ACCELERATED PHENOL DEGRADATION BY BACTERIAL COMMUNITY IN THE RHIZOSPHERE OF *SPIRODELA POLLYRHIZA*

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ABSTRACT

The rhizosphere effect of *Spirodela polyrrhiza* to accelerate the biodegradation of phenol was investigated to show the universality and to elucidate the mechanism focusing on the selective accumulation of phenol-degrading bacteria in its rhizosphere. Phenol degradation tests were performed using 6 environmental water samples with/without *S. polyrrhiza*. During the phenol degradation tests, behaviour of bacterial community was monitored qualitatively and quantitatively by DNA-mediated methods. The phenolic compounds in the root exudates of *S. polyrrhiza* were also determined. Accelerated biodegradation of phenol was confirmed in all the 6 environmental water samples with both non-sterile and sterile *S. polyrrhiza*. *S. polyrrhiza* selectively accumulated aromatic compounds degrading bacteria in its rhizosphere, although taxonomic bacterial compositions depended on the source of the environmental water samples. *S. polyrrhiza* released phenolic compounds into its rhizosphere fraction as root exudates at considerably high specific release rate of 1,520 mg-TOC d^{-1} g^{-1} wet root and 214 mg-phenolic compounds d^{-1} g^{-1} wet root. Thus the rhizosphere effect of *S. polyrrhiza* to accelerate the biodegradation of phenol was confirmed as a universal property. *S. polyrrhiza* secreted root exudates which were rich in phenolic compounds into its rhizosphere. This ability of *S. polyrrhiza* could selectively recruit bacterial populations which possess catechol 1,2- or 2,3- dioxygenase genes and resulted in the accelerated biodegradation of phenol. *S. polyrrhiza* and bacteria in its rhizosphere can be very useful device for the enrichment of bacteria which can degrade organic pollutants, especially phenolic compounds in the aquatic environment.

KEYWORDS

Spirodela polyrrhiza, rhizosphere, accelerated biodegradation of phenol, selective accumulation of phenol degrading bacteria, root exudates

INTRODUCTION

Phytoremediation, which is the use of plants to cleanup the polluted environments, has been mainly applied to remove radionuclides (Zhu and Smolders, 2000) or heavy metals (Salt et al., 1995) in soil environments and to remove nitrogen or phosphorus (Tripathi et al., 1991) in water environments using accumulation and transformation ability of plants themselves for the last several decades. Recently, accelerated degradation of organic chemicals including recalcitrant xenobiotics such as petroleum hydrocarbons (Radwan et al., 1995; Siciliano et al., 2003), chlorinated solvents (Walton and Anderson, 1990; Binet et al., 2000), polycyclic aromatic hydrocarbons (Miya and Firestone, 2001), and pesticides (Singh et al., 2004) has been reported in rhizosphere or rhizoplane of terrestrial plants by the "rhizosphere effect" of plants to transport oxygen and to secrete exudates

physiologically active substances (Shaw and Burns, 2003). Moreover, it is reported that plants could recruit specific microbes responsible for the degradation of herbicides (Shaw and Burns, 2005) and petroleum hydrocarbons (Siciliano et al., 2001; Siciliano et al., 2003) into the rhizosphere and root interior.

On the other hand, there is only one report on the accelerated mineralization of surfactants, linear alkylbenzene sulfonate (LAS), linear alcohol ethoxylate (LAE), and mixed amino acid (MAA) by microbiota of aquatic plants (Federle and Schwab, 1989), although this report showed only the fact that the accelerated mineralization occurred and the mechanisms who contributed in what way to what extent were obscure. Recently, we have reported accelerated biodegradation of synthetic surfactants (Mori et al., 2005) and aromatic compounds (Toyama et al., 2006) in the rhizosphere of *Spirodela polyrrhiza* by the rhizosphere effect such as oxygen supply and selective recruitment or stimulation of aromatic compounds degrading bacteria in the rhizosphere. It was an interesting observation that phenol-degrading bacteria were highly accumulated in the rhizosphere fraction of *S. polyrrhiza* even it was unexposed to phenol (Toyama et al., 2006). Verification of the universality of the rhizosphere effect of *S. polyrrhiza* will motivate the use of the aquatic plant-bacterial associations for the purification of aquatic environment contaminated with organic compounds. Elucidation of the mechanism of *S. polyrrhiza* to selectively recruit phenol degrading-bacteria in its rhizosphere will provide rational strategy for making better use of the rhizosphere effect of aquatic plants for the preservation of aquatic environment.

In the present study, we tried to show the universality of the rhizosphere effect of *S. polyrrhiza* and to elucidate the mechanism of the rhizosphere effect of *S. polyrrhiza* especially focusing on the selective accumulation of phenol-degrading bacteria in its rhizosphere. Phenol degradation tests were performed using 6 environmental water samples with/without *S. polyrrhiza*. During the phenol degradation tests, bacterial community structure and behaviour of total bacteria was monitored by DNA-mediated methods. The phenolic compounds in the root exudates of *S. polyrrhiza* were also determined.

MATERIALS AND METHODS

Spirodela polyrrhiza

The sterile *S. polyrrhiza* was maintained in 300 mL Erlenmeyer flasks containing 200 mL of sterile modified Hoagland nutrient solution until used for experiments (Toyama et al., 2006). They were statically grown in an incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition).

Environmental water samples

Six different environmental water samples were collected from 2 lakes (Lake Biwa [LB] and Lake Amagase-dam [LA]), 2 rivers (Katura River [KR] and Yodo River [YR]), and 2 ponds (Inukai Pond [IP] and Zuion Pond [ZP]) located in Osaka, Kyoto or Shiga Prefecture, Japan. The water samples were filtered (Isopore Membrane Filter, pore size 10.0 μm, Millipore, Tokyo, Japan). These water samples were stored at 4 °C until used for experiments.

Design of phenol degradation test

Three test systems were constructed for each environmental water sample. The test system A was made up of environmental water samples with 20 fronds of non-sterile *S. polyrrhiza* which had been acclimated to each environmental water sample for 7 days before used for experiment. The test system B was made up of environmental water samples with 20 fronds of sterile *S. polyrrhiza*. The test system C was made up of only environmental water samples as a control against test systems A and B. These test systems were constructed using 200 mL of each environmental water sample containing phenol at a final concentration of 10 mg L^{-1} in 300 mL Erlenmeyer flask in triplicate. All the test systems were statically incubated in the incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition) for 3 days. During the phenol degradation test, the phenol concentrations and the bacterial populations were monitored

periodically. The phenol concentration was measured by high-performance liquid chromatography (HPLC) as previously described (Toyama et al., 2006).

Bacterial community structure analysis

To investigate the ability of *S. polyrrhiza* to recruit specific bacteria in its rhizosphere, terminal-restriction fragment length polymorphism (T-RFLP) was performed. Twenty fronds of sterile *S. polyrrhiza* were cultured in 200 mL of 6 different environmental water samples in 300 mL Erlenmeyer flasks. They were statically incubated in the incubation chamber at 28 ± 1 °C under fluorescent lamp at 8,000 lux (16 h of light and 8 h of dark condition) for 7 days. Microbes were collected from bulk water and rhizosphere fractions of *S. polyrrhiza* separately as previously described (Toyama et al., 2006). DNA was extracted from each sample by proteinase K method (Sei et al., 2000) and purified using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). PCR was conducted using a eubacterial universal primer 27F labeled at the 5'-end with 6-carboxyfluorescein (6-FAM) and 1392R (Amann et al., 1995) with condition described elsewhere (Sei et al., 2004). The PCR products were purified using Microcon PCR (Qiagen) and digested with *Hha*I at 37 °C for 5 h. The resulting product was analyzed by electrophoresis using an ABI PRISM 310 Genetic analyzer (Applied Biosystems) following the manufacturer's instruction. The size and the fluorescence intensity of each T-RF were automatically calculated by the GeneScan Analysis Software (version 3.7, Applied Biosystems). Bacterial community similarities were analyzed by cluster analysis with unweighted pair groups with mathematical averages (UPGMA) of Dice coefficient of similarity (S_D) and principal component analysis (PCA) using NTSYS-pc 2.1 software (Exeter software, NY, USA).

Enumeration of 16S rDNA, C12O and C23O DNA

The numbers of 16S rDNA, C12O and C23O DNA in each bacterial community were measured by MPN-PCR as described by Sei et al. (2004). The numbers of DNA were determined as MPN-DNA copies per milliliter for bulk water fraction and MPN-DNA copies per gram of wet root for rhizosphere fraction, respectively. The relative abundance of C12O and C23O DNA were expressed as the ratios of them to 16S rDNA. The rhizosphere effect of *S. polyrrhiza* during phenol degradation test was quantified as previously described (Anderson et al., 1993, Shaw and Burns, 2003), and was defined as RE value. The RE value shows the ratios of the numbers of 16S rDNA, C12O or C23O DNA in test system B to those in test system C. Thus the RE value indicates the effect of *S. polyrrhiza* to accumulate total and C12O/C23O genes possessing bacteria in the test system.

Analysis of root exudates of *S. polyrrhiza*

The root exudates of *S. polyrrhiza* were collected from triplicate sterile cultures. Twenty fronds of sterile *S. polyrrhiza* were gently shaken for 10 min using rotary shaker (120 rpm) 3 times in 200 mL of sterile MilliQ water to flush initial root exudates from root surface. Then they were transferred to a 300 mL Erlenmeyer flask containing 200 mL of sterile MilliQ water and statically incubated in the incubation chamber for 1 day. The root exudate of *S. polyrrhiza* in bulk water and rhizosphere fractions was collected and analyzed separately. TOC was measured using TOC analyzer (TOC-5000A, Shimadzu, Kyoto). Total phenolic compounds was measured by 4-aminoantipyrine method (APHA, 1998). The results were shown as phenol equivalent using calibration curve constructed for standard phenol solution. The ability of *S. polyrrhiza* to secrete phenolic compounds was showed as milligrams of TOC or phenol per gram of wet root per day (mg-C or mg-phenolics d^{-1} g^{-1} wet root).

Data analysis

All results were indicated as the mean values with standard deviations (± 95% confidence interval) of triplicate experiments. Significant difference was determined by Student's t test with $p < 0.05$.

RESULTS AND DISCUSSION

Phenol degradation in various environmental waters with/without *S. polyrrhiza*

Phenol degradation tests were performed in 6 environmental water samples with/without *S. polyrrhiza* (Fig. 1). Phenol degradation profiles showed almost the same tendency regardless of the

difference of the environment. Phenol degradation rate was almost the same among test systems A, B and C, and amended phenol (10 mg/L) was completely removed in all the test systems. However the lag period was completely different among the test systems: approximately 6–15 h, 24 h and 40–48 h in test systems A, B and C, respectively. Thus the accelerated phenol degradation was confirmed in all environmental waters with both sterile and non-sterile *S. polyrrhiza* (Fig. 1). The lag period for the phenol degradation could be shortened with *S. polyrrhiza* although it took a little longer time when sterile *S. polyrrhiza* was used probably due to the period for the accumulation of phenol-degrading bacteria in its rhizosphere.

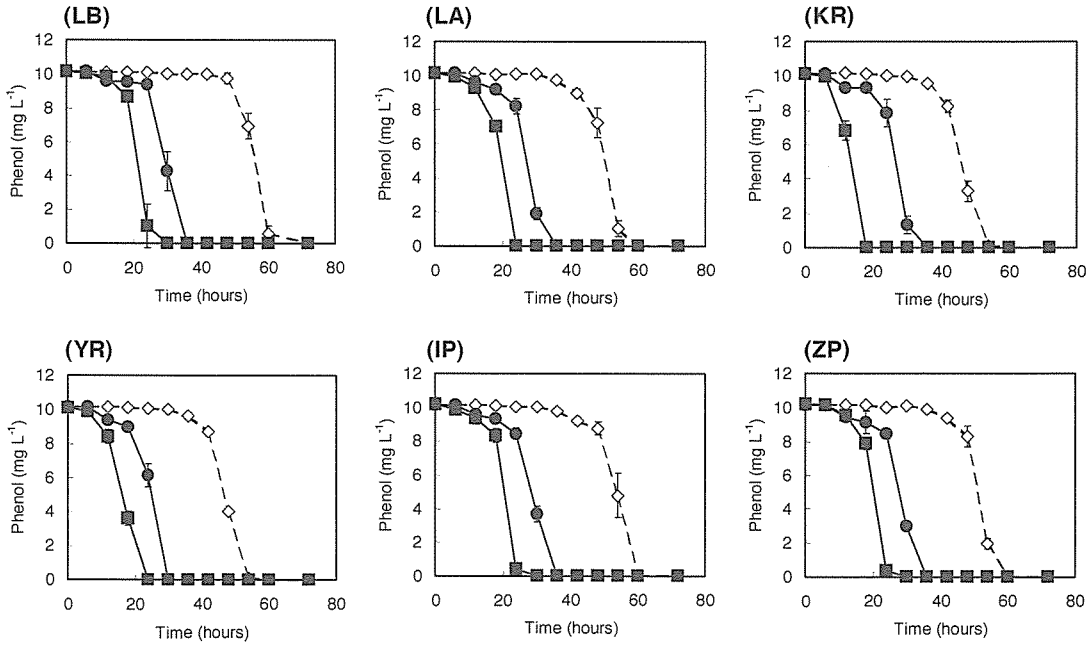

Fig. 1. Degradation profiles of phenol in 6 environmental water microcosms with non-sterile *S. polyrrhiza* (test A, closed squares), sterile *S. polyrrhiza* (test B, closed circles), and without *S. polyrrhiza* (test C, open diamonds). Error bars represent 95% confidence intervals (n=3).

Comparison of the bacterial communities in original environmental water and in rhizosphere fraction of *S. polyrrhiza*

Bacterial communities in the rhizosphere fractions of *S. polyrrhiza* were compared with those in the original environmental water. The 16S rDNA-based T-RFLP analysis was performed against the bacterial community in the original environmental water and the rhizosphere fractions of *S. polyrrhiza* acclimated to each environmental water sample for 7 days. T-RFs patterns in the rhizosphere fractions were significantly different from those in the corresponding original environmental water. The dominant T-RFs in the rhizosphere fractions were different among the environmental water samples (Fig. 2). The dendrogram representing the similarities of the T-RFs patterns among the samples showed the two main clusters drawing a clear distinction between the samples from the environmental water and the rhizosphere fractions (Fig. 3A). PCA score plots again showed the 2 core groups which were clearly distinctive between the environmental water and the rhizosphere fractions by first component axis (PC1). The second component axis (PC2) might represent the difference of the environmental water samples (Fig. 3B). It was an interesting result that the bacterial communities in the rhizosphere fractions of *S. polyrrhiza* differed depending on the source of the environmental water samples. The numbers of C12O and C23O DNA in the test system were almost the same or even higher in the rhizosphere fraction than in bulk water fraction at the beginning of the experiment. It was another interesting point that although taxonomic

bacterial compositions in the rhizosphere of *S. polyrrhiza* varied depending on the environmental waters, selective accumulation of bacteria which possess C12O and C23O DNA was commonly observed regardless of the difference of the origin.

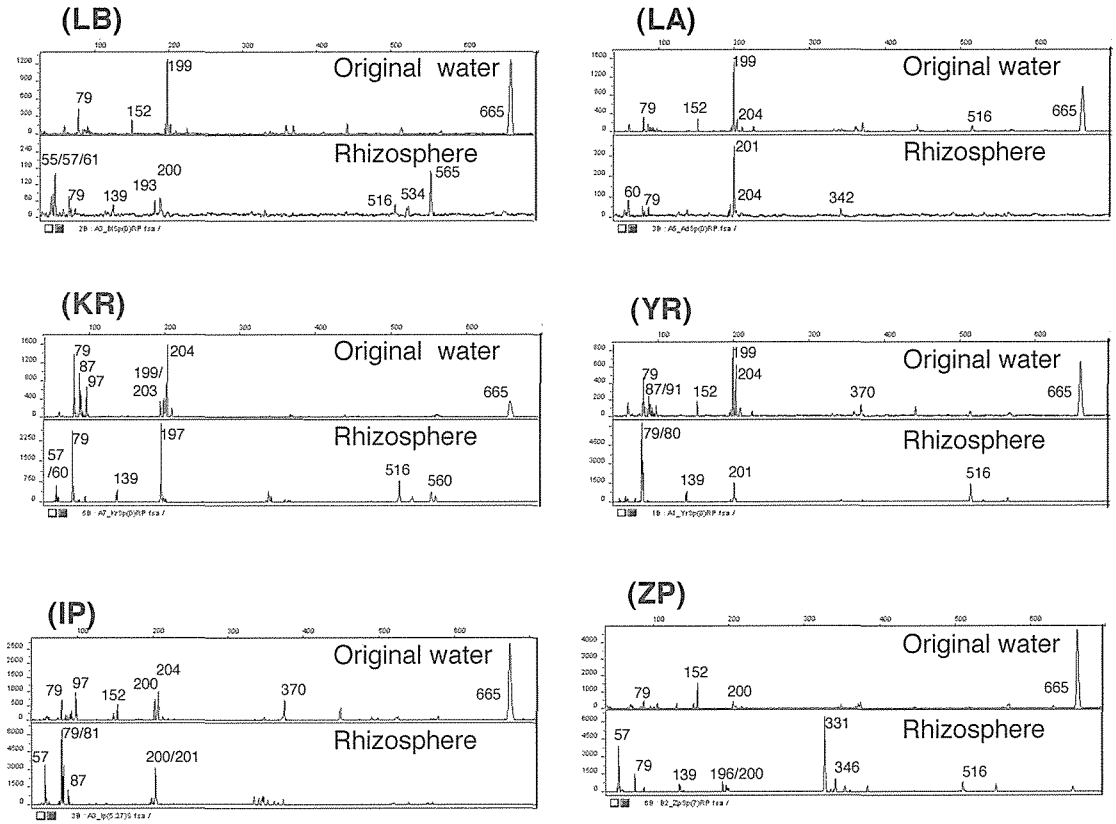

Fig. 2. The 16S rDNA-based T-RFLP patterns of bacterial community in bulk water and in rhizosphere fractions from 6 original environmental water samples with *S. polyrrhiza*. LB: Lake Biwa, LA: Lake Amagase, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond.

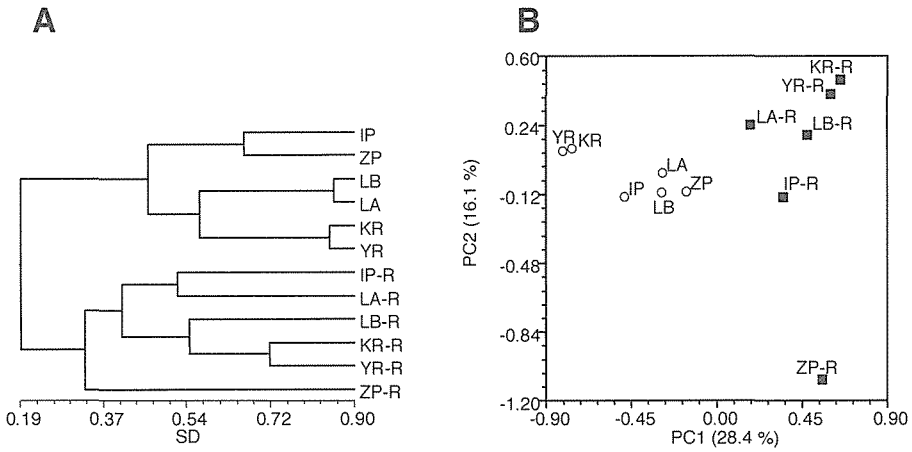

Fig. 3. Cluster analysis (A) and principal component analysis (B) of the microbial community in bulk water and in rhizosphere fractions from 6 original environmental water samples with *S. polyrrhiza*. LB: Lake Biwa, LA: Lake Amagase, KR: Katsura River, YR: Yodo River, IP: Inukai Pond and ZP: Zuion Pond, and corresponding rhizosphre fractions (–R).

Changes in the numbers of 16S rDNA, C12O and C23O DNA in the test systems

Before the phenol degradation test, the relative abundances of C12O and C23O DNA (C12O/16S rDNA and C23O/16S rDNA) in the rhizosphere fractions were 1.0×10^{1}–9.6×10^{3} and 1.4×10^{2}–1.7×10^{4} times higher than those in corresponding environmental water samples, respectively (Table 1).

During 3 days experiments, the numbers of 16S rDNA in bulk water fraction in test system A, remained relatively stable in all the 6 environmental water samples. Those in rhizosphere fraction significantly increased from 1.2×10^{5}–1.2×10^{7} MPN-DNA copies/test system to almost the same level in bulk water fraction, 1.8×10^{7}–1.8×10^{8} MPN-DNA copies/test system. The numbers of C12O and C23O DNA, which were almost the same or even higher in rhizosphere fraction at the beginning of the experiments, increased significantly in both bulk water and rhizosphere fractions within 3 days. It is remarkable that even in test system B, where the sterile *S. polyrrhiza* was used, bacteria were immediately accumulated in rhizosphere fraction. It is another remarkable point that the numbers of C23O DNA were always higher than those of C12O DNA in all the 6 environmental samples.

The relative abundance of C12O and C23O DNA on days 0 and 3 are summarized in Table 1. In bulk water fraction, the relative abundance of C12O and C23O DNA increased from 0–2.3×10^{-5} to 2.1×10^{-6}–2.1×10^{-4} and from 1.7×10^{-6}–2.3×10^{-3} to 5.8×10^{-5}–1.0×10^{-1} during phenol degradation, respectively. In rhizosphere fraction in test system A, the relative abundances of C12O and C23O DNA was 9.1×10^{0}–1.1×10^{2} and 4.1×10^{1}–2.2×10^{3} times higher, respectively, than those in bulk water fraction. In test system B, the relative abundances of C12O and C23O DNA in rhizosphere fraction on day 3 were 1.0×10^{-4}–7.8×10^{-3} and 1.1×10^{-1}–1.0×10^{0}, respectively. These were significantly higher than those in bulk water fraction and the same level as rhizosphere fraction in test system A.

TABLE 1. Relative abundance of C12O DNA and C23O DNA before and after phenol degradation tests

Microcosm	Relative abundance of C12O DNA [a]				Relative abundance of C23O DNA [a]			
	Bulk water		Rhizosphere		Bulk water		Rhizosphere	
	day 0	day 3	day 0	day 3	day 0	day 3	day 0	day 3
LB								
A	0	9.5×10^{-6}	3.2×10^{-4}	1.0×10^{-3}	2.3×10^{-4}	9.5×10^{-3}	3.2×10^{-2}	3.9×10^{-1}
B	0	5.1×10^{-5}	–	3.9×10^{-4}	2.3×10^{-4}	5.1×10^{-3}	–	3.9×10^{-1}
C	0	2.3×10^{-6}	–	–	2.3×10^{-4}	2.3×10^{-3}	–	–
LA								
A	2.7×10^{-7}	1.0×10^{-5}	2.6×10^{-3}	2.6×10^{-4}	1.5×10^{-5}	1.0×10^{-3}	2.5×10^{-1}	4.8×10^{-1}
B	2.7×10^{-7}	2.6×10^{-5}	–	1.0×10^{-4}	1.5×10^{-5}	1.0×10^{-3}	–	4.8×10^{-1}
C	2.7×10^{-7}	3.5×10^{-6}	–	–	1.5×10^{-5}	3.5×10^{-3}	–	–
KR								
A	2.3×10^{-6}	5.8×10^{-5}	5.8×10^{-4}	5.3×10^{-4}	1.1×10^{-4}	1.1×10^{-2}	5.6×10^{-1}	9.3×10^{-1}
B	2.3×10^{-6}	5.9×10^{-5}	–	2.3×10^{-4}	1.1×10^{-4}	5.9×10^{-3}	–	1.0×10^{0}
C	2.3×10^{-6}	2.1×10^{-6}	–	–	1.1×10^{-4}	2.1×10^{-2}	–	–
YR								
A	2.3×10^{-5}	5.3×10^{-5}	5.8×10^{-4}	2.3×10^{-4}	2.3×10^{-3}	3.5×10^{-3}	7.2×10^{-1}	2.3×10^{-1}
B	2.3×10^{-5}	1.1×10^{-5}	–	3.4×10^{-4}	2.3×10^{-3}	1.1×10^{-2}	–	8.6×10^{-1}
C	2.3×10^{-5}	1.7×10^{-5}	–	–	2.3×10^{-3}	1.0×10^{-1}	–	–
IP								
A	0	5.8×10^{-5}	1.3×10^{-4}	6.1×10^{-4}	5.8×10^{-4}	5.8×10^{-5}	2.1×10^{-1}	1.3×10^{-1}
B	0	2.3×10^{-5}	–	1.0×10^{-4}	5.8×10^{-4}	1.1×10^{-3}	–	1.1×10^{-1}
C	0	1.7×10^{-5}	–	–	5.8×10^{-4}	1.7×10^{-2}	–	–
ZP								
A	1.0×10^{-5}	2.1×10^{-4}	1.0×10^{-4}	1.7×10^{-2}	1.7×10^{-6}	1.0×10^{-4}	1.0×10^{-3}	7.8×10^{-2}
B	1.0×10^{-5}	1.7×10^{-4}	–	7.8×10^{-3}	1.7×10^{-6}	1.7×10^{-4}	–	2.6×10^{-1}
C	1.0×10^{-5}	1.0×10^{-4}	–	–	1.7×10^{-6}	5.3×10^{-4}	–	–

[a] Results are shown as MPN–C12O or –C23O DNA copies / MPN-16S rDNA copies.

The RE values for 16S rDNA, C12O and C23O DNA were 1.4–9.4, 12–98 and 13–144, respectively. These results indicated that the rhizosphere effect of *S. polyrrhiza* selectively activated bacteria possessing C12O and C23O DNA, especially those possessing C23O DNA (Table 2).

Estimation of root exudates released by *S. polyrrhiza*

Accelerated biodegradation of the chemicals and accumulation of bacteria in the rhizosphere should depend on the amount of root exudates, especially on phenolic compounds in the root exudates

TABLE 2. Rhizosphere effects on 16S rDNA, C12O and C23O DNA during phenol degradation test

Microcosm	Rhizosphere effect The rhizosphere-to-nonrhizosphere ratio (R:S) [a]		
	16S rDNA	C12O DNA	C23O DNA
LB	1.4	76	53
LA	3.1	45	144
KR	2.1	98	23
YR	59	32	13
IP	9.4	36	30
ZP	2.1	12	51

[a], Results are shown as ratio of the numbers of MPN-DNA copy in test B to those in test C.

(Shaw and Burns, 2003; Kamath et al., 2004). Thus the root exudate released by sterile *S. polyrrhiza* into bulk water and rhizosphere fractions was analyzed (Table 3). Specific release rates of TOC in bulk water and rhizosphere fractions were 1,270 ± 110 and 250 ± 75 mg d^{-1} g^{-1} wet root, respectively while those of phenolic compounds in bulk water and rhizosphere fractions were 16.8 ± 2.8 and 197 ± 29 mg d^{-1} g^{-1} wet root, respectively. The amount of organic carbon and phenolic compounds released by terrestrial plants are generally estimated to be 0.4–27.7 mg-TOC and 0.24–8.5 mg-phenolic compounds g^{-1} wet root (salicylate equivalent), respectively (Kamath et al., 2004). The concentration of phenolic compounds in root exudates of *S. polyrrhiza* was approximately same level as that of previously reported terrestrial plants (Kamath et al., 2004). The results indicated that *S. polyrrhiza* has the great ability to release root exudates which were rich in phenolic compounds. This seemed to stimulate the bacteria in the rhizosphere for the biodegradation of aromatic compounds. In addition, the phenolic compounds on the root surface was significantly higher than those in bulk water fraction. This indicated that phenolic compounds released by *S. polyrrhiza* are hard to be diffused into bulk water and tended to remain on the root surface. This should cause the selective enrichment of bacteria possessing C12O/C23O DNA on the root surface and the accelerated biodegradation of phenol in the rhizosphere.

TABLE 3. Characteristics of root exudates released by sterile *S. polyrrhiza* into the rhizosphere water and on its root surfaces

	Distribution of released root exudates (mg d^{-1} g^{-1} of wet root) [a, b]		Whole root exudates releasing ability (mg d^{-1} g^{-1} of wet root) [b, c]
	Rhizosphere water fraction	Root surface fraction	
TOC	1270 ± 110	250 ± 75	1520
Total phenolics [d]	16.8 ± 2.82	197 ± 29	214

[a] Results are shown as mean ±95% confidence interval (n=3).
[b] Results are shown as mg carbon or phenolics per g of wet root per day (mg d^{-1} g^{-1} of wet root, specific release rate).
[c] Whole exudates releasing ability is shown as rhizosphere water fraction + root surface fraction.
[d] Total phenolics are shown as mg of phenol equivalent

CONCLUSIONS

The rhizosphere effect of *S. polyrrhiza* to accelerate the biodegradation of phenol was confirmed as an universal property. *S. polyrrhiza* secreted root exudates which were rich in phenolic compounds into its rhizosphere. This ability of *S. polyrrhiza* could selectively recruit bacterial populations which possess C12O/C23O DNA and resulted in the accelerated biodegradation of phenol. Thus *S. polyrrhiza* and bacteria in its rhizosphre can be very useful device for the enrichment of bacteria which can degrade organic pollutants, especially phenolic compounds in the water environment.

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