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Bacterial community structure of the marine diatom *Haslea ostrearia*

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ABSTRACT

Haslea ostrearia produces a water-soluble, blue-green pigment, called marennine, with proven economic benefits (as a bioactive compound used to green oysters, which improves their market value). Incomplete knowledge of the ecological features of this marine diatom complicates its cultivation. More specifically, the ecology of bacteria surrounding *H. ostrearia* in ponds is what remains unknown. The structure of this bacterial community was previously analyzed by means of PCR-TTGE before and after isolating *H. ostrearia* cells recovered from 4 localities in order to distinguish the relative parts of the biotope and biocenose and to describe the temporal dynamics of the bacterial community structure at two time scales (2 weeks vs. 9 months). The bacterial structure of the phycosphere differed strongly from that of bulk sediment. The level of similarity between bacteria recovered from the biofilm and suspended bacteria did not exceed 10%. On the other hand, similarities among the bacterial community structures in biofilms were above 90% regardless of the geographic origin of the algal isolates, while the percentages were lower for suspended bacteria. The differences in bacterial structures of two *H. ostrearia* isolates (HO-R and HO-BM) resulted in specific metabolomic profiles. The non-targeted metabolomic investigation revealed more distinct profiles in the case of this bacteria-alga association than for the *H. ostrearia* monoculture. At the culture cycle scale under laboratory conditions, the bacterial community depended on the growth stage. When *H. ostrearia* was subcultured for 9 months, a shift in the bacterial structure was observed as of 3 months, with the bacterial structure stabilizing afterwards (70%–86% similarities), in spite of the size reduction of the *H. ostrearia* frustule. Based on these results, an initial insight into the relationships between *H. ostrearia* and its surrounding bacteria could be drawn, leading to a better understanding of the ecological feature of this marine diatom.

1. Introduction

Haslea ostrearia is a cosmopolitan species of diatoms commonly found on the French Atlantic coast, especially in oyster ponds of the Bay of Marennes-Oléron and Bay of Bourgneuf [1]. This diatom has long been the subject of curiosity [2] and became a topic of investigations due to its water-soluble, blue-green pigment, called marennine, which is responsible for the greening of oysters. This blue-green pigment, produced when *H. ostrearia* blooms, is released into the seawater, at which point the ponds turn green. At this stage, *H. ostrearia* is the dominant diatom species in ponds, and oyster farmers take advantage of this phenomenon by immersing their oysters in these shallow waters for 'refinement' (fattening) and greening, since these last two stages of raising oysters guarantee product quality and improve a farm's profits. Beyond its benefit in aquaculture for greening oysters [3], marennine has been shown to possess several biological functions with potential biotechnological applications, namely: i) antibacterial, anticoagulant and antiviral activities [4,5]; ii) antioxidant activity [6]; and iii) antitumor and antiproliferative effects of the aqueous extract from *H. ostrearia* on solid tumors (lung and kidney carcinoma and melanoma cell line molecules). Until now however, an incomplete knowledge of the ecological conditions under which this microalgae develops in its natural ecosystem has complicated controlling the cultivation of *H. ostrearia*, even though dedicated photobioreactors adapted to the physiological specificity of this microalga - through the use of artificially immobilized cells - were designed at the laboratory scale [7–10]. This microalga indeed exhibits several types of behavior, primarily benthic, occasionally planktonic but also epiphytic [11,12], thus making immobilization a relevant course of action. The ecophysiology of *H. ostrearia* is complex and moreover not yet completely understood. In oyster ponds, *H. ostrearia* can outcompete other microalgae yet is also being consumed by oysters [13–15]. The *H. ostrearia* biotope has also been studied; it was demonstrated that: i) this diatom is extremely tolerant to high irradiance (thus offering an ecological advantage over the other main diatoms encountered in oyster ponds, e.g. *Skeletonema costatum* [16,17]); and ii) the greening phenomenon is controlled by the nutrient composition in oyster-pond waters (see [18] for

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conditions). Recently, a more detailed genetic characterization of *H. ostrearia* was undertaken by developing genetic molecular tools, which led to identifying new strains of *H. ostrearia* [19] along with a second species of blue diatom called *Haslea karadagensis*.

Surprisingly, only a few studies have focused on bacteria-microalgae interactions, in noting that some bacteria may increase the microalgal biomass while offering potential applications in aquaculture. For now, nothing is still known for the specific case of *H. ostrearia*, yet for instances regarding bacteria and diatoms overall, "they have co-occurred in common habitats throughout the oceans for more than 200 million years, fostering interactions between these two groups over evolutionary time scales" [20]. The link between bacterioplankton and phytoplankton dynamics was recently demonstrated by Rooney-Varga et al. [21]. The habitat of phytoplankton-associated bacteria has been depicted by the concept of "phycosphere", i.e. the area around algal cells where bacteria feed on extracellular products of the algae [22]. The phycosphere is thus the aquatic analog of the rhizosphere in soil ecosystems and has direct implications for nutrient fluxes to and from algal cells. Bacteria-microalgae interactions have been studied for several diatom species, including *Guinardia delicatula*, *Pseudo-nitzschia pungens*, *Thalassiosira rotula*, *Skeletonema costatum* [23], *Ditylum sp.*, *Thalassiosira sp.*, *Asterionella sp.*, *Chaetoceros sp.*, *Leptocylindrus sp.*, *Coscinodiscus sp.* [24], *Pseudo-nitzschia multiseries* [25] and *Nitzschia microcephala* [26]. Some of these common species are frequently encountered in oyster-pond waters and sediments [11,18]. On the whole, the bacterial biodiversity of the phycosphere was shown to be limited in comparison to the complexity of bacterial assemblages in bulk seawater [24]. The structure of the bacterial community related to microalgae is specific to the microalgae species [20,24,27], though some bacterial phylotypes, such as bacteroides, are known to play a significant role in nutrient cycling by degrading algal macromolecules; moreover, such species attach to growth and are then recovered in most phycospheres [28]. To demonstrate this specific bacterial-algal interaction, Schäfer et al. [24] attempted, using two algal cultures, to associate each culture with the "satellite" bacterial assemblage of the other culture and proved such an association infeasible.

Based on these considerations, i.e. an incomplete knowledge of the ecological features of *H. ostrearia*, the present work has been intended to: i) characterize the structure of the bacterial community by means of PCR-TTGE both before and after *H. ostrearia* isolation from oyster ponds in different localities; ii) compare the bacterial community of the *H. ostrearia* phycosphere vs. free cells within the culture medium; iii) distinguish the relative portion of the biotope and biocenose based on the bacterial structure composition; and iv) describe the temporal dynamics of the bacterial community structure at the time scale of one culture cycle under laboratory conditions and after several subculturing steps. A metabolic fingerprinting (untargeted approach) was aimed at assessing the global metabolic profile of *H. ostrearia* cultures, whether or not associated with the phycosphere bacteria. Additional clarifications on the bacteria-*H. ostrearia* associations were provided, as well as on the role of the geographic origin of *H. ostrearia*. The compound class or classes potentially affected under these study conditions were not anticipated; moreover, specific compounds were not necessarily identified or quantified.

2. Materials and methods

2.1. Sampling location

The test samples were collected in oyster ponds from four localities along the French Atlantic coast, along the following north-to-south gradient: Bouin (46.96°N; 2.04°W), La Barre-de-Monts (46.90°N; 2.11°W), Isle of Ré (46.22°N; 1.45°W), and La Tremblade (45.80°N; 1.15°W) (see Fig. 1). One liter of each sample was collected at the seawater-sediment interface on the bottom of the oyster ponds in order to obtain both sediment and seawater. The samples were immediately stored at 4 °C. In the laboratory, the presence of *H. ostrearia* was verified before isolation.

2.2. Isolation of H. ostrearia from environmental samples and cultivation

Monospecific cultures of *H. ostrearia* were obtained by isolating a single cell of *H. ostrearia* from the raw samples. The specimen was recovered using a capillary pipette and an inverted microscope; it was then washed by successive cell subculturing in filtered seawater (0.22 μm) to remove contaminants (e.g. bacteria, other microalga, flagellate, larvae). Among the *H. ostrearia* isolates, six were selected for the following studies: HO1 and HO2 Bouin (HO1-B and HO2-B), HO La Barre-de-Monts (HO-BM), HO Isle of Ré (HO-R), and HO1 and HO2 La Tremblade (HO1-T and HO2-T).

For the ensuing experiments, the aforementioned isolates were grown in 250 mL-Erlenmeyer flasks filled with 150 mL of the modified Provasoli [29] medium (ES1/3: [30]) to obtain sufficient biomass. The monospecific isolates of *H. ostrearia* were transferred during the exponential growth stage (every 7–10 days) into a fresh ES 1/3 medium. The cultures were incubated in a culture chamber at 16 °C under 120 μmol photons m^{-2} s^{-1} and a 14:10 h light: dark regime.

2.3. Algal fingerprints and the bacterial community structure

2.3.1. Sample preparation

2.3.1.1. Raw sediments. The seawater and sediment of the raw samples recovered in the oyster ponds were separated by overnight sedimentation in a culture chamber at 16 °C. The samples were then frozen at −20 °C. 0.5 g of sediment was used for DNA extraction purposes.

2.3.1.2. Supernatant and biofilm from monospecific H. ostrearia cultures. To compare the bacterial community structure in the algal biofilm, which entails comparing the bacteria embedded in exopolysaccharides forming biofilm and epiphytic bacteria of *H. ostrearia* with that of the suspended cells in the culture medium, the biofilm and supernatant from the culture of *H. ostrearia* were separated. From cultures during the exponential growth stage in 250-mL Erlenmeyer flasks filled with 150 mL of ES 1/3 medium, 100 mL of the liquid - attached at the bottom of the Erlenmeyer flasks - were carefully collected to avoid contact with the biofilm, and the few free alga (possibly associated with bacteria) were removed by centrifugation (SIGMA 3K30 Fisher Bioblock Scientific: 900*g*, 90 s, 16 °C) to ensure recovering in the supernatant just the bacteria in suspension within the culture medium. The supernatant was filtrated through a 0.22-μm filter (cellulose nitrate membrane, Sartorius) so as to concentrate the bacteria on the filter, which was then frozen at −20 °C. Before DNA extraction, each filter was cut into small pieces of about 4 mm^2.

From this same culture, the remaining 50 mL were eliminated, and 30 mL of fresh ES 1/3 medium were added to the Erlenmeyer flask; the algal biofilm was recovered by means of the mechanical action of a sterile bar magnet. Microalgae and bacteria (epiphytic and those embedded in the biofilm) were both recovered by centrifugation (SIGMA 3K30 Fisher Bioblock Scientific: 6000*g*, 5 min, 16 °C), and the samples were frozen at −20 °C prior to DNA extraction.

2.3.1.3. Cultures of H. ostrearia at various growth stages and generations. For both experiments, approx. $1.5 \cdot 10^6$ algal cells were collected once the culture had been homogenized and then centrifuged (Universal 320 Hettich: 6000*g*, 10 min, 16 °C); the pellets containing suspended cells and cells of the biofilm were frozen at −20 °C prior to DNA extraction.

The bacterial community structures of the HO-BM and HO-R isolates were studied at the time scale of one culture cycle. Samples were collected at the time of transferring *H. ostrearia* into the fresh ES 1/3