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Research Article

First Report of the Endophytic Bacteria Associated with *Phormidium* sp.

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Abstract: Recent molecular studies on endophytic bacterial diversity have revealed a large richness of species. Associations between endobiotic bacterial-algae interactions have been studied for more than 40 years but were, up to now, never molecularly analyzed within the filamentous Cyanobacteria *Phormidium*. Therefore, the endophytic bacteria associated with fresh microalgae *Phormidium*, a group of ubiquitous photosynthetic organisms that play an important role in aquatic ecosystems, has been investigated. To study this partnership, *Phormidium* sp. was cultured in BG-11 medium using optimal conditions, and after the incubation period, cell biomass was obtained. Total genomic DNA from biomass was extracted and used for endophytic bacteria determination by using the 16S rRNA gene. Sequencing results revealed that a total of seven endophytic bacteria living within the cytoplasm of the host *Phormidium* sp. have been identified, including six bacteria belonging to three genera, namely *Sphingomonas*, *Sphingopyxis,* and *Stenotrophobacter* and while one bacteria remained unidentified due to low sequence homology in the GenBank database. The results highlighted the importance of endophytic bacteria associated with *Phormidium* sp. for the first time by using sequence-based identification.

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1. Introduction

Endophytic microbial communities, which reside in symbiotic associations inside the cell, particularly bacteria and fungi, are known that live in algae or plants without inducing disease in the development and growth of various host organisms (Yaish et al., 2015; Gouda et al., 2016). Among endophytic microorganisms, especially bacteria, have an ingenuity for living in internal alga or plant tissues and performing beneficial impacts to host growth by having a symbiotic association and having co-evolved an intimate ecological relationship that helps hosts adapt to biotic and abiotic stress (Nouh et al., 2021). Similarly, the surface of algae organisms like often represents a highly active association between hosts and microbes. In some cases, microorganisms play significant roles in hosts. In other cases, endophytic bacteria help supply the defense chemical and metabolism in algae with vitamins, heat, salinity, drought, fatty acids, pathogens, infections, and pollutants (Wahl et al., 2012; Singh and Reddy, 2014; Flewelling et al., 2015; Manomi et al., 2015; Karthick and Mohanraju, 2018; Mandelare et al., 2018; Ismail et al., 2020).

For over 40 years, the relationships between microalgae and bacteria have been widely studied by culturing in the laboratory. In recent studies, it is seen that there are methods using bacterial gene sequence analysis obtained from DNA isolated from host cells without culturing bacteria. (Reiter et al., 2002; Miyamoto et al., 2004). Over the past two decades, three revolutionary techniques, the development of the polymerase chain reaction (PCR), Denaturing Gradient Gel Electrophoresis (DGGE), and the establishment of a classification system for bacteria based on the phylogeny of 16SrRNA, have changed our understanding of the microbial world (Rappé and Giovannoni, 2003; Muyzer et al., 2004).

Phormidium sp. (phylum cyanobacteria) is a genus of blue-green microalgae that is single-cell, filamentous, unbranched, and about 3 to 4 μm in diameter. Used extensively in biotechnology processes due to its endurance and simple nutritional requirements, a few species of this genus live in extreme environments like contaminated areas, hot springs, and desert lands (Guiry and Guiry, 2016).

The major objective of the present study was to explore the potential of endophytic bacterial communities within *Phormidium* microalgae by using the modified molecular method. *Sphingomonas* sp., *Sphingopyxis* sp., and *Stenotrophobacter* sp. as endophytic bacteria associated with *Phormidium* sp. were the first time identified. The outcome of this study will open a framework for controlling which of the endophytic bacterial members likely maintain an endosymbiotic relationship with the algae host.

2. Material and Methods

2.1. Algal material and culture condition

Phormidium sp. (BDCC 002) used in this study was obtained from Manisa Celal Bayar University Culture Collection of Biology Department in Manisa, Turkey. The algal biomass is grown in 250 mL Erlenmeyer flasks containing 100 mL of BG-11 medium. The aqueous biomass is incubated in a light incubator at 26 °C, 36 μmol m^{-2} sec^{-1} irradiances, 110 rpm magnetic stirring, and 2000-3000 lux fluorescent light on 16:8 h photoperiod. The medium was fixed to pH 5-7 using 1 M NaOH or 1 M HCI. *Phormidium* cells were harvested after the 12th and 15th days of incubation (Stanier et al., 1971). After the completion of incubation, the algal biomass was harvested from the media by centrifugation (6000 rpm, 5 min, 25 °C) and then subjected to total genomic DNA isolations.

2.2. Total genomic DNA extraction

The collected culture biomass was washed with distilled water and centrifuged to remove the remnants of the medium. The culture biomass was collected and then washed with distilled water and centrifuged for the removal of the medium. Afterward, the filamentous thalli were cut into 1–2 cm pieces and washed with double distilled water 2 times between the time interval of 5 min. The sample in test tubes was initially surface sterilized with 0.5% (w/v) EDTA solution for 5 min, followed by 70% (v/v) ethanol for 5 min, and later washed with PCR grade water for 1.5 min. This procedure was repeated at least 4-5 times. Sections about 1 mm thick were cut with a sterile lancet, and biomass was placed in a new sterilized 2 mL microtube and centrifugated for 5 min at 15000 rpm. Total genomic DNA from this sample was extracted using the WizardR Genomic DNA Purification Kit (Promega, A1120) by following per under the conditions specified by the Promega instructions. The total DNA samples were checked by 1% (w/v) agarose gel electrophoresis, by adding 10 μL DNA dye (Invitrogen, SYBR, Safe DNA Gel Stain) and with a 1 kb DNA marker (Geneaid DL006) using TAE buffer.

2.3. The 16S rRNA gene amplification

The 16S rRNA gene was amplified by PCR reaction using two universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991). A reaction mixture optimized for PCR was done in a total volume of 50 μL, and each reaction contained 5 μL of 10X DreamTaq PCR buffer (with $MgCI_2$), 2 μL of 100% DMSO, 1 μL of 10 mM dNTPs, 0.75 μL of each 20 μM primer, 0.4 μL of DreamTaq DNA polymerase (ThermoScientific, EP0702), 39.6 μL of PCR grade water and 0.5 μL of template DNA (approximately 50 ng).

PCR amplification was carried out with a thermal cycler (Applied Biosystems Veriti Thermal Cycler, USA) and conditions were as follows: 4 min at 95 °C, 36 cycles of 30 sec at 95 °C, 30 sec at 49 °C, and 1 min at 72 °C, followed by 10 min final extension at 72 °C, then cooling at 4 °C. PCR products

were electrophoresed in 0.8% agarose gels containing SYBR (Invitrogen) using TBE buffer. Afterward, the PCR products were examined in a UV transilluminator (gLite gel scanner), and those that formed a single pure bank were separated for sequence analysis and stored at -20 °C. The PCR products were commercially sequenced by the GATC biotech company in Germany.

2.4. Phylogenetic analysis

The raw sequence data were edited with the BioEdit Sequence Alignment Editor program (V, 7.2.5.) (Hall, 1999) for the counterpart of degenerative bases, and the forward and reverse reading sequences were combined using the same program. The edited sequences were then blasted to compare with the data in the gene bank to identify possible bacteria. Using CLUSTALW, the 16S rRNA gene sequence was aligned with multiple sequences from the GenBank database. Phylogenetic trees were created with representative sequences using algorithms with a bootstrap test (1000 replicates) (Felsenstein, 1985; Saitou and Nei, 1987; Tamura et al., 2004) in the MEGA X software (V, 10.0.4) (Kumar et al., 2018).

3. Results and Discussion

Molecular characterization of endophytic bacteria is the first step in differentiating them at the strain level. 16S rRNA gene sequencing allowed accurate identification of endophytes from various host species. In the current research, the identification of endophytic bacteria from *Phormidium* sp. was performed using 16S rRNA gene sequencing. The phylogenetic tree was constructed based on the obtained sequences along with the closely related taxa from GenBank (NCBI).

All of the endophytic bacteria were classified to the genus level because of the low sequence coefficient of similarity between 83-96% of that the bacterial species collected with GenBank.

According to the bioinformatics analysis, seven endophytic bacteria were identified from *Phormidium* sp. (BDCC 002) by using a sequence-based method into three genera: *Sphingomonas* sp. (3), *Sphingopyxis* sp. (2), and *Stenotrophobacter* sp. (1) and an unidentified bacteria (1) (Figure 1).

On the other hand, five endophytic bacteria (three of *Sphingomonas* sp. and two of *Sphingopyxis* sp.) and one bacteria (*Stenotrophobacter* sp.) belonged to the largest group of bacteria- phylum Proteobacteria and Acidobacteria, respectively.

The sequences of seven endophytic bacteria were deposited at the GenBank with accession numbers (MW759557–MW759563). Previous research has shown that these bacteria are isolated and identified from different hosts (Zhang et al., 2014; Battu et al., 2017; Wang et al., 2020; Cheng et al., 2021). However, these genera were not found in the *Phormodium* sp. before.

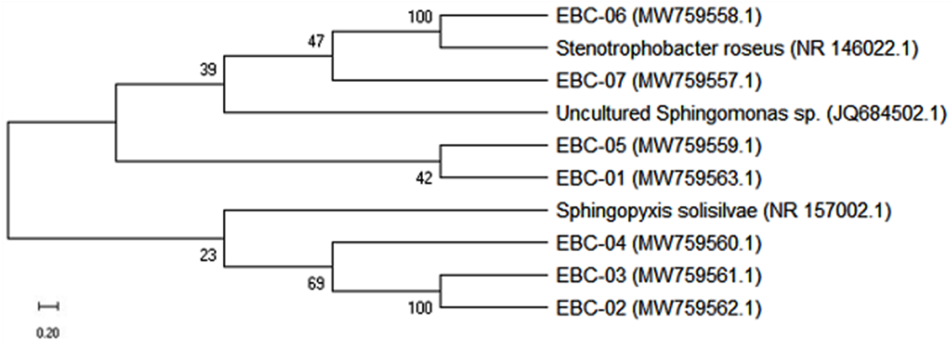

Figure 1. Neighbour-joining phylogenetic tree based on analysis of the 16S rRNA gene sequences showing the phylogenetic relationships between endophytes and representatives. Bootstrap values calculated for 1000 replications are indicated. GenBank accession numbers are given in parentheses. Only bootstrap values >50% are shown. Bar, 0.20 substitutions per nucleotide position.

The genus *Sphingomonas* is a Gram-negative, rod-shaped, chemoheterotrophic, strictly aerobic bacteria that typically produce colonies with yellow pigment and belongs to the class Alphaproteobacteria. Phylogenetically, *Sphingomonas* is well delineated from other genera, *Sphingobium*, *Sphingopyxis,* and *Novosphingobium* of the sphingomonads group, family

Sphingomonadaceae of Proteobacteria. This microorganism was defined by Yabuuchi et al. (2002) as generally non-pathogenic to humans (Glaeser & Kampfer, 2014). *Sphingomonas* sp. was previously isolated and identified in different plants such as *Sedum alfredii* (Bao et al., 2014), *Solanum lycopersicum* (Halo et al., 2015), *Solanum pimpinellifolium* (Khan et al., 2017), *Allium tuberosum* (Feng et al., 2017), *Glycine max* L. (Bilal et al., 2018) and *Oryza sativa* (Cheng et al., 2021). According to these researches, *Sphingomonas* sp. can be considered to be the dominant genus and may be represented in large numbers in the host plant. Table 1 shows the occurrence of related 3 endophytic bacteria in some plants. They appear more of *Sphingomonas* type than the other endophytic bacteria.

Table 1. Examples of reported bacterial endophytes and organisms harboring them

Endophytes	Host organisms	Reference
Sphingomonas SaMR12	*Sedum alfredii*	Bao et al., 2014
Sphingomonas sp. C40	*Oryza sativa*	Cheng et al., 2021
Sphingomonas sp. LK11	*Solanum pimpinellifolium*	Khan et al., 2017
Sphingomonas sp. LK11	*Solanum lycopersicum*	Halo et al., 2015
Sphingomonas sp. LK11	*Glycine max* L.	Bilal et al., 2018
Sphingomonas strain HJY	*Allium tuberosum*	Feng et al., 2017
Uncultured *Sphingomonas* sp. EBC-01	*Phormidium* sp.	In this study
Uncultured *Sphingomonas* sp. EBC-04	*Phormidium* sp.	In this study
Uncultured *Sphingomonas* sp. EBC-07	*Phormidium* sp.	In this study
Sphingopyxis granuli	Rice cultivar RP Bio-226	Battu et al., 2017
Sphingopyxis sp.	Potato tubers	Liu et al., 2011
Uncultured *Sphingopyxis* sp. EBC-02	*Phormidium* sp.	In this study
Uncultured *Sphingopyxis* sp. EBC-05	*Phormidium* sp.	In this study
Stenotrophobacter sp.	Korean Pine Forests	Wang et al., 2020
Uncultured *Stenotrophobacter* sp.EBC-06	*Phormidium* sp.	In this study
uncultured bacterium EBC-03	*Phormidium* sp.	In this study

On the other hand, the genus *Sphingopyxis* belongs to the class Alphaproteobacteria and the family Sphingomonadaceae. *Sphingopyxis* cells are aerobic Gram-negative, chemoheterotrophic bacteria that typically produce rod colonies with yellow pigment (Peter Kampfer et al., 2002). The genus *Sphingopyxis* was first proposed by Takeuchi et al. (2001). Relatively less studied strains of *Sphingopyxis* are mostly found in environments such as anaerobic sludge cover, seawater, wastewater treatment plant, hydrocarbon contaminated soil, and hexachlorocyclohexane contaminated soil (Jindal et al., 2013; Glaeser and Kampfer, 2014; Verma et al., 2015).

Sphingopyxis was isolated and identified by Battu et al. (2017) on rice culture and by Liu et al. (2011) on potato tubers. Battu et al. (2017) reported the use of high throughput plant genomic data to identify *Sphingopyxis granuli* endophytic bacteria colonizing rice plants by using novel next-generation sequencing-based computational methods.

Stenotrophobacter is a rod feeding on a few substrates. *Stenotrophobacter* belongs to the class Acidobacteria and the family Blastocatellaceae. *Stenotrophobacter* Gram-negative, nonmotile, short rods. Based on the 16S rRNA gene sequence analysis, *Stenotrophobacter* occurs in various habitats such as freshwater and marine water, microbial mats, hot springs, human body, as well as additional uncultured representatives in bulk soils, rhizosphere of wild and crop plants (Pascual et al., 2017). *Stenotrophobacter* sp. was identified as an endophytic species only in Korean pine forests by Wang et al. (2020).

It has been stated in different studies that the identified endophytic bacteria contain chlorophyll a, and it is predicted that these bacteria can affect development and growth positively by nitrogen fixation and production of some growth factors such as indole-3-acetic acid, HCN production, or synthesis of 1-ACC-deaminase to the host *Phormidium.* From the existing literature, it is known that these bacteria might also have the potential to adapt the host to extreme conditions such as desert soils, thermal springs, and polluted environments. The genus *Sphingomonas* and *Sphingopyxis* are commonly isolated from freshwater and marine habitats, soils, plant rhizosphere, or activated sludge. Some are antagonistic against plant pathogens and can produce exopolysaccharides (sphingans), which are gelling

agents that are used for pharmaceutical, food, or industrial applications including bioremediation of wastewater or contaminated dumping sites (Glaeser and Kampfer, 2014).

It should be noted that attempts to the isolation of these bacteria, depending on the growth media in both solid and liquid growth media such as R2A, Luria–Bertani agar, Tryptic Soy Agar, and conditions could not be cultured. It is thought that the reason for this is that endosymbiotic bacteria cannot be cultured in individually artificial growth conditions and media without a host.

This study, which provides the first information on the identity and phylogenetic diversity of bacterial communities within *Phormidium*, although they cannot be cultured in different synthetic media, shows that *Phormidium* harbors endophytic bacteria that are not very complex but taxonomically diverse, including members of *Sphingomonas, Sphingopyxis,* and *Stenotrophobacter*.

Conclusion

Endophytic bacteria associated with *Phormidium* sp. (BDCC 002) algae have been identified for the first time. The molecular approach revealed three bacteria, *Sphingomonas* sp., *Sphingopyxis* sp., and *Stenotrophobacter* sp. belonging to endophytic. As a future perspective will be to focus on the biological function and the potential biotechnological relation between endophytic bacteria and host *Phormidium* microalgae, such as the production of phytohormones and siderophores, as well as nitrogen fixation. The results highlight the importance that *Phormidium* is closely associated with well-defined endophytic bacterial communities.

Data Availability

The nucleotide sequences obtained in this study have been deposited in the GenBank database with accession numbers: MW759557–MW759563.

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