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Bacterial Diversity in the Rhizosphere of Halophyte *Phragmites communis* at the Western Coastal Mudflats of Korea

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ABSTRACT: This study investigated the population densities and diversity of heterotrophic bacteria, and the rhizosphere-to-soil ratios (R/S) in the rhizosphere soil of halophyte *Phragmites communis* at the western coastal mudflats of Korea. The population densities of aerobic heterotrophic bacteria on the rhizosphere soil of *P. communis* were in the range of $3.3 \pm 0.9 \times 10^7$~$1.2 \pm 0.5 \times 10^8$ cfu g^{-1} dry weight (d. wt.). Population densities of amylolytic bacteria ranged from $1.1 \pm 0.2 \times 10^6$ to $3.0 \pm 1.2 \times 10^6$ cfu g^{-1} d. wt., while those of cellulolytic bacteria and proteolytic bacteria ranged from $5.6 \pm 2.3 \times 10^6$ to $1.5 \pm 0.3 \times 10^7$ cfu g^{-1} d. wt. and from $1.4 \pm 0.3 \times 10^6$ to $3.5 \pm 2.3 \times 10^7$ cfu g^{-1} d. wt., respectively. The R/S ratios ranged from 2.26 to 6.89. Genetic (16S DNA) analysis of fifty-one isolates from the roots of *P. communis* suggested that the dominant species were closely related to the γ-proteobacteria group (18 clones) and the α-proteobacteria group (14 clones). We found that halophyte species and mudflat environment both affected the rhizosphere bacterial communities.

Key words: 16S rDNA, Bacterial population density, Halophyte, *Phragmites communis*, γ-proteobacteria group, R/S ratio, Rhizosphere

INTRODUCTION

Among the 2,815 km^2 of mudflats in South Korea, 2,393 km^2 are found on the western and southern coasts of Korea. Mudflats in coastal estuaries act as protective filters and final repositories for runoff pollutants (Teal and Howes 2000), pathogens (Grant et al. 2001), and nutrients (Howes et al. 1996). They also play a vital role in the development of many marine populations and are essential as breeding grounds or nursery areas for many species.

Halophytes in mudflat areas function as primary producers, and their roots and stalks stabilize the sediments, preventing erosion. They also provide habitats for various organisms and have the ability to remove contaminants from ecosystem (Choi and Lee 1996). However, because of recent large-scale land reclamation projects, halophyte habitats have been reduced rapidly, threatening the functioning of coastal ecosystems.

Rhizospheres, generally defined as the soil adjacent to and influenced by plant roots, are regarded as "hot spots" for microbial colonization and activity (Metting 1993). In contrast to bulk soil, where organic carbon is available only at low concentrations, rhizospheres are supplied with higher concentrations of nutrients generated during plant photosynthesis (Duineveld et al. 1998, 2001).

Rhizosphere microbial communities are mainly determined by the plant species (Marshner et al. 2001) and soil characteristics (Degens et al. 2000, Gelsomino et al. 1999), and rhizosphere microbial communities influence plant nutrition, growth, and disease (Assigbetse et al. 2005).

Microbiological studies of mudflat environments in Korea have mainly investigated microbe distribution (Lee et al. 1996), enzyme activities (Kim and Lee 1992, Choi and Lee 1996), and diversity (Lee et al. 2001, Kim et al. 2004, Kim et al. 2005). However, microbial diversity on the rhizosphere of halophytes in mudflat environments in Korea was also studied recently by Park and Lee (2006).

In this study, we investigated the R/S ratios, population densities, and diversities of bacteria in the rhizosphere of *Phragmites communis* communities on mudflats in western Korea as baseline data for a restoration project for halophyte habitats in Korea.

MATERIALS AND METHODS

Sampling and Counting of Bacteria

Samples of rhizosphere soil (R) and soil remote from roots (S) of *P. communis* were collected from 3 stations on the western coastal mudflats of Korea during July and October, 2004 (Fig. 1). Soils were collected using a soil auger, stored at 5℃, and processed within a few hours of collection.

The effects of the rhizosphere were measured by calculating at the ratio of the number of microorganisms in rhizosphere soil (R) to the number of microorganisms in soil remote from roots (S), or the R/S ratio.

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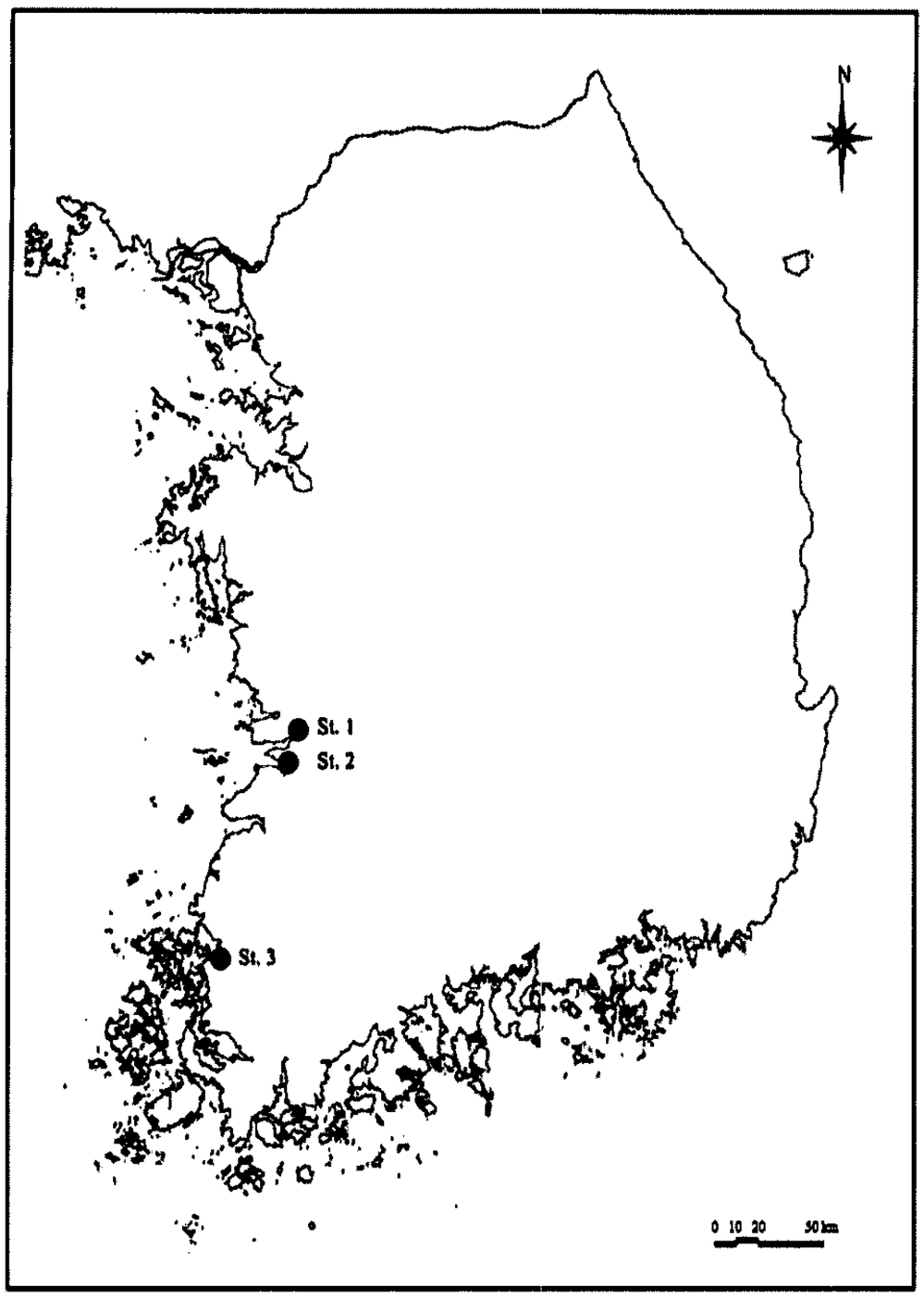

Fig. 1. Map showing the sampling sites in the western mudflats of Korea (St. 1: Seocheon, St. 2: Mankyung R., St. 3: Muan bay).

We estimated of population densities following the methods of Paul and Clark (1988). To determine R/S ratios and the population densities of the aerobic heterotrophic bacteria, one gram of rhizosphere soil and one gram of soil remote from roots were suspended in 10-mL sterile saline solution (0.85% NaCl), and shaken for 5 min at 100 rpm. Then serial decade dilutions were made with sterile saline water and 0.1 mL of each was plated on Nutrient agar (Difco) and Marine agar 2216 (Difco). To determine the number of aerobic physiological groups of heterotrophic bacteria, soluble starch (0.2%) for amylolytic bacteria, carboxymethyl cellulose (0.5%) for cellulolytic bacteria, or gelatin (0.4%) for proteolytic bacteria was added as the sole carbon source to the Trytic soy broth (Difco, USA) as the basal culture medium (Wollum 1982). After incubation at 25 ± 2℃ for 72 hrs, we counted the resulting colonies using the methods of Holding and Collee (1971). Final estimated population densities were expressed as $\log_{10}$ colony-forming units (CFU) g^{-1} oven-dried sediment.

PCR Amplification of 16S rDNA

We amplified 16S rDNA fragments of isolates from the rhizosphere of the halophyte *P. communis* using PCR with the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1522R (5'-AAGGAGGT GWTCCARCC-3'). The PCR reaction mixture contained 5 μL of 10X PCR amplification buffer (final concentration: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl, pH. 9.0), 4 μL of 2.5 mM $MgCl_2$, 1 μL of 10 mM dNTP, 1 μL of each 10 pmol oligonucleotide primer, and 1 U of Taq polymerase (TaKaRa, Japan) in 50 μL of PCR mixture. DNA was amplified with a GeneAmp PCR system 2700 (Applied Biosystems, USA) thermal cycler using the following program: initial denaturation at 94℃ for 10 min, followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 5 min, and a final extension at 72℃ for 7 min. PCR products were either used immediately or stored at 4℃ prior to subsequent analyses. Two replicate reactions were run for each sample.

16S rDNA Sequencing and Phylogenetic Analysis

Ribosomal DNA sequences were determined by the Genotech Company (Daejon, Korea) using an ABI PRISM 3700 DNA analyzer (Applied Biosystem, USA). We then compared the sequences directly to all known sequences in the GenBank database using the basic local alignment search tool (BLAST)(Altschul et al. 1997), and constructed phylogenetic trees with the neighbor-joining (NJ) method using the NEIGHBOR program (PLYLIP, version 3.5) (Saitou and Nei 1987).

RESULTS AND DISCUSSION

Population Densities of Heterotrophic Bacteria

Estimated population densities of aerobic heterotrophic bacteria inhabiting the rhizosphere of *P. communis* ranged from $3.3 \pm 0.9 \times 10^7$ to $1.2 \pm 0.5 \times 10^8$ cfu g^{-1} dry weight (d. wt.) during the sampling periods (Fig. 2). The population densities of aerobic heterotrophic bacteria were the highest on the mudflats near Mankyung River, and lower near Seocheon and Muan. Although the differences in microbial population density among sampling stations were not explored in detail in this study, we suspect that bacterial population densities may be influenced by fine-scale environmental variables in the soil.

Previous studies have reported diversity in the microbial populations in the rhizospheres of different plants (Neal et al. 1970, 1973). In our study the population densities of aerobic heterotrophic bacteria were higher in the rhizosphere of *P. communis* than those reported for the *Suaeda japonica* rhizosphere (Park 2004), but were lower than those in the *Suaeda maritima* and *Salicornia herbacea* rhizospheres, which suggests that rhizosphere microbial communities are mainly determined by root exudates, which vary among plant species (Marshner et al. 2001). Soil microorganisms have been shown to respond to plant exudates and different plant species have different patterns of root exudation (Brimecombe et al. 2001).

Heterotrophic bacteria on the mudflats decompose organic matter derived from plant litter and transform pollutants (Benoit et al. 2003, Smith and Hollibaugh 1993). The population densities of physiological groups of heterotrophic bacteria on the roots of *P. communis* ranged

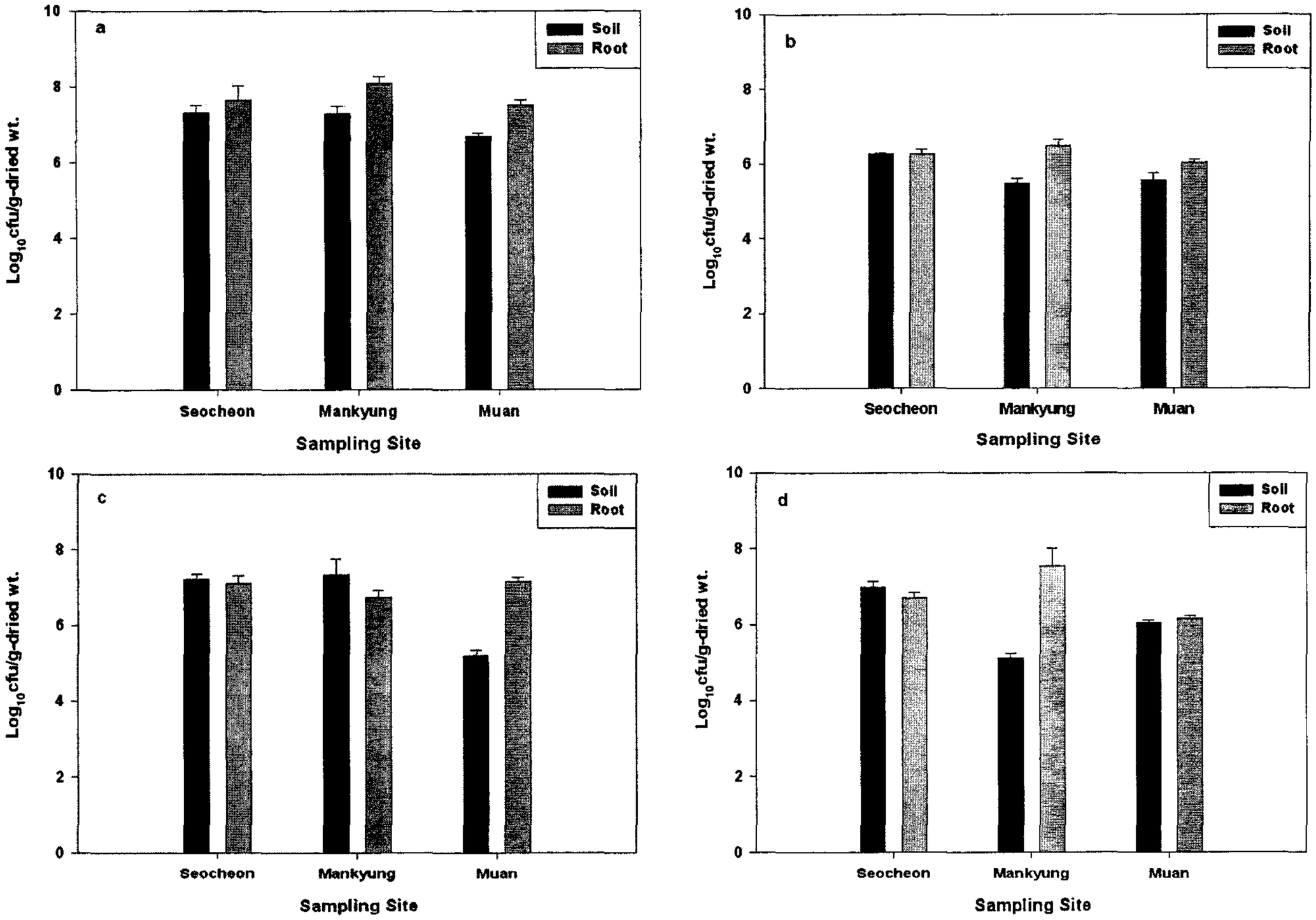

Fig. 2. Population densities of (a) aerobic heterotrophic bacteria, (b) amylolytic heterotrophic bacteria, (c) cellulolytic heterotrophic, and (d) proteolytic bacteria in the rhizosphere soil and soil remote from roots of the halophyte *Phragmites communis* from mudflats in western Korea in July and October, 2004.

from $1.1 \pm 0.2 \times 10^6$ to $3.0 \pm 1.2 \times 10^6$ cfu g^{-1} d. wt. for amylolytic bacteria, from $5.6 \pm 2.3 \times 10^6$ to $1.5 \pm 0.3 \times 10^7$ cfu g^{-1} d. wt. for cellulolytic bacteria, and from $1.4 \pm 0.3 \times 10^6$ to $3.5 \pm 2.3 \times 10^7$ cfu g^{-1} d. wt. for proteolytic bacteria (Fig. 2). Population densities were similar among the different physiological groups of heterotrophic bacteria. However, during the sampling period, bacterial population densities of amylolytic and proteolytic bacteria were highest at Mankyung, while densities of cellulolytic bacteria were highest and densities of amylolytic bacteria were lowest at Muan. It is likely that the materials released by plants into the soil (Atlas and Bartha 1992) had a direct influence on the composition and density of the mudflat microbial community. Accordingly, root exudation is an important ecological phenomenon that affects succession in the plant and root microbial communities (Singh and Mukerji 2006).

Rhizosphere Effects on Microbial Populations

The rhizosphere is chemically, physiologically and biologically complex due to the influence of plant roots on various types of microorganisms. The interaction of soil and rhizosphere microbes results in stimulation of microorganisms known as the "rhizosphere effect".

The R/S ratios in the rhizospheres of *P. communis* ranged from 2.26 to 6.89 (Table 1), which are similar to the R/S values reported for rhizospheres of other halophytes (Park 2004), but lower than those of common terrestrial environments (Gray and Parkinson 1968, Woldendorp 1978). This result suggests that nutrient availability and soil texture have an important effect on the growth of rhizosphere bacteria

Table 1. Ratios of the number of microorganisms in the rhizosphere soil (R) to the number of corresponding microorganisms in soil remote from roots (S) (R/S ratios) of the halophyte *Phragmites communis* from mudflats in western Korea

Station \ Halophyte	*Phragmites communis* (R/S Ratio)
Seocheon	2.26
Mankyung	6.30
Muan	6.89

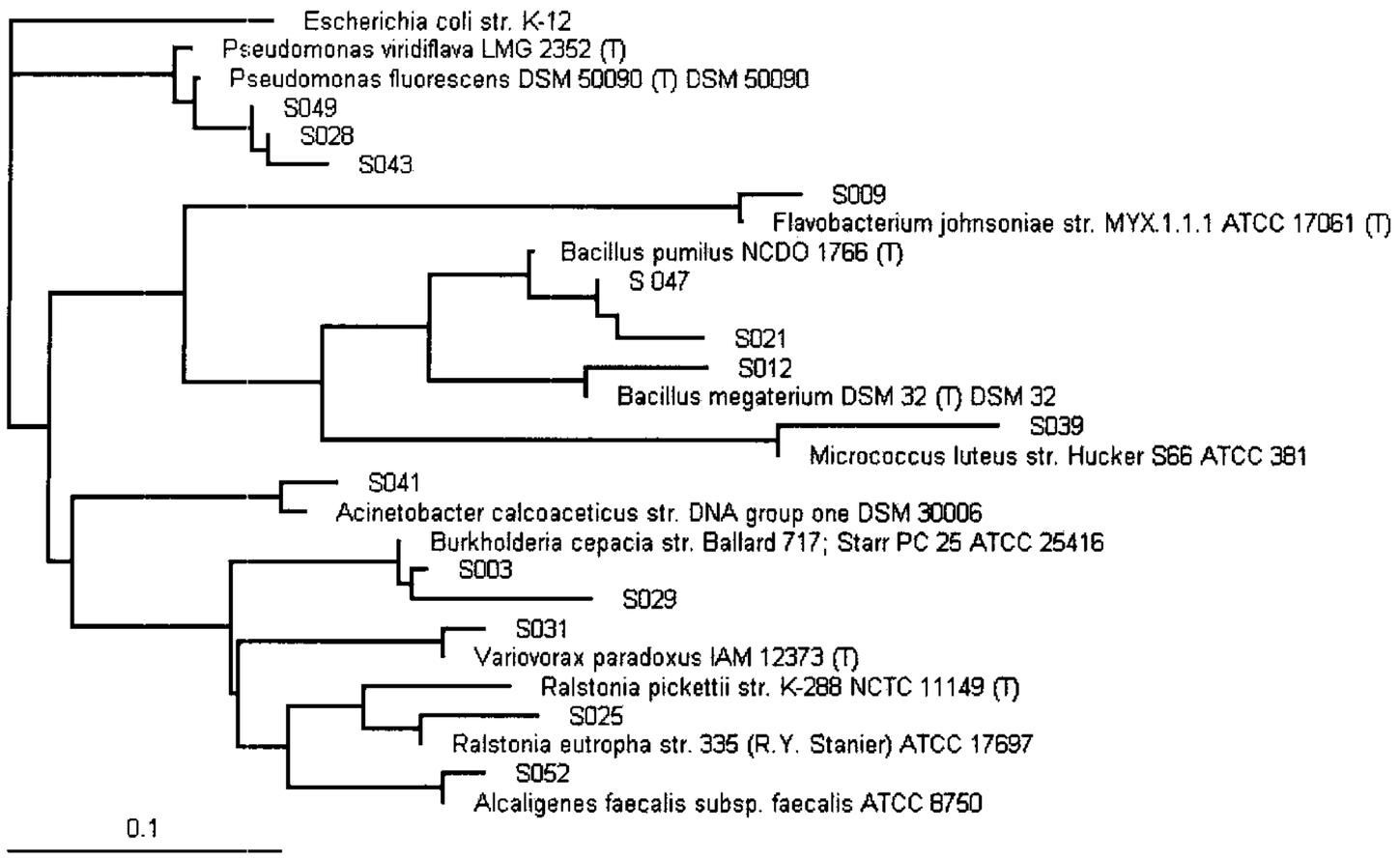

Fig. 3. Phylogenetic tree showing the affiliation of 16S rDNA sequences of heterotrophic bacteria sampled from the rhizosphere of *Phragmites communis* from Seocheon to selected reference sequences. The tree was constructed from a distance matrix by the neighbour-joining analysis. The bar represents 0.1% estimated sequence divergence.

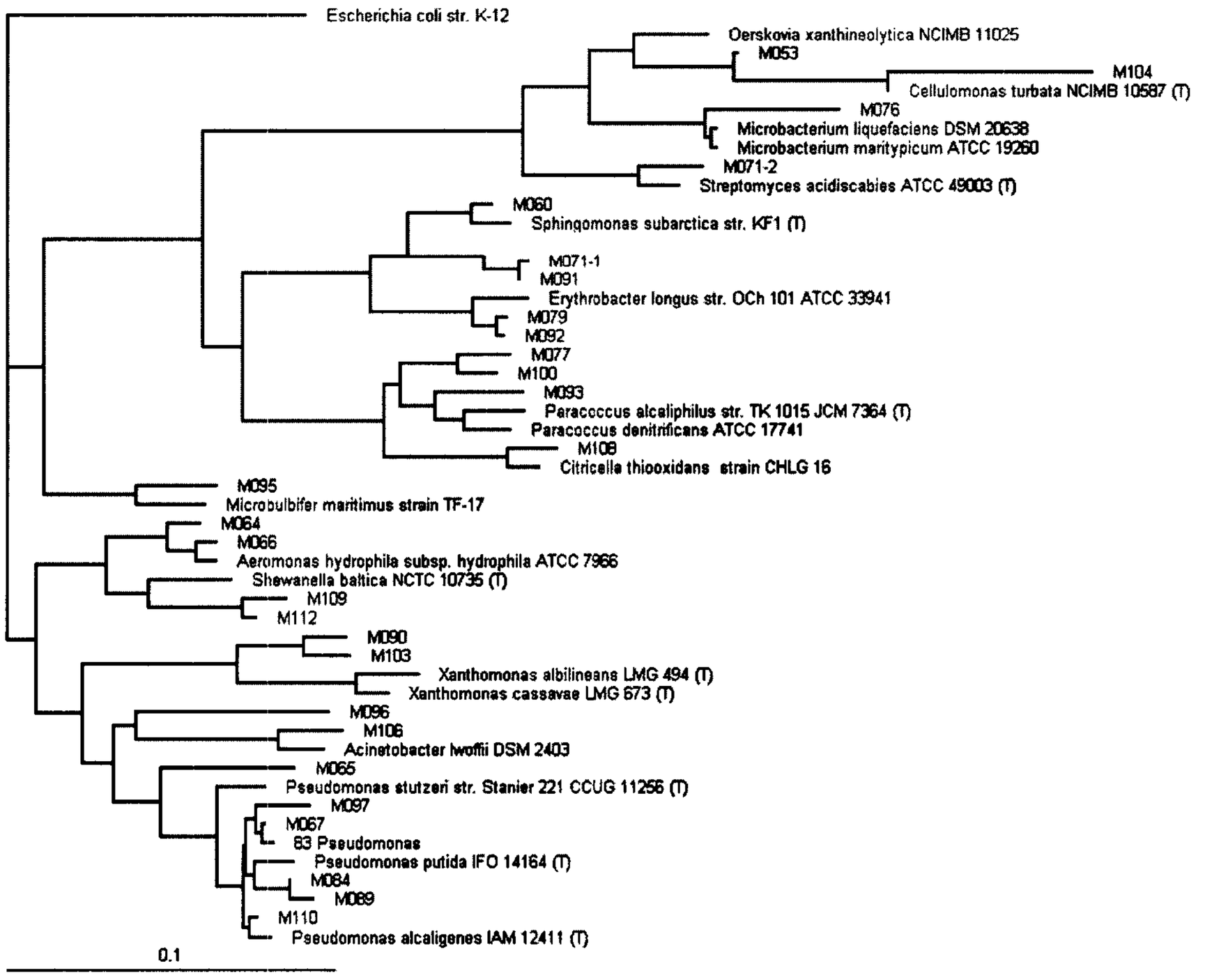

Fig. 4. Phylogenetic tree showing the affiliation of 16S rDNA sequences of heterotrophic bacteria sampled from the rhizosphere of *Phragmites communis* from Mankyung to selected reference sequences. The tree was constructed from a distance matrix by the neighbour-joining analysis. The bar represents 0.1% estimated sequence divergence.

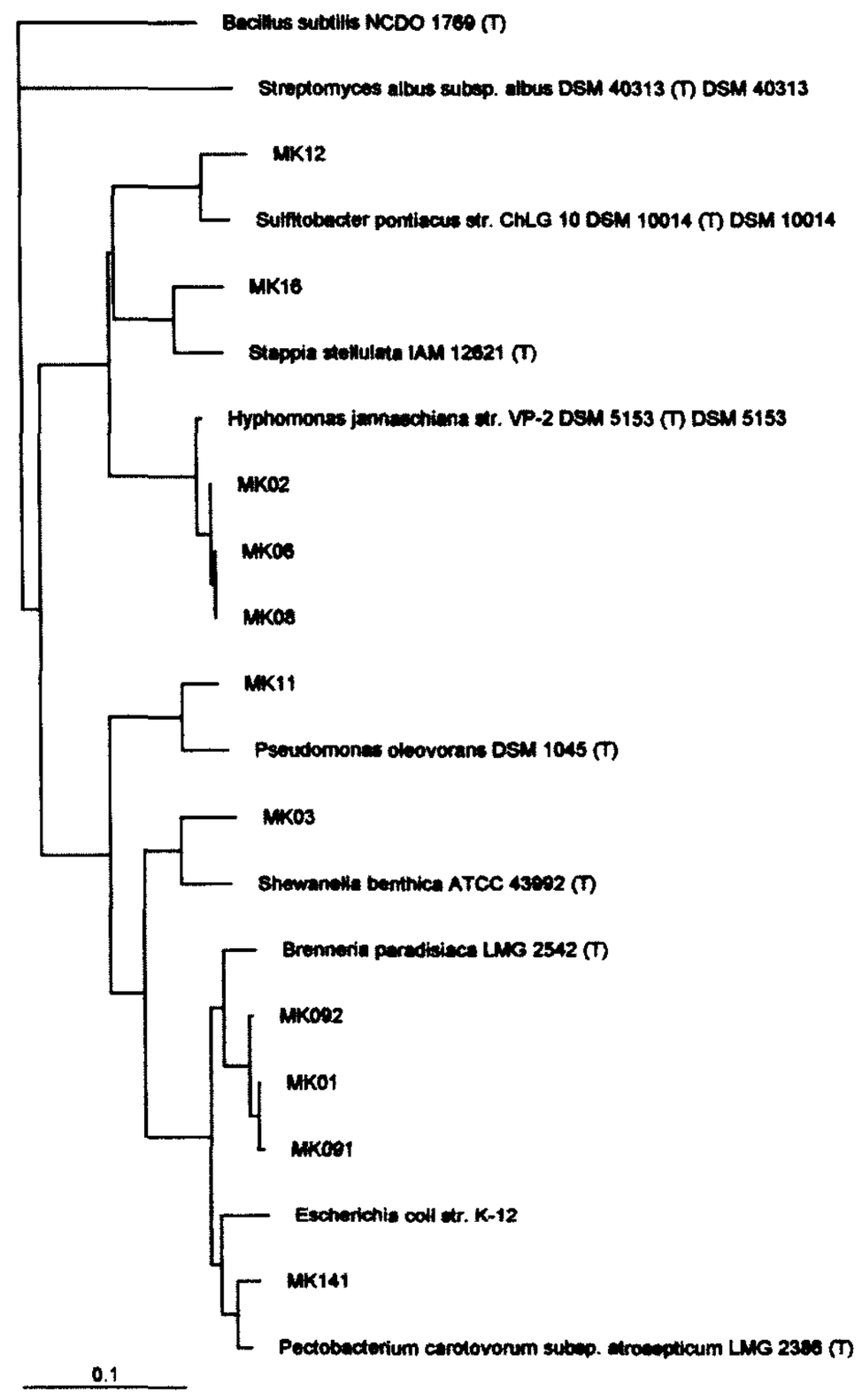

Fig. 5. Phylogenetic tree showing the affiliation of 16S rDNA sequences of the heterotrophic bacteria on the rhizosphere of *Phragmites communis* sampled from Muan to selected reference sequences. The tree was constructed from a distance matrix by the neighbour-joining analysis. The bar represents 0.1% estimated sequence divergence.

on mudflats, and therefore on R/S values.

Sequencing and Phylogenetic Analysis

Bacterial 16S rDNA clones were characterized by partial sequencing and phylogenetic analysis. We identified thirteen isolates from the rhizosphere of *P. communis* from Seocheon: five clones, including *Burkholderia* spp., *Alcaligenes* sp., *Varovorax* sp., and *Ralstonia* sp., were determined to belong to the β-proteobacteria group and four clones, including *Pseudomonas* spp. and *Acinetobacter* sp. were determined to belong to the γ-proteobacteria group (Table 2). Among the twenty-six isolates from Mankyung, twelve clones were determined to belong to the γ-proteobacteria group, and ten were determined to belong to the α-proteobacteria group (Table 2). We identified twelve isolates from Muan, six of which were determined to belong to the γ-proteobacteria

Table 2. List of heterotrophic bacteria isolated by 16S rDNA analysis from the rhizosphere of the halophyte *Phragmites communis* from mudflats in western Korea

Site	Closet Genebank library	Group
Seocheon	*Flavobacterium* sp.	Flavobacteria
	Bacillus spp. (2 species)	Firmicutes
	Varovorax sp.	Betaproteobacteria
	Psedomonas spp. (3 species)	Gammaproteobacteria
	Burkholderia spp. (2 species)	Betaproteobacteira
	Alcaligenes sp.	Betaproteobacteira
	Ralstonia sp.	Betaproteobacteira
	Micrococcus sp.	Actinobacteria
	Acinetobacter sp.	Gammaproteobacteira
Mankyung	*Cellulomonas* sp.	Actinobacteria
	Sphingomonas sp.	Alphaproteobacteira
	Aeromonas spp. (2 species)	Gammaproteobacteira
	Pseudomonas spp. (5 species)	Gammaproteobacteira
	Streptomyces sp.	Actinobacteria
	Sphingomonas sp.	Alphaproteobacteira
	Microbacterium sp.	Actinobacteria
	Paracoccus spp. (4 species)	Alphaproteobacteira
	Erythrobacter spp. (2 species)	Alphaproteobacteira
	Xanthomonas sp.	Gammaproteobacteira
	Citricella sp.	Alphaproteobacteira
	Sulfitobacter sp.	Alphaproteobacteira
	Microbulbifer sp.	Actinobacteria
	Oceanospirillum sp.	Gammaproteobacteira
	Shewanella spp. (2 species)	Gammaproteobacteira
	Acinetoacter sp.	Gammaproteobacteira
Muan	*Erwinia* spp. (4 species)	Gammaproteobacteira
	Hyphomonas spp. (3 species)	Alphaproteobacteira
	Shewanella sp.	Gammaproteobacteira
	Bacillus sp.	Firmicutes
	Arthrobacter sp.	Actinobacteria
	Pseudomonas sp.	Gammaproteobacteira
	Stappia sp.	Alphaproteobacteira

group, and four to the α-proteobacteria group (Table 2). Among the total of fifty-one isolates from the three sampling stations, the dominant group were the γ-proteobacteria (43.1%), followed by the α-proteobacteria (27.5%), and the Actinobacteria (11.8%). According to Gray and Herwing (1996), the γ-proteobacteria group is dominant in marine sediments. The microorganisms prevailing in the rhizosphere originate in the soil. However, because of variation in root exudates characteristics between plant species, the specific organisms constituting the rhizosphere microbial community vary with both plant species and soil type (Grayston et al. 1998, Latour 1996, Maloney et al. 1997). In our study, most sequences had < 97% sequence similarity to previously cultivated microorganisms and phylogenetic analyses of isolated bacteria from the rhizosphere of *P. communis* revealed that the majority of bacteria detected were closely related to the γ-proteobacteria and the α-proteobacteria. However, further study will be required to better understand the diversity, density, and functions of rhizosphere bacteria of halophytes on mudflat environments.

We found higher bacterial densities in the *P. communis* rhizosphere than in soils remote from the rhizosphere. Variation in rhizosphere bacterial populations among localities and plant communities suggests that halophyte species and soil type affects the rhizosphere bacterial communities in mudflat environments (Grayston et al. 1998, Latour 1996, Maloney et al. 1997).

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