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Full Length Article

Growth and Metagenomics Analysis of *Ulva prolifera* after Antibiotic Treatment

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Abstract

To study the growth and metagenomics of *Uiva prolifera* after antibiotic treatment, a portion of bacteria was removed from *U. prolifera* through mixed antibiotics culture to observe morphologic alternation of *U. prolifera*. Then the composition of typical symbiotic bacteria was analyzed by plate culture, separate sequencing and metagenomics, respectively. The results showed that mixed antibiotics (0.1 g/L *neomycin sulphate*, 0.03 g/L *polymyxin* B and 0.1 g/L *penicillin* G) removed main epiphytic bacteria on *U. prolifera*; the control group contained kinds of *Bacteroidetes*, varying in different culture mediums. *U. prolifera* treated with mixed antibiotics grew loosely with uneven cell size. By metagenomics analysis, 90 species of bacteria shared in epiphyte and antibiotics treated strains, mainly involved in *Bacteroidetes*, *Planophyta* and *Proteobacteria*. Besides, 19 strains unique in epiphyte and 48 in antibiotics treated strains (some belong to *Cyanobacteria* and *Chlorobi*) were detected. Overall, Alteromonas and Hyunsoonleella dominanted bacteria species of epiphyte and antibiotics treated strains, respectively. © 2019 Friends Science Publishers

Keywords: Antibiotics; Metagenome; Morphogenesis; Phycosphere; Symbiotic bacteria; *U. prolifera*

Introduction

Phycosphere is the mini-ecosystem where algae interact with bacteria for material exchange, information transfer and energy flow (Zhou *et al.*, 2016). The inducers secreted by bacteria can promote the growth or rhizosphere formation of algae (*Bryopsis*, *Caulerpa* and *Ulva*) (Evans and Trewavas, 2010). Without bacteria, *Ulva lactuca* (Provasoli and Pintner, 2010), *Ulva mutabilis* (Weiss *et al.*, 2017) and *Ulva intestinalis* (Ghaderiardakani *et al.*, 2017) become callus rather than thallus.

Ulva is a model system to study morphogenesis (Thomas *et al.*, 2015). In previous research on growth and thallus morphogenesis of *Ulva mutabilis*, accompanied microorganisms were collected by streaking thallus fragments and plated on agar medium for culture (Spoerner *et al.*, 2012). To study the influence of bacteria on algae morphogenesis, algae and bacteria should be isolated. The identification of bacteria involved plate cultivation (Pace, 1996), followed by molecular techniques employing DGGE electrophoresis and 16S rDNA molecular probes (Ashen and Goff, 1996; Fisher *et al.*, 1998). Nowadays, metagenomics provides a powerful tool for batch microbial identification (Dittami *et al.*, 2014).

U. prolifera is the dominant species of the green tide in South Yellow Sea of China used as natural resource (Cai *et al.*, 2016, 2018a, 2018b). To study the bacteria involved in *U. prolifera* growth, this work first removed typical epiphytic bacteria from *U. prolifera* using mixed antibiotics culture and then morphologic alternation of *U. prolifera* was observed. Plate culture, separate sequencing and metagenomics were used to analyze the composition of typical epiphytic bacteria on *U. prolifera* and that from antibiotics treated strains, respectively.

Materials and Methods

Experiment Materials

The *U. prolifera* thallus was sampled from Rudong sea area near Jiangsu Province. After confirmation according to ITS and 5S DNA, they were culturing in aerobic VSE medium under the conditions of 18ºC, 25–30 salinity, 130–160 μmol m^{-2} s^{-1} photon flux and D:L=12:12 photoperiod. Then the thallus was cut off to disperse sporophyte. Bacterial medium named *Zobell 2216E*, *R2A* and *Cytophaga* were from Boster Biological Technology Co. Ltd.; Ezup column bacteria genomic DNA purification kit and antibiotics were from Sangon Biotech (Shanghai) Co., Ltd.

Algae Cultured under Antibiotics

The cultured *U. prolifera* sporophyte was added with

penicillin G, *neomycin sulfate* or *polymyxin* B solution. The final concentrations of each antibiotic were set at six gradients of 0, 0.02, 0.04, 0.06, 0.08 and 0.10 g/L, respectively. All the groups were observed daily, while culture medium refreshed once every three days. The observation lasted for 30 days.

Algae Treated with Mixed Antibiotics

Ulva prolifera sporophyte was cultured in mixed antibiotic containing VSE medium (0.1 g/L *neomycin sulphate*, 0.03 g/L *polymyxin* B and 0.1 g/L *penicillin* G) for three days. *U. prolifera* cultured in antibiotic free VSE medium was taken as control group. In both groups, the thallus was gathered by centrifugation. Then they were cultured in antibiotic free VSE medium, or plate of agar medium (*Zobell 2216E*, *R2A* or *Cytophaga*) streaked for observation, respectively. Three parallels were taken in each group.

Identification of Bacteria on Agar Medium

Agar medium plate streaked with *U. prolifera* were placed under 30ºC. Single colonies grown on plate were purified, followed by identification of 16S rDNA sequencing. Wherein, PCR primers were

27F: AGAGTTTGATCMTGGCTCAG

1492R: GGTTACCTTGTTACGACTT

The PCR reaction was as follows: with predegeneration at 94ºC for 3 min; with 35 cycles at 94ºC for 30 s, 55ºC for 30 s and 72ºC for 100 s; at 72ºC for 10 min as reaction termination. The PCR product was sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

Metagenomics Analysis of Bacteria

U. prolifera thallus in antibiotic free VSE medium was suspended in TE buffer and vortex shocked for 5 min, followed by centrifugaton at 10,000 rpm for 5 min at 4ºC. Bacteria on the inner wall of centrifugal tube were gathered as sample of typical epiphytic bacteria of *U. prolifera* (group I). *U. prolifera* in VSE medium containing mixed antibiotic was suspended in TE buffer, followed by homogenating to disrupt cells. The homogenate was centrifuged to gathered bacteria as group II. Both groups were used to extract genomic DNA by Ezup column bacteria genomic DNA purification kit, followed by PCR amplification with barcode contained primers. The PCR product was qualitatively detected by electrophoresis and quantitatively analyzed by QuantiFluor™-ST Blue Fluorescent Quantitation System (Promega Co.). Then Illumina PE250 library was constructed for high-throughput sequencing by ABI 3730 XL sequencer. There are three parallels in each sample.

Statistical Analysis

All the data were shown as mean ± standard deviation. Statistical differences between the experimental groups were determined by one way ANOVA. Bray-Curtis algorithm was used for mcrobial community barplot and heatmap analysis. Vegdist and Hclust were used for distance calculation and cluster analysis.

Results

Lethal Effect of Antibiotics on *U. prolifera*

Penicillin G, *neomycin sulfate* and *polymyxin* B has no lethal effect on *U. prolifera* sporophyte under concentration below 0.1 g/L. They could be used as alternative reagents for sterilization of algae.

Morphology Effect of Antibiotics on *U. prolifera*

The *U. prolifera* sporophyte in antibiotic free culture medium grows, with the body length of almost four centimeters and the cells arranged in a tight order after 30 days (Fig. 1a). However, *U. prolifera* sporophyte treats with mixed antibiotic grown into clumps. The arrangement of the cells is loose and there were uneven size and unobvious boundaries between cells (Fig. 1b). The normal grow of *U. prolifera* needs epiphytes. Under long-term sterile conditions, the pseudo-roots of algae also grow slowly and disperse easily; the dead thallus does not rot (Fig. 1c).

Epiphytic Bacteria Identified by Cultivation

After cultivation, bacterial colonies were only in agar medium plate dealt with *U. prolifera* from antibiotic free culture medium. According to sequencing and blasting with 16S rDNA, most of them were *Bacteroidetes* varied in different culture mediums. Specifically, there were *Rhodobacteraceae* on Zobell2216E, *Marinobacter* spp. on *Cytophaga* and *Marivita* spp. and *Roseviovarius mucosus* on R2A.

Bacteria Analyzed by Metagenomics

Lots of endophytic bacteria in mixed antibiotic treated *U. prolifera* were indentified according to 16S rDNA sequence, with average length of 351–400 bp. It was in line with that from metagenomics. Specifically, 90 species of bacteria, shared in group I and group II, involved in *Bacteroidetes*, *Planophyta* and *Proteobacteria*. In addition, 19 strains (*Methylotenera*, *Sphingobacteriales*, *Phycisphaera*, *Rhodobacteraceae*, *Alphaproteobacteria* and *Cyclobacteriaceae*) were unique in group I and 48 (*Oligollexaceae norank* and *Peredibacter*) in group II were also detected. The extra bacteria in group I included *Cyanobacteria* and *Chlorobi*. *Alteromonas*, which was in the majority, followed by *Hyunsoonleella*, *Marivita* and

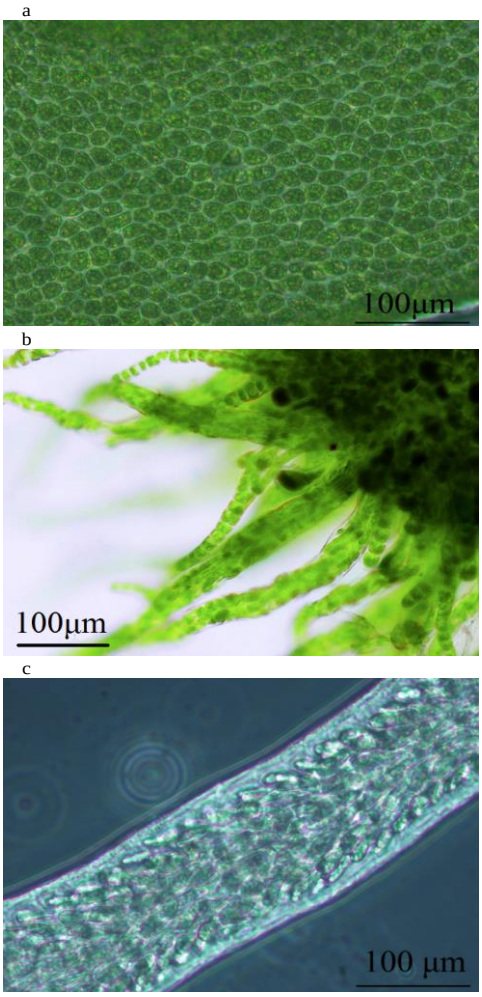

Fig. 1: Morphology effect of antibiotics on *U. prolifera*
a. The *U. prolifera* sporophyte in antibiotic free culture medium grew rapidly, with the cells arranged in a tight order after 30 days. **b**. *U. prolifera* sporophyte treated with mixed antibiotic grown into clumps slowly. The arrangement of the cells was loose, with uneven size and unobvious boundaries between cells. **c**. Under sterile conditions, the thallus did not rot a week after death

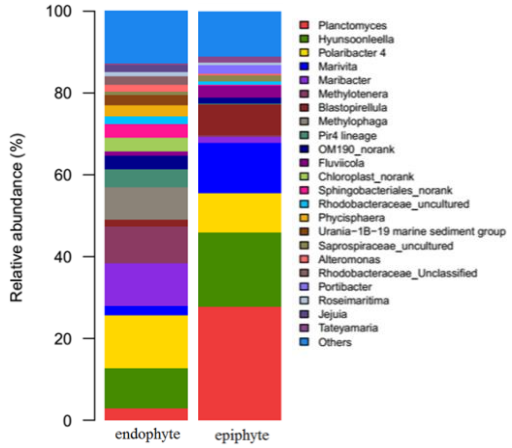

Fig. 2: Mcrobial community barplot of endophytic and epiphytic bacteria from *U. prolifera*

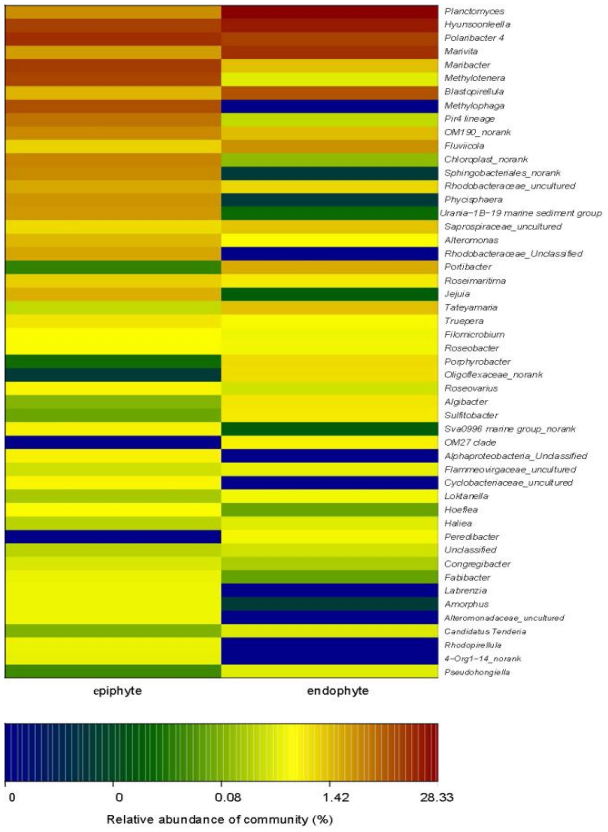

Fig. 3: Mcrobial community heatmap analysis of endophytic and epiphytic bacteria from *U. prolifera*

Polariclaer. These bacteria also existed in group II; however, most of bacteria in group II were *Hyunsoonleella*, *Polaribacter*, *Maribacter* and *Rhodobacteraceae* in sequence (Fig. 2 and 3).

Discussion

Sterilization of algae to get rid of symbiotic bacteria is the premise to study the phycosphere. Comparing with higher plants, it is more difficult to obtain the sterile algae due to the complex environment (Bradley *et al.*, 1988). For microalgae, it is achieved by combination of washing and ultrasonic to remove adnate wild algae and the microbial community of its slime layer (Wiedeman *et al.*, 2011). Since this technique is not suitable for macroalgae (*e.g.*, *Monostroma oxyspermum* (Tatewaki *et al.*, 1983), addition of antibiotics is an alternative way (Lin, 2010), which inhibits or sterilize some bacteria (Ferguson *et al.*, 2000). Microbial communities vary according to marine environments, while antibacterial spectrum varies according to antibiotic (Bradley *et al.*, 1988). So multiple antibiotics are employed, and the antagonism to algae needs to be tested. *U. prolifera* sporophyte is sensitive to *chloramphenicol* and

hygromycin, instead of *polymyxin* B, *penicillin*, *streptomycin*, *kanamycin* and *neomycin sulfate* (Wu *et al.*, 2015). In the work, three antibiotics have no effect on the survival of algae, consistent with the report. We further mixed these antibiotics to remove the typical epiphytic bacteria in *U. prolifera*.

When plate cultures were employed to test the sterilizing effect, sterility is confirmed when the culture medium remains clear after bacterial inoculation for 2 weeks. It is because marine bacteria grow slowly (Fries, 1963). However, it is not reliable to depend on inoculating agar plate or liquid medium, because only less than 1% of microorganism can be screened with the standard medium (Amann *et al.*, 1995). It explains why only *Bacteroides* exist in the three medium used in this work. To improve detection, Spoerner *et al.* (2012) used 16S rDNA molecular probe. Therefore, combination of plate culturing, microexamination and fluorescent staining was applied to eliminate false negative caused by the secondary inhibition of medium. In this experiment, plate culture, 16S rDNA and metagenomics were used for complementation.

After sterilizing, *U. prolifera* alter the leaf morphology soon, due to the absence of some indispensable bacteria for its morphogenesis. In previous research, *Cytophaga* spp. of marine *Bacteroidetes* induced the differentiation of *Monostroma oxyspermum* by secreting the metabolite of Thallusin. The minimum effective concentration reached 1×10^{-18} g/mL (Matsuo *et al.*, 2005). In addition, it promoted the sterile sporophyte germination of *Ulva pertusa* and *Ulva intestinalis*. Although no *Phagocytes* were detected in our experiment, four strains of *Bacteroidetes* such as *R. Bacterium* and *R. muscosus* were isolated in plate culture. They belonged to the *Cytophaga-Flavobacterium-Bacteroides* group based on 16S rDNA sequence. It needs to further study whether the secreted analogues is similar to *Thallusin* and whether it affects the morphologenesis of *U. prolifera*.

The microflora on algae surface are composed of bacteria (90%), where *Bacteroidetes* accounts for 13% and *α-Proteobacteria* for 70% (Tujula *et al.*, 2010; Wu, 2012). The latter contains *Rhodobacteraceae*, *Marinobacter* spp., *Marivita* spp. *Maribacter* and *Roseovarius mucosus*, which function on morphogenesis and are included in plate culture of this work (Kessler *et al.*, 2018).

According to metagenomics, *Alteromonas* of *Gammaproteo* is the main extracellular bacterium of *U. prolifera*. As a gram negative bacterium widely around the ocean, *Alteromonas* can promote the germination of algal zoids (Wang *et al.*, 2011). So far, nine type species have been reported (Xi *et al.*, 2005). Wherein two species of *A. macleodii* have been reported as the whole genome sequence (Dai, 2015). It can be used as model organisms for the interactions between algae and bacteria.

Thirteen strains out of 38 marine bacterial stimulate the zoids of *Ulva linza* into a tubular structure, but none of the bacteria functions independently (Marshall *et al.*, 2006). These bacteria have a synergistic effect on morphogenesis. Thus some special bacteria should be considered, such as the extra bacteria of *U. prolifera* endophyte (*Cyanobacteria* and *Chlorobi*).

Conclusion

U. prolifera treated with mixed antibiotics grew loosely with uneven cell size. Epiphytic bacteria of *U. prolifera* contained kinds of *Bacteroidetes*, varying in different culture mediums. Through metagenomics analysis, 90 species of bacteria, shared in epiphyte and antibiotics treated strains, involved in *Bacteroidetes*, *Planophyta* and *Proteobacteria*. Besides, 19 strains in epiphyte and 48 in antibiotics treated strains were detected. Overall, Alteromonas and Hyunsoonleella dominanted bacteria species of epiphyte and antibiotics treated strains, respectively.

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