Microhabitats within venomous cone snails yield diverse actinobacteria

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Abstract:

Actinomycetes can be symbionts in diverse organisms, including both plants and animals. Some actinomycetes benefit their host by producing small molecule secondary metabolites; the resulting symbioses are often developmentally complex. Actinomycetes associated with three cone snails were studied. Cone snails are venomous tropical marine gastropods which have been extensively examined because of their production of peptide-based neurological toxins but for which no microbiological studies have been reported. A microhabitat approach was used in which dissected tissue from each snail was treated as an individual sample in order to explore bacteria in the tissues separately. Our results revealed a diverse, novel and highly culturable cone snail-associated actinomycete community with some isolates...
showing promising bioactivity in a neurological assay. This suggests that cone snails may represent an underexplored reservoir of novel actinomycetes of potential interest for drug discovery.

Introduction:

Interest in natural products and drug discovery has been a major driving force for the study of microbial communities associated with marine invertebrates. Sponges, which have provided more bioactive metabolites than any other marine invertebrate group (reference (4) and previous in the series), have been the main focus of these investigations, yielding numerous reports of associated bacteria and complex microbial communities (17, 44). Other examples of marine invertebrate-associated microbes explored for their involvement in natural products include bryozoans (9, 42), ascidians (38), and shipworms (47). Thus far, there are literature reports of diverse bacterial taxa involved in natural product biosynthesis in marine animals (9, 10). Notably less well studied are the symbiotic actinomycetes where the biology of host-actinomycete associations is just beginning to be explored in a methodical way. Actinomycetes are known to be important symbionts in a number of biological systems such as plants, insects and marine invertebrates, contributing as nitrogen fixers in plants (40) or utilizing their chemical arsenal for defense purposes (8, 22, 39).

An initial investigation of a cone snail yielded a surprisingly high number of actinomycetes (data not shown), prompting this follow up study on additional samples. Cone snail mollusks belong to the genus Conus, which contains about 500 closely related species (11). These mollusks are well known for their complex neurologically active venoms that they use to
immobilize their prey including fish, worms or other mollusks. The venom of cone snails is extensively studied, but to our knowledge no study of cone snail microbial communities has been reported. Cone snails are part of the larger superfamily Conoidea, comprising ~20,000 species, making it an extremely diverse group (25, 35). Given the initial observation of cultivable actinomycetes from cone snails, this large group seemed like a potentially excellent source of new bacterial natural products and new models of actinomycete symbiosis.

The goal of this study was to examine the actinobacteria community associated with tropical marine snails of the genus *Conus*, using a microhabitat approach where individual organs are treated as independent samples, and to assess the bioactivity of the isolates obtained using a neurological assay. Though similar approaches have previously been used in microbial ecology (3, 43), this is a novel approach in drug discovery for the identification of bioactive bacteria. We report here the association between three cone snails, *Conus pulicarius*, *Conus rolani*, *Conus tribblei* and their actinobacteria as well as the bioactivity of some of these actinobacteria.

**Materials and Methods**

**Collection and processing of snails**

Cone snails were collected in Cebu, Philippines, by professional collectors with appropriate permits. Typically, the water temperature in Cebu ranges between 26 ºC and 29 ºC. The three different snails, *C. pulicarius*, *C. tribblei* and *C. rolani*, were identified according to their shell patterns (Fig 1). Snails were kept in seawater overnight before being processed. Snails were dissected under sterile conditions and each tissue (body,
hepatopancreas, venom duct) was rinsed three times with sterile seawater. Body and hepatopancreas were divided in two, with one-half used for cultivation and the other-half transferred into RNAlater (Ambion) and kept frozen until DNA was extracted for molecular analysis.

Cultivation of actinobacteria

Cone snail tissue extract was obtained by grinding ca. 5 mm$^3$ of tissue in 2 ml of sterile artificial seawater using a sterile mortar and pestle. Three different media types were used: marine agar 2216 (Difco), ISP2 (Difco) and R2A agar (Difco). ISP2 and R2A are media designed for selective growth of actinomycetes and were supplemented with a final concentration of 10 $\mu$g of nalidixic acid ml$^{-1}$, 10 $\mu$g of cycloheximide ml$^{-1}$, 25 $\mu$g nystatin ml$^{-1}$ and 2% NaCl. Serial dilutions of the extracts were plated on the different media and cultures were incubated at 30 ºC for up to 4 weeks. One bacterial colony of each morphotype was selected and subcultured until pure.

Analysis of snail-associated actinobacteria

DNA from preserved snail organs was extracted using the Puregene Tissue Kit (Qiagen) and quantified using a Nanodrop (Thermo Scientific). Bacterial isolates were grown in 50 ml of their medium of isolation and incubated for a week prior to DNA extraction using UltraClean Microbial DNA isolation kit (MoBio). The 16S rRNA genes were amplified using 100 ng/µl of DNA with PrimeStar polymerase (Takara) in a DNA Engine thermocycler (Bio-Rad). Universal bacterial 16S primers 8-27f/1492r (34, 45) were applied to pure cultures and to DNA from whole tissues. To amplify actinomycete
sequences from whole tissues, specific primers 243F (19) and AB1165R (28) were applied

to the universal primer PCR product in a nested PCR strategy. Cycling conditions for the
universal primers were a hot start at 98 °C for 5 min, 30 cycles of 98 °C for 30 s, 48 °C
for 10 s, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. The PCR product
was then gel purified on a 1% (wt/vol) agarose gel and bands of approximately 1500
bp were excised and recovered using a QIAQuick Gel Extraction Kit (Qiagen) and eluted
in a 20 µl volume. PCR amplifications using actinomycete-specific primers 243f/
AB1165R (28) were carried out under the following cycling conditions: a hot start at 98
°C for 5 min, 30 cycles of 98 °C for 30 s, 55 °C for 10 s, 72 °C for 45 s, and a final
extension step at 72 °C for 5 min. The PCR product was then gel purified on a 1%
(wt/vol) agarose gel, gel extracted using the QIAQuick Gel Extraction Kit (Qiagen), and
eluted in 20 µl of water before being used for cloning. Purified PCR products were
cloned with a TOPO-XL cloning kit (Invitrogen) according to the manufacturer’s
instructions. A total of 1017 sequences were obtained, 960 from clone library sequencing
(480 from universal primers generated libraries and 480 from actinobacteria primers
generated libraries) and 57 from isolates.

Sequencing and phylogenetic analysis

Sequencing was performed using an ABI 3730 automated sequencer (PE Applied
Biosystems, Foster City) with M13 forward and reverse primers. Partial sequences were
compared to those in the GenBank database with the Basic Local Alignment Search Tool
(BLAST) algorithm to identify known closely related sequences. Sequences were
examined for the formation of chimeras using the program CHECK_CHIMERA (6).
Sequences were manually compiled and aligned using Phydit software (5). Evolutionary trees were generated using the neighbor-joining (37), Fitch-Margoliash (14), and maximum parsimony (24) algorithms in the PHYLIP package (13). Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes and Cantor (21). The robustness of inferred tree topologies was evaluated after 1,000 bootstrap resamplings of the neighbor-joining data and only values >50% were shown.

**Rarefaction and statistical analysis**

The assemblage of 16S rRNA gene sequences in *C. pulicarius* and *C. tribblei* libraries was analyzed by rarefaction analysis using EcoSim (15) to assess the extent to which the diversity of microbial communities was represented by the library. A 99% and 97% clone-to-clone maximum identity cutoff was used for the generation of the rarefaction curves. The phylogenetic tree branches statistical analysis was done with Unifrac (http://bmf2.colorado.edu/unifrac/index.psp) (27). Using Unifrac significance algorithm, the reported P-value was ≤ 0.06 which is in the suggestive range while a P-value > 0.1 is not significant.

**Fluorescence in situ hybridization (FISH)**

Samples for FISH were fixed in 4% paraformaldehyde in artificial seawater, then stored in 70% ethanol prior to embedding and sectioning as described previously (48). Sections were hybridized with fluorescein-labeled eubacterial probe EUB338 (5’-GCTGCCTCCCGTAGGAGT-3’) (1) and a Cy-3 HG69a actinobacteria probe (5’-GCTGCCTCCCGTAGGAGT-3’).
TATAGTTACCACCGCCGT-3') (36). Nonsense fluorescein-labeled EUB338 (5'-CTCCTACGGGAGGCAGC-3') (1) was used as a negative control. Images were captured by laser scanning confocal microscopy using the FV-300 XY system (Olympus). Final images were merged and artificially colored using FV10-ASW software (Olympus).

**Extraction of cultivated isolates**

Purified actinomycetes were grown in 50 ml of ISP2 or R2A broth for 7 days at 30 ºC. For each isolate, the culture was centrifuged and the supernatant was incubated for 45 min with HP20SS resin. The resin was then washed three times with milliQ water and eluted with methanol. The methanol was dried, and the resulting extract was partitioned three times between ethyl acetate and water. The organic fraction was transferred to a clean vial and dried using a rotovap. The dried extract was resuspended in methanol and passed through a C$_{18}$ column before being dried by rotary evaporation.

**DRG assay**

Dorsal root ganglia (DRG) cells from cervical and lumbar regions were obtained from mice and used in an assay with bacterial culture extracts. DRG cells were suspended in media with additives and loaded with Fura-2 AM (Molecular Probes), a fluorescent dye used to measure intracellular calcium levels. Experiments were performed at RT (20-25 ºC) in 24-well plate format using fluorescence microscopy. Individual cells were treated as single samples, so that the individual responses of diverse neuron subtypes from the DRG could be examined. After baseline measurements, the cells were treated with KCl.
solution, then washed. After return to baseline, bacterial extracts, fractions, or pure compounds were applied. This solution was then later replaced with KCl solution. The use of KCl permits observation of direct, modulatory as well as inhibitory and excitatory effects of the extracts. Full experimental details can be found in the supplemental material section.

Results and Discussion:

Three cone snails, *C. pulicarius*, *C. rolani* and *C. tribblei*, were dissected freshly after collection and three tissue parts — body, hepatopancreas and venom duct — were used to conduct an analysis of the snail-associated actinomycete community. A total of 229 morphologically distinct bacteria were isolated from *C. rolani* (n=121), *C. pulicarius* (n=121) and *C. tribblei* (n=105). Morphologically, 96 actinomycetes were identified: 47 from *C. rolani*, 28 from *C. pulicarius* and 21 from *C. tribblei*. At the end of the isolation and purification process, 57 unique actinomycete strains remained viable (Table S1). Most isolates were recovered from the hepatopancreas (n=27) and the body (n=18) while the venom duct yielded 11 isolates. Phylogenetic analysis of the isolates using the 16S rRNA gene showed that 16 different genera (Fig. 2, S2) were present, representing 11 families. Four isolates have 97% or less maximum identity to their closest relative in GenBank. The snails had in common four actinomycete genera: *Streptomyces*, *Microbacterium*, *Gordonia* and *Brevibacterium*.

The snail-associated actinomycete community was then examined using a non-culture based approach using PCR amplification and cloning of 16S rRNA gene obtained from cone snail
tissues. An initial amplification of 16S rRNA gene using the bacterial universal primer pair 8-189F and 1492r yielded only a limited number of actinomycete sequences while the majority of the clone libraries (480 clones total) were dominated by clones closely related to common marine-invertebrate associated bacteria such as *Vibrio*, *Photobacterium* and *Staphylococcus*. By using a set of specific actinobacteria primers, 243F (19) and AB1165R (28), in a nested PCR approach, the number of actinobacteria sequences in the clone libraries (480 clones total) increased substantially and accounted for 97% of the clone library bacterial sequences. All of the actinobacterial sequences belonged to the Actinomycetales order. Relatively complete data were obtained for the body and the hepatopancreas, while more limited data were available for the venom ducts. Thus, hepatopancreas (*C. pulicarius, C. tribblei*) and body (*C. pulicarius, C. tribblei, C. rolandi*) analyses are presented here. One 96-well plate of clones was analyzed for each tissue type, resulting in 103 unique actinomycete sequences. Rarefaction analysis using 97% and 99% of identity threshold level (Fig. S1) showed that the libraries were appropriately sampled at the 97% level. At the 99% level more clones would need to be sequenced to provide a better bacterial diversity representation. The molecular analysis of the 16S rRNA gene actinobacteria clone libraries from the body of *C. pulicarius, C. rolandi* and *C. tribblei* and the hepatopancreas from *C. pulicarius* and *C. tribblei* showed representatives of 10 actinomycete families (Fig. 2, S2). The body clone libraries contain members of the *Streptomycetaceae* (*C. tribblei*), *Sporichthyaceae* (*C. rolandi*), *Microbacteriaceae* (*C. pulicarius*), *Nocardioidaceae* (*C. pulicarius*), *Pseudonocardia*ceae (*C. rolandi*), *Dietziaceae* (*C. pulicarius, C. rolandi, C. tribblei*), *Propionibacteriaceae* (*C. tribblei*), and *Corynebacteriaceae* (*C. pulicarius, C. tribblei*). The hepatopancreas libraries of *C. tribblei* and *C. pulicarius* contained members of
Streptomycetaceae (C. tribblei), Micrococcaceae (C. tribblei), Intrasporangiaceae (C. tribblei), Nocardoidaceae (C. pulicarius, C. tribblei), Dietziaceae (C. tribblei) and Corynebacteriaceae (C. pulicarius). No clone sequences were identical to those of isolated actinomycetes although there were representatives of many groups.

Although the purpose of this study was to use the microhabitat approach to obtain bioactive actinomycetes for drug discovery, a few other features were evident in the uncultivated analysis. It was observed that sequences of clones from given snails and tissues appeared to cluster according to their snail of origin. A statistical analysis of these clusters using Unifrac showed that the observed clustering is not random and is statistically suggestive \( p \leq 0.06 \). However, the sample size is not large enough to provide statistically significant results \( p \leq 0.05 \).

During the microbiological and molecular analyses of the snails that yielded over a thousand 16S rRNA gene sequences, only actinomycetes were observed and not other actinobacteria. Since the specific primers used were aimed at the actinobacteria group, this indicates that the samples are strongly dominated by the actinomycetes group.

The reasons for the association between cone snails and actinomycetes remain unclear. From the clone library phylogenetic analysis, it was observed that each snail and each tissue contain members of similar subfamilies. However, at the species level, differences are evident. These differences are statistically suggestive of true differences between the groups. It is possible the bacterial groups common to all snails represent a core of symbionts, performing similar tasks required by all host snails, while other bacterial
groups specific to their host snail, i.e. Streptomycetaceae for *C. tribblei* and Pseudonocardiaceae for *C. rolani*, have been selected to fit the biology of this host. In order to test this hypothesis, more snails should be sampled including multiple replicates of the same species.

Results obtained from cultivation compare favorably to those from other marine invertebrates such as sponges, which are often considered as microbial fermenters (18). For example, Zhang *et al.* (48) identified 63 different actinomycete morphotypes isolated from 5 different sponges, with *Streptomyces* accounting for 90% of the morphotypes. In this study, of the 96 actinomycete morphotypes identified and 57 actinomycetes purified, *Streptomyces* accounted for ca. 38% of the morphotypes.

The analysis of cone snail-associated actinomycete communities from clone libraries and cultivation yielded a total of 12 actinomycete families. This diversity rivals and/or surpasses that seen in other marine invertebrates such as sponges (46). The actinomycetes obtained are novel and, based on 16S rRNA gene identity level, include 3 new *Streptomyces* species.

**FISH of *C. pulicarius***

FISH analysis using the actinobacteria probe HGC69a and a scrambled probe as a control revealed that living actinobacteria are present in both the hepatopancreas and the body tissues (Fig. 3). In the hepatopancreas, actinobacteria are present in moderate numbers with no clear distribution pattern. In the body, actinobacteria formed dense clusters near the body wall, inside large oval cells while other bacteria could be seen in other parts of the tissue. These
large oval cells open towards the outside and are similar in shape and position to mucocytes that produce the mucus lubricating the head and foot of the mollusk (Yuri Kantor, personal communication). This analysis confirmed that actinobacteria are associated with the snail and actively dividing. Snails have been known to use mucus to cover their shells (20), and mucus has been reported to contain bioactive compounds (23), indicating one of many potential roles for these bacteria. Moreover, since actinobacterial sequences in the samples were derived solely from actinomycetales, it is probable that these identified bacteria fall within this group.

Dorsal root ganglion assay

This neurological assay consists of mouse neurons from dorsal root ganglions (DRG) (26, 29). Since the DRG contains many types of neurons, responses on many different channel and receptor types can be observed in real time by microscopy. In this particular assay, freshly obtained DRG cells are treated with a fluorophore that binds to cellular calcium. Using fluorescence microscopy, the calcium response of DRG cells to chemicals and extracts is followed in real time. The protocol is optimized to observe effects that are immediate and are highly biased toward direct interactions with ion channels, or with receptors that are closely coupled to ion channels. Using this DRG assay, we tested a total of 87 extracts from cone snail isolates (actinomycetes and non-actinomycetes) which yielded four leads, CT8 (Fig 5), CP32, CP41, and CR60. Though the active compounds remain to be elucidated, results from the DRG assay suggest that the compounds act directly on channels or receptors. The data support either a decrease in a K⁺ channel activation, and / or an increase in Na⁺ channel activation. The leads are potent molecules on which structure elucidation is ongoing and for which individual active HPLC fractions containing small bioactive molecules have
been obtained. In addition, 6 of the isolates exhibited antifungal activity during isolation. Together, these results indicate that the cone snail microhabitats are suitable sources for drug discovery studies.

Significance

The need for new bioactive compounds has lead to the pursuit of actinomycetes from novel habitats, remote tropical forests on land (2) and coral reefs or benthic sediments at sea (32). It has been increasingly common to examine animals (7, 16, 30) or plants (41) for symbiotic actinomycetes and study the biology of these relationships. The microhabitat approach can bridge both drug discovery and microbial ecology studies. By looking at individual organs and tissue types as independent samples, it is possible to simultaneously explore the natural product components while studying details of the host-symbiont relationship.

In this study, a relatively large number of novel actinomycetes were obtained using the microhabitat approach. From a drug discovery point of view, the novel actinomycete strains are attractive as they are likely to contain new compounds. Additionally, the remarkable accessibility to the snail associated actinomycete community is quite unique and will help circumvent the supply issue which so often plagues natural products. If this diversity truly scales with the 20,000 species in the Conoidean superfamily, this diversity is potentially quite large. At least some of these actinomycetes are actively living and dividing within the snail, as evidenced by FISH.
Cone snails have shown to be an unexpectedly rich and diverse reservoir for novel actinomycetes. The promising bioactivity from snail-associated actinomycetes and important rate of cultivation make cone snails animal of interest for drug discovery while their biology may make them a good model for studying symbiosis.

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References


Figure and table legends:

Fig 1. Radial cladogram showing the evolutionary relationships between C. rolani, C. pulicarius and C. tribblei (12, 31, 33). The tree was generated using mitochondrial 16S sequences from the Olivera database. This figure shows that snails used in this study occupy distinct branches of the Conus radiation.

Fig 2. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences from uncultivated and cultivated actinobacteria. Dots indicate cultivated isolates, while capital letters indicate sequences obtained from the body of C. pulicarius (CPB), C. rolani (CRB) and C. tribblei (CTB) and hepatopancreas of C. tribblei (CTHP) and C. pulicarius (CPHP). Colors indicate source snails: black (C. tribblei), blue (C. pulicarius) and red (C. rolani). Reference strains from GenBank are marked by an asterix. f and p indicate branches that were also found using the Fitch-Margoliash or maximum parsimony methods, respectively. The numbers at the nodes are percentages indicating the levels of bootstrap support, based on a neighbor-joining analysis of 1,000 re-sampled data sets. Only values > 50% are shown. Scale bar represents 0.01 substitutions per nucleotide position. All actinobacteria identified fall into the actinomycete group.

Fig 3. Epifluorescence micrograph section of Conus pulicarius tissue visualized by FISH. A: Body region hybridized with fluorescein labeled non-EUB338. B: Body region hybridized with fluorescein labeled EUB338 and Cy-3 labeled HGC69a. C: Hepatopancreas region hybridized with fluorescein labeled non-EUB338. D:
Hepatopancreas region hybridized with fluorescein labeled EUB338 and Cy-3 labeled HGC69a.

Fig 4. Dorsal root ganglion assay of CT8 extract. The initial KCl injection and first wash provides the control. Once the cells have return to their original state, a series of injections follow, starting with injection of the CT8 extract. In panel C, some cells respond with influx of calcium directly upon injection of the extract, while others attenuate the response to a second KCl injection (panels B and C). Other samples show no response to compound (panel A).
Fig 3.