



The Occurrence of *Bacillus cereus* in the Pink Line Syndrome Infected *Porites lutea* Coral

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 12-08-2018
Accepted: 09-12-2018

Key Words:

Bacillus cereus
Coral diseases
Kondang Merak
Pink line syndrome
Porites lutea

ABSTRACT

Corals in the genus *Porites* are among the most dominant scleractinian corals in the coral reef ecosystems yet highly vulnerable to climate change impacts and diseases. In the Kondang Merak and Sempu Strait waters of Indian Ocean, it has been reported that more than 45% of the corals were infected with Pink Line Syndrome (PLS). The objectives of this study are to isolate, morphologically and molecularly characterize, and identify the PLS associated bacteria that infected *Porites lutea* coral. The sampling was performed at the Kondang Merak waters in June 2018 by snorkeling during low tide. In order to isolate and purify the dominant bacterial colony, quadrants streak-plating technique was performed using ZoBell marine agar culture media. 16S rRNA sequencing and BLAST homology were performed for the molecular identification of the bacterial colony. Results showed that the bacterial colony associated with PLS was of *Bacillus cereus*, which was closely related to *Bacillus pseudomycoloides*, *Bacillus toyonensis* and *Bacillus thuringensis* with 97% similarity. The occurrence of *B. cereus* bacterium in the PLS infected *P. lutea* demonstrated that this opportunistic pathogen might be responsible for the PLS in the *P. lutea* coral.

INTRODUCTION

Coral reefs are among the most productive and diverse ecosystems on earth, second only to tropical rainforests. They play an important role in providing spawning ground and shelter to a wide range of marine organisms (Veron et al. 2009). Coral reefs also protect shorelines, regulate carbon dioxide, and support local and global economies via fishing, recreation and tourism (Asadi & Andrimida 2017, Elliff & Kikuchi 2017, Luthfi 2017).

However, the health of these reefs has been declining over the past 50 years due to accumulation of stresses driven by anthropogenic threats such as destructive fishing practice, oil slicks, sedimentation, and organic pollution (Wear 2016). Moreover, the relentless rise of carbon dioxide induces the increase of sea surface temperature and the decrease of ocean pH, which in turn elevates the collapse of coral reef ecosystems (Asadi & Khoiruddin 2017, Bruno 2013, Orr et al. 2005). These factors trigger coral diseases and subsequently elevate the decline of abundance and diversity of coral reef ecosystems (Caldwell et al. 2016, Wear 2016).

The outbreaks of coral reef diseases and syndromes were initially widespread in the Caribbean, affecting some

scleractinian corals. The geographic distribution of coral diseases was then widespread in the Indo-Pacific coral reefs (Weil et al. 2009). In Ryukyu archipelago, pink block disease (PBD) infected *Porites* corals, and was also likely associated with the same disease that affected massive *Porites* in Hawaii (Weil et al. 2012). In Indonesia, the abundance and distribution of white syndrome (WS) and black band diseases (BBD) had been recorded in Kepulauan Seribu Marine National Park (Johan et al. 2015). In Pramuka Island, Jakarta, a total of 61.32% of Fungiidae was infected with coral diseases (Subhan et al. 2011).

Furthermore, study on Kondang Merak waters of Indian Ocean revealed that 47% of *P. lutea* was infected with PLS (Asadi et al. 2017b). It also infected scleractinian corals in many areas from the Caribbean to the Indo-Pacific coral reefs (Ravindran & Raghukumar 2006, Weil et al. 2012). However, the study of PLS associated bacteria is still scarce. Therefore, this study aimed to identify the dominant bacteria associated with PLS in Kondang Merak waters using molecular characterization as well as morphological observation of the bacteria isolates.

MATERIALS AND METHODS

Sampling and description of the study sites: Surveys and

sample collections of *P. lutea* infected with PLS were performed on the Kondang Merak waters (8°23'50.74"S, 112°31'6.00"E), which is located on the southern Malang's coast of Indian Ocean. The sampling area is a shallow reef flat that is often exposed during low tide. The sample collection was performed using snorkeling in June 2018. The sampling map is presented in Fig. 1.

Furthermore, in brief, fragments of *P. lutea* infected with PLS were carefully separated using a hammer and sterilized chisel and directly placed separately into polyethylene plastic bags filled with sterilized seawater to avoid contact with air. The coral samples were stored in a cooler box containing ice and directly carried to the laboratory for further laboratory experiments.

Bacteria isolation and purification: In the laboratory, the coral fragments were sprayed with autoclaved seawater to remove any material that might attach on the coral surface. The coral tissues that contained PLS were then scrapped using an ethanol sterilized scalpel blade. In order to suspend the tissues, each 1 gram of the coral tissues was diluted with 9 mL of sterilized seawater and mixed thoroughly using a vortex mixer. The resultant of PLS infected coral tissues were then fivefold serially diluted. In a triplicate, 500 µL of the resultant was spread on ZoBell 2216E marine agar using quadrants streak-plating technique and incubated for 7 days at 35-37°C. Based on the morphological feature, the dominant colony was selected for bacterial purification using agar slant tubes. The purified colony sample was then

incubated for 96 hours at 35-37°C. After the incubation, the colony was harvested, vortexed, and inoculated using marine broth (ZoBell 2216E) for 48 hours for the molecular identification (Asadi et al. 2017a, Sanders 2012).

Molecular identification: Using an inoculating loop, approximately 1 µL of the bacterial colony was picked off the tube and put into Eppendorf in which 100 µL of double-distilled water was previously poured into each Eppendorf. The sample was vortexed and subjected to five cycles of freezing (-80°C) and thawing (-95°C), and Chelex-100 from BioRad was then used for the bacterial extraction (Silva et al. 2012). 27F universal primers (5'- AGAGTTTGATCM TGGCTCAG-3') and specific primer 1492R (5'-TACGG YTACCTTGTT ACGACTT-3') were then used for 16S rRNA PCR amplification. The procedures were followed by sequencing of 16S rRNA gene fragment and BLAST homology analysis using MEGA 5.7 software (Asadi et al. 2017a, Weisburg et al. 1991).

RESULTS AND DISCUSSION

PLS infected coral sample and morphological observation of the bacteria isolates: *P. lutea* is a dominant scleractinian coral in Kondang Merak waters. The coral colonies are widespread across this intertidal shallow reef-flat environment. The corals are often in massive size and form microatoll that grow laterally up to 3 meters in diameter. As response to environmental variabilities, *P. lutea* in this reef-flat often has a distinct colour from yellowish in the

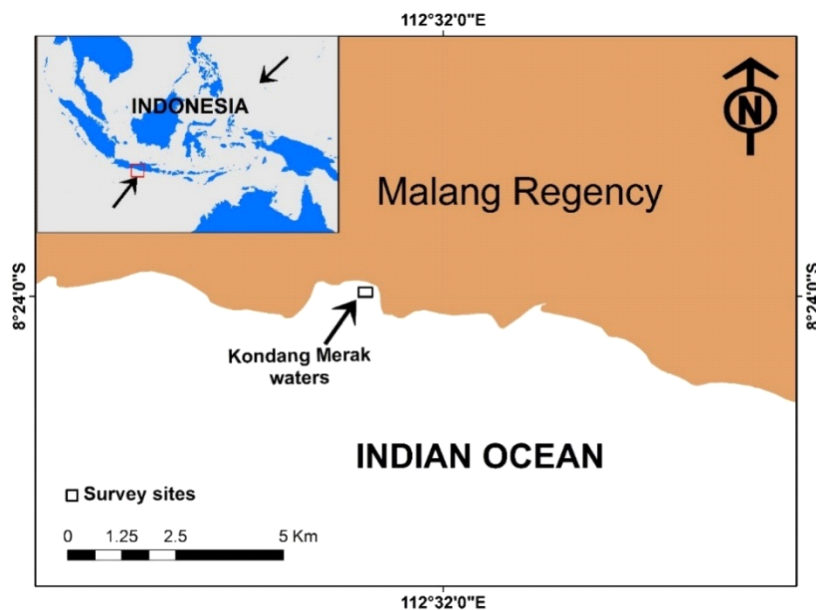


Fig. 1: The map of research stations, the Kondang Merak waters.

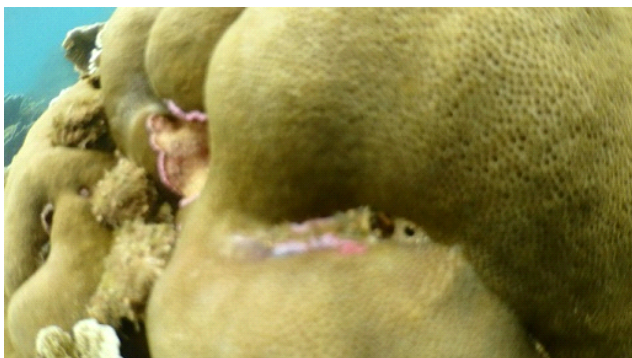


Fig. 2: PLS infected *P. lutea* of Kondang Merak waters. This PLS infected coral tissues was used for the study.

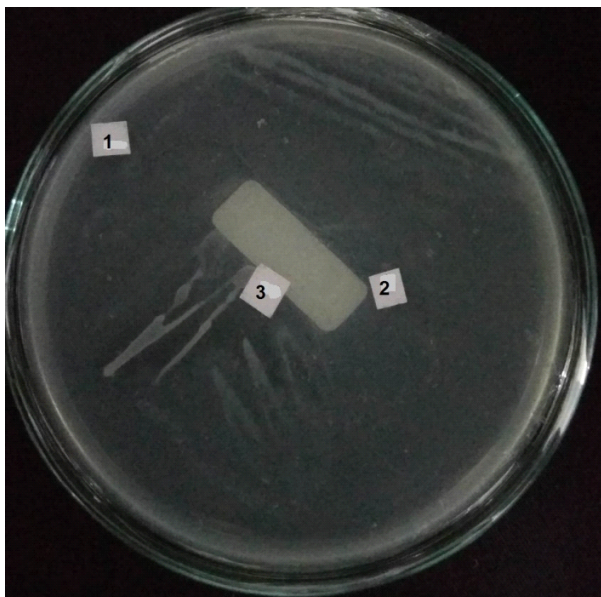


Fig. 3: Bacterial colonies of PLS-infected *P. lutea* inoculated using quadrants streak-plating technique.

shallower areas to brownish in the deeper areas. These colour patterns may occur due to the pigment and protein of algae that interact through interspecific cooperation or symbiosis mutualism with the coral tissues (Dove et al. 2001, Luthfi et al. 2016).

The unique characteristic of the reef-flat of Kondang Merak has been threatened due to tourism activities as well as coral diseases that has infected many *P. lutea* colonies in the area. It had been reported that 47% of *P. lutea* colonies were infected with PLS (Asadi et al. 2017b). Physical and other stressors may trigger this pigmentation responses, which are often characterized by dead tissue areas bordering by pink ring-shaped lines and are surrounded by healthy coral tissues (Fig. 2).

Table 1: Morphological characteristic of the PLS associated bacterial colony based on the quadrants streak-plating technique.

Code	Size	Shape	Elevation	Margin
P.D1	Small	Irregular	Raised	Entire
P.D2	Pinpoint	Circular	Raised	Entire
P.D3	Moderate	Irregular	Raised	Lobate

The pigmentation is a response mechanism of illness condition that may be influenced by the elevation of $p\text{CO}_2$ close to the polyp tissues through cyanobacterial carbon concentration mechanism that triggers the presence of mucus or cyanobacterial film. It excretes toxins and leads competition for the DO, which in turn cause hypoxia to the coral polyps during the night-time (Yellowlees et al. 2008).

Furthermore, during isolation and purification of the PLS associated bacteria, the colony morphology of the bacteria isolates was observed for their size, shape, elevation and margin. Bacteria cells in the same colony in agar plate were originated from a single mother cell and, therefore, it represents a single and pure isolate. Bacterial colony with P.D1 code was chosen for the molecular identification. The size of the colony is small with irregular shape, raised elevation, and entire margin. The morphological characteristics and appearance of the PLS associated bacterial colonies are presented in Table 1 and Fig. 3 respectively.

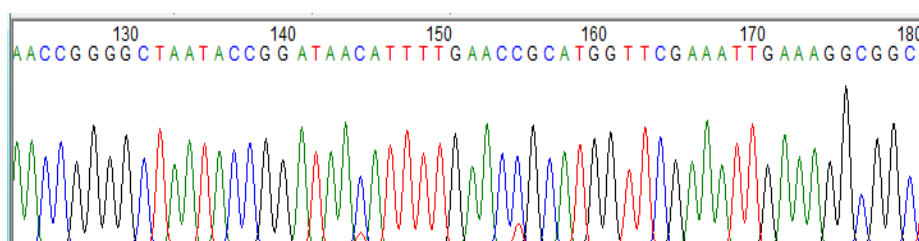
Molecular identification: In order to obtain sequence data, the bacterial isolate was identified by sequencing the 16S rRNA genes. The genes are reliable for phylogenetic reconstruction because the genes of the region have slow rates of evolution (Smit et al. 2007). 16S rRNA sequences are also demonstrated to have similar functionalities with distantly related bacterial lineages and therefore 16S rRNA gene provide species-specific signature sequences and has function as a reliable molecular clock (Case et al. 2007).

16S rRNA approach and implementation are useful to explore phenotype trait of the bacteria. It increases the insight on the biochemical, genetic, physiological and molecular properties of coral bacteria (Sabdono et al. 2015). The length of the DNA fragments was visualized using a plot of DNA sequencing or an electropherogram. In order to reduce the uncertainty of the sequences, MEGA 5.7 software was used to remove gaps and noises by editing the forward and reverse of the sequences. A BLAST program was used to compare the data to NCBI (National Center for Biotechnology Information) database and to calculate the statistical significance. The electropherogram of the sequence data and the top 10 Hit BLAST result against NCBI Database are presented in Fig. 4 and Table 2 respectively.

The BLAST program compared the nucleotide of PLS

Table 2: The top 10 Hit BLAST result against NCBI database of PLS associated bacteria sequences.

NCBI Code	Species	Max score	Total score	Query cover	E-value	Similarity
NR_074540.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_113266.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_115714.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_112630.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_114582.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_115714.1	<i>Bacillus cereus</i>	1686	1729	79%	0.0	97%
NR_113991.1	<i>Bacillus pseudomycooides</i>	1971	1741	79%	0.0	97%
NR_113991.1	<i>Bacillus toyonensis</i>	1663	1706	79%	0.0	97%
NR_114581.1	<i>Bacillus thuringensis</i>	1663	1706	79%	0.0	97%
NR_041248.1	<i>Bacillus anthracis</i>	1663	1663	68%	0.0	97%

Fig. 4: The sequencing electropherograms of PLS associated bacteria on *P. Lutea*.

associated bacteria to sequence databases of bacteria on NCBI database and calculates the statistical significance. The PLS associated bacteria was identified as *Bacillus cereus* and closely related to *Bacillus pseudomycooides*, *Bacillus toyonensis* and *Bacillus thuringensis* with 97% similarity.

A study using a multilocus sequence typing analysis suggests that *B. cereus* is highly diverse and ubiquitously found in marine environments and some of *B. cereus* bacteria demonstrated niche specificity to some extent (Liu et al. 2017). *B. cereus* groups have tremendous contributions for removal of persistent organic pollutants and various heavy metals (Chen et al. 2016), production of numerous metabolites and enzymes (Kevany et al. 2009). Yet, some of the group bacteria are opportunistic pathogen and responsible for serious invasive infections (Kotiranta et al. 2000).

B. cereus bacterium isolated from the PLS affected *Porites* is a large, 3-4 μm , marine strain that is facultative aerobic bacterium and capable of producing endospore, formed when nutrients are scarce. The ability to form biofilms on the coral surfaces and the facultative aerobic characteristic of *B. cereus* elevate pCO_2 leading to oxidative stress and hypoxia to the corals (Majed et al. 2016, Yellowlees et al. 2008). Moreover, the synergistic interaction of elevated temperature and pCO_2 is likely to increase the growth of the opportunistic pathogen bacterium *B. cereus*, leading to the collapse of the coral reef's ecosystem (Hoegh-Guldberg 2005)

CONCLUSION

Based on the molecular study, it was revealed that the bacterium associated with PLS in Kondang Merak waters was identified as *B. cereus*. The bacterium was closely related to *B. pseudomycooides*, *B. toyonensis* and *B. thuringensis* with 97% similarity. *B. cereus* has never been reported to be associated with PLS; therefore, this finding gives a better understanding of the ecology of bacterium that may cause the PLS on *Porites* coral. *B. cereus* as opportunistic pathogen in many environments could also be a serious invasive bacterium in *P. Lutea* coral.

ACKNOWLEDGEMENT

This work was supported by grants from the University of Brawijaya (contract number: 712.13/UN10.C10/PN/2018). The authors acknowledge the laboratory and field work support from Miranti Herdiutami, Arizal Mahendra, Rifqi Novakandi.

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