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Inhibition of bacterial fouling by soft coral natural products

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ABSTRACT

Six common soft coral species (*Sarcophyton* sp., *Sinularia* sp.1 and sp.2, *Cladiella* sp., *Scleronephthya* sp. and *Dendronephthya* sp.) from Bandar Al-Khayran (Sultanate of Oman) had significantly lower bacterial density in comparison with surfaces of empty shells. Methanol: chloroform (1:1) extracts of these species were tested against Gram positive (*Micrococcus luteus, Staphlococcus aureus, Bacillus subtilis, Bacillus* sp.) and Gram negative (*Salmonella enterica, Pseudomonas aeruginosa, Escherichia coli, Cytophaga* sp., *Pseudomonas* sp., *Shewanella* sp.) marine biofouling and pathogenic bacterial strains. All tested extracts had some activity against human pathogens and the highest antimicrobial activity was observed for extracts of *Sinularia* sp.1 and *Cladiella* sp. (inhibited 50% and 60% of the strains, respectively). Only ethyl acetate extracts of *Cladiella* sp. inhibited growth of biofouling bacteria. The active fraction was purified and identified as a mixture of hexadecyl palmitate and hexadecyl stearate. Pure hexadecyl palmitate inhibited growth (*Bacillus* sp. and *Psychrobacter* sp.) and attachment (*Bacillus* sp., *Cytophaga* sp., *Pseudomonas* sp., *Psychrobacter* sp., *Shewanella* sp.) of the marine biofouling bacteria. The results of this study suggest that soft corals have developed mechanisms to combat microbial infections and inhibit bacterial fouling.

Introduction

Any natural or artificial substrata submerged into the marine environment are quickly colonized by micro- and macro-organisms in a process known as “biofouling” (Clare, 1996). Biofouling causes serious problems for marine industries and navies around the world (Yebra et al., 2004). Usually biofouling is prevented by biocidal antifouling and fouling release coatings (Rittschof, 2000, 2001). Modern antifouling biocides (Sea-Nine, Irgarol, isothiazolone and copper) are harmful to marine organisms (Karlson et al., 2010). These biocides are not effective against some marine microbes and their biofilms (Molino et al., 2009; Dobretsov and Thomason, 2011), which increase drag, fuel consumption and affect the performance of antifouling coatings (Yebra et al., 2006). Moreover, micro-fouling on the surface of reverse osmosis membranes of desalination plants cannot be prevented by toxic biocides or chlorination. Thus, there is a need for the development of “environmentally friendly” non-toxic antifouling protection effective against marine microbes.

Marine organisms, especially sessile ones, are faced with the problem of biofouling. They evolved chemical and physical defences to combat marine biofouling (Wahl, 1989; Rittschof, 2000, 2001). Thus, natural products from marine organisms can be potentially used for antifouling defence (Clare, 1996). Soft bodied sessile organisms, such as soft corals (*Alcyonacea*), are of particular interest as they have higher chemical diversity of natural products compared to other marine organisms (Gerhart et al., 1990; Paul and Ritson-Williams, 2008). Soft corals have been shown as an affluent resource of terpenoids, eicosanoids, steroids and cembranoids that are responsible for a range of biological activities that include

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anticancer (Shen et al., 2008; Lin et al., 2010; Xio et al., 2013), antimicrobial (Gerhart et al., 1990; Aceret et al., 1998; Vanisree and Subbaraju, 2002; Kumar and Lakshmi, 2006; Hunt et al., 2012), antiviral (Cheng et al., 2010; Mayer et al., 2013), and anti-inflammatory activities (Cheng et al., 2009; Tai et al., 2013).

Most studies on soft corals metabolites are based on isolation of novel compounds (see reviews: McClintock and Baker, 2001; Paul et al., 2011) and only limited studies have demonstrated that extracts of soft corals and their symbionts are able to inhibit growth of environmental microbes (Slattery et al., 1997; Aceret et al., 1998; Kelman et al., 1998; Bhosale et al., 2002; Harder et al., 2003; Hunt et al., 2012). Only few anti-microfouling compounds from soft corals have been isolated and characterized so far (see reviews: Gerhart et al., 1990; Fusetani, 2003; Qian et al., 2010). A waterborne compound homarine produced by Antarctic soft corals *Alcyonium paessleri* and *Gersemia antarctica* inhibited growth of biofouling bacteria (Slattery et al., 2001). The soft coral *Juncella juncea* produced juncellin 1 and 2 which inhibited growth of bacteria associated with the barnacle *Balanus amphitrite* (Avelin Mary et al., 1993). Desoxyhavannahine isolated from the soft coral *Xenia macrospiculata* inhibited growth of environmental bacteria with MIC 1.25 mg ml^{-1} (Kelman et al., 1998).

This study was designed to investigate the antimicrobial activity of soft corals in Oman waters as a possible source of bacterial fouling inhibitors. The main aims of this study were: (1) to investigate the presence of microorganisms on the surface of various octocorallia from Bandar al-Khayran, Oman; (2) to quantify the antimicrobial activity of extracts of octocorals against bacterial pathogens and fouling bacteria; and (3) to identify the substances responsible for the observed activity.

Material and methods

Soft corals collection

Six abundant soft coral (Octocorallia: *Alcyonacea*) species were collected by SCUBA divers from 10 to 15 m at Bandar Al-Khayran area (Muscat, Sultanate of Oman) (N 23.5227°, E 58.7475°). The soft corals were immediately placed into separate plastic bags with seawater and kept on ice until they were processed in the laboratory. A voucher sample for each soft coral was taken and kept in ethanol for taxonomic identification. Another sample was used for scanning electron microscopy and preparation of soft coral extract (see below). The soft coral specimens were identified by Mr. Kaveh Samimi-Namin (Nationaal Natuurhistorisch Museum, Leiden, Netherlands).

Scanning electron microscopy

Small pieces of each soft coral ($n = 5$) and empty shells ($n = 5$) from Bandar Al-Khayran were fixed in 5% buffered formalin and dehydrated in an increasing ethanol series, dried by the critical point procedure, and sputtered with gold (for details see Dobretsov and Qian, 2002). The specimens were examined by a JEOL 6300F (70 eV) scanning electron microscope (SEM) at magnifications of 1000x and 5000x. Bacteria were counted in 10 selected fields of view (8000 μm^2) per replicate.

Preparation of soft coral extracts

The wet weight of the coral and its volume were measured. Then, the soft coral whole tissues were chopped into small pieces and soaked in 1:1 methanol: chloroform solutions at +25 °C. After one week, extracts were filtered through the filter (Whatman No1) and the filtrates were collected. The same extraction and filtration steps were repeated three times and the resulting crude extracts for each species were combined. These extracts were dried under reduced pressure by a rotary evaporator (Büchi, Switzerland) and weighed on an analytical balance to the nearest 0.001 g. Dried extracts were re-dissolved in 1:1 methanol: chloroform and kept in the fridge at +4 °C until used for separation and bioassays.

Antimicrobial bioassays

Two types of bioassays were performed. First, we tested the ability of the extracts to inhibit growth of pathogenic and biofouling bacteria. Second, we investigated inhibition of attachment of biofouling bacteria by purified compounds. All extracts were tested at the tissue level concentrations.

A disc diffusion assay was performed on several Gram positive (*Micrococcus luteus, Staphlococcus aureus, Bacillus subtilis, Bacillus* sp.) and Gram negative (*Salmonella enterica, Pseudomonas aeruginosa, Escherichia coli, Cytophaga* sp., *Pseudomonas* sp., *Shewanella* sp.) marine biofouling and pathogenic bacterial strains according to Dobretsov and Qian (2002). Pathogenic bacteria were obtained from the culture collection at Sultan Qaboos University hospital. Biofouling bacteria (*Bacillus* sp., *Cytophaga* sp., *Pseudomonas* sp., *Shewanella* sp.) were isolated from biofilms covering stones in the area of the investigation. Before the bioassay, each bacterium was cultivated in marine broth (Oxoid, USA) for 48 h at +25° C (for biofouling bacteria) or at +37° C (for pathogens). Sterile paper discs (diameter = 5.5 mm and surface area 1 cm^2) made of Whatman No1 paper with extracts, fractions or pure compounds were used in the bioassay. Two μl of extracts or pure compounds were applied to disks and solvent were evaporated prior the bioassay. The standard antibiotic streptomycin (Sigma–Aldrich) was used as a positive control at a concentration of 1 g L^{-1}. 1:1 methanol: chloroform was used as a negative control at a concentration of 10 μl $disk^{-1}$. After 48 h, the diameter of the inhibition zone around the paper disks was measured with a ruler to the nearest 0.5 mm.

Purified compounds, hexadecanoic and octadecanoic acid (SC6-FA), hexadecanol (SC6Al) and hexadecyl palmitate (Sigma–Aldrich) (see below) were used in the disk diffusion experiments (see above) and in the bacterial attachment bioassays. The experiments were performed with biofouling bacteria *Bacillus* sp., *Psychrobacter* sp., *Cytophaga* sp., *Pseudomonas* sp., *Psychrobacter* sp. and *Shewanella* sp. Bacterial attachment assays were performed in 96-well plates. Firstly, bacteria were cultured in marine broth until the exponential phase of growth. Series of dilutions of the compounds were made and the concentrations ranging from 3×10^{-5} to 10 mg ml^{-1} were tested. In each well 100 μl of bacterial culture was inoculated at an optical density of 0.01 at 600 nm (OD_{600}). After 2 h of incubation at 25 °C, the wells were emptied and washed with distilled water. Bound cells were stained with 0.2% (wt/vol) crystal violet solution in ethanol at room temperature for 10 min. The wells were then washed with water three times, and dried at room temperature. The dye was solubilized with 95% ethanol. Alteration between the control and the treatment colour indicated differences in attachment of tested bacteria. Minimum inhibitory concentrations (MIC) were determined. The experiments were repeated 3 times.

Fractionation of soft coral extracts

Crude soft coral extracts were partitioned by liquid–liquid extraction using a separating funnel (Ebada et al., 2008). Differences were based on the properties of the soft corals extracts and their fraction solubility. Crude extract of *Sarcophyton* sp. was subsequently partitioned using hexanes, chloroform, butanol and water. Extract of *Sinularia* sp.1 was subsequently partitioned between ethyl acetate, butanol, and water. Extract of *Sinularia* sp.2 was subsequently