacetic acid. Strain DA05 (=NNIBR2019641BA6) was isolated from water of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MN602457.

Description of *Polaromonas glacialis* SS10

Cells are Gram-staining-negative, non-flagellated, cocci-shaped, and aerobic. Colonies are round and convex, and cream white colored after 3 days on R2A at 25°C. Positive for oxidase. Does not hydrolyze esculin and gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Does not possesses activity of arginine dihydrolase, β-galactosidase, and urease. Assimilates arabinose, mannose (weakly), capric acid and trisodium citrate, but not glucose, mannitol, *N*-acetyl-glucosamine, maltose, potassium gluconate, adipic acid, malic acid, and phenylacetic acid. Strain SS10 (=NNIBR2019641BA7) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MN602465.

Description of *Herbaspirillum seropedicae* GS32

Cells are Gram-staining-negative, non-flagellated, rod-shaped, and aerobic. Colonies are moist and smooth, and cream white colored after 3 days on R2A at 25°C. Positive for oxidase. Does not hydrolyze esculin and gelatin. Does not reduces nitrate to nitrite. Cannot produce acid from glucose. Does not produce indole. Possesses activity of β-galactosidase, but not arginine dihydrolase and urease.

Assimilates glucose, arabinose, mannose (weakly), mannitol, potassium gluconate, capric acid, malic acid, trisodium citrate, but not *N*-acetyl-glucosamine, maltose, adipic acid, and phenylacetic acid. Strain GS32 (=NNIBR 2019641BA8) was isolated from sediment of the Nakdong River. The GenBank accession number for the 16S rRNA gene sequence is MN602463.

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