2012

Potential of Marine Bacteria as a Source of New Biofilm Formation Inhibiting Compounds

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MARINE NATURAL PRODUCTS: EXPLORING THE POTENTIAL OF MARINE BACTERIA AS A SOURCE OF NEW BIOFILM FORMATION INHIBITING COMPOUNDS

by

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A thesis submitted to the Honors Program

in partial fulfillment of the requirements for

Honors in the Major – Biology

UNIVERSITY OF NORTH FLORIDA

HONORS PROGRAM

August, 2012

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Dedication

Mom, Dad, [redacted] *Aeneas, DLX3, Addis, and Keke* – thanks for everything.

Acknowledgements

I would like to first thank my professor, mentor, and research supervisor, Dr. Amy Lane for her patience, encouragement, and guidance. You have equipped me the necessary skills and tools to effectively communicate with not only the scientific community, but also professionals and the general population, which will prove invaluable as I continue my education. Thank you for transforming my undergraduate experience and making it one of the most memorable and valuable moments of my life and career.

I would also like to express my deepest appreciation for Louanne Hawkins. Your genuine passion and drive to help students succeed is inspirational. I thank you for giving me the confidence and skills to pursue an Honors Thesis, as well as presenting at poster sessions, honing interview skills, and preparation for professional school. I am so grateful to have met you.

I am so grateful to have met and been under the supervision of Dr. Emil Boganmenn during my National Institute of Diabetes and Digestive and Kidney Diseases Short-Term Education Program for Underrepresented Persons (NIDDK STEP-UP) internship. You opened my world to laboratory research and sparked my lifelong appreciation for scientific research. I am blessed to have met such a committed and uplifting person.

Additionally, I thank Dr. Cliff Ross and his lab, Stephen Naso, Dana Bis, and Elle James for collection assistance, Dr. Terii Ellis and her lab for identification, and Dr. Corey Causey for equipment assistance.

This work would not be possible without the financial support from the Office of Undergraduate Research for the Student Mentored Academic Research Team (SMART) Grant (awarded to J. Han and S. Naso), The Dean's Council Transformational Learning Opportunity Scholarship (awarded to J. Han), and "Diving for Drugs" Transformational Learning Opportunity (awarded to Dr. A. Lane).

Finally, I would like to thank the support and patience from my family, pets, friends, and customers, Mr. Darnell Smith and Kevin. Thank you for your unconditional love, patience, and for always believing in me.

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Abstract

New antibacterial drugs are needed to keep up with the alarming increase in infections and incidence. Historically, the majority of clinically useful drugs have been obtained from *terrestrial* natural sources such as plants and microorganisms. Today such terrestrial sources of drugs are diminishing, owing to rediscovery of known compounds. Fortunately *marine* microorganisms are an emerging and underdeveloped source of novel compounds with promising pharmaceutical potential. We explored the hypothesis that Florida marine bacteria produce compounds that may inhibit bacterial growth/survival or biofilm formation, an aggregation of bacteria tightly adhered to a surface. Through repetitive plating, 57 pure bacterial cultures were obtained from marine sediment samples from coastal northeast Florida and the Florida Keys, chemically extracted, and these extracts subjected to assays for inhibition of bacterial growth and biofilm formation in the model human pathogenic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa*. While these chemical extracts exhibited minimal inhibition of *P. aeruginosa* and *S. aureus* growth, seven of the 57 marine bacterial extracts significantly inhibited formation of *P. aeruginosa* biofilms relative to no extract controls. Through sequencing of 16S rRNA genes, four of these natural product-producing marine bacterial strains were assigned as *Bacillus* spp. while one was proposed as a *Microbacterium* sp. Through bioactivity-guided fractionation of chemical extracts, the natural products most strongly inhibiting biofilm formation will be isolated using chromatography and identified using analytical techniques. These compounds may prove valuable as agents for treatment of biofilm-based infections of *P. aeruginosa*.

Introduction

Bacteria produce a variety of compounds that play many roles during their life cycle. These genetically encoded organic molecules may play a direct and vital role in growth and/or reproduction. These required metabolites, including nucleic acids and proteins, are examples of primary metabolites. Bacteria also produce organic compounds that do not play a direct role in growth and survival; these are referred to as adaptive metabolites or secondary metabolites. Secondary metabolites play various ecological functions, such as interspecies chemical-based communication or chemical defense against other microorganisms.

Secondary metabolites are also known as natural products. These chemical compounds occupy an important niche in drug discovery and design. Historically, the majority of clinically useful drugs have been obtained from natural sources such as microorganisms associated with plants and soil as well as terrestrial macroorganisms (Newmann and Cragg 2007). Compounds from terrestrial organisms play a major role in treating bacterial infections (Table 1). Today such terrestrial sources of drugs are diminishing, due to re-discovery of known compounds and increasing bacterial resistance to existing drugs.

Table 1 Selected antibacterial drugs from terrestrial sources

Drug	Source	Origin	Microorganism
Daptomycin	Streptomyces roseosporus	soil	bacteria
Streptomycin	Streptomyces griseus	soil	bacteria
Ampicillin	Penicillium chrysogenum	soil	fungus
Chloramphenicol	Streptomyces venezuelae	soil	bacteria
Penicillin	Penicillium chrysogenum	soil	fungus
Vancomycin	Amycolatopsis orientalis	soil	bacteria

Due to the exhaustive and widespread use of antibiotics, bacteria are evolving antibiotic resistance via genetic mutations, making existing drugs less effective and creating a public health crisis (Levy and Marshall 2004). Methicillin-resistant *Staphylococcus auerus* (MRSA) is an example of a virulent pathogen that has developed multi-drug resistance. New antibiotics are sought to combat a full array of bacterial strains that are resistant to several overused antibiotics.

Marine microorganisms are an emerging and underdeveloped source of antibacterial compounds that show promising pharmaceutical potential (Fenical and Jensen 2006). Like terrestrial microorganisms, marine microorganisms produce antibacterial compounds that act by killing or inhibiting growth of human pathogenic bacteria (Blunt and others 2004). Marine actinobacteria, which are gram positive bacteria that are prevalent in many environments, are of interest as natural product drug sources because terrestrial actinobacteria (e.g. *Streptomyces* sp.) are well-known producers of several novel medicinally useful compounds (Goodfellow 1988). Limited studies on these marine microorganisms have suggested that marine actinobacteria produce secondary metabolites that are often structurally distinct from those produced by terrestrial bacteria (Jensen and others 2005). In addition, other groups of marine bacteria such as bacilli and proteobacteria have not been extensively explored as sources of new antibacterials and hold promise as sources of new drugs (Kawulka and others 2003, Clardy and others 2006). The prospect of discovering new antibacterial drugs from the natural products of marine microorganisms is great as the ocean contains a great vast of biodiversity covering much of the planet. Marine microorganisms represent a promising, but relatively untapped source of novel bacteria-fighting molecules.

In addition to producing antibiotic compounds lethal to bacteria, marine microorganisms may also produce biologically active natural products that are not lethal but instead modulate bacterial behaviors, such as biofilm formation. A biofilm is an aggregation of bacteria to surfaces including those of lung and other vital tissues. In a response to changes in population size, bacteria use autoinducers, chemical signals, to communicate in quorum sensing to coordinate behaviors such as biofilm formation among groups of bacteria. Quorum sensing results when a population of bacteria reaches a threshold, with a chemical signal causing a change in gene expression and behavior. Biofilms arise when a mixed culture of bacteria or a pure culture releases a polymeric matrix to adhere to abiotic and/or biotic surfaces (Brenda and others 2005, Hall-Stoodley and Stoodley 2009). Biofilms are composed of three essential elements: (1) microorganisms, (2) glycocalyx, a complex of exopolysaccharides produced by the bacteria and substances from the environment, and (3) the surface. Planktonic bacteria attach to a proximal surface either randomly or directed by chemotaxis and motility. Bacteria assemble in nutrient-rich environments concentrated at a surface due to the equilibrium of bacteria, electrostatic and hydrophobic interactions, and van der Waals forces (An and others 2000, Carpentier and Cerf 1993, Lawrence and others 1987). It is the exopolysaccharides and exogenous substances gathered from nearby sources that make the association and adhesion irreversible. The synergistic association of bacteria has led to the universal presence of ingeniously formed biofilms.

The sophisticated formation of biofilms has led to several beneficial and detrimental effects on bacteria and organisms, including humans. Although bacterial biofilms may be useful for bioremediation and waste management in filtration systems as the bacteria in the biofilm interact and rid harmful bacteria, the formation of biofilms is especially detrimental in bacterial

infections of humans. *P. aeruginosa* is a common bacterial strain that forms a biofilm in the airway of patients with cystic fibrosis, and may aggravate nearby cells and cause mortality (Moreau-Marquis and others 2008). The different behaviors between planktonic bacteria and bacteria in biofilms, as well as genetically encoded antibiotic resistance have led to low antibiotic susceptibility of *P. aeruginosa*, exemplifying the difficulty in controlling biofilms (Bagge and others 2004, Vuong and others 2004, Pearson and others 2006, Jurcisek and Bakaletz 2007, Lenz and others 2008, Zhang and Mah 2008). Biofilm-based infections caused by *Streptococcus mutans* are also the root of dental caries and gingivitis. Biofilms on abiotic surfaces such as heart valves, pacemakers, and catheters have posed a serious threat in hospital settings (Trautner and Darouiche 2004). The potentially destructive effects of biofilms are cause for concern in human health.

Discovering anti-biofilm compounds is of great interest as controlling biofilms is difficult and few biofilm inhibiting compounds are known. Known biofilm inhibitors effective at clinically safe concentrations have yet to be discovered (Brown and others 1988). Biofilms are many times more resistant to antibiotics than planktonic cells because bacteria adhered to a surface are encased in a thick extracellular matrix that impedes antibiotic penetration and biofilm-associated bacteria behave differently than planktonic bacteria through quorum sensing via autoinducers. Additionally, the heterogeneous mixture of bacteria and nutrients from the environment in the biofilm matrix make it likely multiple resistance mechanisms exist (Mah and O'toole 2001). Compounds that act as non-lethal inhibitors of biofilm formation are expected to pose less selective pressure for evolution of antibacterial resistance as compared to compounds that are lethal to bacteria. It is important to find compounds that control biofilms as inhibition may thwart the detrimental impacts of biofilm-associated bacteria on the health of humans.

Thus, biofilm inhibitors represent especially promising therapies to be used in conjunction with traditional bacteriocidal compounds.

There is a critical need for new drugs to treat biofilm-based infections. Recent research has uncovered the pharmaceutical potential of marine microorganisms, but much is left to be discovered regarding the capacity of these organisms to produce compounds that inhibit bacterial survival or biofilm formation (Amador and others 2003). Thus, we set out to explore the hypothesis that Florida marine bacteria produce compounds that may inhibit bacterial growth/survival and/or biofilm formation.

Materials and Methods

Collection and isolation of marine bacteria

A total of 68 (31 from Northeast Florida and 37 from Key West) 2-3 mL sediment samples were collected in sterile 15 mL or 50 mL polypropylene Falcon tubes in coastal northeast Florida and the Florida Keys on two separate research expeditions in May 2011 and July 2011, respectively. Samples were collected at depths varying from 0.5 to 3.5 meters and kept frozen at -20°C until plated on nutrient media for isolation of bacterial strains.

Samples were thawed at room temperature, seawater was decanted from sediment samples, and 5 mL of sterile seawater was added to samples in a biosafety cabinet to avoid introduction of environmental contaminants. Samples were heat shocked at 55°C for 6 minutes to select for heat-resistant bacteria such as actinobacteria, and samples were vortexed gently to dissociate bacteria from sand grains. Fifty µl aliquots of 1:1 and 1:2 dilutions of these samples in sterile seawater were deposited on M1 agar plates appended with salt to select for marine bacteria (1% potato starch, 0.4% yeast extract, 0.2% peptone, 1.8% agar, 0.0005%

cycloheximide, 2.8% Instant Ocean sea salt), ISP2 agar plates (0.4% yeast extract, .1% malt extract, 0.4% glucose, 0.18% agar, 0.000001% nalidixic acid, 0.0000001% cycloheximide, and 0.00000025% nystatin, 2.8% Instant Ocean), and/or Actinomycete Isolation Agar plates (2% sodium caseinate, 0.001% asparagine, 4% sodium propionate, 0.005% dipotassium phosphate, 0.001% ferrous sulfate, 15% agar, 5% glycerol, and 2.8% Instant Ocean sea salt). The Instant Ocean sea salt used in all media contained sodium chloride and other salts: calcium chloride, potassium chloride, sodium sulfate, and magnesium chloride. All media were prepared using deionized and filtered water at 18.2 MΩ·cm. Plates were either kept at room temperature or incubated at 30°C and monitored for observation of colonies on plates. Individual colonies observed on plates were repetitively plated onto fresh agar-based media until pure colonies were isolated. For each colony, all antibiotics were omitted from the final media to ensure samples were pure cultures and free from contamination with other microorganisms.

Extraction of natural products from isolated marine bacterial strains

Two to three isolated colonies were transferred to liquid M1 liquid media (15 mL in a sterile 100 mL Erlenmeyer flask covered with a foam stopper and aluminum foil), and incubated with shaking at 175-200 rpm for 7-10 days at 30°C. M1 liquid media consisted of 1% starch, 0.4% yeast extract, 0.2% peptone, 2% Instant Ocean sea salt 0.004% $Fe_2(SO_4)_3x5H_2O$, 0.01% KBr, and 0.1 % $CaCO_3$. To chemically extract organic compounds produced by bacteria, 1 g of Amberlite XAD-7 HP Resin was added to the 15mL liquid cultures and samples incubated with shaking (175-200 rpm) at 30°C for 4-6 hours. The purpose of the resin was to bind with natural products present in the liquid bacterial culture. After incubating samples with resin, the liquid media and resin were separated from one another by filtering through 8 layers of 10 cm x 10 cm cheesecloth. Approximately 100 mL of deionized and filtered water at 18.2 MΩ·cm was then

poured over the resin and cheesecloth to wash the salts away. The resin and cheesecloth were submerged in 40-60 mL of acetone and incubated overnight at 3°C to elute the organic molecules from the resin and into the acetone solution.

A rotary evaporator was used to remove acetone and residual water by evaporation under reduced pressure in a round bottom flask. Approximately 10-15 mL of methanol was added to the remaining traces of liquid extract, extracts were transferred to tared vials, and all remaining solvent was evaporated using a centrifugal evaporator (speedvac). The organic compound mixtures were stored at -20°C until ready for bacterial assays.

Bacterial growth and biofilm inhibition assays

Each bacterial chemical extract was solubilized with dimethyl sulfoxide (DMSO) to yield a concentration of 200 mg of extract per mL of DMSO for bacterial assays to explore the ability of the marine organisms to produce growth or biofilm inhibitory compounds. Tryptic soy broth, prepared according to manufacturer's instructions by MP Biomedicals, LLC, was used to culture *Pseudomonas aeruginosa* (ATCC 27853) or *Staphylooccus aureus* (ATCC 25923), selected human pathogenic bacterial strains. A few frozen cells of *P. aeruginosa* or *S. aureus* were removed from storage at -80°C and aseptically transferred into 2-3 mL of tryptic soy broth media and incubated with shaking for 14-24 hours at 30°C and 175-200 rpm.

These overnight cultures of *S. aureus* or *P. aeruginosa* were diluted by transferring 100 µl of the bacterial culture to 10 mL of fresh tryptic soy broth. Four mL of this diluted culture were subsequently further diluted by dispensing into 40 mL of tryptic soy broth. Two-hundred µl of this final diluted mixture of media and bacteria was aliquoted to rows A and E of a 96-well microtiter plate, and 100 µl of the mixture was aliquoted to remaining wells of a non-tissue

culture treated polystyrene 96-well microtiter plate (Corning Costar 3370). Four µl of 200 mg/mL chemical extract stocks were aliquoted in duplicate to rows A and E to prepare a final concentration of 0.4mg chemical extract per mL of media and cells (Figure 1). One hundred µl of extract/media/cells in rows A and E were removed and added to sequential wells (rows B and F) to reduce the concentration of chemical extract by ½, yielding a test concentration of 0.2 mg extract/mL of cells and media. Further aliquoting of 100 µl of material from rows B and F into sequential wells of rows C and G, respectively, yielded 0.1 mg extract/mL of cells and media in these wells. Similarly, 0.05mg extract/mL of cells and media were present in rows D and H of the microtiter plate. All dilutions were prepared by mixing via pipetting. Hence, all extracts were tested in duplicate at four concentrations each (0.4, 0.2, 0.1, and 0.05 mg/mL). No extracts were placed in column 11 as it was the dedicated DMSO control containing only bacteria, media, and DMSO at the same concentrations as in corresponding treatment wells DMSO with bacteria and media prepared the same as the extract dilutions. No extracts were placed in column 12 as it was the dedicated antibiotic control containing 1 µl of gentamicin sulfate (10 mg/mL stock concentration) in rows A and E, giving diluted concentrations of 0.1, 0.05, 0.025, and 0.00125 mg/mL prepared similarly as the extract and DMSO dilutions. Wells H 11 and 12 were blanks that only contained media, to control for background absorbance of media and ensure no contamination of media. The 96-well plates were wrapped in parafilm and incubated overnight at 30°C without shaking. The following day plates were read at 630 nm with a microplate reader to quantify bacterial growth/death (Figure 1).

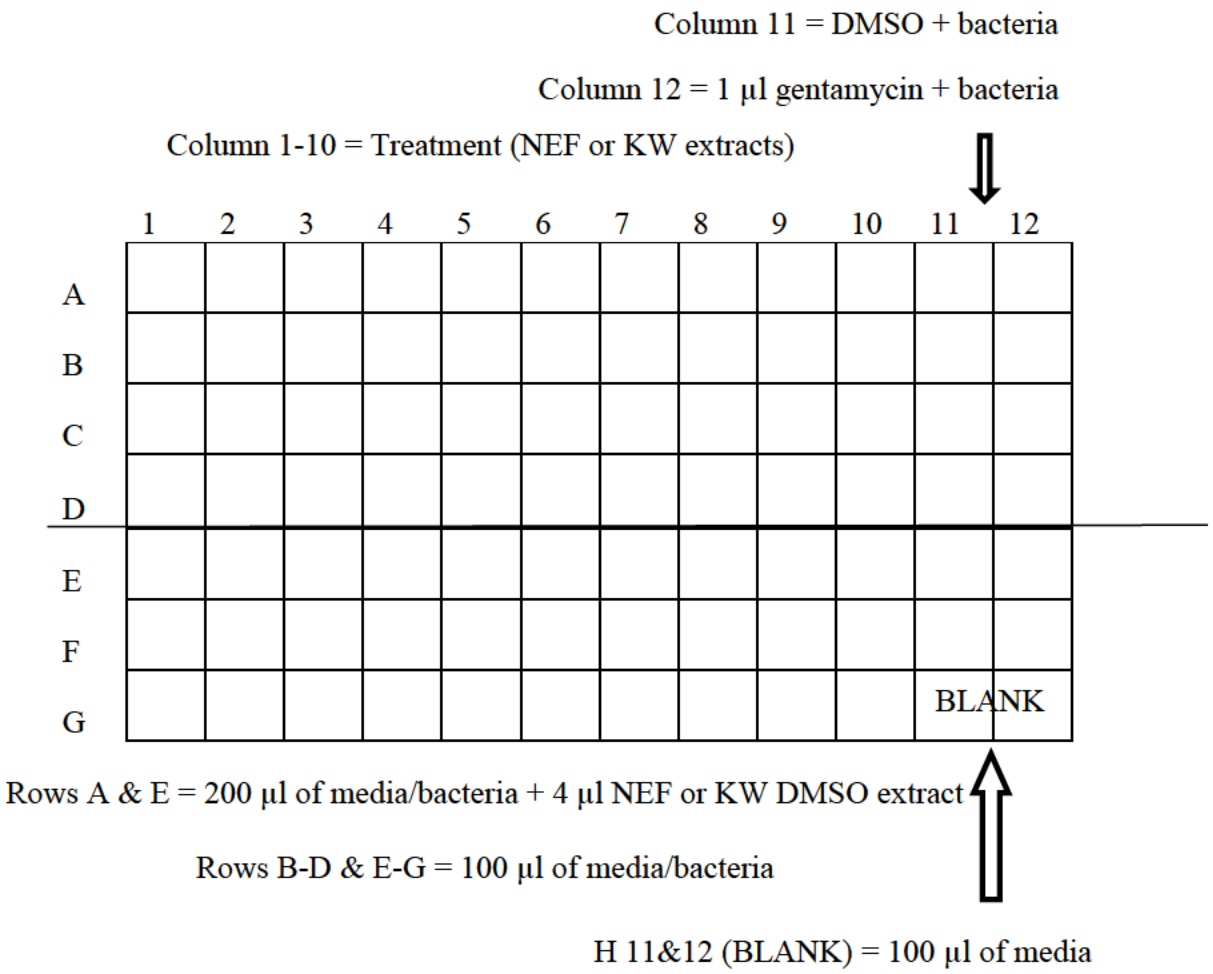


Figure 1 Template of 96-well plate used to test for bioactivity of NEF and KW chemical extracts against pathogenic strains of *P. aeruginosa* or *S. aureus*

All above-described assays were conducted in duplicate on at least two separate occasions. Average inhibition of bacterial growth was calculated by comparing the absorption at 630 nm for all replicate treatment samples to the absorption recorded for replicate controls according to the formula below, and standard deviations computed using Microsoft Excel. For purposes of pursuing the most promising potential drug leads, only chemical extracts inhibiting bacterial

growth or biofilm formation by at least 25% relative to controls were deemed sufficiently bioactive for pursuit of compounds responsible for observed biological activities.

% growth inhibition = (1 - average absorption of treatments/average absorption of controls) x 100%

Chemical extracts were evaluated for capability to inhibit biofilm formation by following a previously reported protocol (Meritt and others 2011). Planktonic bacteria were removed from each of the above microtiter wells by shaking to remove liquid from microtiter plates and then submerging plates in tap water. The water was then removed by shaking, and remaining surface-adhered bacteria were then stained with crystal violet. To stain, 125 µl of 0.1% (m/v) aqueous crystal violet solution was aliquoted to each well and incubated at room temperate for 10-15 min. Crystal violet solution not bound to cells was removed by inverting the plate and shaking, followed by washing wells by dipping into a basin of water. Plates were then air dried and inverted at an angle at 23°C overnight at room temperature. Two hundred µl of a 30% acetic acid solution was added to each well and incubated for 10-15 minutes at room temperature to solubilize the crystal violet dye. After mixing by pipetting, 125 µl of the acetic acid/crystal violet solution from each well was transferred to an optically clear flat-bottom 96-well plate. The optical density (OD) of treatments and controls was measured at 540 nm to assess the amount of crystal violet present in each well and thus quantify percent inhibition of biofilm formation for chemical extract treatments relative to no extract controls. For all biofilm inhibition assays, average inhibition of bacterial biofilm formation was calculated analogously to growth inhibition assays. Compounds that inhibited growth of bacteria were expected to also inhibit biofilm formation in this assay, since lower cell numbers correspond to reduced quantities of bacteria available for biofilm formation. Hence, for all chemical extracts found to inhibit

biofilm formation, we confirmed that no growth inhibition was observed (as determined by the growth inhibition assay described above).

Identification of bacterial strains producing biofilm inhibitors

The genomic DNA of bacteria producing bioactive compounds (as determined by above-described assays) was purified using the Qiagen Blood and Tissue DNA Isolation kit, following the manufacturer protocol for gram-positive bacteria and eluting DNA with 100 uL of Buffer AE instead of the manufacturer-recommended 200 uL of AE Buffer. This reduction in buffer volume was used for bacterial strain identification (Gontang and others 2007). These universal primers had the following sequences: "27F" GAGTTTGATCCTGGCTCA and "1385R" CGGTGTGTRCAAGGCCC.

The PCR reaction to amplify 16S rDNA in identification of bacterial species was carried out with a total volume of 25 uL and PCR reaction mix consisting of 4.5 uL of New England Biolabs (NEB) Taq polymerase, 2.7 uL of each primer (concentration of stock?), 5.58 uL of DNA template, 22.5 uL of NEB Thermopol buffer. The thermocycler was programmed to run the following cycles: 2 min at 95°C, followed by 30 cycles of 1 min at 95 °C, 1 min at 52°C, and 1.5 min at 72°C, and a final extension step at 72°C for 10 minutes. PCR success was confirmed by detection of ~1500 bp bands of amplified 16S rRNA gene fragments through gel electrophoresis. Five µl of the PCR reaction product and 2 µl of loading dye was loaded onto a 0.7% agarose gel with ethidium bromide at a voltage of 100-110 V for 30 minutes. Six mL of DNA ladder (GeneRuler 1kB Plus, ThermoScientific) were also run on the gel.

PCR products of ~1500 bp were purified using the QIAGEN QIAquick PCR Purification kit, following manufacturer protocols. Selected purified PCR products were submitted to Eurofins Operon (Huntsville, Alabama) for sequencing, using the same primers for sequencing

as were used in PCR. DNA sequences were assembled using Geneious software (Drummond and others 2011). To identify bacterial strains, this DNA sequence information was searched against the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) nucleotide database of 16S rRNA sequences (Altschul and others 1990).

Results

Northeast Florida and the Florida Keys are both rich in culturable marine microorganisms. A total of 22 bacterial strains were isolated from coastal waters of northeast Florida and 35 bacterial strains from the Florida Keys were isolated (Figures 2a, 2b, Table 2) using the three medias employed in the current study.

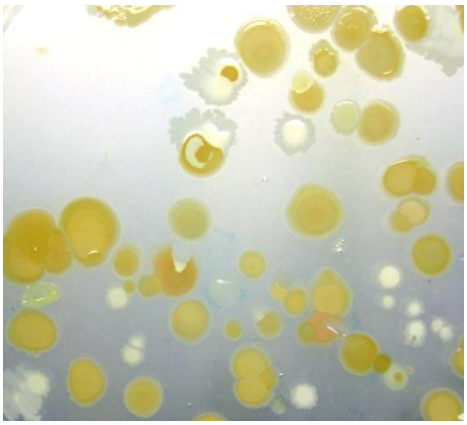
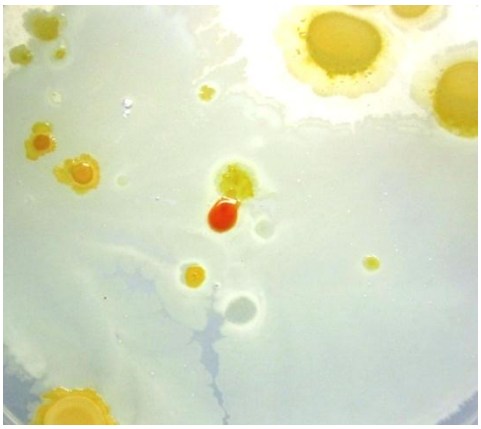
a  b 

Figure 2a, 2b Mixed colonies of KW bacteria grown on M1 media without antifungal agents

Table 2 Location and gross morphology of Northeast Florida and Key West isolated bacterial colonies (NEF = Northeast Florida, KW = Key West)

Sample ID:	Location (GPS)	Gross morphology on M1 agar media
NEF 1	Not recorded	off white, smooth, glossy
NEF 2	30° 23' 39" N, 81° 24' 4" W	peach, glossy, smooth
NEF 3	30° 23' 39" N, 81° 24' 4" W	off white, smooth, cloudy
NEF 4	30° 23' 39" N, 81° 24' 4" W	off white, smooth, glossy
NEF 5	30° 24' 60" N, 81° 24' 23" W	brown, fuzzy
NEF 6	Not recorded	hunter green, fuzzy, powdery
NEF 7	30° 23' 39" N, 81° 24' 4" W	off white, smooth, glossy
NEF 8	30° 23' 39" N, 81° 24' 4" W	yellow, smooth
NEF 9	30° 23' 39" N, 81° 24' 4" W	hunter green, fuzzy
NEF 10	30° 24' 60" N, 81° 24' 23" W	peach, smooth, glossy
NEF 11	30° 23' 39" N, 81° 24' 4" W	hunter green, powdery, fuzzy
NEF 12	30° 23' 39" N, 81° 24' 4" W	off white, cloudy, glossy
NEF 13	30° 23' 39" N, 81° 24' 4" W	brown, flat, powdery
NEF 14	30° 24' 60" N, 81° 24' 23" W	brown dots, flat, powdery
NEF 15	30° 27' 32" N, 81° 24' 47" W	off white, smooth
NEF 16	30° 24' 60" N, 81° 24' 23" W	pale green, fuzzy
NEF 17	30° 23' 39" N, 81° 24' 4" W	brown, powdery
NEF 18	30° 24' 26" N, 81° 24' 36" W	white, clear
NEF 19	Not recorded	peach, cloudy, glossy
NEF 20	30° 23' 39" N, 81° 24' 4" W	peach, smooth, glossy
NEF 21	Not recorded	off white, smooth, glossy
NEF 22	30° 23' 39" N, 81° 24' 4" W	yellow, smooth
KW 1	24° 37' 40" N, 82° 52' 22" W	yellow, fuzzy
KW 2	24° 37' 40" N, 82° 52' 22" W	clear, smooth, branching
KW 3	24° 31' 20" N, 82° 58' 11" W	light hunter green, fuzzy
KW 4	24° 3' 45" N, 82° 7' 28" W	cloudy white, branching
KW 5		white, fuzzy
KW 6	24° 37' 40" N, 82° 52' 22" W	light hunter green, fuzzy
KW 7	24° 31' 20" N, 82° 58' 11" W	cloudy white, branching
KW 8	24° 37' 40" N, 82° 52' 22" W	off white, smooth, glossy
KW 9	24° 31' 20" N, 82° 58' 11" W	yellow, smooth
KW 10	24° 3' 45" N, 82° 7' 28" W	white, smooth, branching, dots
KW 11	24° 31' 20" N, 82° 58' 11" W	orange, smooth, glossy
KW 12	24° 37' 40" N, 82° 52' 22" W	pale yellow, cloudy
KW 13	24° 3' 45" N, 82° 7' 28" W	light hunter green, fuzzy

KW 14	24° 37' 40" N, 82° 52' 22" W	orange, smooth, glossy
KW 15	24° 31' 20" N, 82° 58' 11" W	white, cloudy, fuzzy
KW 16	24° 37' 40" N, 82° 52' 22" W	light hunter green, fuzzy
KW 17	24° 37' 40" N, 82° 52' 22" W	off white, smooth, cloudy
KW 18	24° 37' 40" N, 82° 52' 22" W	pale yellow, cloudy
KW 19	24° 37' 36" N, 82° 52' 3" W	white, smooth, cloudy
KW 20	24° 37' 40" N, 82° 52' 22" W	white, smooth, cloudy, glossy
KW 21	24° 3' 45" N, 82° 7' 28" W	white, smooth, cloudy
KW 22	24° 37' 40" N, 82° 52' 22" W	white, clear
KW 23	24° 3' 45" N, 82° 7' 28" W	white, clear, smooth, dots
KW 24	24° 37' 40" N, 82° 52' 22" W	white, smooth, clear, skinny lines
KW 25	24° 3' 45" N, 82° 7' 28" W	off white, smooth, cloud
KW 26	24° 3' 45" N, 82° 7' 28" W	hunter green, fuzzy, powdery
KW 27	24° 37' 40" N, 82° 52' 22" W	brown, fuzzy
KW 28	24° 31' 20" N, 82° 58' 11" W	brown, fuzzy, powdery
KW 29	24° 37' 40" N, 82° 52' 22" W	off white, cloudy, branching
KW 30	24° 37' 40" N, 82° 52' 22" W	brown, fuzzy
KW 31	24° 37' 40" N, 82° 52' 22" W	clear white, smooth ,dots
KW 32	24° 37' 40" N, 82° 52' 22" W	off white, cloudy, smooth
KW 33	24° 37' 40" N, 82° 52' 22" W	off white, clear, smooth
KW 34	24° 31' 20" N, 82° 58' 11" W	brown, fuzzy
KW 35	24° 3' 45" N, 82° 7' 28" W	brown, fuzzy

All 57 isolated strains were chemically extracted and resulting extracts evaluated for inhibition of growth of two model human pathogens, the gram-positive bacterium *S. auerus* and gram-negative *P. aeruginosa*. No chemical extracts showed significant inhibition of bacterial growth in *S. auerus* or *P.aeruginosa* assays (Table 2, data not shown). Further, none of the 57 chemical extracts significantly inhibited biofilm formation of *S. aureus*. However, significant inhibition of biofilm formation was observed for chemical extracts of 7 bacterial strains against *P. aeruginosa*. These strains correspond to NEF 3, NEF 4, NEF8, NEF 20, KW 2, KW 13, and KW 17 (Table 2, Figure 3). Of the 7 marine bacterial strains producing compounds inhibitory toward

biofilm formation, 5 of the strains most strongly inhibitory toward biofilm formation were identified (Table 3).

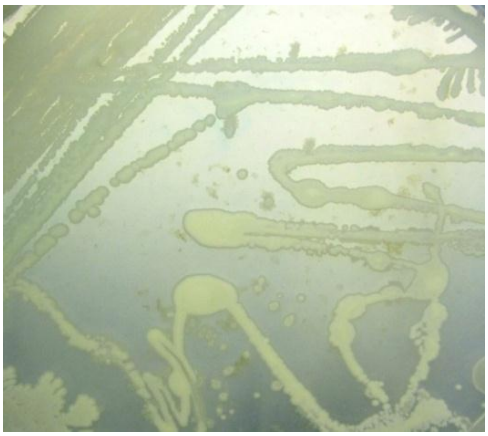


Figure 3 Representative isolated pure colony of NEF bacteria grown on M1 media without antifungal agents

Table 3 Percent inhibition of *P. aeruginosa* (PA) and *S. aureus* (SA) biofilm formation of at least one bacterium by chemical extracts from NEF and KW marine microorganisms (SA n=4, *n=2; PA n=4). (Only those chemical extracts inhibiting biofilm formation by at least 25% (relative to no-extract controls) on average are included. None of the evaluated 57 marine chemical extracts inhibited bacterial growth by at least 25%)

Chemical extract sample #	Species assignment	avg % biofilm inhibition in SA ± 1 SD	avg % bacteria growth inhibition in SA± 1 SD	avg % biofilm inhibition in PA ± 1 SD	avg % bacteria growth inhibition in PA± 1 SD
NEF 3	Bacillius sp.	0 ± 123	2.5 ± 21	61± 14	0 ± 24
NEF 4	Bacillius sp.	0 ± 129	0 ± 19	62± 17	0 ± 17
NEF 8	Microbacterium sp.	4 ±16*	0 ± 71*	52± 18	7 ± 28
NEF 20	Bacillius sp.	0 ± 896	7 ± 7	51± 14	0 ± 5
KW 2	NA	0 ± 83	0 ± 42	30± 20	4 ± 22
KW 13	NA	0 ± 59	0 ± 30	39± 17	0 ± 14
KW 17	Bacillius sp.	0 ± 22*	0 ± 8*	36± 24	0 ± 16

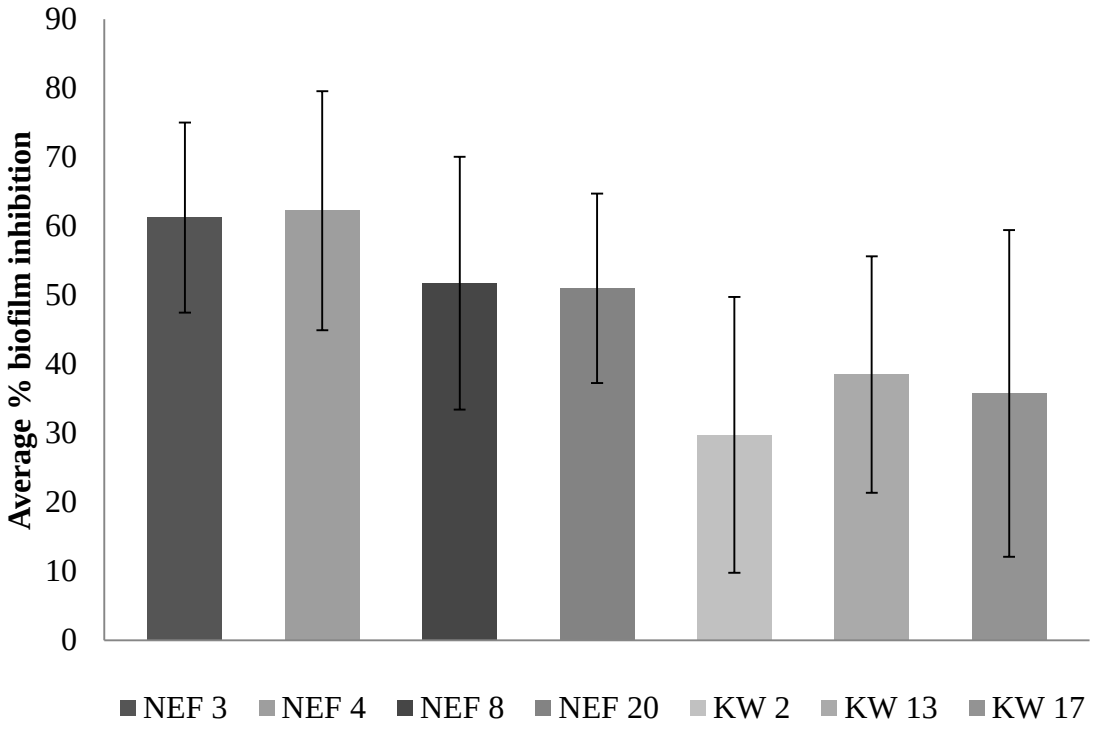


Figure 4 Average percent inhibition of biofilm formation of *P. aeruginosa* by chemical extracts from NEF and KW microorganisms (n=4, chemical extracts tested at final concentration of 0.4 mg/mL of bacteria and media)

Genomic DNA was isolated from all strains and this DNA subjected to PCR using well-established primers for amplification of 16S rRNA gene sequences (Gontang and others 2007). Agarose gel electrophoresis indicated PCR amplicons at the expected size of ~1500 bp, indicating successful amplification of 16S rDNA (Figure 3). This DNA was purified and commercially sequenced for five strains producing compounds strongly inhibitory toward biofilm formation (Table 3). Using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), the amplified sequences were found to match most closely with strains shown in Table 3 (Altschul and others 1990).

Table 4 16S rRNA sequence-based identification of select samples producing compounds strongly inhibitory toward *P. aeruginosa* biofilm formation

Chemical extract sample #	Species assignment	Closest relative	% identity
NEF 3	*Bacillius sp.*	*Bacillus idriensis*	98
NEF 4	*Bacillius sp.*	*Bacillus idriensis*	98
NEF 8	*Microbacterium sp.*	*Microbacterium esteraromaticum*	96
NEF 20	Bacillius sp.	Bacillus idriensis	98
KW 17	Bacillius sp.	Bacillus idriensis	98

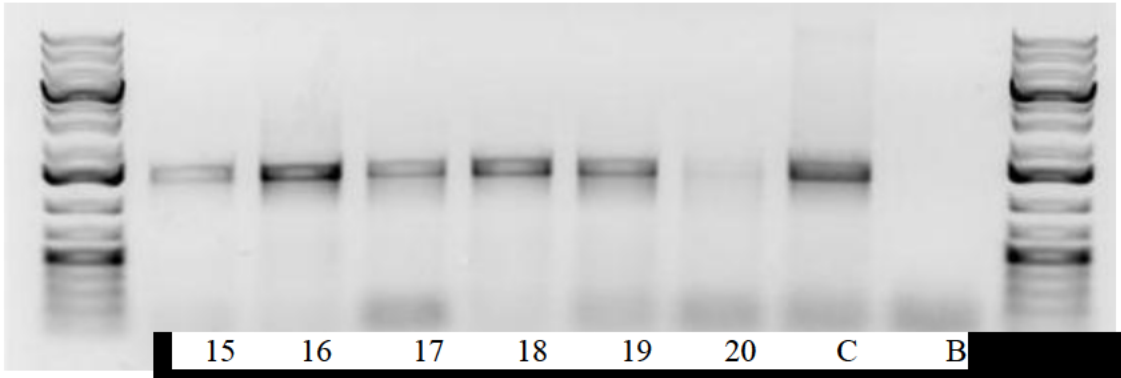


Figure 5 0.7% agarose gel showing amplification of a targeted ~1500bp 16S rDNA sequence for representative KW bacteria with DNA Ladder Fermentas Gene Ruler Plus in first and last lanes of gel (Samples KW 15-20, C=control of 16S rDNA amplified from *Nocardiopsis* sp. genomic DNA, B=blank)

Discussion

A total of 57 bacterial strains were isolated from Florida marine sediments: 22 from northeast Florida and 35 from Key West, which alludes to the substantial marine microbial diversity present in these regions. Upon further investigation of the capacity of these marine organisms to produce compounds inhibitory toward human pathogenic bacteria growth and/or biofilm formation, seven marine strains were found to produce compounds reducing *Pseudomonas aeruginosa* biofilm formation by at least 30% relative to controls. This corresponds to 12.3% (7 out of 57) marine strains in our study yielding chemical extracts capable

of impeding biofilm formation of *P. aeruginosa*, a human pathogen highly prevalent in lung infections of patients with cystic fibrosis and pneumonia. Our findings give insight to the biomedical potential of marine microorganisms as sources of biofilm-inhibiting compounds that may prove useful as human therapeutics, and suggest that further pursuit of Florida marine microorganisms as sources of human therapeutics is in order. Our results also raise the question of the ecological function of these biofilm-inhibiting compounds. It is plausible that the organisms employ biofilm-inhibiting compounds as chemical defenses, to ward off competing co-occurring microorganisms. Future studies may explore such hypotheses.

Sessile biofilm-associated bacteria behave differently than the same bacteria in planktonic states, and traditional growth inhibitory antimicrobial agents are far less effective in eradicating infections of biofilm-associated bacteria than in combatting planktonic bacterial infections. Biofilms are inherently resistant to antibiotics due to three possible explanations: (1) the agent not penetrating the biofilm, (2) the slow-growth rate or starved state of biofilm-associated bacteria, and/or (3) extreme microenvironmental conditions and altered gene expression patterns (Bagge *and others* 2004, Vuong *and others* 2004; Pearson *and others* 2006; Jurcisek and Bakaletz, 2007; Lenz and others 2008; Zhang and Mah 2008). The first explanation postulates that the glycocalyx retards or prevents the antibiotic agent from fully penetrating the biofilm and reaching the bacterial cells. The penetration of the biocide is dependent upon not only the agent, but also the biofilm structure, which is why the discovery of an effective anti-biofilm agent is expected to be of immense value (Ceri and others 1999, Schierholz and others 1999). The second hypothesis suggests that due to a higher density of cells in the biofilm, nutrients and resources are limited. This causes a slow-growing rate or starved state in which potent biocides are less effective due to low susceptibility (Brown and others 1988). The final

hypothesis explains the inherent resistance of biofilms is due to the attached bacteria in biofilms behaving differently as a result of different gene expression in an extreme microenvironment (Bagge *and others* 2004, Vuong *and others* 2004; Pearson *and others* 2006; Jurcisek and Bakaletz, 2007; Lenz and others 2008; Zhang and Mah 2008), and these gene expression differences enhancing resistance to antibiotics. Addressing the former has spurred the search for compounds helpful for the treatment of biofilm-associated bacteria, including *P. aeruginosa*. Thus, our results contribute to efforts to identify biofilm-inhibiting drugs by supporting marine bacteria as a promising source of biofilm inhibitors. Our study is one of only a handful exploring marine bacteria as sources of biofilm inhibitors, and expands to explore sources of anti-biofilm compounds (Assmann and others 2000, Chanas and others 1997, Sullivan and others 1981, Manzo and others 2011).

In addition to providing potential drug leads, our findings may also help us to understand how *P.aeruginosa* forms biofilms. The glycocalyx production is thought to be surface activated, which is responsible for biofilm formation (Davies and others 1993). The *alg*C gene is an important regulation point thought to control attachment. The upregulation and expression of *algC* induced by environmental factors, such as osmoregularity and high oxygen tensions, after attachment is 19 fold greater in attached bacteria than free floating bacteria (Davies and others 1993, Davies and Greesey 1995, Devault and others 1989, Devault and others 1990). Because it is assumed that the *algC* gene is important to quorum sensing and biofilm formation, the compounds produced by the 7 Florida microorganisms may be involved in modulating the *algc* gene and/or products to disassociate *P. aeruginosa* biofilms.

Marine microorganisms may produce anti-biofilm compounds due to selective pressures that favored the evolution of pathways for production of secondary metabolites to inhibit biofilm

formation. Marine sponges present in association with marine sediment have been founds to produce compounds that inhibit biofilms (Assmann and others 2000, Chanas and others 1997). It is predicted that selective pressures, including predators, competition for space and resources, and surface fouling (biofilms), favored the evolution of biochemical pathways for the generation of bioactive secondary metabolites (Assmann and others 2000, Chanas and others 1997, Sullivan and others 1981, Manzo and others 2011). This supports the possibility of marine bacterial bioactive secondary metabolites as chemical defenses against biofilms, a hypothesis that may be explored in the future for our marine bacteria.

Further analysis and identification of specific biofilm-inhibitory compounds from the seven select extracts may give insight to how biofilms are inherently resistant to traditional antibiotics and how the integrity of the biofilm is formed and disassembled. Identifying the bioactive compounds may also facilitate determination of how gene expression is altered during biofilm formation and dispersal or the mechanism of an effective compounds to cause autodispersion, displacing the aggregation of bacteria from the adhered surface and disassembling the biofilm, to increase antibiotic sensitivity to biofilm bacteria. The natural products may also enable us to better understand the autoinducers involved in the chemical signaling (e.g. quorum sensing) for planktonic bacteria to coordinate behaviors and form biofilms and how the bacteria in the biofilm behave differently than free-living bacteria. These findings offer great biomedical applications as they may be used in drug design to develop an effective natural product biofilm inhibitor.

The microorganisms exhibiting the strongest ability to produce biofilm inhibiting compounds (Figure 4) will be further explored to isolate and identify specific components of the mixtures using various analytical tests: chromatography to isolate bioactive compounds, and

NMR spectroscopy and mass spectrometry to determine the complete chemical structure of such compounds. Additionally, bacteria from more locations throughout Florida may be isolated and explored for biofilm inhibitory compounds, as well as subjecting bacteria to various conditions (e.g. different media and antifungal agents) and co-cultivation with other bacteria in an effort to stimulate production of further bioactive natural products. These co-cultivation and media manipulation strategies have previously proven effective in activating unexpressed biochemical pathways and hold promise for further tapping into the potential of marine bacteria to yield new drugs (Onaka and others 2011, Pettit 2009, Scherlach and Hertweck 2009, Zuck and others 2011, Angell and others 2006). Further computer aided analysis and design may be used to identify the biochemical pathway of how the natural products are produced by microorganisms and synthesis of lead compounds responsible for inhibiting biofilm formation.

This study supports natural product drug discovery from marine bacteria and highlights the immense untapped potential of coastal Florida marine bacteria as sources of biofilm inhibitory compounds. Finding 7 marine microorganisms producing natural products that inhibit biofilm formation may help to address the multifaceted problem of understanding biofilm formation mechanisms and discovering effective and clinically useful antimicrobial agents to inhibit biofilm formation. Much has yet to be discovered in this area, and the forecast is clear for finding novel marine natural products to combat biofilm-based infections.

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Vita

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