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Research Article

RESEARCH ARTICLE

Community dynamics of duckweed-associated bacteria upon inoculation of plant growth-promoting bacteria

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One sentence summary: Duckweed microbiome can be modified for accelerated biomass production.

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ABSTRACT

Plant growth-promoting bacteria (PGPB) have recently been demonstrated as a promising agent to improve wastewater treatment and biomass production efficiency of duckweed hydrocultures. With a view to their reliable use in aqueous environments, this study analysed the plant colonization dynamics of PGPB and the ecological consequences for the entire duckweed-associated bacterial community. A PGPB strain, *Aquitalea magnusonii* H3, was inoculated to duckweed at different cell densities or timings in the presence of three environmental bacterial communities. The results showed that strain H3 improved duckweed growth by 11.7–32.1% in five out of nine experiments. Quantitative-PCR and amplicon sequencing analyses showed that strain H3 successfully colonized duckweed after 1 and 3 d of inoculation in all cultivation tests. However, it significantly decreased in number after 7 d, and similar bacterial communities were observed on duckweed regardless of H3 inoculation. Predicted metagenome analysis suggested that genes related to bacterial chemotactic motility and surface attachment systems are consistently enriched through community assembly on duckweed. Taken together, strain H3 dominantly colonized duckweed for a short period and improved duckweed growth. However, the inoculation of the PGPB did not have a lasting impact due to the strong resilience of the natural duckweed microbiome.

Keywords: duckweed; plant growth-promoting bacteria; plant colonization; bacterial community assembly; phytoremediation; predicted metagenome analysis

INTRODUCTION

Some plant-associated microorganisms, called plant growth-promoting bacteria (PGPB), are capable of improving growth, pathogen resistance and the phytoremediation ability of the host plant (Kuiper *et al.* 2004; Lugtenberg and Kamilova 2009). Inoculation of such beneficial microorganisms as microbial inoculants is a prospective method to facilitate plant cultivation

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with reduced use of chemical fertilizer and pesticides without genetic modification technology (Adesemoye, Torbert and Kloppper 2009; Berg 2009; Özilgen 2017). To be effective, microbial inoculants must colonize the plants at high density and persist long enough (Holl and Chanway 1992; Ishizawa *et al.* 2020). Due to the intense competition with indigenous microbes, the persistence of microbial inoculants is considered one of the biggest bottlenecks of this technology (Trabelsi and Mhamdi 2013). A profound understanding of the fate of microbial inoculants within plant-associated bacterial communities is a crucial requisite for their reliable use.

Recently, aquatic plants have been increasingly cultivated for energy-independent wastewater treatment, biomass production and their combined systems. A family of small floating macrophytes called duckweed (family Lemnaceae) is particularly competent for the aforementioned purposes because of its extremely high growth rate, high fuel-conversion rate and ease of harvesting (Xu *e.* 2011; Cui and Cheng 2015; Toyama *et al.* 2018). Studies have also revealed the potential application of duckweeds in the conversion of wastewaters or industrial byproducts including CO_2 into more profitable products such as animal feed, human food and bioplastics (Leng, Stambolie and Bell 1995; Zeller *et al.* 2013; Appenroth *et al.* 2017a). These facts have led researchers to seek the possibility of utilizing beneficial microbial inoculants even for duckweed hydroculture systems. In the last decade, a number of potential microbial inoculants have been isolated and some of them effectively improved duckweed production and/or phytoremediation capacity (Yamaga, Washio and Morikawa 2010; Stout *et al.* 2010; Kristanti *et al.* 2014; Tang *et al.* 2015; Toyama *et al.* 2017; Yamakawa *et al.* 2018).

Previously, we reported that *Acinetobacter calcoaceticus* P23 enhanced duckweed growth in pond water as long as it remained on the plant surface at detectable densities (Ishizawa *et al.* 2020). This corroborates the importance of the colonization and persistence of microbial inoculants. However, the colonization dynamics of microbial inoculants applied to duckweed cultivation systems, with careful attention to indigenous bacterial communities, has not been comprehensively studied. Aquatic plants may attract various bacteria via secretion of oxygen and carbon compounds (Soda *et al.* 2007; Wu, Wang and He 2017), and hence, colonization and persistence of microbial inoculants should be analysed in the context of competition with indigenous bacteria. For example, it would be crucial to understand how fundamental ecological factors, such as initial species abundance (Wright and Vetsigian 2016), order of species arrival (Fukami 2015) and diversity of indigenous communities (van Elsas *et al.* 2012; Vila *et al.* 2019), affect the competition among PGPB and indigenous bacteria for ensuring inoculation success. The impact of microbial inoculants on the overall structure of duckweed-associated bacterial communities would also be an important aspect in plant performance and the environmental risk associated with their application. However, only a few studies have examined rhizobacterial community dynamics in hydroponic systems for crop plants so far (Fujiwara *et al.* 2013; Sheridan *et al.* 2017).

This study aims to understand the colonization dynamics of microbial inoculants applied to duckweed cultivation systems and the ecological consequences for the entire duckweed-associated bacterial community. *Aquitalea magnusonii* H3 was used as the model microbial inoculant because of its efficient plant colonization and robust growth-promotion ability observed in gnotobiotic experiments (Ishizawa et al. 2017, 2019a). In this study, strain H3 was inoculated to duckweed in the presence of three environmental bacterial communities, and its colonization, plant-growth promotion and impacts on residual bacterial communities were studied. The results shown here provide the first detailed analysis of the community dynamics of an aquatic plant microbiome upon the application of microbial inoculants.

MATERIALS AND METHODS

Plants and growth conditions

A laboratory stock of common duckweed (*Lemna minor* L. RDSC #5512; http://www.ruduckweed.org) was used in this study. The plants were previously sterilized using 0.5% sodium hypochlorite (Suzuki *et al.* 2014), and successively cultured in a growth chamber (28°C, photon flux of 80 μmol m^{-2} s^{-1}, 16 h-light cycle) with sterile modified Hoagland medium (all concentrations in mg L^{-1}: 36.1 KNO_3, 293 K_2SO_4, 3.87 NaH_2PO_4, 103 $MgSO_4{\cdot}7H_2O$, 147 $CaCl_2{\cdot}H_2O$, 3.33 $FeSO_4{\cdot}7H_2O$, 0.95 H_3BO_3, 0.39 $MnCl_2{\cdot}4H_2O$, 0.03 $CuSO_4{\cdot}5H_2O$, 0.08 $ZnSO_4{\cdot}7H_2O$ and 0.254 $H_2MoO_4{\cdot}4H_2O$; pH 7.0). All plant cultivation in this study was conducted under the same light and thermal conditions as above.

Microbial inoculum

A. magnusonii H3 is a PGPB strain isolated from *L. minor* co-cultured with pond-derived bacterial communities (Ishizawa *et al.* 2017). The strain was cultured in 20 mL of LB medium (Lennox) at 28°C with shaking at 120 rpm. The cells at late exponential phase were harvested and washed twice with sterile modified Hoagland medium before plant inoculation.

Collection of environmental bacterial communities

Environmental bacterial communities were prepared for use in the cultivation tests according to the method described in Ishizawa *et al.* (2017). At first, three kinds of environmental waters were collected from the near-surface of freshwater environments located in the northern part of Osaka, Japan, on 27 May 2018. To test the influences of phylogenetically different bacterial communities in the inoculation experiments, the water samples were collected from different types of water bodies (P, stream; Q, pond; R, artificial pond). The properties of the water samples are shown in Supplementary Table S1, see online supplementary material. Larger particles including fungal and microalgal cells were removed by filtration (3.0 μm MF-Millipore filter; Millipore, Billerica, MA) and native bacterial communities within water samples were recovered by centrifugation (15 000 × *g*, 4°C, 5 min). The collected bacterial cells were washed once and re-suspended in the original volume of modified Hoagland medium, which was subsequently used for plant cultivation tests. The densities of recovered bacterial communities in the media were evaluated using a counting chamber (Erma, Tokyo, Japan). DNA samples of recovered bacterial communities were collected on 0.2-μm pore size filters and preserved at –80°C until further use.

Cultivation tests and inoculation of strain H3

Figure 1 illustrates the experimental scheme used for the cultivation tests. Cultivation tests were performed by transplanting 10 fronds of *L. minor* into 60 mL of the modified Hoagland medium containing the environmental bacterial communities. Inoculation of strain H3 to *L. minor* was done by three methods, herein referred to as 'large inoculation', 'small inoculation' and

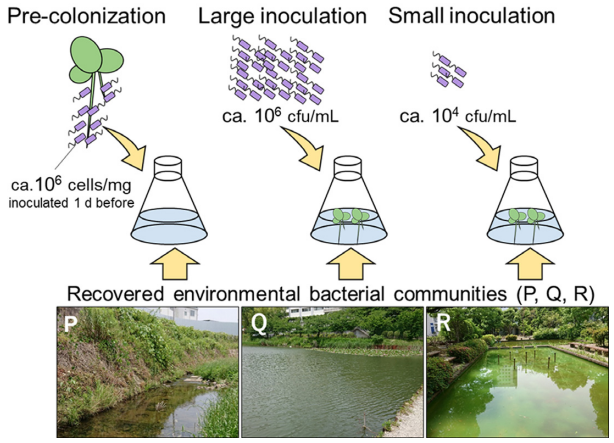

Figure 1. Experimental scheme of duckweed cultivation tests inoculating PGPB strain H3. Strain H3 was inoculated to duckweed by three methods in each of the culture media containing three kinds of environmental bacterial communities. We also performed control experiments without strain H3 inoculation, as well as experiments using axenic media (no environmental bacterial community).

'pre-colonization'. Large inoculation and small inoculation were performed by inoculating strain H3 to the culture media to give a final cell density of ~10^6 and 10^4 colony forming units (cfu) mL^{-1}, respectively, just before the start of experiments. For pre-colonization, *L. minor* was preliminarily co-cultured for 24 h with 10^6 cfu mL^{-1} of strain H3 in modified Hoagland medium, which allowed strain H3 cells to fully adhere to the plants (Ishizawa *et al.* 2019a). Subsequently, cultivation tests were initiated by transplanting the plants to the media containing the environmental bacterial communities.

Nine replicate flasks were prepared for each of the 12 cultivation tests inoculating strain H3 (bacterial communities P, Q, R or axenic media × three inoculation methods). For DNA analyses, plants from each of the three flasks were harvested after the cultivation periods of 1, 3 and 7 d, and immediately preserved at –80°C. Control experiments (bacterial communities P, Q, R or axenic media × without inoculation of strain H3) were also performed in triplicate flasks, and plant samples were collected after 7 d of cultivation. Plant growth speed was evaluated for all cultivation tests by counting the frond numbers after 7 d of cultivation. The growth-promoting effects of the inoculation of strain H3 are expressed as % increase of the frond number in comparison with corresponding control experiments.

DNA extraction

DNA extraction from environmental bacterial communities (filter samples) and duckweed surface bacterial communities (plant samples) were performed using Cica Geneus DNA extraction solution (Kanto Chemical, Tokyo, Japan). Each sample was immersed in 150 μL of extraction mixture and DNA was isolated according to the manufacturer's protocol.

Quantitative-PCR

SYBR Green quantitative-polymerase chain reaction (qPCR) was performed to quantify the amount of strain H3 cells colonized on *L. minor* using primers H3f and H3r (Ishizawa *et al.* 2019a) which were designed to amplify a strain-specific region of strain H3 occurring once in the genome (Table 1). The specificity of this primer pair was evidenced by no amplification of DNA samples extracted from control cultures. The qPCR analysis was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using GeneAce SYBR qPCR Mix α (Nippon Gene, Tokyo, Japan) and 0.25 μM each of forward and reverse primers. qPCR analysis for the quantification of total bacterial colonization was also performed using 0.25 μM of each 534r and 783r primer pair (Rastogi *et al.* 2010) which was designed to amplify bacterial 16S rRNA gene excluding plant organelle DNA (Table 1). The thermal cycling conditions were as follows: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 58°C (strain H3) or 53°C (bacterial 16S rRNA gene) for 1 min. Standard curves were generated with a known copy number of the DNA fragments cloned into the PMD20-T vector (Takara Bio Inc., Shiga, Japan).

Amplification of 16S rRNA gene and Illumina sequencing

Fifteen selected DNA samples were subjected to amplicon sequencing analysis for bacterial community profiling. Amplification of the V5–V6 region of the bacterial 16S rRNA gene was performed in a two-step PCR procedure. The first PCR was conducted with 5-tailed primers designed based on 799f (5-AACMGGATTAGATACCCKG-3; Chelius and Triplett 2001) and 1185mr (5'-GAYTTGACGTCATCCM-3'; Hodkinson and Lutzoni 2009). Barcoding was done by a second PCR with 0.5 μM of the index PCR primers (Illumina, San Diego, CA, USA). The DNA library was then subjected to sequencing on an Illumina Miseq platform (2 × 300 bp paired-end sequencing; Illumina) at the Bioengineering Lab. Co. Ltd (Kanagawa, Japan).

Processing of Illumina sequencing data

Raw sequence reads were demultiplexed and filtered using QIIME (version 1.9.1) based on the following criteria: (i) perfect match with primer sequences, (ii) quality score of >20, (iii) read length of >40. Next, high-quality reads were merged and chimera sequences were removed by the 'uchime' algorithm in QIIME, which generated 19 191–40 902 non-chimera sequences for each sample. *De novo* operational taxonomic units (OTU) clustering was performed for these reads using a 97% similarity cut-off, and phylogenic affiliation was assigned against Greengenes database (version 13.8).

Predicted metagenomic analysis

The abundance of functional genes in each bacterial community was predicted by phylogenetic investigation of communities by the reconstruction of unobserved states (PICRUSt) (Langille *et al.* 2013). For that purpose, non-chimera sequences were re-clustered into OTUs by the closed-reference method using the 'vsearch' algorithm in QIIME2 (version 2018.8) against the Greengenes database (version 13.5) with a similarity threshold of 97%. The obtained OTU table was subjected to PICRUSt using the 'themetagenomics' package in R (version 3.5.1) to obtain a functional table categorized based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologs. For gene categories (tier 3) whose mean relative abundance was >0.4%, the relative abundance in each community was normalized among nine bacterial communities, and the z-scores were plotted by the 'pheatmap' package in R. Clustering analysis for the predicted functional gene abundances was performed with the 'hclust' function. The abundance-weighted nearest sequenced taxon index (NSTI) was calculated for each bacterial community to estimate the accuracy of the functional prediction.

Table 1. Primer pairs used in qPCR analysis.

Primer	Sequences (5'–3')	Products of targeted genes	Amplicon length (bp)	Amplification efficiency (%)	Correlation coefficients (r^2)	Reference
H3f	TCAAGACAGCGGGCTTTTCA	Hypothetical protein	87	108.1–109.4	0.990–0.997	Ishizawa *et al.* (2019a)
H3r	ACAGGGATGGCAATATGCGAA					
534f	CCAGCAGCCGCGGTAAT	16S rRNA	281	109.7–117.0	0.983–0.988	Rastogi *et al.* (2010)
783r	ACCMGGGTATCTAATCCKG					

Statistical analyses

All statistical analyses were performed with R (version 3.5.1). For each set of experiments using the same bacterial community (P, Q, R and axenic media), the statistical significance of growth promotion by strain H3 was examined using one-way analysis of variance (ANOVA) with Dunnett's *post hoc* test. ANOVA was also performed to compare the effects of environmental communities P, Q and R on duckweed growth. Two-way ANOVA with the Tukey honestly significant difference (HSD) test was done to evaluate the effects of the co-existing bacterial community, inoculation methods and their interaction, on the colonization and growth-promoting effects of strain H3. Data on colonization density were log10-transformed before analysis. Enrichment or depletion of gene categories in duckweed-associated bacterial communities was examined by the Wilcoxon (Mann-Whitney U) test.

Alpha diversity of sequenced bacterial communities was evaluated as Shannon index. Beta diversity was ordinated by non-metric multidimensional scaling analysis (NMDS). Because some bacterial communities showed an extremely high proportion of strain H3, the ordination of all 15 bacterial communities was performed using Euclidean dissimilarity on the square root of the OTU's relative abundance data. In addition, for the nine bacterial communities obtained at the start and end of the cultivation, NMDS was conducted using unweighted UniFrac dissimilarity. NMDS was performed with the 'vegan' package in R and UniFrac distances were computed with the 'GUniFrac' package in R. To examine whether the original bacterial community or strain H3 inoculation affected the bacterial community composition, a two-way permutational multivariate ANOVA (PERMANOVA) was performed using the 'adonis' function of R with all the Euclidean, Bray–Cutis and weighted/unweighted-UniFrac distances. Weighted-UniFrac distance was also calculated to compare the phylogenetic distance among each of the three original bacterial communities and duckweed-associated bacterial communities after 7 d.

RESULTS

Growth promoting effects of strain H3

A PGPB strain H3 was inoculated to duckweed plants using three methods (pre-colonization, large inoculation and small inoculation) in the presence of three environmental bacterial communities (P, Q and R) that were obtained from different water environments. To eliminate the effects of abiotic factors on duckweed growth and PGPB performance, recovered bacterial communities P, Q and R were re-suspended in defined media and cultivation tests were performed in the same light and thermal conditions. Microscope counting showed that bacterial communities P, Q and R contained 0.55×10^6, 0.58×10^6 and 3.0×10^6 cells mL^{-1} at the start of the cultivation tests, respectively. Thus, we inoculated a similar amount ($\sim 10^6$ cfu mL^{-1}) and a considerably smaller amount ($\sim 10^4$ cfu mL^{-1}) of strain H3, which represented the large inoculation and the small inoculation methods, respectively.

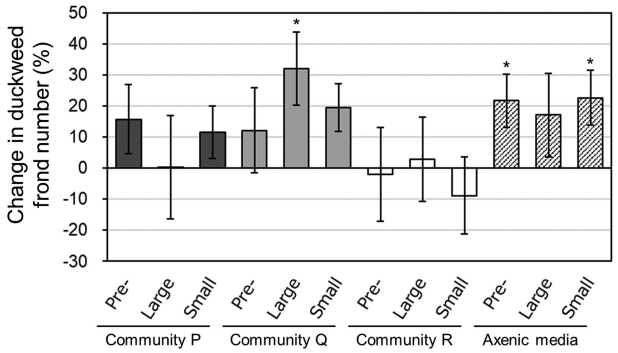

Figure 2. Growth-promoting effects of strain H3. Percent increase or decrease in duckweed frond number after 7 d from the corresponding control (without strain H3 inoculation) are shown. Significant growth promotion (Dunnett's *post hoc* test; $P < 0.05$) from the corresponding control experiment is denoted with an asterisk. Error bars show the standard deviations ($n = 3$).

We counted duckweed frond number after 7 d of cultivation and found that the bacterial communities of P, Q and R themselves affected duckweed growth by 4.3, –13.8 and 1.0%, respectively, compared to aseptic plants, though these differences were not statistically significant (one-way ANOVA, $P = 0.166$). These extents were comparable with the previous report of duckweed growth-promoting and -inhibiting effects of environmental bacterial communities (Ishizawa *et al.* 2017). The growth-promoting effect of strain H3 was evaluated in comparison with the corresponding control experiments, and we found that strain H3 improved average duckweed frond number in five out of nine cultivation tests by 11.7–32.1% (Fig. 2), which was similar to that observed under axenic conditions (17.1–22.7%). On the other hand, the other four cultivation tests showed no plant growth-promoting effects. Such a difference in PGPB performance seemed to be related to the kind of co-existing bacterial community rather than the inoculation method (Table 2).

Colonization of duckweed by strain H3 and total bacteria

Figure 3 shows the colonization densities of strain H3 and total bacteria on duckweed plants after 1, 3 and 7 d of cultivation. We found that the copy numbers of total bacterial 16S rRNA gene ranged between $\sim 10^6$ and 10^7 copies mg^{-1} of plant throughout the cultivation term. Strain H3 colonized the plant at between $\sim 10^5$ and 10^6 copies mg^{-1} of plant after 1 and 3 d, and decreased to between 10^4 and 10^5 copies mg^{-1} of plant after 7 d consistently

Table 2. Summary of two-way ANOVA analysis for the effects of co-existing bacterial community, inoculation method and their interaction on colonization and growth-promotion by strain H3. *F* values and degrees of significance are shown.

Variable	Community (C) df = 2	Method (M) df = 2	C × M df = 4
Growth promotion	8.161**	0.284	1.767
Colonization 1 d	10.30**	55.58***	3.783*
Colonization 3 d	3.882*	1.949	2.062
Colonization 7 d	0.039	0.123	1.663

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

in all nine cultivation tests. The colonization densities after 1 and 3 d were close to those in axenic media, indicating that strain H3 could successfully colonize *L. minor* at the beginning of the cultivation tests. The significant decrease in colonization density between 3 and 7 d is attributed to competition with other bacteria. This is because the decrease in colonization density was not observed in the cultivation tests involving no environmental bacterial communities. Among the three inoculation methods, large inoculation enabled higher colonization density of strain H3 after 1 d (Tukey HSD, $P < 0.001$; Table 2), though the differences from those of the other inoculation methods were within a few fold. Also, the kind of co-existing bacterial community had an effect on strain H3 colonization after 1 and 3 d (Table 2), and here, strain H3 was shown to colonize at higher densities in community Q than in community R (Tukey HSD, $P < 0.05$).

Compositions of bacterial communities formed on duckweed

For the cultivation tests in which strain H3 was inoculated using the large inoculation method, the composition of duckweed-associated bacterial communities was analysed by Illumina Miseq sequencing targeting the V5–V6 region of bacterial 16S rRNA genes. Similar to the results of qPCR, strain H3 (represented by OTU679) showed extremely high dominance after 1 and 3 d, but decreased after 7 d (Fig. 4). Reflecting this, the alpha diversity of duckweed-associated bacterial communities severely decreased after 1 and 3 d, and recovered to a level similar to the original bacterial communities (P0, Q0 and R0) after 7 d (Supplementary Fig. S2, see online supplementary material). It was also found that bacterial communities formed on duckweed after 7 d were quite similar to each other (Fig. 4; Supplementary Fig. S1 for OTU-level relative abundance, see online supplementary material), and neither the kind of original bacterial community nor strain H3 inoculation had a significant effect on the community composition (PERMANOVA, $P > 0.05$). These trends were clearly represented by the NMDS ordination, which showed synchronous community shifts in all the three cultivation tests (Fig. 5A). Another NMDS ordination based on UniFrac dissimilarity, which incorporates phylogenetic distance of community members, also confirmed the similarity of duckweed-associated bacterial communities assembled after 7 d (Fig. 5B). Comparison of weighted Unifrac distance showed that community Q0 was on average more distant from those associated with duckweed, compared to the communities P0 and R0 (Supplementary Fig. S3, see online supplementary material).

In terms of the phylogenetic composition of duckweed-associated bacterial communities, alphaproteobacteria and betaproteobacteria accounted for >75% of the entire population of bacterial communities on the duckweed surface. At bacterial family level (Fig. 4A), Caulobacteraceae, Commamonadaceae and Methylophylaceae are the most predominant bacterial taxa, followed by Oxalobacteraceae, Rhizobiaceae and Sphingomonadaceae (Fig. 4B). Flavobacteriaceae, Hyphomonadaceae, Microbacteriaceae and Rhodocyclaceae were recognized as some of the dominant families in certain communities, but their abundances were quite unstable compared to the other dominant families.

Functional prediction of bacterial communities based on 16S rRNA gene sequences

The abundance of functional genes in sequenced bacterial communities was predicted from their 16S rRNA gene sequences by the PICRUSt pipeline. The accuracy of functional prediction by PICRUSt can be evaluated by the NSTI, which represents a weighted average of phylogenetic distances between OTUs and their nearest sequenced relative. Langille *et al.* (2013) reported that PICRUSt yielded similar results to those of shotgun metagenome analyses for soil bacterial communities with an average NSTI of 0.17. In the present study, the NSTI for each bacterial community ranged from 0.044 to 0.123 (Supplementary

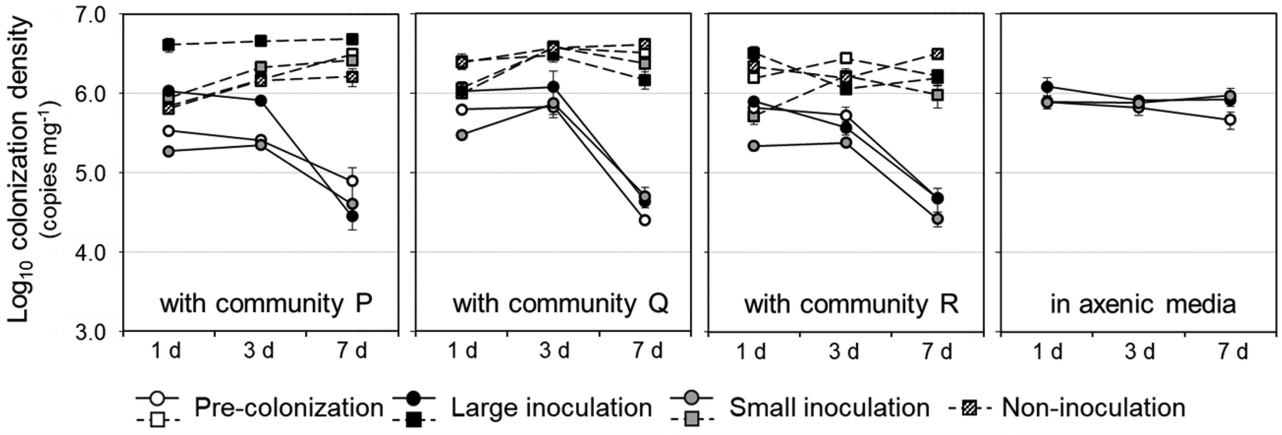

Figure 3. Colonization densities of strain H3 (circles) and total bacteria (squares) on *L. minor* during the cultivation tests. Results of quantitative-PCR targeted on a specific gene of strain H3 and bacterial 16S rRNA gene are shown. Error bars show the standard deviations ($n = 3$).

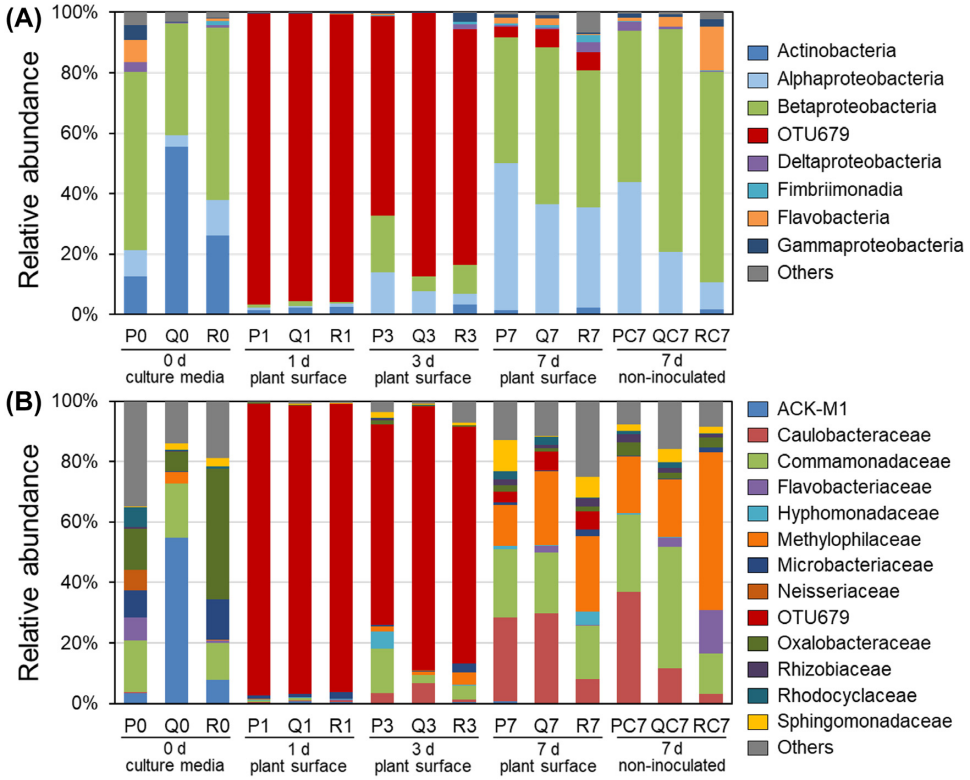

Figure 4. Taxonomic distribution of bacterial communities. (**A**) Relative abundances of bacterial class. (**B**) Relative abundances of bacterial family. OTU679, with representative sequence identical to the gene sequence of *A. magnusonii* H3, is independently shown in red. P0, Q0 and R0 are the original bacterial communities in the culture media (before inoculation of strain H3). P1, Q1, R1, P3, Q3, R3, P7, Q7 and R7 are phytosphere bacterial communities on 1, 3 and 7 d after strain H3 inoculations. PC7, QC7 and RC7 are phytosphere bacterial communities after 7 d without inoculation of strain H3.

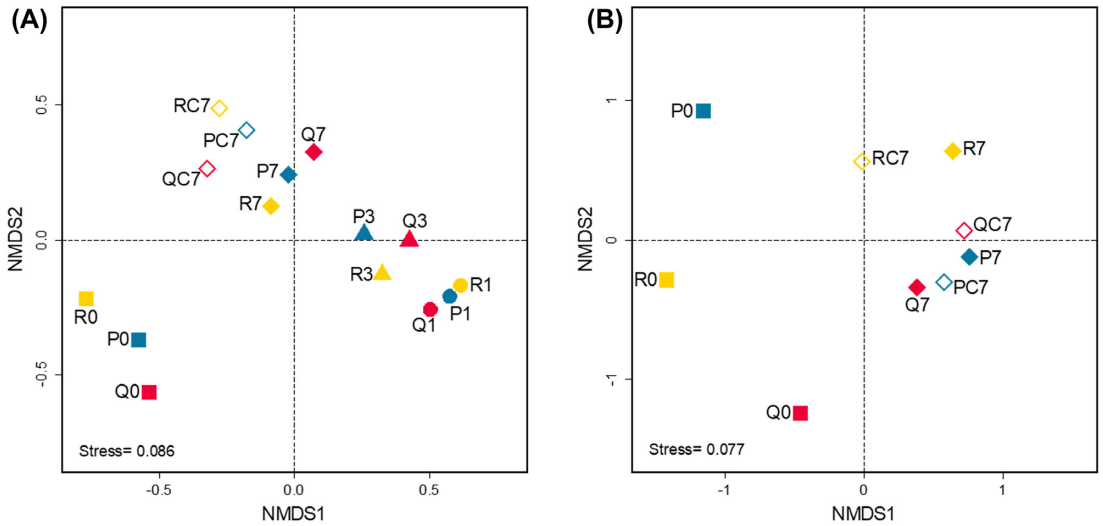

Figure 5. Structure of bacterial community composition. (**A**) NMDS using Euclidean dissimilarity based on the square root of proportional data. (**B**) NMDS based on unweighted UniFrac dissimilarity. Refer to Fig. 4 for a description of the labels.

Table S2, see online supplementary material), indicating the reliability of our functional prediction.

Figure 6 shows the predicted functional properties of original bacterial communities in media (P0, Q0 and R0) and duckweed-associated bacterial communities after 7 d (P7, Q7, R7, PC7, QC7 and RC7). The clustering results in Fig. 6 showed that duckweed-associated bacterial communities had differential functional properties compared to the original bacterial communities, whereas the inoculation of strain H3 had no consistent influence. Five gene categories were consistently more abundant on duckweed surface (bacterial chemotaxis, two-component system, flagellar assembly, bacterial secretion system and biofilm formation–*Vibrio cholerae*; Wilcoxon test, $P < 0.05$). The results on finer gene classification (Supplementary Table S3, see online supplementary material) showed that the increased abundance of the 'two-component system' reflected the increase in receptor

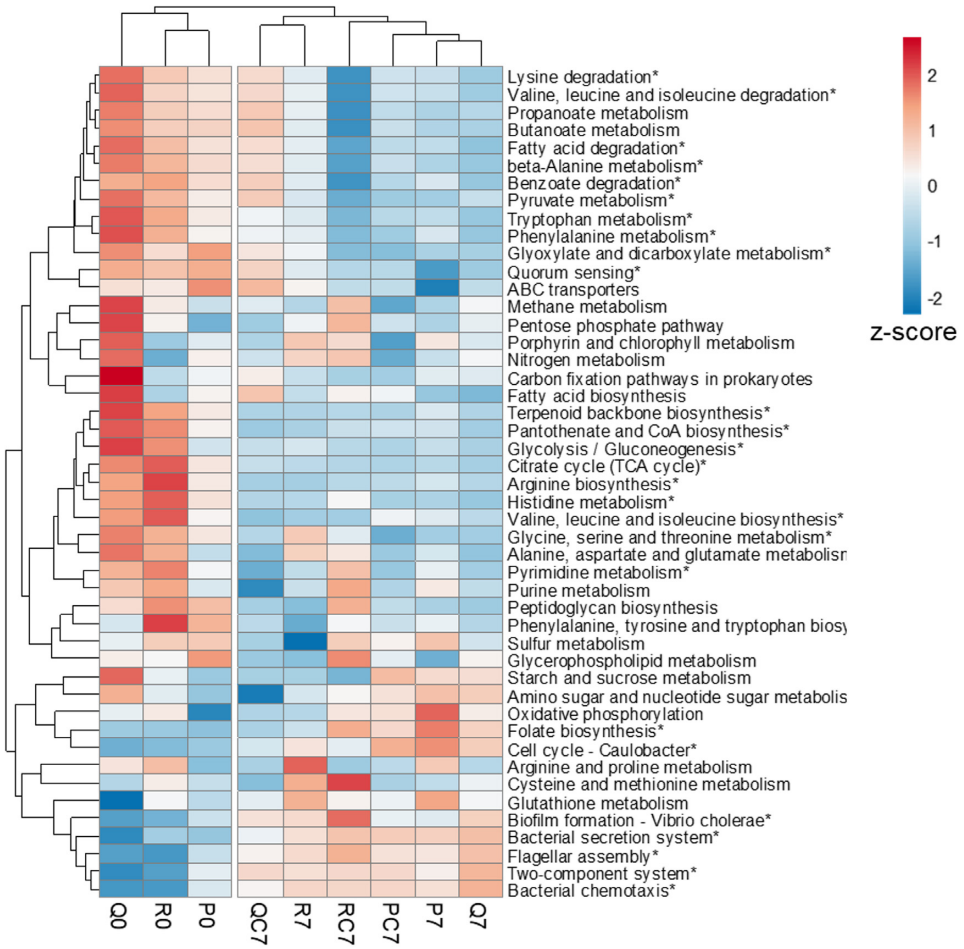

Figure 6. Functional properties of bacterial communities predicted by PICRUSt. Relative abundance of gene categories was compared among the bacterial communities and the z-scores are shown. Refer to Fig. 4 for a description of the labels. An asterisk indicates significant enrichment or depletion of the gene category in the phytosphere of duckweed (Wilcoxon test, $P < 0.05$).

and signal transduction genes for bacterial chemotaxis. 'Biofilm formation–*Vibrio cholerae*' mainly consisted of genes related to extracellular polysaccharide and type IV pilus biogenesis (Supplementary Table S3). Similarly, genes related to the type III and type IV secretion systems in the 'Bacterial secretion system' were especially enriched in duckweed-associated bacterial communities. We also found that genes involved in the degradation of xenobiotic compounds such as nitrotoluene and naphthalene were also consistently enriched on duckweed surface (Supplementary Table S3; Wilcoxon test, $P < 0.05$), which supports the previous reports of enhanced aromatic compound degradation in the phytosphere of duckweed (Toyama *et al.* 2006; Ogata *et al.* 2013). In contrast, the majority of gene categories related to amino acid and carbon metabolism showed significantly lower relative abundances in duckweed-associated bacterial communities (Fig. 6), probably due to the increase in the aforementioned gene categories. Community Q7 and QC7, both of which originated from the same community, showed markedly different functional profiles, while the causality of this change (e.g. in relation to H3 inoculation or growth improvement) could not be clarified.

DISCUSSION

Efficacy of strain H3 as PGPB in the presence of environmental bacterial communities

The present results demonstrated that strain H3 could colonize on duckweed at high abundance even in the presence of the environmental bacterial community, and thereby improves duckweed growth in a few cases (Figs 2 and 3). Our previous studies used large amounts of bacterial cells ($OD_{600} \geq 0.1$) for plant colonization and duckweed growth promotion by a PGPB, *A. calcoaceticus* P23 (Toyama *et al.* 2017; Ishizawa *et al.* 2020), and the minimum amount required for inoculation remains to be determined. It is shown that strain H3 could function with equal or lower inoculation density compared with environmental bacterial communities (10^4–10^6 cfu mL^{-1}; $OD_{600} = 0.0001$–0.01). The cost of preparing the PGPB inoculum is one of the concerns regarding its application in large hydroponic systems (Ishizawa *et al.* 2020). Here, our results suggest the possibility of reducing the cost of the inoculum by several orders of magnitude by using PGPB strains with efficient colonization ability.

Strain H3 could not maintain its full colonization density for a long period, which is possibly the major reason for unsuccessful growth promotion in four out of nine cultivation tests. Colonization density decreased sharply in all nine inoculation experiments, and the insufficient colonization density over the 7-d cultivation was suggested to be the major reason for the unstable growth-promotion effect. The successful growth-promotion observed in community Q could be related to the relatively high colonization density of strain H3 for at least 3 d (Figs 2 and 3; Table 2), confirming the importance of PGPB colonization for its functioning. The decrease in colonization density was due to the strong resilience of the duckweed microbiome which can return to a similar equilibrium state within 7 d (Figs 4 and 5). Rapid budding-based multiplication of duckweed might also act to reduce or dilute the colonization density of strain H3 during cultivation. Considering these observations, the stabilization of PGPB for a long period may not be easy, even when using a highly competitive strain. Thus, repeated inoculation, probably around once per week, would be one rational strategy when using microbial inoculants for duckweed (Ishizawa *et al.* 2020).

Although the reason why strain H3 colonized and functioned more efficiently in the presence of community Q was not fully clarified in this study, our results provide some possible explanations as follows. Among the three communities, community Q was the most distant from those associated with duckweed in terms of phylogenetic structure (Supplementary Fig. S3) and functional properties (Fig. 6). From these facts, it can be inferred that community Q was overall less competitive in the duckweed phytosphere in the beginning; hence, it allowed a high colonization density of strain H3 than the other communities. The relevance of resident community structure and invasion success has often been emphasized in microbial ecology (Kinnunen *et al.* 2016; van Elsas *et al.* 2012; Vila *et al.* 2019). In this context, we suggest that the diversity of the native community, in particular phylogenetic/functional distances to plant-associated ones rather than simple alpha diversity (Supplementary Fig. S2), are important for the inoculation success of PGPB. In addition, community Q itself was inhibitory for duckweed growth, reducing the frond number by –13.8%, as compared with the control experiment. In the past decades, an increasing number of PGPB strains, including strain H3, were reported to exert more significant promotive effects under adverse conditions for host plants (Kumar and Verma 2018; Ishizawa *et al.* 2019b). Thus, such stress-alleviating effects may also help explain the successful growth-promoting effects in community Q.

In our results, interestingly, inoculation methods had only a limited or transient effect on colonization, persistence and growth-promotion by strain H3, even though markedly different amounts of cells were used in the large and small inoculation methods (Figs. 2 and 3; Table 2). This was a somewhat unexpected result because inoculation with higher cell density is believed to increase the performance of microbial inoculants (Hwang *et al.* 2011; Shabir *et al.* 2016), and preliminary inoculation is regarded as an effective strategy to stabilize microbial inoculants (Mahmood *et al.* 2016) according to studies conducted on terrestrial plants. These conflicting results suggest that bacterial duckweed colonization may be governed more by the competitive colonization ability of each bacterium rather than its initial abundance or order of arrival (priority effect), which is consistent with previous observations made for two-membered artificial bacterial communities (Ishizawa *et al.* 2019a).

Resilience and plasticity of duckweed-associated bacterial communities

This study showed that similar bacterial communities are robustly formed on duckweed regardless of the composition of the original bacterial communities or strong disturbance caused by the PGPB inoculation (Figs 4, 5, and Supplementary Fig. S1). The overall community composition was less similar to those in the rhizosphere and phyllosphere of well-studied terrestrial plants such as *Arabidopsis thaliana*, *Zea mays* (Lundberg *et al.* 2012; Niu *et al.* 2017) and the floating macrophyte *Eichhornia crassipes* (Ávila *et al.* 2019), in that there are considerably fewer Gammaproteobacteria, Deltaproteobacteria and Actinobacteria on the surface of *L. minor*. However, the majority of the dominant bacterial families were common to the phyllosphere bacterial communities of a rootless duckweed *Wolffia australiana* (Xie, Su and Zhu 2015), suggesting that core microbial taxa are highly preserved within the duckweed family (Lemnaceae).

Although the composition of the duckweed-associated bacterial communities was generally stable, it should be noted that there is a marked difference in the abundance of some bacterial families among the communities. For example, the relative abundance of family Flavobacteriaceae in duckweed-associated bacterial communities after 7 d ranged from <0.5% (communities P7, R7 and PC7) to >14% (community RC7), without a simple relationship to the original bacterial community and inoculation of strain H3 (Fig. 4B). It is well known that abiotic factors such as climate and fertilization can induce such differences in the plant microbiome (Na *et al.* 2018; O'Brien *et al.* 2018). However, that possibility can be ruled out because this study was performed under strictly controlled culture conditions. Thus, these observations suggest that biotic regulation of the duckweed microbiome is flexible rather than deterministic despite the strong resilience of the overall community composition.

Recently, increasing evidence has emphasized the role of duckweed-associated bacteria in host growth and nutrient cycling in aquatic environments (Stout and Nüsslein 2010; Ogata *et al.* 2013; Xie *et al.* 2014; Sun *et al.* 2016; Zhao *et al.* 2018; Iguchi *et al.* 2019). Researchers are also trying to utilize duckweed as a model plant to study plant–microbial interactions (Fan *et al.* 2012; Appenroth, Ziegler and Sree 2017b, Yamakawa *et al.* 2018). Considering these situations, the resilience and plasticity of the duckweed microbiome observed in this study should be an important starting point for both future basic and applied studies.

Functions presumably enriched in duckweed-associated bacterial communities

To understand the mechanisms behind the robust community assembly on duckweed surface, we tried to uncover functional aspects of duckweed-associated bacterial communities by a PICRUSt pipeline, and identified five gene categories that were consistently enriched on the duckweed surface (Fig. 6). The increased relative abundances of 'flagellar assembly', 'bacterial chemotaxis' and 'two-component system' suggest that flagellar motility towards plant exudate plays important roles in bacterial duckweed colonization, as reported for rhizobacteria of terrestrial plants (Broek, Lambrecht and Vanderleyden 1988; Allard-Massicotte *et al.* 2016). This is quite interesting because the range of bacterial movement and plant exudate diffusion would be far larger in aqueous environments than in soils. Hence, the results imply the possibility that long-distance chemotactic travel is involved in the assembly of the duckweed microbiome. Besides,

another two enriched gene categories, 'biofilm formation–*Vibrio cholerae*' and 'bacterial secretion system', are considered important for the attachment of terrestrial rhizobacteria to plants (Barret, Morrissey and O'Gara 2011). Bacteria would easily be washed off in aqueous phase unless firmly adhered, and this might reflect the importance of surface adherence systems for the persistence of duckweed-associated bacteria. Collectively, several gene functions required for bacterial colonization of terrestrial plants were suggested to confer selective advantages on duckweed-associated bacteria, while their detailed mode of contribution is perhaps different.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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