

# Isolation and 16S rRNA characterization of culturable bacteria derived from fire coral *Millepora intricata* in Morela Coastal Waters, Maluku, Indonesia

MOCHAMAD UNTUNG KURNIA AGUNG<sup>1,2\*</sup>, NUNING KHAIRUNNISA<sup>3</sup>, SRI ASTUTY<sup>1</sup>,  
MUHAMMAD WAHYUDIN LEWARU<sup>1,2</sup>, YUNIAR MULYANI<sup>2,4</sup>

<sup>1</sup>Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia. Tel.: +62-22-87701519, Fax.: +62-22-87701518, \*email: mochamad.untung@unpad.ac.id

<sup>2</sup>Laboratory of Microbiology and Molecular Biotechnology, Faculty of Fisheries and Marine Science, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia

<sup>3</sup>Program of Marine Science, Faculty of Fisheries and Marine Science, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia

<sup>4</sup>Department of Fisheries, Faculty of Fisheries and Marine Science, Universitas Padjadjaran. Jl. Raya Bandung Sumedang Km. 21, Jatinangor, Sumedang 45363, West Java, Indonesia

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**Abstract.** Agung MUK, Khairunnisa N, Astuty S, Lewaru MW, Mulyani Y. 2021. Isolation and 16S rRNA characterization of culturable bacteria derived from fire coral *Millepora intricata* in Morela Coastal Waters, Maluku, Indonesia. *Indo Pac J Ocean Life* 5: 1-7. *Millepora*, well recognized as fire coral, has nematocytes that contain toxins as self-defense, allowing the associated bacteria to have a high level of adaptation to survive in this extreme environment. This research aims to isolate and characterize fire corals *Millepora intricata*-associated bacteria obtained from Morela coastal waters, Maluku, Indonesia using classical culture method. Sampling of *M. intricata* was carried out in August 2017 from a depth of 2-3 meters. The bacteria were grown in sea water-Nutrient Agar (NA) medium, streak plate method then used until pure isolates were obtained. Molecular identification of isolates was performed using 16S rRNA gene marker. The results indicated that nine culturable pure isolates were successfully obtained from *M. intricata*. Three isolates (MI.P1.B; MI.P2.C; and MI.P2.D2.K) then further proceed into molecular identification and sequencing. Alignment result based on the 16S rRNA databases repositored in the genbank (NCBI) showed that isolate MI P1.B has similarity to registered sequence of *Pseudoalteromonas arabiensis* strain k53 (NCBI acc no. NR\_113220.1) with a sequence identity of 98%. Isolate MI.P2.C has a close relationship (98%) to registered sequence of *Halomonas aquamarina* strain DSM 30161 (NCBI acc no. NR\_042063.1). Meanwhile, isolate MI.P2.D2.K has similarity to the registered sequence of *Halomonas zhanjiangensis* strain JSM 078169 (NCBI acc no. NR\_104283.1) with a sequence identity of 99%.

**Keywords:** Culturable bacteria, fire coral, *Millepora intricata*, 16S rRNA gene marker

## INTRODUCTION

Coral-associated bacteria play critical roles in the biogeochemical cycle, material transformation and maintaining health of coral reef ecosystem (Mahmoud and Kalendar 2016). The diversities of bacterial communities associated with corals are significantly affected by factors including species, geography and season (Hong et al. 2009; Li et al. 2014). The coral host physiology also significantly contributed to the composition of their associated bacterial communities, especially for the bacterial community that inhabits the mucus layer. During stress or when repelling predators the chemical composition of the coral mucus changes, concomitant with a shift in the composition of the mucus bacterial communities (Lee et al. 2016). Some corals secreted toxins as self-defense from predators, *Millepora*, is one of them (Veron 2000; Razak and Hoeksema 2003).

*Millepora*, a non-scleractinian coral, member of the family of Milleporidae (Suharsono 2008) have delicate hairs that protrude from the limestone skeleton called

stinging cells or *nematocytes* that contain a cocktail of toxins which is released after appropriate stimulation (Espiritu et al. 2016) and sometimes it can change the composition of their bacterial community. Bacteria surviving inhabit this extreme environment developing the ability to respond to and resist toxin threats from the host and may indicate some unique properties in their adaptation mechanisms. Indonesia has at least six species of *Millepora* namely *Millepora intricata*, *M. platyphylla*, *M. exaesa*, *M. dichotoma*, *M. tenella*, and *M. boschmai* (Suharsono 2008). Various species are spread in several regions in Indonesia, including Maluku. According to Fatkhurrochman (2017), two species of *Millepora* have been found in Morela coastal waters, Maluku, namely *M. intricata* and *M. platyphylla*.

Molecular identification based on 16S rRNA gene is powerful and widely used for decades for prokaryotic cells, due to the slow rates of evolution of this region of the gene (Woese and Fox 1977). This gene is highly conserved between different species of bacteria and archaea (Coenye and Vandamme 2003). Ribosomal RNAs are integral

elements of the protein-synthesizing apparatus, the basic components of which are present in all primary kingdoms, and are among the most highly conserved cellular molecules (Devereux and Wilkinson 2004).

A better understanding of assembly and specificity of bacterial communities surviving with *Millepora* will provide new avenues as a preliminary for further study of the adaptation mechanisms and the uniqueness of metabolites that may be produced by these surviving bacteria as the potential sources for biotechnology and pharmaceuticals purposes. Therefore, the aim of this recent research is to isolate and characterize culturable surviving *M. intricata*-associated bacteria based on 16S rRNA gene marker.

## MATERIALS AND METHODS

### Sample collection

Sampling was performed by snorkeling at 2-3 meters depths in Morela coastal waters, Maluku, Indonesia, spotted at 3°31'25.57"S (Latitude) and 128°12'53.19" E (Longitude) (Figure 1). *M. intricata* fragments (3-5 cm) were taken from coral colony carefully and preserved with 10% glycerol, then transferred to laboratory and stored at -20°C. During sampling, water temperature, salinity and pH were measured.

### Cultivation of *M. intricata*-associated bacteria

The coral skeletons were crushed by using a sterilized pestle and mortar containing 0,85% NaCl. A dilution series was performed until  $10^{-5}$  (P1),  $10^{-6}$  (P2), and  $10^{-7}$  (P3) then 100 µL suspension of each dilution was poured and flattened carefully in Nutrient Agar medium (Oxoid™)

dissolved with seawater, incubated for 1x24 hours at 30°C. Sub-culture and isolates purification was performed using streak plate method in consistent medium (Cappuccino and Welsh 2018). Morphological characteristics and cell forms of colonies were observed. Gram staining also performed to observe and distinguish particular colonies during isolates purification, based on differences in the composition and structure of cell walls (Pelczar and Chan 1986).

### Genome isolation, PCR-amplification and sequencing

Bacterial genome from selected pure isolates was extracted by using Genomic DNA Purification Kit (Promega™), according to the manufacturer's instructions and resuspended in 50 µL Nuclease Free Water (NFW), visualized with 0,8% gel agarose electrophoresis stained with GelRed (Biotum™), and stored at -20°C until required.

Primer pair 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'), especially targeting a 1.465 bp fragment of the genomic bacterial 16S rRNA gene (Lane 1991), were used for PCR amplification of DNA. The PCRs include 1X GoTaq Green Master Mix (Promega™), 10 pmol of each primer, adjusted to a final volume of 25 µL with NFW. The reaction conditions were as follows: 95°C for 120 sec; followed by 30 cycles of 95°C for 45 sec, 51°C for 60 sec, and 72°C for 60 sec; then a final extension of 72°C for 5 min. Amplified PCR products were visualized by electrophoresis on 1% agarose gel stained with GelRed (Biotum™). All PCR products were sent to 1st Base™ in Singapore to be sequenced using Sanger method.



Figure 1. Map of sample collection site (showed by a red node and arrow) at Morela Coastal Waters, Maluku, Indonesia

## RESULTS AND DISCUSSION

**BLAST and phylogenetic analysis**

DNA sequences of 16S rRNA fragments of selected isolates were proceed using BioEdit™. The resulting sequences are then identified by aligning the sequences using Basic Local Alignment Search Tool/ BLAST™, available on NCBI (www.ncbi.nlm.nih.gov), and compared with the reference sequences. Phylogenetic analysis was carried out using MEGA™ 7.0 software. All sequences were aligned using ClustalW™ program. Trees were constructed using Neighbor-joining methods and assessed with 1000 bootstrap replicates.

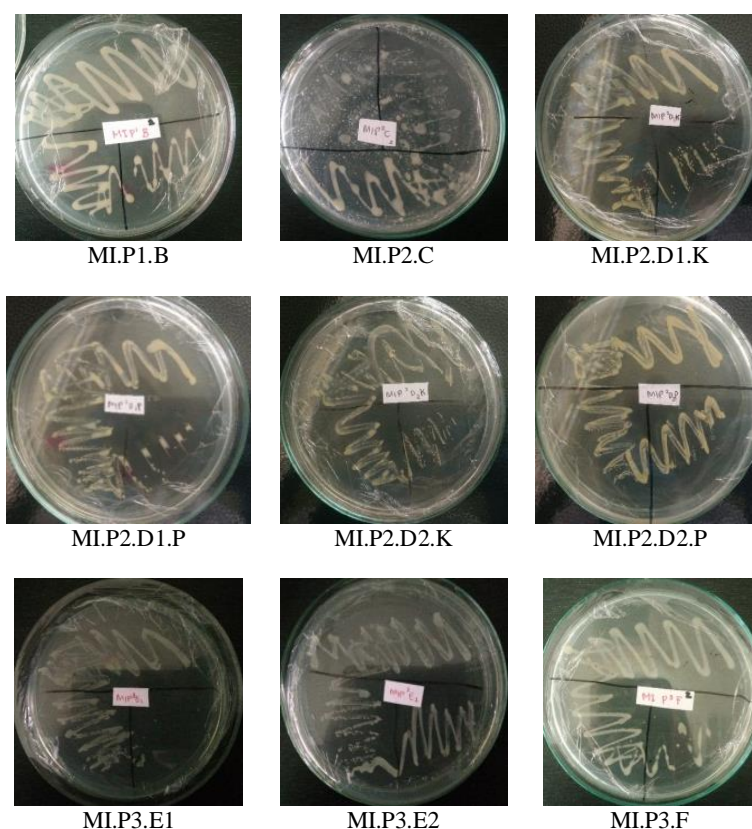
**Colony morphology and cell types of isolated *M. intricata*-associated bacteria**

Nine pure colonies were obtained from 3 dilution series (P1, P2, and P3). The majority of colonies showed similar characteristics, according to Leboffe and Perce (2012) (Table 1, Figure 2).

Pelczar and Chan (1986) stated that 95% of marine bacteria are Gram-negative bacteria. The complex structure of Gram-negative bacteria's cell wall makes this group of bacteria able to withstand extreme environmental conditions such as in the ocean environment.

**Table 1.** Morphological characteristics of colonies and cell types

Isolate name	Colony morphology			Cell type	
	Elevation	Margin	Form	Form	GRAM
MI.P1.B	Convex	Smooth, entire	Round	Rod	Negative
MI.P2.C	Raised	Irregular	Irregular	Rod	Negative
MI.P2.D1.K	Flat	Lobate	Irregular	Spherical	Negative
MI.P2.D1.P	Convex	Irregular	Irregular	Rod	Negative
MI.P2.D2.K	Flat	Smooth, entire	Irregular	Rod	Negative
MI.P2.D2.P	Convex	Irregular	Irregular	Spherical	Negative
MI.P3.E1	Convex	Smooth, entire	Round	Spherical	Negative
MI.P3.E2	Convex	Smooth, entire	Round	Rod	Negative
MI.P3.F	Convex	Smooth, entire	Irregular	Rod	Negative

**Figure 2.** Morphological appearance of isolates on Nutrient Agar (NA) media

Based on the results of Gram staining, two different forms of bacterial cell types were obtained, rod (*bacillus*) and spherical (*coccus*). The *coccus* bacteria do not have flagellum as a tool for its movement, and this group is possible to be associated with *M. intricata*. This is reinforced by the statement of Hutchings and Saenger (1987) that the *coccus* bacteria are generally bound or joined to form a solid surface, because of the mucus material so that the cells are bound together and cause the bacteria to live in algae, seaweed, seagrass, and coral reefs. Meanwhile, the *bacillus* bacteria have a flagellum so that their life defense mechanism by moving in the waters. According to Sidharta (2000), the flagellum allows bacteria to move towards favorable environmental conditions or avoid an environment that is detrimental to their lives.

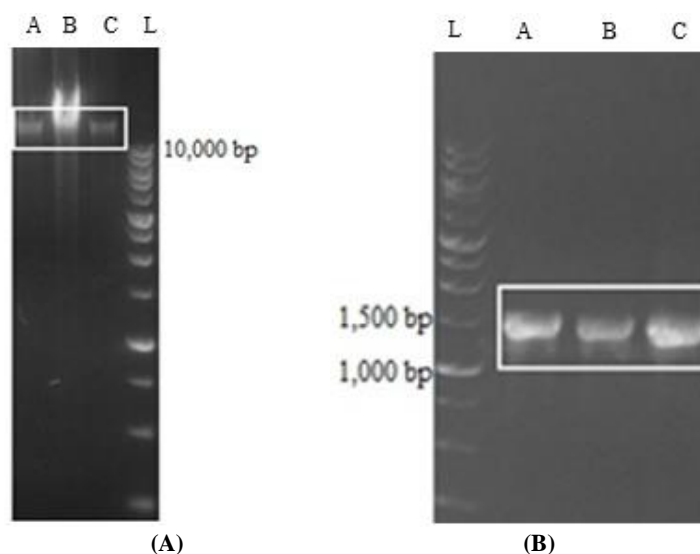
### Bacterial genome and 16S rRNA amplicon

Three of nine pure isolates were selected based on their morphological differences and were administrated for molecular identification. Three bands with size upper than 10 kb were obtained (Figure 3a). Genomes concentration was measured. And three bands spotted around 1500 bp were obtained after PCR amplification (Figure 3b) indicates successful 16S rRNA gene fragments amplification using 27F and 1492R primer pair.

According to the gel electrophoresis visualization, the genomic DNA has been successfully isolated showed by bands rose above 10.000 bp compared to DNA Ladder (Figure 2a). The thickness of bands was varied between three samples and indicated the difference in DNA concentration (Agung et al. 2020). Thus, the 16S rRNA gene amplification showed that the three isolates had a single band that was about 1.500 bp, corresponds to the expected product size of 27F and 1492R primer pair, which is  $\pm 1,465$  bp. Amplification of 16S rRNA encoding gene has become a standard for studying the phylogenetic and diversity of marine bacteria (Radjasa and Sabdono 2003). The 27F and 1492R primer pair are the most widely used primers for amplification of the 16S rRNA bacterial gene (Frank et al. 2008).

### BLAST™ analysis and phylogenetic tree construction

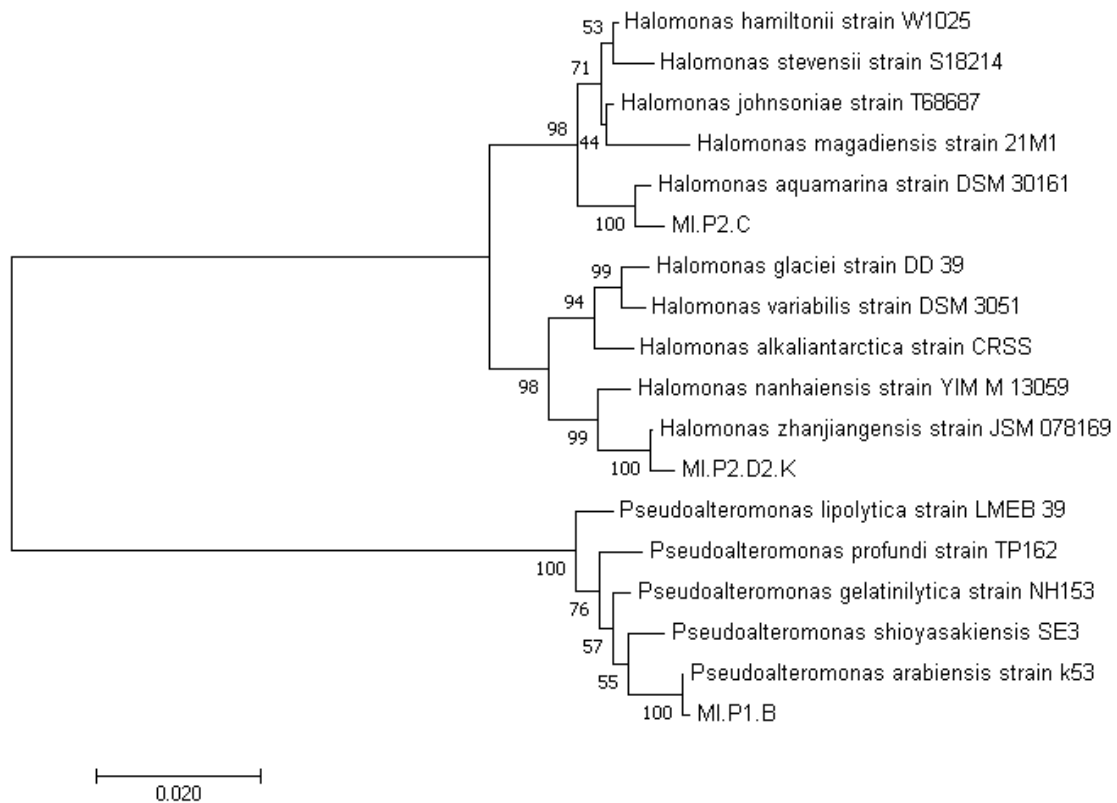
DNA sequencing results of the three selected isolates then be aligned to compare with 16S rRNA database using BLAST™ that was available in NCBI (Table 5). The result showed the high identity of isolates compared with NCBI database of 16S rRNA. And according to the results, all isolates have similarity with bacteria comes from marine environment.



**Figure 3.** Gel electrophoresis visualization of (A) genome DNA and (B) 16S rRNA gene fragments of *Millepora intricata*-associated bacteria performed in 1% agarose gel, L: 1 Kb DNA ladder (Fermentas™), A: isolate of MIP1.B, B: isolate of MIP2.C, C: isolate of MIP2.D2.K

**Table 2.** BLAST™ analysis of 16S rRNA sequences of *M. intricata*-associated bacteria

Isolate Codes	Closest taxonomically related strain	Taxonomic description (class)	Sequence identity (%)	Query cover (%)	NCBI Acc. no
MIP1.B	<i>Pseudoalteromonas arabiensis</i> strain k53	Gammaproteobacteria	98	96	NR_113220.1
MIP2.C	<i>Halomonas aquamarina</i> strain DSM 30161	Gammaproteobacteria	98	95	NR_042063.1
MIP2.D2.K	<i>Halomonas zhanjiangensis</i> strain JSM 078169	Gammaproteobacteria	99	92	NR_104283.1



**Figure 4.** Neighbor-joining tree of 16S rRNA Genes of *Millepora intricata*-associated bacteria (1000x bootstrapping)

The three isolates obtained from *M. intricata* have belonged to the same class of Gammaproteobacteria. Some genus belongs to Gammaproteobacteria have been reported to associate with a large diversity of marine organisms, including cnidarians, poriferans, mollusks, annelids, tunicates, and fish (Morrow et al. 2012). Phylogenetic analysis aims to properly reconstruct the relationship between organisms and estimate differences that occur from one ancestor to their offspring (Li et al. 1996).

According to the phylogenetic tree above, isolate ML.P1.B has a very close relationship with *Pseudoalteromonas arabiensis* strain k53. This is indicated by the bootstrap value between the two sequences which is high at 100%. The percentage of 100% bootstrap means that of the 1,000 times the construction of this phylogenetic tree, the sequence ML.P1.B has a 100% genetic relationship with *P. arabiensis* strain k53. According to Hillis and Bull (1993) bootstrap values  $\geq 70\%$  indicate the possibility of  $\geq 95\%$  of these organisms have a high level of genetic relationship and can be trusted.

Bacterial species *P. arabiensis* strain k53 was first isolated by Matsuyama et al. (2013) from sediments in the Arabian Sea using a culture method. This species grows at temperatures around 6-35°C with optimum pH 7-8. This is similar to the culture approach carried out in this research, cultivation of the sample ML.P1.B also performed at 30°C. This species is an aerobic, Gram-negative bacterium, not an endospore-forming bacterium, is motile, its cell type is bacillic, has smooth colonies, convex, circular, and entire. This species belongs to the genus of *Pseudoalteromonas*

and the class of Gammaproteobacteria. The genus *Pseudoalteromonas* is a genus consisting of aerobic, Gram-negative marine bacteria and has a flagellum to push or attract bacterial cells in a liquid medium (Madigan et al. 2015). Based on a study conducted by Atencio et al. (2018), the genus *Pseudoalteromonas* is widely distributed in the marine environment and associated with marine organisms.

The isolate ML.P2.C has a very close relationship with the *H. aquamarina* bacterial species strain DSM 30161 with a bootstrap value of 100%. Based on data on the Integrated Taxonomic Information System (ITIS), the species of *H. aquamarina* strain DSM 30161 belong to the genus *Halomonas* and the Gammaproteobacteria class. The species is an aerobic, Gram-negative bacterium isolated from seawater and can live in environments that have a wide range of salinity (euhaline) (Dobson and Franzmann 1996). According to Arahal et al. (2002), this species is a bacillic bacterium, is motile, has a flagellum around its cells, can grow at temperatures around 5-40°C with a pH between 5-9.

Meanwhile, the isolate ML.P2.D2.K has a very close relationship with the *H. zhanjiangensis* bacterial species strain JSM 078169 with a 100% bootstrap value. Based on research by Chen et al. (2009) *H. zhanjiangensis* bacteria strain JSM 078169, was first isolated from the species of sea urchin *Hemicentrotus pulcherrimus* in the tidal zone at the Naozhou Island waters, south of Zhanjiang city, China. This bacterium is a halophilic, aerobic, Gram-negative, bacillic, flat surface, circular edge, motile, has a flagellum

around the cell, and is yellow. This species can grow at a pH of about 6-10.5 (optimum 7.5) and a temperature of 4-40°C (optimum 25-30°C). Antibiotics that can inhibit the growth or kill of these bacterial species at certain concentrations include ampicillin, carbenicillin, gentamicin, nalidixic acid, polymixin, rifampicin, and streptomycin (Chen et al. 2009).

### ***Millepora intricata*-bacteria interactions**

The coral holobiont is comprised of the coral animal and its associated microorganisms consisting of bacteria, archaea, fungi, viruses, and protists including the dinoflagellate algae *Symbiodinium* (Rohwer et al. 2002). However, the interactions between coral and bacteria have not been well studied because the bacterial assemblages on corals are complex and dynamic (Ritchie 2012). Research to date indicates that bacterial associates of corals may have critical roles in protecting the host (Rosenberg et al. 2007), supporting its growth, including fixation and passage to the coral host of nitrogen and carbon (Kimes et al. 2010) and stabilizing the coral holobiont as a whole (Zang et al. 2015).

Genus of *Millepora* has been reported secreting a protein toxin called Milleporin (Radwan and Aboul-dahab 2005) and may be used as chemical defenses against fouling and potentially pathogenic microorganisms. Since host metabolites are a strong selective force for controlling its microbiome composition (Foster et al. 2017), bacteria use a range of appendages to attach to a surface that can mediate host specificity (Klemm and Schembri 2000). According to this recent study, *P. arabiensis*, *H. aquamarina*, and *H. zhanjiangensis* were successfully isolated from *M. intricata* based on classical culture method. These three isolates may have developed special mechanisms for both interacting with the host and contributing some beneficial roles to support the host survival.

*Pseudoalteromonas arabiensis* has significant roles in reducing nitrate to nitrite and producing various types of enzymes including trypsin (Matsuyama et al. 2013). *P. arabiensis* which is isolated from the tissues and layers of the Octocoralia mucus is capable of producing exopolysaccharide (EPS), a bioactive molecule that acts as an antifungal. These bioactive molecules make this bacteria able to help corals against pathogenic invasions and be resistant to disease infections (Atencio et al. 2018).

*Halomonas aquamarina* also plays an important function to reduce nitrate in the marine environment and produce urease (Mata et al. 2002). Suantika et al. (2013) reported that *H. aquamarina* is able to inhibit pathogenic bacteria *Vibrio harveyi* in white shrimp intestine, by producing exoenzyme and organic compounds and release it to the intestine mucus as defensive agents. *H. aquamarina* can also secrete bacteriocin to inhibit the growth of other pathogenic bacteria. This bacterial species can produce *aquachelin siderophore* molecules which are Fe<sup>3+</sup> complex compounds or specific iron chelating, which are useful for hiding iron elements in the environment so that the iron is not available for the growth of pathogenic bacteria.

The discovery of compounds or mechanisms carried out by Genus of *Halomonas* to inhibit pathogenic bacterial growth, indicates that *H. aquamarina* and *H. zhanjiangensis* successfully adapt and survive in an environment filled with pathogens or toxins, such as in the *M. intricata* holobiont.

Finally, a detailed understanding of the mechanism and functional role of bacteria in the *M. intricata* holobiont and their ecological role would be valuable both for the management of coral reefs and for the biotechnological prospects for the development of pharmacological bioactive compounds.

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