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Research article

Inhibition of *Nitzschia ovalis* biofilm settlement by a bacterial bioactive compound through alteration of EPS and epiphytic bacteria

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ABSTRACT

Background: Marine ecosystems contain benthic microalgae and bacterial species that are capable of secreting extracellular polymeric substances (EPS), suggesting that settlement of these microorganisms can occur on submerged surfaces, a key part of the first stage of biofouling. Currently, anti-fouling treatments that help control this phenomenon involve the use of biocides or antifouling paints that contain heavy metals, which over a long period of exposure can spread to the environment. The bacterium *Alteromonas* sp. Ni1-LEM has an inhibitory effect on the adhesion of *Nitzschia ovalis*, an abundant diatom found on submerged surfaces.
Results: We evaluated the effect of the bioactive compound secreted by this bacterium on the EPS of biofilms and associated epiphytic bacteria. Three methods of EPS extraction were evaluated to determine the most appropriate and efficient methodology based on the presence of soluble EPS and the total protein and carbohydrate concentrations. Microalgae were cultured with the bacterial compound to evaluate its effect on EPS secretion and variations in its protein and carbohydrate concentrations. An effect of the bacterial supernatant on EPS was observed by assessing biofilm formation and changes in the concentration of proteins and carbohydrates present in the biofilm.
Conclusions: These results indicate that a possible mechanism for regulating biofouling could be through alteration of biofilm EPS and alteration of the epiphytic bacterial community associated with the microalga.

1. Introduction

Benthic diatoms are among the dominant forms reported in marine biofilms [1]. These microorganisms are unicellular algae that excrete extracellular polymeric substances (EPS) with a high carbohydrate content and are used in the formation of biofilms [2,3]. The formation of microbial biofilms on surfaces is one of the most important factors that affects the adhesion and growth of benthic diatoms [4,5,6]. The substrate upon which biofilms grow is determined by the topology [7,8] and chemical composition of the surface, light conditions, and water currents [9,10,11]. The production and composition of EPS differs in response to environmental conditions as well as the growth stage of the diatoms [12,13], suggesting that microalgae modulate their polysaccharide biosynthesis machinery to adapt to environmental conditions and allow them to adhere to surfaces [14]. In diatoms, EPS have different functions, with the most commonly reported ones including the stabilization of habitats, colony formation, adhesion and motility on the substrates [12,15]. The process of adhesion is different for diatoms than for bacteria, since they lack flagella and are therefore incapable of actively approaching a particular surface. However, biofilm-forming diatom species move by gliding via the excretion of extracellular polymeric substances from their raphe, an elongated slit in the cell wall [16,17]. It has been suggested that the observed rotational movements of diatoms can be produced by pulling off one pseudopod or stalk from the substratum with the help of extracellular polymeric substances. When a pseudopod or stalk is adhered to the substratum, the resulting torque causes a whole-cell rotational movement [18]. Although diatoms randomly settle, once in contact with the surface, they begin an active engagement where the initial reversible contact (primary adhesion) determines whether the diatom will continue its life cycle in that location (secondary adhesion) [19]. It has been suggested that the interaction between bacteria and diatoms has an important role in their ecological success [20]. Bacteria and diatoms interact in the phycosphere, where the bacteria can access algal exudates, such as polysaccharides, sugars, proteoglycans, small amino acids and glycoproteins [20]. These exudates can be species-specific and may determine the type of bacteria that associate with a diatom species [4]. The attachment of diatoms on surfaces makes them important fouling organisms due to their adherence to man-made substrates,

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which, in addition to bacteria, constitute a major problem for artificial structures immersed in the marine environment [4]. The recent ban on toxic antifouling biocides has underscored the need for the development of "environmentally friendly" strategies and has stimulated an active search for non-toxic natural marine antifouling compounds [21]. The bacterial strain *Alteromonas* sp. Ni1-LEM, first isolated by our lab from a natural substrate (red macroalgae; *Rhodymenia* sp. [22,23]) in northern Chile, produces an extracellular, thermostable compound that has a peptidic nature and a molecular weight lower than 3500 Da. This inhibitory compound showed antifouling activity against *Nitzschia ovalis*, a cosmopolitan benthic diatom often present in algal blooms [22,23]. The aim of this study was to characterize the antagonistic effect of the bioactive fraction secreted by *Alteromonas* sp. Ni1-LEM on microalgal biofilm development by altering the secretion of microalgal EPS. We also determined the phylogenetic affiliations of the diatom epiphytic communities with and without the antifouling compound by analyzing metagenomic 16S ribosomal DNA (rDNA) sequences.

2. Materials and methods

2.1. Bacterial culturing and isolation of the bioactive fraction

The strain *Alteromonas* sp. Ni1-LEM [23] was incubated in 1 L of M9 minimal medium at 20°C with constant stirring at 120 rpm for 4 d until the stationary phase was reached. The culture was centrifuged at 9632 *g* for 15 min at 4°C (Avanti J-25I, Rotor JA-14, Beckman Coulter, USA). Next, the supernatant was filtered through 0.2- μm nitrocellulose membranes (Sartorius, Germany) and concentrated 10-fold (10×) in a rotary evaporator (R200, Büchi, Switzerland) at 60°C. The concentrated supernatant was dialyzed at 4°C using a 1000-Dalton cut-off dialysis membrane (SpectraPor®, USA). The dialyzate (SN10X) was filtered again through nitrocellulose membranes and stored under sterile conditions at 4°C until its later use. The protein concentration was determined using a BCA Protein Assay kit (ThermoScientific, USA) according to the manufacturer's instructions.

2.2. Diatom culture conditions

The benthic microalgae *Nitzschia ovalis* Arnott (14 μm × 6 μm) was isolated from a commercial abalone (*Haliotis rufescens*) hatchery in Caldera, Chile (27°03′24″S-70°51′30″W) [23]. For culturing, 250- mL flasks containing 150 mL of Guillard f/2 medium [24] were inoculated with 1×10^5 cells mL^{-1} of the non-axenic benthic microalga *N. ovalis* in pre-stationary phase. The monoalgal cultures were incubated for 5 d at 20°C under a light intensity of 100 μmol photons m^2 s^{-1}, after which the cultures were sonicated for 15 s to separate the cells from the flask walls. The optical density of the cultures was determined every 24 h at 560 nm using a Halo20 Dynamica (Australia) spectrophotometer. The number of cells was determined by a direct count of ten fields at 20× magnification. For EPS extraction, 145-mL cultures of *N. ovalis* were grown and 5- mL samples were evaluated by microscopy. All experiments were run in triplicate using independent cultures.

2.3. Extraction of EPS

Two chemical methods (2.3.1 and 2.3.2) and one physical method (2.3.3) were used to extract EPS from *N. ovalis* biofilms.

2.3.1. Extraction with formaldehyde and NaOH (modified from Pan et al. [25])

The cell suspension was first extracted with formaldehyde (36.5%) for 1 h at 4°C followed by NaOH (1 M, 4°C, 3 h). The samples were centrifuged at 4873 g (4600 rpm, Rotanta 460R, Hettich, Germany), 4°C for 15 min. Next, the supernatants were filtered through a 0.45-μm membrane and concentrated in a rotary evaporator (R200, Büchi, Switzerland) at 60°C to a volume of 50 mL, which was subsequently dialyzed under sterile conditions for 24 h using 3500 Da MW cut-off dialysis bags (Snakeskin, Pierce, USA). During the dialysis, the water was replaced after 4, 8, 16 and 24 h. Once dialyzed, the EPS was lyophilized and stored at −20°C until further processed.

2.3.2. Ethanol precipitation (modified from de Brouwer et al. [26])

Soluble EPS was obtained by centrifuging 145 mL of culture at 3500 g for 15 min at room temperature. The supernatant was transferred to a bottle containing 435 mL of cold ethanol (96%), and the soluble EPS was allowed to precipitate overnight at −20°C. After centrifugation (15 min at 3500 g), the EPS pellet was lyophilized.

2.3.3. Extraction by ultrasonication (modified from Pan et al. [25])

The cell suspension was first ultrasonicated at room temperature at 37 kHz for 30 s using an ultrasound generator (Elmasonic S, Germany). The sonicated cell suspension was then centrifuged at 4873 *g* (4600 rpm, Rotanta 460R, Hettich, Germany) for 15 min at 4°C. Next, the supernatants were filtered through a 0.45- μm membrane and concentrated in a rotary evaporator (R200, Büchi, Switzerland) at 60°C to a volume of 50 mL, which was subsequently dialyzed under sterile conditions for 24 h using 3500 Da MW dialysis bags (Snakeskin, Pierce, USA). During the dialysis, the water was replaced after 4, 8, 16 and 24 h. Once dialyzed, the EPS was lyophilized and stored at −20°C until further processed.

2.4. Chemical analysis of EPS

The lyophilized EPS was analyzed for protein and carbohydrate content. The protein concentration was measured with a BCA Protein Assay Reagent kit (Pierce, USA) using bovine serum albumin as a standard. The total amount of carbohydrates in EPS samples was determined by the phenol-sulfuric acid method using glucose as a standard [27].

2.5. Adherence assays

To evaluate the adherence of *N. ovalis* to glass, and the effect of the bacterial supernatant on EPS, 125- mL flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase diatom cultures to a density of 1×10^5 cells mL^{-1}. Concentrated and dialyzed bacterial supernatant (SN10X) was added to a final concentration of 100 μg mL^{-1} of total protein. The microalgae culture was incubated at 20°C under a constant illumination of 100 μmol photons m^2 s^{-1}. As controls, flasks containing 50 mL of Guillard f/2 medium were inoculated with pre-stationary phase cultures of the microalga *N. ovalis* to a density of 1×10^5 cells mL^{-1} without the addition of the concentrated and dialyzed bacterial supernatant. Coverslips were placed at the bottom of the flasks and were later recovered for microscopy analyses. All experiments were performed in triplicate using independent cultures. After diatoms were cultured for 24 and 48 h, EPS were extracted using the most efficient protocol. Each replicate was standardized to 2.5 mg mL^{-1} for protein and carbohydrate analyses. The remaining EPS were stored for subsequent spectroscopy analyses.

2.6. FT-IR spectroscopy

Samples were prepared for by grinding the extracted EPS with potassium bromide and forming the mixture into a pellet. FTIR spectroscopy (Spectrum Two, Perkin Elmer) was used to read the absorbance from 650 to 4000 cm^{-1} (4 cm^{-1} resolution and a laser incidence of 20 repetitions) and detect the major structural groups of EPS present in the biofilm.