Microbial Ecology (2019) 77:440–450
https://doi.org/10.1007/s00248-018-1306-x

PLANT MICROBE INTERACTIONS

Colonization and Competition Dynamics of Plant Growth-Promoting/Inhibiting Bacteria in the Phytosphere of the Duckweed Lemna minor

Hidehiro Ishizawa[1] · Masashi Kuroda[1] · Kanako Inoue[2] · Daisuke Inoue[1] · Masaaki Morikawa[3] · Michihiko Ike[1]

Received: 9 November 2018 / Accepted: 11 December 2018 / Published online: 2 January 2019

Abstract

Despite the considerable role of aquatic plant-associated bacteria in host plant growth and nutrient cycling in aquatic environments, the mode of their plant colonization has hardly been understood. This study examined the colonization and competition dynamics of a plant growth-promoting bacterium (PGPB) and two plant growth-inhibiting bacteria (PGIB) in the aquatic plant *Lemna minor* (common duckweed). When inoculated separately to *L. minor*, each bacterial strain quickly colonized at approximately 10^6 cells per milligram (plant fresh weight) and kept similar populations throughout the 7-day cultivation time. The results of two-membered co-inoculation assays revealed that the PGPB strain *Aquitalea magnusonii* H3 consistently competitively excluded the PGIB strain *Acinetobacter ursingii* M3, and strain H3 co-existed at almost 1:1 proportion with another PGIB strain, *Asticcacaulis excentricus* M6, regardless of the inoculation ratios (99:1–1:99) and inoculation order. We also found that *A. magnusonii* H3 exerted its growth-promoting effect over the negative effects of the two PGIB strains even when only a small amount was inoculated, probably due to its excellent competitive colonization ability. These experimental results demonstrate that there is a constant ecological equilibrium state involved in the bacterial colonization of aquatic plants.

Keywords Duckweed · Plant growth-promoting bacteria · Plant growth-inhibiting bacteria · Colonization · Competition · Aquatic plant

Introduction

Aquatic plants that proliferate in contaminated lakes or wetlands have attracted much attention in environmental technology because they can naturally remove nutrient salts, toxic heavy metals, and organic compounds from water bodies [1, 2]. Recently, they are also emerging as next-generation energy crops for biofuel production that do not compete with terrestrial food crops [3, 4]. Floating aquatic plants such as duckweed and water hyacinth are particularly competent for those purposes because of their extremely high growth rate and ease in harvesting [5, 6]. Therefore, attention has been directed toward establishing industrial-scale cultivation of these plants, offering co-beneficial systems performing wastewater treatment coupled with useful biomass production [7–13].

It has been recognized that bacteria co-existing with aquatic plants significantly affect the performance of wastewater treatment and biomass production through growth-promoting and growth-inhibiting effects on the host aquatic plants [14, 15], detoxifying pollutant elements such as nitrogen and metals by oxidation and reduction reactions [16–19], and degrading various organic pollutants, including recalcitrant ones [20, 21]. Several bacterial strains that are beneficial or efficient in the abovementioned aspects have been isolated from aquatic plants in recent studies [22–25]. These studies indicate the possibility of improved performance of aquatic plant systems by engineering such beneficial plant–bacteria

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00248-018-1306-x) contains supplementary material, which is available to authorized users.

✉ Michihiko Ike
ike@see.eng.osaka-u.ac.jp

[1] Division of Sustainable Energy and Environmental Engineering, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

[2] Research Center for Ultra-High Voltage Electron Microscopy, Osaka University, 7-1 Mihogaoka, Osaka, Ibaraki 567-0047, Japan

[3] Division of Biosphere Science, Graduate School of Environmental Science, Hokkaido University, N10-W5, Kita-ku, Sapporo 060-0810, Japan

interactions. In agriculture, the inoculation of plant growth-promoting bacteria (PGPB) has been intensively researched as a promising technology to increase crop production [26]. Similarly, some recent studies have attempted to introduce beneficial bacteria to aquatic plant systems and have reported enhanced biomass production and/or removal of toxic compounds in lab-scale experiments [27–30].

However, the delivery of those bacterial inoculants to plant surfaces in non-sterile hydrocultures is a big challenge. According to current knowledge of soil–plant–microbe interactions, the plant rhizosphere is a favorable habitat for various bacterial species, and there is intense competition within this habitat [31, 32]. Consequently, the introduction of beneficial bacterial strains, such as PGPB, has often failed to exert the desired effects because of unsuccessful colonization or competition with indigenous microorganisms on the target plants [33, 34]. Detailed understanding about competitive phytosphere colonization by bacteria is thus required to effectively and reliably utilize plant–bacteria symbiosis. To the best of our knowledge, however, no study has specifically examined the colonization and competition of aquatic plant-associated bacteria.

Here, we report the research results of the colonization and competition dynamics of bacterial strains in the phytosphere of *Lemna minor* (common duckweed). *Aquitalea magnusonii* H3, a PGPB isolated from duckweed, was used as a model strain for the inoculant. Because strain H3 is the sole PGPB that presents constant growth-promoting effects in the presence of other bacterial strains, as shown in a study by Ishizawa et al. [15], revealing its population dynamics provides practical knowledge for the effective use of PGPBs in non-sterilized environments. Two plant growth-inhibiting bacteria (PGIB) isolated from *L. minor*, *Acinetobacter ursingii* M3 and *Asticcacaulis excentricus* M6, were used as competitors. A series of gnotobiotic experiments was performed to evaluate the colonization dynamics of the bacterial strains and its relation to the effects on plant growth.

Methods

Plant and Bacterial Strains

Common duckweed (*Lemna minor*, RDSC 5512), collected from the botanical garden of Hokkaido University (Sapporo, Japan), was sterilized as described previously [35]. Plants were successively cultured in modified Hoagland medium [36] in a growth chamber at 28 °C, with a light intensity of 80 μmol m^{-2} s^{-1} and a photoperiod of 16 h/8 h–day/night. All plant cultivations in this study were conducted under the same conditions.

Three gram-negative bacterial strains (*A. magnusonii* H3, *A. ursingii* M3, and *A. excentricus* M6) were previously isolated from the same *L. minor* clone and characterized for their effects on *L. minor* growth [15, 37]. These bacterial strains were kept on solid 1/10 LB medium at 28 °C or in 25% glycerol at − 80 °C. For each experiment, the cells were grown to a late log phase in 20 mL of LB medium at 28 °C with shaking at 120 rpm.

Colonization of Single Bacterial Strains

For colonization assays, bacterial cultures were pelleted (4 °C, 10,000×*g*, 5 min), washed twice with modified Hoagland medium, and inoculated into 60-mL Hoagland medium at cell densities of 10^4, 10^5, and 10^6 CFU mL^{-1}. Then, 10 fronds of *L. minor* were cultured in the cell suspensions for nine growth periods (1 s, 1 h, 3 h, 6 h, 12 h, 1 day, 3 days, 5 days, and 7 days) in separate flasks. At the end of the co-cultivations, plants were harvested, and the excess water was gently absorbed with a sterile paper towel. The fresh weight of the plants was then measured, and the samples were preserved at − 80 °C until further use. The quantities of bacterial cells attached to the plants were determined by quantitative PCR (qPCR) analysis as described below.

Competitive Colonization Assays

Co-colonization assays were performed with the same procedure as described above, using mixtures of strains H3–M3 and H3–M6 prepared in CFU ratios of 100:0, 99:1, 90:10, 50:50, 10:90, 1:99, and 0:100 at a total cell density of 10^5 CFU mL^{-1}. Samples for qPCR analysis were taken 1 h, 1 day, and 7 days after the inoculation. In addition, from the plants that were co-cultivated for 7 days with each of the bacterial species (H3, M3, and M6) and 50:50 mixtures of H3–M3 and H3–M6, samples were taken by separating plant bodies into four parts using tweezers.

Next, to evaluate the influence of inoculation order on the results of co-colonization, similar experiments were conducted by sequentially inoculating bacterial strains. Sterile *L. minor* was first inoculated with 10^5 CFU mL^{-1} of each of the strains H3, M3, or M6 and co-cultivated for 24 h to allow them to fully colonize the plants. Ten fronds of the pre-treated plants were then transplanted to a new medium and inoculated with 10^5 CFU mL^{-1} of each of the strains H3, M3, or M6. The plant samples for qPCR analysis were taken 1 h, 1 day, and 7 days after the second inoculation.

qPCR Analysis

DNA was extracted from the samples by the CTAB method [38] empirically modified as detailed below. First, the samples were homogenized with 0.1 M Tris-HCl buffer, pH 8.0, using a high-power homogenizer (ASG50, ASONE, Aichi, Japan) at 6000 rpm for 30 s. Then, 100 μL of the homogenate

(corresponding to 10-mg fresh weight of plants) was mixed with 500 μL of CTAB buffer (100 mM Tris-HCl, pH 8.0; 1 M NaCl; 50 mM EDTA; 1% polyvinylpyrrolidone k30; 2% CTAB) and 50 μL of 2-mercaptoethanol and incubated at 65 °C for 2 h with shaking at 170 rpm. Extracts were purified twice in one volume of chloroform–isoamyl alcohol (24:1), precipitated in one volume of isopropanol, and washed in 500 μL of 70% ethanol. DNA pellets were finally re-suspended in 100 μL of TE buffer.

SYBR Green qPCR analysis was performed in a 96-well plate using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). The 20-μL reaction mixture contained 10 μL of Power SYBR® Green PCR Master Mix (Applied Biosystems), 2 μL of DNA template, and 0.25 μM of forward and reverse primers specific to each of strains H3, M3, and M6 (Table S1). Positive amplification and specificity of these primer pairs were verified by conventional PCR in relation to DNA templates extracted from each of strains H3, M3, M6, and sterile *L. minor* (data not shown). The PCR cycles comprised initial incubation at 50 °C for 2 min, denaturation at 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C (strains M3 and M6) or 58 °C (strain H3) for 1 min. A standard curve was generated for every run using a known copy number (ca. 10^2 to 10^7) of the DNA fragment cloned into a PMD20-T vector (Takara Bio Inc., Shiga, Japan). The cell numbers of the bacterial strains were determined by dividing the gene copy number (quantified with qPCR analysis) by the number of the target sequence in the complete genome of each strain (Table S1).

Plant Growth Evaluation

During the competitive colonization assays, the frond number of *L. minor* was periodically counted to evaluate the effects of bacterial strains on plant growth. The effects on plant growth (EPG) were calculated using the following formula:

$$EPG(\%) = 100 \times (FNt - FNc)/FNc,$$

where *FNt* is the frond number of bacteria-treated *L. minor* at day 7 and *FNc* is that of bacteria-free control plants. All colonization assays were performed in triplicate. Hence, the standard deviations (SD) for EPG were calculated as follows:

$$SD(EPG) = 100 \times \sqrt{\{SD(FNt)\}^2 + \{SD(FNc)\}^2}/FNc.$$

Scanning Electron Microscopy

For scanning electron microscopy (SEM), *L. minor* plants co-cultivated for 1 day with 10^5 CFU mL^{-1} of bacterial strains were immersed in fixing solution (2% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer) for 12 h at 4 °C and post-fixed with 1% osmium tetroxide for 30 min at room temperature. After fixation, the samples were coated with an osmium coater (HPC-1S; Vacuum Device, Ibaragi, Japan) and observed using SEM (S-4800; Hitachi High-Technologies, Tokyo, Japan) at various magnifications.

Physiological Assays

Strains H3, M3, and M6 were assayed for physiological traits that are known to be associated with plant colonization: biofilm formation, swimming motility, and capacity to utilize carbon sources. In addition, direct growth inhibition among the bacterial strains was also examined to better understand their competitive relationships.

Biofilm formation was assayed principally by the method of Pedersen [39]. First, 10^5 CFU mL^{-1} of each strain was incubated at 28 °C for 24 h in 300 μL of fresh LB medium in 1.5-mL polypropylene test tubes. The tubes were then rinsed once with sterile distilled water and filled with 400 μL of 0.1% crystal violet solution before incubation for 20 min at room temperature. After rinsing twice with 1 mL of distilled water, pigments were extracted with 500 μL of 95% ethanol and quantified by measuring the absorbance at 590 nm (A590) using a spectrophotometer (UV-1850; Shimadzu, Kyoto, Japan).

Swimming motility was tested by spotting 1 μL of log cultures on semisolid 1/10 LB medium supplemented with 0.1% casein hydrolysate and 0.2% purified agar [40]. The expansion of the colonies was observed after incubation at 28 °C for 5 days.

The capacity of bacterial strains to utilize 95 different carbon sources (listed in Table S2) was analyzed using Biolog GN2 MicroPlate (Biolog, Hayward, CA) according to the manufacturer's protocol.

A colony inhibition assay [41] was performed by spotting 5 μL of log cultures of each strain (diluted to 10^6 CFU mL^{-1}) on solid LB medium containing 1.5% (*w*/*v*) purified agar in the vicinity of each other. The morphology of the colonies was observed after incubation at 28 °C for 7 days.

Statistical Analysis

For the data of plant growth and biofilm formation test, significance was analyzed by one-way analysis of variance. Duncan's multiple-range test was performed to separate the means. Significance at $p < 0.05$ was applied. Statistical analyses were performed using R software v3.2.3 (http://www.r-project.org).

Results

Time Course of Single Bacterial Colonizations

The cell number of strains H3, M3, and M6 attached on *L. minor* was monitored by qPCR analysis at several time

points after the inoculation of 10^4, 10^5, and 10^6 CFU mL^{-1}. As shown in Fig. 1, all bacterial strains fully colonized plants within approximately 1 day when separately inoculated to *L. minor*. The time course could be divided into an "initial colonization phase" (where bacterial cells that colonized plants increase almost exponentially) and a "stationary phase" (where the colonized cell density stabilizes at approximately 10^6 cells per milligram plant fresh weight; approximately one frond). It was observed that the inoculation density did not affect the population at the stationary phase, and a larger inoculation density tended to enable quicker colonization at the initial colonization phase, particularly for strains H3 and M6.

Effects of Initial Abundance on Co-colonization

The population dynamics of bacterial strains that colonized *L. minor* plants were monitored after co-inoculating strain H3 and each of two PGIB strains (M3 and M6) in different inoculation ratios. Figure 2 shows the abundance of colonized bacterial cells 1 h, 1 day, and 7 days after inoculation. On the basis of the colonization time course analyzed in the previous experiments, these cultivation terms (1 h, 1 day, and 7 days) would represent the beginning of the initial colonization phase, near-end of the initial colonization phase, and late stationary phase, respectively.

In the co-colonization of strains H3 and M3, strain H3 exhibited a larger population than strain M3 after 1 h, although the proportions of strains H3 and M3 were similar to the inoculation ratios after 1 day (Fig. 2). The relative abundance of strain H3 notably increased after 1 day and reached 44–97% of the total bacterial cells after 7 days. The consistent population shifts may indicate a superior competitive ability of strain H3 to strain M3. In contrast, the proportions of strain H3 and M6 cells that colonized *L. minor* converged to almost equal in all inoculation ratios from 99:1 to 1:99, suggesting that these strains built a constant co-existing relationship. The colonization density of strain H3 was quite stable at approximately 10^6 cells mg^{-1}, irrespective of the presence or absence of strain M6, and the colonization density of strain M6 decreased to approximately half when co-inoculated with strain H3.

We also evaluated the percent increase/decrease of *L. minor* growth compared with the bacteria-free control by the co-inoculations of PGPB and PGIB strains (Table 1). When inoculated separately (100:0 or 0:100), strain H3 promoted and strains M3 and M6 inhibited *L. minor* growth, as expected. In competitive conditions, the plant growth seemed to reflect the relative abundance of PGPB and PGIB strains, as shown in Fig. 2, and strain H3 was found to improve *L. minor* growth or at least negate the negative effects of PGIB strains even when only 1% was inoculated. Despite the significant change in growth speed, change in plant morphology was not observed.

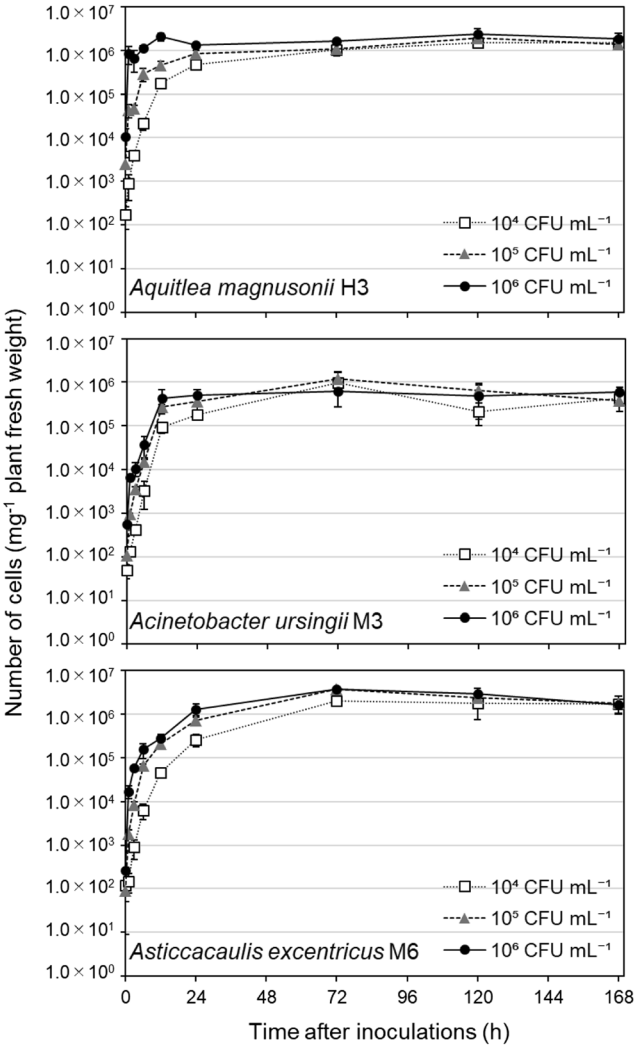

Fig. 1 Colonization time courses of three bacterial strains onto *L. minor* plants. Sterilized *L. minor* was inoculated with 10^4, 10^5, and 10^6 CFU mL^{-1} of strains H3, M3, and M6, and the numbers of bacterial cells attached to plants after 1-s, 1-h, 3-h, 6-h, 12-h, 1-day, 3-day, 5-day, and 7-day cultivations are plotted. Error bars show the standard deviations ($n = 3$)

Influence of Inoculation Order on Colonization of Bacteria

To test if inoculation order affects the competitive outcome, the PGPB and PGIB strains were sequentially inoculated to *L. minor* with a 24-h interval. According to the colonization time course shown in Fig. 1, an interval of 24 h is enough for the bacterial strains to fully colonize the plant. Results showed that noticeable invasions of lately introduced bacteria were observed within 1 day after the second inoculations in all combinations and orders of inoculations (Fig. 3). The population thereby shifted to the dominant colonization of strain H3 over M3 or the co-existence of strains H3 and M6, similar to the previous experiments. The colonization density of strain H3 was quite constant at approximately 10^6 cells mg^{-1} and

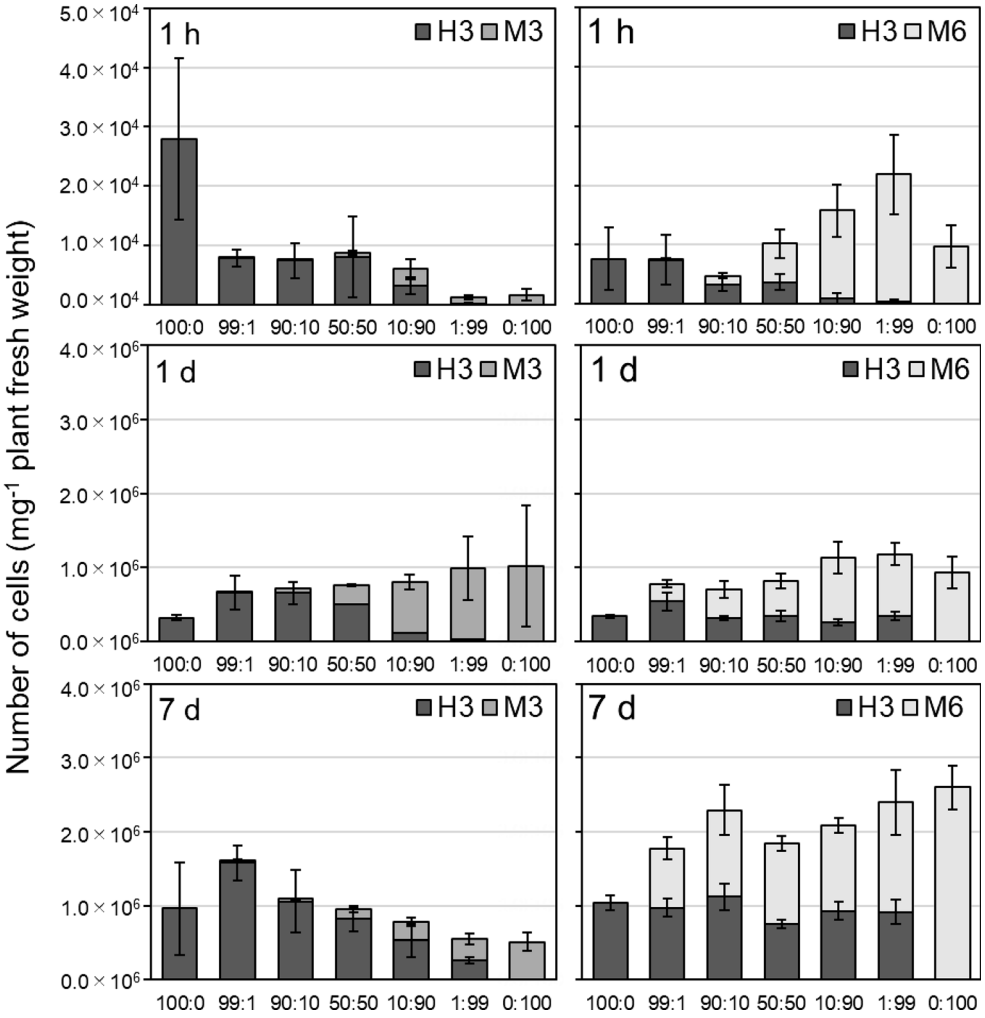

Fig. 2 Competitive plant colonization of strain H3 against strains M3 and M6. A total of 10^5 CFU mL^{-1} of bacterial strains was inoculated to *L. minor* cultures with different inoculation ratios, and the bacterial colonization was quantified by qPCR analysis. Results are shown as the means of cell numbers attached to plants (mg^{-1}) after 1 h, 1 day, and 7 days of co-cultivations. Error bars show the standard deviations ($n = 3$)

that of strains M3 and M6 was significantly decreased under co-inoculation conditions.

As shown in Table 1, the growth-regulating effects of PGPB and PGIB strains were consistently observed when the same bacteria were used in preliminary and post-inoculations. The growth-promoting effects of strain H3 were maintained even in the presence of PGIB strains and were not significantly influenced by the inoculation order.

Distribution of Bacterial Strains on Duckweed

The distribution of the three bacterial strains after the colonization tests was further analyzed by separating plant bodies into four parts: mother frond (MF), daughter frond (DF), upper root (UR), and root tip (RT). In the single-inoculation conditions, strain H3 colonized more frond parts (MF and DF) than root parts (UR and RT) and strains M3 and M6 colonized fronds and roots evenly (Fig. 4). There was no significant difference in the distribution of each strain between frond samples (MF and DF) and between root samples (UR and RT). The results of the co-inoculation showed that the distribution patterns of each bacterial strain were not significantly influenced by the presence and absence of counterparts.

Morphology of Colonized Cells

SEM revealed that strain H3 densely colonized the underside of *L. minor* fronds, forming microcolonies comprising 1–20 cells (Fig. 5a, b). Massive colonization was observed particularly around the root base of the frond. Strain H3 also distributed on the upper side of fronds and roots with a similar appearance. In contrast, strains M3 and M6 colonized plants by forming biofilm structures that were sparsely distributed in both frond and root parts (Fig. 5c, d). Similar appearances of colonized cells were observed even in competitive conditions (data not shown).

Physiological Traits

Strain M6 showed eminently superior biofilm formation ability in the in vitro assay (Fig. 6a). Strain H3, which did not

Table 1 Effects of bacterial inoculation on *L. minor* growth. Mean ± SD ($n = 3$) values of percent increase or decrease in frond number after 7-day cultivation (compared with aseptic control) are shown as EPGs. Values sharing the same letter within the same series of experiments indicate no significant difference. Aseptic control plants were prepared in each of four groups of experiments, and there were 90.3 ± 4.5, 88.7 ± 1.7, 94.3 ± 4.9, and 96.3 ± 1.9 fronds in the controls for the experiments of H3:M3, H3:M6, sequential inoculation of H3 vs M3, and that of H3 vs M6, respectively

H3 vs M3			H3 vs M6		
Treatment	EPG (%)		Treatment	EPG (%)	
H3:M3			*H3:M6*		
100:0	19.2 ± 2.9	ab	100:0	19.9 ± 3.4	a
99:1	13.3 ± 4.6	ab	99:1	12.0 ± 2.9	b
90:10	21.8 ± 11.0	a	90:10	6.4 ± 4.2	bc
50:50	16.6 ± 9.1	ab	50:50	6.8 ± 4.2	bc
10:90	5.2 ± 5.0	b	10:90	5.6 ± 5.9	bc
1:99	6.6 ± 7.9	ab	1:99	0.8 ± 3.0	c
0:100	− 13.7 ± 1.6	c	0:100	− 9.4 ± 4.2	d
First → Second			*First → Second*		
H3 → H3	15.2 ± 9.0	a	H3 → H3	16.6 ± 6.9	a
H3 → M3	9.9 ± 6.2	a	H3 → M6	3.1 ± 4.0	bc
M3 → H3	10.2 ± 7.5	a	M6 → H3	5.5 ± 4.2	b
M3 → M3	− 13.8 ± 5.7	b	M6 → M6	− 5.2 ± 4.4	c

form a biofilm-like structure on plants, showed the least ability in this assay.

The motility assay revealed that strains H3 and M6 possess swimming motility, and strain M3 was non-motile, supporting its identification as an *Acinetobacter* bacterium (Fig. 6b). Because only weak colony expansions were observed without the addition of casein hydrolysate (data not shown), it seems that chemotaxis toward amino acids plays an important role in their motility.

Among 95 different carbon sources included in the Biolog GN2 plate, strains H3, M3, and M6 could utilize 38, 40, and 22 compounds, respectively (Table S2). The pairs H3–M3 and H3–M6 shared 24 and 14 compounds, respectively.

As shown in Fig. 6c, d, no one-sided colony inhibition was observed among the strains used in this study. In contrast, it was found that the colonies of strains H3 and M6 overlapped without recognizable growth impairment, whereas colony development was impaired at the border of H3 and M3 colonies.

Discussion

With the limited impact of environmental context and surrounding bacterial communities, plant species, including aquatic plants, are thought to harbor highly conserved microbiomes [42–44]. This, in turn, suggests that there are large differences in the competitive colonization abilities of environmental bacteria, which principally determine the composition of the phytosphere microbiome [45]. In contrast, other factors such as initial species abundance and colonization order can also confer substantial influence on the phytosphere microbial population [46–48]. The competitive Lotka–Volterra model states that initial species abundance even possibly influences the winner of inter-specific competition [49].

On the basis of these facts, in the present study, a series of experiments was performed to determine the colonization dynamics of three model bacterial strains onto *L. minor* in

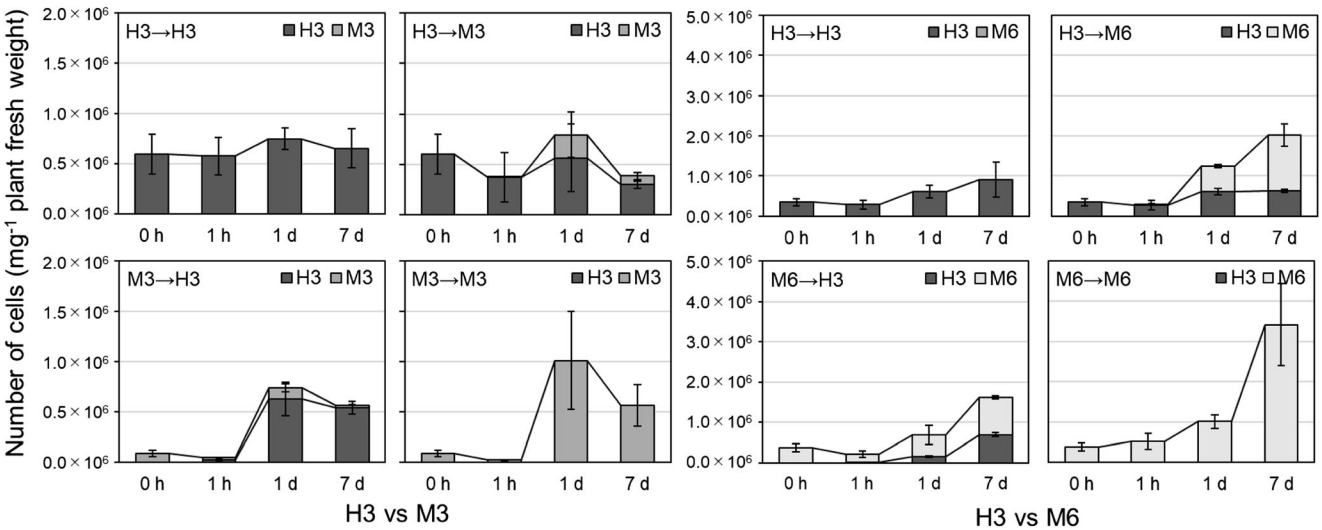

Fig. 3 Competitive plant colonization of strains H3, M3, and M6 that were sequentially inoculated to *L. minor*. *L. minor* was first inoculated with each of strains H3, M3, and M6 for 24 h and then inoculated with the same or another bacterial strain. Text on the top left indicates the bacterial strain used for the first inoculation (before the arrows) and second inoculation (after the arrows). Results are shown as the means of cell numbers attached to plants (mg^{-1}) at the start (0 h) and after 1 h, 1 day, and 7 days of the second inoculation. Error bars show the standard deviations ($n = 3$)

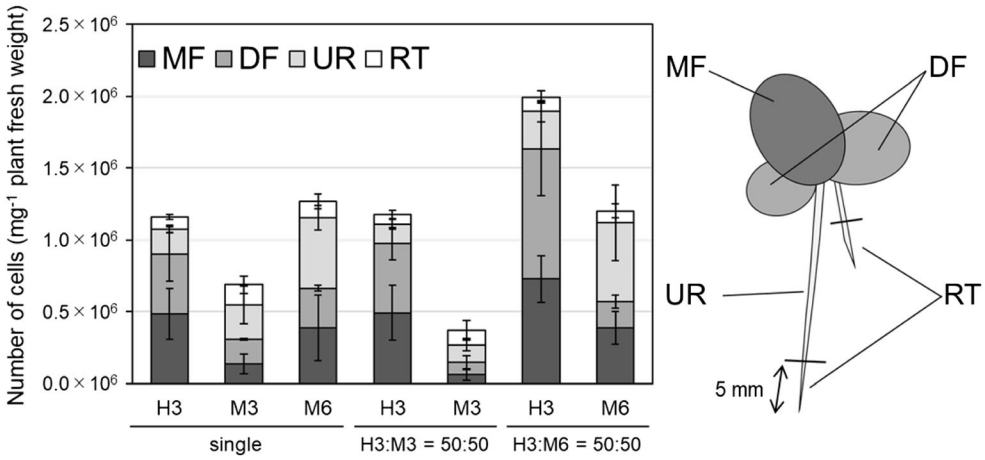

Fig. 4 Distribution of bacterial strains colonized on *L. minor*. The cell numbers of bacterial strains attached on the mother frond (MF), daughter frond (DF), upper root (UR), and root tip (RT) are shown relative to the fresh weight of the whole plant bodies. MF is defined as the oldest frond of the *L. minor* colony with approximately 3–4 fronds, and the remaining fronds are defined as DF. RT indicates the root part within 5 mm from its tip. The roots other than RT are defined as UR. Results are shown as the means of cell numbers attached to plants (mg^{-1}) after 7 days of co-cultivations. Error bars show the standard deviations ($n = 3$)

various initial populations. We began the experiments by investigating the colonization dynamics of each bacterial strain in non-competitive conditions. The bacterial strains used in this study (*A. magnusonii* H3, *A. ursingii* M3, and *A. excentricus* M6) are species that have been frequently isolated from or abundantly detected in the phytosphere of duckweed [22, 43, 50, 51]. They quickly colonized *L. minor* and maintained their populations without the external addition of growth substrates (Fig. 1). Although there were some differences in distribution tendency and morphology of colonized cells (Figs. 4 and 5), the results indicate that all three strains are able to colonize *L. minor* with similar abundance and kinetics. It was estimated from the duckweed frond number and colonization density that bacterial cells attached to plants at 7 days were 10–10^4 times larger than those inoculated. Hence, the cells of each bacterial strain grew during the experiments, probably by utilizing plant exudates. Interestingly, only two bacterial strains that showed relatively high biofilm formation ability (M3 and M6; Fig. 6a) formed biofilm structures on the duckweed surface (Fig. 5), and two motile strains (H3 and M6; Fig. 6b) showed notably more rapid colonization as inoculation density increased (Fig. 1), which suggests the involvement of bacterial biofilm formation ability and swimming motility in plant colonization in the aqueous phase.

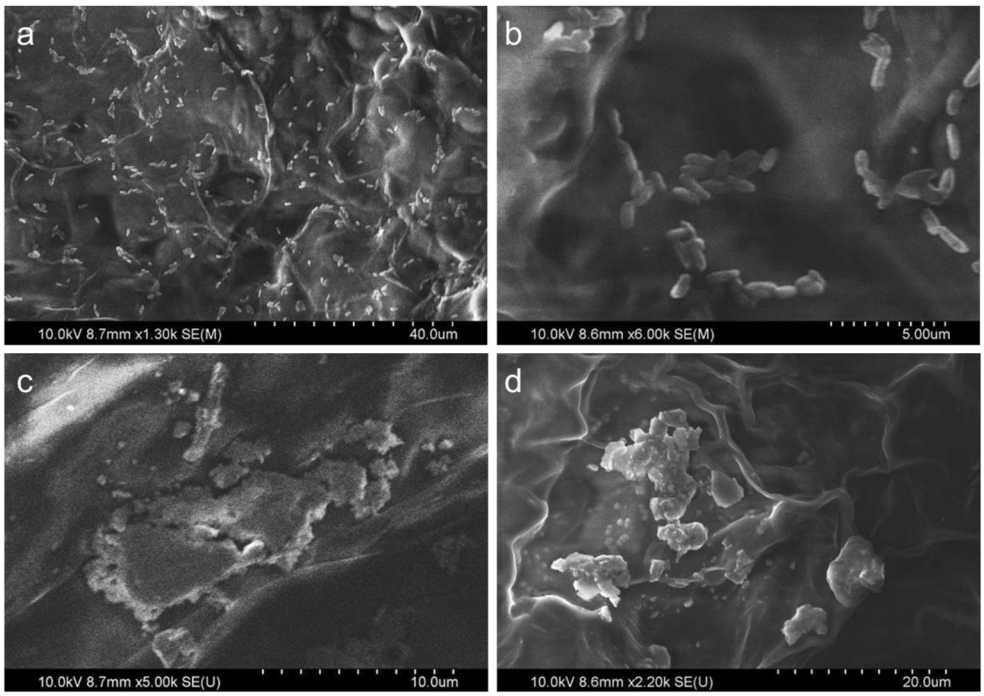

Fig. 5 SEM micrographs of bacterial strains that colonized *L. minor*. **a**, **b** Strain H3 on the underside of the frond. **c** Biofilm of strain M3 on the root. **d** Biofilm of strain M6 on the underside of the frond. Contrasts of images were modified for better visualization

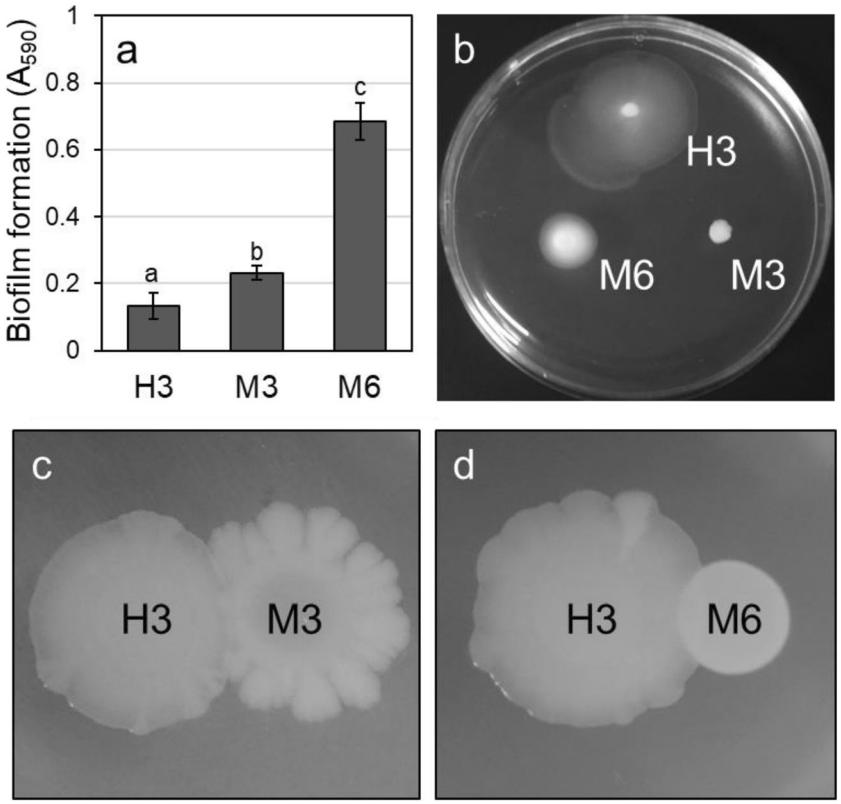

Fig. 6 Phenotypic characterization of bacterial strains. **a** Biofilm-forming abilities assayed in 1.5-mL polypropylene tubes. Different letters indicate significant differences among values. Error bars show the standard deviations ($n = 3$). **b** Colony morphology of bacterial strains grown on 0.2% agar 1/10 LB medium supplemented with 0.1% casein hydrolysate. **c**, **d** Interactions between neighboring colonies of strains H3–M3 and H3–M6 growing on 1.5% agar LB medium

We also found that the outcomes of co-colonization on plants were strongly dependent on the kind of bacteria inoculated to the plant rather than the inoculation regime (Figs. 2 and 3). In particular, in the co-inoculation of strains H3 and M3, strain H3 tended to exclude strain M3 as time passed. Similar population dynamics have frequently been reported in previous studies using terrestrial plants [40, 52], which attributed the phenomena to the hierarchy in bacterial competitive abilities. We also assume a superior competitive plant colonization ability of strain H3 compared with strain M3. In contrast, a quite resilient co-existing relationship was observed between strains H3 and M6. The immediate population shifts shown in Figs. 2 and 3 strongly suggest that strains H3 and M6 occupy different ecological niches, e.g., space and/or substrates, thus reaching constant abundance without intensive competition. Although such niche sharing is theoretically considered a principal mechanism to maintain species diversity in the same environment [53], to the best of our knowledge, there are very few studies that have experimentally demonstrated such phenomena among a pair of plant-associated bacteria. Hence, the interaction of strains H3 and M6 would be a good example to study niche sharing in the phytosphere. Because strains H3 and M6 showed similar distribution patterns on the plant in both single- and co-inoculation conditions (Fig. 4) and a lot of unoccupied space remained on the duckweed surface (Fig. 5), we can speculate that the capacity to utilize plant exudate play a key role in this niche sharing rather than space segregation. Although there was no clear food segregation observed between strains H3 and M6 (Table S2), further analysis of resource utilization by plant-associated bacteria, which is specialized for plant exudate components, could contribute to the deeper understanding of the diversity of the phytosphere microbiome. Additionally, it should be noted that although strain M6 had entirely no effect on the abundance of strain H3, the colonization of strain M6 significantly decreased in competitive conditions (Figs. 2 and 3). Therefore, we consider that there is some niche overlap between these strains, wherein strain H3 is more competent.

Throughout the colonization assays, strain H3, a PGPB of duckweed, efficiently maintained its population and also reduced the abundance of PGIB strains. Because strain H3 did not show one-sided growth inhibition against M3 and M6 colonies (Fig. 6c, d), this may be attributed to the competitive use of available niches in the phytosphere of duckweed. Concerning this, the growth-promoting effect of strain H3 was also observed even in co-inoculation conditions (Table 1). It is worth noting that only 1% inoculation of strain H3 could significantly improve *L. minor* growth, probably both by exerting its beneficial effects and by reducing the negative effects of PGIB strains. In addition, the growth speed of *L. minor* seemed to reflect the abundance or proportion of strain H3 during the cultivation terms (Figs. 2 and 3). Our previous study characterized strain H3 as a robust PGPB

that can exert a growth-promoting effect in the presence of a variety of other bacterial strains [15]. This study further confirms the previous results and, moreover, shows the relevance of competition and colonization ability in its robust growth-promoting ability. Because there are several genes that may contribute to plant colonization in the genome of strain H3 [54], identifying important genes for aquatic plant colonization should be a focus of future research.

This study reports highly consistent exclusive and co-existing relationships among duckweed-associated bacteria. Although only a few examples are shown, the population dynamics observed here demonstrate the important ecological processes that drive bacterial community assembly on aquatic plant surfaces. We believe that the results also have some important implications for the practical use of bacterial inoculants in aquatic plant systems. First, the competition between strains H3 and M3 suggests that poorly competitive species are readily excluded from the phytosphere, even when abundantly inoculated or preliminarily allowed to colonize the targeted plant. Hence, the use of innately competitive inoculants is important; otherwise, the beneficial effects would fade away in shorter periods. Second, the co-existing relationship between strains H3 and M6 suggests that the preferred niche of bacterial species is also a critical factor for the successful colonization of inoculants. Therefore, deciphering the variety of niches in the phytosphere environment would contribute to the deeper understanding and effective engineering of plant-associated microbiomes. For example, the combined use of inoculants [55] may be optimized using species with different preferred niches. Moreover, our study also supports the usefulness of duckweed as a tool to study plant–microbe interactions [56, 57].

Acknowledgments We thank Dr. Takao Sakata and Ms. Minami Tada for their support in experiments.

Funding Information This work was supported by the Advanced Low Carbon Technology Research and Development Program Grant Number JPMJAL1108 of the Japan Science and Technology Agency and the Japan Society for the Promotion of Science KAKENHI Grant Number JP18J10181. A part of this work was supported by the Advanced Characterization Nanotechnology Platform, Nanotechnology Platform Program of the Ministry of Education, Culture, Sports, Science and Technology, Japan, at the Research Center for Ultra-High Voltage Electron Microscopy (Nanotechnology Open Facilities) in Osaka University.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Rezania S, Ponraj M, Din MFM, Songip AR, Sairan FM, Chelliapan S (2012) The diverse applications of water hyacinth with main focus on sustainable energy and production for new era: an overview. Renew Sust Energ Rev 41:943–954. https://doi.org/10.1016/j.rser.2014.09.006
2. Ziegler P, Sree S, Appenroth KJ (2016) Duckweeds for water remediation and toxicity testing. Toxicol Environ Chem 98:1127–1154. https://doi.org/10.1080/02772248.2015.1094701
3. Bayrakci AG, Koçar G (2014) Second-generation bioethanol production from water hyacinth and duckweed in Izmir: a case study. Renew Energ Sust Rev 30:306–316. https://doi.org/10.1016/j.rser.2013.10.011
4. Cui W, Cheng JJ (2015) Growing duckweed for biofuel production: a review. Plant Biol 17:16–23. https://doi.org/10.1016/j.biosystemseng.2011.06.007
5. Cheng JJ, Stomp AM (2009) Growing duckweed to recover nutrients from wastewaters and for production of fuel ethanol and animal feed. Clean (Weinh) 37:17–26. https://doi.org/10.1002/clen.200800210
6. Rezania S, Taib SM, Din MFM et al (2016) Comprehensive review on phytotechnology: heavy metals removal by diverse aquatic plants species from wastewater. J Hazard Mater 318:587–599. https://doi.org/10.1016/j.jhazmat.2016.07.053
7. Fujita M, Mori K, Kodera T (1999) Nutrient removal and starch production through cultivation of *Wolffia arrhiza*. J Biosci Bioeng 87:194–198. https://doi.org/10.1016/S1389-1723(99)89012-4
8. Mishima D, Kuniki M, Sei K, Soda S, Ike M, Fujita M (2008) Ethanol production from candidate energy crops: water hyacinth (*Eichhornia crassipes*) and water lettuce (*Pistia stratiotes* L.). Bioresour Technol 99:2495–2500. https://doi.org/10.1016/j.biortech.2007.04.056
9. Ge X, Zhang N, Phillips GC, Xu J (2012) Growing *Lemna minor* in agricultural wastewater and converting the duckweed biomass to ethanol. Bioresour Technol 124:485–488. https://doi.org/10.1016/j.biortech.2012.08.050
10. Mohedano RA, Costa RHR, Tavares FA, Belli Filho P (2012) High nutrient removal rate from swine wastes and protein biomass production by full-scale duckweed ponds. Bioresour Technol 112:98–104. https://doi.org/10.1016/j.biortech.2012.02.083
11. Zhao Y, Fang Y, Jin Y, Huang J, Bao S, Fu T, He Z, Wang F, Zhao H (2014) Potential of duckweed in the conversion of wastewater nutrients to valuable biomass: a pilot-scale comparison with water hyacinth. Bioresour Technol 163:82–91. https://doi.org/10.1016/j.biortech.2014.04.018
12. Soda S, Ohchi T, Piradee J, Takai Y, Ike M (2015) Duckweed biomass as a renewable biorefinery feedstock: ethanol and succinate production from *Wolffia globosa*. Biomass Bioenergy 81:364–368. https://doi.org/10.1016/j.biombioe.2015.07.020
13. Toyama T, Hanaoka T, Tanaka Y, Morikawa M, Mori K (2018) Comprehensive evaluation of nitrogen removal rate and biomass, ethanol, and methane production yields by combination of four major duckweeds and three types of wastewater effluent. Bioresour Technol 250:464–473. https://doi.org/10.1016/j.biortech.2017.11.054
14. Underwood GJC, Baker JH (1991) The effect of various aquatic bacteria on the growth and senescence of duckweed (*Lemna minor*). J Appl Bacteriol 70:192–196. https://doi.org/10.1111/j.1365-2672.1991.tb02923.x
15. Ishizawa H, Kuroda M, Morikawa M, Ike M (2017) Evaluation of environmental bacterial communities as a factor affecting the growth of duckweed *Lemna minor*. Biotechnol Biofuels 10(62):62. https://doi.org/10.1186/s13068-017-0746-8

16. Stout L, Nüsslein K (2010) Biotechnological potential of aquatic plant-microbe interactions. Curr Opin Biotechnol 21:339–345. https://doi.org/10.1016/j.copbio.2010.04.004
17. Wei B, Yu X, Zhang S, Gu L (2011) Comparison of the community structures of ammonia-oxidizing bacteria and archaea in rhizoplanes of floating aquatic macrophytes. Microbiol Res 166:468–474. https://doi.org/10.1016/j.micres.2010.09.001
18. Tsuji K, Asayama T, Shiraki N, Inoue S, Okuda E, Hayashi C, Nishida K, Hasegawa H, Harada E (2017) Mn accumulation in a submerged plant *Egeria densa* (Hydrocharitaceae) is mediated by epiphytic bacteria. Plant Cell Environ 40:1163–1173. https://doi.org/10.1111/pce.12910
19. Zhao Y, Fang Y, Jin Y, Huang J, Ma X, He K, He Z, Wang F, Zhao H (2015) Microbial community and removal of nitrogen *via* the addition of a carrier in a pilot-scale duckweed-based wastewater treatment system. Bioresour Technol 179:549–558. https://doi.org/10.1016/j.biortech.2014.12.037
20. Toyama T, Furukawa T, Maeda N, Inoue D, Sei K, Mori K, Kikuchi S, Ike M (2011) Accelerated biodegradation of pyrene and benzo [*a*] pyrene in the *Phragmites australis* rhizosphere by bacteria-root exudate interactions. Water Res 45:1629–1638. https://doi.org/10.1016/j.watres.2010.11.044
21. Chen W, Tang Y, Mori K, Wu XL (2012) Distribution of culturable endophytic bacteria in aquatic plants and their potential for bioremediation in polluted waters. Aquat Biol 15:99–110. https://doi.org/10.3354/ab00422
22. Yamaga F, Washio K, Morikawa M (2010) Sustainable biodegradation of phenol by *Acinetobacter calcoaceticus* P23 isolated from the rhizosphere of duckweed *Lemna aoukikusa*. Environ Sci Technol 44:6470–6474. https://doi.org/10.1021/es1007017
23. El-Deeb B, Gherbawy Y, Hassan S (2012) Molecular characterization of endophytic bacteria from metal hyperaccumulator aquatic plant (*Eichhornia crassipes*) and its role in heavy metal removal. Geomicrobiol J 29:906–915. https://doi.org/10.1080/01490451.2011.635764
24. Ogata Y, Goda S, Toyama T, Sei K, Ike M (2013) The 4-*tert*-butylphenol-utilizing bacterium *Sphingobium fuliginis* OMI can degrade bisphenols *via* phenolic ring hydroxylation and *meta*-cleavage pathway. Environ Sci Technol 47:1017–1023. https://doi.org/10.1021/es303726h
25. Tang J, Zhang Y, Cui Y, Ma J (2009) Effects of a rhizobacterium on the growth of and chromium remediation by *Lemna minor*. Environ Sci Pollut Res 22:9686–9693. https://doi.org/10.1007/s11356-015-4138-y
26. Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556. https://doi.org/10.1146/annurev.micro.62.081307.162918
27. Faisal M, Hasnain S (2005) Beneficial role of hydrophytes in removing Cr (VI) from wastewater in association with chromate-reducing bacterial strains *Ochrobactrum intermedium* and *Brevibacterium*. Int J Phytoremediation 7:271–277. https://doi.org/10.1080/16226510500327111
28. Kristanti RA, Toyama T, Hadibarata T, Tanaka Y, Mori K (2014) Bioaugmentation involving a bacterial consortium isolated from the rhizosphere of *Spirodela polyrhiza* for treating water contaminated with a mixture of four nitrophenol isomers. RSC Adv 4:1616–1621. https://doi.org/10.1039/c3ra44892d
29. Xu XJ, Sun JQ, Nie Y, Wu XL (2015) *Spirodela polyrhiza* stimulates the growth of its endophytes but differentially increases their fenpropathrin-degradation capabilities. Chemosphere 125:33–40. https://doi.org/10.1016/j.chemosphere.2014.12.084
30. Toyama T, Kuroda M, Ogata Y, Hachiya Y, Quach A, Tokura K, Tanaka Y, Mori K, Morikawa M, Ike M (2017) Enhanced biomass production of duckweeds by inoculating a plant growth-promoting bacterium, *Acinetobacter calcoaceticus* P23, in sterile medium and non-sterile environmental waters. Water Sci Technol 76:1418–1428. https://doi.org/10.2166/wst.2017.296
31. Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511. https://doi.org/10.1093/jexbot/52.suppl_1.487
32. Raaijmakers JM, Mazzola M (2012) Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. Annu Rev Phytopathol 50:403–424. https://doi.org/10.1146/annurev-phyto-081211-172908
33. Lugtenberg B, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annu Rev Phytopathol 9:461–490. https://doi.org/10.1146/annurev.phyto.39.1.461
34. Liu W, Yang C, Shi S, Shu W (2014) Effects of plant growth-promoting bacteria isolated from copper tailings on plants in sterilized and non-sterilized tailings. Chemosphere 97:47–53. https://doi.org/10.1016/j.chemosphere.2013.10.089
35. Suzuki W, Sugawara M, Miwa K, Morikawa M (2014) Plant growth-promoting bacterium *Acinetobacter calcoaceticus* P23 increases the chlorophyll content of the monocot *Lemna minor* (duckweed) and the dicot *Lactuca sativa* (lettuce). J Biosci Bioeng 118:41–44. https://doi.org/10.1016/j.jbiosc.2013.12.007
36. Toyama T, Yu N, Kumada H, Sei K, Ike M, Fujita M (2006) Accelerated aromatic compounds degradation in aquatic environment by use of interaction between *Spirodela polyrrhiza* and bacteria in its rhizosphere. J Biosci Bioeng 101:346–353. https://doi.org/10.1263/jbb.101.346
37. Ishizawa H, Kuroda M, Morikawa M (2017) Differential oxidative and antioxidative response of duckweed *Lemna minor* toward plant growth promoting/inhibiting bacteria. Plant Physiol Biochem 118:667–673. https://doi.org/10.1016/j.plaphy.2017.08.006
38. Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res 8:4321–4325. https://doi.org/10.1093/nar/8.19.4321
39. Pedersen K (1982) Method for studying microbial biofilms in flowing-water systems. Appl Environ Microbiol 43:6–13
40. Lardi M, de Campos SB, Purtschert G, Eberl L, Pessi G (2017) Competition experiments for legume infection identify *Burkholderia phymatum* as a highly competitive β-rhizobium. Front Microbiol 8(1527). https://doi.org/10.3389/fmicb.2017.01527
41. Xiong H, Li Y, Cai Y, Cao Y, Wang Y (2015) Isolation of *Bacillus amyloliquefaciens* JK6 and identification of its lipopeptides surfactin for suppressing tomato bacterial wilt. RSC Adv 5:82042–82049. https://doi.org/10.1039/C5RA13142A
42. Ofek M, Voronov-Goldman M, Hadar Y, Minz D (2014) Host signature effect on plant root-associated microbiomes revealed through analyses of resident *vs*. active communities. Environ Microbiol 16:2157–2167. https://doi.org/10.1111/1462-2920.12228
43. Xie WY, Su JQ, Zhu YG (2015) Phyllosphere bacterial community of floating macrophytes in paddy soil environments as revealed by Illumina high-throughput sequencing. Appl Environ Microbiol 81:522–532. https://doi.org/10.1128/AEM.03191-14
44. Hamonts K, Trivedi P, Garg A, Janitz C, Grinyer J, Holford P, Botha FC, Anderson IC, Singh BK (2018) Field study reveals core plant microbiota and relative importance of their drivers. Environ Microbiol 20:124–140. https://doi.org/10.1111/1462-2920.14031
45. Barret M, Morrissey JP, O'Gara F (2011) Functional genomics analysis of plant growth-promoting rhizobacterial traits involved in rhizosphere competence. Biol Fertil Soils 47:729–743. https://doi.org/10.1007/s00374-011-0605-x
46. Hatzinger PB, Alexander M (1994) Relationship between the number of bacteria added to soil or seeds and their abundance and distribution in the rhizosphere of alfalfa. Plant Soil 158:211–222. https://doi.org/10.1007/BF00009496

47. Normander B, Prosser JI (2000) Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. Appl Environ Microbiol 66:4372–4377. https://doi.org/10.1128/AEM.66.10.4372-4377.2000
48. Adame-Álvarez RM, Mendiola-Soto J, Heil M (2014) Order of arrival shifts endophyte-pathogen interactions in bean from resistance induction to disease facilitation. FEMS Microbiol Lett 355: 100–107. https://doi.org/10.1111/1574-6968.12454
49. Chesson P (1994) Multispecies competition in variable environments. Theor Popul Biol 45:227–276. https://doi.org/10.1006/tpbi.1994.1013
50. Matsuzawa H, Tanaka Y, Tamaki H, Kamagata Y, Mori K (2010) Culture-dependent and independent analyses of the microbial communities inhabiting the giant duckweed (*Spirodela polyrrhiza*) rhizoplane and isolation of a variety of rarely cultivated organisms within the phylum *Verrucomicrobia*. Microbes Environ 25:302–308. https://doi.org/10.1264/jsme2.ME10144
51. Quisehuatl-Tepexicuapan E, Ferrera-Cerrato R, Silva-Rojas HV et al (2016) Free-living culturable bacteria and protozoa from the rhizoplanes of three floating aquatic plant species. Plant Biosyst 150:855–865. https://doi.org/10.1080/11263504.2014.989282
52. De-Bellis P, Ercolani GL (2001) Growth interactions during bacterial colonization of seedling rootlet. Appl Environ Microbiol 67: 1945–1948. https://doi.org/10.1128/AEM.67.4.1945-1948.2001
53. Levine JM, HilleRisLambers J (2009) The importance of niches for the maintenance of species diversity. Nature 461:254–257. https://doi.org/10.1038/nature08251
54. Ishizawa H, Kuroda M, Ike M (2017) Draft genome sequence of *Aquitalea magnusonii* strain H3, a plant growth-promoting bacterium of duckweed (*Lemna minor*). Genome Announc 5:e00812–e00817. https://doi.org/10.1128/genomeA.00812-17
55. Singh M, Awasthi A, Soni SK, Singh R, Verma RK, Kalra A (2015) Complementarity among plant growth promoting traits in rhizospheric bacterial communities promotes plant growth. Sci Rep 5:15500. https://doi.org/10.1038/srep15500
56. Fan B, Borriss R, Bleiss W, Wu X (2012) Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. J Microbiol 50:38–44. https://doi.org/10.1007/s12275-012-1439-4
57. Appenroth KJ, Ziegler P, Sree S (2017) Duckweed as a model organism for investigating plant-microbe interactions in an aquatic environment and its applications. Endocytobiosis Cell Res 27:94–106