International Journal of PharmTech Research
CODEN (USA): IJPRIF, ISSN: 0974-4304
Vol.8, No.4, pp 730-736, 2015

Phylogenetic Analysis of Bacteria Associated with Ascidian*Phallusia julinea*andInhibitory Activity of Extracellular Protein Against Clinical Isolate

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Abstract: Microbial diversity can be understood through their classification system and phylogenetic identification. The phylogenetic part of ascidian-associated bacteria was used in 16S rDNA sequencing. Ascidians and bacterial interactions occur in many forms. The process of interaction between ascidians and microbes occurs through filter feeding. Bacterial symbiosis has an important role in providing energy and nutrients and inhibiting microbial pathogens. Microbes that are symbiotic with ascidians may produce metabolites that have biological activity like antimicrobial. The objective of this study is to identify bacterial isolates from ascidian based on their 16S rDNA sequencing and producing antimicrobial protein that could inhibit clinical isolates (*Candida albicans*, Enteropathogenic *E. coli* (EPEC), *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA).The methods were isolation using SCW medium and screening antimicrobial compound using double layer method, identification of isolate bassed on 16S rDNA,their extracellularproteins precipited using ammonium sulfate,split into two fraction by percent saturation range fraction 0 - 40% and 40-80%.To determine protein profile using SDS-PAGE. The result showed that isolate Pj-1 was a species *Pseudomonas aeruginosa*.The sequence of the bacteria was submitted in DDBJ, with accession number KF670598, resulting in the highest sequence similarity values of 99%. Pure fraction protein 40 - 80 % can inhibit with inhibition zone EPEC 9±0.6 mm and *C. albicans* 9±1.8 mm. Protein profile pure fraction 40 - 80 % was in range 8 – 98 kDa. *P aeruginosa* could produce antimicrobial protein are knownpyocins.
Keywords: ascidian, 16S rDNA sequencing, *Pseudomonas aeruginosa*, pyocins.

Introduction

Bacteria are ubiquitous and their interaction with other organisms could be beneficial or harmful. Majority of bacteria can contribute to the environmental balance of the ecosystem, especially for human prosperity. Examples of the useful roles of bacteria are the use of bacteria in bioremediation and bio-prospecting [1].

Interactions among organisms living in aquatic environments are very diverse. An important role is played by the interaction of microbes. Microbes are found growing on the surface of some marine organism and, some of which are contained in the digestive organs where there are often a number of bacteria[2].Ascidians are marine filter feeders with a water-filled, sac-like body structure and two tubular opening known as siphons. Microbes have symbiotic relationship with ascidians because their nutrient-rich and host-associated environments form a unique niche for microbial exploitation.

Ascidians and bacterial interactions occur in many forms. For ascidians, different microbes can be interpreted as a source of food, pathogens/parasites or symbionts for mutualism. The presence of symbionts outside the body of marine organisms is called exosymbiosis, outside the cell but inside the body called endosymbiosis, in the cytoplasm of the cell called intracellular symbiosis and in the cell nucleus called intranucleus symbiosis [3].

The process of interaction between ascidians and microbes occurs through filter feeding. Microbial epiphytes attached to the ascidians during the growth period in which microbial symbiosis have a role to maintain the stability ofgrowth and health of the ascidians. Bacterial symbiosis has an important role in providing energy and nutrients as well as inhibiting microbial pathogens thought that symbiosis of microbes with ascidians may product metabolites that have biological activity[3,4],like antimicrobial. The substance of antimicrobial is a biological or chemical compound that can distrupting the growth and the activity of microbial, especially the harmful microbial. Antimicrobial peptides are interesting candidate as a new antimicrobial agents because of their broad spectrum, toxicity is very selective, and adversity for bacteria to expand insensitive to these proteins[5].

Microbial diversity can be understood through the system of classification and identification based on phylogenetic. Characters used to identify some bacterial isolates are only limited to phenotypic characters such as morphology, physiology and biochemistry. The use of phenotypic characters as the basis for identification is feared to lead to confusion, because some of the phenotype characters are the result of interaction between the genotype and the environment. Therefore, methods are developed based on the identification of genomic information, that is comparing the sequences of DNA and RNA. The sequences are obtained through techniques based on polymerase chain reaction (PCR). This technique involves the determination of the variation of the 16S rDNA gene pattern and comparison with several enzymes that can be analyzed on prokaryotic communities found in a particular habitat. The 16S rDNA gene is a part of prokaryotic DNA found in bacteria[6,7,8].

In this paper, we report the isolation of bacteria from Doreri Gulf, Manokwari, that associated with ascidian *Phallusiajulinea* and theiridentification to the levels of genus and species through 16S rDNA sequencingand producing antimicrobial protein that could inhibit clinical isolates.

Materials and Methods

Isolation of bacteria

Ascidian *Phallusia julinea* sample was collected from the DoreriGulf, Manokwari- West Papua. Sampling site was an area of coral reef slope at a depth of 3-17 meters. Theascidian *P.julinea*, 7 cm inlength,was takenfrom a depth of10mat high tide and 2 km offshore in the waters ofManokwari. This sample was collected in September of 2012. The water salinity was 3.5%, temperature 30^{o}C, and pH 7.6. When surfaced, the samples were rinsedwithsterileseawater. For isolation the bacteria were takenfrom the entrysiphons, the out siphonsandthe center tunicusinga sterileswaband thenhomogenizedin10mlPhosphate Buffer Saline (PBS). Suspension from the homogenizationweredilutedserially inPBS. Suspension from eachdilution rateof 75 µL was pouredandleveledintothe SeaWaterComplete(SWC) medium, consisting of 5 g/l bacto peptone, 1 g/l extract yeast and 3 ml/l glycerol, 15 g/l bacto agar[9],andincubated at30^{o}Cfor 24 hours. Eachcolony of isolates waspurified byrepeatedlyscrapingthequadrantinSWCmediumtoobtainsingle pure colony to be subcultured on SWC slants. The isolated strain was identified biochemically.

Screening for antimicrobial activity

In early screening, certainty of the antimicrobial activity from pure isolates was done by the method of double layer[10] using four clinical isolate. They are*Candida albicans*, Enteropathogenic *E. coli* (EPEC), *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA). First step for this method after get pure isolate, pour medium SWC agar in Petri dish up to solidify and than pour medium SWC agar mixed of clinical isolate. Taken the pure of isolate from the subculturedof SWC slants and the scratches on medium SWC agar in Petri dish contain clinical isolate. Culture in Petri dish was incubated for 24 hours at 30 ^{o}C. The result compounds antimicrobial shown on the appear of clear zones around colonies of pure isolates. The medium of grow *C. albicans* used Potato Dextrose Agar (Merck) and the other of assay isolate using

medium SWC agar. After obtain the isolate with inhibition zone is very clear and has a spectrum of inhibition against clinical isolate was selected to assay antimicrobial protein activity.

Identification of the selected bacteria

The bacteria was identified to the species level by PCR amplification of the 16S-rDNA gene, BLAST analysis and comparison with sequences in the GenBank nucleotide database. Specifically, the 16S-rDNA used was universal forward primer fD1 and reverse primer rP2[7]. The DNA was amplified at 32 cycles of denaturation for 1 min at 95^0C, annealied at 50^0 C for 4,5 second and extended at 72^0C for 1,5 min. The final extension at which DNA extended, was 72^0 C for 5 min. The conformation of amplification was done by Agarose Gel Electrophoresis by running a 5 µl of PCR reaction mixture on 1% Agarose gel. The PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Sequence data were analyzed through comparison with 16S rDNA genes in the GenBank database. The nearest relative of the organism was obtained by BLAST searches and aligned with close relatives using the Clustal W software. A phylogenetic tree was constructed with MEGA version 5.03 using maximum likehood algorithm.

Precipitation and antimicrobial proteinactivity assay

After getting the selected of isolate then culture was incubated onmedium Luria Bertani broth (Merck)for 48 hours at the temperatur of 30 ^{o}C, incubated in shaker incubator and the agitation 120 rpm for get optimum growth.Culture isolate were centrifuged (10.000 rpm for 10 min, 4 ^{o}C). Supernatant was fractionated by ammonium sulfate, and split into two fraction by percent saturation range, fraction 0-40% and 40-80% [11]. Ammonium sulfate was added little by little until approached was saturated, and store in cooling for 25 min. Spin at 10.000 rpm for 10 min, 4 ^{o}C. Pour supernatant and resuspend precipitate with Phosphate Buffer 0.1 M pH 7. Ammonium sulfate can be removed by dialysis for one night in Phosphate Buffer Saline solution. Dialysis membranes with pore size > 5 kDa.

Pure fraction protein 40-80 % used to assay inhibitory activity against clinical isolate by disc diffusion method. Taken 100 µL clinical isolate (MRSA, *S. aureus*, EPEC dan *C. albicans*) was spread on the medium Nutrient Agar (Merck) dan PDA. Culture in a Petri dish were incubated at 30 ^{o}C for 24 hours, after the phase incubation, it was observed for the zone of inhibition and diameter was measured.

Determination of protein molecular weight by SDS-PAGE

For separating proteins based on their molecular weight by the method of Laemmli[12].Buffer for SDS-PAGE used Tris-Cl,acrylamide gels 12.5%, and dye with silver stain.The measurement of protein weight with regression analysis among protein marker and logarithm by protein marker.

Result

Isolation and Screening of antimicrobial activity

Marine bacteria were isolated from ascidian *P julinea* from the Doreri Gulf, Manokwari, using SWC agar by serial dilution spread plate method. After 24 hours incubation, obtain 24 isolate colonies were collected and only one isolated that has the ability to inhibit MRSA. The biochemical characteristictics of isolate Pj1 taken from the out siphons (Table 1).To confirm that the selected isolate can producing antimicrobial protein done next selection.

Table 1. Biochemical tests one isolated associated with ascidian *P julinea*could product antimicrobial activity

Test	Result	Test	Result
Gram	-	V-P	-
Motility	+	Citrate	+
Oxidase	+	TDA	-
Nitrate	+	Gelatin	-
Lysine	-	Malonate	+
Ornithine	-	Inositol	-

H_2S	-	Sorbitol	-
Glucose	-	Rhamnose	-
Mannitol	+	Sucrose	-
Xylose	-	Lactose	-
ONPG	-	Arabinose	+
Indole	-	Adonitol	-
Urease	+	Raffinose	-
Salicin	-	Arginine	+

Identification isolate by 16S rDNA

The 16S rDNA sequence analysis indicate that Pj1was most closely related to the member of species Pseudomonas aeruginosa P60. The highest sequence simillarity values was 99.6 % and genbank accession number was KF670598 (Figure 1). Using 1,000 bootstrap resamplings of maximum likehood data to predictions of the branches of the phylogeny topology.

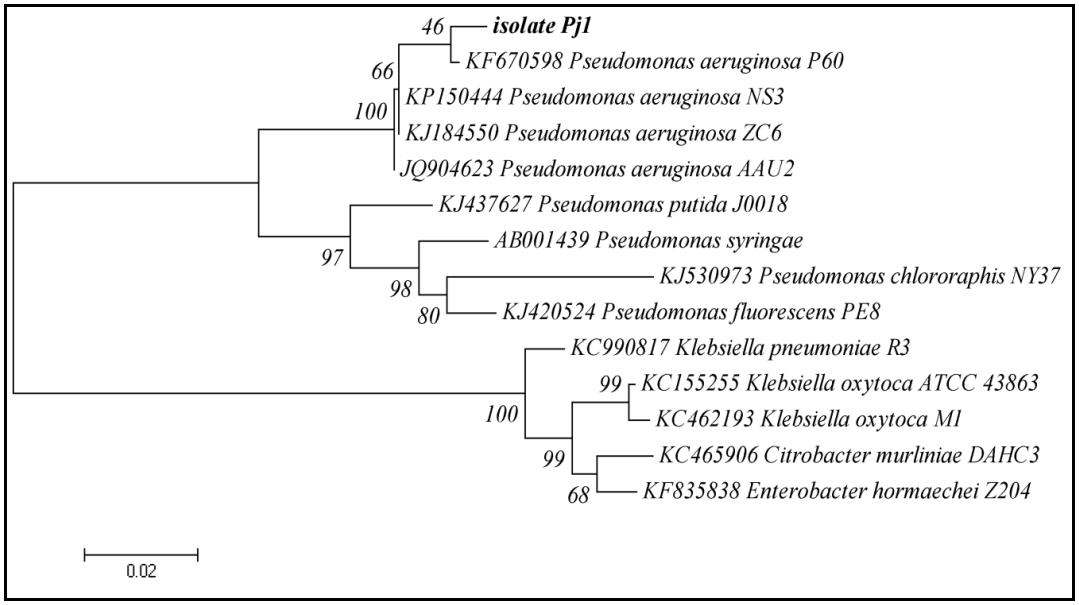

Figure1. Maximum likelihood tree constructed using the aligned partial 16S rDNA gene sequences of bacteria isolate Pj1 analysis of 1432 bp.

Antimicrobial protein activity assay

In Figure 2shows relation growth curve isolate Pj1 and production of antimicrobial compound exist on the exponential phase 18 to 58 hours in medium LB broth and decrease in antimicrobial compound production after 58 hours. For antimicrobial protein activity assay used pure fraction protein 40-80 % to inhibited the growth of clinical isolate (Table 2).

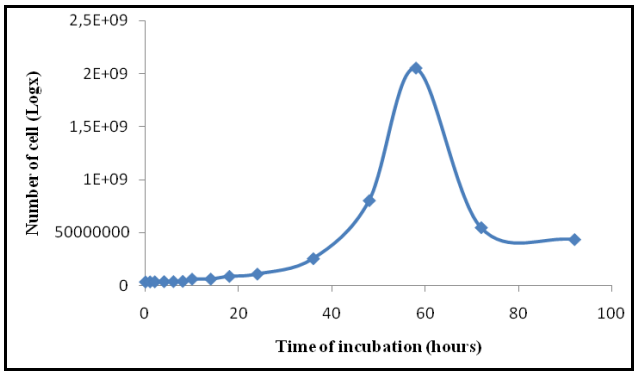

Figure 2. Profile growth curve isolate Pj1 production of antimicrobial compound

Table 2. Antimicrobial pure protein activity from isolate Pj1 inhibit clinical isolate

Clinical isolate	Inhibition zone (mm)*
MRSA	-
S. aureus	-
EPEC	9±0.6
C. albicans	9±1.8

-no inhibition zone
*** The average value and standart deviation and 3x replay**

Description Protein Analysis

Antimicrobial protein pure fraction 40-80 % based on SDS-PAGE have six bands protein with molecular weight in range 8 – 98 kDa shows in Figure 3.

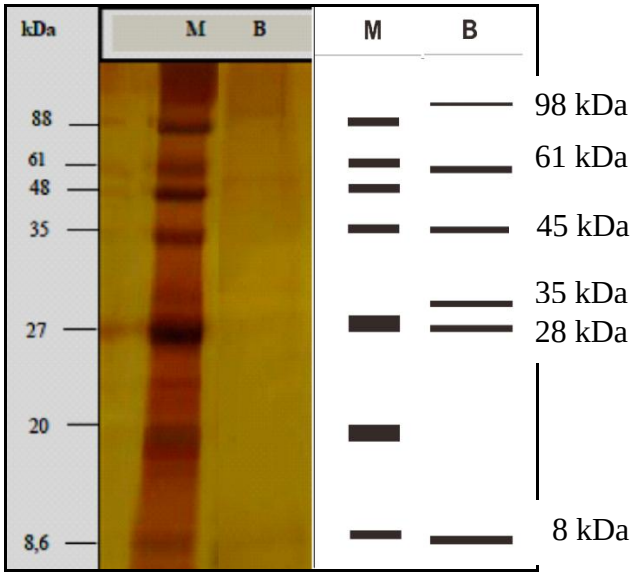

Figure 3. The analysis of SDS-PAGE from antimicrobial protein isolate Pj1

Discussion

Results of screening isolate Pj1 can inhibit MRSA with formed clear zones around colonies. IsolatePj1 can produce antimicrobial compound and type bactericidal. Intensity clear zones influenced many antimicrobial compounds get released. To identification this isolate by 16S rDNA. Amplification of 16S rDNA sequences was carried out using primer fD1 and rP2 generate amplicons of 1500 bp (data no show). The result of this amplication always produce a single band around 1500 bp that consist of highly conserved region and hyper variable region. Highly conserved region is the part that relatively fixed and not affected by changes in environment and therefore it describes the level of phylogenetic relations. Hyper variable region is the part of different nucleotide composition for each species so that it can be used to indicate the degree of kinship between prokaryotes[13].

The results of biochemical assayand identification based on 16S rDNA analysis indicated that Pj1 was most closely related to the member of species *Pseudomonas aeruginosa*. The highest sequence similarity values was 99%. The use maximum likehood phylogeny provide the best count of variation of the data sequence and suitable for sequence in small amount[14].Based on the analysis of phylogenetic, the bacteria associated with ascidians belong to the phylum Proteobacteria, class Gammaproteobacteria, Gram negative and shaped rod.*P. aeruginosa* could be found anywhere including soil, water and plants. This is bacteria has a simple nutrient needs but can grow slowly under anaerobic conditions if available NO_3 as a source of electron acceptors. The result of partial 16S rDNA sequences showed that isolate Pj1 was phylogenetically related to *P. aeruginosa* P60, *P. aeruginosa* NS3 and *P. aeruginosa* ZC6 with a similarity value of 99,6 % and the evolutionary distance of 0,004. Isolates that have 16S rDNA sequence with similarity ≥ 99 % can be grouped into bacterial species[13].

Antimicrobial compounds produced by *P.aeruginosa* consists of primary metabolite (protein) and secondary metabolite (antibiotic)[15]. Curve growth showed that the antimicrobial protein production in exponential phasefrom18 to 58 h. During the exponential phase of metabolism this bacteria the most active, the synthesis of cell material very quickly with a constant amount until nutrients run out.

To make sure *P.aeruginosa* produce antimicrobial protein,the harvest of antimicrobial protein at 48 h and precipitation by ammonium sulfate.After get pure protein by dialysis to assay inhibit clinical isolate. Pure fraction protein 40 - 80 % can inhibit with inhibition zone EPEC 9±0.6 mm and *C. albicans* 9±1.8 mm. Antimicrobial protein or peptide can produced by bacteria and known as bacteriocins, which are usually just toxic that are nearly related to the producing strain[16].*Pseudomonas* bacteriocins known as pyocins[17].

The result of SDS-PAGE show antimicrobial protein pure fraction 40-80 % have six protein bands with molecular weight in range 8 – 98 kDa. Bacteriocins of Gram-negative bacteriaare categorized into four main classes with size: colicins 20 - 80 kDa, colicins-like bacteriocins 20 – 80 kDa, phage-tail like bacteriocins > 80 kDa and microcins < 10 kDa[18]. From the research of antimicrobial protein pure fraction 40-80 % into classes microcins, colicins-like bacteriocins and phage-tail like bacteriocins.

Conclusion

In this present study isolate Pj1 was phylogenetically related to *Pseudomonas aeruginosa* species with 99 % similarity in 16S rDNA gene sequence. Antimicrobial protein pure fraction 40-80 % from *P. aeruginosa*could inhibit EPEC and *C. albicans*.

Acknowledgements

Authors are thankful to Directorate General of Higher Education, Ministry of Education and Culture, Indonesia for support of this research; and the authors would like to thank Professor M. Kubo head of Laboratory of Bioengineering and Adhikari Dinesh, Ph.D candidate at the Laboratory of Bioengineering, Faculty of Life Science, Ritsumeikan University, Japan, for the identification of this bacteria using 16S rDNA.

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