ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

Original Paper

BIOPROSPECTING OF BACTERIAL SYMBIONT OF *Tunicate Didemnum molle* FROM SAMBANGAN, KARIMUNJAWA ISLANDS

Miftahuddin Majid Khoeri [1,3], Ocky Karna Radjasa[2,3], Agus Sabdono[2], and Herawati Sudoyo[3]

[1)] Magister of Biology, Satya Wacana Christian University, Jl. Diponegoro 52-61 Salatiga 50711, Central Java, Indonesia
[2)] Faculty of Fishery and Marine Science, Diponegoro University, Semarang 50275, Central Java, Indonesia
[3)] Marine Microbiology Unit, Eijkman Institute for Molecular Biology, Jl. Diponegoro 69, Jakarta 10430, Indonesia

Received : February, 10, 2011 ; Accepted : May, 30, 2011

ABSTRACT

Coral reef is a productive ecosystem with high biodiversity in the sea and being targeted to find a useful bioactive compound. However, the serious problem in development of bioactive compounds from marine invertebrate is the supply problem, because to get a small amounts of active compounds a massive numbers of sea organisms are needed. Tunicate is an animal in coral reef ecosystem that produces many bioactive compounds with pharmacological activities, such as, antibacterial, antitumor, and anticancer compounds. It has been reported that bacterial symbionts of coral reef invertebrates may synthesize the same compounds as the host. The purposes of this research are to isolate and to identify microbes which have antibacterial activity against MDR bacteria based PCR 16S rRNA and to detect the existence of PKS and NRPS biosynthetic gene fragments from tunicate bacteria of Didemnum molle. Out of 15 bacterial isolates, one isolate showed antibacterial potential against Escherichia coli and Staphylococcus sp. Molecular identification result showed that TS2A5 bacterium has a homology of 99 % with Virgibacillus sp. strain GSP17 16S ribosomal RNA gene. This isolate was also capable of amplifying NRPS gene fragment.

Key words : Tunicate ; PCR 16S rRNA ; gen NRPS and PKS ; antibacterial ; *Escherichia coli; Staphylococcus* sp.
***Correspondence**: Tel: +62-24-7474698; Fax: +62-24-7474698; email : majid@eijkman.go.id

INTRODUCTION

Antimicrobial resistance has become a major health problem worldwide, both in hospitals and the community. The emergence of antimicrobial resistance is correlated with selective pressure from the use, often inappropriate, of antimicrobial agents and results in increased mortality, morbidity, and health care costs (Cohen, 1994; Lestari *et al*., 2007).

Utilization of antibiotics is directly or indirectly will increase the prevalence of resistance of bacterial pathogens or normal flora that will develop into a multi-drug resistant strains (MDR). The alternative for antibiotic compounds that have been resistant bacteria has become a very urgent need (Hunt and Vincent, 2006).

The ocean is a major source of organisms that produce bioactive compounds (Bernan *et al*., 1997; Proksch *et al*., 2002). Prospectors of bioactive compounds look for them in slow moving or sessile marine invertebrates that have no protective structures such as spines or shells (Flam, 1994). However, in most instances the amounts of the compounds produced by these organisms are relatively small and thus not likely to satisfy any industrial demands (Proksch *et al*., 2002).

The oceans are the source of a large group of structurally unique natural products that are mainly accumulated in invertebrates such as sponges, tunicates, bryozoans, and molluscs. Several of these compounds such as the tunicate metabolite ET-743, a potent anti-cancer agent, is obtained from *Ecteinescidia turbinata*, a tunicate from the Caribbean sea (Rinehart *et al*., 1990) show pronounced pharmacological activities and are interesting

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

candidates for new drugs primarily in the area of cancer treatment (Proksch *et al*., 2002).

Recent evidence shows that at least some of the bioactive compounds isolated from invertebrates are of microbial origin (Davidson *et al*., 2001; Lim and Haygood, 2004; Schmidt *et al*., 2005). Since bacteria are known to produce metabolites similar to ET-743, isolation and identification of the putative metabolite-producing bacteria associated with *E. turbinata* may reveal a new approach for the production of this compound (Proksch *et al*., 2002).

In this work, we report the potential of marine bacterium associated with tunicate *Didemnum molle* for the production of secondary metabolites against pathogenic *Staphylococcus* sp. and *E. coli* coupled with PCR based-screening for the presence of non-ribosomal polypeptide synthetases.

MATERIALS AND METHODS

Collection of samples and bacterial isolation

Colonies of tunicate *Didemnum molle* were collected from Sambangan waters, Karimunjawa islands, North Java Sea, Indonesia by scuba diving from a depth of approximately 2 meter. Upon collection colonies were put into sterile plastic bags (Whirl-Pak, Nasco, USA) and put into cool-box. The tissues were then rinsed with sterile seawater and cut with a sterile knife. The resultant tissues were serially diluted, spread on ½ strength ZoBell 2216E marine agar medium and incubated at room temperature for 2x24 hours. On the basis of morphological features, colonies were randomly picked and purified by making streak plates (Madigan *et al*., 2000).

Inhibitory interaction test

Inhibitory interaction test of bacterial isolates against pathogenic *Staphylococcus* sp. and *E. coli*, was performed by using the agar disk diffusion method. One hundreed microliter culture of target microorganism in the logaritmic phase (ca.10^9 cells mL^{-1}) was spread on to agar medium. (Paper disks 8 mm; Advantec, Toyo Roshi, Ltd, Japan) containing 25 µl of the tunicate bacterial strain were placed on the respective agar surface. The plates were then incubated at room temperature for 48 h. Antibacterial activity was defined according to Radjasa *et al*., (2007a) by the formation of inhibition zones greater than 9 mm around the paper disk.

The following MDR strain bacteria *Staphylococcus* sp. and *E. coli*, obtained from Dr. Kariadi Hospital in Semarang, Central Java, Indonesia as described Lestari *et al*., (2007) were used as a tested strains.

Polymerase Chain Reaction 16S rRNA

Genomic DNA of strains for PCR analysis were obtained from cell materials taken from an agar plate, suspended in sterile water (Sigma, Germany) and subjected to five cycles of freeze (-80^{o}C) and thaw (95^{o}C). PCR amplification or partial 16S rRNA gene of tunicate associated bacteria and subsequent sequencing analysis were performed according to method of Radjasa *et al*., (2007b). The determined DNA sequences of strains were then compared for homology to the BLAST database.

PCR based screening of NRPS producing bacterial strains

Non-ribosomal peptide synthetases (NRPS) primers were prepared and amplification of peptide synthetase gene fragments was carried out with the NRPS degenerated primers as described (Radjasa *et al.*, 2007a). PCR amplification of partial 16S rRNA gene of marine bacterium TS2A5, purification of PCR products and subsequent sequencing analysis were performed according to the methods of Thiel and Imhoff (2003), respectively. The determined DNA sequences of strains were then compared for homology to the BLAST database.

RESULTS AND DISCUSSION

Screening among 15 marine bacteria associated with tunicate *Didemnum molle* by using test organism revealed that only one isolate, TS2A5 capable of inhibiting the growth of *Staphylococcus* sp. *and E. coli*, while the rest of isolates showed no activity. The measurement of inhibition zone as indicator of the antibacterial potential of isolate TS2A5 against pathogenic bacterium is presented in **Table 1.**

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

As shown in the F**ig. 1**, the isolate was also capable of amplifying the gene fragments of Non-ribosomal peptide synthetases (NRPS) indicated by the presence of single band having similar height to the positive control of *Pseudomonas fluorescens* DSM 50117.

Table 1. Antibacterial activity of isolate TS2A5 against pathogenic *Staphylococcus* sp. and *Escherichia coli*

Isolate	Tested pathogen	Inhibition Zone (cm)	Tested pathogen	Inhibition Zone (cm)
TS2A5	*Escherichia coli*	1.05 ± 0.87	*Staphylococcus* sp.	1.79 ± 0.534

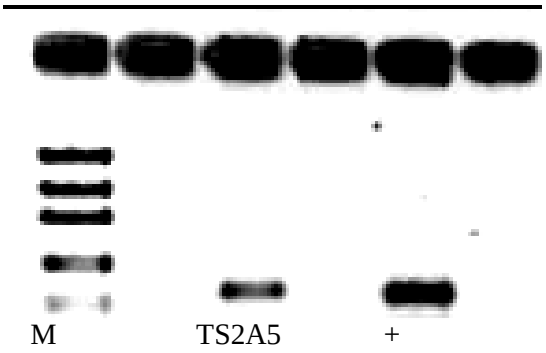

Fig.1. PCR amplification of NRPS gene fragments; + control *Pseudomonas fluorescens* DSM No. 50117; M is DNA markers

Molecular identification of actives isolates of marine bacteria associated with tunicate based on 16S rRNA, revealed that active strain TS2A5 was closely related to *Virgibacillus* sp. (**Table 2**). Phylogenetic tree shown in **Fig.2.** shows the phylogenetic affiliation of bacterial isolate with other microorganisms.

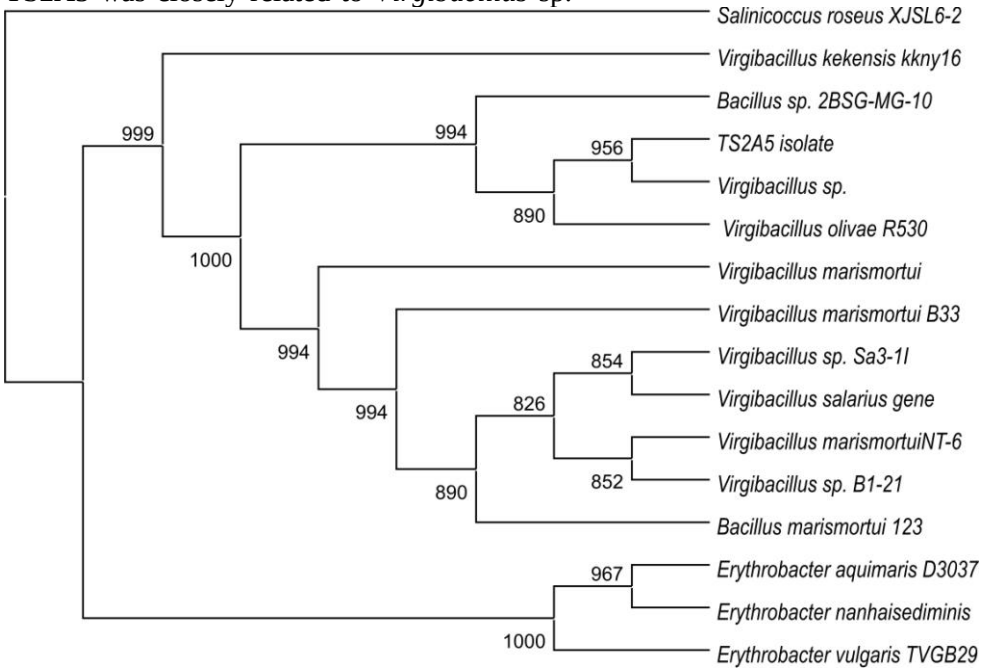

Fig 2. Neighbor joining tree showing phylogenetic topology of strain TS2A5 with other species and related taxa based on 16S rRNA gene sequence. Bootsrap values from 1000 resamplings are indicated.

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

Table 2. Molecular identification of active isolate obtained from marine tunicate.

No	Isolate	Length	Closest Relative	Similarity (%)	Accession
1	TS2A5	1333 bp	*Virgibacillus* sp.	99 %	EU868858.1

An attempt was carried out to estimate the potential of marine bacteria associated with tunicate *Didemnum molle* from Sambangan island as the source of antibacterial compounds in particular against the pathogenic *Staphylococcus* sp. and *E. coli.*

Tunicates have been known to produce secondary metabolites such as ET-743 a potent anti-cancer agent, obtained from *Ecteinescidia turbinata*, a tunicate from the Caribbean sea. Since ET-743 has been found from tunicate *E. turbinate* samples both in the Caribbean and in the Mediterranean and the compound is likely to be bacterial in origin. Thus, it is possible that the bacteria responsible for its production are associated with the tunicate from both regions (Rinehart *et al.*, 1990 ; Mendola 2000).

A number of compounds with biomedical potential have been isolated from marine invertebrates, especially sponges and tunicates. There is now evidence that some of these compounds are synthesized by bacteria associated with the invertebrates (Schmidt *et al.*, 2000). For other biologically active compounds there is only circumstantial evidence that they could come from bacteria associated with the invertebrate (Koenig *et al*., 2005).

It is interesting to note that tunicate bacterium *Virgibacillus* sp. TS2A5 showed strong growth inhibition against *Staphylococcus* sp. and *E. coli*. This raises the possibility the use of tunicate bacteria as the source of antibacterial compounds for controlling the pathogenic bacteria *Staphylococcus* sp. and *E. coli*.

Our results highlight tunicate-associated bacterium (TS2A5) carrying the NRPS gene. Growth inhibition of pathogenic bacterium by NRPS strain TS2A5 demonstrates the uncharacterized secondary metabolites produced by strain TS2A5 lead to antagonistic activity.

TS2A5 is characterized by Gram-positive and motile by means of peritrichous flagella. Terminal or subterminal spore position. Colonies are irregular to regular, flat and translucent, and milky white in colour. Optimal growth occurs at 35-40 ^{o}C.

The genus *Virgibacillus* was first proposed by Heyndrickx *et al*., (1998). Recently, the 12 members of genus *Virgibacillus* sp. have been validly described: *V. pantothenticus, V. proomii, V. carmonensis, V. necropolis, V. picturae, V. marismortui, V. salexigens, V. halodenitrificans, V. dokdonensis* (Yoon *et al*., 2005), *V. koreensis* (Lee *et al*., 2006), *V. olivae* and *V. halophilus* (An *et al*., 2007).

This genus is characterized by A gram-positive, motile, endospore-forming, halophilic bacterial strain, DNA G+C content (mol%) 36.7 (*V. dokdonensis*)- 42.8 (*V. marismortui*) (Wang *et al*., 2008).

The genus Virgibacillus has been implicated in the production of a number of biotechnologically relevant biomolecules. A typical example of enzymes would include proteases. The enzyme proteases are found to be produced or secreted most by the genus Virgibacillus and are of particular interest due to their wide applications in, protein recovery or solubilization, organic synthesis, meat tenderization, detergents, food industry, and pharmaceuticals (Gupta *et al*., 2008).

Other studies have reported amylases bioflocculant compound, and antifungal compound production by some Virgibacillus species (Essghaier *et al*., 2009). Although as mentioned a number of bioactive compounds have been documented to be produced by Virgibaccillus species (Cosa *et al*., 2011).

CONCLUSION

In conclusion, the present study highlighted the potential use of tunicate associated bacteria in inhibiting the growth of pathogenic bacteria *Staphylococcus* sp and *E. coli*. This finding can be developed further in order to obtain sustainable source of marine marine antimicrobial compounds.

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

ACKNOWLEDGEMENTS

This work was partially funded by a grant from The US Department of State's Biosecurity Engagement Program (BEP), USA . (Grant # IDB1-21003-JA-08 through the US Civilian Research &Development Foundation (CRDF)).

We acknowledge the Ministry of National Education of Indonesia for the support through Scholarship Program (Beasiswa Unggulan) 2009 in Satya Wacana Christian University, Salatiga. (Contract No. 89211/A2.5/LN/2009).

REFERENCES

An, S. Y., M. Asahara, K.Goto, H.Kasai, and A. Yokota. 2007. *Virgibacillus halophilus sp*. nov., spore-forming bacteria isolated from soil in Japan. *Int J. Syst Evol Microbiol.* (57) 1607–1611.

Atschul, S.F., T.L. Madden., A.A. Schaffer., J. Zhang., Z. Zhang., W. Miller and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs. *Nucleid Acid Res*. (25): 3389-3402.

Bernan VS., M. Greenstein, W.M. Maiese. 1997. Marine microorganisms as a source of new natural products. *Adv Appl Microbiol.* 43:57–89

Bowman, J.P. 2007. Bioactive compound synthetic capacity and ecological significance of marine bacterial genus *Pseudoalteromonas*. *Mar. Drugs*. 5: 220-241.

Cohen ML. 1994. Antimicrobial resistance: prognosis for public health. Trends Microbiol 2:422–425

Cosa S., V. Leonard, I.Mabinya, O. Ademola Olaniran., Omobola O. Okoh., Kim Bernard., Shaun Deyzel and I. Anthony Okoh. 2011. Bioflocculant Production by *Virgibacillus* sp. Rob Isolated from the Bottom Sediment of Algoa Bay in the Eastern Cape, South Africa. *J. Mol*, (16) : 2431-2442

Davidson SK., S.W, Allen, G.E. Lim, C.M. Anderson, M.G. Haygood. 2001. Evidence for the biosynthesis of bryostatins by the bacterial symbiont ‘‘*Candidatus Endobugula sertula*’’ of the bryozoan Bugula neritina. *Appl Environ Microbiol* 67 (10):4531–4537

Essghaier, B., M.L.Fardea, J.L. Cayol, M.R. Hajlaoui, A. Boudabous, H.Tijakli, N. Sadfi-Zouaoui. 2009. Biological control of grey mould in strawberry fruits by halophilic bacteria. *J. Appl. Microbiol*. (106) : 833-846.

Flam F.1994. Chemical prospectors scour the seas for promising drugs. *J. Sci* 266 (5189):1324–1325.

Gupta, A., B. Josheph, A. Mani. 2008. Biosynthesis and properties of an extracellular thermostable serine alkaline protease from *Virgibacillus pathothenticus*. *World J. Microbiol. Biotechnol.* (24) : 237-243.

Heyndrickx, M., L. Lebbe, K. Kersters, P. De Vos, G. Forsyth and N. A. Logan.1998. Virgibacillus: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. Int *J. Syst Bacteriol* 48:99–106.

Hunt, B.H. and A.C.J. Vincent. 2006. Scale and sustainability of marine bioprospecting for pharmaceuticals. *Ambio.* (35) : 57-64

Holmstrom, C., S. James, B. A. Neilan, D. C. White, and S. Kjelleberg. 1998. *Pseudoalteromonas tunicata* sp. nov., a bacterium that produces antifouling agents. *Int. J. Syst. Bacteriol*. 48 (Pt. 4) : 1205–1212.

Ivanova, E. P., L. S. Shevchenko, T. Sawabe, A. M. Lysenko, V. I. Svetashev, N. M. Gorshkova, M. Satomi, R. Christen, and V. V. Mikhailov. 2002. *Pseudoalteromonas maricaloris* sp. nov., isolated from an Australian sponge, and reclassification of [*Pseudoalteromonas aurantia*] NCIMB 2033 as

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

Pseudoalteromonas flavipulchra sp. nov. *Int. J. Syst. Bacteriol. Evol. Microbiol.* (52) : 263–271.

Koenig, G.M., S. Kehraus, S.F. Seibert, A. D. Abdel-Lateff Mu¨ller. 2005. Natural products from marine organisms and their associated microbes. *J. Chem BioChem.* 6 :1–10

Lestari, E. S., P. M. G. Filius., K. Kuntaman., D. O. Duerink., U. Hadi, and H. A. Verbrugh. 2005. The Antimicrobial Resistance in Indonesia; Prevalence and Prevention (AMRIN) study group. Antimicrobial resistance among Staphylococcus aureus and Escherichia coli isolates in the Indonesian population inside and outside hospitals. *J. Clin. Microbiol Infect. Dis.*

Lim G.E and M.G. Haygood. 2004. ‘‘*Candidatus Endobugula glebosa*,’’ a specific bacterial symbiont of the marine bryozoan Bugula simplex. *Appl Environ Microbiol* 70(8):4921–4929

Lee, J. S., J. M. Lim, K. C. Lee, J. C. Lee, Y.H. Park,. and C. J. Kim. 2006. *Virgibacillus koreensis* sp. nov., a novel bacterium from a salt field, and transfer of *Virgibacillus picturae* to the genus Oceanobacillus as *Oceanobacillus picturae* comb. nov. with emended descriptions. *Int J. Syst Evol Microbiol* (56) : 251–257.

Madigan M.T, J.M. Martinko, J. Parker, and T.D. Brock. 2000. Biology of microorganisms. Prentice-Hall, Inc., New Jersey, USA

Mendola D .2000. Aquacultural production of bryostatin 1 and ecteinascidin 743. In: Fusetani N (ed) Drugs from the sea. *Karger, Basel*, pp 120–133

Proksch, P., R.A. Edrada and R. Ebel. 2002. Drugs from the Seas – Current Status and Microbiological Implications. *J. Appl. Microbiol. Biotechnol*. (59) : 125-124.

Radjasa, O.K., T. Martens., H-P. Grossart., T. Brinkoff., A. Sabdono., and M. Simon. 2007a. Antagonistic activity of a marine bacterium *Pseudoalteromonas luteoviolacea* TAB4.2 associated with coral *Acropora* sp. *J. Biol. Sci*. 7(2):239-246.

Radjasa, O.K., S.I.O. Salasia, A. Sabdono, J. Weise, J.F. Imhoff, C. Lammler and M.J. Risk. 2007b. Antibacterial Activity of Marine Bacterium *Pseudomonas* sp. Associated with Soft Coral *Sinularia polydactyla* Against *Streptococcus equi* Subsp. *Zoopidemicus*. *Int J. Pharmacol*. 3 (2) : 170-174.

Rinehart, K.L., T.G. Holt., N.L. Fregeau., J.G. Stroh., P.A. Keifer., F. Sun. L.H. L.I and D.G. Martin. 1990. Ecteinascidins 729, 743, 745, 759B, and 770 : potent antitumor agents from the Caribbean tunicate, Ecteinascidia turbinate. *J. Org. Chem.* 55 : 4512.

Schmidt EW, Obraztsova AY, Davidson SK, Faulkner DJ, Haygood MG. 2000. Identification of the antifungal peptide-containing symbiont of the marine sponge *Theonella swinhoei* as a novel d-proteobacterium ‘‘*Candidatus Entothenonella palauensis*’’. Mar Biol 136:969–977

Schmidt EW, J.T. Nelson, D.A. Rasko, S. Sudek J.A.Eisen M.G. Haygood, J. Ravel. 2005. Patellamide A and C biosynthesis by a microcin-like pathway in Prochloron didemni, the cyanobacterial symbiont of Lissoclinum patella. *Proc. Natl. Acad. Sci.USA* .102 (20):7315–7320

Skerratt, J. H. 2001. Bacterial and algal interaction in a Tasmanian estuary. PhD Thesis. University of Tasmania, Hobart, *Tasmania, Australia*, 2001; pp. 219-235.

Thiel,V and J.F. Imhoff. 2003. Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. *Biomol. Eng*. 20: 421-423.

Wang. C.Y., C.C.Chang, C.N. Chang, T.W. Chen. and Y.T.Shyu. 2008. *Virgibacillus chiguensis* sp. nov., a novel halophilic

ISSN : 1410-5217
Accredited : 83/Dikti /Kep/2009

bacterium isolated from Chigu, a previously commercial saltern located in southern Taiwan. *Int J. Syst and Evol. Microbiol.* 58 : 341–345

Yoon, J. H., S. J. Kang, S. Y.Lee, M. H. Lee, and T.K.Oh. 2005. *Virgibacillus dokdonensis* sp. nov., isolated from a Korean island, Dokdo, located at the edge of the East Sea in Korea. *Int J Syst Evol Microbiol.*(55) : 1833–1837.