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DISCOVERY OF NEW ANTIMICROBIAL COMPOUNDS FROM MARINE ACTINOBACTERIA ASSOCIATED WITH SPONGES

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This project looks at the diversity of antimicrobial-producing actinobacteria in sponges. Sponges are known to produce natural products such as antimicrobial compounds and are known to harbor actinobacteria which are a known source of antibiotics. In this project, we have collected six sponge samples by SCUBA diving and isolated 416 bacterial strains (203 from site 1, 134 from site 2, and 79 from site 3.) Of the strains from site 1, 33 were found to be gram positive and genetically analyzed. DNA sequencing of the partial 16s ribosomal RNA gene revealed that 17 of these 33 matched up to known actinobacteria. The DNA of all strains from sites 2 and 3 have been isolated and prepared for sequencing. Glycerol stocks of all identified actinomycetes will be preserved at -80°C and made available for antimicrobial activity assays by AFRL

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1. SUMMARY

Actinobacteria are capable of producing secondary metabolites exhibiting pharmaceutically relevant properties (i.e. anti-biotic, anti-tumor), but the discovery of new species of these microbes, and further new secondary metabolites, has slowed in terrestrial species. This has prompted investigation into the existence of actinobacteria in marine ecosystems, specifically within marine sponges. It has been found that marine sponges harbor a multifarious collection of bacteria, with several sponge species yielding new genera of actinobacteria. However, there is still a vast number of sponge species to study, which will enable a continued discovery of new actinobacteria species and secondary metabolites. Herein, we investigate the diversity of antimicrobial-producing actinobacteria in sponges. Six sponge samples were collected by SCUBA diving and 416 bacterial strains were isolated from those samples (203 from site 1, 134 from site 2, and 79 from site 3). Of the strains from site 1, 33 were found to be gram positive and genetically analyzed. Deoxyribonucleic acid (DNA) sequencing of the partial 16s ribosomal ribonucleic acid (rRNA) gene revealed that 17 of these 33 strains are homologous to members of the actinobacteria phylum. The DNA of all strains from sites 2 and 3 have been isolated and prepared for sequencing. Glycerol stocks of all identified actinomycetes will be preserved at -80°C and made available for antimicrobial activity assays by AFRL. It is recommended that the isolated strains of actinobacteria determined from site 1 be screened for antimicrobial activity against *Staphylococcus aureus*, *Streptococcus* spp., *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa.* Further, future collection of sponge samples should continue, with protocols written for culturing and isolation of marine Actinobacteria.

2. INTRODUCTION

2.1. Background

Natural compounds produced by microbes are an important commercial source of both existing and new drugs (Solanki et al., 2008). About 60% of the drugs available in the market are derived from natural products (Valliappan et al., 2014). For this reason, there is a continuous search for antimicrobial compounds that can be effective against Gram-negative bacteria, fungi, viruses, and mycobacteria (Flora et al., 2015). Since the discovery of penicillin in the mid-twentieth century (Fleming, 1929), antimicrobial agents have proven to be useful tools to save lives and ease the suffering of millions of people (Bibi et al., 2016).

The phylum Actinobacteria contains a wide range of Gram-positive bacteria with a high G+C DNA content (Choi et al., 2015; Montalvo et al., 2005; Sun et al., 2010) and includes both rod or coccoid-shaped and filamentous bacteria that are increasingly recognized for their capacity to produce new secondary metabolites with diverse biological activities (Zotchev, 2012). These prokaryotes can thrive as free-living microbes in soil environments and in marine and freshwater ecosystems (van der Meij et al., 2017). Members of the order Actinomycetales, commonly known as actinomycetes, are responsible for the production of approximately half of the discovered bioactive secondary metabolites, notably antibiotics, anti-tumor agents, immunosuppressive agents (Lam, 2006), which highlights their importance for human health, agriculture, and biotechnology. Representative genera of actinomycetes include *Streptomyces*, *Actinomyces*, *Arthrobacter*, *Corynebacterium*, *Frankia*, *Micrococcus*, *Micromonospora*, among others (Manivasagan et al., 2014; Solanki et al., 2008), and about 75% of these metabolites are produced by species belonging to the genus *Streptomyces* (Solecka et al., 2012).

This astounding biotechnological potential of actinomycetes for drug discovery has been observed since the 1960s (Vicente et al., 2013). In the 1940s, the first report of streptomycin, a substance produced by a member from the *Streptomyces* group, encouraged researchers and pharmaceutical companies to focus on the search for microbial natural products (Schatz et al., 1944) and by the 1980s, approximately 70% of the world's naturally occurring antibiotics were attributed to actinomycetes (Li and Liu, 2006). Because of the excellent track record of actinomycetes in this regard, a significant amount of effort has been focused on isolating terrestrial specimens for drug screening programs (Lam, 2006; Montalvo et al., 2005). However, the rate of discovery of new compounds has decreased in the past four decades. Since the nineties, no new species of bacteria have been detected when using soil habitats as a source of actinomycetes for bioactive compound discovery and known compounds have been re-isolated frequently (Bérdy, 2012; Lam, 2006). With the intense exploitation of terrestrial actinobacteria over many years, the discovery rate of novel bioactive compounds has decreased to around 95% rediscovery rate of known compounds (Zhang et al., 2006). Additionally, the number of drug-resistant pathogens has increased drastically. Approximately more than 70% of pathogenic bacteria are resistant to most of the commercially available antibiotics (Bérdy, 2012). As a result, this trend has incited interest in exploring the actinomycete diversity in marine ecosystems (Sun, et al, 2010; Vicente et al., 2013; Zhang et al., 2006). Actinomycetes were once considered to be primarily soil microbes and rare in marine environments, but in recent years they have been found widely distributed (Jiang et al., 2007). Skepticism regarding the existence of obligate marine species of actinomycetes has contributed to a lack of effort spent in exploring these

prokaryotes. It is known that terrestrial bacteria can produce resistant spores that are transported from land into sea, where they can remain available but dormant for many years (Lam, 2006; Ward and Bora, 2006). However, the descriptions of *Rhodococcus marinonascens*, along with other marine species, have demonstrated that certain actinomycetes are indigenous to the marine environment (Jensen et al., 1991).

In the last four decades, drug discovery efforts have shifted to the marine environment where invertebrates (i.e., sponges, zoantharians, soft corals, tunicates, sea slugs, bryozoans) and plants have been subject to elaborate screening programs (Tabares et al., 2011). More than 10,000 bioactive compounds have been isolated from marine organisms (Bibi et al., 2016) and marine invertebrates have been reported to be the largest contributors to marine natural compound production (Brinkmann et al., 2017). Nevertheless, it is well known that these marine organisms have a distinct relationship with numerous microorganisms, including archaea, bacteria, fungi, and viruses (Abdelmohsen et al., 2014). These mutualistic relationships offer protection, support, nitrogen fixation and nitrification, and nutritional requirements to both the microbial symbionts and the host organism, but it also provides defense mechanisms for the host and the symbiotic microorganisms alike against predators and pathogens and the ability to compete for space (Brinkmann et al., 2017). It has also been hypothesized that these compounds have a role in antifouling, anti-overgrowth, and protection against UV rays (Puyana et al., 2015). Many bioactive compounds result from the involvement of specific functions in their respective hosts, as in the case of marine organism-associated bacteria. It has been shown that marine organism-associated bacteria produce secondary metabolites that were previously attributed to their hosts (Valliappan et al., 2014). However, the exact origin of many bioactive compounds is still under investigation due to the lack of cultivation of *bona fide* sponge symbionts (Abdelmohsen et al., 2014).

Marine sponges (phylum Porifera) are sessile multicellular invertebrates that have gained significance as a rich source of bioactive secondary metabolites owing in part to their phylogenetically diverse microbial groups (Li and Liu, 2006). The two-layer structure of the sponge's outer and inner endosome and their strategy for sequestering food by filtering large volumes of seawater through a unique aquiferous system make them an ideal habitat for microorganisms (Li et al., 2006). The microbes that are not digested by the sponge may survive and inhabit the host (Zhang et al., 2008). This mode of sustenance is portrayed as a selective process, which signifies that some prokaryotes are being favored over others, creating a form of symbiotic relationship with the sponge (Santos-Gandelman et al., 2013). This selection is evidenced by the diverse groups of microorganisms that are found intracellularly within sponge cells and extracellularly within the sponge matrix, which can consist of up to 60% of their biomass (Bibi et al., 2016). In fact, the bacterial concentration in sponges may exceed those present in the surrounding water by about two to four orders of magnitude (Zhang et al., 2008). For this reason, sponges that have high levels of bacteria are classified as "high microbial abundance" (HMA) sponges (Hentschel et al., 2003). It is still unclear how exactly sponges acquire and maintain their symbionts and the specificity of the microbial symbionts to the sponges (Montalvo et al., 2014). Nevertheless, it has been found that distantly related sponges from separate geographical locations harbor comparable microbial groups that have not been detected in the surrounding seawater or other marine habitats, which indicates that these microorganisms probably are sponge-specific (Brinkmann et al., 2017).

With culture-dependent and culture-independent techniques, such as denaturing gradient gel electrophoresis (DGGE) of amplified 16S rRNA gene fragments, amplified fragment length polymorphism (AFLP) analysis, fluorescent *in situ* hybridization (FISH), amplified ribosomal DNA restriction analysis (ARDRA), and clone libraries (Bibi et al., 2016), our knowledge about marine sponge-associated microbial diversity has been enhanced greatly. At least 32 bacterial phyla and candidate phyla discovered in marine sponges have been described so far, with the most common phyla being Acidobacteria, Chloroflexi, Nitrospira, Cyanobacteria, Bacteriodetes, Gemmatimonadetes, Planctomycetes, Spirochaetes, Proteobacteria (Alpha-, Gamma-Proteobacteria), and Actinobacteria (Abdelmohsen et al., 2014; Thomas et al., 2010). Using molecular approaches, members of the phylum Actinobacteria and specifically the order Actinomycetales have been identified as abundant microorganisms of sponge-associated microbial communities (Abdelmohsen et al., 2010), which happen to be the dominant group in the production of natural compounds, followed by Proteobacteria (Bibi et al., 2016). This has been demonstrated by the presence of biosynthetic genes encoding polyketide synthases (PKSs) and non-ribosomal polyketide synthetases (NRPSs), which are the hallmarks of secondary metabolite production (Manivasagan et al., 2014). This suggests that sponge-associated actinobacteria are beneficial to their hosts (Valliappan et al., 2014). At least 60 genera of actinobacteria have been identified from marine sponges (Sun et al., 2015), but the number of descriptions of new genera and species of actinomycetes from marine sponges is continuously rising (Abdelmohsen et al., 2014). Nevertheless, research on sponge-associated actinobacteria is limited to a few sponges out of the approximately 15,000 marine species, which fails to provide a general comprehension of these bacteria regarding their diversity, distribution, and ecology, as well as for further exploitation of this reservoir of actinobacteria (Jiang et al., 2007).

The Caribbean region contains more than 300 species of sponges, but only a scarce amount of these sponges has been examined for the presence of culturable actinomycetes (Vicente et al., 2013). It has been established through a series of surveys that the Caribbean sponges of the genus *Aplysina* are among the most chemically defended species against sponge-eating fishes (Puyana et al., 2015) and the presence of actinomycetes has been reported in *Aplysina* sponges (Tabares et al., 2011). Additionally, Abdelmohsen et al. (2014) demonstrated that actinomycete abundance is significantly high in *Aplysina fistularis* and in other sponge species by comparing copy numbers of 16S rRNA genes of actinobacteria to other environmental samples. But even though these sponges are classified as HMA sponges, it is not clear whether the sponge microbiome is responsible for the production of secondary metabolites found in the sponge tissue (Puyana et al., 2015).

3. METHODS, ASSUMPTIONS, AND PROCEDURES

3.1. Methodology

This research was designed to sample bacteria associated with sponges (Figure 1) at three sites in southwestern Puerto Rico at various distance from the coast and thus with differing levels of terrigenous influence. Sampling of all three sites (in triplicates) for each of the 2 sponge species (i.e., *Aplysina fistularis* and *Aplysina fulva*) has been completed (Figure 2). Samples were collected at depth of 5 to 18 meters. Some morphological characteristics of the two species of sponges include having branching tube, tough surface, and yellow (*A. fistularis*) or yellow-green (*A. fulva*) color.

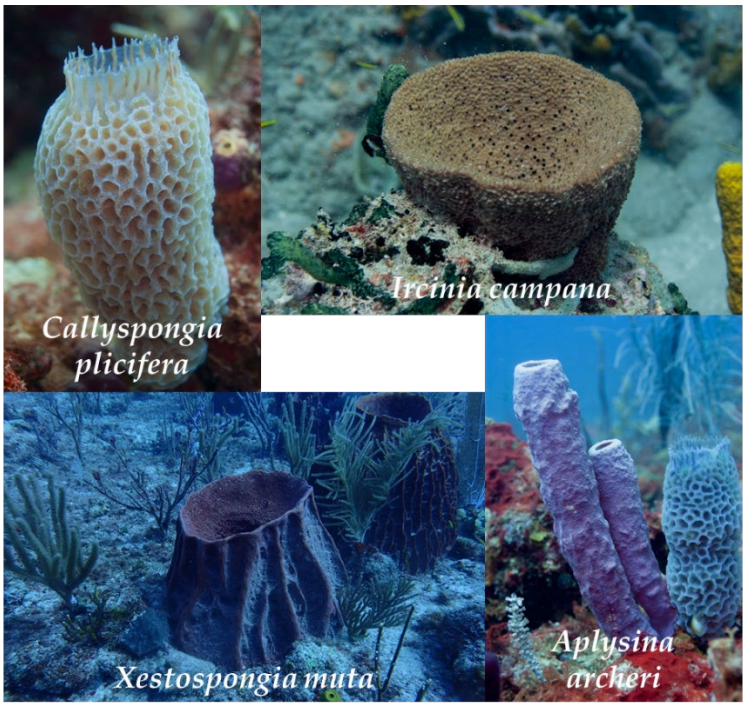


Figure 1. Photographs of sponge species from Caribbean Sea. Photos taken by Jaaziel E. Grarcia Hernandez during SCUBA diving.

Figure 2. SCUBA diving sampling of *Aplysina fistularis* and *Aplysina fulva* sponges from Caribbean Sea.

From the collected six sponge samples by SCUBA diving (Figure 2), 416 bacterial strains were isolated (203 from site 1, 134 from site 2, and 79 from site 3). To effectively grow and isolate the marine Actinobacteria species in the laboratory, a suitable growth medium that met the salinity and nutrition requirement of the isolates was developed. Below we describe the bacteria cultivation media and culturing protocols that successfully allow the isolation of pure colonies in the laboratory.

3.1.1. Protocol for preparation of International Streptomyces Project-2 (ISP-2) media

Table 1. Component list for ISP-2 Generation

	Volume of filtered natural seawater (NSW) or autoclaved artificial seawater (ASW)			
Components	***1 L***	***750 mL***	***500 mL***	***250 mL***
Yeast extract	4 g	3 g	2 g	1 g
Malt extract	10 g	7.5 g	5 g	2.5 g
Dextrose	4 g	3 g	2 g	1 g
Agar*	20 g	15 g	10 g	5 g

*When preparing agar plates.

Media preparation instructions:

1. Add all the components (except agar) in a flask. Heat (if possible) and mix the contents until everything is dissolved.
2. Adjust pH to 7.0 – 7.5. In the same flask, add the agar.
3. Autoclave (121°C / 15 minutes).
4. OPTIONAL: Place flasks in a water bath at 50° – 55°C after autoclaving to cool down.
5. Pour on plates or prepare slants. Wait until media is solidified.
6. Store at 4°C.

3.1.2. Protocol for preparation of Starch Casein Agar (SCA) media

Table 2. Component list for SCA generation

	Volume of 1:1 ratio of dH2O and NSW or ASW			
Components	***1 L***	***750 mL***	***500 mL***	***250 mL***
Soluble starch	10 g	7.5 g	5 g	2.5 g
Casein	1 g	0.75 g	0.50 g	0.25 g
Agar*	20 g	15 g	10 g	5 g

*Media must be prepared with NSW or ASW and dH2O at a 1:1 ratio (refer to the media preparation instructions).

**When preparing agar plates.

Media preparation instructions:

1. Casein is insoluble in water and will only dissolve in an alkaline environment. To prepare SCA, media must be prepared with NSW or ASW and dH_2O at a 1:1 ratio. Casein must be dissolved in dH_2O and starch and agar must be dissolved in NSW or ASW. The quantities of each component correspond to the final volume of the media. For example, to prepare 1 L of SCA, 1 g of casein will be dissolved in 500 mL of dH_2O and starch and agar will be dissolved in 500 mL of NSW or ASW.
2. Add casein to dH_2O (half the total volume of media) in a flask and add slowly drops of NaOH until pH>10. Heat (if possible) and mix the contents with a magnetic stirrer. Wait until casein dissolves completely. Adjust pH to 7.0 – 7.5.
3. Add soluble starch to filtered NSW or autoclaved ASW (half the total volume of media) in a separate flask. Heat (if possible) and mix the contents with a magnetic stirrer. Wait until starch dissolves completely. Adjust pH to 7.0 – 7.5. Add the agar.
4. Autoclave each flask (121°C / 15 minutes).
5. OPTIONAL: Place flasks in a water bath at 50° – 55°C after autoclaving to cool down.
6. Mix the contents of the flasks.
7. Pour on plates or prepare slants. Wait until media is solidified.
8. Store at 4°C.

3.1.3. Actinobacteria isolates maintenance/cultivation protocol

1. Transfer bacteria to new plates once every month.

2. Incubation: Temperature: 28° - 30°C; Time: varies (check plates every two days)
3. Storage: 4°C

Protocol develop by: Nicolle E. Lebrón López | 2021 Marine Molecular Biology Laboratory Department of Marine Sciences University of Puerto Rico – Mayagüez

4. RESULTS AND DISCUSSION

4.1. Results

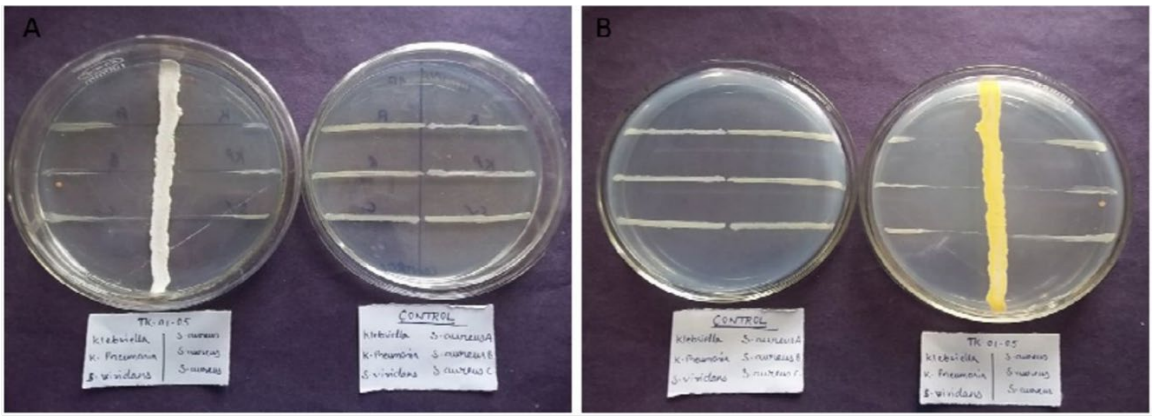
A total of 203 strains were isolated from sponge samples collected from both Aplysina fistularis and Aplysina fulva at the first site (Cayo Enrique) using the selective media previously described. Of the strains from site 1, 33 were found to be Gram-positive bacteria and were genetically analyzed. DNA sequencing of the partial 16s ribosomal RNA gene revealed that 17 (Table 1) of these 33 matched up to known Actinobacteria, while 16 isolates were potentially new species not previously described. Several of the 17 known species are known to produce antimicrobial but further characterization is needed to demonstrate what types of compounds might be produced. The 16 unknown species present an opportunity to isolate completely new classes of antimicrobial compounds. While detail characterization of antimicrobial activity and identification of compounds is expected to be performed by collaborators at AFRL in the future, we developed a "quick and dirty" assay to initially test potential antimicrobial activity.

Antagonism is a native property of certain microorganisms, which can be exhibited by them through the production of secondary metabolites. The rapid screening method of microorganisms for antagonistic activity was described by Balouiri et al. (2016). Based on the determination of antagonistic activity, secondary metabolites from such microorganisms are further isolated, characterized, and explored for the development of antibiotics. In general, antagonism through the production of secondary metabolites is the native property of filamentous organisms like actinobacteria, fungi, and cyanobacteria. Methods like crowded plate technique, cross-streak method, cross-spot method, broth dilution method, agar dilution method, and agar plug method are commonly used to determine the antimicrobial activity of actinobacteria.

The cross-streaking (Figure 3) is an easy and relatively rapid method for screening cultures in search for new antibiotics and thus establish a spectrum of inhibiting properties of any bacterium, mold, or actinobacteria, which will grow discretely on an agar plate. Studies have revealed that the 'cross streak method' resulted in higher inhibition zones on indicator bacteria than those obtained by agar well diffusion method, but the major drawback of the 'cross streak method' was difficulty in obtaining quantitative data, since the margins of the zone of inhibition were usually very fuzzy and indistinct.

Table 3: 16S rRNA gene sequences of actinobacterial strains isolated from the marine sponges *Aplysina fistularis* and *Aplysina fulva* from Site 1 (Cayo Enrique)

ID	Host	Sequence Length (bp)	Identity (%)	Closest relative and its accession number
1	*A. fulva*	509	99%	*Dietzia* sp. "Mali 159" (AY211169)
2	*A. fulva*	519	97%	*Mycolicibacterium poriferae* JCM 12603 (AP022570)
3	*A. fulva*	513	99%	*Kocuria* sp. strain APBSWPTB157 (MG733630)
5	*A. fulva*	511	99%	*Micrococcus* sp. strain zg-1185 (MN938194)
6	*A. fulva*	510	99%	*Ornithinimicrobium* sp. EQH20 (FJ999949)
12	*A. fulva*	509	99%	*Micrococcus* sp. strain GP2 (MF101699)
13	*A. fulva*	504	99%	*Aeromicrobium* sp. OTB50 (KX022841
16	*A. fulva*	506	100%	*Micrococcus* sp. SS13.30 (KC160768)
18	*A. fulva*	509	99%	*Brachybacterium* sp. 4131 (JX566597)
20	*A. fulva*	506	100%	*Cellulosimicrobium* sp. NEAU-Z02 (HM623867)
21	*A. fulva*	508	99%	*Micrococcus* sp. "Mali 39" (AY211133)
24	*A. fulva*	505	99%	*Gordonia* sp. RL-JC02 (MK787328)
28	*A. fulva*	506	99%	*Micrococcus* sp. strain zg-1185 (MN938194)
29	*A. fulva*	510	100%	*Marihabitans asiaticum* strain UMBR4119 (MH915434)
30	*A. fulva*	502	99%	*Dietzia* sp. strain PRIM-49 (MH191256)
34	*A. fistularis*	503	99%	*Streptomyces* sp. 1A01518 (EF056493)
37	*A. fistularis*	506	99%	*Gordonia* sp. VCM12 (KJ700463)



(Srivastav and Pofali, 2018)

Figure 3. Antimicrobial Screening of Actinobacteria using a Modified Cross-Streak Method (Velho-Pereira and Kamat, 2011)

5. CONCLUSIONS

A total of 203 strains were isolated from sponge samples collected from both Aplysina fistularis and Aplysina fulva at the first site (Cayo Enrique) using selective media. Furthermore, 33 of these strains were selected on the basis of Gram staining results and observed restriction fragment length polymorphism (RFLP) analysis patterns (to be able to differentiate between specimens with similar colony morphology) for further examination. Genomic DNA was extracted from each of the chosen strains and a partial segment of the 16S rRNA gene was amplified and sequenced using the universal primer F8 and R534. The BLAST analysis of the 16S rRNA gene sequences (approximately between 500 and 520 bp) of the 33 strains compared to submitted sequences in the GenBank database revealed that 17 strains in total showed homologies of 97% or greater to members of the phylum Actinobacteria (**Table 1**). However, in most cases, identification of the isolates only reached up to genus level. These strains are now available to be screened in detail for antimicrobial activity by AFRL. It is recommended that this screening should include the test microorganisms Staphylococcus aureus, Streptococcus spp., Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa. A total of 213 strains were isolated from sites 2 and 3. Genomic DNA from all 213 of these strains has been extracted and prepared for sequencing. A qualified and credentialed carrier has not been identified to ship live cultures to WPAFB but we are in conversation with AFRL to determine the best way to ship the identified isolates to AFRL.

We proposed to perform SCUBA diving expeditions to collect samples of sponges from the Caribbean Sea, develop protocols for culturing and isolation of marine Actinobacteria from sponges with the potential to produce antimicrobials (based on their genetic sequence), and produce pure isolate stocks for shipment to AFRL for further characterization of antimicrobial activity. All of previous tasks have been successfully completed.

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LIST OF ACRONYMS, ABBREVIATIONS, AND SYMBOLS

AFLP	Amplified fragment length polymorphism
ARDRA	Amplified ribosomal DNA restriction analysis
ASW	Artificial seawater
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescent *in situ* hybridization
HMA	High microbial abundance
ISP-2	International Streptomyces Project-2
NRPSs	Non-ribosomal polyketide synthetases
NSW	Natural seawater
PKSs	Polyketide synthases
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SCA	Starch Casein Agar