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A report of 11 unrecorded bacterial species in Korea, isolated from Hapcheonho Lake and Jinyangho Lake

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In order to investigate the indigenous prokaryotic species diversity of the Nakdong River system in Korea, fresh water samples from Hapcheonho Lake and Jinyangho Lake were analyzed for bacterial taxonomic diversity. The isolated bacterial strains were identified based on 16S rRNA gene sequences, and those exhibiting at least 98.7% sequence similarity with known bacterial species, but never reported in Korea, were selected as unrecorded species. Eleven unrecorded bacterial species were discovered in this study. The isolates were identified as *Aquabacterium citratiphilum*, *Clostridium ghonii*, *Curvibacter delicates*, *Deinococcus depolymerans*, *Eubacterium moniliforme*, *Flavobacterium nitrogenifigens*, *Kineosporia mesophila*, *Luteibacter jiangsuensis*, *Microbacterium terricola*, *Rhizobium larrymoorei*, and *Sediminicoccus rosea* belonging to the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Deinococcus-Thermus*. The selected isolates were further characterized for cellular and colonial morphologies, growth conditions, physiological properties, and enzymatic activities. Descriptive information of these previously unrecorded species is also provided.

Keywords: Hapcheonho Lake, Jinyangho Lake, Nakdong River, unrecorded bacterial species

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Introduction

Bacteria constitute the overwhelming majority of living organisms. There are approximately 5×10^{30} bacteria on Earth (Whitman *et al*., 1998), and the number of bacterial species is estimated at about 1×10^{6}-1×10^{9} (Pedros-Alio, 2006). Because of the enormous diversity and unculturability of bacteria, however, the majority of bacterial species have not been reported or characterized to date. For example, the Domain Bacteria contained 29 phyla as of November 2016, but only half of the phyla are represented by species that can be grown in the laboratory (Rappe and Giovannoni, 2003). Recent molecular techniques, such as metagenomics, have made significant advances in describing the enormous genetic diversity of previously uncultured microorganisms, but it is impossible to understand the nature of bacterial species from sequence data alone. Never the less, cultivation of isolates is essential in order to to understand the physiology and ecological roles of bacteria and use bacteria for natural product production (Stewart, 2012). Because prokaryotic microorganisms are valuable genetic resources, it is important to isolate indigenous bacterial species that can be cultivated. To this end, the Korean government is conducting a program to investigate and collect indigenous bacterial species in Korea. Through years of research and exploration funded by the Korean Ministry of Environment, the enormous prokaryotic diversity in Korea has begun to be described.

As part of this research, we aimed to discover indigenous prokaryotic species diversity and collect specimen of previously unreported bacterial resources from the Nakdong River system in Korea. In particular, Hapcheonho Lake and Jinyangho Lake were selected as survey sites in this study. Both are large artificial lakes generated by dam construction for hydroelectric power generation, located in Gyeongsangnam-do Province. Hapcheonho Lake was constructed in 1984 with 925 km^2 of drainage area. Jinyangho Lake was constructed in 1970 with 2,285 km^2 of drainage area. According to previous reports, Hapcheonho Lake is mesotrophic (Seong *et al*., 2011) and Jinyangho Lake is mesotrophic

or hypertrophic depending on measurement index (Lee *et al*., 2006). As neither lake has been surveyed for bacterial diversity, the composition of unique indigenous species that exist in those lakes are of interest.

Materials and Methods

Bacterial strains were isolated from fresh water samples from two lakes belonging to the Nakdong River system during the summer season. Fresh water samples from surface and middle layer water (depth of 30 m) were collected from Hapcheonho Lake (N35°31′40″; E128°1′20″) on July 16, 2016. Fresh water samples from surface and middle layer water (depth of 5 m) were collected from Jinyangho Lake (N35°10′3″; E128°1′56″) on July 17, 2016. To isolate aerobic bacteria, R2A (BD) was inoculated with a freshwater sample using a spread plating technique and incubated at 25°C for 2-7 days. To isolate anaerobic bacteria, Anaerobe Basal Agar (Oxoid) was used and incubated under anaerobic gas conditions created by AnaeroPack (Mitsubishi Gas Chemical) at 25°C for 2-7 days. Once a purified single colony was obtained, for some of the strains, the routine culture media were replaced by marine agar 2216 (MA; BD) or MRS (BD) to enhance growth. The designation of strains, source of isolation, culture media, and incubation conditions are summarized in Table 1. Isolated strains were stored as 20% glycerol suspension at −80°C and as lyophilized ampoules.

Bacterial DNA extraction, PCR amplification, and gene sequencing were performed using standard procedures (Shin *et al*., 2016). Universal primers 27F and 1492R were used for PCR and sequencing of the 16S rRNA gene. The 16S rRNA gene sequences were compared with other bacterial species with published names using the EzTaxon-e server (Kim *et al*., 2012). A cutoff value of 98.7% sequence similarity was employed for identification. Strains exhibiting 98.7% or higher sequence similarity with known bacterial species but never reported in Korea were selected as unrecorded species. For phylogenetic analyses, sequence alignments between the 16S rRNA gene sequences of the isolates and those of the reference type strains were carried out using EzEditor (Jeon *et al*., 2014). Evolutionary distances were calculated using the Jukes-Cantor distance model (Jukes and Cantor, 1969) and the phylogenetic trees were constructed by using the neighbor-joining (Saitou and Nei, 1987) and maximum-likelihood algorithms (Felsenstein, 1993) implemented in MEGA 6.0 (Tamura *et al*., 2013). The robustness of the inferred trees was evaluated by bootstrap analysis (Felsenstein, 1985) based on 1,000 resamplings.

Colonial morphology was observed on agar plates

Table 1. Taxonomic affiliations and summary of strains isolated from fresh water in Hapcheonho Lake and Jinyangho Lake.

Order	Family	Genus	Strain ID	NIBR ID	Most closely related species	Similarity (%)	Isolation source	Medium	Incubation conditions
Lysobacterales	*Rhodanobacteraceae*	*Luteibacter*	HYN0015	IHBA_24	*Luteibacter jiangsuensis*	99.5	Fresh water	R2A	25°C, 3 d
Burkholderales	Unassigned	*Aquabacterium*	HYN0035	IHBA_32	*Aquabacterium citratiphilum*	98.9	Fresh water	R2A	25°C, 3 d
Burkholderales	*Comamonadaceae*	*Curvibacter*	HYN0016	IHBA_25	*Curvibacter delicatus*	99.5	Fresh water	NA	25°C, 3 d
Rhodospirillales	*Acetobacteraceae*	*Sediminicoccus*	HYN0003	IHBA_22	*Sediminicoccus rosea*	99.2	Fresh water	R2A	25°C, 5 d
Rhizobiales	*Rhizobiaceae*	*Rhizobium*	HYN0033	IHBA_30	*Rhizobium larrymoorei*	99.9	Fresh water	R2A	25°C, 3 d
Clostridiales	*Clostridiaceae*	*Clostridium*	HYN0019	IHBA_26	*Clostridium ghonii*	99.8	Fresh water	CM	37°C, 3 d
Clostridiales	*Eubacteriaceae*	*Eubacterium*	HYN0057	IHBA_34	*Eubacterium moniliforme*	98.9	Fresh water	ABA	25°C, 3 d
Deioncoccales	*Deinococcaceae*	*Deinococcus*	HYN0037	IHBA_33	*Deinococcus depolymerans*	99.3	Fresh water	R2A	25°C, 3 d
Flavobacteriales	*Flavobacteriaceae*	*Flavobacterium*	HYN0034	IHBA_31	*Flavobacterium nitrogenifigens*	99.8	Fresh water	R2A	25°C, 3 d
Micrococcales	*Microbacteriaceae*	*Microbacterium*	HYN0002	IHBA_21	*Microbacterium terricola*	99.1	Fresh water	R2A	25°C, 2 d
Kineosporales	*Kineosporaceae*	*Kineosporia*	HYN0032	IHBA_29	*Kineosporia mesophila*	100.0	Fresh water	R2A	25°C, 3 d

after the cells were cultivated to their stationary phase. Cellular morphology and cell size were examined by transmission electron microscopy. Gram staining was performed using a Gram-reaction kit. Biochemical characteristics were tested by using API 20NE or API 20A galleries (bioMérieux) according to the manufacturer's instructions.

Results and Discussion

Through taxonomic investigation of culturable bacteria isolated from Hapcheonho and Jinyangho Lakes, a number of bacterial strains were isolated. Eleven of the isolates were identified as members of known bacterial species by exhibiting higher than 98.7% 16S rRNA gene sequence similarity. The similarity based identification was further supported by phylogenetic trees (Fig. 1). Each isolate formed rigid monophyletic clades with the type strain of identified bacterial species, confirming the proper assignment of the isolate to the species with published names. The tree topology of the maximum-likelihood method was not different from that of the neighbor-joining tree. For convenience, based on sequence similarity and the phylogenetic trees, we identified the eleven bacterial isolates as members of previously described bacterial species. The strain information and identification results are summarized in Table 1.

The eleven strains belonged to the class *Actinobacteria* (2 strains) of the phylum *Actinobacteria*, the class *Flavobacteriia* (1 strain) of the phylum *Bacteroidetes*, the class *Deinococci* (1 strain) of the phylum *Deinococcus-Thermus*, the class *Clostridia* (2 strains) of the phylum *Firmicutes*, and the class *Alphaproteobacteria* (2 strains), *Betaproteobacteria* (2 strains), and *Gammaproteobacteria* (1 strain) of the phylum *Proteobacteria*. At generic and family level, those isolate assigned to 11 different genera of 11 families, namely *Kineosporia* of *Kineosporiaceae*, *Microbacterium* of *Microbacteriaceae*, *Flavobacterium* of *Flavobacteriaceae*, *Deinococcus* of *Deinococcaceae*, *Clostridium* of *Clostridiaceae*, *Eubacterium* of *Eubacteriaceae*, *Rhizobium* of *Rhizobiaceae*, '*Sediminicoccus*' of *Acetobacteriaceae*, *Curvibacter* of *Comamonadaceae*, *Luteibacter* of *Rhodanobacteraceae*, and *Aquabacterium* of an unassigned family.

The isolates were Gram-reaction-negative or positive, rod- or coccoid-shaped, flagellated or non-flagellated bacteria (Fig. 2). Colonies were white, yellow, or red colored. None of the isolates produced diffusible pigment on agar plates (NA, R2A, ABA, or CM). Some strains possessed enzymatic activities of oxidase, catalase, urease, arginine dihydrolase, and/or β-galactosidase. Several isolates could hydrolyze high molecular weight compounds such as esculin and gelatin. Nitrate reduction and glucose fermentation varied depending on strains. None of the isolate produced indole from L-tryptophan. The detailed morphological and physiological characteristics of each isolate are given in the strain descriptions.

Based on the results obtained in this study, the eleven fresh water isolates were identified as members of *Aquabacterium citratiphilum*, *Clostridium ghonii*, *Curvibacter delicates*, *Deinococcus depolymerans*, *Eubacterium moniliforme*, *Flavobacterium nitrogenifigens*, *Kineosporia mesophila*, *Luteibacter jiangsuensis*, *Microbacterium terricola*, *Rhizobium larrymoorei*, and *Sediminicoccus rosea*. However, the presence of the above mentioned eleven bacterial species has not been previously reported in Korea (Kang and Yoon, 2015). Accordingly, the following 11 species are reported as unrecorded species in Korea.

Description of *Aquabacterium citratiphilum* HYN0035

Cells are Gram-reaction-negative, non-flagellated, and rod-shaped. Colonies are circular with entire margin and white colored after 3 days of incubation on R2A at 25°C. Possesses oxidase and urease activities, but not arginine dihydrolase or β-galactosidase activities. Does not hydrolyze esculin or gelatin. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Uses adipic acid and malic acid as sole carbon source, but not D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, trisodium citrate, or phenylacetic acid. Strain HYN0035 (=IHBA_32) was isolated from a fresh water sample, Hapcheonho lake, Korea.

Description of *Clostridium ghonii* HYN0019

Cells are Gram-reaction-positive, flagellated, and rod-shaped. Colonies are irregular, opaque, undulate, and white colored after 3 days of incubation on Clostridial medium at 37°C. Catalase negative. Hydrolyzes gelatin and esculin. Does not produce indole. Does not possess urease activity. Does not produce acid from D-glucose, D-mannitol, D-lactose, sucrose, D-maltose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-mannose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose, or D-trehalose. Strain HYN0019 (=IHBA_26) was isolated from a fresh water sample, Jinyangho lake, Korea.

Description of *Curvibacter delicatus* HYN0016

Cells are Gram-reaction-negative, flagellated, and rod-shaped. Colonies are punctiform, flat, and white colored after 3 days of incubation on NA at 25°C. Possesses oxidase and urease activities, but not arginine dihydrolase or β-galactosidase activities. Reduces nitrate. Does not

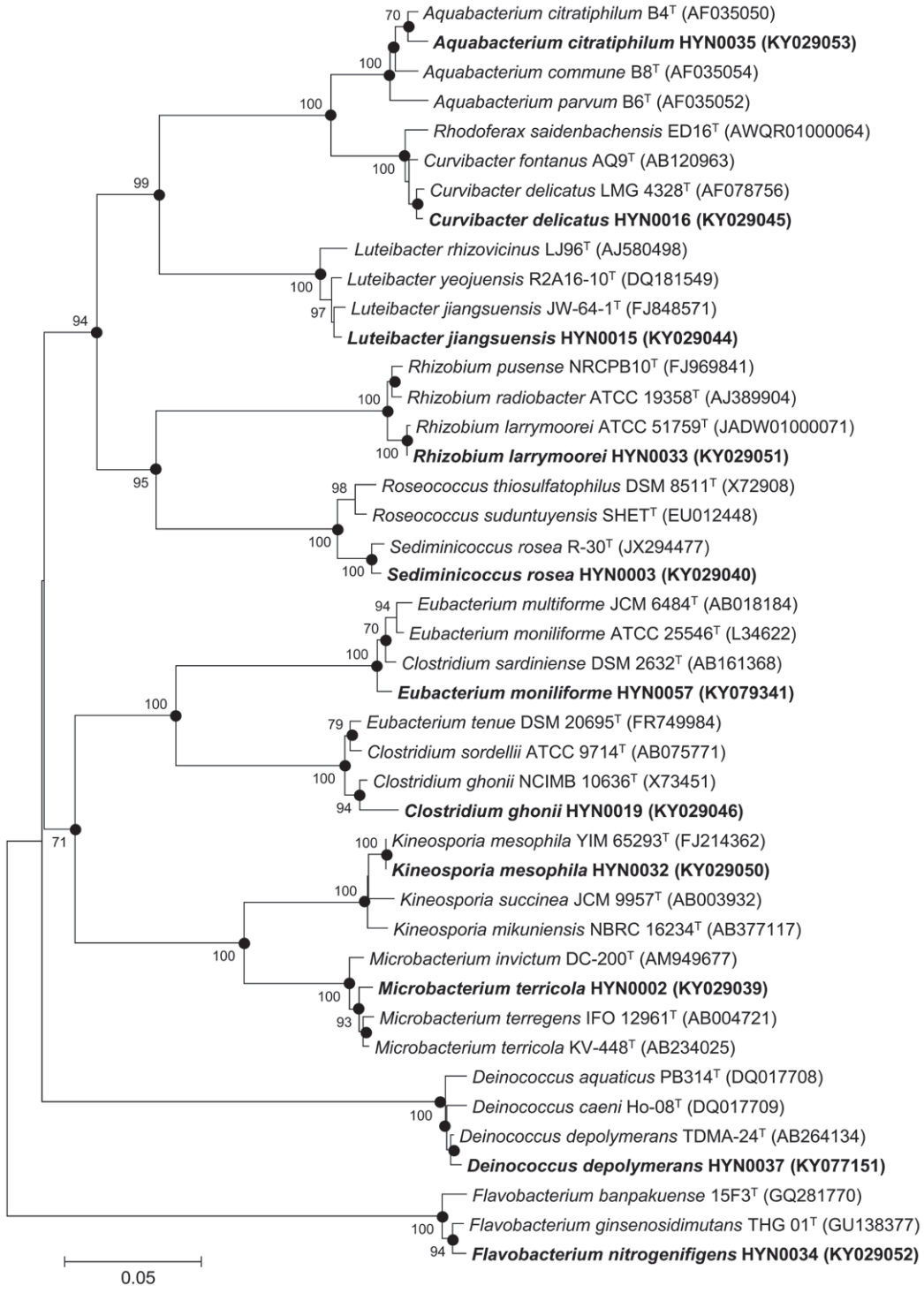

Fig. 1. Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationships among the eleven isolates and their closest relatives. Filled circles indicate the nodes that were also recovered in the maximum-likelihood tree. Bootstrap values are shown above nodes.

produce indole. Does not ferment glucose. Does not hydrolyze esculin or gelatin. Uses adipic acid as sole carbon source, but not D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, malic acid, trisodium citrate, or phenylacetic acid. Strain HYN0016 (=IHBA_25) was

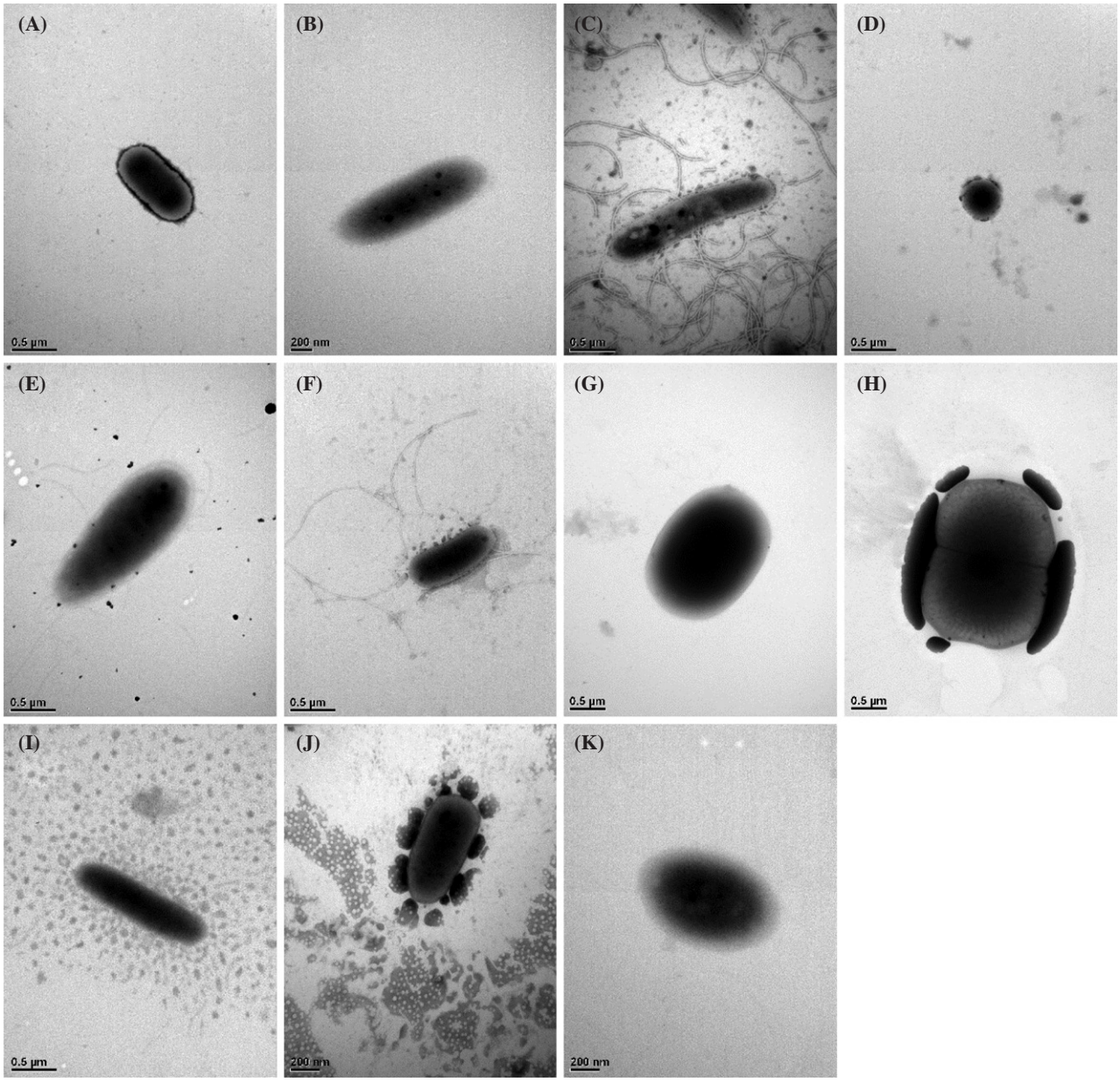

Fig. 2. Transmission electron micrographs of the isolates. Strains: (A) HYN0015; (B) HYN0035; (C) HYN0016; (D) HYN0003; (E) HYN0033; (F) HYN0019; (G) HYN0057; (H) HYN0037; (I) HYN0034; (J) HYN0002; (K) HYN0032.

isolated from a water sample, Jinyangho lake, Korea.

Description of *Deinococcus depolymerans* HYN0037

Cells are Gram-reaction-positive, non-flagellated, and cocci-shaped. Colonies are circular with entire margin, convex, and pink-red colored after 3 days of incubation on R2A agar at 25°C. Possesses oxidase, urease, and β-galactosidase activities, but not arginine dihydrolase activity. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Hydrolyzes esculin and gelatin. Uses D-glucose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, and malic acid as sole carbon source, but not L-arabinose, capric acid, adipic acid, trisodium citrate, or phenylacetic acid. Strain HYN0037 (=IHBA_33) was isolated from a water sample, Hapcheonho lake, Korea.

Description of *Eubacterium moniliforme* HYN0057

Cells are Gram-reaction-positive, anaerobic, non-flagellated, and cocci-shaped. Colonies are circular with entire margin, raised, and yellow colored after 3 days of incubation on Anaerobe Basal Agar at 25°C. Catalase-

and urease-negative. Does not hydrolyze gelatin or esculin. Does not produce indole. Produces acid from D-glucose, D-lactose, and D-mannose, but not from D-mannitol, sucrose, D-maltose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose, or D-trehalose. Strain HYN0057 (=IHBA_34) was isolated from a water sample, Jinyangho lake, Korea.

Description of *Flavobacterium nitrogenifigens* HYN 0034

Cells are Gram-reaction-negative, non-flagellated, and rod-shaped. Colonies are irregular, opaque, mucoid, and yellow colored after 3 days of incubation on R2A at 25 °C. Possesses oxidase and β-galactosidase activities, but not urease or arginine dihydrolase activities. Hydrolyzes esculin and gelatin. Reduces nitrate. Does not produce indole. Does not ferment glucose. Uses D-glucose, L-arabinose, D-mannose, D-maltose, *N*-acetyl-glucosamine, potassium gluconate, adipic acid, malic acid, and trisodium citrate as carbon sources, but not D-mannitol, capric acid or phenylacetic acid. Strain HYN0034 (=IHBA_31) was isolated from a water sample, Hapcheonho lake, Korea.

Description of *Kineosporia mesophila* HYN0032

Cells are Gram-reaction-positive, non-flagellated, and cocci-rod-shaped. Colonies are circular with entire margin and white colored after 3 days of incubation on R2A at 25°C. Oxidase-positive. Hydrolyzes gelatin, but not esculin. Reduces nitrate. Does not produce indole. Does not ferment glucose. Does not possesses arginine dihydrolase, urease, or β-galactosidase activities. Uses D-glucose as carbon sources, but not L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid. Strain HYN0032 (=IHBA_29) was isolated from a water sample, Hapcheonho lake, Korea.

Description of *Luteibacter jiangsuensis* HYN0015

Cells are Gram-reaction-negative, non-flagellated, and rod-shaped. Colonies are circular opaque, and yellow colored after 3 days of incubation on R2A at 25°C. Possesses oxidase and β-galactosidase activities, but not urease or arginine dihydrolase activities. Hydrolyzes esculin and gelatin. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Uses D-glucose, D-mannose, *N*-acetyl-glucosamine, and malic acid as sole carbon source, but not L-arabinose, D-mannitol, D-maltose, potassium gluconate, capric acid, adipic acid, trisodium citrate, or phenylacetic acid. Strain HYN0015 (=IHBA_24) was isolated from fresh wager, Jinyangho lake, Korea.

Description of *Microbacterium terricola* HYN0002

Cells are Gram-reaction-positive, flagellated, and rod-shaped. Colonies are circular, glistening, mucoid, and yellow colored after 2 days of incubation on R2A at 25 °C. Possesses oxidase and β-galactosidase activities, but not arginine dihydrolase or urease activities. Hydrolyzes esculin and gelatin. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, potassium gluconate, and malic acid as sole carbon source, but not *N*-acetyl-glucosamine, capric acid, adipic acid, trisodium citrate, or phenylacetic acid. Strain HYN 0002 (=IHBA_21) was isolated from fresh water, Jinyangho lake, Korea.

Description of *Rhizobium larrymoorei* HYN0033

Cells are Gram-reaction-negative, flagellated, and rod-shaped. Colonies are circular with entire margin, raised, and white colored after 3 days of incubation on R2A at 25°C. Possesses oxidase, urease, and β-galactosidase activities, but not arginine dihydrolase activity. Hydrolyzes esculin, but not gelatin. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Uses D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, malic acid, and trisodium citrate as carbon sources, but not capric acid, adipic acid, or phenylacetic acid. Strain HYN0033 (=IHBA_30) was isolated from a fresh water, Jinyangho lake, Korea.

Description of *Sediminicoccus rosea* HYN0003

Cells are Gram-reaction-negative, non-flagellated, and coccus-shaped. Colonies are circular, translucent, and red colored after 5 days of incubation on R2A at 25°C. Possesses oxidase and urease activities, but not arginine dihydrolase or β-galactosidase activities. Does not reduce nitrate. Does not produce indole. Does not ferment glucose. Does not hydrolyze esculin or gelatin. Does not use D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, or phenylacetic acid as a sole carbon source. Strain HYN 0003 (=IHBA_22) was isolated from a fresh water, Jinyangho lake, Korea.

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