Journal of Species Research 10(1):1-11, 2021

Isolation and identification of 18 unrecorded prokaryotic species from the intestinal tracts of aquatic animals in Korea

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Evidence highlighting the importance of gut microbiota in biodiversity conservation is growing; however, gut bacteria in South Korean wildlife have not been well identified. Using a culture-dependent isolation method, we identified the gut bacteria from Korean aquatic wildlife: the gazami crab (*Portunus trituberculatus*), Korean striped bitterling (*Acheilognathus yamatsutae*), oily bitterling (*Acheilognathus koreensis*), leopard mandarin fish (*Siniperca scherzeri*), Korean dark chub (*Zacco koreanus*), diving beetle (*Cybister lewisianus*), spotted steed (*Abbottina springeri*), and Korean spotted sleeper (*Odontobutis obscura interrupta*). We identified 18 strains previously unrecorded in South Korea by comparing 16S rRNA gene sequences of isolates against the EzBioCloud and National Institute of Biological Resources (NIBR) databases. The isolated strains belong to the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria. We also assessed for phylogenetic relatedness, Gram-stain reaction, colony and cell morphology, and biochemical characteristics. Basic information and 16S rRNA gene sequences of the isolates were registered in NIBR, and NIBR accession numbers are provided.

Keywords: 16S rRNA gene sequences, gut microbiota, Korean aquatic wildlife, unidentified Korean gut bacteria

DOI:10.12651/JSR.2021.10.1.001

Introduction

Since the adoption of the Convention on Biological Diversity (CBD) at the United Nations Conference on Environment and Development (UNCED) in 1992, the urgency of conserving biodiversity and protecting biological resources is being globally realized. Furthermore, in 2010, the Nagoya Protocol was adopted by the CBD given the growing economic value of the genetic resources defined as "genetic material of actual or potential value." Hence, understanding indigenous biodiversity is essential to appropriate protection of a nation's sovereign rights over biological resources.

Owing to its wide-ranging potential functional capacities (Qin *et al*., 2010), the microbiome has received most attention as a biological resource. In particular, the gut microbiome is considered crucial because it has been shown to be closely related to host health (Shin *et al*., 2014), development (Koropatnick *et al*., 2004), and reproductive behavior (Sharon *et al*., 2010). However, bacterial members that comprise the gut microbiome of Korean wildlife have not yet been fully studied. We aimed to identify domestically unrecorded bacterial species from the gastrointestinal tracts of Korean aquatic animals, including fish, crustaceans, and insects, using a culture-dependent isolation method. Furthermore, using the 16S rRNA gene sequence-based identification, we isolated 18 strains previously unrecorded in South Korea belonging to the phyla Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria.

Materials and Methods

Culture-dependent isolation and identification

Gastrointestinal tracts of eight aquatic species were dissected and their contents were immediately resuspended in sterile phosphate-buffered saline (PBS) to prepare sample inoculums: Korean dark chub (*Zacco koreanus*), gazami crab (*Portunus trituberculatus*), Korean striped bitterling (*Acheilognathus yamatsutae*), oily bitterling (*Acheilog-*

nathus koreensis), spotted steed (*Abbottina springeri*), leopard mandarin fish (*Siniperca scherzeri*), Korean spotted sleeper (*Odontobutis obscura interrupta*), and diving beetle (*Cybister lewisianus*). Then, the inoculums were serially diluted with sterile PBS at concentrations of 10^{-1}, 10^{-2}, and 10^{-3}. Each concentration of the inoculum was individually spread onto Reasoner's 2A (R2A) agar, Marine agar (MA), Tryptic soy agar (TSA; supplemented with 0.5 % yeast extract or not), Lactobacilli MRS agar (MRSA), Brain heart infusion (BHI), and MacConkey agar plates; these were incubated at 10°C, 15°C, 25°C, and 30°C. Emerging colonies were separately transferred to fresh media and were sub-cultured at least 3 times to obtain pure cultures. Each pure culture was routinely cultivated under isolation conditions and used for further experiments.

We used the 16S rRNA gene-based identification to identify the bacterial pure cultures. The 16S rRNA gene was amplified using C1000 Touch Thermal Cycler (Bio-Rad) with 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGYTACCTTGTTACGACTT-3′) primers, and the PCR products were sequenced using the PRISM 3730xl DNA analyzer (Applied Biosystems) with universal bacterial primers (Lane, 1991). The 16S rRNA gene sequence fragments were then assembled using SeqMan 5.0 (DNASTAR) to obtain full-length 16S rRNA gene sequences. EzBioCloud and National Institute of Biological Resources database were used to assign the obtained 16S rRNA gene sequences to corresponding taxa (Yoon *et al*., 2017).

Phylogenetic analysis

To understand the phylogenetic relationships of the isolates, phylogenetic trees were reconstructed using the isolates in the same phylum, the most closely related species, and the type species in the genus using MEGA 7 software (Kumar *et al*., 2016) using the neighbor joining (NJ) (Saitou and Nei, 1987), maximum likelihood (ML) (Tamura and Nei, 1993), and maximum parsimony (MP) (Nei and Kumar, 2000) algorithms. Multiple sequence alignment of 16S rRNA gene sequences was performed using ClustalW (Thompson *et al*., 1994). Clade credibility was assessed with a bootstrap test with 1000 replicates.

Assessment of morphologic and biochemical characteristics

For biochemical characterization of the isolated strains, we tested the cells from the strain isolates using API 20NE, API ID 32GN, API ZYM (bioMérieux) and GEN III MicroPlate (Biolog) kits. Specifically, API 20NE, API ID 32GN, and GEN III MicroPlate demonstrated carbon source utilization, and API 20NE and API ZYM demonstrated enzymatic activity. To verify their Gram-staining responsiveness, we used a commercial Gram-staining kit (bioMérieux). All the experiments were conducted in accordance with the manufacturers' instructions. Cell morphology was observed using LIBRA120, an energy-filtering transmission electron microscope (Carl Zeiss). Colony morphology was determined by observing a single colony of each strain incubated on appropriate media at isolation temperatures for 48 h.

Results and Discussion

On the basis of 16S rRNA gene-based phylogenetic analysis, 18 strains, designated K33R8, K33T6, G12R3, R13S1, R23R4, R33M6-1, R33M4, K11M4, O13M9, 719, 176, 775, 765, 684, A52, 771, A79 and 772, were assigned to the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria. The strains were confirmed as chemoheterotrophic rod- or coccoid shaped bacteria (Fig. 1). The detailed phylogenetical, morphological and biochemical characteristics of the strains are described below.

In the 16S rRNA gene sequence comparison among the 18 isolated strains, K33R8, K33T6, G12R3, R13S1, R23R4, R33M6-1, R33M4, K11M4, O13M9, 719, 176, 775, 765, 684, A52, 771, A79 and 772 showed the highest 16S rRNA gene sequence similarities with *Chryseobacterium oleae* DSM 25575^T (jgi.1085839; 99.42% similarity), *Buttiauxella agrestis* ATCC 33320^T (JMPI01000079; 99.93 %), *Leucobacter triazinivorans* JW-1^T (KT439069; 100%), *Lysinibacillus macroides* DSM 54^T (LGCI01000008; 99.52%), *Flavobacterium cutihirudinis* DSM 25795^T (jgi. 1108034; 99.58%), *Gordonia didemni* B204^T (JN615417; 98.92%), *Mycolicibacterium fortuitum* subsp. *acetamidolyticum* JCM 6368^T (BCSZ01000080; 100%), *Yersinia kristensenii* subsp. *rochesterensis* EPLC-04^T (KJ606916; 99.86%), *Paracoccus sanguinis* 5503^T (JRKQ01000154; 99.86%), *Aerococcus viridans* ATCC 11563^T (ADNT 01000041; 99.93%), *Alcaligenes faecalis* subsp. *parafaecalis* G^T (AJ242986; 100%), *Aliarcobacter faecis* AF1078^T (JARS01000021; 100%), *Citrobacter portucalensis* A60^T (MVFY01000035; 100%), *Citrobacter gillenii* CDC 4693-86^T (AF025367; 99.66%), *Cryobacterium arcticum* GCJ 02^T (GQ406814; 99.56%), *Comamonas odontotermitis* Dant 3-8^T (DQ453128; 99.86%), *Comamonas nitrativorans* 23310^T (AJ251577; 99.24%) and *Enterococcus phoeniculicola* ATCC BAA-412^T (AJAT01000017; 100%), respectively (Table 1). Phylogenetic consensus trees of the 18 strains and related taxa also robustly support that the isolated strains are most closely related to the strains which showed the highest 16S rRNA gene sequence similarities, establishing monophyletic clades each other (Figs. 2−5).

Description of *Chryseobacterium oleae* K33R8

Cells are Gram-stain-negative, non-flagellated and rod

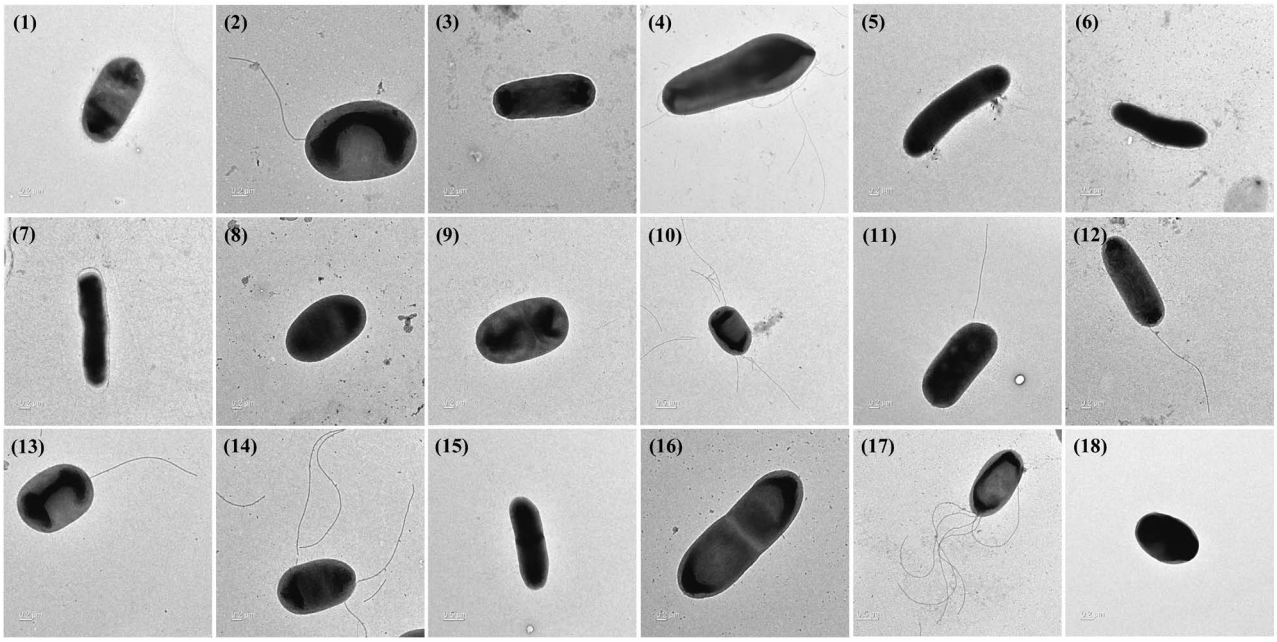

Fig. 1. The energy-filtering transmission electron micrographs of the isolated strains. Strains: 1, K33R8; 2, K33T6; 3, G12R3; 4, R13S1; 5, R23R4; 6, R33M6-1; 7, R33M4; 8, K11M4; 9, O13M9; 10, 719; 11, 176; 12, 775; 13, 765; 14, 684; 15, A52; 16, 771; 17, A79; 18, 772.

shaped. Colonies are circular, convex, entire and yellowish on R2A agar medium after 48 h incubation at 30°C. Cells reduce nitrate to nitrogen and are positive for arginine dihydrolase, β-glucosidase, protease, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, *N*-acetyl-β-glucosaminidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, gluconate, adipate, malate, citrate, L-histidine, L-proline, D-sucrose, acetate, glycogen (API 20NE/ID 32GN) and utilize dextrin, gentiobiose, D-turanose, D-melibiose, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, D-glucose, D-fructose, gelatin, L-histidine, galacturonic acid, galactonic lactone, D-glucuronic acid, glucuronamide, Tween40, acetoacetic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain K33R8 (=NIBRBAC000503075) was isolated from the gut of Korean dark chub (*Nipponocypris koreanus*) at Hwasun, Jeollanam Province, Korea. The DNA G+C content of the type strain of the species is 38 mol%.

Description of *Buttiauxella agrestis* K33T6

Cells are Gram-stain-negative, flagellated and coccus shaped. Colonies are circular, convex, entire and beige colored on TSA medium after 48 h incubation at 30°C. Cells reduce nitrate to nitrite and are positive for arginine dihydrolase, β-glucosidase, β-galactosidase, alkaline phosphatase, leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, malate, citrate, phenyl-acetate, salicin, D-melibiose, L-fucose, L-histidine, 2-ketogluconate, 4-hydroxy-benzoate, L-proline, L-rhamnose, D-ribose, D-sucrose, malonate, acetate, lactate, L-alanine, 5-ketogluconate, L-serine (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-D-glucoside, D-salicin, *N*-glucosamine, β-mannosamine, *N*-galactosamine, neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, glycerol, D-glucose, D-fructose, D-serine, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, methyl pyruvate, L-lactic acid, citric acid, L-malic acid, succinic acid, α-butyric acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain K33T6 (=NIBRBAC000503076) was isolated from the gut of Korean dark chub (*Nipponocypris koreanus*) at Hwasun, Jeollanam Province, Korea. The DNA G+C content of the type strain of the species is 47–51 mol%.

Table 1. Taxonomic affiliations and isolation sources of the 18 isolated strains.

Phylum	Class	Order	Family	Genus	Strain ID	NIBR accession number	Most closely related species		Similarity (%)	Isolation source
							Closest type strain	Accession number		
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae*	*Leucobacter*	G12R3	NIBRBAC000503077	*Leucobacter triazinivorans* JW-1(T)	KT439069	100	Gazami crab (*Portunus trituberculatus*)
Actinobacteria	*Actinomycetes*	*Actinomycetales*	*Gordoniaceae*	*Gordonia*	R33M6-1	NIBRBAC000503080	*Gordonia didemni* B204(T)	JN615417	98.92	Korean striped bitterling (*Acheilognathus yamatsutae*)
Actinobacteria	*Actinobacteria*	*Corynebacteriales*	*Mycobacteriaceae*	*Mycolicibacterium*	R33M4	NIBRBAC000503081	*Mycolicibacterium fortuitum* subsp. *acetamidolyticum* JCM 6368(T)	BCSZ01000080	100	Korean striped bitterling (*Acheilognathus yamatsutae*)
Actinobacteria	*Actinobacteria*	*Micrococcales*	*Microbacteriaceae*	*Cryobacterium*	A52	NIBRBAC000503090	*Cryobacterium arcticum* GCJ02(T)	GQ406814	99.56	Leopard mandarin fish (*Siniperca scherzeri*)
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Chryseobacterium*	K33R8	NIBRBAC000503075	*Chryseobacterium oleae* DSM 25575(T)	jgi.1085839	99.42	Korean dark chub (*Zacco koreanus*)
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	*Flavobacterium*	R23R4	NIBRBAC000503079	*Flavobacterium cutihirudinis* DSM 25795(T)	jgi.1108034	99.58	Korean striped bitterling (*Acheilognathus yamatsutae*)
Firmicutes	*Bacilli*	*Bacillales*	*Planococcaceae*	*Lysinibacillus*	R13S1	NIBRBAC000503078	*Lysinibacillus macroides* DSM 54(T)	LGCI01000008	99.52	Korean striped bitterling (*Acheilognathus yamatsutae*)
Firmicutes	*Bacilli*	*Lactobacillales*	*Aerococaceae*	*Aerococcus*	719	NIBRBAC000503085	*Aerococcus viridans* ATCC 11563(T)	ADNT01000041	99.93	Leopard mandarin fish (*Siniperca scherzeri*)
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococcaceae*	*Enterococcus*	772	NIBRBAC000503082	*Enterococcus phoeniculicola* ATCC BAA-412(T)	AJAT01000017	100	Diving beetle (*Cybister lewisianus*)
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Buttiauxella*	K33T6	NIBRBAC000503076	*Buttiauxella agrestis* ATCC 33320(T)	JMPI01000079	99.93	Korean dark chub (*Zacco koreanus*)
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Yersiniaceae*	*Yersinia*	K11M4	NIBRBAC000503083	*Yersinia kristensenii* subsp. *rochesterensis* EPLC-04(T)	KJ606916	99.86	Oily bitterling (*Acheilognathus koreensis*)
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Paracoccus*	O13M9	NIBRBAC000503084	*Paracoccus sanguinis* 5503(T)	JRKQ01000154	99.86	Spotted steed (*Abbottina springeri*)
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Alcaligenaceae*	*Alcaligenes*	176	NIBRBAC000503086	*Alcaligenes faecalis* subsp. *parafaecalis* G(T)	AJ242986	100	Korean spotted sleeper (*Odontobutis obscura interrupta*)
Proteobacteria	*Epsilonproteobacteria*	*Campylobacterales*	*Campylobacteraceae*	*Aliarcobacter*	775	NIBRBAC000503087	*Aliarcobacter faecis* AF1078(T)	JARS01000021	100	Korean spotted sleeper (*Odontobutis obscura interrupta*)
Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Citrobacter*	765	NIBRBAC000503088	*Citrobacter portucalensis* A60(T)	MVFY01000035	100	Korean spotted sleeper (*Odontobutis obscura interrupta*)
Proteobacteria	*Gammaproteobacteria*	*Enterobacterales*	*Enterobacteriaceae*	*Citrobacter*	684	NIBRBAC000503089	*Citrobacter gillenii* CDC 4693-86(T)	AF025367	99.66	Leopard mandarin fish (*Siniperca scherzeri*)
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	771	NIBRBAC000503091	*Comamonas odontotermitis* Dant 3-8(T)	DQ453128	99.86	Diving beetle (*Cybister lewisianus*)
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*	A79	NIBRBAC000503092	*Comamonas nitrativorans* 23310(T)	AJ251577	99.24	Diving beetle (*Cybister lewisianus*)

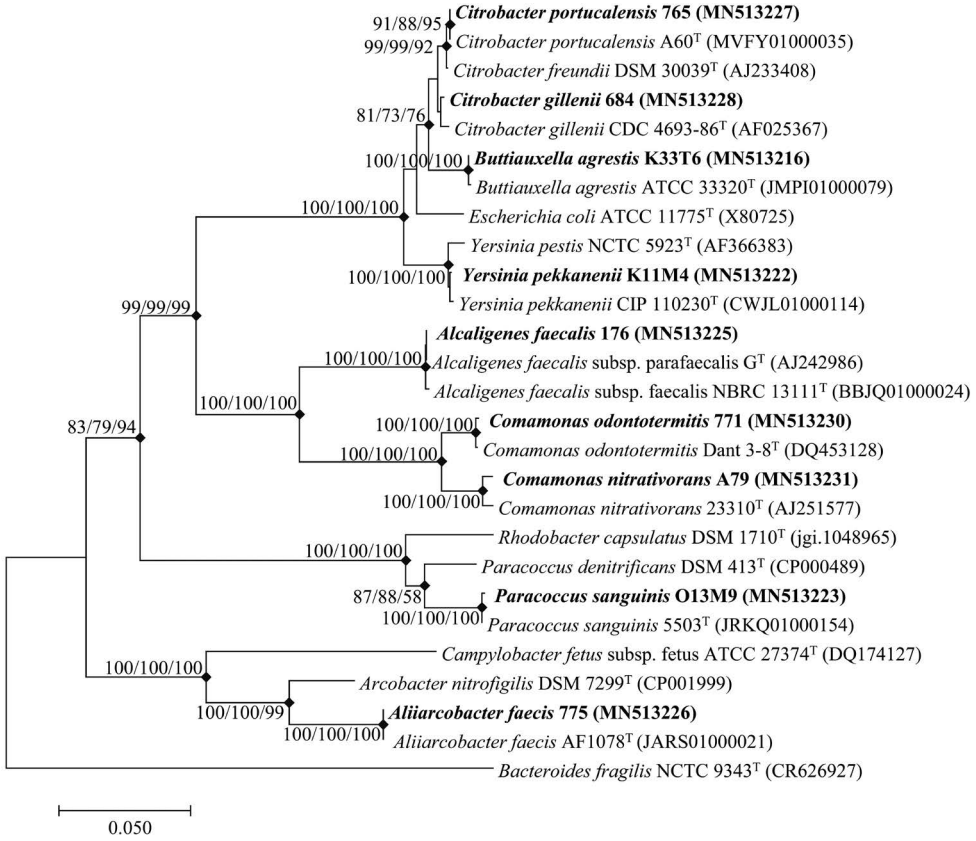

Fig. 2. The phylogenetic tree based on 16S rRNA gene sequence showing the phylogenetic relatedness between the nine isolated strains belonging to the phylum Proteobacteria and the type species from each genus. The tree was mainly reconstructed using the neighbor joining (NJ), maximum likelihood (ML), and maximum parsimony (MP) algorithms. The Filled diamonds indicate the branches present in the phylogenetic trees reconstructed using three different tree reconstruction methods. Numbers on the nodes represent bootstrap values with 1000 replicates (NJ/ML/MP). *Bacteroides fragilis* NCTC 9434^{T} was used as an outgroup. The bar indicates 0.05 accumulated substitutions per nucleotide.

Description of *Leucobacter triazinivorans* G12R3

Cells are Gram-stain-positive, non-flagellated and rod shaped. Colonies are circular, convex, entire and yellow colored on TSA medium after 48 h incubation at 25°C. Cells are positive for esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM) and assimilate D-glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, D-maltose, adipate, citrate, phenyl-acetate, L-fucose, propionate, L-histidine, L-proline, L-rhamnose, inositol, acetate, L-alanine (API 20NE/ID 32GN). Utilize dextrin, phenylacetic acid, methyl pyruvate, Tween40, α-butyric acid, acetoacetic acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain G12R3 (=NIBRBAC000503077) was isolated from the gut of gazami crab (*Portunus trituberculatus*) at Taean, Chungcheongnam Province, Korea. The DNA G+C content of the type strain is 73 mol%.

Description of *Lysinibacillus macroides* R13S1

Cells are Gram-stain-positive, flagellated and rod shaped. Colonies are irregular, raised and beige colored on lactobacilli MRS agar medium after 48 h incubation at 30°C. Cells are positive for arginine dihydrolase, protease, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API 20NE/ZYM) and assimilate *N*-acetyl-D-glucosamine, gluconate, malate, propionate, valerate, L-histidine, 3-hydroxy-butyrate, L-proline, acetate, lactate, L-alanine, glycogen, L-serine (API 20NE/ID 32GN). Utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, *N*-glucosamine, β-mannosamine, D-fructose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, D-arabitol, glycerol, D-glucose, D-fructose, D-aspartic acid, D-serine, gelatin, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, pyroglutamic acid, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, mucic

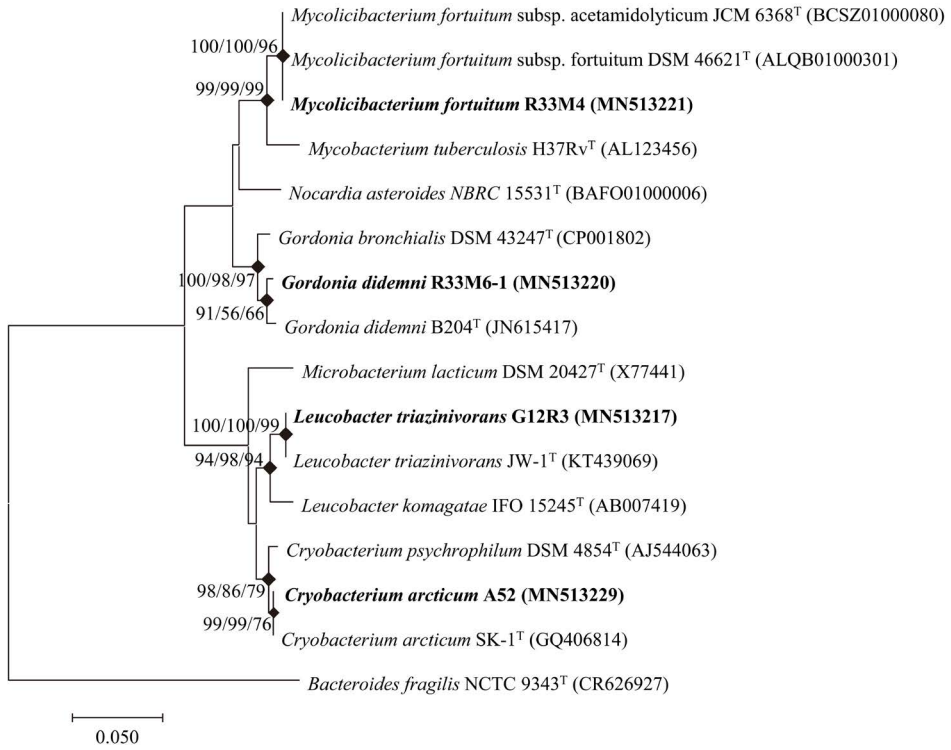

Fig. 3. The phylogenetic tree based on 16S rRNA gene sequence showing the phylogenetic relatedness between the four isolated strains belonging to the phylum Actinobacteria and the type species from each genus. The tree was mainly reconstructed using the NJ, ML, and MP algorithms. The filled diamonds indicate the branches present in the phylogenetic trees reconstructed with three different tree reconstruction methods. Numbers on the nodes represent bootstrap values with 1000 replicates (NJ/ML/MP). *B. fragilis* NCTC 9434^T was used as an outgroup. The bar indicates 0.05 accumulated substitutions per nucleotide.

acid, quinic acid, D-saccharic acid, phenylacetic acid, methyl pyruvate, D-lactic acid, L-lactic acid, α-glutaric acid, L-malic acid, Tween40, γ-butyric acid, α-butyric acid, β-butyric acid, α-butyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid as sole carbon sources (Biolog GEN III).

Strain R13S1 (=NIBRBAC000503078) was isolated from the gut of Korean striped bitterling (*Acheilognathus yamatsutae*) at Yeongwol, Gangwon Province, Korea. The DNA G+C content of the type strain is 38 mol%.

Description of *Flavobacterium cutihirudinis* R23R4

Cells are Gram-stain-negative, non-flagellated and rod shaped. Colonies are irregular, raised and yellow colored on R2A agar medium after 48 h incubation at 30°C. Cells reduce nitrate to nitrogen and positive for β-glucosidase, β-galactosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-fucosidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, D-maltose, malate, D-sucrose, glycogen (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, *N*-glucosamine, β-mannosamine, *N*-galactosamine, neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, L-fucose, D-fructose, gelatin, glycyl-L-proline, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, galacturonic acid, galactonic lactone, D-glucuronic acid, glucuronamide, Tween40, acetoacetic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain R23R4 (=NIBRBAC000503079) was isolated from the gut of Korean striped bitterling (*Acheilognathus yamatsutae*) at Yeongwol, Gangwon Province, Korea. The DNA G+C content of the type strain is 34 mol%.

Description of *Gordonia didemni* R33M6-1

Cells are Gram-stain-positive, non-flagellated and rod shaped. Colonies are circular, flat, entire and apricot colored on MA medium after 48 h incubation at 30°C. Cells are positive for alkaline phosphatase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase,

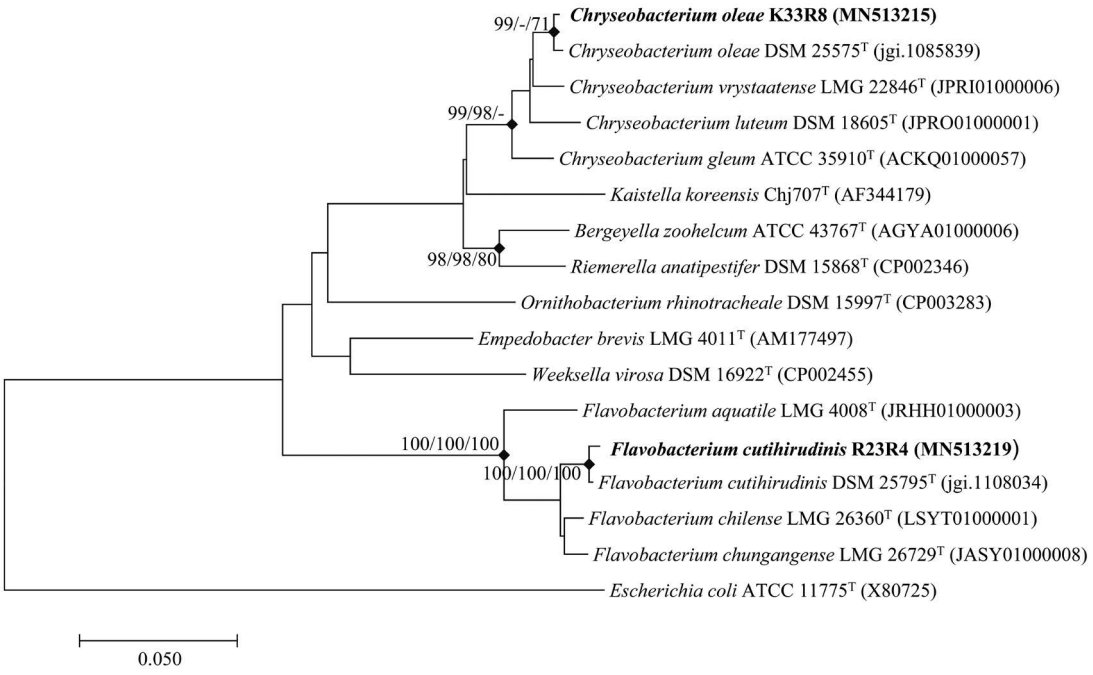

Fig. 4. The phylogenetic tree based on 16S rRNA gene sequence showing the phylogenetic relatedness between the two isolated strains belonging to the phylum Bacteroidetes and the type species from each genus. The tree was mainly reconstructed using the NJ, ML, and MP algorithms. The filled diamonds indicate the branches present in the phylogenetic trees reconstructed using three different tree reconstruction methods. Numbers on the nodes represent bootstrap values with 1000 replicates (NJ/ML/MP). *Escherichia coli* ATCC 11775^T was used as an outgroup. The bar indicates 0.05 accumulated substitutions per nucleotide.

naphthol-AS-BI-phosphohydrolase, α-galactosidase, α-glucosidase, β-glucosidase (API ZYM) and D-glucose, L-arabinose, D-mannose, *N*-acetyl-D-glucosamine, D-maltose, gluconate, adipate, citrate, salicin, D-melibiose, propionate, valerate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, inositol, D-sucrose, acetate (API 20NE/ID 32GN). Utilize D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, *N*-glucosamine, neuraminic acid, α-D-glucose, D-mannose, D-fructose, 6-methyl-glucose, D-fucose, myo-inositol, glycerol, D-glucose, gelatin, L-glutamic acid, pectin, D-glucuronic acid, citric acid, α-glutaric acid, L-malic acid, succinic acid, α-butyric acid, β-butyric acid, acetoacetic acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain R33M6-1 (=NIBRBAC000503080) was isolated from the gut of Korean striped bitterling (*Acheilognathus yamatsutae*) at Yeongwol, Gangwon Province, Korea. The DNA G+C content of the type strain is 71 mol%.

Description of *Mycolicibacterium fortuitum* subsp. *acetamidolyticum* R33M4

Cells are Gram-stain-positive, non-flagellated and rod shaped. Colonies are circular, flat and beige colored on MA medium after 48 h incubation at 30°C. Cells reduce nitrate to nitrite and are positive for β-glucosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, gluconate, adipate, malate, propionate, valerate, 3-hydroxy-butyrate, L-proline, acetate (API 20NE/ID 32GN) and utilize α-D-glucose, D-fructose, D-fructose, L-aspartic acid, L-serine, D-gluconic acid, D-glucuronic acid, glucuronamide, citric acid, L-malic acid, succinic acid, Tween40, β-butyric acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain R33M4 (=NIBRBAC000503081) was isolated from the gut of Korean striped bitterling (*Acheilognathus yamatsutae*) at Yeongwol, Gangwon Province, Korea. The DNA G+C content of the type strain is 66 mol%.

Description of *Yersinia kristensenii* subsp. *rochesterensis* K11M4

Cells are Gram-stain-negative, non-flagellated and rod shaped. Colonies are circular, convex, entire and beige colored on MA medium after 48 h incubation at 10°C. Cells reduce nitrate to nitrite and are positive for β-glucosidase, β-galactosidase, alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glu-

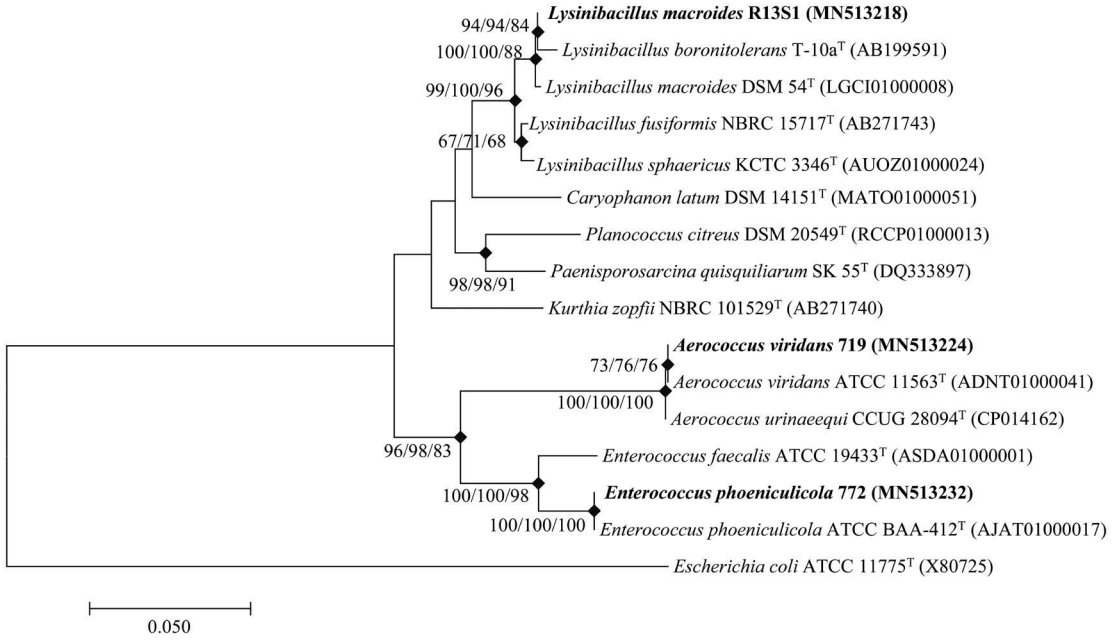

Fig. 5. The phylogenetic tree based on 16S rRNA gene sequence showing the phylogenetic relatedness between the three isolated strains belonging to the phylum Firmicutes and the type species from each genus. The tree was mainly reconstructed using the NJ, ML, and MP algorithms. The filled diamonds indicate the branches present in the phylogenetic trees reconstructed using three different tree reconstruction methods. Numbers on the nodes represent bootstrap values with 1000 replicates(NJ/ML/MP). *E. coli* ATCC 11775^T was used as an outgroup. The bar indicates 0.05 accumulated substitutions per nucleotide.

cosaminidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, salicin, L-fucose, D-sorbitol, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, L-proline, L-rhamnose, D-ribose, inositol, D-sucrose, acetate, lactate, L-alanine, 5-ketogluconate, glycogen (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-raffinose, D-melibiose, β-D-glucoside, D-salicin, *N*-glucosamine, β-mannosamine, *N*-galactosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, myo-inositol, glycerol, D-glucose, D-fructose, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, methyl pyruvate, D-lactic acid, citric acid, D-malic acid, L-malic acid, succinic acid, Tween40, acetoacetic acid, acetic acid and formic acid as sole carbon sources (Biolog GEN III).

Strain K11M4 (=NIBRBAC000503083) was isolated from the gut of oily bitterling (*Tanakia koreensis*) at Cheongju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 48 mol%.

Description of *Paracoccus sanguinis* O13M9

Cells are Gram-stain-negative, non-flagellated and rod shaped. Colonies are circular, convex, entire and yellowish on MA medium after 48 h incubation at 30°C. Cells reduce nitrate to nitrogen and are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM). Assimilate D-glucose, L-arabinose, gluconate, adipate, malate, L-fucose, propionate, valerate, L-histidine, 3-hydroxy-butyrate, L-proline, D-ribose, itaconate, suberate, acetate, lactate, L-alanine, 3-hydroxy-benzoate, L-serine (API 20NE/ID 32GN) and utilize β-D-glucoside, α-D-glucose, D-galactose, D-fucose, L-fucose, inosine, myo-inositol, glycerol, gelatin, glycyl-L-proline, L-alanine, L-aspartic acid, L-glutamic acid, pyroglutamic acid, L-serine, D-gluconic acid, quinic acid, methyl pyruvate, L-lactic acid, α-glutaric acid, D-malic acid, L-malic acid, Tween40, β-butyric acid, acetoacetic acid, acetic acid and formic acid as sole carbon sources (Biolog GEN III).

Strain O13M9 (=NIBRBAC000503084) was isolated from the gut of spotted steed (*Biwia springeri*) at Yeongwol, Gangwon Province, Korea. The DNA G+C content of the type strain is 70 mol%.

Description of *Aerococcus viridans* 719

Cells are Gram-stain-positive, flagellated and coccus shaped. Colonies are circular, convex, entire and beige colored on MA medium after 48 h incubation at 25°C.

Cells reduce nitrate to nitrite and are positive for β-glucosidase, β-galactosidase, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucosidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, malate, phenyl-acetate, salicin, D-melibiose, L-fucose, D-sorbitol, 2-ketogluconate, L-rhamnose, D-ribose, lactate, 5-ketogluconate, L-serine (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, stachyose, D-raffinose, α-D-lactose, β-D-glucoside, D-salicin, α-D-glucose, D-mannose, D-fructose, D-galactose, D-mannitol, glycerol, pectin, D-gluconic acid, methyl pyruvate and acetoacetic acid as sole carbon sources (Biolog GEN III).

Strain 719 (=NIBRBAC000503085) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 39 mol%.

Description of *Alcaligenes faecalis* subsp. *parafaecalis* 176

Cells are Gram-stain-negative, flagellated and rod shaped. Colonies are circular, umbonate and beige colored on BHI agar medium after 48 h incubation at 25°C. Cells are positive for alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM) and assimilate D-glucose, caprate, malate, citrate, phenyl-acetate, propionate, valerate, L-histidine, 3-hydroxy-butyrate, L-proline, malonate, acetate, lactate, L-alanine (API 20NE/ID 32GN). Utilize dextrin, D-cellobiose, gentiobiose, D-turanose, stachyose, D-melibiose, β-mannosamine, *N*-galactosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, D-mannitol, D-arabitol, myo-inositol, glycerol, D-fructose, D-serine, gelatin, glycyl-L-proline, L-alanine, L-arginine, L-glutamic acid, L-histidine, pyroglutamic acid, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, phenylacetic acid, methyl pyruvate, D-lactic acid, L-lactic acid, citric acid, α-glutaric acid, D-malic acid, L-malic acid, succinic acid, Tween40, β-butyric acid, acetoacetic acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain 176 (=NIBRBAC000503086) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 56 mol%.

Description of *Aliarcobacter faecis* 775

Cells are Gram-stain-negative, flagellated and rod shaped. Colonies are circular, convex, entire and beige colored on TSA medium supplemented with 0.5% yeast extract after 48 h incubation at 25°C. Cells reduce nitrate to nitrite and are positive for urease, alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API 20NE/ZYM). Assimilate acetate, lactate, glycogen (API ID 32GN) and utilize D-fructose, pectin, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, methyl pyruvate, D-lactic acid, L-lactic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain 775 (=NIBRBAC000503087) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 27 mol%.

Description of *Citrobacter portucalensis* 765

Cells are Gram-stain-negative, flagellated and coccus shaped. Colonies are circular, convex, entire and beige colored on TSA medium supplemented with 0.5% yeast extract after 48 h incubation at 25°C. Cells reduce nitrate to nitrite and are positive for β-galactosidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-D-glucosamine, D-maltose, gluconate, malate, citrate, phenyl-acetate, D-melibiose, L-fucose, D-sorbitol, propionate, valerate, 2-ketogluconate, 3-hydroxy-butyrate, L-proline, L-rhamnose, D-ribose, inositol, D-sucrose, acetate, lactate, L-alanine, 5-ketogluconate, 3-hydroxy-benzoate, L-serine (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-D-glucoside, *N*-glucosamine, β-mannosamine, *N*-galactosamine, neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, myo-inositol, glycerol, D-glucose, D-fructose, D-aspartic acid, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, D-saccharic acid, methyl pyruvate, D-lactic acid, L-lactic acid, citric acid, α-glutaric acid, D-malic acid, L-malic acid, succinic acid, α-butyric acid, β-butyric acid, α-butyric acid, propionic acid, acetic acid and formic acid as sole carbon sources (Biolog GEN III).

Strain 765 (=NIBRBAC000503088) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 52 mol%.

Description of *Citrobacter gillenii* 684

Cells are Gram-stain-negative, flagellated and rod shaped. Colonies are circular, raised, entire and pink colored on MacConkey agar medium after 48 h incubation at 25°C. Cells reduce nitrate to nitrite and are positive for urease, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API 20NE/ZYM). Assimilate D-glucose, D-mannose, *N*-acetyl-D-glucosamine, gluconate, malate, propionate, L-histidine, L-proline, D-ribose, lactate, L-alanine, L-serine (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, D-raffinose, α-D-lactose, D-melibiose, β-D-glucoside, D-salicin, *N*-glucosamine, β-mannosamine, *N*-galactosamine, neuraminic acid, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, inosine, D-sorbitol, D-mannitol, myo-inositol, glycerol, D-glucose, D-fructose, D-serine, glycyl-L-proline, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, pyroglutamic acid, L-serine, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, D-saccharic acid, methyl pyruvate, D-lactic acid, L-lactic acid, citric acid, α-glutaric acid, L-malic acid, succinic acid, γ-butyric acid, α-butyric acid, β-butyric acid, α-butyric acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain 684 (=NIBRBAC000503089) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea.

Description of *Cryobacterium arcticum* A52

Cells are Gram-stain-positive, non-flagellated and rod shaped. Colonies are circular, convex, entire and yellow colored on TSA medium supplemented with 0.5% yeast extract after 48 h incubation at 15°C. Cells reduce nitrate to nitrite and are positive for β-glucosidase, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase (API 20NE/ZYM). Assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, gluconate, salicin, D-melibiose, L-fucose, 2-ketogluconate, 3-hydroxy-butyrate, L-rhamnose, D-ribose, D-sucrose, lactate (API 20NE/ID 32GN) and utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, sucrose, D-turanose, stachyose, D-raffinose, α-D-lactose, D-melibiose, β-D-glucoside, D-salicin, β-mannosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, D-sorbitol, D-mannitol, glycerol, D-fructose, L-arginine, L-glutamic acid, pyroglutamic acid, pectin, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, D-lactic acid, Tween40, acetoacetic acid, propionic acid and acetic acid as sole carbon sources (Biolog GEN III).

Strain A52 (=NIBRBAC000503090) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 68 mol%.

Description of *Comamonas odontotermitis* 771

Cells are Gram-stain-negative, flagellated and rod shaped. Colonies are irregular, raised and beige colored on TSA medium supplemented with 0.5% yeast extract after 48 h incubation at 25°C. Cells reduce nitrate to nitrite and are positive for alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase (API ZYM). Assimilate D-mannitol, gluconate, caprate, adipate, malate, phenyl-acetate, propionate, valerate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, D-ribose, itaconate, suberate, acetate, lactate, L-alanine (API 20NE/ID 32GN) and utilize dextrin, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose, D-fructose, D-aspartic acid, L-alanine, L-arginine, L-aspartic acid, L-glutamic acid, L-histidine, pyroglutamic acid, L-serine, galacturonic acid, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, phenylacetic acid, methyl pyruvate, D-lactic acid, L-lactic acid, α-glutaric acid, D-malic acid, L-malic acid, succinic acid, Tween40, γ-butyric acid, α-butyric acid, β-butyric acid, α-butyric acid, acetoacetic acid, propionic acid, acetic acid and formic acid as sole carbon sources (Biolog GEN III).

Strain 771 (=NIBRBAC000503091) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 62 mol%.

Description of *Comamonas nitrativorans* A79

Cells are Gram-stain-negative, flagellated and rod shaped. Colonies are circular, convex, entire and pink colored on MacConkey agar medium after 48 h incubation at 15°C. Cells are positive for esterase (C4), leucine arylamidase, valine arylamidase, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase (API ZYM) and assimilate caprate, malate, propionate, valerate, 3-hydroxy-butyrate, L-proline, acetate, lactate, L-alanine (API 20NE/ID 32GN). Utilize dextrin, gentiobiose, D-melibiose, α-D-glucose, D-mannose, D-fructose, D-galactose, 6-methyl-glucose, D-fucose, L-fucose, L-rhamnose, D-fructose, D-serine, gelatin, glycyl-L-proline, L-aspartic acid, L-glutamic acid, pectin, galacturonic acid, galactonic lactone, D-glu-

curonic acid, glucuronamide, L-lactic acid, L-malic acid and acetoacetic acid as sole carbon sources (Biolog GEN III).

Strain A79 (=NIBRBAC000503092) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea.

Description of *Enterococcus phoeniculicola* 772

Cells are Gram-stain-positive, non-flagellated and coccus shaped. Colonies are circular, convex, entire and white colored on TSA medium supplemented with 0.5% yeast extract after 48 h incubation at 25°C. Cells are positive for arginine dihydrolase, β-galactosidase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase (API 20NE/ZYM) and assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, D-maltose, salicin, D-ribose, D-sucrose (API 20NE/ID 32GN). Utilize dextrin, D-maltose, D-trehalose, D-cellobiose, gentiobiose, D-turanose, stachyose, α-D-lactose, D-melibiose, β-D-glucoside, D-salicin, *N*-glucosamine, *N*-galactosamine, α-D-glucose, D-mannose, D-fructose, D-galactose, L-fucose, L-rhamnose, D-fructose, pectin, galactonic lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, citric acid, L-malic acid, succinic acid, β-butyric acid, α-butyric acid and acetoacetic acid as sole carbon sources (Biolog GEN III).

Strain 772 (=NIBRBAC000503082) was isolated from the gut of Leopard mandarin fish (*Siniperca scherzeri*) at Chungju, Chungcheongbuk Province, Korea. The DNA G+C content of the type strain is 36 mol%.

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Submitted: September 21, 2020
Revised: January 22, 2021
Accepted: January 22, 2021