**Improved cultivation and isolation of diverse endophytic bacteria inhabiting *Dendrobium* roots by using simply modified agar media**

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Well done for the article and the investigation. Your results showed that we need to seek for new strategies to overcome some difficulties in cultivating recalcitrant species. Just need to point on line 308 is written dada2, I supposed was to be data.

Reviewer #2 (Comments for the Author):

Comments

1. Line 32: "...contribute to clarify their unknown role in the growth and development of Dendrobium plants." Please justify the above statement as the study did not deal with the role of endophytes in the growth and development of Dendrobium plants.
2. Was the endophytic diversity also analyzed from other parts of the plant except roots?
3. Line 218: "These results support that the......" The findings shows that application of PS and GG media is effective in isolating more than 50% of the predominant endophytic bacteria in Dendrobium. Give an explanation for the effectiveness of the
Suggestions
The discussion section should explain a comparison of the diversity of endophytes observed in Dendrobium plants from culture-dependent and culture-independent approach.

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Thank you for submitting your paper to Microbiology Spectrum.
Improved cultivation and isolation of diverse endophytic bacteria inhabiting *Dendrobium* roots by using simply modified agar media

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Abstract

*Dendrobium* plants are members of the family *Orchidaceae*, many of which are endangered orchids with ornamental and medicinal values. *Dendrobium* endophytic microbes has attracted attention to develop strategies for the plant protection and utilization of medicinal principles. However, the role of endophytic bacteria is poorly elucidated due to the lack of their successful cultivation. This study obtained a total of 749 endophytic isolates from *Dendrobium* roots using solid media prepared by simple modified methods (“PS”; separate sterilization of phosphate and agar, and “GG”; use of gellan gum as a gelling reagent) and a conventional “PT” (autoclaving the phosphate and agar together) method. Notably, based on comparison of 16S rRNA gene sequences between the isolates and *Dendrobium* root endophyte community, we successfully retrieved more than 50% (17 out of 30) of the predominant endophytic bacterial OTUs using PS and GG media, which is much higher recovery rate than that of PT media (16.7%). We further found that a number of recalcitrant bacteria including phylogenetically novel isolates and members of even rarely cultivated phyla *Acidobacteria* and *Verrucomicrobiota* were obtained only when using PS and/or GG media. Intriguingly, majority of these recalcitrant bacteria formed colonies faster on PS or GG medium than on PT medium, which may have contributed to their successful isolation. Taken together, this study succeeded in isolating a wide variety of *Dendrobium* endophytic bacteria including predominant ones using PS and GG media and could contribute to clarify their unknown role in the growth and development of *Dendrobium* plants.
Importance

*Dendrobium* endophytic bacteria are of great interest since their functions may contribute to protect the endangered orchids with ornamental and medicinal values. To understand and reveal “true roles” of the endophytes, obtaining those axenic cultures is necessary even in the metagenomic era. However, no effective methods for isolating a variety of endophytic bacteria have been established. This study first demonstrated that a use of the simply modified media is quite effective and indeed allows to isolate more than half of predominant endophytic bacteria inhabiting *Dendrobium* roots. Besides, even phylogenetically novel and/or recalcitrant endophytic bacteria were successfully obtained by the same strategy. The obtained endophytic bacteria could serve as “living material” for elucidating their unprecedented functions related to the conservation of endangered orchid plants. Furthermore, the culture method used in this study may enable to isolate various endophytic bacteria dominating not only in orchid plants but also in other effective plants.

Introduction

*Orchidaceae* is the largest family of flowering plants, together with *Asteraceae*, with approximately 800 genera and more than 28,000 species, which include almost 10% of all flowering plant species (1–3). The genus *Dendrobium* is the second largest genus after *Bulbophyllum* in the *Orchidaceae* and comprises over 1,100 species of epiphytic orchids which is distributed across Asia, New Guinea, and Australia (4, 5). Although species of this genus are well known for their ornamental and medicinal value, many of them are threatened with extinction (5, 6).

The orchid roots are strongly associated with a wide variety of endophytic microbes (2, 5–7). The function of endophytic microbes of orchids, especially fungi, has attracted attention, and a number of research have supported those endophytic fungi play important roles in growth and development of orchids throughout their life (6, 8–10). A prime example is that orchid seeds lack essential nutrients to maintain plant growth and thus depend on fungi for germination and carbon supply (6, 11). On the other hand, very little is known about the role of endophytic bacteria in growth and development of orchid (7, 11). In general, endophytic bacteria play a crucial role in promoting plant growth and yield through nitrogen fixation, phytohormone production, nutrient acquisition, and biocontrol activities, and also have a potential to create novel natural products like pharmaceutically relevant compounds (12–14). Therefore, understanding the interaction between *Dendrobium* plants and the endophytic bacteria is a significant issue in order to develop new strategies for orchid protection and better utilization of its medicinal principles (2).

Predominant endophytic bacteria are likely to have profound effects on their hosts; thereby analyzing their function may lead to a detailed understanding of plant-bacteria
interactions. Recent advances in culture-independent methods, e.g. 16S rRNA gene amplicon sequencing, has made it possible to reveal the composition and diversity of endophytic bacterial community of orchids (2, 7, 15). Metagenomic sequencing approach further allows to predict their functional and metabolic potential. Even though, to understand and verify “true” function and/or discover unprecedented functions of the endophytic bacteria, cultivation and isolation is ultimately essential. To date, however, no effective method obtaining pure culture of a wide variety of bacteria from the interior of plants (not just orchids) has been established. The fact that few environmental bacteria can grow in the laboratory (<1%) has been a limiting factor for unveiling the role of endophytic bacteria (7, 16).

Several simple methods for preparing media have been devised for widely isolating bacterial groups from the environmental samples, which includes the use of gellan gum as a gelling reagent instead of agar (termed “GG” medium) (17) and separate autoclave sterilization of phosphate and agar (termed “PS” medium, where “S” represents “separately”) (18). Both methods are very simple, but those have been found to improve the efficacy of diverse bacterial isolation from several environmental samples including soil, sediment and/or freshwater.

Furthermore, many reports showed that these techniques are also effective for culturing recalcitrant bacteria inhabiting various environments (17–22). Nonetheless, these cultivation methods have not been adopted for obtaining the axenic cultures of endophytic bacteria in plants including orchid.

We hypothesized that these simple modified media (GG and PS) could be useful for isolating diverse indigenous endophytic bacteria of *Dendrobium* plants, including predominant and/or novel bacterial taxa. To verify this hypothesis, we isolated more than 700 endophytic bacteria from the surface-sterilized *Dendrobium* roots using the different media and compared the phylogenetic compositions of the isolates with those of endophytic bacterial community obtained from 16S rRNA gene amplicon sequencing. Besides, the present study further validated the effectiveness of the modified media for growing the phylogenetically novel and/or recalcitrant endophytic isolates.

**Results**

*Dendrobium* roots-endophytic bacterial community analysis by 16S rRNA gene amplicon sequencing

Endophytic bacterial communities of the two strains of *Dendrobium moniliforme* ("GS"; green stem strain and "WS"; white stem strain; Fig. 1A and B) were analyzed using 16S rRNA gene amplicon sequencing. The sequencing generated a total of 1,132,902 raw reads from the orchid root DNA samples (Supplementary Table S1). After merging forward and reverse reads using *dada2* and removing OTUs classified into chloroplasts, mitochondria, and archaea, the
number of merged reads for each of the samples ranged from 43,557 to 51,299. The number of 98% similarity OTUs varied from 772 to 934. The rarefaction curve indicated that the number of reads was sufficient to assess the diversity of the endophytic bacterial communities (Supplementary Fig. S1).

A comparison of the endophytic bacterial community composition at the phylum level showed that the following 8 of top 10 abundant phyla were detected in both GS and WS: Proteobacteria, Actinobacteriota, Verrucomicrobiota, Planctomycetota, Bacteroidota, Chloroflexi, Cyanobacteria, and Acidobacteriota (Fig. 1C). Remaining two of the top 10 phyla of GS were Patescibacteria and Firmicutes, while those of WS were Dependencia and Myxococca. To clarify the bacterial groups dominating inside the Dendrobium roots, OTUs with a relative abundance of more than 1% were extracted as predominant OTUs (Fig. 2). Consequently, a total of 30 predominant OTUs were found, with 7 OTUs common to GS and WS, 11 OTUs specific to GS, and 12 OTUs specific to WS. These OTUs were classified into the following 9 phyla: Proteobacteria (11 OTUs), Actinobacteriota (8 OTUs), Bacteroidota (3 OTUs), Chloroflexi (2 OTUs), Verrucomicrobiota (2 OTUs), Planctomycetota (1 OTU), Cyanobacteria (1 OTU), Patescibacteria (1 OTU), and Dependencia (1 OTU).

Isolation efficacy of PT, PS and GG media for endophytic bacteria of Dendrobium roots

The isolation efficacy of DTS and DR2A media prepared by the simply modified methods of PS (separate autoclave sterilization of phosphate and agar) or GG (the use of gellan gum as a gelling agent instead of agar) was compared with the conventional one (PT; the well-known conventional method, autoclaving all nutrients including phosphate and agar together). The numbers of endophytic bacterial isolates obtained from Dendrobium roots using six different media (PT-DTS, PS-DTS, GG-DTS, PT-DR2A, PS-DR2A and GG-DR2A) were 151, 128, 114, 140, 117 and 99, respectively (Table 1). Based on the 16S rRNA gene sequencing analysis, a total of 749 isolates were classified into 97 "groups" using 95% similarity cut off value (corresponding to genus level), which were designated instead of OTUs to avoid confusion with data from the MiSeq amplicon sequencing. Interestingly, regardless of the type of medium compositions, the alpha diversity indices (Simpson and Shannon) of PS- and GG-derived isolates were much higher compared with the PT-isolates (Table 1). Isolates belonging to the phyla Actinobacteriota, Bacteroidota, Proteobacteria (Alphaproteobacteria and Gammaproteobacteria) and Firmicutes were retrieved from all examined media (Fig. 1C). Intriguingly, the isolates of the phyla Acidobacteriota and Verrucomicrobiota were obtained only from the simple modified media (PS-DTS, GG-DTS, and/or GG-DR2A), but not from conventional ones (all the PT media) at all. The six isolates of the Acidobacteriota were classified into two groups: one is related to the genus Edaphobacter (1 isolate) and the other related to the genus Terriglobus (5 isolates) (Table 2). An
isolate of the Verrucomicrobiota was related to the genus Chthoniobacter. In addition, the novel endophytic bacterial isolates (<95% similarity to the validly described strains) were successfully obtained only from PS and GG media (Table 2). All novel isolates were classified into 9 groups based on 98% similarity, which were associated with either Alphaproteobacteria, Gammaproteobacteria, or Bacteroidota. Four or more groups of novel isolates were retrieved from any PS or GG medium. These results indicated that the PS and GG media were more effective for isolating a wide variety of Dendrobium roots-endophytic bacteria including recalcitrant endophytic bacteria such as phylogenetically novel isolates and the rarely cultured phyla Acidobacteriota and Verrucomicrobiota isolates.

We further verified how much predominant bacteria of the interior of the Dendrobium roots were retrieved with the media used in this study. For this, the sequence homology between all isolates and the predominant endophytic bacterial OTUs revealed by 16SrRNA gene amplicon sequencing was calculated using BLAST+ (23) and then the isolates exhibiting high homology (>98%) was selected as the isolates affiliated with the predominant OTUs. As a result, a use of PS or GG media allowed to isolate the predominant bacterial groups more widely than the corresponding PT medium (Fig. 2). The recovery rate of the predominant endophytic bacteria by PT-DTS, PS-DTS, GG-DTS, PT-DR2A, PS-DR2A, and GG-DR2A were 13.3%, 46.7%, 23.3%, 13.3%, 26.7%, and 23.3%, respectively. Surprisingly, the use of PS and GG media resulted in culturing and isolating more than 50% (17 out of 30) of the predominant OTUs (Fig. 2). Besides, all predominant endophytic isolates obtained using PT media were also retrieved from PS and GG media. The 17 OTUs obtained here consisted of 8 OTUs belonging to Proteobacteria (72.7%), 6 OTUs belonging to Actinobacteriota (75%), and 3 OTUs belonging to Bacteroidota (100%), although the predominant OTUs of the phyla Chloroflexi, Verrucomicrobiota, Planctomycetota, Cyanobacteria, Patescibacteria, and Dependenciae were not obtained in this study. Notably, these predominant isolates included the novel endophytic bacterial isolates which were affiliated with OTU404 and OTU1984 (Table 2). Using PS media, we more efficiently isolated members of the predominant bacterial OTUs than using GG media (Fig. 2). However, several predominant isolates (compatible with OTU1537 and OTU1581) were obtained only from GG media. Taken together, we found that high isolation efficacy of the predominant endophytic bacteria could be achieved by using both PS and GG media.

Effect of PS and GG methods on growth of recalcitrant bacterial isolates

To clarify a reason why the recalcitrant bacterial isolates such as novel isolates (<95% similarity to valid strains) and Acidobacteriota and Verrucomicrobiota isolates were successfully retrieved with PS or GG media but not with PT media (Table 2), we further investigated the effect of PS and GG methods on the colony formation of the endophytic recalcitrant bacterial isolates.
Although all tested recalcitrant isolates formed colonies on even PT media, 25 of novel isolates (63.0% of the total 46 isolates), 3 isolates affiliated with phylum Acidobacteriota (50.0% of the 6 isolates), and 1 isolate affiliated with phylum Verrucomicrobiota (100% of the 1 isolate) formed visible colonies on PS or GG plates at least twice faster than on their corresponding PT plate (Fig. 3A). In particular, the isolate GSA-72 of the phylum Verrucomicrobiota formed visible colonies on PS-DTS plate (18 h incubation) more than eighth as fast as on PT-DTS plate (162 h incubation) (Fig. 3B and C). Novel isolates belonging to the remaining seven groups, except for novel groups 2 and 3, were found to form colonies more rapidly on PS or GG plates than on each PT plate. These results suggested that such a rapid colony formation on PS and GG plates is one of the plausible reasons for successful isolation of these recalcitrant bacterial isolates.

Discussion

Cultivation and isolation of a wide range of endophytic bacteria including predominant ones is a critical issue that must be addressed to investigate their unidentified functions and biology which may lead to elucidation of plant-endophytic bacteria interactions. In the present study, we demonstrated that the simply modified cultivation approaches (i.e., a use of modified media and the improved recipe, PS and GG methods, proposed by our previous studies) (17, 18) improved the isolation efficacy of even endophytic bacteria of Dendrobium roots. In fact, the alpha diversity indices (Simpson and Shannon) of PS- and GG-isolates were much higher than those from their corresponding PT-isolates (Table 1). Besides, the endophytic bacterial isolates obtained from Dendrobium roots using PS and GG media (PS-DTS, PS-DR2A, GG-DTS, and GG-DR2A) were distributed across six different phyla including two rarely cultivated phyla such as Acidobacteriota and Verrucomicrobiota (other four phyla are Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria), whereas those from media prepared by conventional methods (PT-DTS and PT-DR2A) belonged to only four phyla (Actinobacteriota, Bacteroidota, Firmicutes, and Proteobacteria) (Fig. 1C). So far, several studies have attempted to isolate endophytic bacteria of Dendrobium plants (7, 16, 24, 25). Wang and colleagues isolated endophytic bacteria from the interior of Dendrobium plant using a total of 11 different media (e.g., R2A agar, 10% nutrient agar, and humid acid agar) supplemented with 1% plant extracts of Dendrobium bodies (7). Despite such vigorous efforts, the isolated bacterial taxa were only three phyla (Actinobacteriota, Firmicutes, and Proteobacteria). Another studies have also isolated endophytic bacteria from inside Dendrobium bodies using media of nutrient agar, oatmeal agar, and/or ISP4 agar, but bacterial taxa other than the three phyla (Actinobacteriota, Firmicutes, and Proteobacteria) have not been isolated (16, 24, 25). In these previous studies, no phylogenetically novel bacteria were obtained. Furthermore, using PS and GG methods, we successfully isolated more than 50% (17 out of 30) of the predominant endophytic bacterial OTUs of Dendrobium
roots with a relative abundance of more than 1.0% revealed by the MiSeq amplicon sequencing (Fig. 2). To our knowledge, this is an unprecedented level of isolation efficacy of predominant plant endophytic bacterial taxa. These results support that the application of the PS and GG methods is effective for isolating a wide variety of endophytic bacteria including predominant ones from the interior of *Dendrobium* plants.

The previous studies including ours indicated that the application of PS and GG methods is effective for culturing recalcitrant bacteria in soil, sediment, sludge, and/or freshwater (17–21). In accordance with these reports, this study showed that a number of phylogenetically novel isolates (<95% similarity to valid strains) affiliated with phyla *Proteobacteria* and *Bacteroidota*, and members of even rarely cultivated phyla *Acidobacteriota* and *Verrucomicrobiota* were successfully isolated with PS and/or GG media, whereas these bacteria were not obtained using PT media (Fig. 1C and Table 2). Notably, the isolates that were affiliated with the novel groups 3 and 6 belonged to the predominant endophytic bacterial OTUs (OTU404 and OTU1984) (Table 2). Among nine novel groups, isolates of group 3, 4, 6, and 8 are closely related to plant-derived 16S rRNA gene sequence. These suggest that our novel isolates are likely plant-associated (not only with orchids but also with other plant species). Such success of isolating predominant endophytic bacteria might be brought by growth promotion of the modified cultivation methods adopted in this study. Indeed, the majority of the recalcitrant endophytic bacterial isolates formed visible colonies on PS or GG plates faster than on their respective PT plate (Fig. 3A). In particular, the visible colony formation of the isolate of the phylum *Verrucomicrobiota* was found at least eight times faster on the PS plate than on the PT plate (Fig. 3B and C). Similarly, our previous studies reported that the recalcitrant bacterial isolates obtained from soil, sediment, and freshwater grew only or well on PS or GG plates (18–20). For instance, the isolates of rarely cultivated phylum *Gemmatimonadota* showed a dramatic difference in colony formation on PS/GG plates compared to the corresponding PT plates (18, 20). Collectively, it was suggested that the application of the PS and GG methods might enable cultivation and isolation of recalcitrant bacterial isolates not only from soil, sediment, sludge, and freshwater but also from plant-associated (e.g. endophyte) by facilitating their colony formations.

To the best of our knowledge, we first succeeded in obtaining bacterial isolates of the phyla *Acidobacteriota* and *Verrucomicrobiota* from the internal of orchid plants (Fig. 1C). In particular, *Verrucomicrobiota* has been rarely isolated from not only the interior of orchids but also that of whole plants. Only two isolates (both belong to subdivision 4, out of seven subdivisions proposed for phylum *Verrucomicrobiota*) recovered from the root endosphere of *Oryza sativa* and *O. longistaminata* were reported (26). The isolate obtained in this study belongs to subdivision 2 (the closest relative is the genus *Chthoniobacter*) and is phylogenetically different from the abovementioned two isolates (26). This isolate together with the previous ones
would be useful for elucidating functional roles of *Verrucomicrobiota* in plant bodies that has long been largely unknown. The phylum *Acidobacteriota* is composed of diverse members spanning 26 subdivisions and recently has attracted much attention due to its members that are associated with soil-plant ecosystems worldwide (27). For instance, recent studies showed that the non-endophytic isolates affiliated with subdivisions 1, 3, and 6 of phylum *Acidobacteriota* possessed growth-promoting effects on plants such as *Arabidopsis thaliana* and duckweed species (28, 29). Since the six isolates obtained in this study belong to subdivision 1 and related to the genera *Edaphobacter* or *Terriglobus*, perhaps these isolates might contribute to the growth and development of orchid plants. Accordingly, the endophytic bacterial isolates affiliated with phyla *Verrucomicrobiota* and *Acidobacteriota* obtained in the present study must be valuable resources for comprehensive functional analysis of the endophytes of these two phyla.

In conclusion, the present study demonstrated that the DTS and DR2A media prepared by PS and GG methods enables the endophytic bacteria isolation from the interior of *Dendrobium* roots as follows: i) more than 50% of the predominant endophytic bacterial taxa of *Dendrobium* roots, ii) some phylogenetically novel isolates (<95% similarity to valid strains) affiliated with phyla *Proteobacteria* and *Bacteroidota*, and iii) bacteria affiliated with the rarely cultivated phyla *Verrucomicrobiota* and *Acidobacteriota* were successfully obtained. These results suggest that the PS and GG methods is effective for widely isolating endophytic bacteria from interior of plant bodies, in addition to soil, sediment and/or freshwater (17, 18, 20). Future studies revealing functions of the endophytic bacterial isolates obtained in this study would contribute to unveiling the unknown roles of endophytic bacteria in the growth and development of *Dendrobium* plants, and perhaps may shed light on developing new strategies for their protection and better utilization of their medicinal principles.

### Materials and methods

**Dendrobium** roots sampling

This study used two strains of *Dendrobium moniliforme*, which had been kindly provided by Mr. Hiroshi Noda and Ms. Hiroko Noda. They characteristically had green stem ("GS" strain) and white stem ("WS" strain), respectively (Fig. 1A and B), and were cultivated on sphagnum moss under natural light with regular watering for more than two years. Root sections were collected from three plants of each strain and then immediately surface-sterilized as following methods. The roots were treated with 75% ethanol for 30 s; 1 % (v/v) tween 20 for 1 min; 3% sodium hypochlorite for 10 min; 75% ethanol for 30 s, and then were rinsed with sterile distilled water (SDW) three times and cut into ca. 0.5 cm long sections with a sterile scalpel. To confirm whether the sterilization process was successful, roots were rolled on the plates of six different media used for the bacterial isolation experiment as described below, and also 100 μL
of the final water rinse was inoculated and spread on the same plates, which consistently yielded no bacterial colonies incubated at 25 °C for four weeks. For the isolation of endophytic bacteria, one gram of roots was homogenized gently in 9 ml of SDW using a mortar and pestle. To extract DNA from surface-sterilized roots, 0.5 gram of roots was homogenized in liquid nitrogen using a mortar and pestle, and the homogenate was immediately stored at −80°C until use.

**Endophytic bacterial community analysis by 16S rRNA gene amplicon sequencing**

A 16S rRNA gene amplicon sequencing was performed to determine predominant endophytic bacterial taxa of both *Dendrobium* (GS and WS) roots. Genomic DNA was extracted from each homogenized root using FastDNA SPIN Kit for soil (MP Biomedicals, CA, USA) according to the manufacturer’s protocol. The DNA extraction was repeated three times. Amplifications of V4 region of 16S rRNA genes were performed using primer set specific for V4 region (505F: GTGCCAGCMGCCGCGGTAA; and 806R: GGACTACHVGGGTWTCTAAT). Ex Taq® DNA polymerase (Takara Bio, Shiga, Japan) was used for PCR amplification and the thermal cycle step was performed with a denaturation step at 94°C for 2 min, followed by 23 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s and a final extension step at 72°C for 5 min. The 16S rRNA gene amplicon libraries were paired-end sequenced on an Illumina MiSeq platform using 2 × 250 bp overlapping paired-end reads (Illumina, CA, USA). Sequence processing was conducted using Qiime2 pipeline (version 2019.7). The paired-end fastq files were demultiplexed with demux-summarize and then were processed by quality filtering, merging of the paired ends and chimera removal with dada2 (30). In dada2 processing, we used options to do the following, 1) primer sequences were removed, 2) forward and reverse reads were truncated to 194 bp and 125 bp, respectively, and 3) the reads containing the base with quality score less than or equal to 15 were truncated. Each read was clustered into operational taxonomic units (OTUs) at 98% similarity using VSEARCH (31). Taxonomy was assigned to each OTU using the SILVA database (silva-138-99) using feature-classifier. Subsequently, reads classified into chloroplasts, mitochondria, and archaea were removed. OTUs with a relative abundance of more than 1.0% either in the interior of GS or WS root were defined as the predominant endophytic bacterial OTUs. Predominant bacterial OTUs were further identified using EzBioCloud server (32), and the corresponding OTUs were excluded when there were no hits. Furthermore, a phylogenetic tree of the predominant endophytic bacterial OTUs was constructed using maximum likelihood (ML) methods. Sequences were aligned using MAFFT with default settings and then a ML tree was constructed using RAxML-NG (version 0.9.0) with 100 bootstrap replicates (33). Tree results were viewed using Figtree (version 1.4.4) (http://tree.bio.ed.ac.uk/software/figtree/).

**Isolation of endophytic bacteria from *Dendrobium* roots**
To isolate endophytic bacteria from *Dendrobium* roots, two types of basal agar media supplemented with the fungicide cycloheximide (50 μg mL⁻¹) were used: diluted R2A (DR2A; yeast extract 0.05 g L⁻¹, peptone 0.05 g L⁻¹, dextrose 0.05 g L⁻¹, starch 0.05 g L⁻¹, casamino acids 0.05 g L⁻¹, dipotassium phosphate 0.3 g L⁻¹, magnesium sulfate heptahydrate 49.2 mg L⁻¹, sodium pyruvate 0.3 g L⁻¹, agar 15 g L⁻¹) and diluted tryptic soy (DTS: tryptone 0.17 g L⁻¹, soytone 0.03 g L⁻¹, dextrose 0.025 g L⁻¹, sodium chloride 0.05 g L⁻¹, dipotassium phosphate 0.025 g L⁻¹, agar 15 g L⁻¹). Both basal media were prepared by simple modified methods ("PS" and "GG"), and conventional "PT" (autoclaving all nutrients including phosphate and agar together) method. In the PS method, all nutrients including phosphate and agar were separately autoclaved and mixed. In the GG method, gellan gum is used instead of agar as a gelling agent and CaCl₂ was added at a final concentration of 3 mM. A 100-μL aliquot of each serial dilution of the *Dendrobium* roots suspension was spread onto the surface of each plate (90 mm in diameter) in triplicate. Each plate was incubated at 25°C in the dark for 21–28 days. After incubation, colonies appeared on each plate were randomly selected and streaked using quadrant streaking on fresh plates for further purification.

### Phylogenetic analysis of the endophytic isolates

The genomic DNA of each isolate was prepared using InstaGene matrix (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions. The 16S rRNA gene of each isolate was amplified with 27f (AGAGTTTGATCMTGGCTCAG) and 1492r (GGYTACCTTGTTACGACTT) primers and PrimeSTAR HS DNA polymerase (Takara Bio, Shiga, Japan). The amplification conditions were 30 cycles at 98°C for 10 s, at 55°C for 5 s, and at 72°C for 90 s. The PCR products were purified using the ExoSAP-IT Express PCR Cleanup Reagents (Takara Bio, Shiga, Japan). Cycle sequencing was performed using 907r (CCGTCAATTCMTTTRAGTTT) primer with the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., Japan) according to manufacturer's instructions. The fluorescent-labeled fragments were purified using the BigDye X Terminator™ Purification Kit (Thermo Fisher Scientific) and were analyzed by an ABI 3730xl DNA Analyzer (Thermo Fisher Scientific). To compare the isolation efficacy of each medium, the sequences obtained were assigned to OTUs by using the CD-HIT-EST program (34) with a cutoff value of 95% (corresponding to genus level) (35), and alpha-indices (shannon and simpson) were calculated using the vegan 2.5-6 package in R 3.6.1 software. To avoid confusion with data from the Miseq amplicon sequencing, the OTU of the isolate was designated as group. The partial 16S rRNA gene sequences of each group were identified using a EzBioCloud server. The isolates obtained in this study which exhibit high homology (>98%) to the predominant endophytic bacterial OTUs were shown as the isolates affiliated with the predominant OTUs.
Effect of PS and GG methods on growth of recalcitrant endophytic bacterial isolates

In order to clarify part of reasons why the recalcitrant endophytic bacterial isolates including the phylogenetically novel isolates (<95% similarity to valid strains based on their partial 16S rRNA gene sequence) and the isolates affiliated with members of even rarely cultivated phyla (e.g. Acidobacteriota and Verrucomicrobiota) were obtained from PS or GG media but not from PT media, the effect of PS and GG methods on the colony formation of the recalcitrant isolates were examined based on the criteria of at least twice faster visible colony formation on PS or GG plates than on their respective PT plate, as described by our previous report (20). Each isolate was precultured on the medium plate used for the isolation experiment and then suspended with SDW. Each suspension was spread onto the surface of the medium plate used for the pre-culture and the corresponding PT medium, and then incubated at 25°C under dark conditions. The colony formation was monitored every 18 h by the naked eye and finally by using stereomicroscope.

Data availability

Sequence data were deposited in the Sequence Read Archive database under accession numbers DRR354706–DRR354711.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

We thank Mr. Hiroshi Noda and Ms. Hiroko Noda for providing two strains of Dendrobium moniliforme. This work was mainly supported by Grant-in-Aids for Young Scientists (grant no. 20K15643), Scientific Research on the Innovative Area “Post-Koch Ecology” (MEXT KAKENHI no. JP19H05683, and JSPS Fellows (grant no. 19J01859) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Figure 1. Relative abundances of the endophytic bacterial phyla (or class) in the roots of *Dendrobium* strains (“GS”; green stem strain, and “WS”; white stem strain) according to the Miseq 16S rRNA gene amplicon sequencing and isolation using basal agar media (“DTS”; diluted tryptic soy, and “DR2A”; diluted R2A) prepared by simple modified methods (“PS”; separate sterilization of phosphate and agar, and “GG”; use of gellan gum as a gelling reagent) and conventional “PT” (autoclaving the phosphate and agar together) method. A and B. Photographs of the roots of *Dendrobium* strains GS (A) and WS (B). C. Phylum *Proteobacteria* was shown at class level (*Alphaproteobacteria* and *Gammaproteobacteria*) and additional 15 phyla are included as other phyla. The data from Miseq 16S rRNA gene amplicon sequencing and isolation were shown for each *Dendrobium* strain (GS and WS) and each medium, respectively.
Figure 2. The legend is shown in the next page.
Figure 2. Isolation recovery rate of predominant endophytic OTUs by each medium (PT-DTS, PS-DTS, GG-DTS, PT-DR2A, PS-DR2A, and GG-DR2A) or method (PT, PS, and GG). The predominant endophytic bacterial OTUs comprise more than 1.0% of relative abundance either in the interior of roots of *Dendrobium* strains (GS or WS) according to the Miseq amplicon sequencing. Each OTU is followed by taxonomy (Family; Genus) which was determined using the SILVA database (silva-138-99). The isolates obtained in this study which exhibit high homology (>98%) to the predominant endophytic bacterial OTUs were shown as the isolates affiliated with the predominant OTUs. Maximum likelihood phylogenetic tree of 16S rRNA genes V4 regions of the predominant endophytic bacterial OTUs was prepared using RAxML-NG (version 0.9.0) and Figtree (http://tree.bio.ed.ac.uk/software/figtree/). The accession number of each reference sequence was shown and the accession number HM243822 was used as an outgroup. ML bootstrap values were obtained using 100 bootstrap replicates and are shown on branches if above 50%.
Figure 3. Effect of PS and GG methods on visible colony formation of the phylogenetically novel endophytic isolates, and Acidobacteriota and Verrucomicrobiota isolates. (A) The number of the isolates showing at least twice faster colony formation on PS or GG media than their respective PT medium. Novel group was defined as described in Table 2. Colony formation of the isolate GSA-72 belonging to Verrucomicrobiota on the PS-DTS plate (B) or PT-DTS plate (C) after 7 days incubation. White arrows indicate the colonies.
Table 1. Comparison of alpha diversity values of the *Dendrobium* endophytic isolates obtained from PT, PS, and GG media

| Medium    | No. of isolates | No. of groups | α-diversity indices | Simpson | Shannon |
|-----------|-----------------|---------------|---------------------|---------|---------|
| PT-DTS    | 151             | 29            | 0.77                | 2.21    |         |
| PS-DTS    | 128             | 46            | 0.95                | 3.36    |         |
| GG-DTS    | 114             | 41            | 0.94                | 3.24    |         |
| PT-DR2A   | 140             | 26            | 0.79                | 2.22    |         |
| PS-DR2A   | 117             | 41            | 0.94                | 3.19    |         |
| GG-DR2A   | 99              | 34            | 0.94                | 3.14    |         |

* The bacterial isolates obtained in this study were classified with 95% similarity (corresponding to genus level) into 97 "groups".

* The indices of α-diversity based at group level were averaged using 10 replicates of data rarefied to the lowest number of isolates obtained on a single medium.
Table 2. Recalcitrant endophytic bacterial isolates obtained on the six different media from the interior of *Dendrobium* roots.

| Recalcitrant bacterial isolates | The No. of isolates from | Representative isolate | Closest match valid strain<sup>a</sup> | Closest match accession<sup>b</sup> | Genus | Similarity | Accession | Phylum/Class | Genus | Related predominant OTUs<sup>d</sup> | (Similarity) | Source<sup>d</sup> | (Similarity to OTUs)<sup>d</sup> |
|---------------------------------|--------------------------|-------------------------|-------------------------------|--------------------------|-------|------------|------------|--------------|-------|-------------------------------|----------------|---------------|------------------------|
| Novel isolates                  |                          |                         |                               |                          |       |            |            |              |       |                                |                |               |                        |
| Novel group 1                   | 0                        | 2                       | 1                             | 0                        | 0     | WSC-37     | AB091581   | *Alphaproteobacteria Rhizobium* | 91.5% | PAC000263 (97.3%) | Unknown       | Not applicable       |
| Novel group 2                   | 0                        | 0                       | 2                             | 0                        | 1     | GSC-63     | DQ672568   | *Alphaproteobacteria Skermanella* | 91.5% | PAC000228 (98.3%) | Unknown       | Not applicable       |
| Novel group 3                   | 0                        | 1                       | 0                             | 0                        | 0     | GSA-66     | JX412366   | *Gammaproteobacteria Acidobacter* | 93.9% | FJ554396 (97.9%) | *Allium rhizosphere* | OTU404 (98.4%) |
| Novel group 4                   | 0                        | 0                       | 0                             | 0                        | 0     | GS1-61     | KM083135   | *Gammaproteobacteria Sapientia* | 92.6% | JQ798403 (97.3%) | Maize straw   | Not applicable       |
| Novel group 5                   | 0                        | 0                       | 1                             | 0                        | 0     | GSC-66     | JX412366   | *Gammaproteobacteria Acidobacter* | 92.2% | PAC001319 (99.7%) | Unknown       | Not applicable       |
| Novel group 6                   | 0                        | 11                      | 2                             | 0                        | 2     | WSA-37     | jgi.1048941 | Bacteroidetes Chaaeobacteria | 92.7% | AB240469 (93.6%) | *Phragmites rhizosphere* | OTU1984 (100%) |
| Novel group 7                   | 0                        | 1                       | 3                             | 0                        | 1     | WSA-10     | JQ638910   | *Bacteroidetes Asinibacter* | 92.9% | JN656858 (93.7%) | Water         | Not applicable       |
| Novel group 8                   | 0                        | 0                       | 2                             | 0                        | 2     | GSC-53     | DQ244076   | Bacteroidetes Niastella | 94.9% | FJ479490 (98.6%) | Grass         | Not applicable       |
| Novel group 9                   | 0                        | 0                       | 0                             | 0                        | 0     | GSD-26     | JX458466   | Bacteroidetes Helimonas | 94.1% | JQ684312 (98.1%) | Permafrost soil | Not applicable       |
| Isolates affiliated with Acidobacteriota |              |                          |                               |                          |       |            |            |              |       |                                |                |               |                        |
| Group 1                         | 0                        | 3                       | 1                             | 0                        | 0     | GSA-29     | CP003379   | *Acidobacteriota Terriglobus* | 97.7% | JUGR01000001 (98.2) | Soil          | Not applicable       |
| Group 2                         | 0                        | 0                       | 1                             | 0                        | 0     | WSC-45     | KI9507088  | *Acidobacteriota Edaphobacter* | 99.2% | KN505788 (99.2%) | Forest soil   | Not applicable       |
| Isolate affiliated with Verrucomicrobiota |             |                          |                               |                          |       |            |            |              |       |                                |                |               |                        |
| Group 1                         | 0                        | 1                       | 0                             | 0                        | 0     | GSA-72     | ABVL01000001 | *Ferrumicrobiota Chthoniobacter* | 96.2% | JF17605 (97.7%) | Human skin   | Not applicable       |

<sup>a</sup> Recalcitrant bacterial isolates include novel isolates whose sequences had less than 95% similarity to valid strains and isolates affiliated with phyla *Acidobacteriota* and *Ferrumicrobiota*. These isolates were classified with 98% similarity into 9 groups of novel isolates, 2 groups of *Acidobacteriota*, and 1 *Ferrumicrobiota* group.  
<sup>b</sup> Taxonomic identity of each group to valid strains was determined using the EzBioCloud server.  
<sup>c</sup> Taxonomic identity of each group to all accessions was determined using the EzBioCloud server.  
<sup>d</sup> Representative isolates with more than 98% similarity to the predominant endophytic bacterial OTUs, as described in Fig. 2, were shown.
Supplementary Figure S1. Rarefaction curves for endophytic bacterial operational taxonomic units (OTUs) from each Dendrobium sample. GS: green stem-Dendrobium root and WS: white stem-Dendrobium root.
Supplementary Table S1  Statistics of Miseq amplicon sequencing of endophytic bacterial communities in the *Dendrobium* roots

| Root sample | No. of raw reads | No. of reads after Qiime2 process | No. of OTUs |
|-------------|------------------|----------------------------------|-------------|
| GS-1        | 183219           | 45725                            | 843         |
| GS-2        | 200196           | 51299                            | 934         |
| GS-3        | 179852           | 43557                            | 837         |
| WS-1        | 189484           | 46691                            | 787         |
| WS-2        | 183392           | 43614                            | 772         |
| WS-3        | 196759           | 50086                            | 792         |

GS: green stem-*Dendrobium* root and WS: white stem-*Dendrobium* root.
August 18, 2022

Dear Dr. Courtney Robinson,

Thank you very much for serving as the editor for our manuscript (Manuscript #: Spectrum02238-22) and giving us the opportunity to revise it. We also greatly appreciate the careful review and constructive comments from two reviewers. We would like to submit the revised version of manuscript. According to the constructive comments, we have thoroughly revised our manuscript and addressed all the issues raised. We truly believe that we have improved the quality of the manuscript to meet the journal's standards of *Microbiology Spectrum*. For more information, please confirm the responses to all the comments following this letter.

We hope the revised manuscript is now suitable for publication in *Microbiology Spectrum*.

Yours sincerely,

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Point-by-point responses to Reviewer 1

Reviewer's comments in black
Responses by authors in green

Well done for the article and the investigation. Your results showed that we need to seek for new strategies to overcome some difficulties in cultivating recalcitrant species. Just need to point on line 308 is written dada2, I supposed was to be data.

----- We greatly appreciate your positive evaluation. We sincerely apologize for having caused confusion. “dada2” is an error-corrected clustering method, namely Divisive Amplicon Denoising Algorithm 2. Also, dada2 should have been written as DADA2. Therefore, we have changed “dada2” to “divisive amplicon denoising algorithm 2 (DADA2)”.

(Lines 109, 323, and 324 in the revised manuscript).
Point-by-point responses to Reviewer 2

Reviewer's comments in black
Responses by authors in green

We deeply appreciate your valuable comments. According to the suggestions, we have revised the manuscript as described below.

Comments
1. Line 32: "...contribute to clarify their unknown role in the growth and development of Dendrobium plants." Please justify the above statement as the study did not deal with the role of endophytes in the growth and development of Dendrobium plants.
   ----- We agree with this comment. As you pointed out, this study did not deal with the role of endophytes in the growth and development of Dendrobium plants. Here, we just intended to mention that the obtained endophytes should be useful living materials for future study clarifying the unknown roles in growth and development of Dendrobium plants, but our description seems to mislead the readers as you suggested. To clarify this point, we have revised this sentence as follows.

   “…makes it possible to conduct future studies to clarify their unknown roles associated with growth of Dendrobium plants.”
   (Line 32-33 in the revised manuscript).

2. Was the endophytic diversity also analyzed from other parts of the plant except roots?
   ----- In this study, we did not analyze the endophytic diversity of other parts of the plant except roots. As the future study, we would like to investigate endophytes in other parts by both culture-dependent and culture-independent approaches.

3. Line 218: "These results support that the......" The findings shows that application of PS and GG media is effective in isolating more than 50% of the predominant endophytic bacteria in Dendrobium. Give an explanation for the effectiveness of the aforesaid media.
   ----- As suggested, we have revised the sentence as follows:

   “These results support that the application of the PS and GG methods is effective for isolating a wide variety of endophytic bacteria including more than 50% of predominant ones from the interior of Dendrobium plants.”
4. Line 230: "These suggests that our novel isolates......" Give reference
----- As suggested, we have added “(Table 2)” for reference.
(Line 242 in the revised manuscript).

5. Line 331: instead of "all nutrients" please give details on experimental methods and be precise.
----- As suggested, we have changed “all nutrients“ to “all medium nutrient components”.
(Lines 131, 348, and 349 in the revised manuscript).

6. Line 251: add space in "abovementioned"
----- Corrected.

7. Line 524: Remove the comma after "isolates"
----- Corrected.

Suggestions
The discussion section should explain a comparison of the diversity of endophytes observed in Dendrobium plants from culture-dependent and culture-independent approach.
----- As suggested, we have added and revised the sentences as follows:

Original sentence:
To our knowledge, this is an unprecedented level of isolation efficacy of predominant plant endophytic bacterial taxa.

Revised sentences:
On the other hand, endophytic bacterial communities of Dendrobium roots revealed by MiSeq amplicon sequencing were higher diversity than those by culture dependent methods (Fig. 1). The predominant OTUs belonging to phyla Chloroflexi, Verrucomicrobiota, Planctomycetota, Cyanobacteria, Patibacteria, and Dependentiae were not isolated in this study (Fig. 2). These bacterial groups are known to be difficult to isolate, and their isolation and culture will be the next challenge. However, to our knowledge, the successful isolation of more than half is an unprecedented level of isolation efficiency for the dominant plant endophytic bacterial taxa.
(Line 222-229 in the revised manuscript).
September 30, 2022

Dr. Tomoki Nishioka  
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Japan

Re: Spectrum02238-22R1 (Improved cultivation and isolation of diverse endophytic bacteria inhabiting *Dendrobium* roots by using simply modified agar media)

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