

A report of nine unrecorded bacterial species in the phylum *Bacteroidetes* collected from freshwater environments in Korea

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During a comprehensive study of indigenous prokaryotic species in South Korea, nine bacterial species in the phylum *Bacteroidetes* were isolated from freshwater environmental samples that were collected from three major rivers in the Republic of Korea. High 16S rRNA gene sequence similarity ($\geq 98.7\%$) and robust phylogenetic clades with the closely related species suggest that each strain was correctly assigned to an independent and predefined bacterial species. There were no previous reports of these nine species in Korea. Within the phylum *Bacteroidetes*, four species were assigned to the genus *Flavobacterium*, order *Flavobacteriales*, and five species to three genera of two families in the order *Cytophagales*. Gram reaction, colony and cell morphology, basic biochemical characteristics, isolation source, and strain IDs are described in the species description section.

Keywords: 16S rRNA gene, *Bacteroidetes*, *Flavobacteriales*, *Cytophagales*, unrecorded species

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INTRODUCTION

The phylum *Bacteroidetes* (Ludwig and Klenk, 2001), also known as the *Bacteroides-Cytophaga-Flexibacter* group, are widely distributed over a diverse range of ecological niches including soil, marine, freshwater, and the gastrointestinal tracts of animals (DeLong *et al.*, 1993; Bowman *et al.*, 1997; Glöckner *et al.*, 1999; O'Sullivan *et al.*, 2002). This phylum consists of four classes, with most environmental *Bacteroidetes* belong to the *Flavobacteriia*, *Cytophagia*, and *Sphingobacteriia* classes, while the gastrointestinal tract microbiota is primarily composed of members in the *Bacteroidia*. These bacteria are Gram-stain-negative and rod-shaped that do not form endospores (Ludwig *et al.*, 2010).

From 2015-2016, we collected freshwater environmental samples from three major rivers, the Han River, Nakdong River and Seomjin River in (Republic of) Korea, and isolated novel and unrecorded bacterial species in Korea. The isolates belonged to the phyla *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Actinobacteria* and *Verrucomicrobia*. As a subset of this study, we report nine unrecorded bacterial species belonging to the orders *Flavobacteriales* and *Cytophagales* in the phylum *Bacteroidetes*.

MATERIALS AND METHODS

Samples of freshwater, brackish water, and sediment were collected from the Han River, Nakdong River, and Seomjin River. Each sample was processed separately, spread onto diverse culture media including R2A agar and Marine Agar 2216 (Difco), and then incubated at 15-30°C for 14 days. All strains were purified as single colonies and stored as 20% glycerol suspension at -80°C. The designated strain IDs, sources, culture media, and incubation conditions are summarized in Table 1.

Colony morphology of the strains was observed on agar plates with a magnifying glass after the cells grew to stationary phase. Cellular morphology and cell size were examined by transmission electron microscopy (CM200, Philips). Gram staining was performed using a Gram-staining kit (BD). Biochemical characteristics were tested by using API 20NE (bioMérieux) according to the manufacturer's instructions.

The phylogenetic position of the strains assigned to the phylum *Bacteroidetes* was investigated using 16S rRNA gene analysis. The 16S rRNA genes were amplified by PCR and sequenced. The 16S rRNA gene sequences of the strains assigned to the phylum *Bacteroidetes* were analyzed using the EzTaxon-e server (Kim *et al.*, 2012)

Table 1. Summary of strains belonging to the phylum *Bacteroidetes* and their taxonomic affiliations.

Order	Family	Genus	Strain ID	NNIBR ID	Mostly related species	Similarity (%)	Isolation source	Medium	Incubation condition
<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	SJ-153	NNIBRBA 38	<i>F. ahnfeltiae</i> 10Alg 130 ^T	99.3	Brackish water	MA	25°C, 3 d
		<i>Flavobacterium</i>	SSI-37	NNIBRBA 8	<i>F. indicum</i> GPTSA100-9 ^T	99.5	Sediments	R2A	30°C, 3 d
		<i>Flavobacterium</i>	BK-550	NNIBRBA 47	<i>F. macrobrachii</i> an-8 ^T	98.7	Freshwater	R2A	25°C, 3 d
		<i>Flavobacterium</i>	KSI-10	NNIBRBA 55	<i>F. reichenbachii</i> WB 3.2-61 ^T	98.8	Sediment	R2A	25°C, 2 d
<i>Cytophagales</i>	<i>Cyclobacteriaceae</i>	<i>Algoriphagus</i>	SJ-361	NNIBRBA 40	<i>A. alkaliphilus</i> AC-74 ^T	99.7	Brackish water	MA	25°C, 3 d
		<i>Algoriphagus</i>	ESI-03	NNIBRBA 61	<i>A. zhangzhouensis</i> 12C11 ^T	99.9	Sediments	MA	25°C, 2 d
	<i>Cytophagaceae</i>	<i>Flectobacillus</i>	BK-168	NNIBRBA 26	<i>F. roseus</i> GFA-11 ^T	99.2	Freshwater	R2A	30°C, 3 d
		<i>Hymenobacter</i>	04KSI-21	NNIBRBA 58	<i>H. algorigicola</i> VUG-A23a ^T	98.8	Sediments	R2A	20°C, 2 d
		<i>Hymenobacter</i>	SI-175	NNIBRBA 39	<i>H. chitinivorans</i> Txc1 ^T	98.8	Brackish water	R2A	25°C, 3 d

and confirmed using GenBank database (<http://ncbi.nlm.nih.gov>). For phylogenetic analyses, the 16S rRNA gene sequences were aligned using EzEditor (Jeon *et al.*, 2014). A phylogenetic tree was constructed using the neighbor-joining (NJ) (Saitou and Nei, 1987), maximum-parsimony (MP) (Fitch, 1971) and maximum-likelihood (ML) (Felsenstein, 1981) methods in MEGA version 6 (Tamura *et al.*, 2013). The topology of the phylogenetic tree was evaluated via a bootstrap analysis (Felsenstein, 1985), based on 1,000 replications.

RESULTS AND DISCUSSION

On the basis of 16S rRNA gene sequence comparisons and phylogenetic analyses, all nine strains were assigned to the phylum *Bacteroidetes*. The nine strains were distributed in two orders: four strains in the order *Flavobacteriales* and five strains in the order *Cytophagales* (Table 1). These strains were Gram-staining-negative, chemoheterotrophic and rod-shaped bacteria except for strain SJ-153 and BK-168 showing a coccoid or oval shape (Fig. 1). Cultural, morphological and physiological characteristics are also shown in the species description section.

Based on 16S rRNA gene sequence similarity, nine strains were identified as unrecorded bacterial species in Korea. A phylogenetic tree of bacterial strains assigned to the order *Flavobacteriales* and *Cytophagales* in the phylum *Bacteroidetes* are shown in Figs. 2 and 3 respectively. The four species that were assigned to the order *Flavobacteriales* belonged to the genus *Flavobacterium* in the family *Flavobacteriaceae* (Fig. 2). Another five species were assigned to the families *Cyclobacteriaceae* and *Cytophagaceae* in the order *Cytophagales*. Among these species, two species that were assigned to the family *Cyclobacteriaceae* which belongs to the genus *Algoriphagus*. Three species that were assigned to the family *Cytophagaceae* belonged to the genera *Flectobacillus* (1 species) and *Hymenobacter* (2 species) (Fig. 3).

There are no previous reports of these nine species in Korea. In this study, we propose that these species are unrecorded bacterial species: *Flavobacterium ahnfeltiae*, *F. indicum*, *F. macrobrachii*, *F. reichenbachii*, *Algoriphagus alkaliphilus*, *A. zhangzhouensis*, *Flectobacillus roseus*, *Hymenobacter algorigicola*, and *H. chitinivorans* in Korea.

Description of *Flavobacterium ahnfeltiae* SJ-153

Cells are Gram-staining-negative, non-flagellated and coccus-shaped. Colonies are circular, convex with entire edge and yellow-colored after 3 days on MA at 25°C. Positive for esculin hydrolysis and indole production, but negative for nitrate reduction, glucose fermentation, argi-

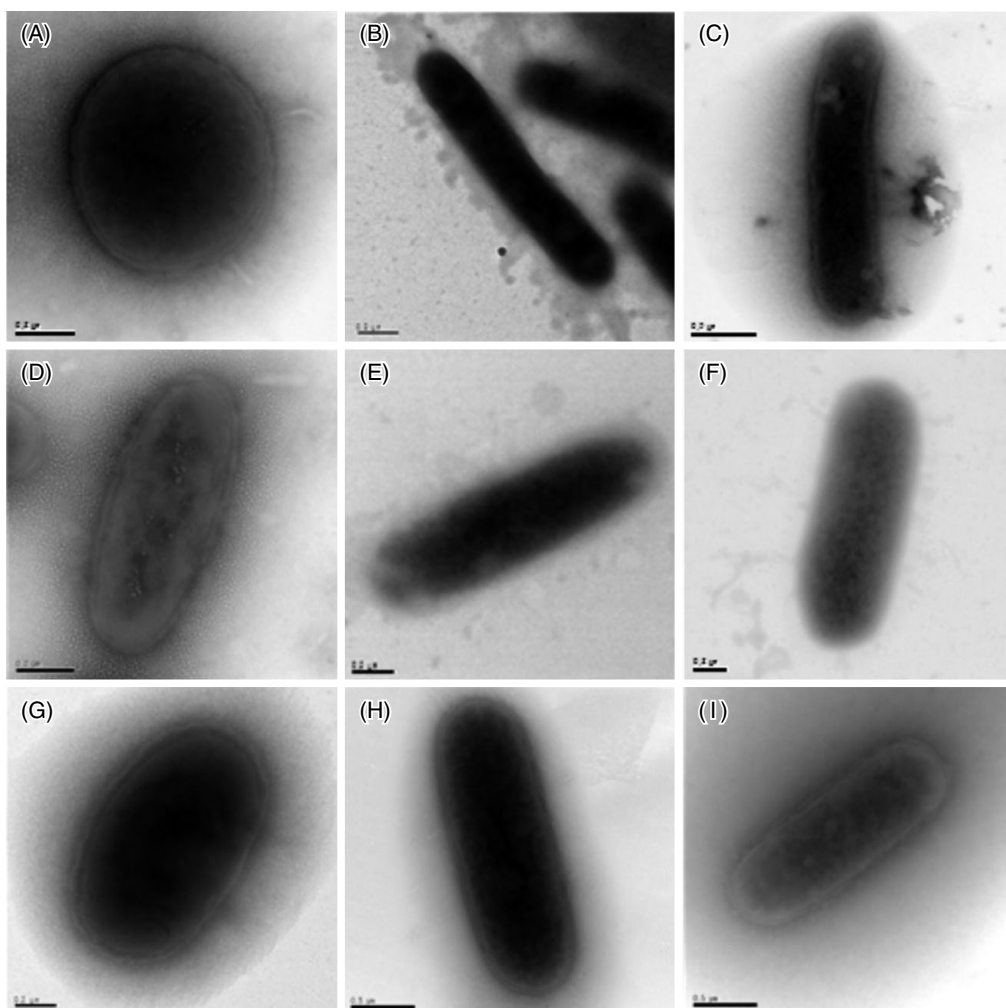


Fig. 1. Transmission electron micrographs of the strains isolated in this study. Strains: A, SJ-153; B, SS1-37; C, BK-550; D, KS1-10; E, SJ-361; F, ES1-03; G, BK-168; H, 04KS1-21; I, SJ-175. Bar: 0.2 μm (A-G), 0.5 μm (H and I).

nine dihydrolase, urease, gelatinase and β -galactosidase. Does not utilize D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The strain SJ-513 (=NNI BRBA 38) was isolated from brackish water of Seomjin River, Gwangyang, Korea.

Description of *Flavobacterium indicum* SS1-37

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex to umbonate with irregular margin and yellow-colored after 3 days on R2A agar at 30°C. Positive for esculin hydrolysis, gelatinase and β -galactosidase, but negative for nitrate reduction, indole production, urease, arginine dihydrolase and glucose fermentation. D-Mannose is utilized. Does not utilize D-glucose, L-arabinose, D-mannitol, malic acid,

adipic acid, D-maltose, *N*-acetyl-glucosamine, potassium gluconate, capric acid, trisodium citrate and phenylacetic acid. The strain SS1-37 (=NNIBRBA 8) was isolated from a riverside sediment of Nakdong River, Gyeongcheon-island, Sangju, Korea.

Description of *Flavobacterium macrobrachii* BK-550

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex, opaque with entire edge and yellow-colored after 3 days on R2A agar at 25°C. Positive for nitrate reduction, esculin hydrolysis, urease and β -galactosidase, but negative for indole production, gelatinase, glucose fermentation, arginine dihydrolase. D-Glucose, D-mannitol and malic acid are utilized. Does not utilize L-arabinose, D-mannose, D-maltose, *N*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid.

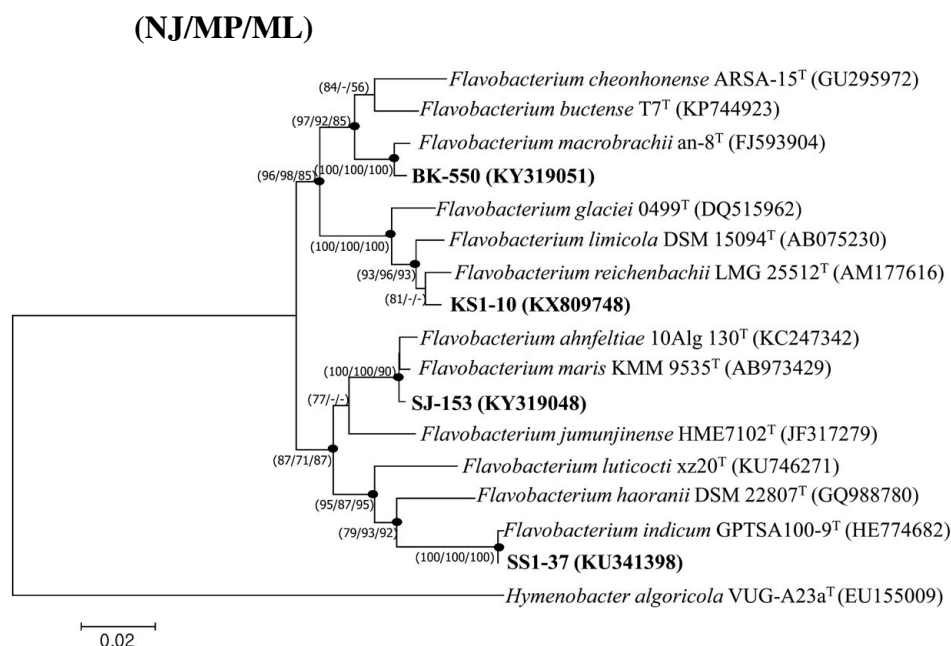


Fig. 2. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships among the isolates and related taxa in the order *Flavobacteriales*. The values above each branch indicate the percentage levels of bootstrap support based on 1,000 resamplings (NJ/MP/ML). The closed circles indicate that the corresponding nodes were recovered by all treeing algorithms (NJ, MP and ML). Bar, 0.02 changes per nucleotide position.

The strain BK-550 (= NNIBRBA 47) was isolated from freshwater, Eulsuk-island at the end of Nakdong River, Busan, Korea.

Description of *Flavobacterium reichenbachii* KS1-10

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex, smooth and light yellow-colored after 3 days on R2A agar at 25°C. Positive for urease and arginine dihydrolase, but negative for nitrate reduction, esculin hydrolysis, β -galactosidase, indole production, gelatinase and glucose fermentation. D-Glucose, L-arabinose, D-mannitol, malic acid and adipic acid are utilized. Does not utilize D-mannose, D-maltose, *N*-acetyl-glucosamine, potassium gluconate, capric acid, trisodium citrate and phenylacetic acid. The strain KS1-10 (= NNIBRBA 55) was isolated from freshwater sediment of Geomyeongso (origin of Han River), Taebaek, Korea.

Description of *Algoriphagus alkaliphilus* SJ-361

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex with entire edge and orange-colored after 3 days on MA at 25°C. Positive for esculin hydrolysis, gelatinase and β -galactosidase, but negative for urease, nitrate reduction, glucose fermentation and arginine dihydrolase. *N*-Acetyl-glucosamine, D-mannose, D-maltose and potassium gluconate are uti-

lized. Does not utilize D-glucose, L-arabinose, D-mannitol, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The strain SJ-361 (= NNIBRBA 40) was isolated from brackish water of Seomjin River, Gwangyang, Korea.

Description of *Algoriphagus zhangzhouensis* ES1-03

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex and smooth and reddish-orange colored after 3 days on MA at 25°C. Positive for nitrate reduction, esculin hydrolysis, gelatinase and β -galactosidase, but negative for indole production, urease, glucose fermentation and arginine dihydrolase. D-Glucose, D-mannose, *N*-acetyl-glucosamine and D-maltose are utilized. Does not utilize L-arabinose, D-mannitol, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The strain ES1-03 (= NNIBRBA 61) was isolated from brackish marsh sediment of Eulsuk-island at the end of Nakdong River, Busan, Korea.

Description of *Flectobacillus roseus* BK-168

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex, entire margins and pale pink-colored after 3 days on R2A agar at 30°C. Positive for esculin hydrolysis and β -galactosidase, but negative for nitrate reduction, indole production, urease,

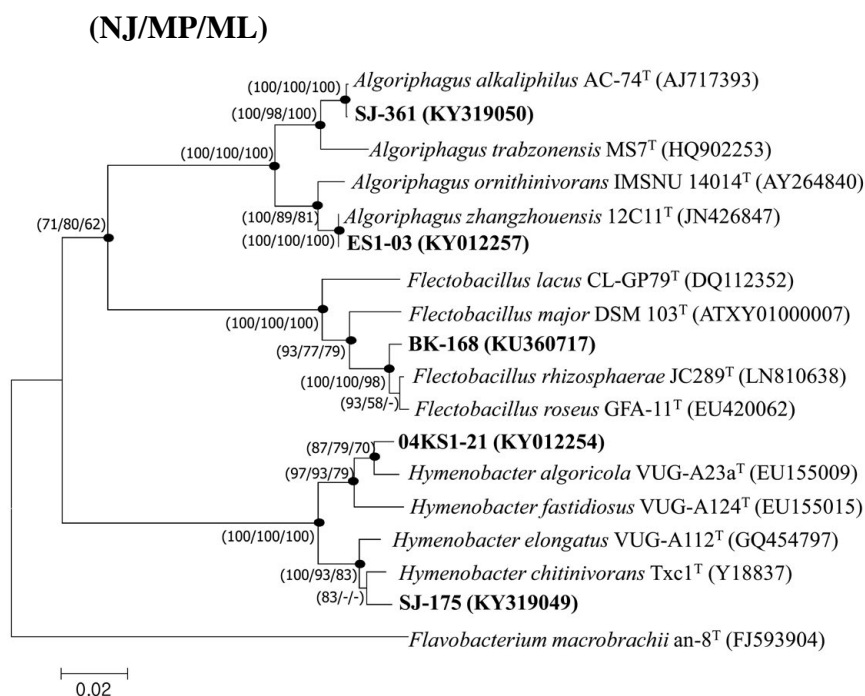


Fig. 3. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships among the isolates and taxa in the order *Cytophagales*. The values above each branch indicate the percentage levels of bootstrap support (> 70%) based on 1,000 resamplings. The closed circles indicate that the corresponding nodes were recovered by all treeing algorithms (NJ, MP and ML). Bar, 0.02 changes per nucleotide position.

arginine dihydrolase, gelatinase and glucose fermentation. D-Glucose, D-mannose, *N*-acetyl-glucosamine, D-maltose and potassium gluconate are utilized. Does not utilize L-arabinose, D-mannitol, malic acid, adipic acid, capric acid, trisodium citrate and phenylacetic acid. The strain BK-168 (= NNIBRBA 26) was isolated from freshwater of Nakdong River, Sangju, Korea.

Description of *Hymenobacter algicola* 04KS1-21

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, smooth and pink-colored after 3 days on R2A agar at 20°C. Positive for esculin hydrolysis, gelatinase and β -galactosidase, but negative for indole production, nitrate reduction, glucose fermentation, arginine dihydrolase, and urease. D-Glucose and D-maltose are utilized. Does not utilize L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The strain 04KS1-21 (= NNIBRBA 58) was isolated from freshwater sediment of Geomryeongso (origin of Han River), Taebaek, Korea.

Description of *Hymenobacter chitinivorans* SJ-175

Cells are Gram-staining-negative, non-flagellated and rod-shaped. Colonies are circular, convex with entire edge

and orange-colored after 3 days on R2A agar at 25°C. Positive for esculin hydrolysis, urease and gelatinase, but negative for indole production, nitrate reduction, glucose fermentation, arginine dihydrolase and β -galactosidase. D-Glucose and D-maltose are utilized. Does not utilize L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid. The strain SJ-175 (= NNIBRBA 39) was isolated from brackish water of Seomjin River, Gwangyang, Korea.

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