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Bacterial Diversity in the Rhizosphere of Halophyte *Suaeda japonica* in Western and Southern Mudflats of Korea

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ABSTRACT: This study was carried out to investigate the population densities, R/S ratios, and identification of heterotrophic bacteria on the rhizosphere soil of halophyte *Suaeda japonica* found on the western and southern mudflats of Korea. The population densities of aerobic and anaerobic heterotrophic bacteria on the rhizosphere soil of *Suaeda japonica* were in the range of $1.3 \pm 0.3 \times 10^6 \sim 6.3 \pm 3.3 \times 10^7$ and $2.8 \pm 1.3 \times 10^4 \sim 1.8 \pm 0.7 \times 10^7$ cfu g^{-1} d. wt., respectively. In case of physiologically specific bacteria, population densities of amylolytic bacteria on the rhizosphere soil of *Suaeda japonica* were in the range of $4.4 \pm 0.6 \times 10^6 \sim 2.5 \pm 1.2 \times 10^7$ cfu g^{-1} d. wt., those of cellulolytic bacteria were from $8.5 \pm 6.0 \times 10^4 \sim 2.3 \pm 1.6 \times 10^6$ cfu g^{-1} d. wt., and those of proteolytic bacteria were from $3.8 \pm 1.8 \times 10^5 \sim 4.2 \pm 2.9 \times 10^6$ cfu g^{-1} d. wt., respectively. The R/S ratios were ranged from 2.33 to 2.39. Among eleven isolates from the roots of halophyte *Suaeda japonica* of Goheung bay by using 16S rDNA analysis, five clones were closely related to γ-Proteobacteria group and six clones were closely related to α-Proteobacteria group. Among four isolates from Suncheon bay, two strains were related to γ-Proteobacteria group and another two were related to *Actinobacteria* and Bacilli group, respectively.

Key words: Halophyte, Population density, Rhizosphere, R/S ratio, *Suaeda japonica*, 16S rDNA

INTRODUCTION

The area of mudflats in South Korea is approximately 2,815 km^2, which occupies 3% of the gross area of Korean Peninsula. Among them, 2,393 km^2 is distributed in western and southern coast of Korea. Because of its relatively shallow, protected waters, and availability of nutrients, it is exceptionally rich in biological activity and provides commercially most valuable marine products. Recently, due to the large-scale land reclamation project, mudflat area reduced rapidly, which threatened the coastal ecosystem in this area seriously (Lee et al. 2003).

Halophytes inhabited in mudflat area are functionally acted as primary producers and their roots and stalks induce solidity and stability of sediments, which prevent land from erosion and from accumulation of sand by wind. Moreover, halophytes have the ability to purify the sewage wastes and place various organisms as habitats (Choi 1998).

Microbiological studies on the mudflats were mainly about the distribution, enzyme activities (Kim and Lee 1992, Choi and Lee 1996, Lee et al. 1996, Lee et al. 2001), and diversity (Kim et al. 2004, Kim et al. 2005) on the sediment. However, little is known about microbial diversity on the rhizosphere of halophytes in Korea. Soil microorganisms associated with plant roots have an important influence on plant nutrition, growth promotion, and disease interaction (Assigbetse et al. 2005). The structure and function of plant root system contributes to the establishment of the rhizosphere microbial population (Nye and Tinker, 1977, Russell 1977, Lynch 1982) and rhizosphere microbial communities are mainly determined by plant species (Ibekwe and Kennedy 1998, Marshner et al. 2001, Miethling et al. 2000) and soil characteristics (Degens et al. 2000, Gelsomino et al. 1999). The interactions of plant roots and rhizosphere microorganisms are based largely on the composition of root cell components and root exudates (Grayston et al. 1996, Ibekwe and Kennedy 1998). Within the rhizosphere, plant roots have a direct influence on the composition and density of the soil microbial community, known as the rhizosphere effect (Atlas and Bartha 1992). Therefore, microbial distribution and composition on the rhizosphere can be grasped by estimating the rhizosphere effects.

In this study, as a baseline survey data for the restoration of halophytes on the mudflat area in Korea, we compared the population densities of aerobic and anaerobic heterotrophic bacteria, physiological groups of heterotrophic bacteria, and R/S ratios on *Suaeda japonica* as a first step among the halophyotes inhabited on western and southern mudflats in Korea. At the same time, we isolated rhizosphere bacteria from the roots of *Suaeda japonica* and identified them by 16S rDNA analysis.

MATERIALS AND METHODS

Sampling and Counting of Bacteria

Samples of rhizosphere soil (R) and soil remote from root (S)

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of halophyte *Suaeda japonica* were collected from July to October, 2003 from 4 stations on the mudflats of western and southern mudflats of Korea (Fig. 1). Soils were collected by using a soil auger and were processed within a few hours after collection and were maintained at 5℃ during storage.

Methods in microbiological study deal with isolation and estimation of numbers were followed by Paul and Clark (1988). For the determination of the aerobic heterotrophic bacteria, one gram of rhizosphere soil sample and one gram of soil sample remote from roots were suspended in 10-mL sterile saline solution (0.85% NaCl), respectively and shook 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 marine ager 2216 (Difco). After incubation at 25 ± 2℃ for 72 hrs, colonies were enumerated. Viable anaerobic heterotrophic bacteria were determined in a similar way to aerobic heterotrophic bacteria, but their incubation took place in an anaerobic jar (BBL Anaerobic System, USA). To determine the number of aerobic physiological groups of heterotrophic bacteria, soluble starch (0.2%) foramylolytic bacteria, carboxymethyl cellulose (0.5%) for cellulolytic bacteria, and gelatin (0.4%) for proteolytic bacteria were added, respectively, as the sole carbon source to the Tryticase soy broth (Difco, USA) as for the basal medium (Wollum 1982). After incubation at 25 ± 2℃ for 72 hrs, colonies were counted by the methods of Holding and Collee (1971). The final population densities were expressed as $\log_{10}$ colony-forming units (CFU) g^{-1} oven- dried sediment.

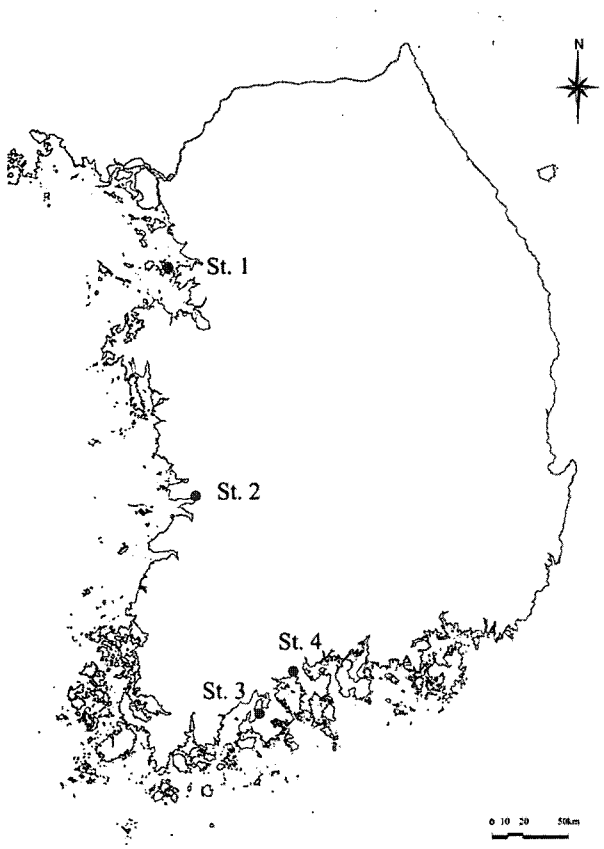

Fig. 1. A map showing the sampling sites in the western and southern mudflats of Korea (St. 1: Daebu-do, St. 2: Mankyung River, St. 3: Goheung bay, St. 4: Suncheon bay).

PCR Amplification of 16S rDNA

For the specific amplification of 16S rDNA fragments of isolates from the roots of halophyte *Suaeda japonica*, a nested PCR protocol was applied using primers 27F (5'-AGA GTTTGATCMTGG CTCAG-3') and 1522R (5'-AAGGAGGTGWTCCARCC-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 cycle using the following programme: initial denaturation at 94℃ for 10 min, followed by 30 cycle 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 using an ABI PRISM 3700 GENETIC analyser (Applied Biosystem, USA) at the Genotech Company (Daejon, Korea). The sequences were compared directly to all known sequences deposited in GeneBank database using the basic local alignment search tool (BLAST). The phylogenetic trees were constructed by the neighbor-joining (NJ) method (Saitou and Nei 1987) using the NEIGHBOR program (PLYLIP, version 3.5).

Accession Numbers

The sequence data obtained in this study have been submitted to the GenBank database under accession numbers from AY690670 to 690684.

RESULTS AND DISCUSSION

Population Densities of Heterotrophic Bacteria

Bacterial population densities of aerobic heterotrophic bacteria inhabited on the roots of *Suaeda japonica* were in the range of 5.3 ± 1.8 × 10^5 ~ 6.3 ± 3.3 × 10^7 cfu g^{-1} dry weight (d. wt.) during sampling periods at 4 stations (Fig. 2a). Examining the population densities according to sampling stations, the population densities at Daebu-do (St. 1) fluctuated between 5.3 ± 1.8 × 10^5 and 1.3 ± 0.3 × 10^6 cfu g^{-1} d. wt., and those at Mankyung (St. 2) between 1.1

± 0.3 × 10^7 and 2.6 ± 0.6 × 10^7 cfu g^{-1} d. wt., those at Goheung bay (St. 3) between 1.4 ± 0.8 × 10^7 and 3.3 ± 1.2 × 10^7 cfu g^{-1} d. wt., and those at Suncheon bay (St. 4) between 3.1 ± 1.3 × 10^7 and 6.3 ± 3.3 × 10^7 cfu g^{-1} d. wt., respectively. Bacterial population densities of aerobic heterotrophic bacteria at Suncheon bay were shown the highest values among 4 sampling stations. When comparing the population densities of aerobic heterotrophic bacteria on the roots of *Suaeda japonica* with those of other halophytes investigated by Park (2005), they showed lower values than those on *Suaeda maritima* and *Salicornia herbacea* during sampling periods, which indicated rhizosphere microbial communities were mainly determined by root exudates according to plant species (Ibekwe and Kennedy 1998, Marshner et al. 2001, Miethling et al. 2000).

Bacterial population densities of anaerobic heterotrophic bacteria on the roots of *Suaeda japonica* range from 2.8 ± 1.3 × 10^4 to 1.8 ± 0.7 × 10^7 cfu g^{-1} d. wt. during sampling periods at 4 stations (Fig. 2b). Population densities by sampling stations were 3.2 ± 0.7 × 10^4 ~ 4.1 ± 2.0 × 10^4 cfu g^{-1} d. wt. at Daebu-do (St. 1), 1.4 ± 0.7 × 10^5 ~ 1.6 ± 0.4 × 10^5 cfu g^{-1} d. wt. at Mankyung (St. 2), 2.8 ± 1.3 × 10^4 ~ 6.0 ± 4.5 × 10^4 cfu g^{-1} d. wt. at Goheung bay (St. 3), and 1.2 ± 0.3 × 10^6 ~ 1.2 ± 0.7 × 10^7 cfu g^{-1} d. wt. at Suncheon bay (St. 4), respectively (Fig. 3). Comparing the population densities by sampling stations, those at Suncheon bay showed the maxima during sampling periods, which indicated that the soil environment at St. 4 was more anaerobic condition than other sampling stations.

The physiological groups of heterotrophic bacteria on the roots of *Suaeda japonica* ranged from 4.4 ± 0.6 × 10^6 to 2.5 ± 1.2 × 10^7 cfu g^{-1} d. wt. for amylolytic bacteria, from 8.5 ± 6.0 × 10^4 to 2.3 ± 1.6 × 10^6 cfu g^{-1} d. wt. for cellulolytic bacteria, and from 3.8 ± 1.8 × 10^5 to 4.2 ± 2.9 × 10^6 cfu g^{-1} d. wt. for proteolytic bacteria, respectively (Fig. 3). Amylolytic and proteolytic bacteria at St. 3 showed maxima, while cellulolytic bacteria at St. 4 showed maxima during sampling periods. According to the results of measured population densities of physiological groups of heterotrophic bacteria on the roots of *Suaeda japonica*, population densities of amylolytic bacteria showed higher values than those of proteolytic and cellu-

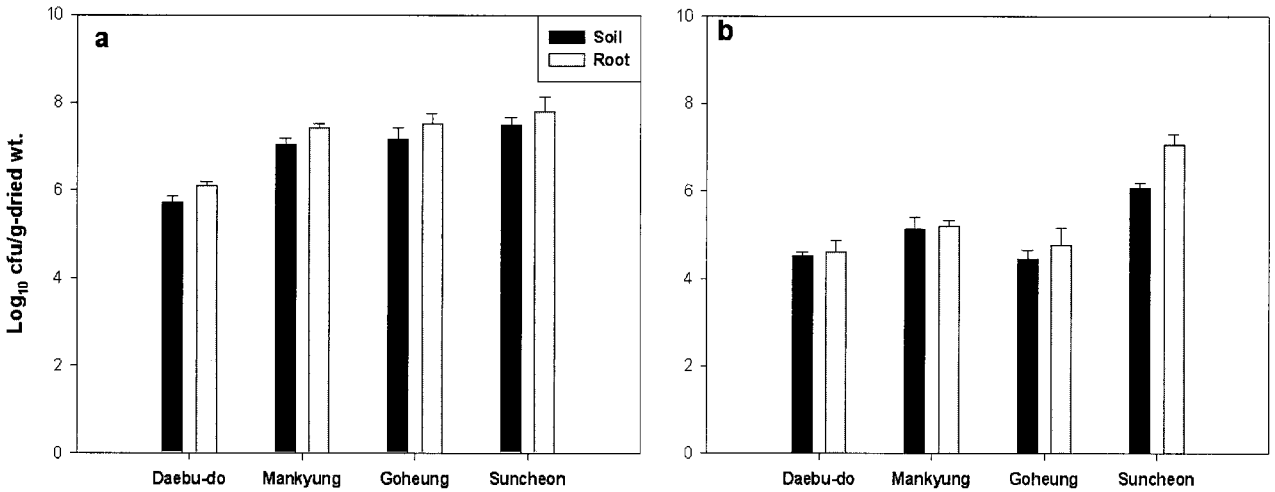

Fig. 2. Population densities of (a) aerobic and (b) anaerobic heterotrophic bacteria in the rhizosphere soil and soil remote from roots of halophyte *Suaeda japonica* sampled from western and southern mudflats of Korea during July and October, 2003.

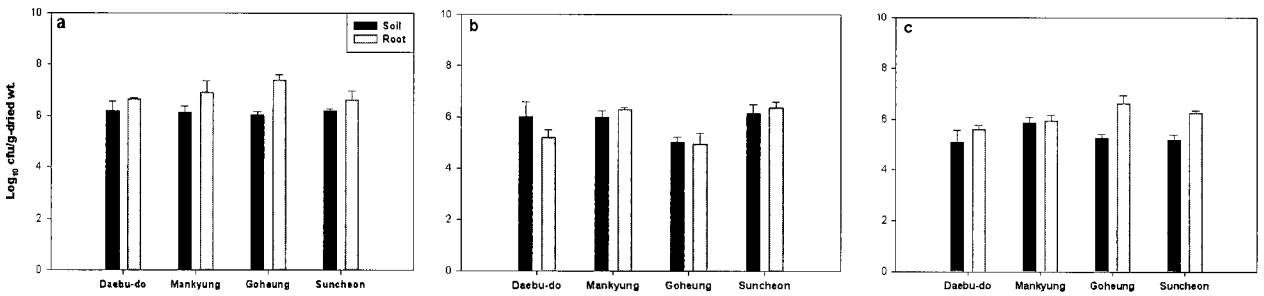

Fig. 3. Population densities of (a) amylolytic heterotrophic bacteria, (b) cellulolytic heterotrophic, and (c) proteolytic bacteria in the rhizosphere soil and soil remote from roots of halophyte *Suaeda japonica* sampled from western and southern mudflats of Korea during July and October, 2003.

lolytic bacteria, which indicated that plant species (Miethling et al. 2000), plant age-dependent effects (Gomes et al. 2001, von der Weid et al. 2000), and the materials released by plant into the soil (Atlas and Bartha 1992) had a direct influence on the composition and density of the soil microbial community. And root exudation is an important ecological phenomenon which manipulates the plant and root microbial succession (Singh 2006).

Plant Root Effects on Microbial Population

The rhizosphere is a soil ecological region where soil is subjected to specific influenced by plant root due to the exudates from root cells and sloughing of tissue (Curl and Truelove 1986, Giddens and Todd 1984, Harley and Russell 1968). The population and functional dynamics of soil microorganisms differ from rhizosphere to non-rhizosphere zone due to the rhizosphere effect (Johnson et al. 1959). The metabolic state of the plant and the nature of soil appear to influence the extent of the rhizosphere effect (Rovira 1991). Factors such as soil type, soil moisture, pH, temperature, plant age, relative humidity and several other factors are known to influence the rhizosphere effect. The rhizosphere effect can be seen by looking at the ratio of the number of microorganisms in the rhizosphere soil (R) to the number of corresponding microorganisms in soil remote from roots (S) (Katznelson 1946, Timonin 1966). Activities of rhizosphere bacteria is related to the types and amounts of root exudates/ rhizodeposition.

The R/S ratios on the roots of *Suaeda japonica* were measured-from 2.33 to 2.39 by sampling stations (Table 1). Comparing R/S values of *Suaeda japonica* with those of other halophytes measured by Park (2005), similar R/S values were measured. Generally R/S ratios range from 5 to 20, but it is common to find an R/S ratio of 100 (Gray and Parkinson 1968, Woldendorp 1978). However, the R/S ratios on the roots of *Suaeda japonica* of sampledmudflat area showed lower R/S values than those of ordinary terrestrial environments. It suggests that, apart from halophyte specificity, mudflat environments condition played an important role for deciding R/S values for the rhizosphere bacteria to grow.

Table 1. The R/S ratios of halophyte *Suaeda japonica* sampled from western and southern mudflats of Korea by looking at the ratio of the number of microorganisms in the rhizosphere soil (R) to the number of corresponding microorganisms in soil remote from roots (S)

Halophyte / Site	*Suaeda japonica* (R/S Ratio)
Daebu-do	2.39
Mankyung River	2.35
Goheung bay	2.36
Suncheon bay	2.33

Sequencing and Phylogenetic Analysis

Bacterial 16S rDNA clones were characterized by partial sequencing and phylogenetic analysis. There were eleven total clones. As a result of the identification of eleven isolates from the roots of *Suaeda japonica* from Goheung bay by molecular methods, five clones such as *Pseudomonas* sp. (GC06, GC07) and *Marinobacter* sp. (GC10-1, GC10-2, GC11) were determined to belonging to γ -Proteobacteria group and six clones such as *Sulfitobacter* (GC01, GC02), *Mesorhizobium* sp. (GC08, GC15), and *Sphingomonas* sp. (GC13, GC14) belonging to α-Proteobacteria group were also identified (Fig. 4). Whereas among four isolates from Suncheon bay,

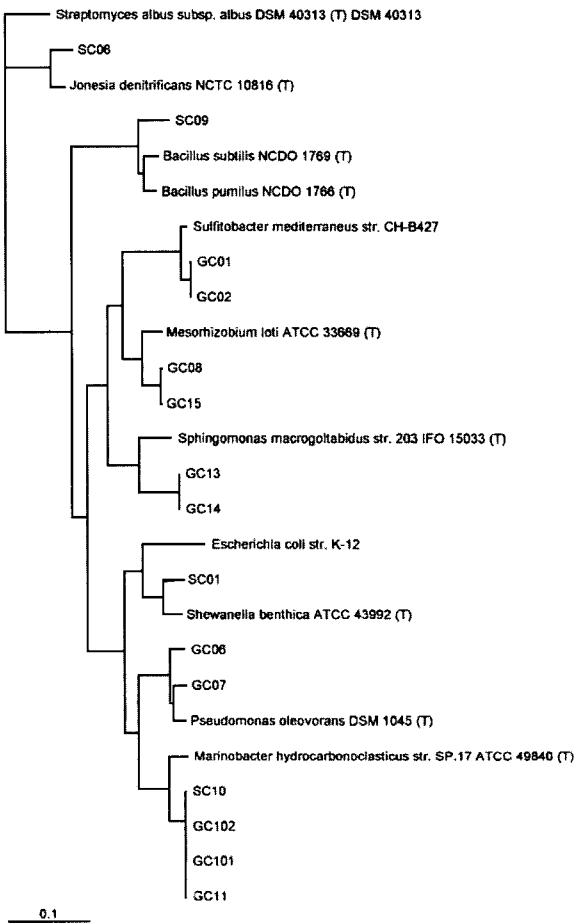

Fig. 4. Phylogenetic tree showing the affiliation of 16S rDNA sequences to selected reference sequence of the heterotrophic bacteria on the rhizosphere of *Suaeda japonica*. The tree was constructed from a distance matrix by the neighbour-joining analysis. The bar represents 10% estimated difference in nucleotide sequences per 16S rDNA position.

Table 2. List of 16S rDNA genes for heterotrophic bacteria isolated from root of *Suaeda japonica*

Halophyte	St.	Serial No.	Accession No.	Closet Genebank library and accession No.	Similarity (%)
Suaeda japonica	Goheung bay	GC01	AY690670	*Sulfitobacter mediterraneus* Y17387	1343/1371 (97%)
		GC02	AY690671	*Sulfitobacter mediterraneus* Y17387	1364/1392 (97%)
		GC06	AY690672	*Pseudomonas alcalophila* AJ550466	1402/1431 (97%)
		GC07	AY690673	*Pseudomonas pseudoalcaligenes* Z76666	1403/1432 (97%)
		GC08	AY690674	*Mesorhizobium* sp. AY258096	1373/1378 (99%)
		GC10-1	AY690675	*Marinobacter aquaeolei* AF173969	1430/1450 (98%)
		GC10-2	AY690676	*Marinobacter flavimaris* AY517632	1447/1468 (98%)
		GC11	AY690677	*Marinobacter aquaeolei* AF173969	1430/1450 (98%)
		GC13	AY690678	*Marinobacter aquaeolei* AF173969	1427/1427 (100%)
		GC14	AY690679	*Sphingomonas flavimaris* AY554010	1405/1405 (100%)
		GC15	AY690680	*Mesorhizobium* sp. AY258096	1373/1378 (99%)
	Suncheon bay	SC01	AY690681	*Shewanella marisflavi* AY485224	1486/1487 (99%)
		SC06	AY690682	*Jonesia qinghaiensis* AJ626896	1036/1045 (99%)
		SC09	AY690683	*Bacillus aquaemaris* AF483625	1451/1464 (99%)
		SC10	AY690684	*Marinobacter aquaeolei* AF173969	1448/1469 (98%)

two clones (SC01, SC10) were determined to belonging to γ-Proteobacteria group, and another two clones (SC06, SC09) belonging to *Actinobacteria* and Bacilli group, respectively, were found (Table 2). According to Gray and Herwing (1996), γ-Proteobacteira group were dominated in the marine sediment. In our study, most identified sequences had < 97% sequence similarity to previously cultivated microorganisms and phylogenetic analyses of isolated bacteria from the roots of *Suaeda japonica* were revealed that the major group of bacteria detected in this study were closely related to γ-Proteobacteria and Bacilli group, and α-Proteobacteria and *Actinobacteria* group were also found.

Recently, the rapid development of molecular tools for the analysis of bacterial communities has greatly enhanced the sensitivity and potential of microbial ecology. However, despite the large amount of data concerning the molecular identification of bacteria in the environment, understanding the function of microbial communities is still a major objective in microbial ecology (Wellington et al. 2003). In the present study, although we can not understand the exact function of rhizosphere bacteria isolated in this study, we found higher bacterial densities on rhizosphere than in soils remote from rhizosphere, and halophyte species and mudflat environments affected the rhizosphere bacterial communities. In order to understand the rhizosphere bacteria and mudflat environments, further studies are needed. However, It is expected that the results in this study will contribute to understand the rhizosphere environment of halophytes, and provide a framework for future molecular ecological study for the restoration of halophytes on the mudflat area in Korea.

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