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ORIGINAL PAPER

Antifouling activity of sessile bacilli derived from marine surfaces

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Abstract Marine biofilms are a virtually untapped source of bioactive molecules that may find application as novel antifoulants in the marine paint industry. This study aimed at determining the potential of marine biofilm bacteria to produce novel biomolecules with potential application as natural antifoulants. Nine representative strains were isolated from a range of surfaces and were grown in YEB medium and harvested during the late exponential growth phase. Bacterial biomass and spent culture medium were extracted with ethanol and ethyl acetate, respectively. Extracts were assayed for their antifouling activity using two tests: (1) antimicrobial well diffusion test against a common fouling bacterium, *Halomonas marina*, and (2) anti-crustacean activity test using *Artemia salina.* Our results showed that none of the ethanolic extracts (bacterial biomass) were active in either test. In contrast, most of the organic extracts had antimicrobial activity (88%) and were toxic towards *A. salina* (67%). Sequencing of full 16 S ribosomal DNA analysis showed that the isolates were related to *Bacillus mojavensis* and *Bacillus firmus*. Matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS) profiling of ethyl acetate extracts of culture supernatants showed that these species produce the bioactive lipopeptides surfactin A, mycosubtilin and bacillomycin D.

Keywords Biofilm · *Bacillus* sp. · Antibiotics · Biotechnology · Lipopeptides

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Introduction

Surfaces immersed in aqueous environments are rapidly colonized by organisms. This biological phenomenon, known as biofouling, can cause enormous damage to marine infrastructure such as ship hulls and offshore platforms, leading to economic loss [5, 11]. Antifouling technology that has been currently applied to cope with biofouling include the use of broad-spectrum biocides such as tri-n-butyl tin (TBT)-bearing paints [30], along with fouling-release coatings. However, environmentally adverse consequences of using TBT-containing coatings and the often unsatisfactory performance of fouling-release

coatings have prompted research on natural, environmental friendly antifouling products [5].

Natural compounds derived from marine algae have been the subject of recent research to identify novel antifoulants, mainly based on the hypothesis that secondary metabolites produced by macroalgae deter colonization by fouling organisms [36]. However, it has recently been shown that certain epibiotic bacteria living associated with higher organisms (including algae) produce inhibitory extracellular agents that may benefit the host by deterring the colonization process of common fouling organisms [12, 29]. This suggests that under certain circumstances, a chemical protection is conferred to the host by the epibiotic biofilms and not as a result of constitutive chemical defense afforded by the host itself.

Therefore, it was the goal of this work to assess the ability of biofilm bacteria isolated from a tropical marine intertidal environment to produce antifouling compounds, identify the most active producing-bacteria and to perform a preliminary characterisation of the main compounds displaying antifouling activity.

Materials and methods

Isolation of biofilm bacteria and growth conditions

Leaves of the turtle grass *Thalassia testudinum* and limestone fragments were aseptically collected from a rocky intertidal shore in the State of Campeche (Mexico), chilled on ice and transported to the laboratory. To remove loosely attached bacteria the samples were gently washed with sterile seawater. Biofilm bacteria were recovered from the remaining attached biomass by placing the samples in 10 ml of sterile seawater, and then vortexing for 1 min to obtain cell suspensions. These suspensions were serially diluted and plated on Marine Agar 2216 (Difco, Detroit) and incubated at 30 °C for 5 days. Plates were checked daily, representative colonies were randomly picked, and successively restreaked for purification. Isolates were preserved as glycerol suspensions 20% (w/v) at −80 °C. Working cultures were maintained on Marine Agar 2216 slants at 4 °C.

Nine isolates were selected on the basis of colony characteristics and grown in liquid culture in flasks containing 500 ml of Yeast Extract Broth (YEB) [2] at 25 °C and 150 rpm in a rotary shaker (2 days). Bacterial growth was determined spectrophotometrically at 520 nm. During the late exponential growth phase, the cultures were centrifuged at 3,200*g* (5 min, 4 °C) to obtain bacterial cells and fermented culture broth, the latter being subsequently filtered [sterile Whatman (GF/C) filter paper] and treated as described below. The cells were washed with deionized water, dried and stored at −80 °C.

Preparation of extracts of bacterial biomass and culture fermentation broths

Ethanolic extraction of 100 mg dry bacterial biomass in 100 ml was carried out with constant shaking (24 h at 24 °C). Crude ethanolic extracts were obtained after evaporation at 40 °C under reduced pressure. The fermented culture broth was subjected to two liquid–liquid extractions with ethyl acetate (1:1, v/v). After extraction,the solvent was removed under reduced pressure at 40 °C to yield the corresponding organic extracts.

Antifouling bioassays

Determination of antibacterial activity

To determine the potential of antifouling activity of both, ethanolic and organic extracts, the marine bacterium *H. marina* was used as a model organism, given its role in primary colonization of surfaces and mediation of invertebrate attachment in fouling communities [21, 36]. All bacterial extracts were screened for production of antibacterial substances, using the well diffusion method [38]. *Halomonas marina* CCUG16095 obtained from the Culture Collection of the University of Göteborg, Sweden, was cultivated in modified YEB medium [2] at 26 °C and maintained on marine agar medium 2216 slants at 4 °C. Antibacterial tests were carried out in duplicate in Petri dishes containing 15 ml of Marine Agar 2216, inoculated with 0.2 ml of a bacterial suspension of *H. marina* with an $OD_{520\,nm}$ of 0.3 ($\sim 2 \times 10^9$ cells/ml). The bacterial suspension was homogeneously swabbed on the agar surface and five wells of 6 mm diameter were made on the agar using a sterile Pasteur Pipette [15]. The solutions of extracts were prepared at a concentration of 5% in dimethyl sulfoxide (DMSO, w/v; Sigma). Positive controls were included with solutions of 1% copper sulphate (w/v) and gentamicine at a concentration of 100 µg/ml [25] in DMSO. Pure DMSO was used as negative control. Fifty microliters of extract or control solutions were placed in the wells, and the plates were incubated at 30 °C for 24 h. The zones of inhibition around the wells were measured with a Vernier caliper (Table 1).

Determination of anti-crustacean activity

Brine shrimp eggs (Microfeast®) were hatched in seawater prepared with sea salts (Coralife®) at 38 g/l and supplemented with 6 mg/l of dried yeast under continuous air-bubbling with an aquarium pump at 27 °C under illuminated conditions [34]. Extracts were dissolved in a minimum amount DMSO at a final concentration of 1% and diluted with seawater to concentrations of 1,000, 100 and 10 µg/ml for triplicate. Copper sulphate was used as

Table 1 Antibacterial activity of extracts of biofilm marine bacteria against *Halomonas marina*

Isolate	Diameter mean of inhibition zone (mm)[a,b]	
	Ethanolic extract	Organic extract
MC3B01	–	–
MC6B02	–	9
MC1B03	–	14
MC3B04	–	9
MC6B06	–	10
MC6B12	–	10
MC1B14	–	13
MC1B16	–	10
MC1B17	–	11
Controls		
Copper sulphate (1% w/v)	14	
Gentamicine (100 μg/ml)	20	
Dimethyl sulphoxide	–	

[a] Very active: >15 mm; active: 8–14 mm; inactive (–): <7 mm in diameter

[b] Mean with standard deviation lower than 10%

positive control at a range of concentrations (100, 10 and 1 μg/ml). After 48 h, ten nauplii were placed in each test tube containing the test concentrations and controls. Lethal concentration of 50% (LC_{50}) values were determined by counting the dead nauplii after an incubation period of 24 h. Data were analyzed with the Finney computer program as described previously [22].

Identification of selected bacterial isolates

Bacterial isolates designated as MC1B-03, MC1B-14 and MC1B-17 were selected for molecular identification. The 16 S ribosomal DNA genes of overnight cultures of isolates MC1B03, MC1B14, MC1B17 were analyzed. DNA was extracted using the Wizard Genomic DNA Purification kit, according to the manufacturer's protocol (Promega, Madison, WI, USA). The 16 S rDNA gene was amplified by PCR using universal primers (Fd1 5′-CAGAGTTTG ATCCTG GCTCAG-3′) and (Rd1 5′-AAGGAGGTGATC CAGCC-3′) [40].

The PCR mixture contained 50 μl final volume: 5.0 μl of 10× buffer (Sigma), 5 μl of 25 mmol/l, $MgCl_2$ (Sigma), 0.5 μl of 10 mmol/l, dNTP mixture, 0.5 μl of a 50 pmol/μl solution of each primer, 0.5 μl of Taq polymerase (5 U/μl; Sigma), qsp. Water (Sigma). The amplification of DNA was performed using a Perkin Elmer Gene Amp PCR, System 2400. The PCR program was as follows: 95 °C for 1 min, 30 cycles of 20 s at 95 °C, 30 s at 55 °C, 1.30 min at 72 °C, and final extension of 5 min at 72 °C. PCR products were visualized under UV light after electrophoresis on a 0.8% (w/v) agarose gel containing ethidium bromide. PCR products were cloned using the pGEM-T-easy cloning kit and chemically competent *Escherichia coli* JM109 cells, according to the manufacturer's protocol (Promega). The clone library was screened by direct PCR amplification from a colony using the vector-specific primers SP6 (5′-ATTTAGGTGACACTATAGAA-3′) and T7 (5′-TAAT ACGACTCACTATAGGG-3′) and the same reaction conditions as described above, was used. A plasmid containing the right length insert was isolated using the kit Wizard Plus SV Minipreps DNA Purification System (Promega) as described in the protocol. Purified plasmids were sent for sequencing to Genome Express (Grenoble, France).

The nucleotide sequences of the 16 S rDNA genes were aligned using Clustal W 1.5 c [37] and then manually adjusted. Reference sequences were obtained from the Ribosomal Database Project II [20] and from EMBL and GenBank databases [3]. Positions of sequence and alignment uncertainty were omitted from the analysis. The pairwise evolutionary distances based on more than 1,400 unambiguous nucleotides was computed using the Jukes and Cantor method [16]. The dendrogram was constructed by the neighbor-joining method [32]. Confidence in the tree topology was determined by bootstrap analysis using 100 replicates of the sequences [7]. The full 16 S rDNA sequences of the three isolates were deposited into the GenBank database under the accession numbers AY833569 (MC1B-03), AY833571 (MC1B-14) and AY833572 (MC1B-17).

MALDI-TOF mass spectrometry analysis of bioactive molecules

Fermentation of *Bacillus* species was carried out in 2-l Erlenmeyer flasks containing 800 ml YEB and incubated as described earlier. Culture supernatants were obtained by centrifugation (3,200*g*, 25 min, 4 °C) and split into two aliquots of 400 ml. One aliquot was extracted twice (2 × 600 ml) with ethyl acetate. The second aliquot was brought to a final pH of 2.0 with 6N HCl, and the acid precipitate obtained after centrifugation (3,200*g* for 25 min at 4 °C) was extracted with ethyl acetate. Approximately 0.5–1 μl aliquots of the samples were mixed with 0.5 μl of matrix (5 mg/ml α-cyano-4-hydroxycinnamic acid; Bruker, Bremen, Germany) in 50% ACN/0.5% (v/v) directly on stainless steel targets (Applied Biosystems (ABI; Darmstadt, Germany) and dried under ambient conditions. Matrix assisted laser desorption (MALDI), time-of-flight (TOF) mass spectra were recorded by using a Perspective Biosystems MALDI-TOF instrument containing a 337-nm nitrogen laser for desorption and ionization [35].

Table 2 Anti-crustacean activity of extracts of marine biofilm bacteria against the crustacean *Artemia salina*

Isolate	Ethanolic extract LC_{50} (µg/ml)[a]	Organic extract LC_{50} (µg/ml)[a]
MC3B01	>1,000	340.1
MC6B02	>1,000	>1,000
MC1B03	>1,000	265.9
MC3B04	>1,000	>1,000
MC6B06	>1,000	416.9
MC6B12	>1,000	542.1
MC1B14	>1,000	701.9
MC1B16	>1,000	>1,000
MB1B17	>1,000	334.9
Controls		
Copper sulphate[b] (1% w/v)	3.2	
Dimethyl sulphoxide[c] (1% v/v)	>1,000	

[a] Lethal concentration of 50% (LC_{50}): >1,000 µg/ml: inactive; <1,000 a 200 µg/ml: active; <200 µg/ml: very active

[b] Positive control

[c] Negative control: 50 µL of DMSO in 5 ml of seawater

Results

Antifouling activity displayed by selected isolates

In contrast to the lack of activity in the ethanolic extracts, almost all organic extracts (88%) were active towards *H. marina*. Isolates MC1B03 and MC1B014 displayed levels of antibiotic activity against *H. marina* in the range of those obtained in the positive control of copper sulphate (Table 1). The anti-crustacean activity bioassay against the brine shrimp *A. salina* has been used as a preliminary assessment to determine the biocide activity against crustacean foulers [10, 28]. Similarly to the antimicrobial test, the ethanolic extracts had lethality values higher than 1,000 µg/ml and were thus indicative of inactivity (Table 2). Conversely, most of the organic extracts (67%) were active at concentrations lower than 1,000 µg/ml against *Artemia salina*. Isolates MC1B03 (LC_{50} = 265 µg/ml) and MC1B17 (LC_{50} = 334.9 µg/ml) were particularly active.

16 S rDNA sequencing of bioactive isolates

Full 16 S ribosomal DNA sequencing analysis of the three most active isolates from rock surfaces (epilithic biofilms) MC1B-03 (1561 pb), MC1B-14 (1579 pb) and MC1B-17 (1381 pb) showed that they all belonged to the genus *Bacillus* and were specifically related to *B. mojavensis* (MC1B-03 and MC1B-17), and *Bacillus firmus* in the case of isolate MC1B-14 (Fig. 1).

MALDI-TOF analyses of bioactive extracts

MALDI-TOF mass spectrometry (MS) profiling of ethyl acetate extracts of the culture supernatant of isolates MC1B-03 and MC1B-17 resulted in signals at *m/z* 1008.7, 1022.7 (1044.7), 1036.7 (1058.7; 1074.7) that correspond exactly to the calculated masses of protonated species of the lipoheptapeptide surfactin A with fatty acid chain lenghts of 13–15 carbon atoms (C_{13}–C_{15}, values in brackets represent corresponding Na^+- and K^+-adducts). Additional signals at *m/z* 1071.8, 1085.8 and 1099.8 are good hints for the presence of protonated lipoheptapeptides mycosubtilin/iturin (C_{16}–C_{18}), or bacillomycin F (C_{15}–C_{17}) species. Within extracts of MC1B-14 several peak clusters were detected; however, they do not fit to known antimicrobial active agents.

Discussion

This study shows that intertidal biofilm bacteria from tropical shores produce extracellular non-polar compounds with significant inhibiting activities against the crustacean *A. salina* and the marine fouling bacterium *H. marina*. Of the nine bacteria tested, three isolates produced inhibitory compounds in both tests. This is in agreement with recent studies that bioactive metabolite production is more common among surface-associated bacteria (both epibiotic and those derived from non-living substrata) compared to planktonic isolates; this phenomenon has been explained as ecological strategy exhibited by bacteria growing on the surfaces, those living in a highly competitive environment in which space and access to nutrients are limited [4, 12, 18, 19], and epilithic bacteria may deter colonization of substrata by bacterial and crustacean competitors by producing antibiotics.

Diverse surface-associated bacteria have been found to produce antifouling (antibiotic) compounds. These include the best-studied antifoulant-producing bacterium *Pseudoalteromonas tunicata* (commonly associated with algal, invertebrate and inanimate surfaces) along with a species of *Streptomyces* isolated from the surface of a jellyfish [4, 10, 13, 29]. In our study, the most active isolates were shown to be closely related to *B. mojavensis* (MC1B-03 and MC1B-17) and *B. firmus* (MC1B-14). Previous reports have shown that some culturable Gram-positive bacteria from marine environments belong to several species of the genus *Bacillus*, among these *B. firmus*, but not *B. mojavensis* [9, 14, 33]. As *B. mojavensis* was originally isolated from desert soil in

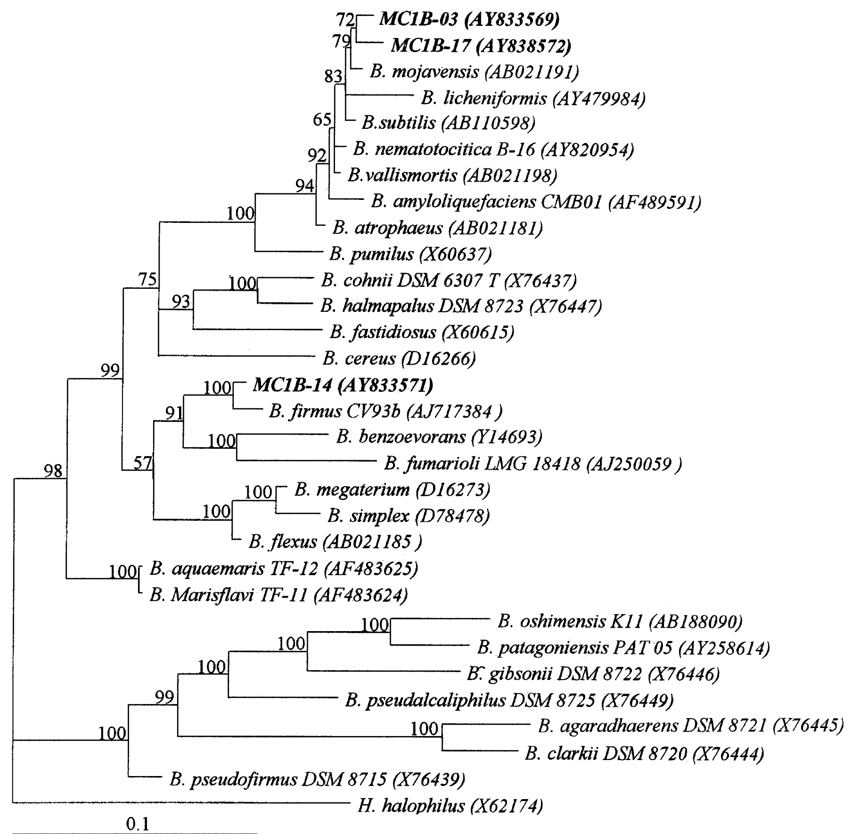

Fig. 1 Phylogenetic dendrogram based on 16 S rDNA sequence data indicating the position of isolates MC1B-03 (AY833569), MC1B-14 (AY833571) and MC1B-17 (AY833572) amongst members of the genus *Bacillus*. Accession numbers of 16 S rDNA gene sequences of reference organisms are included in the dendrogram. Bootstrap values, expressed as a percentage of 100 replications, are shown at *branching points*. Only values above 80% are shown. Bar 0.02 nucleotide substitutions per 100 nucleotides

the Mojave Desert [31], and subsequently found as endophyte [1], it is reasonable to hypothesize that this bacterium may have been transported into the coasts by run-off [27]. The unpublished preliminary data of our *Bacillus mojavensis* indicating their halotolerant behavior reinforces the hypothesis of a terrestrial origin, because true marine bacteria have an obligate physiological requirement for marine ions such as Na^+ [23]. In addition, from the reported *Bacillus* spp. inhabiting marine environments, only bacteria related to *B. pumilus, B. subtilis* and *B. licheniformis* were shown to produce antibiotics [17, 27, 41]. In agreement with our findings, a *Bacillus licheniformis* JF-2 recently renamed *Bacillus mojavensis* based on a polyphasic approach has been shown to be halotolerant and capable of producing a biosurfactant of lipopeptidic nature [8]. It is also tempting to speculate that our *Bacillus mojavensis* isolates are true intertidal biofilm-forming bacteria (cells were detached after mechanical removal by vortexing) and not only transient planktonic bacteria adsorbed to the stone surface, as suggested by their ability to grow confluently on solid media and capable of synthesizing exopolymers, the latter being biomolecules with known attachment function [26].

HCl precipitability and presence of characteristic peak clusters in the MALDI-TOF MS suggest the production of several classes of non-ribosomally biosynthesized lipoheptapeptide antibiotics from the surfactin and mycosubtilin/iturin, or bacillomycin class [35] by the *B. mojavensis* isolates. Presumably these molecules were also responsible for the cytotoxic activity detected in the *Artemia salina* test, probably by their detergent-like mode of action [39]. However, we cannot rule out the presence of other antibiotics, and further isolations and detailed structural analyses are necessary to characterize the antibacterial activities, especially those produced by *B. firmus* (MC1B-14).

Our studies along with previous reports show that marine biofilm bacteria are an underexploited source of natural compounds with antifouling potential. Progress has to be made by including other microbial groups or by increasing the number of microbial isolates from as-yet uncultured microflora to screen for bioactivity and production of novel metabolites. This is particularly important because marine epilithic biofilms are dominated (in terms of biomass) by cyanobacterial populations [24], and these organisms are known to produce a wide array of bioactive compounds [6]. In addition, other production strategies need to be explored such as "niche-mimicking" culturing methods, a useful method to increase the production of bioactive compounds, or to elicit this reponse in non-previous bioactive isolates [41]. The relevance of this work is that it expands our knowledge about the diversity and biotechnological potential of marine Gram-positive bacteria, and encourages further bioprospecting efforts as much biological novelty still remains to be discovered [9].

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