

Endophytic bacteria producing antibacterial against methicillin-resistant *Staphylococcus aureus* (MRSA) in seagrass from Rote Ndao, East Nusa Tenggara, Indonesia

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Abstract. Fitri DS, Pangastuti A, Susilowati A, Sutarno. 2017. Endophytic bacteria producing antibacterial against methicillin-resistant *Staphylococcus aureus* (MRSA) in seagrass from Rote Ndao, East Nusa Tenggara, Indonesia. *Biodiversitas* 18: 733-740. Methicillin-resistant *Staphylococcus aureus* (MRSA) are bacteria that resistant to the various type of antibiotics and yet cannot be handled comprehensively. The discovery of new antibiotic from endophytic bacteria in seagrass of Rote Island is an option to overcome the resistance. The aims of this research were to screen endophytic bacteria inhibit MRSA from seagrass, to determine the species of the endophytic bacteria and the genetic relationship. Isolation of endophytic bacteria has carried out by inoculating surface sterilization seagrass leaves on Marine Agar (MA) medium. Selection of potential endophytic bacteria-producing anti-MRSA has done using overlay method against MRSA, gram-positive *Bacillus subtilis*, and gram-negative *Escherichia coli*. Identification of the endophytic bacteria based on the sequence of 16S rRNA encoding gene. The results showed that there were eight isolates of endophytic bacteria which have antibacterial activity against MRSA of seagrass *Enhalus acoroides*, *Thalassia hemprichii*, and *Cymodocea rotundata* in the Litanak and Oeseli Beaches, Rote Ndao. The diameter of inhibition zone was between 0.65-18.27 mm with narrow spectrum with broad spectrum antibacterial activity. The eight potential endophytic bacteria identity were Bacillaceae E2M1, Bacillaceae E2M3, *Bacillus* E2M4, *Bacillus* E2M7, Bacillaceae E2M8, Pseudomonadaceae C1M7, Shewanellaceae C2M3, and Rhodobacteraceae T1M3. Most of the isolates can be said to be a new species as the percent similarity of 16S rRNA gene sequence was less than 95% and promising to produce new antibacterial compounds. Phylogenetic relationship showed some isolates clustering in different groups that present the diverse groups of endophytic bacteria were found.

Keywords: Antibacteria, *Bacillus subtilis*, endophytic bacteria, *Escherichia coli*, MRSA, seagrass

INTRODUCTION

Since the imposition of antibiotics, bacteria have evolved mechanisms to resist the effects of antibiotics or often referred to as resistant bacteria. The evolution and the increasing spread of resistant bacteria species caused by several factors such as the widely used antibiotic and inaccurate application of antibiotics, as well as the use of antibiotics in animal feed extensively (Karchmer 2006). *Staphylococcus aureus* is one of the human pathogen most commonly found in various diseases ranging from mild skin infections, severe infections until disease that can threaten human safety such as endocarditis, pneumonia, and sepsis (Paterson et al. 2014). *S. aureus* resistance to the antibiotic penicillin was first reported in 1940. Then, methicillin-resistant strains of *S. aureus* (MRSA) was first reported in the early 1960s as the second leading cause of infection patients in hospitals and community; and become endemic worldwide including in Indonesia. Until now part of the problem of bacterial resistance that was never solved completely (Chamber 2001; Karchmer 2006).

Exploration of new sources becomes very important to find bioactive compounds that can be used as a new antibiotic (Clardy et al. 2006). Microorganisms have been the source of many useful compounds in medicine, the

pharmaceutical industry, and agriculture. However, most of these organisms derived from terrestrial habitats. After intensive research on terrestrial microorganisms, researchers are now focusing attention on other ecosystems, such as the sea. Microorganisms that associated by marine organisms has become a major target for the discovery of new bioactive compounds (Penesyant et al. 2010, 2011; Kang et al. 2015). One of them is the utilization of microbial endophyte. Endophyte is defined as microorganisms contained in asymptotically in the plant tissue (Schulz et al. 2006). Some marine microorganisms survive in habitats with high-stress levels, undersea with low temperatures, limited light conditions with high pressure, grazing by herbivores on host plants, predation, and their competitors. These factors have led to the development of a unique metabolism, resulting in the production of new metabolites that differ from terrestrial microorganisms. Some endophytic microorganisms including bacteria can contribute to host defense by excretion of antibiotics and other bioactive substances. Thus, marine microorganisms offer an excellent resource for the discovery of new compounds with interesting biological activities, including antimicrobial, antifungal, antiprotozoal, antituberculosis and antiviral (Lu et al. 2010; Rahman et al. 2010).

Seagrass is an aquatic plant that can adapt to live and grow in the marine environment. Seagrass adapt to the unstable condition of the beach; seagrass vegetation provides many ecological functions for macroorganisms and microorganisms associated. One factor organisms to settle interest in the environment is classified as seagrass habitat productive, so as to provide food, a shelter for the survival of organisms associated (Gufran 2011).

In Rote Ndao, seagrass is distributed almost in all coastal areas such as Litanak and Oeseli beaches. Ecologically both beaches have differences, Litanak beach is a sandy beach and a bit muddy, while the Oeseli beach is rocky beaches mixed with sand. Although the presence of seagrass on both coasts knew their existence, both research and information about endophytic bacteria and potential in every beach have not been performed. Unique habitat of the beach is one of the important parameters to obtain new sources of endophytic bacteria that can produce secondary metabolites as antibiotics. The discovery of new antibiotic metabolites of endophyte is an important alternative to cope with increasing levels of drug resistance by human pathogens such as MRSA and the limited number of antibiotics that are effective against a variety of bacterial species. Therefore, it is necessary to do research on the potential of bioactive compounds owned by endophytic bacteria in seagrass in the Litanak and Oeseli beaches, Rote Ndao, East Nusa Tenggara, Indonesia.

MATERIALS AND METHODS

Media and culture condition

Methicillin-resistant *Staphylococcus aureus* (MRSA), *Bacillus subtilis* and *Escherichia coli* were obtained from the Laboratory of Clinical Microbiology, Faculty of

Medicine, Universitas Indonesia, Depok, West Java, Indonesia. The bacteria were stored on nutrient agar slant (Himedia). Endophytic bacteria were stored on marine agar media (Himedia). Stock cultures of the bacteria stored at 4°C. The bacteria were refreshed and stored at room temperature for anti-bacterial testing.

Seagrass sampling

Seagrass was collected at Litanak and Oeseli Beaches, Rote Island of the Rote Ndao District, East Nusa Tenggara Province, Indonesia (Figure 1). *Enhalus acoroides*, *Thalassia hemprichii*, and *Cymodocea rotundata* were taken as a source of endophytic bacteria. Each seagrass was put in plastic bags and labeled contains information of species and the location, and then was placed in an ice box to be brought to the laboratory. The seagrass was identified by comparing the morphological character with a guide book of seagrass in tropical seagrasses in the Indo-West Pacific by Lanyon (1986) and Waycott et al. (2004).

Isolation of seagrass endophytic bacteria

The seagrass leaves from each species were cleaned with aquadest and then cut into 5 cm long. Surface sterilization was done by keeping a piece of the seagrass leaves into 70% ethanol for 1 minute, continued into 5.25% sodium hypochlorite solution for 1 minute, and washed with 70% ethanol for three times. The sterile leaves were sliced then planted in the marine agar media and incubated at room temperature (28-30°C) for 1-2 days. The plates were observed every day until some colony of the bacteria emerged (Ravikumar et al. 2010). Every single colony of the endophytic bacteria that showed different morphological colony was purified and stored as a pure culture.



Figure 1. Study site in Rote Island of the Rote Ndao District, East Nusa Tenggara Province, Indonesia. 1. Litanak Beach, 2. Oeseli Beach

Screening for endophytic bacteria producing bioactive anti-MRSA

Screening for seagrass endophytic bacteria which produced antibacterial conducted using overlay method (Sulistiyani et al. 2015). The pure culture of endophytic bacteria was inoculated into marine agar (MA) media and incubated at room temperature for six days or until the colony was formed with a diameter of ± 6 mm. Tested bacteria i.e., Methicillin-resistant *S. aureus* (MRSA), Gram-positive *B. subtilis* and Gram-negative *E. coli* were grown in nutrient broth medium and then incubated overnight in incubator shaker 100 rpm. A total of 0.1 mL nutrient broth culture with cell density $\pm 10^8$ cells/mL put in 5 mL of Nutrient Agar media that have not been solidified (about 60°C), was homogenized and poured over MA media which has been overgrown with the endophytic bacteria and incubated for 24 hours at a temperature of 37°C. If the endophytic bacteria produced anti-MRSA inhibition zone would be observed. The endophytic bacteria showed inhibition zone against the tested bacteria regarded as potential isolates.

Amplification of 16S rRNA encoding gene

Genomic DNA of potential isolates of the endophytic bacteria was extracted using Presto™ Mini gDNA kit (Geneaid). Concentration and purity of the extracted bacterial genomic DNA were analyzed using biophotometer. 16S rRNA gene amplification was conducted in a thermocycler machine (Veriti, Applied Biosystem) using a forward primer of 63f (5'-CAGGCCTAACACAT-GCAAGTC-3') and reverse primer of 1387r (5'-GGGCGGAWGTGTACAAGGC-3') (Marchesi et al. 1998). The PCR product was 1300 bp. PCR mixing reactions consisted of 12.5 μ L of KAPA 2G Fast Ready Mix (buffer, dNTP mix, and DNA polymerase) (Kapa Biosystems), 1.25 μ L of 63 forward primer (10 pmol), 1.25 μ L of reverse primer 1387 (10 pmol), 2-5 μ L of DNA template (genomic DNA of potential endophytic bacteria with concentrations <25 ng/ μ L); 1 mL of DNA template (for genomic DNA of potential endophytic bacteria with concentrations > 25 ng/ μ L); ddH₂O up to 25 μ L. The process of denaturation, annealing, and elongation consist of 30 cycles. Stages of the each cycle were comprised of pre-denaturation at a temperature of 94°C for 3 minutes, denaturation at 94°C for 15 seconds, annealing

at 55°C for 15 seconds, elongation of 72°C for 30 seconds, and finalizing at a temperature of 72°C for 2 minutes. The PCR products were determined by electrophoresis in 1% agarose on 85 volt power supply voltage, an amperage of 300 A for 60 minutes. Agarose gel was stained with ethidium bromide. DNA visualization was done using UV transilluminator and documented using gel documentation. The PCR product was then purified and sequenced at the 1stBase Laboratory, Singapore.

Data analysis

The nucleotide sequences were aligned with the GenBank data used the program of BLAST-N (Basic Local Alignment Search Tool Nucleotides) at the site of NCBI (National Center for Biotechnology Information) to determine the similarity of each of potential endophytic bacteria with 16S rRNA gene database that were stored in GenBank. Phylogenetic analysis of potential endophytic bacteria with other references bacteria was done by phylogenetic tree construction using Molecular Evolutionary Genetics Analysis (MEGA) 7 software (Kumar et al. 2015).

RESULTS AND DISCUSSION

Endophytic bacteria in seagrass with antibacterial activities towards MRSA

The isolation of endophytic bacteria of each seagrass species taken from Litanak Beach and Oeseli Beach, Rote Ndao District, East Nusa Tenggara, Indonesia results in 32 isolated endophytic bacteria and 8 of them have antibacterial activity against MRSA, *B. subtilis*, and *E. coli*. The result shows isolate of endophytic in seagrass has a potential as the antibacterial source against both irrisistant and resistant pathogenic bacteria. From eight potential endophytic bacteria, five of them are isolated from seagrass *E. acoroides*, two of them are isolated from *C. rotundata* and 1of them are isolated from *T. hemprichii*. The eight screened isolates of endophytic bacteria in seagrass species i.e., *E. acoroides*, *T. hemprichii*, and *C. rotundata* from Litanak Beach and Oeseli Beach, which has antibacterial activities to MRSA bacteria, *B. subtilis*, and *E. coli* were provided in Table 1.

Table 1. Seagrass endophytic bacteria which have antibacterial activity against MRSA, *Bacillus subtilis*, and *Escherichia coli*.

Isolate name	Origin of isolate (Seagrass species)	Sampling area	Clear Zone Diameter (mm)		
			MRSA	<i>Bacillus subtilis</i>	<i>Escherichia coli</i>
E2M1	<i>Enhalus acoroides</i>	Oeseli	915	119	-
E2M3	<i>Enhalus acoroides</i>	Oeseli	347	278	-
E2M4	<i>Enhalus acoroides</i>	Oeseli	875	316	-
E2M7	<i>Enhalus acoroides</i>	Oeseli	521	092	-
E2M8	<i>Enhalus acoroides</i>	Oeseli	235	065	-
C2M3	<i>Cymodocea rotundata</i>	Oeseli	167	124	-
T1M3	<i>Thalassia hemprichii</i>	Litanak	563	-	-
C1M7	<i>Cymodocea rotundata</i>	Litanak	1827	1653	1341

The difference amount of potential isolate of endophyte among three species of host plants from those two beaches caused by the difference of host plants and plant area which support endophytic bacteria in producing the antibacterial compound. The host plants are habitat for endophytic bacteria due to symbiotic mutualism as endophytic bacteria get nutrition which is the vitamin, polysaccharide and fatty acid from its host plant. On the other hand, the bacteria produce several products such as amino acid, antibiotics, and the toxin which are beneficial for growth, metabolism, and increasing chemical resistant of the host plant (Jeganathan et al. 2013). The infection due to pathogenic bacteria making the chemical resistant which have a self-protect for host plant become necessary. Thus, the antibacterial bioactive compound which produces by endophytic bacteria will be affected by host plant's condition.

Oeseli and Litianak Beaches have different characteristic. Oeseli Beach is a rocky and sandy beach; the seagrass spreads more evenly and densely if it is compared to Litianak Beach which is sandy and muddy. The seagrass density indicates the more bacteria associated with the plant, the more space available for the bacteria to associate as well as endophytic bacteria, therefore the competition for getting space and nutrition for bacteria's growth is bigger. This condition becomes one of the elements that boost the bacteria to produce bioactive compound as antibacterial which uses against the competitor.

Clear zone diameter that seen as antagonistic activity by the potential isolates between 0.65-18.27 mm (Table 1). This value is considered quite promising. For comparison, the previous research conducted by Handayani et al. (2015) shows an antagonistic activity of six strain of *Bacillus* sp. and four strain of *Corynebacterium* sp. sponge bacterial symbionts, *Haliclona fascigera* against MRSA bacteria,

shows minimum clear zone is 6.67 mm, and the maximum is 15.17 mm. Besides, Susilowati et al. (2015) find that *B. subtilis* strain, bacterial symbionts of brown algae *Sargassum* sp., shows clear zone diameter 3.9 mm to MRSA. *Virgibacillus salarius* and *Virgibacillus marismortui* strain, bacterial symbionts of *Sinularia* sp. also have activity against MRSA, and the width of a clear zone are 1.9 mm and 2.8 mm. However, both of bacteria do not have antibacterial activity against *E. coli* (Sulistiyani et al. 2010). Based on that matter, it is known that potential endophytic bacteria which found in this research have a bigger and wider antibacterial ability not only to MRSA but also Gram-positive bacteria and Gram-negative bacteria if it is compared with the other research. The kind of antagonistic bacteria used against endophytic bacteria affects the forming of the clear zone. It is found that there are one isolated bacteria which forms clear area to MRSA only, two isolated bacteria to *B. subtilis* only, six isolated bacteria to both MRSA (Figure 1) and *B. subtilis*, and one isolated bacteria forms clear zone to MRSA, *B. subtilis*, and *E. coli*. Those results indicated that antibacterial produced by the bacteria shows narrow spectrum which is only active against Gram-positive bacteria and broad spectrum which is active against not only Gram-positive but also Gram-negative bacteria. Similar research undertaken by Ravikumar et al. (2010) shows five isolated endophytic bacteria in seagrass *Syringodium isoetifolium* and *Cymodocea serrulata* have antibacterial with broad spectrum against bacteria that are *S. aureus*, *Klebsiella* sp., *Streptococcus pneumoniae*, *Streptococcus aeruginosa*, and *Pseudomonas aeruginosa*. Sulistiyani et al. (2015) also finds 1 of 9 isolated symbionts bacteria in seagrass *Enhalus* sp. that belongs to *Bacillus* sp., has antibacterial activity against bacteria of Multi Drugs Resistant Tuberculosis (MDR-TB) came from their bioactive crude extracts.



Figure 1. Clear zone showing antibacterial activity formed by seagrass endophytic bacteria (isolate E2M1, left and E2M4, right) against MRSA bacteria in overlay method, incubation overnight at 37 C

Antibacterial produced by the isolate of endophytic bacteria in seagrass in this research are suspected having different mechanism action regarding the activity against resistant bacteria such as MRSA, Gram-positive and Gram-negative bacteria. However, the mechanism involved in the activity against the bacteria as synergy from several bioactive compounds or one of the compounds is not determined yet because the bioactive compound extraction does not undertake yet to find out bioactive compound contained in antibacterial that produced by the bacteria. The previous research conducted by Darabpour et al. (2012) shows that bacteria *Pseudoalteromonas piscicida* strain PG-01 isolated from Persian Gulf waters had an antibacterial activity against MRSA. The antibacterial is bactericidal with a target to lyse cell wall of MRSA; it is known from TEM (Transmission Electron Micrographs) study on a dead MRSA bacteria. Moreover, Ling et al. (2015) have found new antibiotics named teixobactin from uncultured bacteria by using in situ method, and it can be used to overcome MRSA infection and *Mycobacterium tuberculosis* without creating resistance. Based on the 16S rRNA gene, the microorganism is *Eleftheria terrae* which belong to β -proteobacteria class. The mechanism of teixobactin regarding the activity against resistant bacteria is by obstructing cell wall synthetic by binding to lipid II (peptidoglycan precursor) and Lipid III (cell wall of the teichoic acid precursor) of the bacteria.

Identity of endophyte bacteria based on 16SrRNA encoding gene

The amplicon of 16S rRNA gene is analyzed by electrophoresis of agarose gel 1% (c/v) and it is known that 16S rRNA gene fragment size is \pm 1300 bp. The electrophoregram is provided in Figure 2.

The determination of potential endophytic bacteria is based on 16S rRNA gene sequence similarity percentage \geq 99% indicated same species, similarity percentage \geq 95% to $<$ 99% indicates same genus, and similarity percentage $<$ 95% indicates same family (Bosshard et al. 2003). Based on those criteria, it is known that six of eight isolated bacteria have a low similarity, they are E2M1, E2M3, E2 4, E2M8, T2M3, C2M3, C1M7, and T1M3. Moreover, two isolates of potential endophytic bacteria having high similarity $>$ 95% they are E2M4 and E2M7 (Table 2).

The low similarity indicates there is the possibility that the six isolate of potential endophytic bacteria is new bacteria. According to Pangastuti (2006), usually if the similarity degree of the sequencing of 16S rRNA gene less than 97%, it can be considered as a new species. Stackebrandt and Goebel (1995) also state that if the sequencing of 16S rRNA gene shows low similarity degree between two taxa, a definition of a taxon can be undertaken. In this research, the seagrass endophytic bacteria are successfully isolated. Moreover, it is discovered the identity is isolated seagrass bacteria and unexplored habitat as the bioactive source before, which is Litanak and Oeseli Beaches, Rote Ndao District, East Nusa Tenggara. It becomes one of low similarity factors in BLAST-N test by comparing isolate sequencing of 16S

rRNA gene to GeneBank database. Moreover, that factor raises a presumption the existence of new bioactive compound from new bacteria found and it can be used to fight against resistant pathogenic bacteria. Environment factors as geographic and host plant type affects the variation of symbiont bacteria and the bioactive compound produced. According to Brader et al. (2014), the ability of endophytic bacteria in producing bioactive antibacterial compound are affected by the host plant's environment, as in very competitive environment and have many predators. Thus bacteria tend to produce bioactive compound as itself-protector.

The blast-n analysis shows most of the potential endophytic isolates, as E2M1, E2M3, E2M4, E2M7, and E2M8 are identified have similarity sequencing of 16S rRNA gene with bacterial genus *Bacillus* as *Bacillus megaterium*, *Bacillus flexus*, and *Bacillus* sp. Bacterial symbionts from genus *Bacillus*, *Pseudomonas*, *Pseudoalteromonas*, and *Paracoccus* are known having high antibacterial activity (Ali et al. 2012). According to Stein (2005), genus *Bacillus* is an active antibacterial producer. Gram-positive bacteria knew its ability forming spore in a severe condition, as encountered in marine ecosystems, for secure its life in plants or animals cells which are its habitat (Hentschel et al. 2001). According to Marahiel et al. (1993), spore-formation is regulated simultaneously with antibiotic production. The bioactive compound produced by bacteria genus *Bacillus* isolated from the deep-sea environment are different with the terrestrial one (Sulistiani et al. 2015). The species from that genus produces bioactive compound lipopeptide cyclic, peptide cyclic, and thiopeptide (Nagai et al. 2003). Several *Bacillus* spp. which has been isolated are reported having high antibacterial activity against fouling bacteria (Burgess et al. 2003). *Bacillus pumilus* sponge symbionts *Ircinia* sp., produces surfactin, acyldepsipeptide (ADEP), bacircine 2, 3, 4, 5, and 5A (Itokawa et al. 1994). Related research also conducted by Susilowati et al. (2015) which has isolated brown algae bacteria symbionts that are *B. subtilis* and it has an ability to obstruct pathogenic bacteria like MRSA and *Staphylococcus epidermidis*.

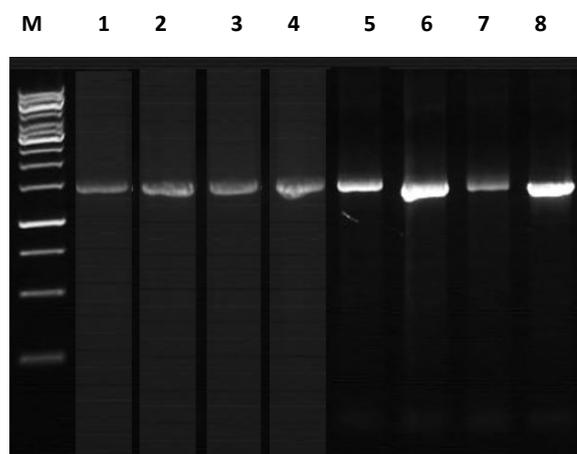


Figure 2. Electrophoregram 16S rRNA gene amplicon of eight potential endophytic isolated bacteria M= Marker DNA ladder 1 kb, 1= E2M1, 2= E2M3, 3= E2M4, 4= E2M7, 5= E2M8, 6= C2M3, 7= C1M7, 8= T1M3

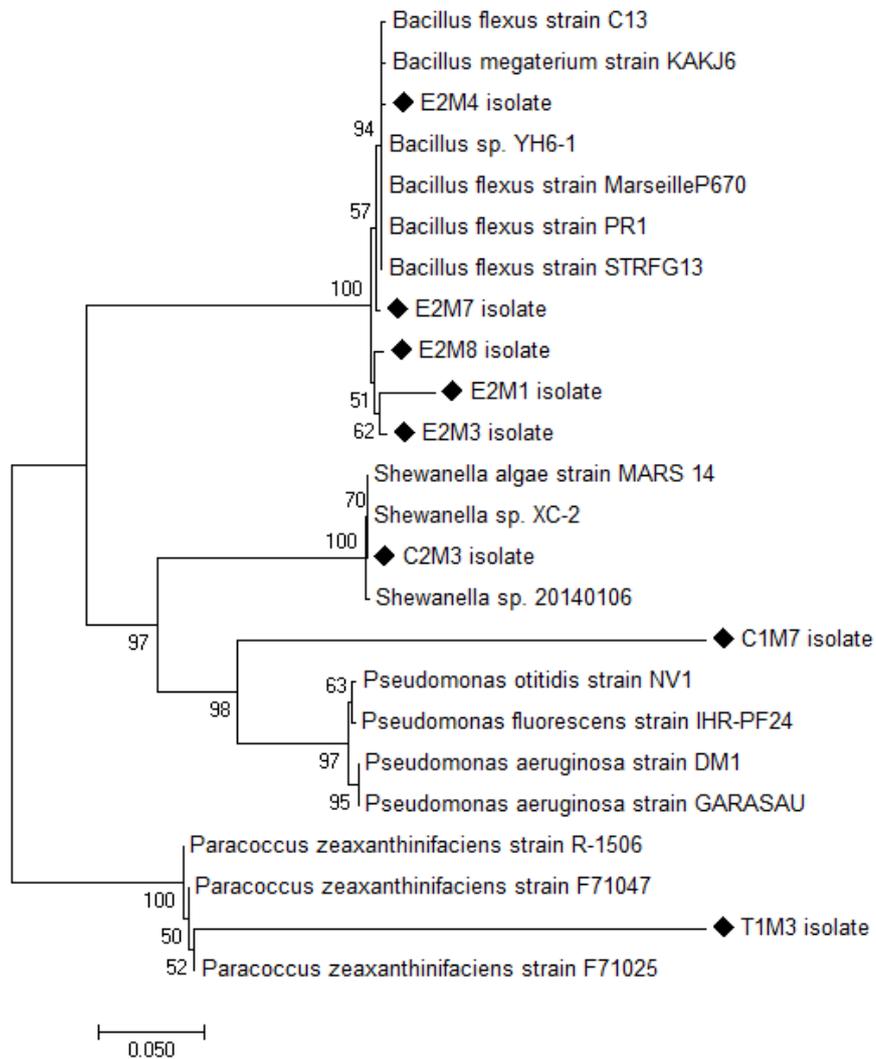


Figure 3. The phylogenetic relationship of potential endophytic bacteria and the nearest species of bacteria based on 16S rRNA gene sequence by using Neighbor Joining (NJ) methods. Isolate E2M1, E2M3, E2M4, E2M7, E2M8, C2M3, T1M3, and C1M7 (indicated by diamond shape) are potential endophytic bacteria; *Bacillus megaterium* strain KAKJ6, *Bacillus sp.* YH6-1, *Bacillus flexus* strain C13, *Bacillus sp.* BAB-4161, *Bacillus flexus* strain KJ1-5-982, *Bacillus flexus* strain PR1 are the nearest species of isolate E2M1, E2M3, E2M4, E2M7, and E2M8; *Shewanella algae* strain MARS 14 are the nearest species of isolate C2M3; *Pseudomonas otitidis* strain NV1, *Pseudomonas fluorescens* strain IHR-PF24, *Pseudomonas aeruginosa* strain DM1, *Pseudomonas aeruginosa* strain GARASAU are the nearest species of isolate C1M7; *Paracoccus zeaxanthinifaciens* strain F71025, *Paracoccus zeaxanthinifaciens* strain F71047, *Paracoccus zeaxanthinifaciens* strain R-1506 are the nearest species of isolate T1M3

Table 2. Similarity of the 16SrRNA gene sequence of potential endophytic bacteria using BLAST-N (Basic Local Alignment Search Tool-Nucleotides) at the NCBI (National Center for Biotechnology Information)

Isolate name	Nearest species	% Similarity	Access no.	Potential endophytic bacteria identity
E2M1	<i>Bacillus megaterium</i> strain KAKJ6	91	LC085342.1	Bacillaceae E2M1
E2M3	<i>Bacillus sp.</i> BAB-4161	93	KJ794118.1	Bacillaceae E2M3
E2M4	<i>Bacillus flexus</i> strain Marseille-P670	98	LT223626.1	<i>Bacillus</i> E2M4
E2M7	<i>Bacillus flexus</i> strain PR1	97	KP889216.1	<i>Bacillus</i> E2M7
E2M8	<i>Bacillus flexus</i> strain STRFG 13	94	KT893317.1	Bacillaceae E2M8
C1M7	<i>Pseudomonas aeruginosa</i> strain DM1	76	KF952773.1	Pseudomonadaceae C1M7
C2M3	<i>Shewanella algae</i> strain MARS 14	94	LN795823.1	Shewanellaceae C2M3
T1M3	<i>Paracoccus zeaxanthinifaciens</i> strain F71025	80	JF281760.1	Rhodobacteraceae T1M3

Moreover, C1M7 isolate has a similarity with bacteria family Pseudomonadaceae. *Pseudomonas* belongs to Gram-negative bacteria, non-fermentative, was found live in terrestrial and also on the deep-sea environment. *Pseudomonas* from deep-sea produces bioactive such as pyrroles, pseudopeptide, pyrrolidinedione, phthalate, andrimid, moiramides, zafrin, and bushrin, and also several bioactive components as antibacterial agents (Isnansetyo and Kamel 2009). Some species of that genus have been reported have antibacterial activity both against resistant bacteria and the irrisistant one such as *Pseudomonas bromoutilis* (Burkholder et al. 1966), *P. aeruginosa* (Darabpour et al. 2010), and *Pseudomonas fluorescens* (Needham et al. 1994).

C2M3 has similarity with *Shewanella algae*. Nowadays, Shewanellaceae only has one genus that is *Shewanella* (MacDonell and Colwell 1985). *S. algae* is an anaerobe facultative bacteria which mostly has stem shape, Gram-negative, with oxidase-positive, and it is usually found in the deep-sea environment all over the world. This group of bacteria can be found in various sources such as active mud, sea invertebrate, red algae, seawater, sediment, and clinical sample (Lee et al. 2006). *S. algae* which are isolated from *Callyspongia diffusa* sponge has a high antibacterial activity to human and fish pathogenic bacteria as *E. coli*, *E. cloacae*, *P. vulgaris*, *Salmonella typhi*, and *Vibrio* sp. (Rachanamol et al. 2014).

However, T1M3 isolate has similarity with *Paracoccus zeaxanthinifaciens*. *Paracoccus* genus is Gram-negative bacteria short basil shaped, oxidase-positive and catalase-positive. Genus *Paracoccus* is from Rhodobacteraceae family and consists of 30 species. Members of genus *Paracoccus* can be isolated from soil, active mud, plant root, animal, and sea plants (Chen et al. 2011). The members of *Paracoccus* produce a beneficial metabolism for human as carotenoid zeaxanthin which is produced by *P. zeaxanthinifaciens*, and it has a significant role in preventing macular degeneration regarding aging which is the primary cause of blindness (Sajilata et al. 2008).

Phylogenetic relationship based on 16S rRNA gene sequence

The phylogenetic relationship of potential endophytic bacteria of seagrass in this research and nearest species depicted in Figure 3. The construction of phylogenetic tree or known as dendrogram conducted using Molecular Evolutionary Genetics Analysis (MEGA) program version 7.0 (Kumar et al. 2015) by using Neighbor Joining (NJ) methods (Saitou et al. 1987).

According to a phylogenetic tree, isolated bacterial E2M1, E2M3, E2M4, E2M7, and E2M8 have a very close phylogenetic relationship since clustering in one group. Those six of the endophytic bacteria were isolated together from the same seagrass *E. acoroides* which came from Oeseli Beach, and it belongs to genus *Bacillus*, the Bacillaceae family. Those six potential isolated endophytic bacteria have the furthest phylogenetic relationship with isolate T1M3 which is indicated as Rhodobacteraceae family. T1M3 is isolated from different species of seagrass and different growing area that is *T. hemprichii* from

Litianak Beach. Isolate C2M3 which belongs to Shewanellaceae family has near phylogenetic relationship and belongs to the same group with isolated C1M7 which belongs to Rhodobacteraceae even though each isolate are isolated from the different growing area and different species of seagrass. It is because the three group of the isolates belong to Gram-negative bacteria.

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