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A report on five unrecorded bacterial species belonging to the phyla *Actinomycetota*, *Bacillota* and *Pseudomonadota* in Korea isolated in 2020

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During an investigation into the indigenous prokaryotic species diversity in Korea, a total of five bacterial strains were isolated from various environments in Korea. The isolated bacterial strains were identified by analyzing their 16S rRNA gene sequences, and those with a minimum of 98.7% sequence similarity with known bacterial species but not reported in Korea were designated as unrecorded species. These isolates were assigned to three phyla, five orders, five families, and five different genera. The isolates were identified as *Cumulibacter manganitolerans* (99.1%) and *Myolicibacterium tusciae* (98.7%) of the class *Actinomycetes*; *Bacillus marasmi* (99.9%) of the class *Bacilli*; and *Novosphingobium mathurense* (100%) and *Microvirga ossetica* (98.8%) of the class *Alphaproteobacteria*. Gram reaction, colony and cellular morphology, basic biochemical characteristics, and phylogenetic position of theses isolates are also described.

Keywords: 16S rRNA, bacterial diversity, unreported species

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Introduction

In 2020, five unrecorded bacterial species were isolated from various environmental samples collected in Korea and identified as members of phyla *Actinomycetota*, *Bacillota* and *Pseudomonadota*.

Phylum *Actinomycetota* (Oren *et al*., 2021) is the newly validated name of the phylum *Acitnobacteria*, which was originally proposed by Goodfellow (2012). The phylum *Actinomycetota* is currently classified into six classes including *Acidimicrobiia*, *Actinomycetes*, *Coriobacteriia*, *Nitriliruptoria*, *Rubrobacteria*, and *Thermoleophilia* (List of Prokaryotic Name with Standing in Nomenclature, as of March 2023). The phylum *Actinomycetota* is a diverse group of aerobic and Gram-staining-positive bacteria with high G+C content in their DNA. Members of the phylum *Actinomycetota* are widespread in nature and can be found in various habitats such as soil, water, and plants. *Actinomycetota* have been found in all layers of soil, but their abundance decreases with increasing depth (Takahashi and Omura, 2003). Some *Actinomycetota* members can cause diseases in humans, animals, and plants (Amin *et al*., 2020). *Actinomycetota* has diverse morphological features including simple rods and cocci, or complex mycelial structures similar to fungi (Amin *et al*., 2020). Although they were once considered an intermediate group between bacteria and fungi, they are now recognized as prokaryotic organisms (Amin *et al*., 2020). *Actinomycetota* play important roles in various ecological processes, such as decomposition of organic matter, nitrogen fixation, and symbiotic relationships with plants (Zhang *et al*., 2019). Some species are also pathogenic to humans, causing diseases such as tuberculosis and leprosy. *Actinomycetota* are of great interest due to their significant contributions to various fields such as agriculture, ecology, industry, and medicine. They are known for their ability to produce a wide range of secondary metabolites, which have important applications in drug discovery, agriculture, and industry. For example, *Actinomycetota* are responsible for the production of many antibiotics, anticancer agents, immunosuppressants, and other bioactive compounds that

are used in medicine. In agriculture, some *Actinomycetota* are involved in nitrogen fixation and plant growth promotion. *Actinomycetota* also plays important roles in soil ecology by decomposing organic matter and recycling nutrients (Van der Meij *et al*., 2017). Overall, the vast diversity of *Actinomycetota* and their ability to produce a wide range of biologically active compounds make them an important group of microorganisms for research and development.

Phylum *Bacillota* is the newly validated name of the phylum *Firmicutes* (Oren *et al*., 2021). The phylum *Bacillota* is currently classified into seven classes including *Bacilli*, *Clostridia*, *Culicoidibacteria*, *Erysipelotrichia*, *Limnochordia*, *Negativicutes*, and *Thermolithobacteria*. The phylum *Bacillota* is composed of gram-positive bacteria with low G+C content in their DNA. They have a rigid or semi-rigid cell wall containing peptidoglycan (Gibbons and Murray, 1978). Cells of the phylum are mainly rod or sphere-shaped, reproducing through binary fission. Some species can form endospores and have flagella for motility. These bacteria are widely distributed in soil and aquatic environments, where they play a role in decomposing and recycling organic matter (Baik *et al*., 2008). Certain members of the phylum *Bacillota* are also of industrially valuable, as they are used in the production of antibiotics, enzymes, and dairy products (Liu *et al*., 2012).

Phylum *Pseudomonadota* is the newly validated name of the phylum *Proteobacteria* (Oren *et al*., 2021) and currently classified into eight classes including *Acidithiobacillia*, *Alphaproteobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Gammaproteobacteria*, *Hydrogenophilia*, and *Oligoflexia*. *Pseudomonadota* is a major phylum of Gram-staining-negative bacteria and reported among the predominant in natural environments. Members of the phylum *Pseudomonadota* encompass both pathogenic genera, such as *Escherichia*, *Salmonella*, *Vibrio*, *Yersinia*, and *Legionella*, and free-living bacteria including bacteria responsible for nitrogen fixation. Nitrogen-fixing bacteria play a crucial role in the ecosystem as they convert atmospheric nitrogen into a form that plants can use, making them important for plant growth and soil fertility.

During an investigation into the indigenous prokaryotic species diversity in Korea in 2020, various soil samples were collected from agricultural fields and forests in Korea. An automobile air-conditioning system was collected from a car operated at least one year in Korea. Phylogenetic analysis of 16S rRNA gene sequences has led to the identification of five unrecorded species as being previously unrecorded in Korea. Here, we report phylogenetic and phenotypic characteristics of these bacterial species.

Materials and methods

A total of five bacterial strains were isolated from various environments including agricultural soil, forest soil and car air conditioner collected in Korea. Agricultural soils were collected from Seongsan-eup, Seogwipo-si, Jeju-do (N 33°24′35.7, E 126°52′41.1) and Taean-gun, Chungcheongnam-do (N 36°45′00.8, E 126°16′29.8); and forest soils were collected from Seoul (N 37°27′42.4, E 126°57′32.6) in South Korea. Each sample was independently processed as the following, serially diluted, spread onto R2A, nutrient agar (NA), and Tryptic Soy Agar (TSA), and Peptone-Tryptone-Yeast extracts-Glucose (PTYG) agar medium, and incubated at 25−28°C for three days. The strain name, growth media, isolation source, incubation conditions are summarized in Table 1. All strains were purified as single colonies and stored as 15−17% glycerol suspension at −80°C as well as lyophilized ampoules. After the cells reached the stationary phase, colony morphology of the strains was observed using a magnifying lens. The cellular morphology and size of the strains were examined using transmission electron microscopy (80 Kv, JEM1010, Fig. 1). Gram reaction was performed according to the Gram procedure described by Doetsch (1981). Biochemical characteristics were tested by using API 20NE, API 32GN, API ZYM galleries (bioMérieux) according to the manufacturer's instructions.

Genomic DNA was extracted and 16S rRNA gene was amplified by PCR with 27mf and 1492R universal bacterial primers (Lee and Kim, 2022). The 16S rRNA gene sequences of the closely related strains were obtained from EzBioCloud server (Yoon *et al*., 2017). The obtained 16S rRNA gene sequences were aligned using the SILVA alignment tool according to the SILVA seed alignment (Presse *et al*., 2012). Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987) algorithm in MEGA11 program (Tamura *et al*., 2021). The evolutionary distances were calculated using the two-parameter model (Kimura, 1983). Tree topologies were evaluated on the basis of bootstrap values calculated based on 1,000 replications (Felsenstein, 1985).

Results and discussion

Strains with 16S rRNA gene sequence similarity of 98.7% or more were selected as candidates for the unrecorded species. Based on the analysis of the Korean Peninsula Biodiversity Database (https://species.nibr.go.kr/server/gatePage.do), strains selected as candidates for unrecorded species were assigned as unrecorded species in Korea. Through a comparison of the 16S rRNA gene

Table 1. The taxonomic affiliations of isolated strains belonging to the phyla *Actinomycetota*, *Bacillota* and *Pseudomonadota*.

Phylum	Class	Order	Family	Genus	Strain name	NIBR ID	Most closely related species	Similarity (%)	Isolation source	Medium	Incubation conditions
Actinomycetota	*Actinomycetes*	*Geodermatophilales*	*Antricoccaceae*	*Cumulibacter*	ID2628S	NIBRBAC000506101	*Cumulibacter manganitolerans*	99.1	Air conditioner	R2A	28°C, 3d
		Mycobacteriales	*Mycobacteriaceae*	*Mycobacterium*	SY1(iv-2)	NIBRBAC000506104	*Mycolicibacterium tusciae*	98.7	Paddy soil	PTYG	25°C, 3d
Bacillota	*Bacili*	*Caryophanales*	*Bacillaceae*	*Bacillus*	CN1234	NIBRBAC000506105	*Bacillus marasmi*	99.9	Paddy soil	R2A	28°C, 3d
Pseudomonadota	*Alphaproteobacteria*	*Sphingomonadales*	*Erythrobacteraceae*	*Novosphingobium*	EM833	NIBRBAC000506102	*Novosphingobium mathurense*	100.0	Forest soil	R2A	28°C, 3d
		Hyphomicrobiales	*Methylobacteriaceae*	*Microvirga*	SY1(iii-1)	NIBRBAC000506103	*Microvirga ossetica*	98.8	Paddy soil	PTYG	28°C, 3d

sequence and phylogenetic analysis, five unrecorded bacterial species were identified. These strains were assigned to three phyla (*Actinomycetota*, *Bacillota*, and *Pseudomonadota*) and classified into three classes, five orders, five families, and five genera (Table 1) Among these strains, ID2628S was coccus-shaped, and the others were rod-shaped (Fig. 1). Colony morphology and physiological characteristics are presented in the species description section. In the order *Geodermatophilales*, a strain was assigned to the family *Antricoccaceae* which includes the genus *Cumulibacter*. In the order *Mycobacteriales*, a strain was assigned to the family *Mycobacteriaceae* which includes the genus *Mycolicibacterium*. In the order *Caryophanales*, a strain was assigned to the family *Bacillaceae* which includes the genus *Bacillus*. A strain was assigned to genus *Novosphingobium* of the family *Erythrobacteraceae* in the order *Sphingomonadales* and one strain was assigned to the genus *Microvirga* of the family *Methylobacteriaceae* in the order *Hyphomicrobiales*. The identification of the five strains based on 16S rRNA sequence similarity was supported by the phylogenetic trees (Fig. 2). The detailed morphological and physiological characteristics are given in the strain descriptions.

Description of *Cumulibacter manganitolerans* ID2628S

Cells are Gram-staining-positive, flagellated, and coccus-shaped with 0.6−0.8 × 1.0−1.5 μm in size. Colonies are circular, smooth, entire and yellow colored after three days of incubation at 30°C on R2A. The strain is positive for β-glucosidase (esculin hydrolysis) and β-galactosidase (PNPG); but negative for reduction of nitrates to nitrite, reduction of nitrates to nitrogen, indole production, glucose acidification, arginine dihydrolase, urease, and protease (gelatin hydrolysis). The strain assimilates D-glucose, D-maltose, malate and phenyl-acetate; but does not assimilate L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, gluconate, caprate, adipate, and citrate. The strain utilizes D-glucose, salicin, D-sucrose, D-maltose, acetate, lactate, and glycogen; but does not utilize D-mannitol, D-melibiose, L-fucose, D-sorbitol, L-arabinose, propionate, caprate, valerate, citrate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, inositol, itaconate, suberate, malonate, L-alanine, 5-ketogluconate, 3-hydroxy-benzoate, and L-serine. The strain produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, and β-glucosidase; but does not produce lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosamini-

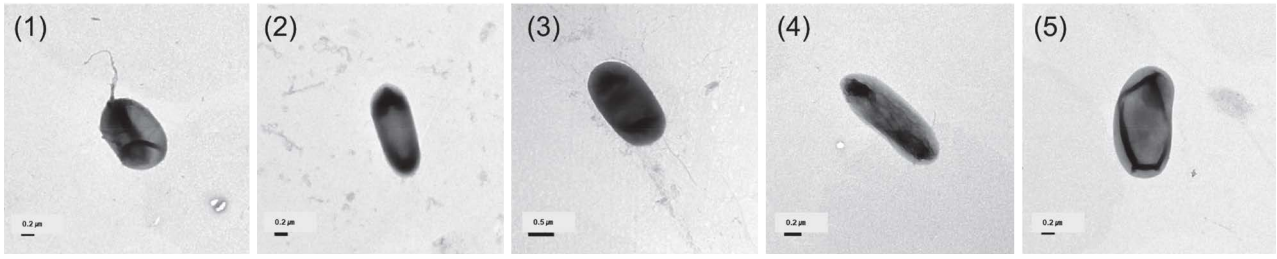

Fig. 1. Transmission electron microscopy of cells of the strains isolated in this study. The cells were cultured at their optimal growth conditions. Strains: 1, ID2628S; 2, SY1 (iv-2); 3, CN1234; 4, EM833; 5, SY1 (iii-1).

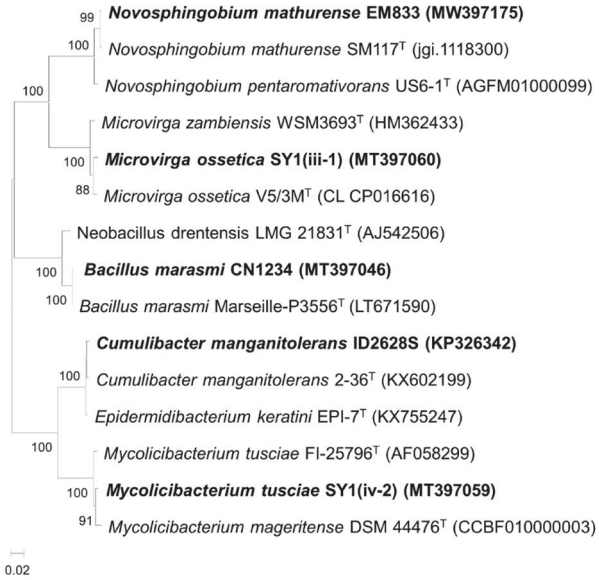

Fig. 2. A neighbor-joining phylogenetic tree based on 16S rRNA gene sequences shows the relationship between the strains isolated in this study and their relatives. Bootstrap values (>70%) are shown above nodes for the neighbor-joining methods. Bar: 0.02 substitutions per nucleotide position.

dase, α-mannosidase, and α-fucosidase. Strain ID2628S (=NIBRBAC000506101) was isolated from the surface of an automobile air conditioner system.

Description of *Mycolicibacterium tusciae* SY1 (iv-2)

Cells are Gram-staining-positive, non-flagellated, and rod-shaped with 0.9−1.0 × 1.4−1.6 μm in size. Colonies are circular, moist, undulate and white colored after three days of incubation at 25°C on R2A. The strain is positive for reduction of nitrates to nitrite, assimilation of D-mannitol, gluconate, and malate; but negative for indole production, glucose acidification, arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), β-galactosidase (PNPG), D-glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, D-maltose, caprate, adipate, citrate, and phenyl-acetate. The strain utilizes D-mannitol, D-sorbitol, propionate, valerate, 3-hydroxy-butyrate, inositol, and acetate; but does not utilize D-glucose, salicin, D-melibiose, L-fucose, L-arabinose, caprate, citrate, L-histidine, 2-ketogluconate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, D-sucrose, D-maltose, itaconate, suberate, malonate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine. The strain produces esterase (C4), esterase Lipase (C8), leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, trypsin (weak); but does not produce alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain SY1 (iv-2) (=NIBRBAC000506104) was isolated from a paddy soil sample, Jeju Island, Korea.

Description of *Bacillus marasmi* CN1234

Cells are Gram-staining-positive, flagellated, and rod-shaped with 0.7−0.8 × 1.6−1.8 μm in size. Colonies are smooth, translucent and white colored after three days of incubation at 30°C on R2A. The strain is positive for reduction of nitrates to nitrite, glucose acidification, β-galactosidase (PNPG), assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, and malate; but negative for indole production, arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), caprate, adipate, citrate, and phenyl-acetate. The strain utilizes D-mannitol, D-glucose, D-melibiose, L-fucose, 2-ketogluconate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, D-maltose, acetate, lactate, L-alanine, glycogen, and L-serine; but does not utilize salicin, D-sorbitol, propionate, caprate, valerate, citrate, L-histidine, 3-hydroxy-butyrate, 4-hydroxy-benzoate, inositol, D-sucrose, itaconate, suberate, malonate, 5-ketogluconate, and 3-hydroxy-benzoate. The strain produces alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphtol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase, α-glucosidase, and

cystine arylamidase (weak); but does not produce esterase (C4), esterase lipase (C8), lipase (C14), α-chymotrypsin, α-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain CN1234 (=NIBRBAC000506105) was isolated from a paddy soil sample, Taean County, Korea.

Description of *Novosphingobium mathurense* EM833

Cells are Gram-staining-negative, non-flagellated, and rod-shaped with 0.6−0.8 × 1.0−1.5 μm in size. Colonies are circular, smooth, entire and pale-yellow colored after three days of incubation at 30°C on R2A. The strain is positive for nitrate reduction, glucose fermentation, arginine dihydrolase, and β-galactosidase (esculin hydrolysis); but negative for indole production, and protease (gelatin hydrolysis). The strain assimilates D-glucose, L-arabinose, D-maltose, and citrate; but does not assimilate D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, gluconate, caprate, adipate, malate, and phenylacetic acid. The strain utilizes D-glucose, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, propionate, caprate, valerate, citrate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, inositol, D-sucrose, and D-maltose; but does not utilize D-mannitol, D-melibiose, L-fucose, D-sorbitol, propionate, caprate, valerate, L-histidine, 2-ketogluconate, *N*-acetyl-D-glucosamine, D-ribose, inositol, itaconate, suberate, malonate, acetate, lactate, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine. The strain produces alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphtol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, and β-glucosidase; but does not produce lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain EM833 (=NIBRBAC000506102) was isolated from a forest soil sample, Seoul, Korea.

Description of *Pseudorivibacter rhizosphaerae* SY1 (iii-1)

Cells are Gram-staining-negative, non-flagellated, and rod-shaped with 0.8−1.0 × 1.5−1.8 μm in size. Colonies are circular, smooth, entire and cream white colored after three days of incubation at 30°C on R2A. The strain is positive for reduction of nitrates to nitrite, urease, D-glucose, and D-mannose; but negative for indole production, glucose acidification, arginine dihydrolase, β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), and β-galactosidase (PNPG). The strain assimilates D-glucose, D-mannose, L-arabinose, D-mannitol, D-maltose, gluconate, *N*-acetyl-D-glucosamine, caprate, adipate, malate, citrate, and phenyl-acetate; but does not utilize D-mannitol, salicin, D-melibiose, L-arabinose, propionate, caprate, valerate, citrate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, *N*-acetyl-D-glucosamine, D-ribose, inositol, D-sucrose, D-maltose, itaconate, suberate, malonate. lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine. The strain produces esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphtol-AS-BI-phosphohydrolase; but does not produce lipase (C14), valine arylamidase, cystine arylamidase, trypsin (weak), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Strain SY1 (iii-1) (=NIBRBAC000506103) was isolated from a paddy soil sample, Jeju Island, Korea.

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