Survey of Antibiotic-producing Bacteria Associated with the Epidermal Mucus Layers of Rays and Skates

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Elasmobranchs represent a distinct group of cartilaginous fishes that harbor a remarkable ability to heal wounds rapidly and without infection. To date very little work has addressed this phenomenon although it is suggested that antibiotic capabilities associated with epidermal surfaces may be a factor. The study of benefits derived from mutualistic interactions between unicellular and multicellular organisms is a rapidly growing area of research. Here we survey and identify bacterial associates of three ray and one skate species in order to assess the potential for antibiotic production from elasmobranch associated bacteria as a novel source for new antibiotics.

Keywords: antibiotic producing bacteria, antibacterial screening, pathogens, epidermal mucus, stingray, skate, beneficial bacteria

INTRODUCTION

Marine bacteria are genetically and metabolically diverse, capable of producing a wide range of chemical compounds, and are known to establish symbioses with a range of marine organisms (Bhatnagar and Kim, 2010; Sobhana, 2015). As a result, there is a growing interest in mutually beneficial associations between microbes and their hosts (Zamioudis and Pieterse, 2012). Increasing studies illustrate that bacteria produce chemical compounds that were previously ascribed to marine hosts, providing bioactive compounds for utilization in host defenses (Chau et al., 2013; Abdelmohsen et al., 2014). The study of host-associated microbes also provides a unique avenue for the search for novel bioactive compounds that could be utilized in biomedical applications (Vasanthabharathi and Jayalakshmi, 2013).

Studies in corals have shown that antibiotic activity is present in the surface mucus of healthy corals (Ritchie, 2006) Furthermore, greater than 20% of bacteria isolated from the mucus of the elkhorn coral, Acropora palmata, demonstrated antibiotic activity against a range of pathogenic test strains and 8% were specifically active against a pathogen that causes disease in this species (Ritchie, 2006). These results suggest that organisms may derive some of their immunity from probiotic bacteria associated with their surface mucus layers.

Aqueous protein extracts of the epidermal mucus layers of several finfishes have been investigated as a source of innate immunity and have demonstrated broad-spectrum antibiotic activity (John and Patterson, 2011). Similar extracts from the mucus layers of Atlantic cod, (Bergsson et al., 2005) and hagfish (Subramanian et al., 2008) have also demonstrated antibiotic activity against several common infectious pathogens, further illustrating that innate immunity...
is an important immune function for aquatic organisms. As part of their normal life activities, notably associated with regular mating events, aggressive behavior, predation, and anthropogenic encounters, sharks and their skate and ray relatives routinely sustain and recover from wounds penetrating the epidermal and dermal layers of the skin (Stevens, 1974; Pratt, 1979; Hoyos-Padilla et al., 2013). Such traumatic wounds heal completely and apparently without infection, even when continuously exposed to an environment rich in pathogens (Towner et al., 2012; Chin et al., 2015). With the regular occurrence of fresh and well-healed wounds on stingrays (Snelson et al., 1988; Kajiura et al., 2000) it is possible that their epidermal mucus may serve an innate immune function to account for the absence of wound-related infection.

Several studies have already investigated the possibility of such an immune function and found that chemical extracts of stingray epidermal mucus display antibiotic activity. Venilila et al. (2011) demonstrated that acidic extracts from the epidermal mucus of Dasyatis sephen, the cowtail stingray, and Himantura gerrardi, the sharpnose stingray, displayed peptide derived antimicrobial action, while Conceição et al. (2012) identified protein based antibiotic activity in the epidermal mucus of Potamotrygon henlei, the bigtooth river stingray. In addition, aqueous spine extracts of Himantura imbricata, the scaly stingray, demonstrated broad spectrum antibiotic activity (Kalidasan et al., 2014). In a microbiobased, human health oriented study, it was found that the epidermal mucus of the ocelate river stingray, Potamotrygon motoro, harbored a number of bacteria that were toxic to human epithelial cells (Domingos et al., 2011). However, no studies have isolated and characterized antibiotic-producing bacteria associated with the epidermal mucus layers of stingrays.

The purpose of this study was to isolate and identify antibiotic producing bacterial strains from the epidermal mucus of skates and rays that may serve a potential probiotic role against wound infections as well as provide a novel source for antibiotics. With the realization that human wound infection pathogens are adapting to existing antibiotic drugs and becoming increasingly resistant to treatment over time, the United States Department of Defense is interested in developing new antibiotic compounds to treat their wounded warriors. As a result of the recurring observation of infection-free healing in elasmobranch fishes, the data presented here are part of a study funded by the Department of Defense to establish the feasibility of identifying novel compounds from stingray epidermal mucus with potential application in treating wound infection pathogens and promoting wound healing.

**MATERIALS AND METHODS**

**Experimental Animals**

Animals used as sources of epidermal mucus included cownose ray, *Rhinoptera bonasus*, Atlantic devil ray, *Mobula hypostoma*, marine and freshwater Atlantic stingrays, *Dasyatis sabina*, and clearnose skate, *Raja eglanteria*. All marine species are inhabitants of Gulf of Mexico waters off the coast of Sarasota, FL, United States, at various times of the year. Freshwater *D. sabina* are permanent inhabitants in lakes near Orlando, FL, United States.

**Specimen Collection**

Cownose rays and devil rays were collected passively by surrounding schools in shallow water with a seine net and transferring individual rays with dip nets to an onboard live-well. Marine Atlantic stingrays were captured in shallow nearshore water using cast nets, while deeper water clearnose skates and freshwater Atlantic stingrays were collected using baited set lines. Cownose rays, devil rays, and freshwater Atlantic stingrays were sampled at time of capture and released unharmed, while marine Atlantic stingrays and clearnose skates were returned to the laboratory where samples were collected. All animals were collected following guidelines specified in Special Activities Licenses issued by the Florida Fish and Wildlife Conservation Commission. Numbers of individuals from each species are as follows: Freshwater *D. sabina*: *n* = 3 (1 male; 2 females), Marine *D. sabina*: *n* = 12 (3 males; 9 females), *M. hypostoma*: *n* = 30 (20 males; 10 females), *R. bonasus*: *n* = 74 (27 males; 47 females), *R. eglanteria*: *n* = 3 (all female).

**Mucus Collection**

Epidermal mucus was sampled from individual rays and skates by sterile seawater surface rinses followed by passive scraping of the pectoral fin surfaces with a sterile scoopsula and transferred to sterile culture tubes. Mucus was separated by centrifugation (2,600 × g; 30 min, 4°C) into an aqueous supernatant and a viscous pellet (Figure 1).

**Bacterial Isolation**

Aliquots (100 µL) of freshly collected mucus pellets and aqueous supernatants were serially diluted in sterile seawater (ranging in concentration relative to the original sample from 1:10 to 1:1,000,000). One hundred microliters of each dilution was plated onto marine agar (Sigma Chemical Co) with sterile 2 mm ColiRoller glass beads. Cultures were grown at 25°C for 3–5 days for development of bacterial colonies. Bacterial colonies exhibiting a unique morphology were sub-cultured 3 times to purify under the same set of growth conditions. Purified isolates were cryopreserved at −80°C in 25% glycerol in marine broth and stored in 96-well microtiter plate culturable libraries for antibiotic screening. Most of the mucus-associated bacteria were isolated from the mucus pellet fraction rather than the supernatant.

**Antibacterial Screening**

Isolated colonies were analyzed for antibacterial properties against a number of pathogenic and non-pathogenic test strains using an agar overlay assay. Purified elasmobranch bacterial libraries were inoculated onto single well plates (VWR) of 1.5% marine agar using a 96-prong well inoculator (V&P Scientific, Inc.). Library plates were allowed to grow at 25°C for 2 days and were UV-irradiated for 30 min to prevent further growth and cross-contamination of isolates. Test strains were grown overnight at 25°C (marine pathogens) or 37°C (human pathogens) for 2 days and were UV-irradiated for 30 min to prevent further growth and cross-contamination of isolates. Test strains were grown overnight at 25°C (marine pathogens) or 37°C (human pathogens) for 2 days and were UV-irradiated for 30 min to prevent further growth and cross-contamination of isolates. Test strains were grown overnight at 25°C (marine pathogens) or 37°C.
pathogens) in 2 mL of strain specific culture broth and included *Bacillus subtilis* (ATCC 6633 Km resistant), *Enterococcus faecalis* (ATCC 29212), *Vancomycin-resistant Enterococcus* (VRE, ATCC MP-1), *Methicillin-sensitive Staphylococcus aureus* (MSSA, ATCC 29213), *Methicillin-resistant S. aureus* (MRSA, ATCC 43300), *Escherichia coli* O157 *Serratia marcescens* PDL100 and *Vibrio shilonii* BAA-91. Aliquots of each broth culture were inoculated into 0.8% agar containing marine broth, Luria broth or tryptic soy broth, depending on the test strain. Elasmobranch library plates were overlaid with approximately 10 mL of inoculated agar. Following overnight growth, plates were analyzed for antibacterial activity and zones of inhibition were measured in millimeters.

**Antibacterial Compound Characterization (Blood Agar and Proteinase K Assays)**

A subset of antibiotic-producing isolates, chosen based on an ability to inhibit the growth of a varying range of pathogens as well as their stability upon repeated subculturing, was further characterized by testing for blood lysis potential and production of antimicrobial peptides. Eleven *R. bonasus* isolates displaying broad spectrum antibacterial activity in the initial screening process were inoculated onto 1.5% marine agar plates containing 5% sheep blood by volume. Following overnight growth, these strains were analyzed for the ability to lyse red blood cells by identifying and measuring zones of hemolysis (clearing zones) in millimeters. The antibacterial compounds produced by these isolates were further characterized by testing antibacterial activity in the presence of Proteinase K. Overlays were carried out as described with the addition of 100 µg/mL of Proteinase K to the test strain-appropriate 0.8% agar. Following overnight growth, zones of pathogen growth inhibition were identified and measured in millimeters. Results from this assay were then compared to initial overlay assays to determine which strains retained antibacterial properties in the presence of Proteinase K.

**Phylogenetic Characterization of Bacterial Strains**

DNA was extracted from pure cultures of all antibiotic-producing isolates from stingrays using a Power Soil DNA Extraction Kit (Mo Bio, Inc.). The 16S rRNA genes were amplified using a 25 µL polymerase chain reaction (PCR) mixture as follows: 12.5 µL of Taq master mix (Qiagen), 1 µL of 0.5 µg/mL bovine serum albumin, 8.5 µL of molecular grade water, 1.0 µL (50 ng) of template DNA and 1 µL (10 µM) each of forward oligonucleotide primer U9F (5′-GAGTTTGATYMTGGCTC) and reverse primer U1502R (5′-GYTACCTTGTTACGACTT; Weidner et al., 1996). Cycling conditions included an initial denaturation at 94°C for 120 s followed by 35 rounds of 94°C for 80 s, 54°C for 60 s, and 72°C for 90 s with a final 72°C extension for 180 s. PCR products were identified via 1% agarose gel electrophoresis, visualized on an Alphalmager 3300, and purified using a Qiagen PCR purification kit (Qiagen, Inc). DNA from purified PCR samples was sequenced at the UIUC Core Sequencing Facility, University of Illinois, Urbana-Champaign. Consensus sequences of forward and reverse strands generated through sequencing were analyzed using the GenBank Basic Local Alignment Search Tool (BLAST) to determine percent similarity to other strains in the international bacteria sequence database (Altschul et al., 1997).

**Phylogenetic Tree of Antibiotic-Producing Bacteria**

A phylogenetic tree was constructed using QIIME (Caporaso et al., 2010b). Consensus sequences for each strain found in Table 2, as well as four reference sequences from GenBank

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**FIGURE 1**

(A) Epidermal mucus is collected by passive scraping of the pectoral fin surface of an Atlantic stingray, *Dasyatis sabina* with a sterile scoopula. Fresh mucus (B) can be separated into an aqueous supernatant and a mucus pellet (C) following centrifugation at 2,600 × g for 20 min at 4°C.
(Pseudoalteromonas tunicata strain D2: Z2552, B. circulans: FJ581445, V. coralliilyticus: HM771346, and Photobacterium halotolerans: AY551089) were aligned with each other with the function align_seqs.py in QIME using the PyNAST method (Caporaso et al., 2010a). Following the initial alignment, the QIME function filter_alignment.py was used to remove gaps in the alignment shared by all sequences. Using this alignment, the phylogeny was created using FastTree 2 (Price et al., 2010), the default method utilized by the QIME function make_phylogeny.py. The FastTree method is an approximately maximum-likelihood method in which a skeleton tree is created, using a neighbor-joining algorithm, to gain an initial topography which is subsequently improved using maximum-likelihood rearrangements. The resulting phylogeny is unrooted by default. The phylogenetic tree was visualized in R using the package ggtree (Yu et al., 2016).

**GenBank Accession Numbers**
16S rRNA gene sequences generated were entered into the GenBank world-wide database (NCBI) under accession numbers KP713443-KP713670.

**Ethics Statement**
This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and outlined in Mote Marine Laboratory’s Animal Welfare Assurance (A4219-01). All experimental protocols were approved by Mote Marine Laboratory’s Institutional Animal Care and Use Committee (IACUC) and by the US Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO). All biological samples were obtained passively with minimal discomfort and no animals died or were euthanized during the project.

**RESULTS**
During this study, 1860 bacteria were isolated from the epidermal mucus of three stingray species and one skate species (Table 1). All bacterial isolates were screened for their ability to produce antibacterial compounds that inhibit the growth of a range of pathogenic test strains. Three hundred and eleven of these isolates demonstrated activities against one or more test strains (Table 1). Of these 311, 57 produced either broad-spectrum antibiotics or activities against VRE or MRSA only (Table 2).

**Antibacterial Screening**
Analysis of zones of inhibition against pathogenic test strains identified a number of bacterial isolates that displayed antibiotic properties. Examples of pathogen inhibition by mucus-associated isolates are shown in Figures 2, 3. Two hundred out of 960 (21%) R. bonasus isolates, 60 of 323 (19%) marine D. sabina isolates, 9 of 96 (9%) of freshwater D. sabina isolates, 35 of 193 (18%) of M. hypostoma isolates and 7 of 288 (2%) of R. eglanteria isolates displayed inhibition against at least one test pathogen (Table 1).

**Phylogenetic Identification of a Subset of Total Ray Isolates**
Table 2 represents a list of representative antibiotic producing bacterial associates from ray species. Not all isolates initially tested for antibiotic activity were phylogenetically identified due to loss of viability during repeated sub-culturing and/or frozen storage, or an inability to obtain adequate template or sequence DNA. Up to 20% of antibiotic producing isolates did not survive repeated freeze thaw upon cryopreservation and are, therefore, not included in phylogenetic identification attempts. Bacteria associated with the clearnose skate, R. eglanteria, were not phylogenetically identified for this study due to loss of strains during long-term storage. Repeated isolates are indicated in Table 2 and were dereplicated based on identical 16S rDNA sequences and antibiotic spectrum profiles.

**Freshwater D. sabina Bacterial Isolates**
Freshwater stingray isolates showed the greatest amount of diversity with 18 genera revealed (data not shown for non-antibiotic producing bacteria) yet had the lowest percentage of antibiotic activities among all the stingray isolates surveyed. Of 96 freshwater D. sabina bacterial isolates screened, only 9 (9%) isolates showed antibiotic activities against one or more test strains (Table 2). Eight isolates were active against only E. coli and consisted mainly of members of the genus Pseudomonas, with one Psychrobacter and one Stenotrophomonas sp. showing similar activities against E. coli. One B. cereus isolate displayed broad-spectrum activity against E. coli, MRSA, MSSA and VRE (Table 2). Other non-antibacterial producing isolates making up the culturable flora of freshwater D. sabina mucus included Gordonia, Mycobacterium, Microbacterium, Caulobacter, Brevundimonas, Chryseobacterium, Staphylococcus, Psychrobacter, Nocardia, Bosea, Rhizobium, Delfia, Stenotrophomonas, Leucobacter, Acinetobacter, and Oscrobactrum spp. (data not included).

**Marine D. sabina Bacterial Isolates**
Of 323 marine D. sabina bacterial isolates screened, 60 (19%) displayed antibiotic activity (Table 1), and only against B. subtilis

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**Table 1 | Compiled stingray and skate antibacterial screening data.**

| Species       | Common name  | Bacterial isolates | # Isolates with antimicrobial activity | % Showing activity |
|---------------|--------------|--------------------|----------------------------------------|--------------------|
| Desyatis sabina (F) | Atlantic stingray | 96                 | 9                                      | 9.4                |
| Desyatis sabina (M) | Atlantic stingray | 323                | 60                                     | 18.6               |
| Rhinoptera bonasus | Cowrnose ray   | 960                | 200                                    | 20.8               |
| Mobula hypostoma | Devil ray     | 193                | 35                                     | 18.1               |
| Raja eglanteria | Clearnose skate | 288                | 7                                      | 2.4                |
| **Totals**     |              | **1,860**          | **311**                                | **16.7**           |
### TABLE 2  
List of representative bacteria isolated from the mucus of rays.

| Strain | Bp  | % ID | Strain identification | Isolate # | AB spectrum (ZI) | Accession # |
|--------|-----|------|------------------------|-----------|------------------|-------------|
| **Freshwater Atlantic stingray, Dasyatis sabina** | | | | | | |
| 845A3  | 780 | 99   | Microbacterium sp.     | B. subtilis (1.0) | KP713518 |
| 845D5  | 847 | 99   | Stenotrophomonas sp.   | E. coli (1.5) | KP713525 |
| 845E11a| 926 | 99   | Pseudomonas stutzeri    | E. coli (1.5) | KP713564 |
| 845E11b| 902 | 99   | Pseudomonas putida     | E. coli (1.5) | KP713565 |
| 845E4  | 539 | 100  | Psychrobacter pacificensis | E. coli (1.0) | KP713562 |
| 845E9  | 896 | 100  | Bacillus cereus         | E. coli (1.0); MRSA (2.5); MSSA (1.5); VRE (2.5) | KP713528 |
| 845F2  | 879 | 99   | Pseudomonas sp.        | E. coli (0.5) | KP713566 |
| **Marine Atlantic stingray, Dasyatis sabina** | | | | | | |
| 846B4  | 839 | 99   | Photobacterium damselae | B. subtilis (1.0) | KP713568 |
| 846C2  | 922 | 99   | Vibrio harveyi         | B. subtilis (1.0) | KP713569 |
| 846C5  | 939 | 99   | Photobacterium sp.     | B. subtilis (1.0) | KP713480 |
| 846F8  | 1003| 99   | Vibrio sp.             | B. subtilis (1.0) | KP713572 |
| **Atlantic devil ray, Mobula hypostoma** | | | | | | |
| 809A8  | 844 | 99   | Vibrio sp.             | VRE (2.0) | KP713464 |
| 809B1  | 893 | 99   | Pseudoalteromonas sp.  | B. subtilis (1.0) | KP713465 |
| 809B6  | 962 | 99   | Pseudoalteromonas sp.  | V. shilonii (1.0) | KP713468 |
| 810E4  | 937 | 98   | Vibrio sp.             | B. subtilis (0.1) | KP713446 |
| 810F6  | 976 | 99   | Alteromonas sp.        | B. subtilis (0.5) | KP713449 |
| 810G1  | 967 | 98   | Vibrio sp.             | MRSA (0.1); B. subtilis (0.2) | KP713451 |
| **Cownose ray, Rhinoptera bonasus** | | | | | | |
| 803A6-1| 880 | 99   | Exiguobacterium sp.    | B. subtilis (0.2) | KP713613 |
| 803B11 | 787 | 99   | Pseudoalteromonas sp.  | B. subtilis (1.75) | KP713582 |
| 803B6-3| 870 | 99   | Bacillus sp.           | MRSA (0.3); MSSA (1.0); B. subtilis (3.5) | KP713633 |
| 803B8-1| 889 | 99   | Exiguobacterium sp.    | MRSA (0.5); MSSA (0.2); B. subtilis (0.3) | KP713634 |
| 803B8-2| 890 | 99   | Bacillus sp.           | MRSA (0.5); MSSA (0.2); B. subtilis (0.3) | KP713630 |
| 803B9-1| 872 | 100  | Bacillus sp.           | MRSA (0.2); B. subtilis (1.2) | KP713627 |
| 803D10-2a| 881| 99  | Lysinibacillus sp.     | MSSA (4.5); MRSA (5.0) | KP713670 |
| 803D10-2c| 955| 99  | Bacillus sp.           | MSSA (4.5); MRSA (5.0) | KP713625 |
| 803D5  | 860 | 99   | Halomonas sp.          | MRSA (10.5) | KP713669 |
| 803D9  | 760 | 99   | Vibrio sp.             | B. subtilis (1.0) | KP713585 |
| 803E8  | 913 | 99   | Pseudoalteromonas sp.  | MRSA (7.5); MSSA (8.5); VRE (4.5); B. subtilis (10) | KP713479 |
| 803G11 | 869 | 99   | Bacillus sp.           | MRSA (6.0); MSSA (4.5); B. subtilis (7.5) | KP713626 |
| 803G9  | 811 | 99   | Pseudoalteromonas sp.  | B. subtilis (0.2) | KP713587 |
| 803H10 | 787 | 99   | Pseudoalteromonas sp.  | MRSA (0.1); B. subtilis (0.1) | KP713588 |
| 804C6  | 787 | 99   | Bacillus cereus        | MRSA (1.25) | KP713589 |
| 804D11-1| 837| 100  | Bacillus sp.           | MRSA (0.1) MSSA (0.1); B. subtilis (0.2) | KP713635 |
| 804D11-2| 897| 99   | Lysinibacillus sp.     | MRSA (0.1) MSSA (0.1); B. subtilis (0.2) | KP713636 |
| 804D11-3| 793| 99   | Vibrio sp.             | MRSA (0.1) MSSA (0.1); B. subtilis (0.2) | KP713637 |
| 804D6  | 802 | 99   | Bacillus sp.           | MRSA (1.25) | KP713591 |
| 804E10 | 765 | 99   | Bacillus sp.           | MRSA (0.5); B. subtilis (0.5) | KP713592 |
| 804E12 | 859 | 99   | Bacillus cereus        | MRSA (2.5) | KP713593 |
| 804G3  | 909 | 99   | Pseudoalteromonas sp.  | MRSA (0.5) | KP713594 |
| 804G9  | 791 | 99   | Bacillus megatentum    | B. subtilis (8.0) | KP713595 |
| 804H10 | 836 | 99   | Bacillus sp.           | MRSA (0.1); B. subtilis (0.1) | KP713476 |
| 805A10 | 738 | 99   | Psychrobacter celer    | B. subtilis (0.75) | KP713598 |
| 805A6  | 902 | 99   | Psychrobacter sp.      | B. subtilis (0.2) | KP713478 |
| 805B1  | 809 | 99   | Marinobacter hydrocarbonoclasticus | B. subtilis (0.1) | KP713599 |

(Continued)
TABLE 2 | Continued

| Strain     | Bp   | % ID | Strain identification | Isolate # | AB spectrum (ZI) | Accession # |
|------------|------|------|------------------------|-----------|-----------------|-------------|
| 805B12     | 883  | 99   | Vibrio sp.             | 5         | B. subtilis (0.5)| KP713600    |
| 805C10     | 737  | 100  | Alteromonas sp.        |           | B. subtilis (0.1)| KP713603    |
| 805C12     | 771  | 99   | Vibrio sp.             |           | B. subtilis (0.25)| KP713575    |
| 805C7      | 851  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (1.5)| KP713601    |
| 805E11     | 570  | 99   | Shewanella sp.         |           | B. subtilis (0.1)| KP713606    |
| 805E12     | 852  | 100  | Bacillus sp.           |           | B. subtilis (0.5)| KP713481    |
| 805E7      | 860  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (4.5)| KP713605    |
| 805F10     | 694  | 99   | Vibrio sp.             |           | B. subtilis (0.2)| KP713609    |
| 805F12     | 845  | 99   | Vibrio sp.             |           | B. subtilis (0.1)| KP713576    |
| 805F4      | 817  | 100  | Marinobacter sp.       |           | B. subtilis (0.1)| KP713607    |
| 805H7      | 1002 | 99   | Vibrio maritimus       | 11        | B. subtilis (0.1)| KP713577    |
| 806B10     | 832  | 99   | Shewanella sp.         |           | MSSA (>10)      | KP713485    |
| 806B11     | 919  | 99   | Alteromonas sp.        |           | MSSA (>10)      | KP713486    |
| 806B12     | 795  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (0.2)| KP713487    |
| 806C11     | 816  | 99   | Alteromonas sp.        |           | MSSA (>10)      | KP713492    |
| 806C12     | 886  | 99   | Pseudoalteromonas sp.  | 2         | B. subtilis (0.2)| KP713493    |
| 806C7      | 875  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (0.5)| KP713489    |
| 806C9      | 775  | 99   | Vibrio sp.             |           | B. subtilis (0.2)| KP713490    |
| 806E11     | 831  | 99   | Vibrio maritimus       |           | MSSA (0.1)      | KP713618    |
| 806F10     | 624  | 100  | Vibrio sp.             |           | MRSA (0.1); B. subtilis (0.2)| KP713631   |
| 807A1      | 965  | 99   | Vibrio sp.             | 13        | B. subtilis (0.1)| KP713496    |
| 807A6      | 875  | 99   | Vibrio parahaemolyticus|           | B. subtilis (0.1)| KP713499    |
| 807E3      | 752  | 99   | Vibrio sp.             |           | B. subtilis (0.2)| KP713501    |
| 807H7      | 851  | 99   | Vibrio sp.             |           | VRE (1.5)       | KP713507    |
| 807H8      | 889  | 99   | Vibrio sp.             |           | VRE (0.5)       | KP713508    |
| 808A12     | 653  | 99   | Pseudoalteromonas sp.  |           | MRSA (0.5); B. subtilis (0.2)| KP713457   |
| 808A7      | 918  | 99   | Psychrobacter sp.      |           | B. subtilis (0.5)| KP713456    |
| 808B1      | 918  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (0.3)| KP713482    |
| 808C1      | 903  | 99   | Psychrobacter sp.      |           | B. subtilis (0.3)| KP713458    |
| 808E1      | 872  | 99   | Psychrobacter sp.      |           | B. subtilis (0.1)| KP713459    |
| 808F11     | 624  | 99   | Pseudoalteromonas sp.  |           | MRSA (0.2); B. subtilis (0.1)| KP713460   |
| 809G11     | 942  | 100  | Vibrio sp.             |           | B. subtilis (1.5)| KP713461    |
| 809H4      | 950  | 99   | Alteromonas sp.        |           | B. subtilis (0.1)| KP713462    |
| 811A6      | 936  | 99   | Bacillus cereus        |           | MRSA (1.0); MSSA (2.0)| KP713578   |
| 815D3      | 512  | 99   | Paracoccus sp.         | E. coli (2.0)| KP713579        |
| 815G7      | 834  | 99   | Pseudoalteromonas sp.  |           | B. subtilis (0.1)| KP713581    |
| 816C8      | 854  | 99   | Bacillus sp.           |           | MRSA (2.0); MSSA (1.0); B. subtilis (4)| KP713573   |
| 823Bb-2c   | 804  | 99   | Exiguobacterium sp.    |           | MRSA (0.3); MSSA (1.0); B. subtilis (3.5)| KP713622   |

Not all isolates initially tested for antibiotic activity are included in this table due to loss of viability during repeated sub-culturing and/or frozen storage or an inability to obtain adequate template or sequence DNA. Test strains include: Bacillus subtilis (ATCC 6633 Km resistant), Enterococcus faecalis (ATCC 29212), Vancomycin-resistant Enterococcus (VRE, ATCC MP-1), Methicillin-sensitive Staphylococcus aureus (MSSA, ATCC 29213), Methicillin-resistant Staphylococcus aureus (MRSA, ATCC 43300), Escherichia coli O157, Seneta marcescens PDL100, and Vibrio shilonii BAA-91. The closest match and %ID are reported as the most similar bacterial isolates from the NCBI’s GenBank database. Antibacterial spectrum (AB spectrum) was measured as the distance from the edge of the colony (mm) to the end of the zone of inhibition (ZI). Values are reported for inhibition of any tester strain. bp = number of DNA base pairs blast searched. Isolate # illustrates the number of purified isolates with identical sequences and antibiotic production range/spectrum. Accession # = Genbank accession number of the representative bacterial isolate.

(Table 2). Of eight active isolates genetically identified, 16S rDNA sequence analysis revealed 3 bacterial genera including Vibrio spp. (n = 4), Photobacterium spp. (n = 3), and a Staphylococcus sp. (n = 1).

M. hypostoma Bacterial Isolates
Of 193 M. hypostoma bacterial isolates screened, 35 (18%) produced antibiotic activities against one or more test strains (Table 1). Of 20 isolates genetically identified, only three bacterial genera were revealed (Table 2). Twelve Vibrio isolates showed activities against B. subtilis; one showed activity against VRE; 1 showed activity against both MRSA and B. subtilis; and 1 isolate showed no antibacterial activity against any pathogenic strain tested. Ten isolates were identified as Pseudoalteromonas species with 4 isolates showing activities against B. subtilis, 4 against V. shilonii, and 2 showing no activities to any pathogenic strain tested. One Alteromonas species showed activity against B. subtilis only (Table 2).

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Ritchie et al. Antibiotic-producing Elasmobranch Bacteria
**Lysinibacillus** One Shewanella B. subtilis (8.5 mm), VRE (4.5 mm) and large zones of inhibition against MRSA (7.5 mm), MSSA of inhibition exceeding 10 mm. Bacillus (803D5) displayed antibiotic activity against MRSA with a zone of inhibition exceeding 10 mm. Bacillus sp. 803E6, displayed large zones of inhibition against MRSA (7.5 mm), MSSA (8.5 mm), VRE (4.5 mm) and B. subtilis (10 mm) (Table 2). One Shewanella sp. (806B10) and one Alteromonas sp. (806B11) were active against MSSA with zones of inhibition over 10 mm. Two Vibrio sp. demonstrated antibacterial activities against VRE (Table 2).

**Phylogenetic Tree of Antibiotic-Producing Bacteria**
To better visualize the phylogenetic relatedness of antibiotic producing strains found in this study (Table 2) a phylogenetic tree (Figure 4) was constructed. In order to address relatedness to other antibiotic-producing bacteria, we included reference sequences from P. tunicata strain D2: Z2552, B. circulans, FJ581445, V. coralliilyticus, HM771346, and P. halotolerans, AY551089). R. bonasus bacteria were the most abundant isolates genetically identified in this study and formed the basis of the three phylogenetic clusters illustrated in Figure 4. M. hypostoma isolates clustered within groups II and III and marine D. sabina clustered entirely within group II. Isolates from fresh water and marine D. sabina did not cluster together. The freshwater D. sabina isolates clustered between group I and II with a single isolate positioned within the R. bonasus dominated group I Bacillus isolates. This fresh water Atlantic stingray isolate, B. cereus 845E9, showed inhibition against numerous test strains (E. coli, MRSA, MSSA, VRE; Table 2). The R. bonasus isolates positioned closest to strain 845E9 in Figure 4 showed similar inhibitory properties against MSSA and MRSA with varying inhibitory properties against other test strains (Table 2). Alteromonas and Shewanella isolates derived from R. bonasus produced the strongest zones of inhibition observed against both MSSA and MRSA (heatmap, Figure 4 and Table 2).

**Antibacterial Compound Characterization of a Subset of Broad-Spectrum Antibacterial-Producing Isolates**
Eleven strains from M. hypostoma and R. bonasus were selected for further characterization based on activities against a range of nosocomial pathogens, as well as an ability to survive repeated subculturing and cryopreservation. Compound characterization included hemolytic activity testing and testing for activity in the presence of Proteinase K. Hemolysis tests were performed in order to identify antibiotics with potential toxicity to eukaryotic cells and proteinase K tests were used to specifically determine which activities rely on the presence of small peptide antibiotics (Table 3). Seven of 9 strains tested from R. bonasus were found to be hemolytic. In contrast, both strains isolated from M. hypostoma did not display hemolytic activity. The activity of both M. hypostoma strains was inhibited by the presence of Proteinase K in antibacterial assays against MRSA and B. subtilis suggesting that the active compound is a peptide antibiotic. Antibiotic activities in 3 of 9 R. bonasus strains were inhibited by the presence of Proteinase K, suggesting that the active compound(s) in these strains are peptide antibiotics.

**DISCUSSION**
As elasmobranchs display impressive wound healing capabilities, the intent of this study was to survey culturable ray and skate bacterial associates for antibiotic activity with the ultimate goal of identifying a new marine source for novel anti-infective compounds. Results of antibiotic overlays demonstrated that a

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**R. bonasus Bacterial Isolates**
Of 960 bacteria isolated and screened, 200 (21%) were active against one or more test strain (Table 1). Eleven different bacterial genera were identified that produce antibacterial compounds, with members of the genera Bacillus, Exiguobacterium, Lysinibacillus, Vibrio, and Pseudoalteromonas displaying the broadest spectra of activity (Table 2). One Halomonas sp. (803D5) displayed antibiotic activity against MRSA with a zone of inhibition exceeding 10 mm. Bacillus sp. 803E6, displayed large zones of inhibition against MRSA (7.5 mm), MSSA (8.5 mm), VRE (4.5 mm) and B. subtilis (10 mm) (Table 2). One Shewanella sp. (806B10) and one Alteromonas sp. (806B11) were active against MSSA with zones of inhibition over 10 mm. Two Vibrio sp. demonstrated antibacterial activities against VRE (Table 2).
number of mucus associated bacteria display antibiotic activity against common pathogens. In other studies, protein extracts of the stingray mucus layers have presented similar antibiotic activity (Vennila et al., 2011; Conceição et al., 2012). Collectively over 16% of bacterial isolates of the epidermal mucus from three stingray and one skate species displayed antibiotic activity against one or more pathogenic test strain. Previous studies have illustrated that roughly 3% of bacteria cultured from seawater and up to 13% of bacteria isolated from abiotic or biotic surfaces are inhibitory (Gram et al., 2010). Up to 20% of bacteria isolated from corals produce antibiotic activities (Ritchie, 2006). In the present study, roughly 3% showed either broad-spectrum antibacterial activity or activities against VRE or MRSA, only, suggesting that these isolates, and representative compounds, may be promising candidates for future drug discovery initiatives. Broad-spectrum antibiotic-producing bacterial strains isolated from stingrays, or strains active against important nosocomial pathogens (MRSA and VRE), were identified within 5 different genera including Bacillus, Vibrio, Exiguobacterium, Lysinibacterium, and Pseudoalteromonas. This information will help in targeting bacterial growth conditions for future antibiotic screens. It is important to note that some small zones of inhibition reported...
could be caused by factors other than antibiotic production, such as absence of nutrients, changes in pH or other variables that were not measured in this study.

Because of their ability to osmoregulate over a broad range of salinities, both marine and freshwater populations of the Atlantic stingray inhabit Florida waters. This provided a unique opportunity to consider the influence of environment on mucus-associated microbial communities in freshwater and marine D. sabina. We phylogenetically identified a large pool of total isolates from both freshwater and marine Atlantic stingrays among both antibiotic-producing and non-antibiotic-producing isolates (Table 2 and Supplementary Table 1). A predominance of Vibrio and Photobacteria spp. were associated with marine D. sabina (Supplementary Table 1). In comparison we isolated a wide range of bacterial types associated with freshwater D. sabina, including members of the genera Brevundimonas, Psychrobacter, Gordonia, Chryseobacterium, Staphylococcus, Microbacterium, Acinetobacter, Caulobacter, Mycobacterium, Bosea, Nocardia, Rhizobium, and others (Supplementary Table 1). This is the first research to our knowledge to address a comparison of D. sabina bacterial host specificity in fresh vs. marine waters. Other studies have shown host species-specific bacterial associations regardless of differences in environmental locations (Knowlton and Rohwer, 2003; Taylor et al., 2003). Our limited culture-based data suggest a lack of host-driven specificity in Atlantic stingrays that is likely driven by the extreme salinity differences in freshwater vs. marine environments.

Of note is the lack of overlap in antibiotic producing and non-antibiotic producing bacteria associated with freshwater D. sabina, where antibiotic producers fall within the genera Pseudomonas, Stenotrophomonas, Psychrobacter, and Bacillus. Representative active isolates derived from fresh water D. sabina were all active against E. coli with Bacillus strain 845E9 being the only strain with activity against gram-negative E. coli as well as gram-positive MRSA, MSSA and VRE (Table 2). In contrast, antibiotic-producing bacteria isolated from marine D. sabina fall within the genera Vibrio, Photobacterium, and Staphylococcus. These isolates were predominantly active against B. subtilis and illustrated a distinct overlap with similar isolates that did not show antibiotic-producing capabilities.

Perhaps as a reflection of their tannic acid river and lake environment, mucus from the freshwater rays is noticeably darker than mucus from marine specimens, with the majority of pigment remaining in the microbe-rich mucus pellet. In addition, in their relatively confined lake environment, freshwater D. sabina are exposed to anthropogenic influences of pollution from stormwater runoff and contamination from sewage (Gelsleichter et al., 2006). It is interesting to note that freshwater stingray isolates showed the greatest diversity (with 18 genera revealed among all antibiotic and non-antibiotic producing isolates genetically identified; Supplementary Table 1) and the lowest percentage of antibiotic activities among all the ray isolates surveyed (Table 1). That all nine of the isolates from freshwater ray mucus were active against E. coli may reflect an adaptive innate immune mechanism driven by beneficial bacterial associates. These data illustrate different bacterial community associations as well as differences in bacterial genera capable of producing antibiotic compounds associated with the epidermal mucus layers of stingrays that have adapted to dramatically different environments. Future efforts using molecular techniques will be necessary to thoroughly address differences in Atlantic stingray bacterial host specificities and potential functional redundancies of freshwater and marine bacterial associates.

The higher total number of bacteria isolated from the cownose ray, R. bonasus, is reflected in the higher sample size of 74 individuals from this ray species. In contrast to Atlantic stingray isolates, identification of active isolates from R. bonasus revealed that a majority are members of the genera Bacillus, Pseudoalteromonas, and Vibrio, with other active isolates belonging to the genera Alteromonas, Exiguobacterium, Psychrobacter, Lysinibacillus, Halomonas, Marinobacter, Shewanella, and Paracoccus. With the exception of Vibrio spp., different genera are represented among the antibiotic producing isolates from this ray species. This higher apparent diversity is likely due to the higher animal sample size. Overall, although there is apparent overlap in antibiotic producing bacterial species across all ray species analyzed, different isolates were considered unique due to host source and different colony or antibiotic spectra phenotypes and may represent bacteria in normal associations with these animals. Additional biases may include different animal capture methods. Cownose rays and devil rays were collected in natural habitats using a seine net and dip nets to access animals for sampling. While animals were rinsed with sterile seawater prior to mucus sampling, these collection techniques may have removed adhering bacteria while contributing non-associated bacteria to our isolate pools.

The phylogenetic tree shown in Figure 4 reflects stingray-derived bacterial isolates that display varying degrees of antibiotic activities. As noted above, isolates from fresh water and marine D. sabina did not cluster together. Although this study did not address total microbial community differences between hosts, this finding suggests that habitat type can drive culturable host communities even in the same host species. This is likely reflective of the long established inland freshwater versus marine habitats of these Atlantic stingray groups that influences bacterial associates. Marine Bacillus species have been shown to produce lipopeptides that are active against a range of gram positive and gram negative pathogenic test strains (Das et al., 2008) which could be a source of activity seen in our active Bacillus isolates. Pseudoalteromonas species associated with a variety of marine eukaryotes are also known to produce a range of bioactive antagonistic extracellular compounds, including antifouling agents, which may enable them to colonize surfaces (Holmström and Kjelleberg, 1999). Wietz et al. (2010) chemotyped over 300 marine Vibrionaceae and Photobacterium species revealing the presence of the previously described antibiotics, andrimid (from Vibrionaceae) and holomycin (from Photobacterium). Andrimid is a broad-spectrum peptide antibiotic originally isolated from a bacterial endosymbiont of a plant-associated arthropod (Fredenhagen et al., 1987). Holomycin also exhibits broad-spectrum activity and belongs to pyrrothine class of antibiotics that acts to interfere with RNA synthesis (Wietz et al., 2010).
Lastly, we have sub-classified active compounds from 11 candidate antibiotic-producing bacterial strains. We selected this subset of active isolates from *R. bonasus* and *M. hypostoma* based on the range of test strains against which they were active, as well as their ability to retain stable activity upon repeated subculture. Proteinase K assays were performed in order to characterize compounds from ray associated bacterial isolates as peptide or non-peptide antibiotics (Table 3). Roughly half of the active isolates tested produce peptide antibiotics as the active compound, as assayed based on loss of antibiotic activity in the presence of proteinase K. In addition blood agar assays indicate that compounds produced by the majority of isolates tested, both peptide and non-peptide, are able to lyse blood cells (Table 3), indicating a potential toxicity to eukaryotic cells and providing another criteria from which to sub classify and prioritize active strains for future studies.

This study represents an initial search for antibiotic producing bacteria associated with ray and skate species and many isolates will be pursued for drug discovery efforts. Future studies will include molecular determination of a baseline of bacterial associates of elasmobranchs to provide a more comprehensive understanding of the stability of microbial associates across species and habitats. Future work will also contribute to an understanding of the potential role of these associates in the health and wound healing capabilities of elasmobranchs.

**AUTHOR CONTRIBUTIONS**

KR, CL, and CW designed the study. CL and CW collected all epidermal mucus samples. KR, MS, JM, VL, and DM carried out bacterial culturing, antibiotic assays, genetic analysis, and helped prepare tables. CL prepared Figures 1–3. MS prepared Figure 4. KR, JM, MS, and CL drafted the manuscript. All authors read, edited and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01050/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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