Isolation and Characterization of Novel Plant Growth-Promoting Bacteria from the Fronds of Duckweed

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Abstract

To obtain novel plant growth-promoting bacteria that inhabit duckweed fronds, 25 bacterial strains isolated from the fronds of three duckweed species were subjected to two-level screening, whereupon strain SP-2-C10 was selected as the most effective plant growth-promoting bacterium for the common duckweed *Lemna minor*. As the first-ever plant growth-promoting bacterium isolated from duckweed fronds, strain SP-2-C10 was found to belong to the rarely reported and taxonomically novel genus *Aquidulcibacter* (Class: *Alphaproteobacteria*). In an aseptic medium, this strain increased the growth of *L. minor* by 3.6-fold that of the bacteria-free plant. It even increased the growth of *L. minor* by 2.1-fold in a non-sterile secondary sewage effluent. Strain SP-2-C10 colonized the fronds and roots of *L. minor* at almost similar cell densities under sterile conditions. By contrast, under non-sterile conditions (sewage effluent), the strain colonized the fronds more strongly than it did the roots, which was likely due to its isolation source—the fronds. This result might be useful information for constructing a functional water purification and/or biomass production system using duckweeds, whose functions were enhanced by inoculating useful microbes such as PGPB.

Key Words: duckweed, frond, plant growth-promoting bacteria, *Aquidulcibacter*

INTRODUCTION

Lemnoideae, commonly known as duckweed, is a subfamily of floating aquatic plants that are generally made up of two parts: fronds (fusion of the leaf and stem) and roots. Since duckweed are fast growing and easy to collect, they have been used for the removal of nutrients (nitrogen and phosphorus) from

wastewater¹⁾⁻⁶⁾. Duckweed also possess the ability to stimulate the microbial degradation of harmful recalcitrant organic compounds (e.g., nitrophenols, bisphenols, and nonylphenols) in their rhizosphere⁷⁾⁻⁹⁾. Additionally, because duckweed grown under optimal conditions contain a high ratio of starch (ranging from 40% to 65%) and protein (ranging from 15% to 45%)¹⁰⁾⁻¹²⁾, they can potentially be used as

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a biofuel (bioethanol and biogas) or feedstuff resource^{10),13)}. The microbes associated with duckweed are a key factor for enhancing the water purification ability and biomass productivity of the plants. Indeed, previous studies have reported the isolation of pollutant-degrading microbes and plant growth-promoting bacteria (PGPB) from the rhizosphere of duckweed and demonstrated how their re-inoculation into the rhizosphere or whole plant bodies of the host duckweed leads to enhancements in pollution removal and plant biomass productivity^{8),9),14),15)}.

Acinetobacter calcoaceticus strain P23, which was the first plant growth-promoting bacterial strain isolated from the whole body of *Lemna aoukikusa*, almost doubled the biomass productivity of this duckweed species^{14),16)}. To date, over 15 strains of PGPB belonging to the genera, such as *Pseudomonas*, *Acinetobacter*, *Sphingomonas*, *Azospirillum*, and *Aeromonas* have been isolated from duckweed roots or whole plant bodies^{14),15),17)}. However, to the best of our knowledge, PGPB have never been isolated from the fronds of duckweeds. We have previously reported that the microbial communities in the fronds of three species within the subfamily *Lemnoideae* (viz., *Spirodela polyrhiza*, *Lemna minor*, and *Lemna aequinoctialis*) were distinct from those growing in the roots¹⁸⁾, suggesting that the plant part (i.e., fronds or roots) is an important factor to consider when screening for key PGPB that will enhance the functional abilities of duckweed. Moreover, the fronds and roots of duckweed harbor a wide variety of taxonomically novel bacteria (i.e., showing less than 97% of 16S rRNA gene sequence similarity to known type species), including the rarely cultivated phyla *Acidobacteria*, *Armatimonadetes*, and *Verrucomicrobia*¹⁸⁾. If some of these taxonomically novel and rarely cultivated bacteria are PGPB, they might possess novel plant growth-promoting factors. Therefore, in this study, we aimed to identify novel PGPB using a bacterial library of taxonomically novel strains that we had previously isolated from the fronds of *L. minor*, *L. aequinoctialis*, and *S. polyrhiza*¹⁸⁾.

MATERIALS AND METHODS

Plant materials The plant *L. minor* was cultured aseptically in plant cultivation pots (420 mL; 70-mm diameter; IWAKI, Tokyo, Japan) containing 200 mL of Hoagland medium (36.1 mg/L KNO₃, 293 mg/L K₂SO₄, 3.87 mg/L NaH₂PO₄, 103 mg/L MgSO₄·7H₂O, 147 mg/L CaCl₂·H₂O, 3.33 mg/L FeSO₄·7H₂O, 0.95 mg/L H₃BO₃, 0.39 mg/L MnCl₂·4H₂O, 0.03 mg/L CuSO₄·5H₂O, 0.08 mg/L ZnSO₄·7H₂O, and 0.254 mg/L H₂MoO₄·4H₂O; pH 7.0). The pots were kept in a growth chamber at 25°C (16,000 lux; 16:8 h light: dark cycle).

Taxonomically novel bacterial strains An in-house bacterial library was used to source for strains that could grow rapidly and easily on R2A agar. The library was composed of taxonomically novel bacterial strains obtained from the fronds of the three duckweed species (*L. minor*, *L. aequinoctialis*, and *S. polyrhiza*), including strains that had been isolated in our previous study¹⁸⁾. Within this library, 25 strains were determined to be suitable for testing in this study (Table 1).

First screening for PGPB Each novel bacterial strain was cultured on an R2A plate at 25°C for 7 days, following which the cells were suspended in 40 mL of Hoagland medium (final OD₆₆₀ = 0.1) in a plant culture tube (150 mL; 40-mm diameter; IWAKI). Next, one aseptic *L. minor* plant, which is commonly used for plant growth promotion testing^{15)–17)}, was placed in the tube for co-culture with the bacterial strain. The tube was incubated in a growth chamber at 25°C (16,000 lux; 16:8 h light: dark cycle) for 14 days (*n* = 1 for each bacterial strain), after which the number of fronds was counted. As a negative control, an aseptic (bacteria-free) *L. minor* plant was also cultured in the same medium in the same growth chamber. The plant growth-promoting effect (PGPE) was evaluated as the ratio of the number of fronds on the co-cultured plant to that on the negative control plant. Strains showing a PGPE ratio of greater than 2.5 were passed the first screening.

Second screening for PGPB The strains that had passed the first screening test were subjected to a second more thorough screening of their plant growth-promoting abilities. In

Table 1 Taxonomically novel bacterial strains isolated from the fronds of duckweed based on 16S rRNA gene sequences (as of January 2015)

Strain	Closest species	Phylum (Class)	Similarity (%)	Compared length (bp)	Isolation source	Accession number
SP-L9*	<i>Hyphomicrobium hollandicum</i>	Proteobacteria (Alpha)	92	780	<i>S. polyrhiza</i>	LC586318
SP-L7A7*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	748	<i>S. polyrhiza</i>	LC586319
SP-L6	<i>Flavitalea gansuensis</i>	Bacteroidetes	94	742	<i>S. polyrhiza</i>	LC586320
SP-L45D9*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	93	796	<i>S. polyrhiza</i>	LC586321
SP-L38D2*	<i>Paucimonas lemoignei</i>	Proteobacteria (Beta)	94	703	<i>S. polyrhiza</i>	LC586322
SP-L18*	<i>Hydromonas duriensis</i>	Proteobacteria (Beta)	94	813	<i>S. polyrhiza</i>	LC586323
SP-L15B3*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	784	<i>S. polyrhiza</i>	LC586324
SP-2-C10*	<i>Phenylobacterium kunshanense</i>	Proteobacteria (Alpha)	90	761	<i>S. polyrhiza</i>	LC594658
LM-L39G5*	<i>Methylibium petroleiphilum</i>	Proteobacteria (Beta)	94	833	<i>L. minor</i>	LC586325
LM-L37E5	<i>Opitutus terrae</i>	Verrucomicrobia	93	831	<i>L. minor</i>	LC586326
LM-L29E4	<i>Opitutus terrae</i>	Verrucomicrobia	93	831	<i>L. minor</i>	LC586327
LM-L2*	<i>Deinococcus radiotolerans</i>	Deinococcus-Thermus	93	780	<i>L. minor</i>	LC586328
LM-L18B3*	<i>Methylibium petroleiphilum</i>	Proteobacteria (Beta)	94	830	<i>L. minor</i>	LC586329
LM-L49A7	<i>Methylovirgula ligni</i>	Proteobacteria (Alpha)	94	793	<i>L. minor</i>	LC586330
LA-L44D6*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	748	<i>L. aequinoctialis</i>	LC586331
LA-L33A5*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	784	<i>L. aequinoctialis</i>	LC586332
LA-L30F4*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	93	796	<i>L. aequinoctialis</i>	LC586333
LA-L26B4	<i>Flavitalea gansuensis</i>	Bacteroidetes	95	838	<i>L. aequinoctialis</i>	LC586334
LA-L24H3*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	796	<i>L. aequinoctialis</i>	LC586335
LA-L19C3*	<i>Methylibium petroleiphilum</i>	Proteobacteria (Beta)	94	833	<i>L. aequinoctialis</i>	LC586336
LA-L10B2*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	748	<i>L. aequinoctialis</i>	LC586337
LA-34B5*	<i>Hyphomicrobium nitrativorans</i>	Proteobacteria (Alpha)	92	748	<i>L. aequinoctialis</i>	LC586338
LA-3-E1*	<i>Phyllobacterium trifolii</i>	Proteobacteria (Alpha)	93	701	<i>L. aequinoctialis</i>	LC594659
LA-3-B1*	<i>Phyllobacterium trifolii</i>	Proteobacteria (Alpha)	93	701	<i>L. aequinoctialis</i>	LC594660
LA-3-B2*	<i>Phyllobacterium trifolii</i>	Proteobacteria (Alpha)	93	701	<i>L. aequinoctialis</i>	LC594661

*The closest species and similarity of the strains are changed now along with updating the 16S rRNA database.

this second screening level, the inoculation concentration of bacterial cells was $OD_{660} = 0.05$, which was half of that used in the first screening, and the PGPE test was conducted in triplicate. Except for these differences, the co-cultivation conditions were the same as those for the first screening. The strains showing a PGPE ratio of greater than 2.0 were considered to have passed the second screening level. Additionally, *A. calcoaceticus* strain P23 was tested as a positive control. This strain, which was isolated from the whole body of *L. aoukikusa*, was reported as a plant growth-promoting bacterium for duckweed^{14), 16)}. From this second screening, strain SP-2-C10 was selected as the best plant growth-promoting bacterium.

Indole-3-acetic acid production The ability of strain SP-2-C10 to produce indole-3-acetic acid (IAA) was evaluated according to a

previously described method¹⁹⁾, with some modifications. The bacterial cells were dispensed ($OD_{660} = 0.05$) into each well of a 96-well microplate containing 500 μ L of R2A medium supplemented with 0.5% (w/v) L-tryptophan, and the plate was then incubated for 2 days at 25°C with shaking (550 rpm). Thereafter, 500 μ L of the culture broth was centrifuged (20°C, 1,000 rpm, 5 min), and 450 μ L of the resultant supernatant was mixed with 600 μ L of Salkowski reagent (made up of 98 mL of 35% $HClO_4$ and 2 mL of 0.5 M $FeCl_3$) and the mixture was incubated for 30 min at 25°C. The formation of a pink to red color would indicate that the bacterium produced IAA.

Siderophore production The ability of the strain to produce siderophores was determined using the chrome azurol S (CAS)-agar plate assay²⁰⁾. In brief, a SP-2-C10

colony growing on R2A agar was streaked onto the CAS agar and the plate was then incubated for 7 days at 25°C in the dark. The formation of a yellow to orange halo around each resultant colony would indicate that the bacterium produced siderophores.

Phosphate-solubilizing activity The ability of the strain to solubilize phosphate was measured using Pikovskaya's agar plate test²¹⁾. In brief, a SP-2-C10 colony growing on R2A agar was streaked onto Pikovskaya's agar and the plate was then incubated for 7 days at 25°C. The formation of a clear zone around each resultant colony would indicate that the strain had solubilized the phosphate in the agar.

PGPE of strain SP-2-C10 at different cell inoculation concentrations The cell inoculating concentration at which strain SP-2-C10 shows effective PGPE was tested using concentrations of OD₆₆₀ = 0.01, 0.03, 0.1, and 0.3, with the PGPE test conducted in triplicate. All other conditions of co-cultivation were the same as those used for the first screening.

PGPE in the secondary sewage effluent Ten *L. minor* plants were added to 40 mL of Hoagland medium in a plant culture tube containing strain SP-2-C10 (OD₆₆₀ = 0.3), and co-culture was carried out for 3 days in the growth chamber described above. Strain P23 was co-cultured with the duckweed in a similar way as a positive control. After the cultivation period, one plant from the co-culture was transferred to 40 mL of a secondary effluent sample in a 150-mL tube. This sewage sample had been collected from a municipal wastewater treatment plant in Kofu city, Yamanashi, Japan. As a negative control, an aseptic *L. minor* plant was also cultured in the same secondary sewage effluent. After culturing the *L. minor* plant in the sewage sample for 14 days in the growth chamber, the PGPE was evaluated.

Test of strain SP-2-C10 colonization on the fronds and roots of duckweed Strain SP-2-C10 was inoculated (OD₆₆₀ = 0.1) into two plant culture tubes containing 40 mL of Hoagland medium. Then, 15 *L. minor* plants were placed in each tube (total 30 plants) and incubation of the co-cultures was carried out in the growth chamber at 25°C. After 3 days of incubation, five of the inoculated

plants were transferred to new plant culture tubes containing 40 mL of Hoagland medium or secondary sewage effluent (two tubes for each medium were prepared) and cultivated for 3 or 7 days in the growth chamber. Each of the five plants from the bacterial inoculation and co-cultivation steps were gently washed twice with 10 mL of Hoagland medium in a 15-mL conical tube. After the washing steps, a sterilized scalpel was used to cut off the fronds and roots. Total DNA was then extracted from these plant parts using the Cica Geneus DNA Extraction Reagent (Kanto Chemical Co., Tokyo, Japan) and purified using a Zymo-Spin column I (Funakoshi, Tokyo, Japan). The purified DNA was then amplified with the SYBR Green-based real-time polymerase chain reaction (qPCR) using the primer sets SP-2-C10 F1 (5'-AGCCCATACCGCGGTGTGAT-3') and SP-2-C10 R1 (5'-TCGCCGTTTTGACG GGCTTC-3'), which were designed to amplify a specific 156-bp part of the RNA polymerase subunit beta (*rpoB*) gene of strain SP-2-C10 (amplification efficiency based on the standard curve was 97%). The thermal cycling conditions used were as follows: 95°C for 10 s, followed by 35 cycles of 94°C for 15 s, 57°C for 30 s, and 72°C for 20 s. Ten-fold serial dilutions (1.27×10^3 to 1.27×10^7 copies/ μ L) of amplicons targeting the *rpoB* gene of SP-2-C10 (388 bp) were used as standards and had been amplified with the universal primer sets *rpoB* 1675 (5'-TGYCCGATYGAAACACC KGARGG-3') and *rpoB* 2063 (5'-TGACGYTG CATGTTCGMNCCCAT-3'), using the same thermal cycling conditions described in previous studies^{22), 23)}. To estimate the surface areas of the fronds and roots, images of *L. minor* plant samples were taken through a stereomicroscope and analyzed using ImageJ software (<https://imagej.nih.gov/ij/>).

RESULTS AND DISCUSSION

First screening for PGPB Twenty-five taxonomically novel strains (Table 1) isolated from the fronds of three duckweed species (*L. minor*, *L. aequinoctialis*, and *S. polyrhiza*) were subjected to the first screening test for PGPB. Five strains (*viz.*, LA-L26B4, SP-2-C10, LA-L10B2, LA-L33A5, and LA-L24H3) elevated the growth of *L. minor* by

more than 2.5-fold that of the aseptic plant, with strain LA-L26B4 showing the highest PGPE (3.7-fold) (Fig. 1). These five strains were therefore selected for the second screening.

Among the 25 strains examined, only two strains (*i.e.*, LM-L29E4 and LM-L37E5) showed an inhibitory effect on the growth of the plants (0.6- to 0.8-fold). These two strains were members of *Verrucomicrobia*, a rarely cultivated bacterial phylum. Although the members of *Verrucomicrobia* are known to be widely distributed in nature^{24–27} and abundant in the fronds and roots of duckweed^{18), 28)}, how they interact with the plants and whether they affect the health and growth of the plants remain unclear. Therefore, our results provide an insight into the relationship (positive, neutral, or negative effect for the growth of plants) between the *Verrucomicrobia* and duckweeds.

Second screening for PGPB The five strains selected through the first screening process were subjected to a more thorough second screening of their PGPEs. Among them, only strain SP-2-C10 met the criterion for passing this screening step; that is, a 2.0-fold increase in PGPE, almost the same level as that of *A. calcoaceticus* strain P23 (Fig. 2). Although the high SD value of PGPE for strain SP-2-C10 was observed in the experiment, this strain showed the stable PGPEs with low SD values in the after described experiment (see below; Fig. 3).

Fig. 1 Plant growth-promoting effect (PGPE) of 25 taxonomically novel bacterial strains isolated from the fronds of three species of duckweeds: *Spirodela polyrhiza*, *Lemna minor*, and *Lemna aequinoctialis* ($n = 1$ for each bacterial strain).

Therefore, strain SP-2-C10 was selected for further studies of its effectiveness in enhancing the functional activities of duckweed.

At the time of this experiment, strain SP-2-C10 (belonging to the class *Alphaproteobacteria*) was determined to be a phylogenetically novel bacterium at the genus level based on its 16S rRNA gene sequence similarity to other strains, where it showed a relatively low sequence similarity of 90% with its most closely related species, *Phenylobacterium kunshanense*. However, since that time, another bacterial strain has been isolated from cyanobacterial aggregates and proposed

Fig. 2 Plant growth-promoting effect (PGPE) of five bacterial strains that passed the first screening and *A. calcoaceticus* strain P23 at the low bacterial concentration ($n = 3$). Error bars represent the standard deviation ($\pm$ SD).

Fig. 3 Plant growth-promoting effect (PGPE) of strain SP-2-C10 inoculated at four different bacterial cell concentrations ($n = 3$). Error bars represent the standard deviation ($\pm$ SD).

as a novel genus and species (*Aquidulcibacter paucihalophilus* strain TH1-2^T)²⁹⁾; the 16S rRNA gene sequence of this species has 100% identity with that of strain SP-2-C10. To date, no other bacterial species or strains belonging to the new genus *Aquidulcibacter* have been isolated and there are no reports indicating its ecological roles though the organism perhaps may be associated with cyanobacteria to some extent. Therefore, the data obtained in this study would be useful for speculating the functions of this microbial group in nature, especially in aquatic environments. We speculate that at least some of the members of this genus might construct a beneficial relationship with duckweed in supporting plant growth.

Characterization of strain SP-2-C10 The plant growth-promoting factors (siderophore production, IAA production, and phosphate solubilization) of strain SP-2-C10 were examined. The strain showed positive siderophore production and phosphate solubilization activities, but no IAA production activity was detected (data not shown). Since duckweed can take up dissolved phosphate from the Hoagland medium or sewage effluent, phosphorus solubilization is unlikely to be involved in the bacterium's PGPE in these aquatic plants. Instead, siderophore production or other known (e.g., NH_3 production, ACC deaminase production, and N_2 fixation) or unknown plant growth-promoting factors might contribute to the PGPE of this strain.

Figure 3 shows the effects of different cell inoculation concentrations of SP-2-C10 on its PGPE in duckweed, where an OD_{660} value of 0.03 (equal to 3.9×10^8 CFU/mL) or higher was shown to have the significant effects. The results suggest that an OD_{660} value of 0.03 could be the minimum threshold for obtaining effective PGPE from this strain.

PGPE in the secondary sewage effluent

The PGPE of strain SP-2-C10 in a secondary sewage effluent that contained a wide variety of indigenous microbes was determined. As shown in Fig. 4, strain SP-2-C10 increased the frond number by 2.1-fold, and its average PGPE was almost equal to that of strain P23. Therefore, strain SP-2-C10 would definitely be able to realize the previously

Fig. 4 Plant growth-promoting effect (PGPE) of strain SP-2-C10 and *A. calcoaceticus* strain P23 in secondary sewage effluent ($n = 3$). Error bars represent the standard deviation ($\pm$ SD).

conceptualized co-benefits of enhancing the water purification activity and biomass productivity of duckweed in wastewater systems^{30)–32)}.

Ability of strain SP-2-C10 to colonize the fronds and roots of duckweed

To evaluate the ability of strain SP-2-C10 to colonize duckweed fronds and roots, whole duckweed plants inoculated with the strain were cultured in sterile Hoagland medium or the non-sterile secondary sewage effluent, and the number of SP-2-C10 cells on the fronds and roots were measured by qPCR targeting of the *rpoB* gene (The *rpoB* gene of SP-2-C10 was not detected in DNA extracted from the secondary sewage effluent). In the sterile Hoagland medium, the number of SP-2-C10 cells on the fronds was almost the same as that on the roots throughout the experiment (Fig. 5A). By contrast, in the non-sterile secondary sewage effluent, the number of SP-2-C10 cells on the fronds was more than 10-fold higher than that on the roots (Fig. 5B), most likely because the strain was originally isolated from the frond of duckweed. In wild duckweed with indigenous microbial communities, strain SP-2-C10 might mainly colonize the fronds rather than the roots. Taken together, our findings suggest that the isolation source (e.g., duckweed fronds or roots) may be a crucial factor to consider when searching for duckweed PGPB that will stably and sustainably enhance the plant biomass productivity both in practical field

Fig. 5 Copy numbers of *rpoB* in strain SP-2-C10 on the fronds and roots of duckweed cultured in (A) sterile Hoagland medium and (B) secondary sewage effluent ($n = 3$). Error bars represent the standard deviation ($\pm$ SD).

conditions and in sterile laboratory ones.

CONCLUSIONS

In this study, we succeeded in obtaining a plant growth-promoting bacterial strain (SP-2-C10) for duckweed. This strain is the first-ever plant growth-promoting bacterium to be isolated from the fronds of duckweed and belongs to a rarely reported bacterial genus, *Aquidulcibacter*. Under the non-sterile conditions, strain SP-2-C10 increased the growth of *L. minor* by 2.1-fold and showed stronger colonization of the fronds than of the roots. These data suggest that the fronds are the most suitable habitat for this strain and that the isolation source (e.g., duckweed fronds or roots) would be an important factor to consider in the selection of PGPB for enhancing the co-benefit water purification and biomass production system using duckweeds.

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