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Short Communication

Isolation of Symbiotic bacteria from Sponge *Raspaciona aculeata*

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ABSTRACT

Introduction: Microbes of sponges have diverse associations, including true symbiosis. Sponges, being evolutionarily ancient sessile filter feeders, host diverse and abundant microbial species that play crucial roles in host metabolism. Although the microbial symbionts of sponges are widely distributed within the organism (up to 40% of their volume), the ecological relationships and interactions between bacteria and their sponge host remain largely unexplored for many species. The present study was one of the first attempts to isolate symbiotic bacteria from the sponge *Raspaciona aculeata.*

Materials and Methods: After isolation on marine agar medium, the isolates were characterized for different colony morphology. The 16S rDNA taxonomic analysis was carried out on bacteria isolates.

Results: Following an incubation period of two weeks at 25°C, only 13 bacterial strains were isolated with a very low rate of genetic biodiversity. All strains belonged to the Gammaproteobacteria class (Pseudomonadaceae family), except one (isolate AL-18ra) belonging to the Bacilli class (Bacillaceae family).

Conclusion: The obtained results are of great importance for advancing the understanding of symbiosis phenomena within the sponge species *Raspaciona aculeata* to study its bioapplication potential.

1. Introduction

Sponges serve as models for different microbial associations, where microorganisms from the surrounding seawater flow through their pores and channels, becoming symbiotic bacteria, pathogens, or sources of nutrition. The presence of bacteria inside sponges can be of a species-specific origin or connected to the environment surrounding. Bacteria can make up to 40% of the total biomass of these organisms and are found at densities exceeding around the 10^1 microbial cells per cm^2 of (host) tissue (approximately 3-4 orders of magnitude greater than the density of bacteria present in surrounding seawater[3]). This study was an initial endeavor to better understand the adaptability strategies of these organisms in a marine ecosystem. Specifically, bacteria associated with the sponge *Raspaciona aculeata*[4] were isolated and characterized.

At present, there has been no prior investigation into the bacterial communities associated with *R. aculeata*. In this regard, this study could represent a pioneering study aimed to advance ecological understanding by delving into the relationships relationships between bacteria and this organism (sponges).

2. Materials and Methods

2.1. Sampling

All specimens of *R. aculeata* were collected in June 2023

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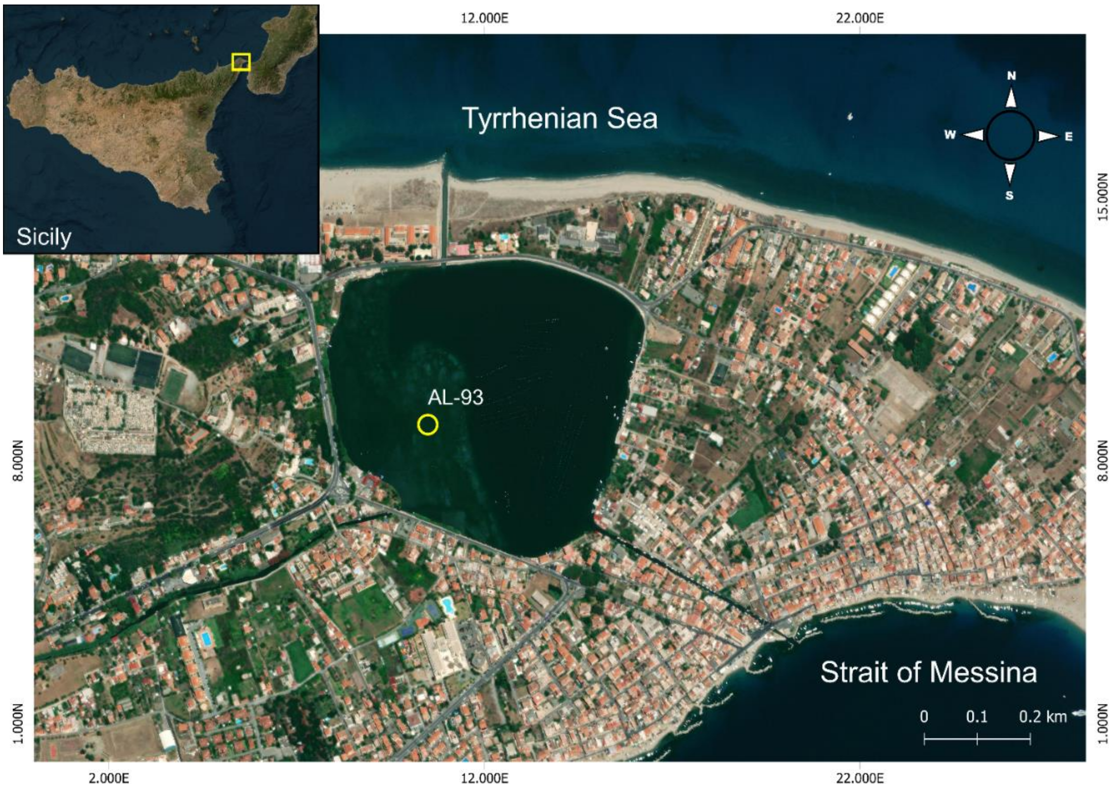

Figure 1. Location and visual appearance of Lake Faro (Messina, Sicily, Italy) and indication (yellow circle) of AL-93 station.

from Lake Faro (Messina, Sicily, Italy; Figure 1) at the station named AL-93 (Latitude: 38°16' N; Longitude: 15°38' E). Organisms were collected manually by scuba diving at a depth of approximately 3 meters in the aforementioned area. The collected samples were transported in a refrigerated box (4 ±1°C) to the IRBIM-CNR laboratories in Messina within 30 minutes.

2.2. Treatment samples

After removing foreign material (associated organisms) with the support of a stereomicroscope (Stemi SV11, Apo, Zeiss, Göttingen, Germany), organisms were washed with sterile seawater (filtered twice at 0.22 µm Millipore) and stored at 4±1°C.

2.3. Bacteria isolation

Fresh tissue samples were homogenized in sterile phosphate buffered saline (PBS 1×, pH = 7.4, Sigma-Aldrich, Milan, Italy) using a ground glass tissue grinder (Omni Tissue Homogenizer). After appropriate dilutions, aliquots of the homogenates were spread with serial dilution, onto Marine Broth plates (Difco S.p.a., Milan, Italy) and incubated at 25 ± 1°C for 7 days. After growth, morphologically distinct bacterial colonies were separated and re-plated in a fresh medium.

2.4. Molecular identification

Genomic DNA was extracted from each isolated bacterial strain using the DNeasy Blood & Tissue Kit (50), QIAGEN, Germany, following the procedures described by the manufacturer. PCR was carried out as previously described[2].

PCR products of the isolated strains were purified and sequenced (Sanger's Method) by Macrogen Inc. (Amsterdam, The Netherlands) using only the reverse primer (1492R- 50-TACGGYTACCTTGTTACGACT-30). The identification of the 16S rDNA sequences together with the construction of the phylogenetic tree were carried out as previously described[2].

3. Results and Discussion

Various approaches exist to determine the composition of microbial communities and their role in different environments. However, microbial availability for the study is constrained by growing conditions, such as artificial growth media and culture conditions, which are often not sufficient to fully recreate the true natural conditions required for microbial growth[5]. Further limitations to their growth and isolation are related to the heterogeneity of the sample matrices, the physical shape, the different viscosity due to the fat and phenol content as well as the non-uniform distribution of bacteria in the environment or samples[1]. Furthermore, in more specific cases, such as in the specific case of the symbiotic bacteria-sponge relationship, other limiting factors are represented by the physic-chemical properties of the sponge, since the interactions of the bacteria in a community as well as between bacteria and sponge can affect the requirements for microbial growth[5]. Despite significant discoveries in the past decade that have increased our knowledge of composition, host specificity, and spatio-temporal

Table 1. Closest relatives of the 16S rDNA gene sequences of bacteria isolated in this study

Code Isolate	Closest hit / Accession N°	%, ID
AL-01ra	*Stutzerimonas stutzeri* strain Atecer4D (MT386141)	99.78
AL-02ra	*Stutzerimonas stutzeri* strain OsEnb_ALM_D31 (MN889370)	99.78
AL-03ra	*Stutzerimonas stutzeri* strain JB4 (OR058633)	99.78
AL-04ra	*Stutzerimonas stutzeri* strain XH03 (KX585258)	99.46
AL-05ra	*Pseudoalteromonas carrageenovora* strain B1 (OL825027)	99.79
AL-06ra	*Pseudoalteromonas tetraodonis* strain A2 (OL825025)	99
AL-07ra	*Pseudoalteromonas tetraodonis* strain GFC (OL875284)	99.78
AL-08ra	*Pseudoalteromonas haloplanktis* strain SQA-70 (MT114591)	100
AL-09ra	*Pseudomonas knackmussii* strain SeaQual_P_B540 (MT626801)	100
AL-10ra	*Stutzerimonas stutzeri* strain 4+5-1 (PP131286)	100
AL-11ra	*Ornithinibacillus composti* strain GSS05 (NR_148296)	99.68
AL-12ra	*Stutzerimonas stutzeri* strain IRQNWYF2 (MT261835)	99.68
AL-13ra	*Stutzerimonas zhaodongensis* strain 7° (MK379595)	99.78

dynamics of microbial communities associated with sponges[6,7], there still exists a considerable gap in knowledge regarding their microbial symbionts. While more than 60 bacterial phyla have been reported to be associated with sponges[8,9], the majority of their microbial symbionts remain unidentified.

Artificial growth media and culture conditions are often not fully suited to mimic natural environmental conditions, such as the physicochemical properties of sponges and the complex interactions among bacteria within a community and between bacteria and sponge hosts, which are required for microbial growth[5].

The findings are corroborated by the fact that only thirteen bacterial strains were isolated following a period of incubation at 25°C.

The sequences of 16S cDNA of isolates were submitted to the Genetic Sequence Database at the National Center for Biotechnical Information (NCBI). As shown in Table 1, all strains belonged to the Gammaproteobacteria class (Pseudomonadaceae family), except one (isolate AL-18ra) belonging to the Bacilli class (Bacillaceae family).

Phylogenetic reconstruction was conducted using the neighbor-joining method with MEGA 4.1[10] based on multiple sequence alignments, and the bootstrap statistical value was calculated with 1.000 replicates to estimate the reliability of the tree (Figure 2).

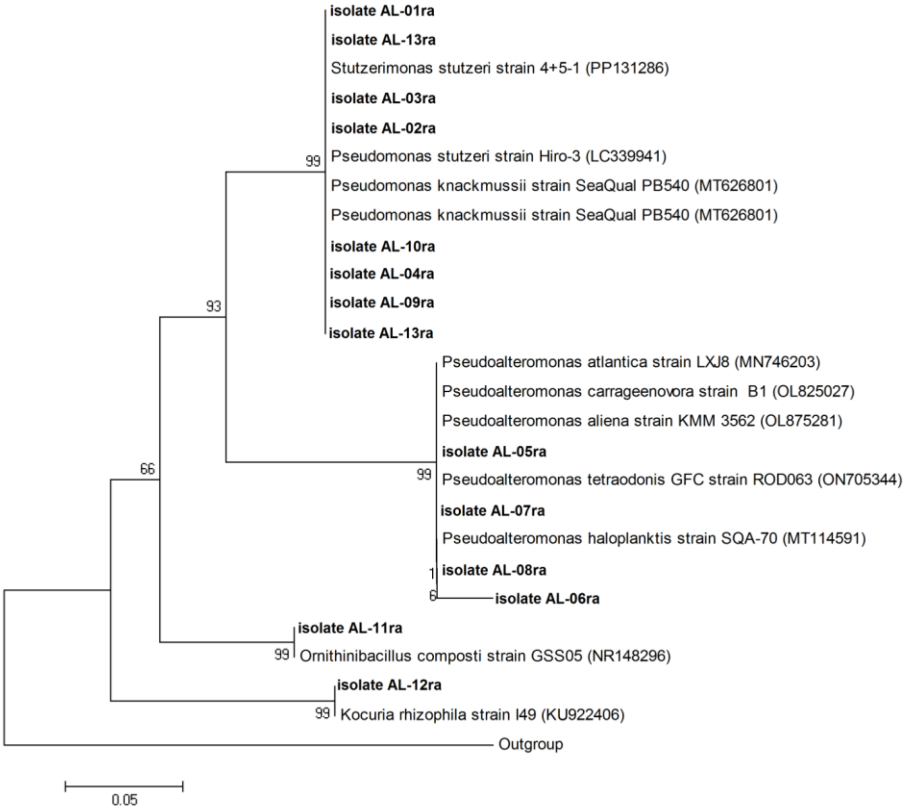

Figure 2. Rooted phylogenetic tree clustered by maximum likelihood union showing the affiliation of partial sequences of the bacterial 16S rDNA gene to closest sequences of members of different bacterial clusters. Isolates obtained in the present study are indicated in bold. The percentages of 1000 bootstrap resamples that supported branching orders in each analysis are shown at or near the relevant nodes (only 50% P values are shown). The number in parentheses indicates the number of times the gene was detected in the analysis. A sequence from an uncultured archaeon clone Methanococcus jannaschii (M59126) was used and indicated in the figure as "Outgroup".

Analyzing the bacteria related to the Pseudoalteromonas genus, the *P. atlantica* and *P. carrageenovora* strains are bacteria with agarolitic [11], associated with marine eukaryotic hosts (such as algae and crabs)[12] present in sea water[13]. The bacterium *P. tetraodonis,* isolated from the mucosa of puffer fish, produces a neurotoxin, tetradotoxin, which causes puffer fish poisoning[14]. Similarly, *P. haloplanktis* produces trypsin-like proteases that are believed to cause fish spoilage[15]. All bacteria are commonly found in the seawater column or from saline and alkaline soils and have different peculiarities, for instance, *P. aliena* has an arginine dihydrolase system[16], *P. knackmussii* can degrade 3-chlorobenzoate and is used for numerous innovative studies on the enzymology and genetics of the degradation pathway of haloaromatic compounds[17].

Of the Stutzerimonas genus, *Stutzerimonas stutzeri* also known as *P. stutzeri* has been identified[18,19] as a denitrifying bacterium widely distributed in the environment and has been isolated as an opportunistic pathogen from humans[24].

Finally, the AL-11ra strain is the only representative of the Bacilli class, specifically belonging to the Bacillaceae family. This strain shares lineage with various species, including *Ornithinibacillus composti* species present in sludge compounds[21], *O. halotolerans* halotolerant actinobacterial strain, isolated for the first time from desert soil[22] and *O. scapharcae* bacterium isolated for the first time from a dead clam ark during a mass mortality event on the southern coast of Korea[23].

The culture media used for this study led to the identification of bacteria belonging to the Pseudomonadacae family. The origin of the isolates obtained was examined in depth through bibliographic research and this contributed to a better strengthening of their finding within sponges belonging to a lagoon system with peculiar chemical-physical characteristics. It should be remembered that, in addition to the limitations of cultivability, some microorganisms exist exclusively as obligate symbionts. Therefore, functions of functional genes can be lost by isolates and instead guaranteed only by the host[24].

The development of new cultivation techniques based on the knowledge of the interactions between sponge cells and bacteria will certainly help both in the cultivability of bacteria that have not been cultivated until now and in the stimulation of the production of bioactive compounds[25].

Collected information regarding the techniques applied for the cultivation and isolation of bacteria associated with sponges provides an overview of the bacteria isolated from sponges[26]. It highlights that cultivation on an agar plate is the most widespread method used for bacteria isolation from sponges, accounting for the isolation of 89.1% of cultivable bacteria. This study also underscores that the diversity of bacteria obtained through cultivation remains much lower than that observed with cultivation-independent methods. Furthermore, it highlights that the isolation methods, culture conditions, and composition of the growth medium significantly influence the composition of the bacterial community that can be cultivated by sponges. The continuous developments of new isolation and cultivability strategies have led to the description of many new taxa in the last two decades. More bacteria have been cultivated and described in the 21st century alone than in all previous years of microbiological research[27]. Therefore, continuous research and development of new selection and growth strategies are necessary to gain better knowledge of microbial diversity, which holds significant potential in terms of phylogenetically and/or metabolically different microorganisms.

4. Conclusion

The results obtained contribute to the exploration and understanding of the biotechnological and bioapplication potential that bacteria associated with the sponge *Raspaciona aculeata* have.

Declarations

Competing interests

There was no conflict of interest.

Authors' contributions

Alessia Lunetta and Simone Cappello collected data by conducting experiments. Salvatore Giacobbe and Maria Genovese designed the study methodology and performed the literature reviews. Mehdi Hassanshahian and Sabrina Patania guided on data analysis and interpretation of results findings, literature search, and previews. All authors read, substantially revised, and approved the final manuscript.

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Availability of data and materials

All data related to the present study can be available upon reasonable requests from authors.

Ethical considerations

The authors verified the absence of plagiarism and provided their consent for the article's publication. Additionally, they conducted a thorough examination of the article to ensure there was no data fabrication, double publication, or redundancy.

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