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Sustainable Biodegradation of Phenol by *Acinetobacter calcoaceticus* P23 Isolated from the Rhizosphere of Duckweed *Lemna aoukikusa*

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Phenol-degrading bacteria were isolated from the rhizosphere of duckweed (*Lemna aoukikusa*) using an enrichment culture method. One of the isolates, P23, exhibited an excellent ability to degrade phenol and attach to a solid surface under laboratory conditions. Phylogenetic analysis revealed that P23 belongs to the genera *Acinetobacter* and has the highest similarity to *Acinetobacter calcoaceticus*. P23 rapidly colonized on the surface of sterilized duckweed roots and formed biofilms, indicating that the conditions provided by the root system of duckweed are favorable to P23. A long-term performance test (160 h) showed that continuous removal of phenol can be attributed to the beneficial symbiotic interaction between duckweed and P23. P23 is the first growth-promoting bacterium identified from *Lemna aoukikusa*. The results in this study suggest the potential usefulness of dominating a particular bacterium in the rhizosphere of duckweeds to achieve efficient and sustainable bioremediation of polluted water.

1. Introduction

Bioremediation is an economically feasible and environmentally friendly technology to remove pollutants from the environment using species from bacteria to plants (*1*, *2*). There is considerable interest in exploring more effective and elegant techniques in this field (*2*). One of the bottlenecks for successful bioremediation is the low survival rates of the microbes and the swift attenuation of their activities, which may be due to competition with other indigenous living organisms or to nutritionally adverse conditions at the contamination sites (*3*). Adding nutrients (such as nitrogen and phosphorus compounds) to overcome nutrient deficiencies at the sites does, however, carry a risk of secondary pollution because of the subsequent growth of undesirable bacteria.

Rhizoremediation is expected to make bioremediation a more effective and durable technology (*4*). Rhizoremediation is one of the bioremediation - utilizing microbial activities in the rhizosphere, which is the area influenced by plant roots. The advantage of rhizoremediation is that the bacteria in the rhizosphere can stably degrade the pollutants while using nutrients from the plant root exudates and oxygen exchange in the root system (*5*). Successful rhizoremediation in soil has already been reported for a variety of pollutants, such as chlorinated ethenes, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls (*4*).

Contamination of environmental water is a serious issue for conservation of the biosphere. Because pollutants in the water are more diffusive and at lower concentrations than those in soils, the immobilization of bacterial cells for long-term biodegradation is critical for complete restoration of contaminated sites (*6*). The rhizosphere is also ubiquitous in natural aquatic environments (*7*). Previous analyses reported accelerated degradation of organic chemicals using the rhizosphere effects of the giant duckweed *Spirodela polyrrhiza* (*8, 9*). These studies were, however, conducted using complex microbial consortia in the rhizosphere of water plants. Individual microbial activities and their interactions that are responsible for effective biodegradation are largely unknown.

Phenolic compounds are common chemicals that are widely used in many manufacturing processes and are one of the major pollutants found in industrial wastewater (*10*). These compounds are toxic to particular aquatic species (*11*) and are also harmful to human health as a result of their ingestion or inhalation even at very low concentrations (*12*). Therefore, it is valuable to develop strategies for the efficient and continuous biodegradation of phenols with minimum impact to the environment. The aim of this study was to conduct an initial survey of phenol-degrading bacteria in the rhizosphere of duckweed and shed light on their beneficial symbiotic interactions.

2. Materials and Methods

2.1. Enrichment and Isolation of Phenol-Degrading Rhizobacteria. Mature duckweed plants (*Lemna aoukikusa*) were collected from a small pond in the botanical garden of Hokkaido University (Sapporo, Japan). The duckweed plants were gently washed with sterile water and cultivated in a 300-mL flask containing 100 mL of Hoagland medium (pH 7.0) (*13*) supplemented with 200 mg L^{-1} phenol (#26719-65, Nacalai Tesque, Kyoto, Japan) at 25 °C with a day/night photoperiod of 16 h/8 h at 8500 lx. After 3 weeks of enrichment culture, ten fronds were washed with sterilized water and then transferred in a 1.5-mL microcentrifuge tube containing 1 mL of 5 mg L^{-1} sodium tripolyphosphate. The duckweed plants were sonicated six cycles of 5 s at 150 W (Model W185, Branson Ultrasonics Corporation, Danbury, CT) to disperse the surface-attached bacteria. The bacterial suspension was serially diluted with sterilized water and spread onto basal salt medium (BM) (*14*) agar plates supplemented with 20 mg L^{-1} phenol as the sole carbon source. Colonies that appeared on the plates were picked and subcultured several times to obtain pure isolates. The bacterial strains were preserved in Luria−Bertani broth (LB) supplemented with 15% sterilized glycerol at −80 °C.

2.2. Phenol Degradation Test. One loop of stock culture maintained on an LB agar plate was inoculated into 5 mL of LB supplemented with 2 mg L^{-1} phenol. Bacterial cells from the late exponential phase were recovered by centrifugation (1900*g*, 15 min, 20 °C) and suspended in BM. This suspension (1 mL) was transferred aseptically to a 100-mL flask with 50 mL of BM (final $OD_{600} = 1.0$) supplemented with 20 mg L^{-1} phenol and incubated at 25 °C with shaking at 150 rpm. At different times, 0.5 mL of culture medium was collected in

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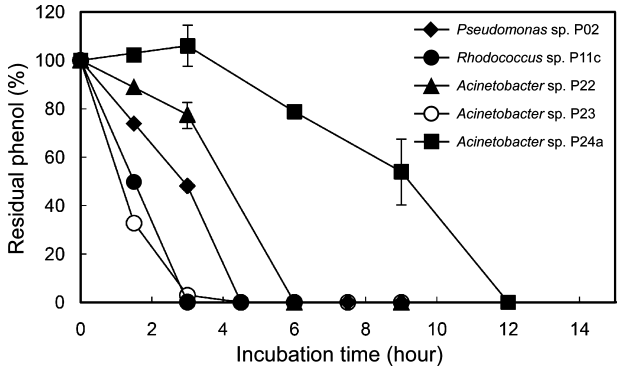

FIGURE 1. Degradation of phenol by bacteria isolated from the surface of duckweed plants. Degradation test of 20 mg L^{-1} phenol was performed at 25 °C with shaking at 150 rpm. Each data point is the average of triplicate experiments. Bars indicate SD.

a 1.5-mL microcentrifuge tube and centrifuged at 13,000*g* for 15 min at 4 °C. Supernatants were used for HPLC analysis. Each sample (100 μL) was separated on a Cosmosil $5C_{18}$-AR II column (4.6 × 150 mm; Nacalai Tesque, Kyoto, Japan) at a flow rate of 0.5 mL min^{-1}. The mobile phase contained 63% (v/v) water and 37% (v/v) acetonitrile. The detector wavelength was set at 254 nm to detect phenol.

2.3. Sequencing of 16S rRNA Gene and Phylogenetic Analysis. Whole-cell lysis PCR amplification was conducted to amplify the 16S rRNA gene using InstaGene Matrix (Bio-Rad, Hercules, CA). Part of the 16S rRNA gene was amplified by KOD Dash DNA polymerase (Toyobo, Osaka, Japan) with the forward primer (5′-GTCCACGCCAACGATG-3′) and the reverse primer (5′-GGCTACCCTTGTTACGACTT-3′), which corresponds to nucleotide positions 804 to 820 and 1510 to 1492, respectively, of most bacterial 16S rRNA genes. The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The nucleotide sequence was determined by BigDye terminator cycle sequencing on an ABI 3100 DNA sequencer (Perkin-Elmer Applied Biosystems, Wellesley, MA). The sequence data have been deposited in the public database under the accession numbers shown in Figure 2. The 16S rRNA gene sequences of related *Acinetobacter* species were retrieved from the public database to construct a phylogenetic tree. Distance trees based on multiple sequence alignments were generated with Clustal X version 2.0 (*15*) using the neighbor-joining method with 1000 bootstrap trials. *Acinetobacter* sp. P23 was physiologically characterized by the ID 32E API system (BioMèrieux Japan Co., Ltd., Tokyo, Japan).

2.4. Biofilm Formation. Biofilm formation was tested as described previously with some modifications (*16*). An overnight culture in LB was diluted to OD_{600} = 0.3 and inoculated (1%) into 300 μL of LB, BM, or Hoagland medium in a 1.5-mL microcentrifuge tube (TC131615; Nippon Genetics, Tokyo, Japan), and the cap of the tube was closed. BM and Hoagland medium were supplemented with 10 mg mL^{-1} phenol. After standing cultivation at 25 °C for 24 h, the pellicles and the medium were removed from the tube. The surface of the biofilm was rinsed with distilled water and stained with 400 μL of 0.1% (w/v) crystal violet (CV) solution for 20 min. The CV solution was removed, and the tube was washed twice with distilled water. The CV attached to the biofilm was dissolved in 500 μL of 95% ethanol and quantified by measuring its absorbance at 590 nm. Each data point is the average of triplicate experiments.

2.5. Preparation of Duckweed Whose Rhizosphere Is Dominated by P23, Duckweed/P23. To eliminate indigenous microorganisms that coexist with duckweeds, ca. 100 fronds were submerged in a 300-mL flask with 100 mL of 0.5% (v/v) NaClO/0.05% (v/v) Triton X-100 and gently stirred for 30

A

Strain	Accession No.	Closely matched species (accession No.)	Max identity (%)
P02	AB538039	*Pseudomonas fluorescens* B59 (EU169162)	100
		Pseudomonas fluorescens B50 (EU169157)	100
P11c	AB538040	*Rhodococcus* sp. GW-2006 (DQ453477)	100
		Rhodococcus erythropolis K22-22 (EU326491)	100
P22	AB538041	*Acinetobacter rhizosphaerae* OCI1 (EU131164)	100
		Acinetobacter calcoaceticus ACI34 (AM410707)	99
P23	AB538042	*Acinetobacter calcoaceticus* YLZZ-1 (EU022688)	100
		Acinetobacter sp. LF-3 (EU316217)	100
P24a	AB538043	*Acinetobacter* sp. P-106 (AM412163)	100
		Acinetobacter sp. TDIW17 (EU000455)	100

B

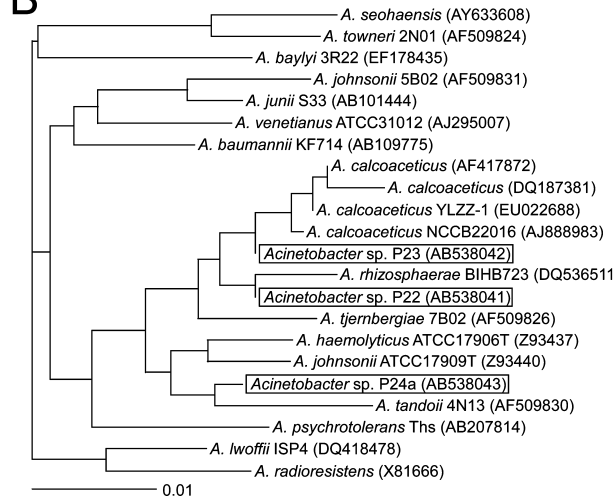

FIGURE 2. Phylogenetic characterization of phenol-degrading bacteria. (A) Taxonomic identity of the phenol-degrading bacteria isolated from duckweed roots. (B) Phylogenetic tree based on the 16S rRNA gene sequence of the three *Acinetobacter* strains and their related species. The scale bar shows 0.01 nucleotide substitutions per site.

min. These surface-sterilized plants were washed twice for 10 min with sterilized water and transferred into Hoagland medium. P23 cells from the late exponential phase grown in LB supplemented with 2 mg mL^{-1} phenol were recovered by centrifugation (1900*g*, 15 min, 20 °C) and suspended in Hoagland medium. This suspension (1 mL) and 30 surface-sterilized fronds were transferred into a 100-mL flask with 50 mL of Hoagland medium supplemented with 20 mg mL^{-1} phenol (final OD_{600} = 0.3) and incubated under the day/night photoperiod for 72 h at 25 °C.

2.6. Sustainable Biodegradation of Phenol by Duckweed/P23. Ten fronds of the duckweed/P23 prepared as described above were transferred into a 100-mL flask with 50 mL of Hoagland medium supplemented with 40 mg mL^{-1} phenol and further cultivated under the photoperiod at 25 °C. For the degradation test by P23 alone, 0.5×10^9 cells (equivalent to the number of P23 cells adhered to ten fronds as counted by an assay of colony-forming units at 72 h) were applied. Culture medium (0.25 mL) was collected at different times and subjected to HPLC analysis for phenol concentration. After 40 and 96 h, 40 mg mL^{-1} phenol was added to the culture volume, and the concentration of phenol was adjusted to 50 mL and 40 mg mL^{-1}, respectively.

2.7. Fluorescence Microscopy. After the cultivation of duckweed carrying P23, the plants were picked and stained in the dark for 15 min with the LIVE/DEAD BacLight Bacterial Viability kit (Molecular Probes-Invitrogen, San Diego, CA) in a 1.5-mL microcentrifuge tube containing 250 μL each of SYTO 9 and propidium iodide solutions. Bacterial cells attached to duckweed roots were observed using a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). Simul-

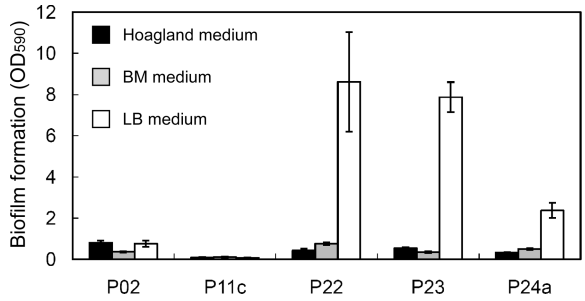

FIGURE 3. Biofilm-forming abilities of phenol-degrading bacteria. Bacterial strains were grown at 25 °C in Hoagland, BM, or LB medium. Biofilm formation was calculated by the crystal violet staining method. The data represent the mean ± SD (n = 3). Cells growth of each strain in Hoagland and BM medium with phenol was significantly less than in LB.

taneous application of both dyes results in green fluorescence of viable cells and intense red fluorescence of dead ones.

3. Results and Discussion

3.1. Isolation and Identification of Phenol-Degrading Rhizobacteria. After 3 weeks of enrichment, culturable bacteria were collected from the surface of the duckweed plants. Bacterial colonies were examined for morphological characteristics, such as color, shape, size, and surface properties on a BM agar plate, and about 100 distinguishable strains were isolated. When tested in BM liquid medium supplemented with 20 mg mL^{-1} phenol, five strains exhibited marked abilities to degrade phenol (Figure 1).

Partial 16S rRNA gene sequencing clearly showed that the five strains belong to γ-Proteobacteria, the genera *Pseudomonas* (P02), *Rhodococcus* (P11c), and *Acinetobacter* (P22, P23, and P24a) (Figure 2A). The nucleotide sequences of the 16S rRNA gene of the three *Acinetobacter* strains were aligned with the reference sequences of their close relatives (Figure 2B). This revealed that P22, P23, and P24a clustered tightly with *A. rhizosphaerae*, *A. calcoaceticus*, and *A. tandoii*, respectively.

The property of adherence is a critical trait of bacteria in the rhizosphere, namely rhizobacteria, grown on the surface of plants (*17*). Biofilm formation, which is an indicator of adherence, was thus examined for the five strains (Figure 3). Each strain was grown in a 1.5-mL polypropyrene tube without shaking for 24 h in Hoagland, BM, and LB medium. The three *Acinetobacter* strains formed robust biofilms in LB, whereas *Pseudomonas* sp. P02 and *Rhodococcus* sp. P11c formed marginal biofilms under all of the tested conditions. These results are in accordance with the classification of the genus *Acinetobacter*, species of which are nonmotile and grow attached to a variety of solid surfaces in nature (*18*). It has been reported that a toluene-degrading *Acinetobacter* sp. Tol5 has a highly hydrophobic cell surface and preferably adheres to a solid phase (*19*).

Because P23 exhibited the best phenol degradation and substantial biofilm formation abilities among the tested strains, we hypothesized that P23 could be a potential candidate for effective biodegradation of phenol. Thus, we used this strain for further experiments. When we examined carbon sources, almost all of the tested compounds, including glucose, maltose, cellobiose, sucrose, trehalose, inositol, 5-ketogluconic acid, pyruvate, arabinose, mannitol, arabitol, sorbitol, galacturonic acid, benzene, toluene, failed to support the growth of P23, except for phenol and ethanol. Because phenolic compounds are the major exudates of plant roots (*5*), the rhizosphere of duckweed should provide favorable growth conditions for P23.

3.2. Strong Adherence of P23 to Duckweed Plants. During incubation of duckweed plants with P23 in Hoagland

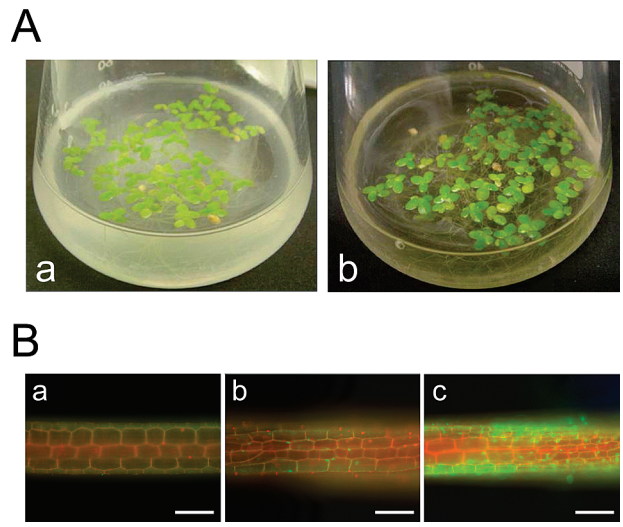

FIGURE 4. Rapid colonization of *A. calcoaceticus* P23 on the surface of duckweed roots. (A) Changes in culture turbidity during the mixed cultivation of duckweed and P23. At 0 h (a), the OD_{600} was 0.3; at 72 h (b), the OD_{600} was 0.02. (B) Staining for live bacteria and observation by fluorescence microscopy of duckweed roots cultivated with P23. (a) A surface-sterilized duckweed root at 0 h. (b) A duckweed root without P23 at 72 h. (c) A duckweed root carrying P23 (duckweed/P23) at 72 h. The high intensity of fluorescent color indicates living bacteria. Scale bars, 50 μm.

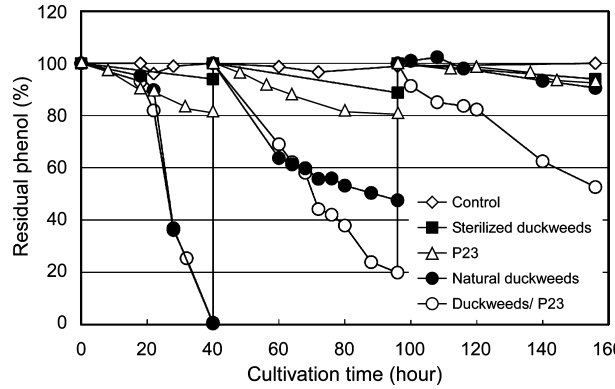

FIGURE 5. Long-term performance test of phenol degradation by duckweed and P23. The elimination of phenol was examined for the different duckweed and P23 combination systems. P23 only was tested using ten times more cells than that of attached to duckweeds. Control was set for the experiment without duckweeds and P23. Each data point is the average of triplicate experiments.

medium, most of the P23 cells adhered to the duckweed, and culture turbidities (OD_{600}) significantly declined from 0.3 to 0.02 at 72 h (Figure 4A). When the duckweed/P23 cultures were stained with the LIVE/DEAD BacLight kit and observed by fluorescence microscopy, many green fluorescent spots, indicating living bacteria, were observed on the surface of the roots at 72 h, whereas there was no obvious green fluorescence on the duckweed plants without P23 (Figure 4B). The bacterial cells were dispersed from the duckweed/P23 plants by sonication and spread on LB agar plates so that we could confirm that P23 was the dominant strain of adhered rhizobacteria (>99%; data not shown). Thus, the small colonies and biofilms formed on the root of duckweeds should be that of P23.

3.3. Sustainable Biodegradation by the Duckweed/P23 System. To determine whether the reinforcement of the specific single species P23 in the rhizosphere of sterilized duckweed plants, namely duckweed/P23 system, could be

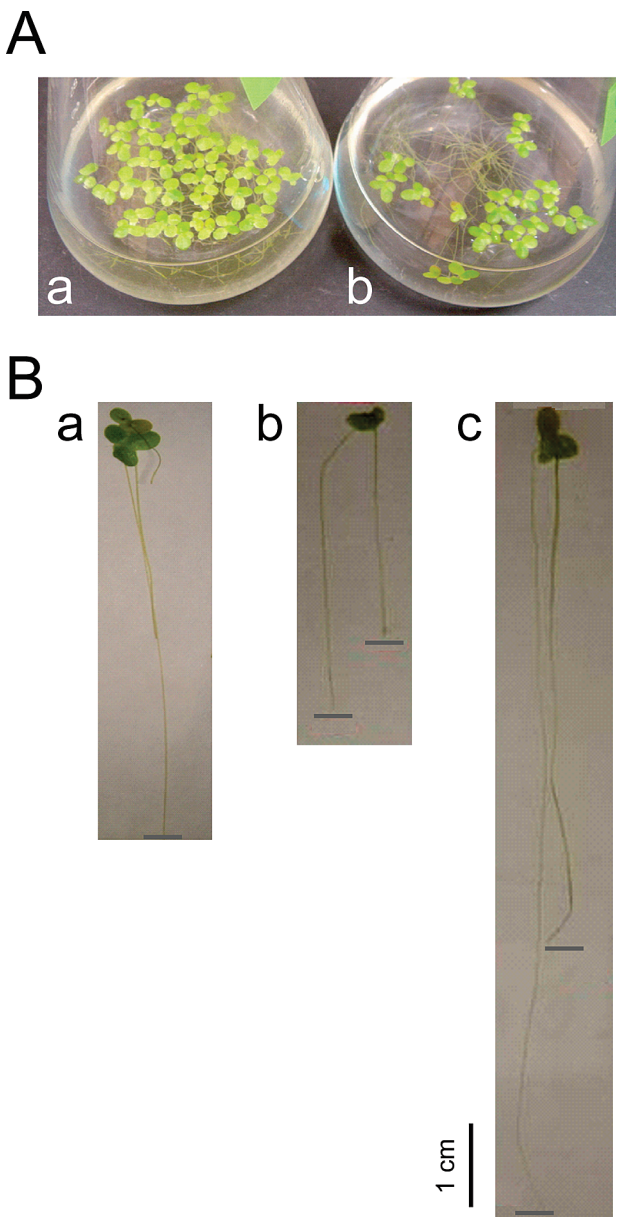

FIGURE 6. The effects of *A. calcoaceticus* P23 on the growth of duckweed. (A) Growth promotion of duckweed fronds resulting from adherence of P23. (a) Surface-sterilized duckweed plants carrying P23 grown for 160 h. (b) Surface-sterilized duckweed plants without P23 at 160 h. (B) Change in duckweed root length resulting from adherence of P23. (a) Natural duckweed at 0 h. (b) Surface-sterilized duckweed plants carrying P23 grown for160 h. (c) Surface-sterilized duckweed without P23 at 160 h. The vertical scale bar indicates 1 cm.

beneficial for biodegradation, several systems of duckweed and P23 were tested for long-term phenol degradation ability (Figure 5). These were natural duckweeds (intact as they are), surface-sterilized duckweeds, P23, and duckweed/ P23. In the first period of 40 h, the intact duckweeds and the duckweed/P23 systems completely eliminated 40 mg L^{-1} phenol in the culture, whereas neither the surface-sterilized duckweed nor P23 alone effectively eliminated phenol. These observations are consistent with a previous report for giant duckweed *Spirodela polyrrhiza* that phenol degradation activity of sterilized duckweed plant is negligible and that indigenous rhizobacteria of natural duckweed are responsible for degrading phenol (*9*). In the second period (40−96 h), there was roughly 80% and 50% elimination of phenol by duckweed/P23 and the natural duckweed, respectively. P23 alone showed less degradation ability, accounting for the elimination of ca. 20% of the phenol. At the end of the third period (160 h), duckweed/P23 maintained a 50% phenol elimination level, whereas the other systems eliminated less than 10% of the phenol. The long-term performance test (96−160 h) under nutrient-starvation conditions resulted in a severe reduction of phenol degradation activity for all combinations except for the duckweed/P23 system. These results also suggest that indigenous rhizobacteria capable of degrading phenol were rapidly attenuated, probably because of competition in the complex microbial community. The exact mechanisms are unclear; however, these experimental results indicate that the durable and continuous removal of phenol by the duckweed/P23 system may be attributed to the beneficial interaction between P23 and the duckweed. Decrease in the phenol degradation activity of duckweed/ P23 system during the periods may be due to accumulation of metabolic wastes in the flasks.

3.4. Growth Promotion of Duckweed by P23. During the long-term performance test of the duckweed/P23 system, we noticed that the number of fronds markedly increased as compared with the system containing surface-sterilized duckweed plants without P23 (Figure 6A). The number of duckweed/P23 fronds increased by 3.7-fold at 160 h, whereas the surface sterilized duckweed plants increased by only 2.2-fold (Table 1). This result clearly indicates that P23 promoted duckweed frond growth, especially multiplication. It should be notable that the growth promoting activity of P23 for duckweeds was also observed in the phenol free Hoagland medium. This result eliminates a possibility that the growth promotion is caused by the removal of toxic phenol by P23. We also found that the root length of the duckweed plants with P23 was substantially shorter than that of duckweed plants without P23 and natural duckweed plants (3.8, 6.5, and 4.2 cm on average, respectively; Figure 6B). Given that duckweeds extend their root length in response to phosphate starvation (*20, 21*), P23 in the rhizosphere presumably formed a healthy and nutritional microenvironment surrounding the duckweed roots while avoiding nutrient starvation.

These pleiotropic effects of *Acinetobacter* sp. bacteria in the rhizosphere are not surprising. *A. calcoaceticus* SE370, that was isolated from soil, produces plant growth-promoting hormones (gibberellins) and also carries phosphate-solubilizing activity (*22*). However, our preliminary result indicates that the plant growth-promoting factor of P23 is not gibberellins (data not shown). Moreover, phosphate-solubilizing activity of the rhizobacteria may be not the case in the liquid culture systems that we adopted. Further studies are needed to clarify the environmental factors and cellular responses of P23 that involve the beneficial rhizospheres of

TABLE 1. Growth Promotion of Duckweed and the Changes of Root Length Directed by Strain P23[a]

	number of fronds				root length (cm)	
treatment	0 h	40 h	88 h	160 h	0 h	160 h
duckweeds without P23	10	14.0 ± 1.0	20.7 ± 2.8	22.0 ± 1.0	4.2 ± 0.7	6.5 ± 1.2
duckweeds caryying P23	10	16.3 ± 2.3	33.0 ± 5.7	37.3 ± 2.3	4.2 ± 0.7	3.8 ± 0.3

[a] The data represent the mean ± SD (n = 3).

duckweed. To our best knowledge, P23 is the first growth-promoting rhizobacteria identified from the duckweed *L. aoukikusa*.

In this study, we demonstrated a beneficial symbiotic interaction between a rhizobacterium *A. calcoaceticus* P23 and the duckweed *L. aoukikusa*. The results suggest the potential usefulness of dominating a particular bacterium in the rhizosphere of duckweed to achieve efficient and sustainable bioremediation. Another advantage of using rhizobacteria that strongly associate with floating water plants, including duckweeds, is the ease of recovering the bacterial cells with the plants after remediation of polluted sites. In fact, the turbidity of the duckweed/P23 culture remained at an OD_{600} of <0.01 during the long-term performance test over 160 h.

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Note Added after ASAP Publication

This paper published ASAP July 21, 2010 with errors throughout the text. The correct version published on July 23, 2010.

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