Journal of Coastal Development
Volume 14, Number 1, October 2010 : 45 - 50

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

Original Paper

ANTIBACTERIAL ACTIVITIES OF BACTERIAL SYMBIONTS OF SOFT CORAL *Sinularia* sp. AGAINST TUBERCULOSIS BACTERIA

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Received : August, 11, 2010 ; Accepted : September, 29, 2010

ABSTRACT

Tuberculosis (TB) is caused by Mycobacterium tuberculosis. Although TB is a curable disease, it continues to be one of the most important infectious causes of death worldwide. Indonesia ranks 3rd on the list of TB high burden countries in the world with 86,000 cases deaths and the Multi Drug Resistant Tuberculosis (MDR TB) estimated cases in Indonesia is 10,000. This research was aimed to isolate and characterize of soft coral Sinularia sp.-associated bacteria having antibacterial activity against Tuberculosis bacteria. There were 109 isolates collected from Sinularia sp. Two isolates from Sinularia sp.-associated bacteria, SC4TGZ3 and SC4TGZ4 were successfully screened for antibacterial against Tuberculosis bacteria. SC4TGZ3 was found to inhibit the growth of MDR TB strain HE, MDR TB strain SR and H37Rv. Whereas, SC4TGZ4 was found to inhibit the growth of MDR TB strain HE. Based on PCR amplification 16S rDNA softcoral bacateria were identified as follows: SC4TGZ3 was closely related to Pseudovibrio sp. and SC4TGZ4 was closely related to Alpha proteobacterium sp.

Keywords: *Mycobacterium tuberculosis; Sinularia* sp.; MDR-TB; Antibacterial activities; associated bacteria

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INTRODUCTION

Tuberculosis (TB) is one of the major health problems in Indonesia. It is estimated that total cases are 528,000 and 86,000 cases deaths. The three major indicators of impact: incidence, prevalence and mortality rates per 100.000 population by further monitoring, MDG target 6.c is to halve prevalence and death rates by 2015 compared with 1990 (WHO, 2009).

Over the past 20 years , it has been seen the worldwide appearance of multidrug-resistant (MDR) TB. MDR TB is caused by *Mycobacterium tuberculosis* that is resistant at least to Isoniazid and Rifampicin, the most effective anti-TB drugs and are considered first-line drugs to be used to treat all persons with TB disease (Gandhi *et al.,* 2010). The MDR TB estimated cases in Indonesia are 10,000 (WHO, 2009). MDR-TB results from either primary infection with resistant bacteria or may develop in the course of a patient's treatment (WHO, 2010).

Resistance to anti-TB drugs occurrs when these drugs are misused or mismanaged. Examples include when patients do not complete their full course of treatment; when health-care providers prescribe the wrong treatment, the wrong dose, or length of time for taking the drugs; when the supply of drugs is not always available; or when the drugs are of poor quality (Center for Diseases Control, 2008). Treatment failures also increase the numbers of infected people moving into the community and thus expose the general population to the risk of contracting a resistant strain of infection. It is an opportunity to find alternative antibiotic to combat the resistant bacteria.

Nature has continuously provided mankind with a broad and structurally diverse arsenal of pharmacologically active compounds that continue to be utilised as highly effective drugs to combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature. 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 mollusks (Proksch *et al.,* 2002).

The development of marine organisms-derived compounds into drugs has been hampered by supply limitations. Symbioses between microorganisms and marine organisms are abundant and widespread in the sea. Most marine invertebrates and algae harbor diverse microbial symbionts including prokaryotic bacteria, archaea, cyanobacteria, and fungi. Increasing evidence implicates microbial symbionts as the true source of many marine organism-derived compounds, which makes marine microbial symbionts a hotspot in the field of marine microbiology and marine natural products because of their potential for solving the bottleneck problem of marine natural product supply (Li, 2009).

Numerous natural products from marine invertebrates show striking structural similarities to known metabolites of microbial origin, suggesting that microorganisms (bacteria, microalgae) are at least involved in their biosynthesis or are in fact the true sources of these respective metabolites. This assumption is corroborated by several studies on natural products from sponges that proved these compounds to be localized in symbiotic bacteria or cyanobacteria (Proksch *et al.,* 2002).

This article describes the characterization of soft coral *Sinularia* sp.-associated bacteria that having antibacterial activity against tuberculosis bacteria (H37Rv, MDR TB strain HE, MDR *TB strain* SR*)*. The characterization is supported by moleculer techniques using 16S rDNA approach.

MATERIAL AND METHODS

Sampling *Sinularia* sp. and isolation bacterial symbionts

Collonies of softcoral *Sinularia* sp. were collected by scuba diving from Tanjung Gelam waters, Karimunjawa islands, North Java Sea, Indonesia. Upon collection, softcorals were put into steril plastic bags (Whirl-Pak, Nasco USA). The tissue were then rinsed with sterile seawater and homogenized with blender. The homogenized tissues were serially diluted, spread on half 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).

Antibacterial test

Antibacterial test of *Sinularia* sp. associated bacteria against tuberculosis bacteria was performed by using an overlay method. Tuberculosis bacteria (H37Rv, MDR TB strain HE, MDR TB strain SR) used in this study were obtained from Health Laboratory of Central Java Province-Semarang. Culture of each bacterium in the logarithmic phase was mixed with Middle brook 7H9+OADC soft agar medium (1% v/v), which were poured on to the respective agar surface previously inoculated with *Sinularia* sp. associated bacteria that had incubated for 4 days at room temperature. Then the plates were incubated at room temperature 2x24 hours. Antibacterial activity was defined by the formation of inhibition zones around the tuberculosis bacterial colonies.

PCR amplification and DNA sequencing

PCR amplification was carried out according to the method of Radjasa *at.al.,* (2007a). Universal primers described by Weisburg *et al.,* (1991) was used for PCR amplification. 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 *Sinularia* sp. associated bacteria and subsequent sequencing analysis were performed according to method of Radjasa *et al.,* (2007b). The determined DNA sequences of strains were compared for homology to the BLAST database.

RESULTS AND DISCUSSION

Results

There were 109 isolates of marine bacteria associated with softcoral *Sinularia* sp, and 2 isolates associated bacteria were found to inhibit the growth of tuberculosis bacteria (H37Rv, MDRTB strain HE, MDR TB strain SR) as shown in **Table 1.**

Table 1. The growth inhibition zone of Tuberculosis Bacteria

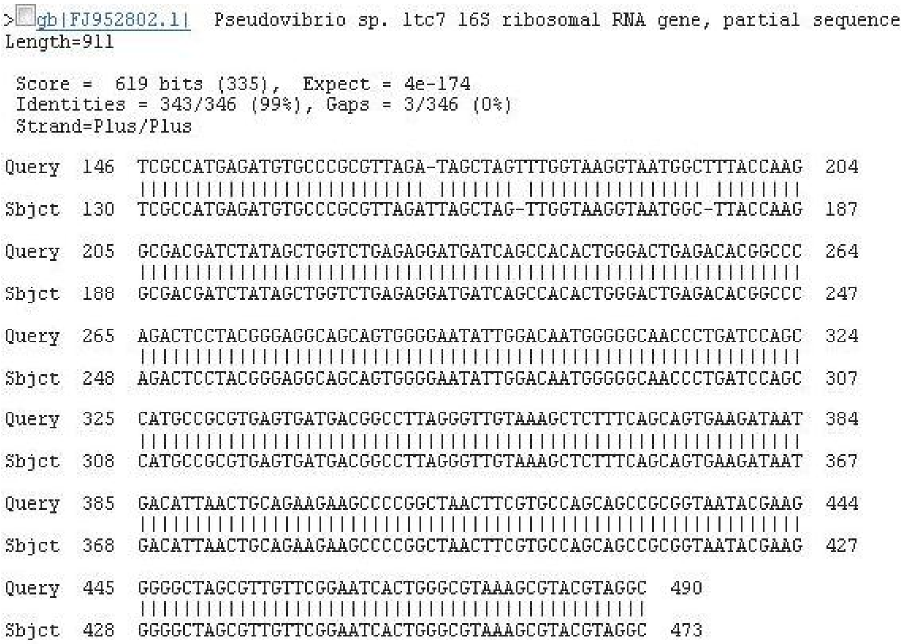
NO	Stram	*Soft Coral*	*H37Rv*	*MDR TB strain HE*	*MDR TB strain SR.*
1.	SC4 TGZ3	*Sinularia* sp.	8.5±0.5	4.533 ± 0.057	6.133±2.307
2.	SC4 TGZ4	*Sinularia* sp.	-	4.95±0.260	6.900 ± 3.61

Moleculer identification of active isolates of marine bacteria associated with softcoral *Sinularia* sp. based on 16S rDNA, revealed that active strains: SC4TGZ3 was closely related to *Pseudovibrio* sp. and SC4TGZ4 was closely related to *Alpha proteobacterium* sp. (**Table 2**).

Table 2. Moleculer identification of active isolates obtained from soft coral Sinularia sp.

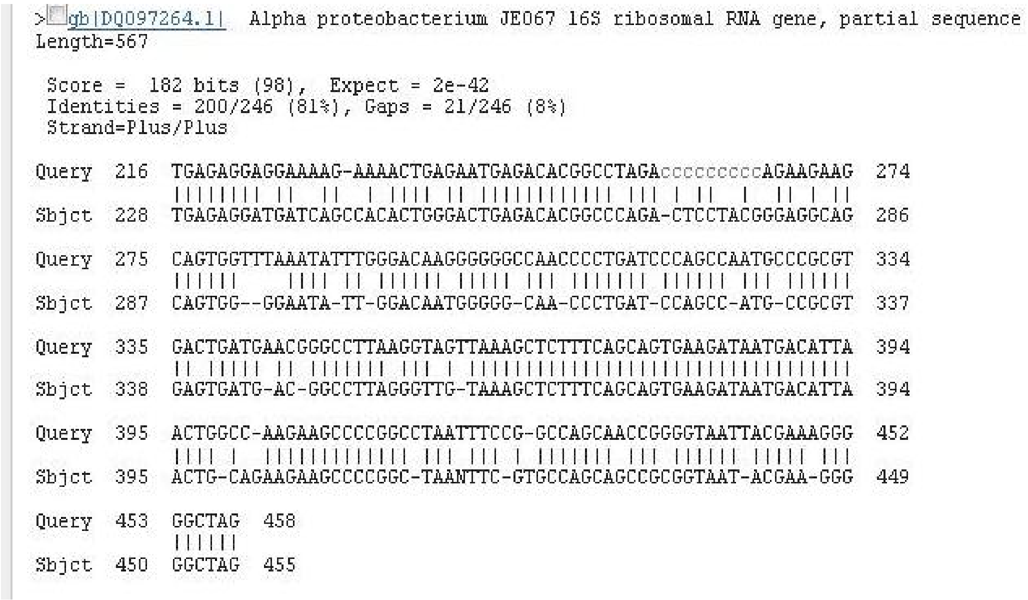
No	Kode Bakteri	Length	Closest Relative	Homology	Accession
1.	SC4TGZA	458	Alpha proteobacterium	81%	DQ097264.1
2.	SC4TGZ3	493	*Pseudovibrio sp.*	99 %	FJ952802.1

The result of BLAST search for SC4TGZ3 and SC4TGZ4 are shown in the following **Fig.1 and Fig. 2.**



```
>☐gb|FJ952802.1|  Pseudovibrio sp. ltc7 16S ribosomal RNA gene, partial sequence
Length=911

 Score =  619 bits (335),  Expect = 4e-174
 Identities = 343/346 (99%), Gaps = 3/346 (0%)
 Strand=Plus/Plus

Query  146   TCGCCATGAGATGTGCCCGCGTTAGA-TAGCTAGTTTGGTAAGGTAATGGCTTTACCAAG  204
             |||||||||||||||||||||||||| ||||||| |||||||||||||||| ||||||||
Sbjct  130   TCGCCATGAGATGTGCCCGCGTTAGATTAGCTAG-TTGGTAAGGTAATGGC-TTACCAAG  187

Query  205   GCGACGATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC  264
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  188   GCGACGATCTATAGCTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCC  247

Query  265   AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGC  324
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  248   AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGGGCAACCCTGATCCAGC  307

Query  325   CATGCCGCGTGAGTGATGACGGCCTTAGGGTTGTAAAGCTCTTTCAGCAGTGAAGATAAT  384
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  308   CATGCCGCGTGAGTGATGACGGCCTTAGGGTTGTAAAGCTCTTTCAGCAGTGAAGATAAT  367

Query  385   GACATTAACTGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG  444
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  368   GACATTAACTGCAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAG  427

Query  445   GGGGCTAGCGTTGTTCGGAATCACTGGGCGTAAAGCGTACGTAGGC  490
             ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  428   GGGGCTAGCGTTGTTCGGAATCACTGGGCGTAAAGCGTACGTAGGC  473
```

Fig. 1 BLAST for SC4TGZ3 bacteria



```
>☐gb|DQ097264.1|  Alpha proteobacterium JE067 16S ribosomal RNA gene, partial sequence
Length=567

 Score =  182 bits (98),  Expect = 2e-42
 Identities = 200/246 (81%), Gaps = 21/246 (8%)
 Strand=Plus/Plus

Query  216  TGAGAGGAGGAAAAG-AAAACTGAGAATGAGACACGGCCTAGAcccccccccAGAAGAAG  274
            |||||||| ||  ||  | |||| || |||||||||||| ||| | ||  |  || | ||
Sbjct  228  TGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGA-CTCCTACGGGAGGCAG  286

Query  275  CAGTGGTTTAAATATTTGGGACAAGGGGGGCCAACCCCTGATCCCAGCCAATGCCCGCGT  334
            ||||||    |||| || |||||| ||||| ||| ||||||| |||||| ||| ||||||
Sbjct  287  CAGTGG--GGAATA-TT-GGACAATGGGGG-CAA-CCCTGAT-CCAGCC-ATG-CCGCGT  337

Query  335  GACTGATGAACGGGCCTTAAGGTAGTTAAAGCTCTTTCAGCAGTGAAGATAATGACATTA  394
            || ||||| || ||||||| ||| | ||||||||||||||||||||||||||||||||||
Sbjct  338  GAGTGATG-AC-GGCCTTAGGGTTG-TAAAGCTCTTTCAGCAGTGAAGATAATGACATTA  394

Query  395  ACTGGCC-AAGAAGCCCCGGCCTAATTTCCG-GCCAGCAACCGGGGTAATTACGAAAGGG  452
            |||| |  ||||||||||||| ||| ||| | ||||||| ||| |||||| ||||| |||
Sbjct  395  ACTG-CAGAAGAAGCCCCGGC-TAANTTC-GTGCCAGCAGCCGCGGTAAT-ACGAA-GGG  449

Query  453  GGCTAG  458
            ||||||
Sbjct  450  GGCTAG  455
```

Fig. 2. BLAST for SC4TGZ4 bacteria

Discussion

MDR tuberculosis has become increasingly prevalent. Treatment regimens for MDR tuberculosis are longer, less effective, less tolerable, and more expensive than is standardised short-course chemotherapy, and include the use of injectable drugs. The percentage of patients with MDR tuberculosis who are cured is estimated to be no more than 69% on the basis of results from retrospective cohort studies, even when treated for more than 18 months with directly observed treatment(Ma *et al.,* 2010). This is an opportunity to investigate new drugs for tuberculosis.

The causative agent of Tuberculosis is *Mycobacterium tuberculosis*. The member of the genus *Mycobacterium* are non-motile and non-sporulated rods, they have high lipid content in the wall, probably the highest among all bacteria. Lipids constitute more than half of the dry weight of the mycobacteria. However, the lipid composition of the tubercle bacillus may vary during the life cycle in culture, depending on the availability of nutrients. The waxy coat confers the idiosyncratic characteristics of the genus: acid fastness, extreme hydrophobicity resistance to injury, including that of many antibiotics, and distinctive immunological properties. It probably also contributes to the slow growth rate of some species by restricting the uptake of nutrients (Barrera, 2007).

This research used sensitive strain from tuberculosis bacteria *Mycobacterium tuberculosis H37Rv* and the resistant strains: MDR TB strain HE (*Mycobacterium tuberculosis* resistant Isoniazid and Etambutol) and MDR TB strain SR (*Mycobacterium tuberculosis* resistant Streptomycin and Rifampicin). Isoniazid, Rifampicin, Etambutol and Streptomycin are the first line drugs for tuberculosis. The *Sinularia* sp. associated bacteria SC4TGZ3 (homolog 99% with *Pseudovibrio* sp.) and SC4TGZ4 (homolog 81% with *Alpha proteobacterium*) had potential with antibacterial activities.

Pseudovibrio sp. and *Alphaproteobacterium* have the same class in Alphaproteobacteria (α-proteobacteria). They are gram negative with an outer membrane mainly composed of lipopolysaccharides. These bacteria also symbionts of plants and animals The α-proteobacteria form one of the largest groups within bacteria that includes numerous phototrophs, chemolithotrophs, chemoorganotrophs and aerobic photoheterotrophs. They are abundant constituents of various terrestrial and marine environments. The intimate association that many α-proteobacteria exhibit with the eukaryotic organisms is of central importance from agricultural and medical perspectives (Gupta and Mok, 2007).

Bacterial symbionts of marine invertebrates have been known to produce secondary metabolites which are potential for medical and pharmalogical fields such as symbionts of coral *Acropora* sp. (Radjasa *et al.*, 2007a) and sponge *Haliclona* sp (Radjasa *et al.*, (2007b). Futher, Radjasa *et al.*, (2007c) reported the antibacterial activity of bacterial symbionts of softcoral *Sinularia polydactyla* against pathogen *Streptococcus equi* subsp. *zooepidemicus.* The present study has confirmed the importance of bacterial symbiont, in particular those having symbioses with softcoral *Sinularia* sp. as the producer of antibacterial compounds against TB bacteria.

CONCLUSION

Soft coral *Sinularia* sp. exhibited secondary metabolite producing marine bacteria with antibacterial activity that potential against tuberculosis bacteria: *Mycobacterium tuberculosis H37Rv* and the resistant strains: MDR TB strain HE (*Mycobacterium tuberculosis* resistant Isoniazid and Etambutol) and MDR TB strain SR (*Mycobacterium tuberculosis* resistant Streptomycin and Rifampicin). Further study, however, is needed to isolate and purify the active compounds that inhibit the growth of TB strains.

ACKNOWLEDGEMENTS

The work was part of a research grant provided by Directorate General of Higher Education with the Skala Prioritas Nasional Batch II Programme (Contract No. 304/SP2H/PP/DP2M/VI/2009). We thank the students Department of Marine Science Diponegoro University for the support in field and laboratory; Mrs. Elly Karlina the head of Microbiology laboratory from the Health Laboratory of Central Java Province-Semarang for the support in this research.

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