A report of four unrecorded *Proteobacteria* species isolated from soil in Korea

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In 2015 and 2017, the National Institute of Biological Resources has isolated four unrecorded prokaryotic species designated as R-1-5, R-2-13, R-2-1, and R-1-8 from the peatland soil of Yongneup. Phylogenetic analysis based on 16S rRNA gene sequence similarity determined the four strains (R-1-5, R-2-13, R-2-1, R-1-8) were most closely related to *Curvibacter lanceolatus* (99.93%), *Massilia brevitalea* (98.7%), *Pseudomonas lini* (99.54%), and *Pseudomonas vancouverensis* (99.93%), respectively. The four unrecorded strains belong to the phylum *Proteobacteria*, in which the genera *Curvibacter* and *Massilia* are assigned to the class *Betaproteobacteria*, and the genus *Pseudomonas* to the class *Gammaproteobacteria*. Since there are no publications or official reports on these four strains, these four species are new records to Korea. The strains were further characterized by Gram reaction, colony and cell morphology, basic biochemical properties, and phylogenetic position. Descriptive information of the four unrecorded species is provided.

Keywords: 16S rRNA sequence, *Burkholderiales*, peatland, *Proteobacteria*, *Pseudomonadales*, unrecorded bacterial species

INTRODUCTION

Proteobacteria is a major phylum of Gram-negative bacteria, which constitute the largest and most phenotypically diverse division among prokaryotes (Gupta, 2000). They represent nearly a half of the partial and complete prokaryotic genomes hosted at the National Center for Biotechnology Information (NCBI; ftp:// ftp. ncbi.nlm.nih.gov/ genomes/ GENOME_REPORTS/ prokaryotes.txt) (Itävaara *et al*., 2016). The phylum *Proteobacteria* comprises 116 validated families according to the List of Prokaryotic Names with Standing in Nomenclature (http://www.bacterio.net/).

The phylum was formally established, using phylogenetic analysis of 16S rRNA gene sequences by Garrity *et al*. (2005a), with five constituent classes containing all known Gram-negative bacteria: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria* and *Epsilonproteobacteria* (William and Kelly, 2013). Further, the molecular analyses using complete multigenome/multiprotein alignments added the sixth (*Zetaproteobacteria*) and seventh class (*Oligoflexis*) to the phylum *Proteobacteria* (Williams *et al*., 2010; Yutin *et al*., 2012). The species of *Proteobacteria* are abundantly present in various ecological niches such as soil (36.5%), plants (62%), the atmosphere (77.9%), seawater (57.9%), and freshwater (61.3%) (Woese *et al*., 1987; Shin *et al*., 2015; Kim *et al*., 2017). Their major functional roles are nitrogen fixation and oxidation of iron, sulfur, and methane (Gupta, 2000).

In Korea, the highest peatland moor is located in the mountainous area of Yongneup (38°12′53′N 128°07′30′E) in Mt. Daeam, Gangwon Province. Yongneup was designated as a natural reserve in July 1973 owing to its rare and unique plant species (Kim *et al*., 2015). The peat layers are about 150 cm thick, which have formed over 4,000-5,000 years. This region is characterized by a basic pH, relatively low temperature, and limited environments characterized by the organic matter, released by the plant species in the peatland (Li *et al*., 2014). The Microor-
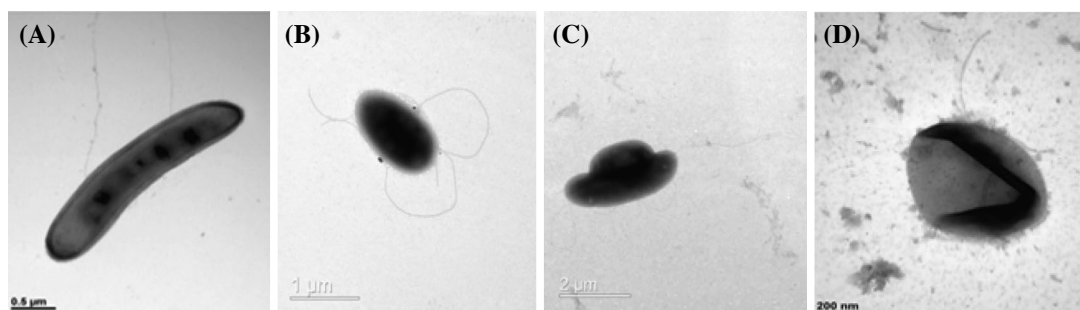
Fig. 1. Transmission electron micrographs of the strains isolated in this study. Strains: (A) *Curvibacter lanceolatus* R-1-5; (B) *Massilia brevitalea* R-2-13; (C) *Pseudomonas lini* R-2-1; and (D) *Pseudomonas vancouverensis* R-1-8.

Table 1. Summary of strains isolatèd belonging to the *Proteobacteria* and their taxonomic affiliations.

Phylum	Class	Order	Genus	Strain ID	NIBRBAC ID	Most closely related species	%	Medium	Incubation conditions
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Curvibacter*	R-1-5	000499694	*Curvibacter lanceolatus*	99.93	R2A	30°C, 3d
			Massilia	R-2-13	000500608	*Massilia brevitalea*	98.7	R2A	30°C, 3d
	Gammaproteobacteria	*Pseudomonadales*	*Pseudomonas*	R-2-1	000500607	*Pseudomonas lini*	99.54	R2A	30°C, 3d
			Pseudomonas	R-1-8	000500609	*Pseudomonas vancouverensis*	99.93	R2A	30°C, 3d

ganism Resources Division of the National Institute of Biological Resources (NIBR) collected soil samples from the Yongneup peatland and isolated unrecorded bacterial species. The 16S rRNA sequence similarity provides robust evidence identifying four bacterial strains belonging to the phylum *Proteobacteria*. In this report, we describe these four bacterial species which are not previously reported in Korea.

MATERIALS AND METHODS

Soil samples were collected from the Yongneup peatland under aseptic conditions and all the samples were processed separately. Each sample was serially diluted, spread on to R2A agar (Difco, Beckton, Dickinson, and Co., Spake, MD, U.S.A.), and incubated at 30°C for three days. The isolates colonies were deposited at NIBR, where they were stored at −70°C until further examinations. Colony morphology and cell size of the strains were observed on R2A agar after cells grown for three days at 30°C by transmission electron microscopy (LIBRA 120, Carl Zeiss). Transmission electron micrographs of the strains are shown in Fig. 1. Gram staining was performed using a Gram-Staining kit (Sigma-Aldrich). Biochemical characteristics were tested by using API 20NE galleries (bioMerieux) according to manufacturer's instructions.

Genomic DNA was extracted using a DNA extraction kit (AccuPrep Genomic DNA extraction kit, Bioneer, Korea) and 16S rRNA was amplified with PCR (primer sets 27F, 5′-AGAGTTTGATCCTGGCTCAG-3′; 1088R, 5′-GCTCGTTGCGGGACTTAACC-3′, 518F, 5′-CCAG-CAGCCGCGGTAATACG-3′; 800R, 5′-TACCAGG-GTATCTAATCC-3′) with 30 cycles of denaturation at 95°C for 30 sec, annealing at 57°C for 30 sec, and polymerization at 72°C for 45 sec, and sequenced by Macrogen (Daejeon, Korea). The 16SrRNA gene sequences of the closely related strains were obtained from Ez-Taxon-e (http://eztaxon-e.ezbiocloud.net). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987; Thompson *et al*., 1997) in MEGA5 (Tamura, 2013) with bootstrap values based on 1,000 replications (Felsenstein, 1985).

RESULTS AND DISCUSSION

The four bacterial species (R-1-5, R-2-13, R-2-1, R-1-8) were isolated from peatland soil samples in Korea. The taxonomic composition and identification results are summarized in Table 1. Morphology and physiological characteristics are shown in the species description section. All the four strains belonged to the phylum *Proteobacteria*, in which R-1-5 and R-2-13 were assigned to
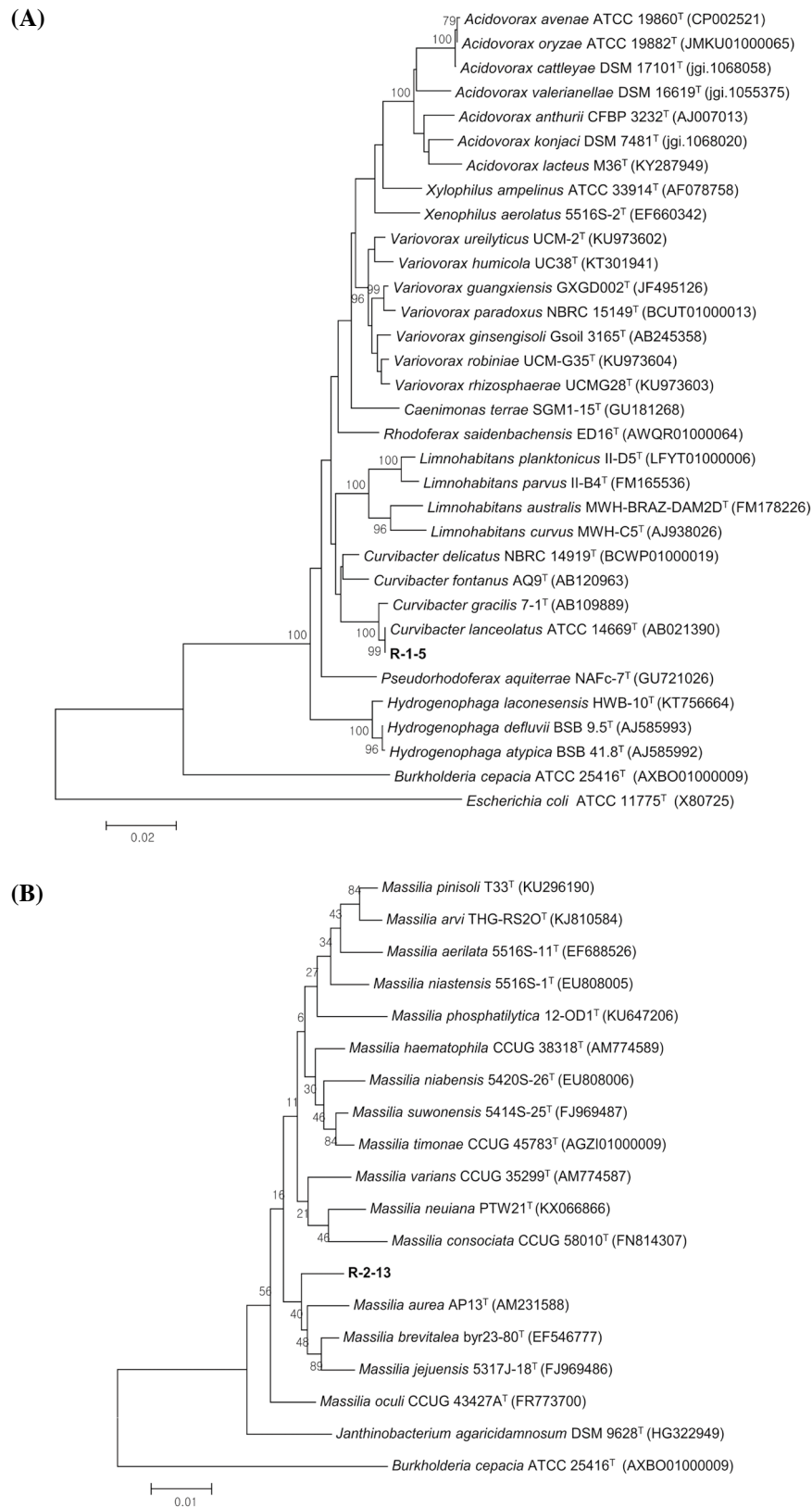
(A)

(B)

Fig. 2. Neighbor-joining phylogenetic tree based on 16S rRNA sequences shows the relationship between the strains isolated in this study and their relatives in the genera *Curvibacter* (A), *Massilia* (B) and *Pseudomonas* (C). Bootstrap values (>70%) are shown above nodes for the neighbor-joining. Scale bar: 0.01 changes per nucleotide.
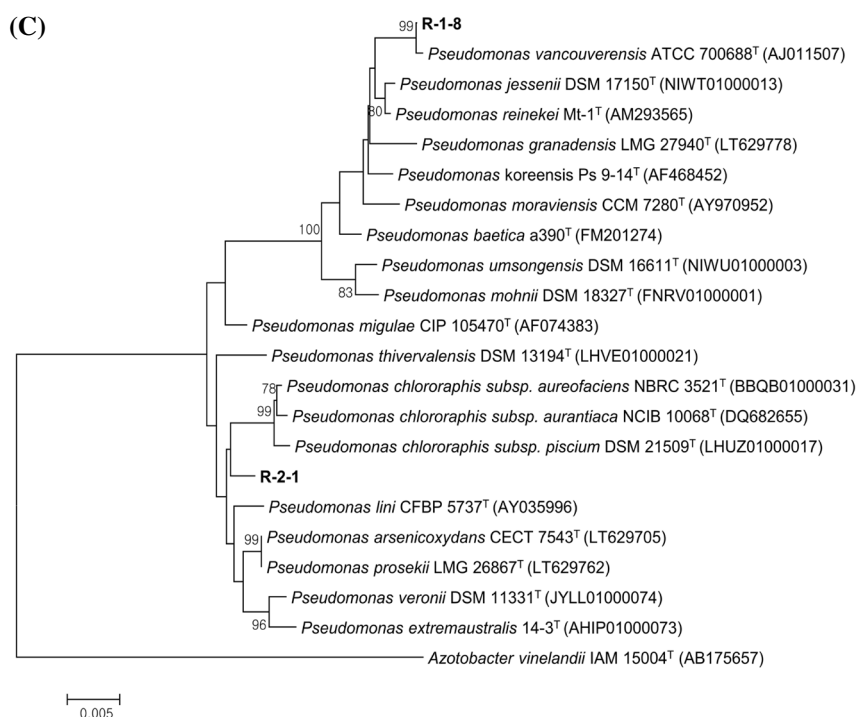
(C)


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99 ┌ R-1-8
   └ Pseudomonas vancouverensis ATCC 700688ᵀ (AJ011507)
     ┌ Pseudomonas jessenii DSM 17150ᵀ (NIWT01000013)
80 └ Pseudomonas reinekei Mt-1ᵀ (AM293565)
     ┌ Pseudomonas granadensis LMG 27940ᵀ (LT629778)
     ┌ Pseudomonas koreensis Ps 9-14ᵀ (AF468452)
     ┌ Pseudomonas moraviensis CCM 7280ᵀ (AY970952)
100  ┌ Pseudomonas baetica a390ᵀ (FM201274)
     ┌ Pseudomonas umsongensis DSM 16611ᵀ (NIWU01000003)
83   └ Pseudomonas mohnii DSM 18327ᵀ (FNRV01000001)
   ┌ Pseudomonas migulae CIP 105470ᵀ (AF074383)
   ┌ Pseudomonas thivervalensis DSM 13194ᵀ (LHVE01000021)
78 ┌ Pseudomonas chlororaphis subsp. aureofaciens NBRC 3521ᵀ (BBQB01000031)
99 └ Pseudomonas chlororaphis subsp. aurantiaca NCIB 10068ᵀ (DQ682655)
   ┌ Pseudomonas chlororaphis subsp. piscium DSM 21509ᵀ (LHUZ01000017)
   ┌ R-2-1
   ┌ Pseudomonas lini CFBP 5737ᵀ (AY035996)
99 ┌ Pseudomonas arsenicoxydans CECT 7543ᵀ (LT629705)
   └ Pseudomonas prosekii LMG 26867ᵀ (LT629762)
   ┌ Pseudomonas veronii DSM 11331ᵀ (JYLL01000074)
96 └ Pseudomonas extremaustralis 14-3ᵀ (AHIP01000073)
   ┌ Azotobacter vinelandii IAM 15004ᵀ (AB175657)
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0.005

Fig. 2. Continued.

the class *Betaproteobacteria*, and R-2-1 and R-1-8 assigned to the class *Gammaproteobacteria*. Based on the 16S rRNA phylogenetic analysis (Fig. 2), the strains R-1-5, R-2-13, R-2-1 and R-1-8 had highest gene sequence similarity with *Curvibacter lanceolatus* ATCC 14669ᵀ (AB021390; 99.93%), *Massilia brevitalea* byr23-80ᵀ (EF546777; 98.7%), *Pseudomonas lini* CFBP 5737ᵀ (AY035996; 99.54%) and *Pseudomonas vancouverensis* ATCC 700688ᵀ (AJ011507; 99.93%), respectively. Here we report on these four unrecorded bacterial species belonging to the phylum *Proteobacteria* isolated from peatland soil in Korea.

Description of *Curvibacter lanceolatus* R-1-5

Cells are Gram-negative, non-flagellated, and rod-shaped. Colonies are circular, raised, entire, and white colored after 3 days of incubation on R2A agar at 30°C. Oxidase and catalase positive. Using the API 20NE kit, this strain was positive for urease, D-glucose, and gluconate; but negative for nitrate reduction, reduction of nitrates to nitrogen, indole production, glucose acidification, arginine dihydrolase, β-glucosidase, protease, β-galactosidase, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, caprate, adipate, malate, citrate, and phenyl-acetate. D-Glucose, propionate, valerate, L-histidine, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, suberate, acetate, DL-lactate, L-alanine, 3-hydroxy-benzoate are utilized. Does

not utilize D-mannitol, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, caprate, citrate, 2-ketogluconate, L-rhamnose, *N*-acetyl-glucosamine, D-ribose, inositol, D-sucrose, D-maltose, itaconate, malonate, 5-ketogluconate, glycogen, and L-serine. DNA G + C mol is 59.1%. Strain R-1-5 (= NIBRBAC000499694) was isolated from peatland soil, Gangwon Province, Korea.

Description of *Massilia brevitalea* R-2-13

Cells are Gram-negative, motile, and rod-shaped. Colonies are circular, raised, entire, and cream colored after 3 days on R2A agar at 30°C. Oxidase and catalase positive. Using the API 20NE kit, this strain was positive for protease; but negative for nitrate reduction, reduction of nitrates to nitrogen, indole production, glucose acidification, arginine dihydrolase, urease, β-glucosidase, β-galactosidase, D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose, gluconate, caprate, adipate, malate, citrate, and phenyl-acetate. In API 32GN, carbon assimilation is positive for salicin, propionate, valerate, L-histidine, 3-hydroxy-butyrate, D-sucrose, itaconate, acetate, DL-lactate, L-alanine, and 5-ketogluconate. Negative for D-mannitol, D-glucose, D-melibiose, L-fucose, D-sorbitol, L-arabinose, caprate, citrate, 2-ketogluconate, 4-hydroxy-benzoate, L-proline, L-rhamnose, *N*-acetyl-glucosamine, D-ribose, inositol, D-maltose, suberate, malonate, glycogen, 3-hydroxy-benzoate, and L-serine. DNA G + C mol

is 64.4%. Strain R-2-13 (=NIBRBAC000500608) was isolated from peatland soil, Gangwon Province, Korea.

Description of *Pseudomonas lini* **R-2-1**

Cells are Gram-negative, motile, and rod-shaped. Colonies are circular, raised, entire, and white colored after 3 days on R2A agar at 30°C. Oxidase and catalase positive. Using the API 20NE kit, this strain was positive for nitrate reduction, arginine dihydrolase, urease, D-glucose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, gluconate, caprate, malate and citrate; but negative for indole production, glucose acidification, β-glucosidase, protease, β-galactosidase, L-arabinose, D-maltose, adipate and phenyl-acetate. In API 32GN, carbon assimilation is positive for D-mannitol, D-glucose, D-sorbitol, propionate, caprate, citrate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, L-Proline, L-rhamnose, *N*-acetyl-glucosamine, acetate, DL-lactate, L-alanine and L-serine. Negative for salicin, D-melibiose, L-fucose, L-arabinose, valerate, 4-hydroxy-benzoate, D-ribose, inositol, D-sucrose, D-maltose, itaconate, suberate, malonate, 5-ketogluconate, glycogen and 3-hydroxy-benzoate. DNA G+C mol is 58.4%. Strain R-2-1 (=NIBRBAC000500607) was isolated from peatland soil, Gangwon Province, Korea.

Description of *Pseudomonas vancouverensis* **R-1-8**

Cells are Gram-negative, non-flagellated, and rod-shaped. Colonies are circular, raised, entire, and cream colored after 3 days on R2A agar at 30°C. Oxidase and catalase positive. Using the API 20NE kit, this strain was positive for arginine dihydrolase, urease, D-glucose, L-arabinose, gluconate, caprate, malate, citrate and phenyl-acetate; but negative for Nitrate reduction, reduction of nitrates to nitrogen, indole production, glucose acidification, β-glucosidase, protease, β-galactosidase, D-mannose, D-mannitol, *N*-acetyl-glucosamine, D-maltose and adipate. In API 32GN, carbon assimilation is positive for D-glucose, L-arabinose, propionate, caprate, valerate, citrate, L-histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, D-ribose, malonate, acetate, DL-lactate, L-alanine, 3-hydroxy-benzoate and L-serine. Negative for D-mannitol, salicin, D-melibiose, L-fucose, D-sorbitol, L-rhamnose, *N*-acetyl-glucosamine, inositol, D-sucrose, D-maltose, itaconate, suberate, 5-ketogluconate and glycogen. DNA G+C mol is 60.8%. Strain R-1-8 (=NIBRBAC000500609) was isolated from peatland soil, Gangwon Province, Korea.

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