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Detection of quorum sensing systems of bacteria isolated from fouled marine organisms

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ABSTRACT

N-Acyl homoserine lactones (AHLs or *N*-AHLs) are a class of signaling molecules involved in bacterial quorum sensing (qs) that have recently been proposed as mediators of the fouling process. In this study, we determined the presence of AHLs in the following marine bacteria strains, which were collected in Santa Marta Bay (Colombia) from heavily fouled surfaces: *Ochrobactrum* sp., *Vibrio* sp. (23-6PIN), *Vibrio campbellii*, *Vibrio* sp. (11-6DEP), *Ochrobactrum pseudogringnonense*, *Shewanella* sp., *Vibrio harveyi* and *Alteromonas* sp. The detection and identification of AHLs was conducted using the microbial biosensor *Escherichia coli* (pSB401) and GC–MS and HPLC-MS analyses. We found that all isolated marine strains had quorum sensing systems mediated by either *N*-butanoyl homoserine lactone or *N*-hexanoyl homoserine lactone and in some cases by both. These results are in agreement with the theory that qs is involved in the fouling process. It is noteworthy to mention that we identified qs systems for the first time in bacteria of the genera *Ochrobactrum* and *Alteromonas*.

1. Introduction

In marine environments, living or inert surfaces that are not protected are rapidly colonized by micro- and macroorganisms through a process known as fouling (Fusetani and Clare, 2006). This process occurs in several stages when different types of micro- and macroorganisms settle. The selection of the surface to be colonized depends on both biotic and abiotic factors (Koehl, 2007). Among the biotic factors, the presence of bacterial biofilms is critical, as they can either stimulate or deter the settlement of some macroorganisms (Kato et al., 2006). These biofilms can be thought of as stratified, sessile bacterial communities attached to the surface in question (Shapiro, 1998). The formation and maturation of biofilms is influenced by many factors (Greenberg, 2003) and quorum sensing plays a fundamental role (Lowery et al., 2008). Recently, it has been proposed that if bacterial quorum sensing is disrupted, biofilm maturation will be affected, thus avoiding (or at least reducing) the occurrence of macrofouling Dobretsov et al. (2009) suggested that qs inhibitors (qsi) directly control the formation of multi-species biofilms and indirectly affect the subsequent larval attachment on those modified biofilms by altering the biofilm properties (Dobretsov et al., 2009). Furthermore, it has been established that bacterial biofilms play an important role in the larval settlement of benthic marine invertebrates. Therefore, the use of qsi could be beneficial in fouling control.

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It is well known that Gram-negative bacteria are dominant in the marine environment (Zhang et al., 2006). This group of bacteria use qs systems regulated by *N*-acyl homoserine lactones (AHLs) (Fig. 1), (Waters and Bassler, 2005) while gram-positive bacteria qs systems are mediated by small peptides. *N*-acyl homoserine lactones consist of a fatty acid coupled to a homoserine lactone (AHL). AHLs can vary in the acyl group chain length (C4-C18), C3 substitution pattern (hydrogen, hydroxyl or oxo group), and the presence or absence of one or more carbon–carbon double bonds in the fatty acid chain (Dobretsov et al., 2009).

There are few studies on the occurrence of AHLs in bacteria isolated from marine sources. One noteworthy study was conducted by Mohamed et al. (2008) who demonstrated the production of AHLs by α- and γ-Proteobacteria isolated from the marine sponges *Mycale laxissima* and *Ircinia strobilina*, which were collected at Conch Reef, Key Largo, Florida. The presence of signaling molecules was detected in both bacterial extracts and the water column surrounding the sponges. However, it was observed that AHLs produced by the bacteria isolated from the sponges were different than the AHLs produced by the bacteria isolated from the water column (Mohamed et al., 2008). In a study carried out by Taylor et al. (2004), samples of the sponge *Cymbastela concentrica* were collected from several coastal locations near Sydney in southeastern Australia. The production of *N*-hexanoyl homoserine lactone (C6-AHL) and *N*-(3-oxo)-hexanoyl homoserine lactone (3-oxo-C6-AHL) by a *Vibrio* sp. strain was found. *Agrobacterium tumefaciens* At-AHL and *Chromobacterium violaceum* CV026 were employed as biosensors, and GC–MS analysis was used to study the AHLs found. In both the methanolic extract of this sponge and the associated bacteria, the same AHLs were identified, suggesting that these bacteria could produce these signaling molecules under natural conditions (Taylor et al., 2004).

In this paper, we present a study carried out with Gram-negative bacteria isolated from biofilms coating the sponges *Aplysina lacunosa* and *Aplysina insularis*, the shell of a *Donax* sp. bivalve, and submerged phytagel dishes. All of these bacteria were collected in Santa Marta Bay (Colombian Caribbean Sea). The aim of this work was to determine the presence of AHLs and subsequently that of qs systems.

2. Experimental

2.1. Materials and methods

TLC analyses were conducted on precoated RP-18 F_{254} plates (Merck), using MeOH/H_2O (6:4 or 7:3) as the eluting solvent. *N*-hexanoyl-DL-homoserine lactone (C6-AHL) (Fluka) was used as a standard, and the spots were visualized using *Escherichia coli* (pSB401) in Luria–Bertani media as a biosensor strain. Bioluminescence photographs were captured using a BIORAD Quantity-One 4.1.0 imager. Column chromatography was performed using LiChroprep RP-18 as the stationary phase (40–63 μm, Merck).

GC analyses were performed using a GC-17A SHIMADZU gas chromatographer, connected to a QP-5050A SHIMADZU mass spectrometer. Samples were prepared by dissolving 1 mg of extract in 1 mL of acetone. Sample injection was performed in split mode (10:1) into a RPX-5 capillary column (30 m × 0.32 mm × 0.25 μm -length, i.d., film thickness, respectively). Helium (UHP grade) was used as the carrier gas at a flow rate of 1.9 mL min^{-1}. The GC injector temperature was set to 200 °C. The oven temperature program was set to hold at 50 °C for 3 min and then increase by 8.3 °C min^{-1} up to 300 °C. Mass spectrometry conditions were as follows: electron ionization source set to 70 eV, with the mass spectrometer run in full scan mode (*m/z* 40–800) and in SIM mode (*m/z* 102 and 143).

HPLC-MS analyses were performed with a LC-10AD SHIMADZU HPLC system connected to a LCMS-2010 EV SHIMADZU mass spectrometer. Reversed phase separation was carried out using a Phenomenex Luna C18 column (particle size 5 μm, 150 × 2.0 mm, -length, i.d., respectively). The flow rate was 0.3 mL min^{-1}. A linear gradient from 15% MeOH in 0.1% formic acid to 100% MeOH, over 15 min, was used. Mass spectrometry conditions were as follows: electrospray ionization (ESI) source set to 1.5 kV, with the mass spectrometer operated in positive ion, full scan mode (*m/z* 100–300). Nitrogen was used as the nebulizer gas at a flow rate of 1.5 L min^{-1}.

2.2. Growth media

Marine broth (MB), marine medium (MM) and modified marine medium (mMM) were used as the culture media for marine bacterial growth. The MB composition was NaCl 2.08%, KCl 0.06%, $MgSO_4$ 0.48%, $MgCl_2$ 0.40%, Rila 0.15%, Tris 0.04%, peptone 0.30%, yeast 0.15% and glycerol 0.15%. The MM composition was the same as MB with agar–agar 1.80%. The mMM

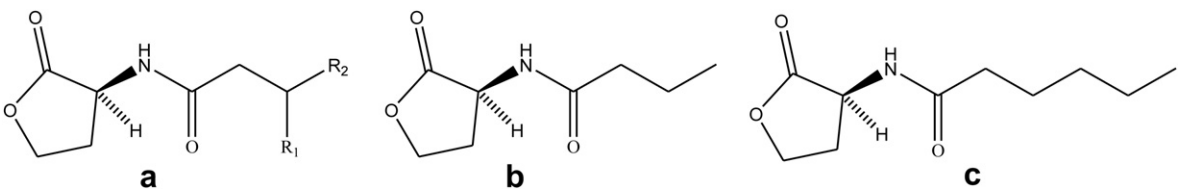

Fig. 1. AHL Structures: a. General Structure: R_1: aliphatic chain, and R_2: H, OH, or =O. b. *N*-butanoyl homoserine lactone. c. *N*-hexanoyl homoserine lactone.