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PHYLOGENETIC IDENTIFICATION OF BACTERIA WITH ANTIMICROBIAL ACTIVITIES OF TUNICATE *Ascidia ornata* FROM DORERI BAY

Mogea, R.A.[1*], Tururaja, T. S.[2], Leatemia, S.P.O.[2], Salosa, Y.Y.[1]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Papua University, Jalan Gunung Salju Amban, Manokwari Papua Barat 98314 Indonesia. [2] Faculty of Fisheris and Marine Sciences, Papua University, Jalan Gunung Salju Amban Manokwari Papua Barat 98314 Indonesia.
E. mail: *r.mogea@unipa.ac.id

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ABSTRACT.
We investigated the interaction between tunicate *Ascidia ornata* and microorganism. This microorganism can be interpreted as a source of food or symbionts for mutualism. Symbiosis of microorganism with tunicate may produce metabolites that have biological activity like antimicrobial. *Ascidia ornate* were collected from hard coral at Lemon Island Doreri Bay Manokwari. Bacteria isolate of tunicate *Ascidia ornata*showed antimicrobial activities against Gram-negative, Gram-positive and the fungus *Candida albicans*. Eight isolates which can have antimicrobial activity like *Bacillus cereus*, *Bacillus* sp, *Bacillus megaterium, Bacillus pumilus*, *Enterobacter* sp, *Enterobacter hormaechei* and *Ochrobactrum* sp. The isolate was identified with 16S rDNA sequencing with 99 – 100 % sequence similarities. *Bacillus* species was identified can against all human pathogenic human isolates including methicillin-resistent *Staphyloccocus aureus*. The marine symbionts bacteria collected has potential to inhibit human pathogenic microbes and could be used as raw material for medicine.

Keywords: Antimicrobial activities, Doreri Bay, Symbionts bacteria, tunicate, 16SrDNA

INTRODUCTION

The discovery of new antimicrobial compound is very important for the development of new drugs. Some diseases from microbial infection are very difficult to cure because they resist to antimicrobe. Therefore, it is important to find new antimicrobes from microorganism which can control pathogenic microbes. Bioactive compound from microorganism is seen to be a source of new antimicrobe compound (Mantaser and Luesch, 2011; Pangestuti and Arifin, 2018).

In Indonesia, infections are one of the causes of death and treatment for infectious diseases is carried out using antibiotics. Until now, Indonesia has not been fully able to find the needs of pharmaceutical raw materials for the pharmaceutical industry, at approximately 96% of antibiotic raw materials required are imported from China and India. Infections can occur in the community or in the hospital. The most common treatment of infections is antibiotic therapy, many are microbes resistant to antibiotics. WHO in 2011 was very concerned about the development of bacterial pathogens and declared war on resistant bacteria (Ministry of Health Republic of Indonesia 2012)

There are many antimicrobial compounds produced by microorganism especially bacteria. Bacteria produces antimicrobe compounds in order to survive and protect themselves toward other microorganisms (Penesyan *et al*., 2009; Jeganathan *et al*., 2013; Sinimol *et al*., 2016;). The *Pseudo alteromonas piscicida* NJ6-3-1 has an association with sponge *Hymeniacidon perleve* to produce β-carboline alkaloid which has antimicrobial activity in a wide spectrum (Zheng *et al*., 2005), *Bacillus licheniformis* SAB1 has an association with *Halicondria* sp. Sponge producing 3-phenylpropionic acid wich also has antimicrobial activity in a wide spectrum (Devi *et al*., 2010). *Pseudo- monas aeruginosa* Pj1 has an association with *Phallusia julinea* ascidian, which produces phenazine compound that has an antimicrobial activity in the wide spectrum (Mogea *et al*., 2015a).

Bacteria have been used in commercial factory to produce bioactive compounds, especially in the pharmaceutical sector, because bacteria have fast reproduction and are easy to isolate. Bacteria can be isolated from different habitats for example from the sea. Marine bacteria have different biological activity than terrestrial bacteria, because of the uniqueness of marine environment (Subramani and Aalbersberg, 2012). Bacteria are the main sources of natural substances such as antibiotic, antitumor, antiparasitic, antiviral, herbicide, insecticide, and inhibitor enzyme (Newman and Cragg, 2010; Vignesh *et al*., 2011). It is necessary to explore the potential of local bacteria to produce antimicrobial compounds. The objective of the current research was to isolate and screen

the bacteria symbionts of tunicate from Lemon Island, Doreri Bay Manokwari-West Papua Indonesia which have antimicrobial activity. In addition the project aimed to identify bacteria symbionts of tunicate *Ascidia ornate* with molecular approaches.

MATERIALS AND METHODS

Collection of samples: Collection of sample *Ascidia ornate* tunicate was undertaken from the marine area of Lemon Island ($00^{0}53'22,8"$ LS, $134^{0}05'02,9"$BT) Doreri Bay Manokwari -West Papua (Figure 1) in 10m depth by using diving tools. By exploration of the coral reef area, sampling was under taken purposively by sterile scalpel in ± 5 cm length then put into sterile bottles for further microbiologicaly analysis at the microbiology laboratory, Faculty of Mathematic and Natural Sciences, Papua University Indonesia.

Marine bacteria isolation: *Ascidia ornata*sample was rinsed by sterile sea water to remove weak bacteria and keep bacteria with strong symbiont. Sample was cut in 1 cm small from tunic, siphon in and siphon out.Thereafter, small piece of tunicate were filledin 10 mL Phosphate Buffered Saline and were homogenized by serial mode. Petri dishes were filled with sea water medium (composition: 5g/L Pepton, 1g/L Yeast extract; 15g/L Bacto agar; 3mL/L glycerol; 750mL sterile sea waterand 250mL purified water (Mogea *et al*., 2015b). The resulting sample was spread in the petridish; and was incubated in for 48 h. Each colony of bacteria was split based on colour, size, and shape of colony. The different colonies were then purified in the sea water medium describe above.

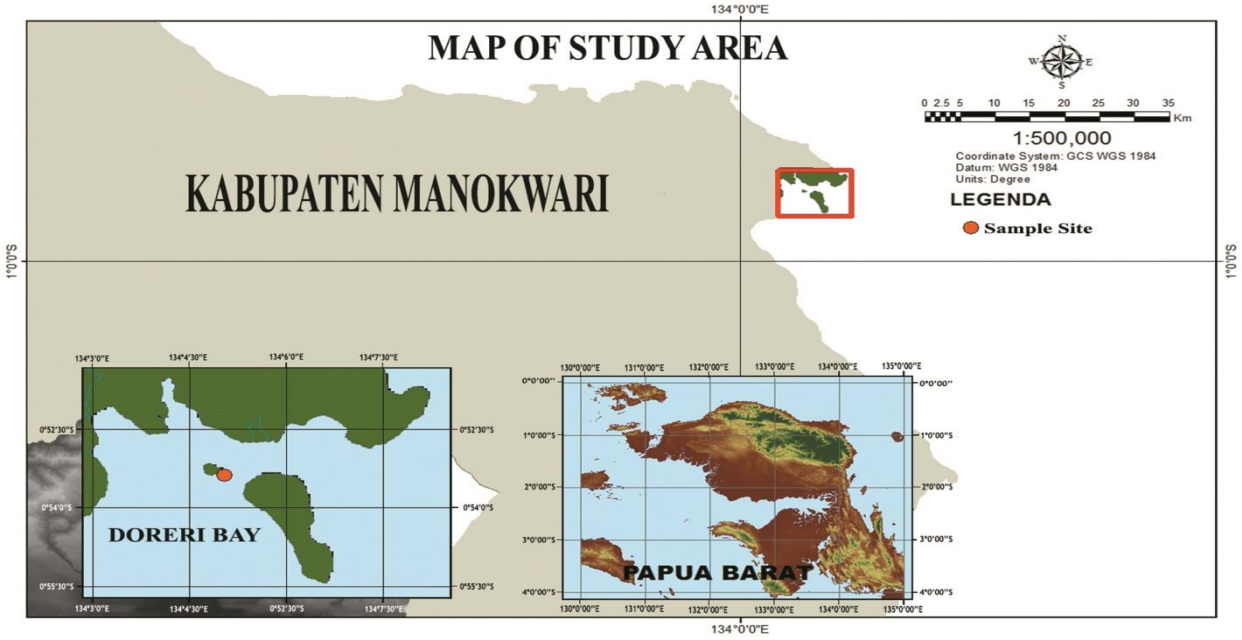

Figure 1: Sampling location for the collection of bacteria symbionts of tunicate *Ascidia ornata* from Lemon Island, Doreri Bay Manokwari-West Papua

Screening of antimicrobial bacteria: Test of antimicrobial activity toward pathogenic bacteria and fungus by qualitative method were carried out by scratching an isolate on top of medium which was spread with the microbial test. Enteropathogenic *E. coli* (EPEC), *Staphylococcus aureus*, methicillin–resistant *Staphylococcus aureus* (MRSA) and *Candida albicans* (eukaryotic fungus), were used as microbial test by overlay technique (Mogea *et al*., 2015b). The culture each bacteria symbiont was incubated for three days at room temperature. The formation of a clear zone around the purified isolate was used to assess antimicrobial activity toward bacteria and fungus. The determination of the antimicrobial activity was carried out using agar diffusion test. 100µL of the overnight cultures of the pathogenic bacteria and fungus (Nutrient broth and Yeast Potato Dextrose broth) were uniformly swabbed on the surface of the Nutrient agar plates and Potato Dextrose agar using L Rod. Several sterile paper discs (6 mm size) containing 25µL of the bacteria symbionts were added to each agar surface. The plates were then incubated at room temperature for 48 h and the zone of inhibition was recorded. Isolates that showed antimicrobial activity against pathogenic microbes were chosen for further studies.

Bacterial DNA extraction: The Aoshima method was used for the extraction. Bacterial cells were grown in liquid Luria Broth medium. 1.0mL of bacteria cell was mixed with 1 mL of SDS 20 % solution and 8 mL of eDNA buffer (100 mM/L; Tris-HCl pH 8; 100 mM/L sodium EDTA, 100m M/L sodium phosphate; 1.5mM/L NaCl; 1.0% CTAB) in the 50 mL centrifuge tube, then stirred at 1500 rpm speed for 20 min. Thereafter 1.5 mL of the suspension was put in the 2mL microtube and centrifuged in at 8000 rpm for 10min. Then 700µL of supernatant was placed into a 2.0mL microtube and 700µL of chloroform-isoamic alcohol (24:1) solution was added. The sample was stirred slowly and centrifuged at 14000rpm for 10 min. Finally, 500µL of supernatant was withdrawn then placed into 2mL microtube and 300µL of isopropanol before centrifugation at 14000rpm for 20 min. Then the supernatant was discarded and 50µL of 10mM TE buffer added to the pellet (Aoshima *et al*., 2006).

PCR amplification 16S rDNA gene sequence: Amplification of 16S rDNA was undertaken by using bacteria genomeDNA as DNA template, using fD1(AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT) as primers (Weisburg *et al*., 1991). Themixed PCR reactant was prepared as follow 3µL Tag-polymerase 10x, 1.5µL dNTPs, 1.5µL primer F, 1.5µL primer R, 3µL DNATemplate, 1.5µL DNA polymerase and 19.5µL free aquabidest nuclease. The PCR process consisted of early denaturation at 94^0C for 2 min, followed by 32 denaturation cycleat 94^0C for1 min, an annealing process at 50 ^{0}C for 1 min, extension at 72^0C for 2 min and the last extension at 72^0C for 15min. PCR products were always analysed with 1% agarose gel and the results obtained by using UVIDocHD5.

DNA sequencing and Phylogenetic Analysis: The The PCR amplification product were purified by using the QIAquick Gel Extraction Kit (Qiagen) and PCR cycle sequencing result samples were sequenced by 3730 XL DNA analyzer. The resulted DNA base result sequence was analoged with 16S rDNA database sequence at the Gen Bank using BLAST from the DNA Data Bank of Japan (DDBJ). Sequencing alignment was done by using Crustal W program and the connectivity visualized by using phylogeny treein MEGA 6software.

RESULTS AND DISCUSSION

Based on the screening of symbiont bacteria, 22 bacteria isolates were collected. Almost all of the colonies grew at the surface of the medium. On the SWC media colonies were yellow, white, and brownish white. Most colonies were circular, followed by filamentous and irregular shape. The other colonies margins were entire, convex and undulate with elevation flat, convex, raised and umbonate.

Based on antimicrobial activity tests only 8 isolate 36% from 22 bacteria symbionts tunicate have antimicrobial activity with different capacity. The choice of the best isolate was performed by using diffusion test and the result showed that *Bacillus, Ochrobactrum* and *Enterobacter* inhibited the growth of pathogenic microbial (Table 1). The antimicrobial activity different from species to species. Four species (AOT9, AOSK15, AOSM17 and AOSM18) showed against both bacteria and fungi. Four species (AOSK12, AOSK13, AOSK14 and AOSM16) just against bacteria.

Table 1: Antimicrobial activity of tunicate *Ascidia ornata* symbionts marine bacteria

Isolate code	Species	Diameters to inhibition zone (mm)			
		MRSA	*S.aureus*	EPEC	*C.albicans*
AOT9	*Bacillus cereus* BAB 6967	8,07±0,02	8,43±0,2	14,64±4,09	8,5±0,39
AOSK12	*Bacillus sp* 111582	12,49±1,07	8,12±1,18	15,3±2,43	-
AOSK13	*Ochrobactrum sp* TN1	-	-	14,35±1,19	-
AOSK14	*Bacillus sp* SPD6	13±2,32	10,9±0,7	11,64±2,5	-
AOSK15	*Bacillus megaterium* P3	9,71±0,95	8,87±0,61	15,84±1,67	7,88±0,94
AOSM16	*Enterobacter sp* TSSAS2-48	-	15,22±2,89	14,64±2,59	-
AOSK17	*Bacillus pumilus* DL006	15,14±4,5	14,04±2,18	12,83±2,29	10,95±0,25
AOSK18	*Enterobacter hormaechei* AO18	-	22,59±0,67	18,85±2,64	14,48±0,65

(-): Indicates no inhibition zone. SD with 3x measurement for each value
SD: standard deviation

By using16S rDNA, identification showed that all isolates were inculuded in the well-defined species until strains since the homolog sequence results were high in 99 to 100 %. Based on morphologicalstudy of isolates, there were 4 isolates in the *Bacillus* species (AOT9, AOSK12, AOSK14, and AOSK15), one isolate in the *Bacillus* strain (AOSM17), one isolate in the *Enterobacter* species (AOSM16), one isolate in the *Enterobacter* strain (AOSM18) and one isolate in the *Ochrobactrum* strain(AOSK13). Then, sequencing results by amplicon 16S rDNA of the 8 isolates were compared to the 16S rDNA data bank to determine the similarity value (Table 2) and phylogenetic tree (Figure 2). The comparison showed that all isolates are related to known species and strain. According to Kim *et al.,* (2014) identified with 16S rDNA sequence with 100% similarity to that bacterial strain, 98-99 % similarity is a species member and 97% similarity to that of a genus.

Tunicate or sea-squirts are sessile marine invertebrates which secrete gelatinous or leathery tunic. These animals are sessile as adults and cannot escape from their predators. Therefore, they secrete bioactive compounds which act as a chemical protection against predation. Tunicate are filter-feeding. Symbiotic bacteria in contact with tunicate potentially produce defensive compound that may help the animal to survive when in contact with pathogens. 22 bacteria from *Ascidia ornata* tunicate were collected in this research. This *Ascidia ornate* was taken from the sea water of Manokwari, West-Papua. Bacteria were taken were the presence of symbionts of *Ascidia ornata* was expected since the sample was watered by sterile sea water to select only strong symbiont bacteria. In this relation, bacteria get nutrition from vitamin, polysacarida and acid from the host. On the other hands bacteria can produces amino acid, antibiotic, and toxin which are usefull for host development and metabolism or for increasing capacity of host chemical security of (Ramanan *et al*., 2016). Also symbiotic relationship of microbe and marine organism play important role to preserve nutrition and to produce bioactive compaunds for self defence (Romanenko *et al*., 2008; Erwin *et al*., 2014).

Table 2: The identification molecular of symbiont isolates *Ascidia ornate*

Isolate code	Base pair	The closest organism	Similarity value	Access number
AOT9	1406	*Bacillus cereus* BAB-6967	99%	MF351827.1
AOSK12	1350	*Bacillus* sp. H1582	99%	JF346664.1
AOSK13	1386	*Ochrobactrum* sp. TN1	100%	KP410395.1
AOSK14	1350	*Bacillus* sp. SPD6	99%	KF878386.1
AOSK15	1424	*Bacillus megaterium* P3	99%	KX768286.1
AOSM16	1387	*Enterobacter* sp. TSSAS2-48	99%	GQ284539.1
AOSM17	1412	*Bacillus pumilus* DL006	100%	KT215541.1
AOSM18	1447	*Enterobacter hormaechei* Ao18	100%	KT215540.1

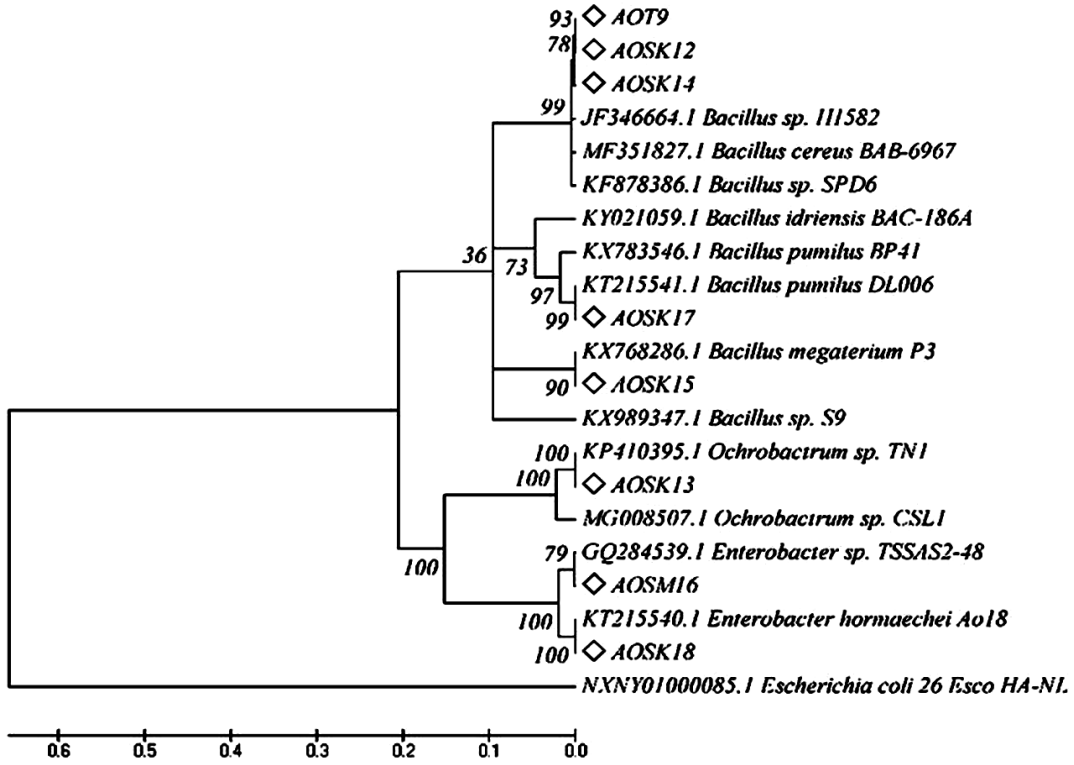

Figure 2: A phylogenetic relationship of tunicate isolates on the basis of 16S rDNA gene sequences. The branching pattern was generated by the Maximum likelihood tree constructed.

In this research, 8 bacteria 36% showing different antimicrobial activity were identified and had a wide spectrum. *Bacillus cereus*, *Bacillus megaterium*, *Bacillus pumilus* and *Enterobacter hormaechei* can inhibit Gram-negative and Gram-positive bacteria and the fungus *Candida albicans*, and *Enterobacter* sp. inhibited Gram-negative and Gram-positive bacteria. The narrow spectrum of *Ochrobactrum* sp. TN1 mode of action inhibits only Gram-negative. We presume it has an inhibitor effect for beta lactamase produced by Enteropathogenic *E. coli* (EPEC). According Al-Dulaimi (2016) antimicrobial sensitivity pattern was varied with time and region from the experiment result *E. coli* resistance to common antibiotics that is used in treatment for infection disease. It is important to find new antibiotic which can control bacterial resistance. Most of the bacteria studied produced bioactive compounds (antimicrobes and antifouling) for protecting their life (Penesyan *et al*., 2010) and to adapt their defensive mechanisms life while competing with other species (Ram anan *et al*., 2016). The antimicrobial substance biosystematic is used to bind, target coloniseand compete in order to have space and food and compete with other microorganism (Romanenko *et al., al*., 2008b). This research revealed *Bacillus* species could inhibit microbial pathogens, especially *Staphylococcus aureus*, including MRSA. *S. aureus* is the mayor's human pathogen that causes a variety of serious illnesses. Infection caused by *S. aureus* is treated with antibiotics, use of antibiotics does not exactly cause resistance. For controlling this problemthe bacteria collected in this research have opportunities to be developed as raw material for medicine and be one of the bioactive components in future. It was evidenced from the research of Mondol *et al.*, (2013) that marine Bacillus isolates have different metabolite compounds with novel mode of action. These compounds have a potential to be used as drugs, pesticides, carotenoids and tool for the bioremediation of heavy metal toxicity. The bacterial were found to produce a highly active compound like antimicrobial substance and could be used as raw material for medicine.

Conclusions

The marine symbionts bacteria collected has antimicrobial activity. This study indicated that antimicrobial activity different between species. The result of the present study shown that 8 active species were identified which four species (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus pumilus* and *Enterobacter hormaechei*) can against both pathogenic bacteria and fungi. Other, four species inhibit only bacteria. In the phylogenetic identification majority of the isolated under the species

Bacillus, *Enterobacter* and *Ochrobactrum* with 99-100% sequence similarity. Report regarding antimicrobial production and phylogenetic identification of tunicate symbiont bacteria found in the sea from Papua are very few, and they are very potential for developing as source of anti-microbial.

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