

Comparative sequence analysis of bacterial symbionts from the marine sponges *Geodia cydonium* and *Ircinia muscarum*

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Abstract:

Marine sponges (Porifera) live in a symbiotic relationship with microorganisms, primarily bacteria. Recently, several studies indicated that sponges are the most prolific source of biologically-active compounds produced by symbiotic microorganisms rather than by the sponges themselves. In the present study we characterized the bacterial symbionts from two Demospongiae, *Ircinia muscarum* and *Geodia cydonium*. We amplified 16S rRNA by PCR, using specific bacterial-primers. The phylogenetic analysis revealed the presence of nine bacterial clones from *I. muscarum* and ten from *G. cydonium*. In particular, *I. muscarum* resulted enriched in *Bacillus* species and *G. cydonium* in *Proteobacterium* species. Since these bacteria were able to produce secondary metabolites with potential biotechnological and biopharmaceutical applications, we hypothesized that *I. muscarum* and *G. cydonium* could be considered as a "gold mine" of natural products.

Keywords: bacterial symbionts, natural products, 16S rRNA, sponges.

Background:

Marine ecosystems represent a large explored reservoir of biodiversity that clearly impose a number of constraints on the cellular processes of the microorganisms, living and surviving within them [1]. Marine sponges are one such unique environmental niche, with sponges playing host to numerically vast and phylogenetically diverse bacterial communities. In fact, sponges are thought to live in a symbiotic relationship with unicellular organisms such as prokaryotes, bacteria, primarily cyanobacteria, eukaryotes, zooxanthellae (yellow symbiotic dinomastogotes) or zoochlorellae (green symbiotic algae). Sponges are sessile filter feeders, provide an ideal habitat for microorganisms and are probably an extreme example of "infested" organisms because, unlike most other invertebrates, there are no sterile areas in a sponge.

With the world-wide increase in antibiotic resistance and the emergence of multidrug-resistant strains of bacteria, the scientific community is facing new challenges to identify and develop novel therapeutic approaches to combat the spread of infectious diseases. Recently, this has induced an increased focus on as-yet under explored environments, such as marine sponges, which are among the animal kingdom's most important producers of bioactive metabolites [2, 3]. While many of these compounds have been isolated from marine sponges themselves, there is ever increasing evidence to suggest that a proportion of them actually originate from bacterial symbionts of these marine invertebrates [2, 4, 6]. During the past two decades, marine natural product chemists have described wonderful array of pharmacologically-active metabolites from marine sponges. Recently, there have been numerous suggestions that these potential pharmaceuticals may be

produced by symbiotic microorganisms rather than by the sponges to which they have been attributed. These findings of interesting natural products in marine sponges and the indication that some of them are produced by bacteria associated with sponges but not by the sponge cells themselves prompted the suggestion that microbial symbionts play a role in the defense of their sponge. As a result, a large number of culture-dependent and culture-independent studies have been undertaken to increase our understanding of the abundance, diversity and specificity of the microbial population associated with marine sponges, with a view to identify and potentially to exploit novel bioactivities [2, 5, 6, 7, 8]. The isolation of endospore-forming bacteria from different sponge samples has been frequently reported, but their abundance seems to vary widely between sponges.

In recent years, several studies have been focused on the isolation of cytotoxic molecules from sponges having active secondary metabolites against broad spectrum of clinical and marine pathogens [9]. More in details, these authors demonstrated that bacteria isolated from sponges have been found to exhibit antibacterial activity. The secondary metabolites of host sponge *D. granulosa* have confirmed significant antimicrobial activity and inhibited the growth of clinical pathogens [9].

In this context, the 16S ribosomal RNA (rRNA) gene provides a powerful tool for the classification of microbes and their phylogenetic analysis because a large number of species occur.

In the present study we examined the composition of the microbial signature of the marine sponges *Ircinia muscarum* and *Geodia cydonium* (belonging to the class of Demospongiae) as potential source of potentially therapeutic compounds by 16S ribosomal RNA and performed a phylogenetic analysis of obtained sequences to classify them.

Methodology:

Sponge collection

The marine sponges *Ircinia muscarum* and *Geodia cydonium* analyzed in this work belong to the class of Demospongiae. *I. muscarum* samples were collected in the bay of Naples by the fishing service of our Institute at a depth of 7; *G. cydonium* was provided by the "Parco Sommerso di Baia" in Naples. Individual specimens were placed separately into plastic bags and kept in seawater basins at a temperature of 15-20°C.

Genomic DNA extraction from sponges

Genomic DNA was extracted from the internal part of the sponge body to avoid contamination of associated epibionts. Sponges were cut into small pieces and 5g of tissue were ground in liquid nitrogen and dissolved in buffer NaCl 100 mM, EDTA 50 mM pH 8. Sodium dodecyl sulfate (SDS) solution (20%) was added to a final concentration of 2% and the mixture heated to 60°C for 30 min. Proteinase K (3 h at 50°C) and RNase (3 h at 37°C) treatments were done. Nucleic acids were extracted with phenol/chloroform, and chloroform/isoamyl alcohol and after precipitation with NaAc 3M pH 5.9 and ethanol. Extracted DNA was dissolved in TE (10 mM Tris-HCl, EDTA 50 mM pH 8), checked on an ethidium bromide-stained 0.7 % agarose gel and visualized on GelDoc 2000

(Biorad) and quantized using a spectrophotometer UV/Vis Spectrometer Lambda Bio40 (Perkin Elmer).

Amplification and sequencing of prokaryotic 16S rDNA

The amplification of prokaryotic 16S rDNA on genomic DNA from both sponges was done using the universal bacterial primers 8f-798rn [9-20] and 27F-1385R that amplify approximately 800-bp and 1400-bp fragments of bacterial 16S rRNA gene sequences, respectively. A 25 ng aliquot of DNA was amplified, using 25 pmol of each primer, 10x buffer, 2 mM dNTP and 2.5 U of Taq High Fidelity PCR System (Roche) in GeneAmp PCR System 9700 (Perkin Elmer). Cycling conditions were as follows: initial denaturation at 95°C, 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, final elongation at 72°C for 10 min. The amplified fragments were purified from agarose gel using QIAquick Gel extraction kit (Qiagen) and specificity of PCR products was checked by DNA sequencing. Sequences were compared to those in databases using the Basic Local Alignment Search Tool (BLAST) algorithm (available at www.ncbi.nih.nlm.gov) to identify known sequences with a high degree of similarity. Multiple alignments of the sequences and the related trees were performed by Clustal W. The related sequence identity percentages were evaluated by FASMA tool, developed in our group [10].

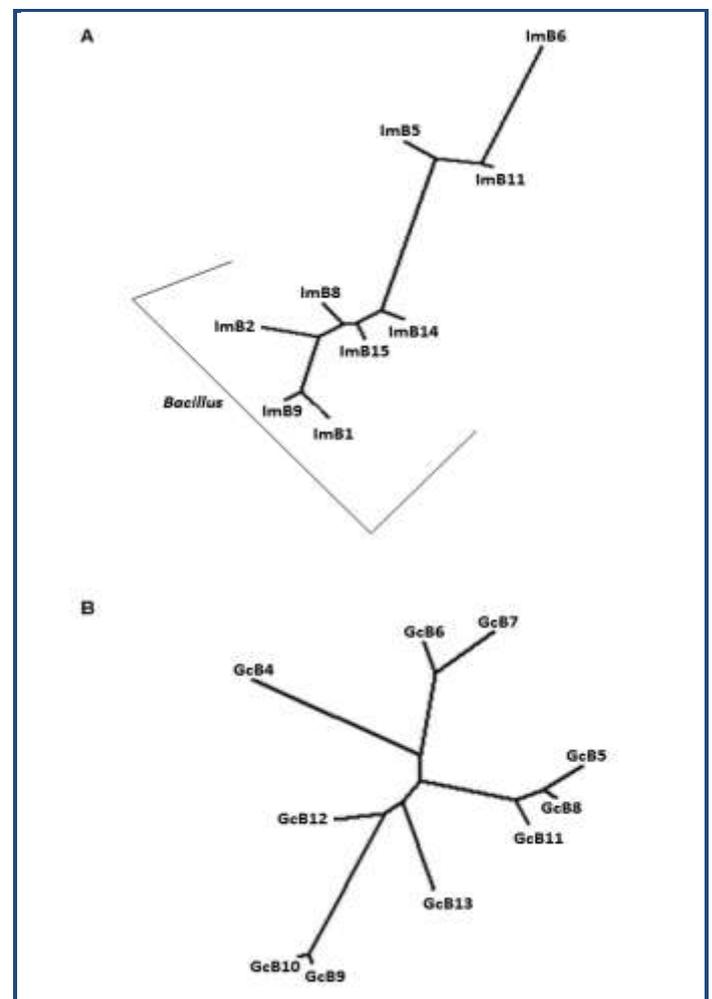


Figure 1: Phylogenetic tree obtained for the bacterial sequences from **A)** *I. muscarum* and **B)** *G. cydonium* by Clustal W.

Results & Discussion:

The bacterial population which lives associated with *I. muscarum* and *G. cydonium* were investigated. The 16S rRNA was amplified with the primers reported in Materials and Methods and sequenced. Blast similarity search revealed that a high diversity of bacterial phylotypes was present within the two sponges **Table 1 (see supplementary material)**. The sponge *I. muscarum* presented a bacterial population enriched in *Bacillus* species. In fact, we isolated from this sponge nine bacterial clones, of which six showed the highest percentage of identity with *Bacillus* species, *Bacillus hwajinpoensis* (ImB1), *Bacillus pumilus* (ImB2), *Bacillus kochii* (ImB8), *Bacillus baekryungensis* (ImB9), *Bacillus sp. CNJ828 PLO4* (ImB14) and *Bacillus sp. 4014* (ImB15); whereas the other three bacterial clones showed the highest identity with *Vibro natriegens* (ImB5), *Marinobacter sp. H-244* (ImB6) and *Uncultured pseualteromonas CI17* (ImB11). On the other hand, *G. cydonium* harbored a more diverse bacterial population, enriched in *Proteobacterium* species. In fact, this sponge revealed the presence of ten bacterial clones, of which five presented the highest identity with *Proteobacterium* species, *Uncultured gamma proteobacterium HOC2* (GcB9), *Uncultured gamma proteobacterium HOC27* (GcB10), *Alpha proteobacterium D323* (GcB5), *Alpha proteobacterium MBIC3368* (GcB11), *Alpha proteobacterium HPC9* (GcB6); whereas the other five bacterial clones showed the highest identity with *Bacillus arsenicus HLS844* (GcB7), *Bacillus hwajinpoensis XJSL2* (GcB4), *Pseudoalteromonas sp.* (GcB8), *Vibrio sp. OS53*(GcB12) and *Vibrio sp. BWDY-62* (GcB13).

All these clones for both sponges were subjected to phylogenetic analysis. The results in **Figure 1A** indicated for *I. muscarum* the presence of two clusters of which one comprised six clones within *Bacillus* (ImB1, ImB2, ImB8, ImB9, ImB14 and ImB15), and the other one comprised three clones phylogenetically closest (ImB5, ImB6 and ImB11). Concerning *G. cydonium* (see **Figure 1B**) two clones clustered within the γ -subdivision of the *Proteobacteria* (GcB9 and GcB10) and were phylogenetically closest, two clustered in *Vibrio* (GcB12 and GcB13), GcB6 and GcB7 even if belonging to different group were phylogenetically closest, as in the case of GcB5, GcB8 and GcB11. GcB4 seems to be the more distant by phylogenetic point of view.

One of surprising findings that come out of this study was the discovery of a sponge-specific, yet phylogenetically diverse, microbial community. The molecular taxonomic analysis of sponge-associated bacteria from *I. muscarum* and *G. cydonium* indicated that there was a diverse assemblage of bacteria residing within these sponges, reflecting also an adaptation to a specific ecological niche in which the two sponges live.

Sponges with enormous diversity of microorganisms have been considered an explicit source of pharmaceutical products [9]. A very interesting result of our work was the fact that the bacterial populations identified from *I. muscarum* and *G. cydonium* were able to produce secondary metabolites with potential biotechnological and biopharmaceutical applications. Phelan *et al.* [11] reported that a bank of aerobic spore-forming bacteria was isolated from the marine sponge *Haliclona simulans*, belonging to the class of Demospongiae. A large diversity of endospore-forming bacteria was distributed through a variety of *Bacillus* species, including ubiquitous

species, such as *B. subtilis*, *B. pumilus* (found by us in *I. muscarum*), *B. licheniformis* and *B. cereus* group, as well as species that are typically associated with marine habitats, such as *B. hwajinpoensis* (found by us in *I. muscarum* and in *G. cydonium*), *B. aquimaris*, *B. algicola*, *B. baekryungensis* (found by us in *I. muscarum*). These endospore-forming bacteria were able to produce proteases and antibiotics with potential biotechnological, biopharmaceutical and probiotic applications. Moreover, species of the genus *Bacillus* are renowned for either the production of chemical agents with antimicrobial properties or enzymes that are of biotechnological interest [12]. Interest in endospore-forming bacteria, particularly *Bacillus*, has seen a comeback in recent years, as spore probiotic preparations are currently being used in human therapy, animal production and aquaculture [13].

We isolated clones that clustered within *Vibrio* and *Pseudovibrio* species both in *I. muscarum* and in *G. cydonium*. Recently Esteves *et al* [14] determined the bacterial richness cultured from two irciniid sponge species, *Sarcotragus spinosulus* and *Ircinia variabilis*, and *Pseudovibrio* and *Vibrio* were classified as the most dominant genera in these two sponges. Several *Pseudovibrio* genotypes showed the presence of polyketide synthase genes. These genes represent a family of multi-domain enzymes or enzyme complexes that produce polyketides, a large class of natural products with diverse biological activities and pharmacological properties. Polyketide antibiotics, antifungals, cytostatics, anticholesteremic, antiparasitics, coccidiostats, animal growth promoters and natural insecticides are in commercial use.

Concerning *Pseudoalteromonas* bacterium found in this work in both sponges, it produces as secondary metabolite the prodigiosin, a well-known tripyrrole red pigment with immunosuppressive and anticancer activities, mainly in human breast cancer [15]. Also *Proteobacteria* species, that we found associated with *G. cydonium*, resulted widespread in the marine environment. In fact, the freshwater sponge *Geodia barretti* harbors bacteria from phylum *Proteobacteria*, corresponding to 6.9% of the microbial biomass [16]. The microbiome of the endemic marine sponge *Arenosclera brasiliensis*, a Demospongiae, was enriched for *Betaproteobacteria* and *Gammaproteobacteria*, compared with the surrounding planktonic microbial communities [17] (Trindade-Silva *et al.* 2012). Moreover, White *et al.* [18] characterized the bacterial symbiont community biodiversity of seven different individuals of the Caribbean reef sponge *Axinella corrugate*, consisting of differentially abundant classes of *Proteobacteria*.

Conclusion:

The advances in molecular biology have provided new and important diagnostic possibilities, not only for the classification of prokaryotes but also for the determination of phylogenetic relationships among animals. The gene sequences, which most commonly have been used, are 16S rRNA for the analysis of bacteria.

In conclusion, this work opens very important perspectives thanks to the world-wide increase in antibiotic resistance and the emergence of multidrug-resistant strains of bacteria. Therefore, the scientific community is facing new challenges to identify and to develop novel therapeutic approaches in order

to combat the spread of infectious diseases. Recently, this has resulted in an increased focus on as-yet under explored environments, such as marine sponges, which are among the animal kingdom's most important producers of bioactive metabolites [2, 7, 19, 20]. While many of these compounds have been isolated from marine sponges themselves, there is ever increasing evidence to suggest that a proportion of them actually originate from bacterial symbionts of these marine invertebrates [5, 6].

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Supplementary material:

Table 1: Isolated bacterial clones from the two sponges under analysis.

Bacterial isolate	Abbreviation	Highest similarity (%)	Accession number
<i>I. muscarum</i>			
<i>Bacillus hwajinpoensis</i>	ImB1	98	GQ903399
<i>Bacillus pumilus</i>	ImB2	99	JX183152
<i>Bacillus kochii</i>	ImB8	100	FR84572
<i>Bacillus baekryungensis</i>	ImB9	96	GQ903400
<i>Bacillus</i> sp. CNJ828PL04	ImB14	99	DQ448751
<i>Bacillus</i> sp. 4014	ImB15	99	JX266326
<i>Vibrio natriegens</i>	ImB5	97	FM99825
<i>Marinobacter</i> sp. H-244	ImB6	98	KF021894
Uncultured <i>pseudoalteromonas</i> CI17	ImB11	99	FJ695538
<i>G. cydonium</i>			
Uncultured <i>gamma</i> <i>proteobacterium</i> HOC2	GcB9	100	AB611839
Uncultured <i>gamma</i> <i>proteobacterium</i> HOC27	GcB10	100	AB054171
<i>Alpha proteobacterium</i> D323	GcB5	98	JX523955
<i>Alpha proteobacterium</i> MBIC3368	GcB11	98	AF218241
<i>Alpha proteobacterium</i> HPC9	GcB6	99	JX523955
<i>Bacillus arsenicus</i> HLS844	GcB7	98	FJ999563
<i>Bacillus hwajinpoensis</i> XJSL2	GcB4	98	GQ903399
<i>Pseudoalteromonas</i> sp.	GcB8	93	AF530129
<i>Vibrio</i> sp. OS53	GcB12	99	AB038028
<i>Vibrio</i> sp. BWDY-62	GcB13	100	DQ328947