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Eight unrecorded bacterial species isolated from soil and marine sediment in Korea

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The Earth contains billions of microbial species, although the vast majority cannot be cultured in laboratories and are thus considered unidentified and uncharacterized. Extremophiles are microorganisms that thrive in extreme conditions, including temperature, salinity, and pH. Extremophilic microorganisms have provided important insights for biological, metabolic, and evolutionary studies. Between 2017 and 2019, as part of a comprehensive investigation to identify bacterial species in Korea, eight bacterial strains were isolated from marine and non-marine environments in Jeju Island. These strains were cultured under extreme salinity or pH conditions. Phylogenetic analysis using 16S ribosomal RNA (rRNA) gene sequencing indicated that all eight strains belonged to the phyla *Gammaproteobacteria*, *Bacilli*, and *Alphaproteobacteria*. Based on their high 16S rRNA gene sequence similarities (>98.7%) and the formation of strong monophyletic clades with their closest related species, all isolated strains were considered as an unrecorded strain, previously unidentified species. Gram stain reaction, culture conditions, colony and cell morphology, biochemical characteristics, isolation source, and National Institute of Biological Resources (NIBR) IDs are described in this article. The characterization of these unrecorded strains provides information on microorganisms living in Korea.

Keywords: 16S rRNA gene sequence, soil, marine, unrecorded species

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Introduction

Extreme conditions include physical extremes such as temperature, radiation, and pressure as well as geochemical extremes including desiccation, salinity, pH, oxidative species, and redox potential (Pikuta *et al*., 2007). Extremophilic microorganisms have been identified in the extreme environmental conditions mentioned above, which are considered to represent early Earth conditions (Merino *et al*., 2019). Generally, extreme conditions are uncomfortable for species (such as humans) that are used to living in more moderate environmental conditions (i.e., mesophiles), but extremophilic microorganisms show optimal growth under extreme conditions. In addition, some extremophilic organisms thrive in more than one extreme environment and are called polyextremophiles (Coker, 2016). An example of a polyextremophile is *Sulfolobus acidocaldarius*, an extreme crenarchaeota that flourishes at pH 3 and 80°C (Rothschild and Mancinelli, 2001). Extremophiles have provided important insight for biological, metabolic, and evolutionary fields. In addition, extremophiles are considered good candidates and/or are thoroughly used for industrial biotechnology such as in agriculture and chemical synthesis.

The addition of the Nagoya Protocol (NP) to the Convention on Biological Diversity has created legal barriers to accessing and using genetic resources, including microorganisms. The NP aims to promote the conservation and sustainable use of biodiversity by protecting the rights of local communities and their traditional knowledge (Rourke, 2018). Locey and Lennon (2016) suggested that the Earth contains up to 10^{12} microbial species. However, most microbial organisms have not yet been cultured in laboratories and are therefore uncharacterized. Thus, it is important to study the cultivation and characterization of the unidentified indigenous microbial strains under the NP (Overmann and Scholz, 2017).

In this study, we isolated and characterized uncultured

and/or unidentified microorganisms related to halophiles and acidophiles or acid-tolerant from non-marine (e.g., freshwater) and marine (e.g., marine sediment or seawater) environments using high salt (10% weight per volume [w/v] NaCl) or alkaline (<pH 5) culture conditions, respectively.

Materials and Methods

To isolate bacterial strains, we harvested diverse environmental samples that were collected from seawater, marine sediment, and freshwater sediment (Table 1). The samples were processed separately, diluted, and spread onto acidic Reasoner's 2A Agar adjusted with Homopiperazine-1,4-bis (2-ethanesulfonic acid) (AR2A, pH 4.0) or marine agar added with final 10% (w/v) of NaCl (HMA, high salt marine agar), and incubated at 30°C for 2 weeks. Single colonies were isolated by secondary culture via transfer to new AR2A or MA plates. Then, to obtain a purified colony, each colony was sub-cultivated on the AR2A or MA plates. All strains were purified as single colonies and stored as 30% glycerol suspensions at −80°C as well as lyophilized ampoules. Colony characteristics of the isolates were assessed on the same agar media used for cultivation. Cellular morphology and cell size were examined using a transmission electron microscope (Fig. 1). Gram staining was performed using standard procedures. Biochemical characteristics were tested using API 20NE, API ZYM, and API 32GN strips (bioMérieux) according to the manufacturer's instructions. In particular, for halophiles, we used basal medium added NaCl (final 3% w/v).

For phylogenetic analysis for each strain in this study using 16S rRNA gene sequences, we extracted genomic DNA (gDNA) using a commercial gDNA extraction kit (GeneAll Biotechnology CO., Ltd., South Korea). Amplification of the 16S ribosomal RNA (16S rRNA) gene sequences was performed using bacterial universal forward and reverse primers (forward primer 27F 5′-AGAGTTTGATCMTGGCTCAG-3′; reverse primer 1492R 5′-TACGGYTACCTTGTTACGACTT-3′) (Weisburg *et al*., 1991). The 16S rRNA gene amplicons were sequenced by a certified service provider (CosmoGenetech, South Korea) using an automated DNA analyzer (Applied Biosystems 3730xl DNA Analyzer). DNA sequences were assembled using SeqMan (DNASTAR) and near full-length 16S rRNA sequences were obtained for all isolates. The closest phylogenetic neighbors of all isolates were identified using the EzBioCloud database (Kim *et al*., 2012). Sequences were edited using the BioEdit program and aligned using Clustal X (Thompson *et al*., 1997). Phylogenetic trees were generated using the neighbor-joining method (Saitou and Nei, 1987) in MEGA 7 (Kumar *et al*., 2016). The phylogenetic trees were evaluated using boot-

Table 1. Summary of isolated strains from the *Proteobacteria* and *Firmicutes* phyla and their taxonomic affiliations. All strains were cultured under 30°C for 2 or 3 days.

Strain ID	NIBR ID	Most closely related species	Similarity (%)	GenBank Accession number	Medium	Isolation source	Taxonomic affiliation (Phylum; Class; Order; Family)
DJ8	NIBRBAC000501577	*Pseudoalteromonas shioyasakiensis*	100	MG561859	HMA	Marine sediment	*Proteobacteria*; *Gammaproteobacteria*; *Alteromonadales*; *Pseudoalteromonadaceae*
GA4	CYQTBAC000000062	*Vibrio pectenicida*	99.8	MK828355	HMA	Seaweed	*Proteobacteria*; *Gammaproteobacteria*; *Vibrionales*; *Vibrionaceae*
GJH2-4	CYQTBAC000000063	*Photobacterium atrarenae*	99.6	MK828358	HMA	Marine sediment	*Proteobacteria*; *Gammaproteobacteria*; *Vibrionales*; *Vibrionaceae*
MSPH4-1	CYQTBAC000000065	*Halomonas pacifica*	99.7	MK828361	HMA	Marine sediment	*Proteobacteria*; *Gammaproteobacteria*; *Oceanospirillales*; *Halomonadaceae*
MSP3	CYQTBAC000000067	*Halomonas alkaliantarctica*	99.6	MK828359	HMA	Marine sediment	*Proteobacteria*; *Gammaproteobacteria*; *Oceanospirillales*; *Halomonadaceae*
MSP4	CYQTBAC000000066	*Marinobacter persicus*	99.0	MK828360	HMA	Marine sediment	*Proteobacteria*; *Gammaproteobacteria*; *Alteromonadales*; *Marinobacter*
SSH18	CYQTBAC000000074	*Falsibacillus pallidus*	100	MN176346	AR2A	Freshwater sediment	*Firmicutes*; *Bacilli*; *Bacillales*; *Bacillaceae*
SSH19	CYQTBAC000000083	*Rhodobacter blasticus*	99.1	MN192128	AR2A	Freshwater sediment	*Proteobacteria*; *Alphaproteobacteria*; *Rhodobacterales*; *Rhodobacteraceae*

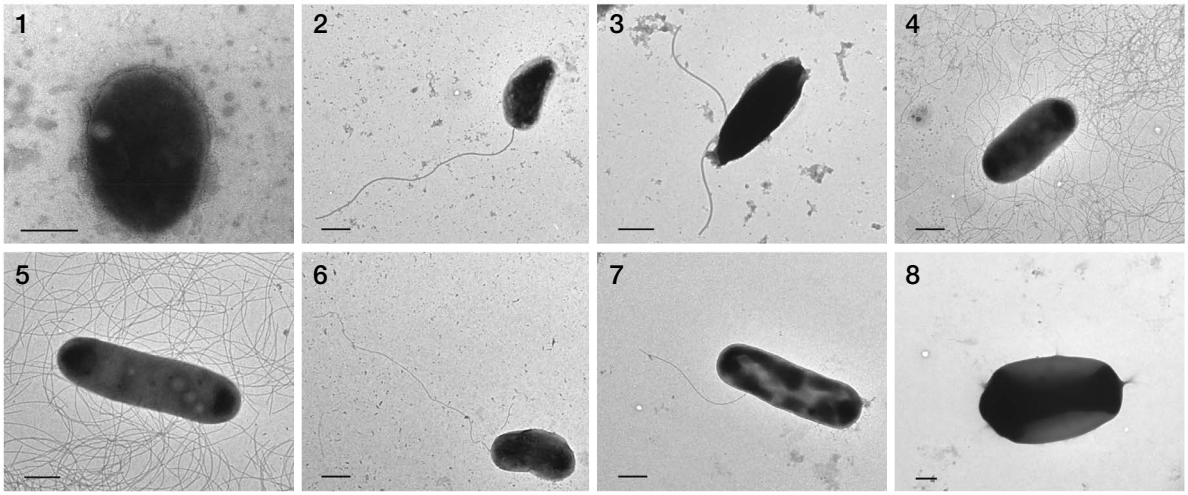

Fig. 1. Transmission electron micrographs of cells from the strains isolated in this study. Strains: 1, DJ8; 2, GA4; 3, GJH2-4; 4, MSPH4-1; 5, MSP3; 6, MSP4; 7, SSH18; 8, SSH19. Bar, 0.5 μm.

strap analysis (Felsenstein, 1985) based on 1,000 resampled datasets.

Results and Discussion

Based on the phylogenetic analysis of the 16S rRNA gene sequences, eight strains (DJ8, GA4, GJH2-4, MSPH4-1, MSP3, MSP4, SSH18, and SSH19) were isolated. All strains were assigned to the three phyla, *Gammaproteobacteria*, *Bacilli*, and *Alphaproteobacteria*, as expected based on high 16S rRNA gene sequence similarities (Table 1 and Fig. 2). The isolated strains were confirmed as putative halophiles or acidophiles. The morphological, physiological, and biochemical characteristics of the isolated strains are described in detail below.

For the identity of the strains isolated in this study, DJ8 was most closely related to *Pseudoalteromonas shioyasakiensis* SE3^T (AB720724; 100% sequence identity), GA4 to *Vibrio pectenicida* A365^T (Y13830; 99.8% sequence similarity), GJH2-4 to *Photobacterium atrarenae* M3-4^T (HM452945; 99.6% sequence similarity), MSPH4-1 to *Halomonas pacifica* NBRC 102220^T (BJUK01000094; 99.72% sequence similarity), MSP3 to *Halomonas alkaliantarctica* CRSS^T (AJ564880; 99.59% sequence similarity), MSP4 to *Marinobacter persicus* IBRC-M 10445^T (jgi.1084702; 99.0% sequence similarity), SSH18 to *Falsibacillus pallidus* DSM 25281^T (QQAY01000036; 100% sequence identity), and SSH1 to *Rhodobacter blasticus* ATCC 33485^T (D16429; 99.1% sequence similarity).

Description of *Pseudoalteromonas shioyasakiensis* DJ8

Strain DJ8 (=NIBRBAC000501577) was isolated from seawater in Daejung, Jeju, Korea. DJ8 cells are Gram-stain-negative and rod-shaped (1.5×1.0 μm). DJ8 colonies are circular, smooth, convex, and white colored after 3 days on HMA at 30°C. Cells are positive for oxidase and catalase activity, and negative for nitrate reduction, indole production, and glucose acidification. Also, cells show negative activity for esterase (C4), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, and α-fucosidase, but positive activity for β-glucosidase (esculin hydrolysis), β-galactosidase (PNPG), arginine dihydrolase, urease, protease (gelatin hydrolysis), alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, and *N*-acetyl-β-glucosaminidase. Moreover, cells are positive for utilization of L-arabinose, gluconate, malate, D-mannitol, D-glucose, L-arabinose, propionate, caprate, citrate, L-histidine, L-proline, *N*-acetyl-D-glucosamine, D-sucrose, D-maltose, acetate, L-alanine, 3-hydroxy-benzoate, and L-serine, and negative for utilization of D-mannose, adipate, phenyl-acetate, salicin, D-melibiose, L-fucose, D-sorbitol, valerate, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-rhamnose, D-ribose, inositol, itaconate, suberate, malonate, lactate, and 5-ketogluconate as energy and carbon source.

Description of *Vibrio pectenicida* GA4

Strain GA4 (=CYQTBAC000000062) was isolated from seawater in Gwakji, Jeju, Korea. GA4 cells are Gram-stain-negative and curved-rod shaped (0.5×2.0 μm). GA4 colonies are circular, convex, and white colored after 2 days of incubation on HMA at 25°C. Cells are positive for oxidase activity, and negative for catalase

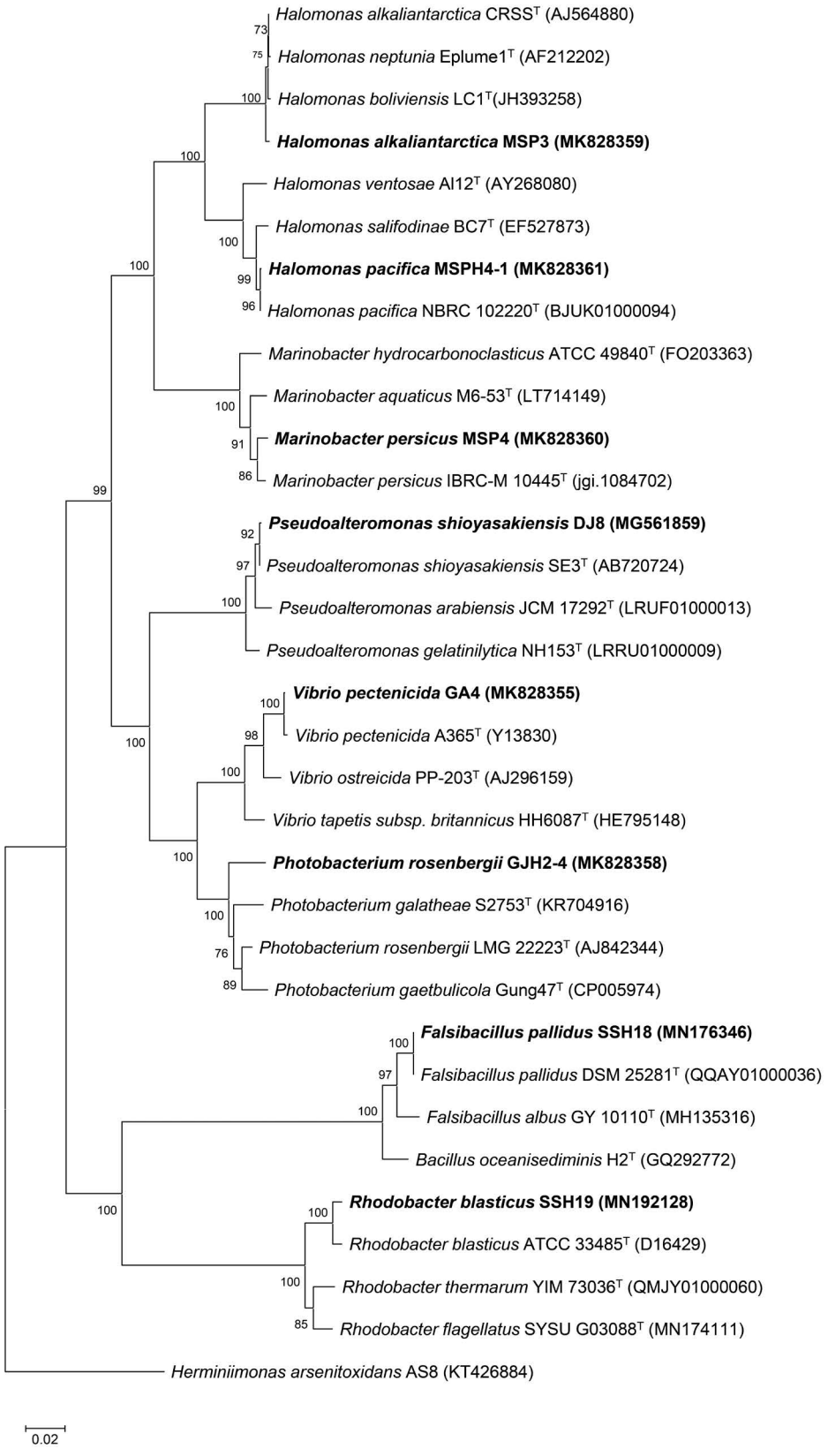

Fig. 2. Neighbor-joining tree based on 16S rRNA gene sequencing showing the phylogenetic relationships between the isolated strains and their closest relatives. The sequence of *Herminiimonas arsenitoxidans* AS8 (KT426884) was used as the outgroup. The GenBank accession numbers are given in parentheses. Bootstrap values (>60%) are shown at each branch. Bar, 0.02 substitutions per nucleotide position.

activity, the reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production, and glucose acidification. Also, cells show positive activity for protease (gelatin hydrolysis), alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, and *N*-acetyl-β-glucosaminidase, but negative activity for arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), β-galactosidase (PNPG), lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, and α-fucosidase. Moreover, cells are positive for utilization of D-glucose, D-mannitol, caprate, D-mannose, adipate, and malate, and negative for utilization of gluconate, phenyl-acetate, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, propionate, 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, D-maltose, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine as energy and carbon source.

Description of *Photobacterium atrarenae* GJH2-4

Strain GJH2-4 (=CYQTBAC000000063) was isolated from marine sediment in Gwakji, Jeju, Korea. GJH2-4 cells are Gram-stain-negative and rod-shaped (0.5×1.2 μm). GJH2-4 colonies are circular, convex, and yellow colored after 2 days of incubation on HMA at 30°C. Cells are positive for oxidase and catalase activity, and negative for the reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production, and glucose acidification. Also, cells show positive activity for protease (gelatin hydrolysis), alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, and Naphthol-AS-BI-phosphohydrolase, but negative activity for arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), β-galactosidase (PNPG), lipase (C14), cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Moreover, cells are positive for utilization of citrate and phenyl-acetate, and negative for utilization of D-mannose, gluconate, adipate, malate, D-mannitol, D-glucose, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, propionate, caprate, valerate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, inositol, D-sucrose, D-maltose, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine as energy and carbon source.

Description of *Halomonas pacifica* MSPH4-1

Strain MSPH4-1 (=CYQTBAC000000065) was isolated from marine sediment in Mosulpo, Jeju, Korea. MSPH4-1 cells are Gram-stain-negative and rod-shaped (1.0×2.5 μm). The colonies are circular, convex, and white colored after 2 days of incubation on HMA at 25°C. Cells are positive for catalase activity, and negative for oxidase activity, reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production, and glucose acidification. Also, cells show positive activity for β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), and β-galactosidase, have low activity for *N*-acetyl-D-glucosamine, but negative activity for arginine dihydrolase, and urease. Moreover, cells are positive for utilization of D-mannose, D-sucrose, D-maltose, glycogen, D-glucose, D-melibiose, and salicin, and negative for utilization of L-arabinose, D-mannitol, gluconate, caprate, adipate, malate, citrate, phenyl-acetate, L-rhamnose, D-ribose, inositol, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, 3-hydroxybenzoate, L-serine, D-fucose, D-sorbitol, propionate, valerate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, and L-proline as energy and carbon source.

Description of *Marinobacter persicus* MSP3

Strain MSP3 (=CYQTBAC000000067) was isolated from marine sediment in Mosulpo, Jeju, Korea. MSP3 cells are Gram-stain-negative and rod-shaped (0.8×2.5 μm). MSP3 colonies are circular, convex, and white colored after 2 days of incubation HMA at 25°C. Cells are positive for catalase activity, and negative for oxidase activity, the reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production, and glucose acidification. Also, cells show positive activity for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, and α-glucosidase, but negative activity for arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), β-galactosidase (PNPG), lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Moreover, cells are positive for utilization of D-glucose, D-mannose, gluconate, adipate, malate, phenyl-acetate, D-mannitol, D-sorbitol, L-arabinose, propionate, valerate, citrate, 2-ketogluconate, 3-hydroxy-butyrate, L-proline, L-rhamnose, *N*-acetyl-D-glucosamine, D-ribose, inositol, D-sucrose, D-maltose, malonate, acetate, lactate, L-alanine, glycogen, and 3-hydroxy-benzoate, and negative for utilization of salicin, D-melibiose, L-fucose, caprate, L-histidine, 4-hydroxy-benzoate,

itaconate, suberate, 5-ketogluconate, and L-serine as energy and carbon source.

Description of *Marinobacter persicus* MSP4

Strain MSP4 (=CYQTBAC000000066) was isolated from marine sediment in Mosulpo, Jeju, Korea. MSP4 cells are Gram-stain-negative and rod-shaped (1.0×1.5 μm). The colonies are circular, convex, and yellow colored after 2 days of incubation on MA at 30°C. Cells are positive for oxidase and catalase activity, and negative for indole production and glucose acidification. Also, cells show positive activity for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, cystine arylamidase, α-chymotrypsin, acid phosphatase, and Naphthol-AS-BI-phosphohydrolase, but negative activity for arginine dihydrolase, urease, β-glucosidase (esculin hydrolysis), protease (gelatin hydrolysis), β-galactosidase (PNPG), lipase (C14), valine arylamidase, trypsin, α-galactosidase, β-glucuronidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Moreover, cells are positive for utilization of D-glucose, L-arabinose, *N*-acetyl-D-glucosamine, gluconate, caprate, malate, citrate, L-histidine, and L-proline, but negative for utilization of D-mannose, D-mannitol, D-maltose, adipate, phenyl-acetate, salicin, D-melibiose, L-fucose, D-sorbitol, propionate, valerate, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-rhamnose, D-ribose, inositol, D-sucrose, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine as energy and carbon source.

Description of *Falsibacillus pallidus* SSH18

Strain SSH18 (=CYQTBAC000000074) was isolated from freshwater sediment in Susan, Jeju, Korea. SSH18 cells are Gram-stain-positive and rod-shaped (1.0×3.0 μm). The colonies are circular, convex, and white colored after 2 days of incubation on AR2A at 30°C. Cells are negative for oxidase and catalase activity, and reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production, and glucose acidification. Also, cells show positive activity for urease, protease (gelatin hydrolysis), esterase (C4), esterase lipase (C8), Naphthol-AS-BI-phosphohydrolase, α-glucosidase, and α-fucosidase, but negative activity for arginine dihydrolase, β-glucosidase (esculin hydrolysis), β-galactosidase (PNPG), alkaline phosphatase, lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, and α-mannosidase. Moreover, cells are positive for utilization of gluconate, and negative for utilization of D-mannose, adipate, malate, phenyl-acetate, D-mannitol, 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, D-maltose, itaconate, suberate, malonate, acetate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine as energy and carbon source.

Description of *Rhodobacter blasticus* SSH19

Strain SSH19 (=CYQTBAC000000083) was isolated from freshwater sediment in Susan, Jeju, Korea. SSH19 cells are Gram-stain-negative, non-flagellated, and rod-shaped (2.0×3.0 μm). The colonies are circular, convex, and red colored after 2 days of incubation on AR2A at 30°C. Cells are positive for oxidase and catalase activity, and glucose acidification, and negative for reduction of nitrates (NO_3^-) to nitrite (NO_2^-), reduction of nitrates (NO_3^-) to nitrogen (N_2), indole production. Also, cells show positive activity for β-glucosidase (esculin hydrolysis), esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, Naphthol-AS-BI-phosphohydrolase, α-glucosidase, and β-glucosidase, but negative activity for arginine dihydrolase, urease, protease (gelatin hydrolysis), β-galactosidase (PNPG), alkaline phosphatase, lipase (C14), trypsin, α-chymotrypsin, α-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. Moreover, cells are positive for utilization of D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, gluconate, adipate, malate, citrate, phenyl-acetate, salicin, L-fucose, D-sorbitol, propionate, valerate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, L-rhamnose, inositol, itaconate, lactate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxy-benzoate, and L-serine, and negative for utilization of *N*-acetyl-D-glucosamine, caprate, D-melibiose, D-ribose, D-sucrose, suberate, malonate, and acetate as energy and carbon source.

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