Preliminary study of myxobacteria as biocontrol agents for panama disease pathogen, tropical race 4 *Fusarium odoratissimum*

S Meliah*, D I Kusumawati and M Ilyas

Research Center for Biology, Indonesian Institute of Science, Cibinong Science Center, Jalan Raya Jakarta-Bogor Km. 46 Cibinong 16911, Indonesia

*Corresponding author: siti.meliah@lipi.go.id

Abstract. Newly described pathogenic fungal *Fusarium odoratissimum* is known to cause severe panama disease on banana and grouped as tropical race 4. The disease constrains banana production throughout the world with no known method to completely manage the disease. This study assayed the ability of myxobacteria to control the pathogen. Myxobacteria were isolated from soil, karst limestone, and decaying wood in Karimun and Simeuleu islands using filter paper and baiting methods. A total of 20 myxobacterial isolates were collected during the process. Based on 16S rRNA gene analysis, they were identified as *Myxococcus*, *Archangium* and *Corallococcus*. To our knowledge, this is the first record of the last two genera in Indonesia. Antifungal assay of two myxobacterial isolates *Corallococcus* KR39b.5 and SLU3.3 extracted using ethyl acetate against *F. odoratissimum* InaCC F936 and *F. odoratissimum* InaCC F946 revealed that both were capable of inhibiting the fungal growth up to 40%. The results suggested the possibility of utilizing myxobacteria as biocontrol agents against pathogenic *F. odoratissimum*.

Keywords: Biocontrol, *Fusarium*, Myxobacteria, Panama disease, TR 4

1. Introduction
Panama disease, also known as *Fusarium* wilt is one of the most virulent and destructive disease on banana. *Fusarium* wilt on banana mainly caused by the pathogenic soil-borne fungi *Fusarium oxysporum* f.sp. *cubense* (Foc) [1, 2]. In 1890s, *Fusarium* wilt was reported to be endemic in the Panama region, until in the 1950s and 1960s the disease was outbreak and destroyed the banana plantations of Gros Michel varieties in Central America and the Caribbean [1, 3]. Thus, in less than 20 years, phytopathogenic Foc was reported infected and devastated almost all of the commercial banana cultivars both in the tropical and subtropical banana plantations in the world[1-3].

In 1990s, the new group of Foc, tropical race 4 (Foc-TR 4) was emerged [4, 5]. At that time, Foc-TR4 devastated the banana plantation of Cavendish cultivar in Southeast Asia, and up until now Foc-TR4 has spread rapidly in the continents of Asia (China, Indonesia, Malaysia, Philippine, Taiwan, Pakistan, Oman, Jordan, Lebanon), Africa (Mozambique), and Australia (Northern territories and Queensland) [2, 4, 5]. *Fusarium odoratissimum*, a new species of *Fusarium* originate from Indonesia, was reported to cause Panama disease TR4 of Cavendish and Gros Michel banana varieties [6].

Pathogenic Foc is difficult to eradicate because of their ability to produce persistent chlamydospores that keep their viability in the soil for decades [7]. Presently there are only limited...
effective methods that have been implemented for controlling the Foc disease [8]. However, biological control integrated with technical control become environmentally sound approach which is suitable to be applied [9]. The biological control could be implemented through introducing the antagonistic agents, introducing natural parasitic or virulent agents, and using endophytic microorganisms to induce host plant resistance systems [9]. One of the promising microorganisms to be developed as biocontrol agents is myxobacteria [10].

Myxobacteria are Gram-negative bacteria, belong to the class δ-proteobacteria [11], and can be found everywhere in soils [12]. Myxobacteria interact and communicate with each other within a swarm [13], move by gliding over agar surfaces and across glass without flagella [14, 15], form myxospores and fruiting body sowing to lack of nutrients [12], and form biofilm [16]. Studies reported the ability of myxobacteria to produce a wide range of antibiotics [15, 17, 18], and lytic enzymes or secondary metabolites [19]. Antibiotics and enzymes produced by myxobacteria are capable of killing their prey and fungal plant pathogen [20]. The role of myxobacteria as biocontrol agents against plant pathogen, particularly Fusarium have been reported by several studies [20-22]. However, the study concerning myxobacteria against F. odoratissimum has not been conducted. Therefore, this research was conducted to isolate of myxobacteria and investigate their ability to control F. odoratissimum the causal agents of fusarium wilt on banana.

2. Materials and methods

2.1. Samples and fungal strains
Soiland decayed wood samples were collected from various places in Great Karimun and Small Karimun Islands, Riau Archipelago as well as Simeuleu Island, Nangroe Aceh Darussalam. Prior isolation procedure, all of the soil samples were air dried for a couple of hours. A collection of fungal strains known as the causal agent of Fusarium Wilt on Banana was used for antifungal assay (table 1).

| Strain          | Origin       | Host          | Pathogenicity | Reference |
|-----------------|--------------|---------------|---------------|-----------|
| F. odoratissimum| Maros, Sulawesi | Musa sp. (Pisang) | TR4          | [6]       |
| InaCC F936      |              |               |               |           |
| F. odoratissimum| Sikka, Flores | Musa sp. (Pisang) | TR4          |           |
| InaCC F946      |              | Barangan, AAA |               |           |

2.2. Isolation of myxobacteria
The isolation of myxobacteria was conducted using baiting method with Escherichia coli on Water Agar media and using sterilized filter paper Whatman No. 1 on Stan 21 agar media as described by Reichenbach and Dworkin [23]. The samples were incubated at 30°C for one to three months. Orange fruiting bodies on isolation media observed under dissecting microscope were transferred to VY/2 agar media using sterilized syringe needle for purification. Pure cultures obtained were stored in 10% glycerol solution at -80°C

2.3. Molecular identification of myxobacteria
Molecular identification was conducted based on 16S rRNA gene analysis. Genomic DNA was obtained by boiling bacterial cells in 20 μL nuclease free water at 98°C for 10 minutes. Cell fragments were then quickly spun down and the upper phase was used as template for polymerase chain reaction (PCR). PCR was carried out in a total volume of 25 μL consisting of 12.5 μL GoTag Green MasterMix (Promega), 0.5 μL 10 μM of each eubacterial primer 27F and 1492R [24], 0.5 μL dimethyl sulfoxide (DMSO), and 1 μL template DNA. Amplification of 16S rRNA gene was performed under the following conditions: pre denaturation at 94°C for 90 seconds, subsequently followed by 35 cycles
of denaturing at 94°C for 30 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 90 seconds, and final extension at 72°C for 10 minutes.

16S rRNA gene sequences were analyzed using BioEdit program [25]. They were subsequently aligned against sequences of type strains in EzBioCloud server (https://www.ezbiocloud.net) [26]. Phylogenetic position of myxobacterial strains was confirmed by phylogenetic tree constructed using neighbor-joining method and Kimura 2-parameter model implemented in MEGA 6 program [27].

2.4. Antifungal assay of myxobacterial strains
Two myxobacterial isolates were subjected to antifungal assay against pathogenic F. odoratissimum. The isolates were cultured in 100 mL Casitone-Yeast Extract medium (CYB; 3 g Casitone, 1.36 g CaCl₂, 1 g yeast extract, 1 L distilled water; pH 7.2) at 25°C for 7 days. The culture was centrifuged at 10,000 rpm for 10 minutes to obtain supernatant, which was subsequently extracted using acetyl acetate in the same volume. The extract was evaporated in 40°C and then the concentrated extract was diluted with 1 ml acetyl acetate. A total of 40 µL of each myxobacterial extract was dropped gradually on the paper disk and air dried. The disk was then exposed to ultraviolet light for about an hour.

Fungal strain was inoculated on the center of Malt Extract Agar (Oxoid). Tested myxobacterial extracts were placed on both side of targeted fungal strain. Acetyl acetate combined with fungal strain anduntreated fungal plate were employed as control. The assay plate was incubated for 6 days at 30°C. This procedure was conducted two times.

3. Results and discussion
Isolation techniques using cellulose membrane and E. coli successfully obtained 20 bacterial isolates having similar phenotypic characteristics with myxobacteria group. The isolates formed swarming colonies and orange to brownish orange fruiting bodies. These fruiting bodies were formed relatively faster on cellulose membrane than on water agar media supplemented with E. coli. They were also easier to collect for direct purification process. This resulted in more myxobacteria cells being recovered from the samples.

In this research, a total of 16 isolates were isolated using filter paper composed by cellulose. They were obtained from decaying wood, soil and karst limestone samples. Meanwhile, as many as 4 isolates were isolated using E. coli strain that treated as prey to lure myxobacteria from the soil sample (table 2). Soil and other terrestrial samples are typical habitat of myxobacteria. A major amount of myxobacteria species were isolated from terrestrial habitat from compost [28] and neutral soils [29] to saline-alkaline soils [30].

The isolates were able to grow well on VY/2 medium contained yeast cells and produce fruiting bodies or swarm colonies. Some isolates produced conspicuous fruiting bodies, big enough to be seen without microscope assistance. They were spherical in shape and solitary. Some isolates produced brownish orange fruiting bodies. Observation under dissecting microscope showed that these fruiting bodies were arranged in clustered. However, not all the recovered isolates in this research produced a distinct fruiting body (table 3). The ability of myxobacteria to form fruiting bodies tends to decrease after several time of transferring process. Some myxobacteria colonies, such as Archangium gephyra and Pyxidicoccus fallax [31] are also reported to produce no fruiting bodies at all.

Based on analysis of 16S rRNA gene sequences, the isolates belong tosuborder Cystobacterinae of myxobacteria group. The length of gene sequences aligned in EzBioCloud server ranged from 1292 to 1378 bp and the percentage of similarity with myxobacteria members ranged from 98.5% to 99.8%. Furthermore, the phylogenetic tree comprised of these twenty isolates exhibited that they were grouped into three genera of myxobacteria, namely Corallococcus, Archangium, and Myxococcus (figure 1). Ten isolates revealed to be closely related to C. exigus, two isolates were closely related to C. coralloides, and one isolate was closely related to C. macrosporus.

Lane and Stackebr separated C. macrosporus from the genus Corallococcus and proposed them to be a member of the genus Myxococcus based on their differences in morphology, consistency of their swarms and fruiting bodies [32]. Taxonomical position of C. macrosporus in the constructed
phylogenetic tree showed that the species was clustered in the genus Myxococcus. Therefore, this present study clearly supports those previous results.

Table 2. Bacterial isolates collected from various samples in Karimun and Simeuleu Islands.

| Isolation technique | Sample source          | Origin                     | Isolate   |
|---------------------|------------------------|----------------------------|-----------|
| Decaying wood       | Mount Betina, Great Karimun | KRDw11.4, KRDw11.5         |           |
|                     | Ex-mining area, Great Karimun | KRDw14.1, KRDw14.3         |           |
|                     |                        |                            | KRDw14.4  |           |
| Cellulose membrane  | Soil                   | Alafan, Simeuleu           | SLU2.3    |           |
|                     |                        |                            | SLU2.6    |           |
|                     |                        |                            | SLU2.7    |           |
|                     |                        |                            | SLU2.8    |           |
|                     |                        |                            | SLU2.9    |           |
|                     |                        |                            | SLU2.14   |           |
| Karst limestone     | Air Pinang, East Simeuleu | SLU3.2, SLU3.3, SLU3.6, SLU3.7, SLU3.8 |           |
| Baiting with E. coli | Soil                   | Ex-mining area, Great Karimun | KR39b.1   |           |
|                     |                        |                            | KR39b.2   |           |
|                     |                        |                            | KR39b.3   |           |
|                     |                        |                            | KR39b.5   |           |

Table 3. Molecular identification of isolates based on 16S rRNA gene analysis.

| Closest relative (EzBioCloud) | Number of isolates | Phenotypic characteristics                                      |
|-------------------------------|--------------------|-----------------------------------------------------------------|
| Corallococcus exigus           | 10                 | Clustered and orange brownish fruiting bodies                   |
| Corallococcus coralloides      | 2                  | Clustered and orange brownish fruiting bodies                   |
| Corallococcus macrosporus      | 1                  | Orange brownish fruiting bodies                                 |
| Archangium gephyra             | 1                  | Translucent swarming colony                                     |
| Myxococcus stipitatus          | 6                  | Spherical and orange fruiting bodies                            |

It is also revealed that one isolate was closely related to A. gephyra. So far, there is no report regarding both Corallococcus and Archangium genera from Indonesian samples. As a matter of fact, the information about myxobacteria in general from Indonesian samples are insufficient. Hence, we suggest that this is the first record of their occurrence in Indonesia. Meanwhile, six isolates were indicated to be closely related to M. stipitatus. The occurrence of this species in Indonesia was first reported from soil samples in Papua [33].

Among domain bacteria, myxobacteria are one of the most prominent groups capable of producing secondary metabolisms with antimicrobial activities. Sorangium cellulosum, known as cellulolytic myxobacteria, was reported to possess antifungal activity against several phytopathogenic fungi, such as Botrytis cinereal, Colletotrichum acutatum, Pyricularia grisea, Penicillium sp. and Phytophthora capsici. Their mode of actions including spore germination blockage and mycelial growth inhibition [34]. Another report suggested that species of Myxococcus from soil sample were able to inhibit Aspergillus fumigatus [29].
Figure 1. Phylogenetic tree of the myxobacteria constructed based on 16S rRNA gene sequences. The bar specifies 1 nucleotide substitution per 200 nucleotides. Bootstrap support in percentage (1000 replicates) is shown for each node. Only values greater than 50% are shown.

Based on antifungal assay, the extract of two \textit{C. exigus} isolates represented by SLU 3.3 and KR39b.5 were able to inhibit mycelial growth of pathogenic \textit{F. odoratissimum} (figure 2). The ability to inhibit fungal growth by SLU 3.3 extracts were 44.6% and 42% against \textit{F. odoratissimum} InaCC F936 and \textit{F. odoratissimum} InaCC F946, respectively. On the other hand, the ability to inhibit fungal growth by KR39b.5 extracts were 50% and 40% against \textit{F. odoratissimum} InaCC F936 and \textit{F. odoratissimum} InaCC F946, respectively. The mycelial growth was not inhibited in all control plates (figure 3).
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Figure 2. Antifungal assay of myxobacterial extracts against pathogenic fungal; control *F. odoratissimum* F936 (a), mycelial growth inhibition of *F. odoratissimum* F936 by SLU 3.3 and KR39b.5 extracts (b), control *F. odoratissimum* F946 (c) and mycelial growth inhibition of *F. odoratissimum* F946 by SLU 3.3 and KR39b.5 extracts (d).

Figure 3. The ability of myxobacterial extract to inhibit fungal growth, measured by the reduction in the radius of fungal mycelia. Growth inhibition of fungal strains treated with ethyl acetate (ea) was not observed on control plates.

Previous studies on *Corallococcus* members also revealed their antifungal activities against *F. culmorum* and *F. oxysporum* known as pathogens for economically important forest trees [22]. Some of the secondary metabolites extracted from the genus including corallopyronins, coralmycins and corallorazines [35–37]. These chemical compounds are responsible for their antibacterial and antifungal activities. However, recent study suggested that enzymatic activity of *Corallococcus* involving endo-chitinase is as important as their secondary metabolite in inhibiting fungal growth [38].

These results provide initial information needed for developing alternative biocontrol agents to control Panama disease pathogen, *F. odoratissimum*, on banana plants. It also indicates the potential employment of myxobacteria to inhibit the growth plant pathogenic fungi. Further research still needs to be done, such as characterization of myxobacteria and their properties to fully utilize these microorganisms.
4. Conclusion
A total of twenty myxobacterial isolates were successfully recovered from soil, decaying wood and karst limestone collected in Karimun and Simeuleu Islands, Indonesia. They were grouped in *Myxococcus*, *Archangium*, and *Coralloccocus* genera. Antifungal extracts of tested *Coralloccocus* revealed that they can inhibit the growth of *Fusarium odoratissimum* TR4, the causal agents of Panama disease on banana plants. Hence, they are potential to be developed as biocontrol agents for the pathogens.

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References
[1] Reid D and R Stover 1964 *Fusarial wilt (panama disease) of bananas and other Musa species* (New York: Springer: John Wiley and Sons) p 115
[2] Ploetz R 2007 *Phytopathology* 96 653
[3] Ploetz R 2015 *Crop Prot.* 73 7
[4] Ploetz R, Freeman S, Konkol J, Al-Abed A, Naser Z, Shalan K, Barakat R and Israeli Y 2015 *Phytoparasitica* 43 283
[5] Ordonez N, Seidl M, Waalwijk C, Drenth A, Kiliian A, Thomma B, Ploetz R, Kema G 2015 *Plos Pathog.* 11 1
[6] Maryani N, Lombard L, Poerba Y S, Subandiyah S, Crous P W and Kema G H J 2019 *Stud. Mycol* 92 155
[7] Dennis R W G and Booth C 1972 *Kew Bull.* 27 371
[8] Dita M, Barquero M, Heck D, Mizubuti E S G and Staver C P 2018 *Frontiers in Plant Science* 9
[9] Cook R J and Baker K F 1983 *The nature and practice of biological control of plant pathogens.* (St Paul: The American Phytopathological Society)
[10] Taylor W J and Draughon F A 2001 *J. Food Prot.* 64 1030
[11] Sharma G, Khatri I and Subramanian S 2016 *Genome Biol. Evol.* 8 2520
[12] Dawid W 2000 *FEMS Microbiol. Rev.* 24 403
[13] Cao P, Dey A, Vassallo C N and Wall D 2015 *J. Mol. Biol.* 427 3709
[14] Wolgemuth C, Hoiczyk E, Kaiser D and Oster G 2002 *Curr. Biol.* 12 369
[15] Reichenbach H, Gerth K, Irschik H, Kunze B and Hfle G 1988 *TIBTECH* 6 115
[16] Mauriello E M F, Mignot T, Yang Z and Zusman D R 2010 *Microbiol. Mol. Biol. Rev.* 74 229
[17] Schaberle T F, Lohr F, Schmitz A and Gabriele M K 2014 *Nat Prod Rep.* 7 953
[18] Gerth K, Bedorf N, Hofle G, Irschik H and Reichenbach H 1996 *J. Antibiot.* 49 560
[19] Wrótniak-Drzewiecka W, Brzezińska A J, Dahm H, Ingle A P, and Rai M 2016 *Ann Microbiol.* 66 17
[20] Bull C T, Shetty K G and Subbarao K V 2002 *Plant Dis.* 86 889
[21] Noren B and Odham G 1973 *Lipids.* 8 573
[22] Dahm H, Brzezińska A J, Wrotniak-Drzewiecka W, Golinska P, Rozycki H and Rai M 2015 *Dendrobiology* 74 13
[23] Reichenbach H and Dworkin M 1992 *The Prokaryotes* (New York: Springer-Verlag)
[24] Lane D J 1991 *Nucleic acid Techniques in Bacterial Systematic* (New York: John Wiley and Sons) p 115
[25] Hall TA 1999 *Nucleic Acids Symp. Ser.* 41 95
[26] Yoon S, Ha S, Kwon S, Lim J, Kim Y, Seo H and Chun J 2017 *Int. J. Syst. Evol. Microbiol.* 67 1613
[27] Tamura K, Stecher G, Peterson D, Filipski A and Kumar S 2013 *Mol. Biol. Evol.* 30 2725
[28] Mohr K I, Stechling M, Wink J, Wilharm E and Stadler M 2016 *Microbiologyopen*. 5 268
[29] Gaspari F, Paitan Y, Mainini M, Losi D, Ron E Z and Marinelli F 2005 *J. Appl. Microbiol*. 98 429
[30] Zhang X, Yao Q, Cai Z, Xie X and Zhu H 2013 *PLoS One*. 8
[31] Kumar S, Yadav A K, Chambel P and Kaur R 2017 3 *Biotech*. 7
[32] Lang E and Stackebrandt E 2019 *Int. J. Syst. Evol. Microbiol*. 59 2122
[33] Meliah S and Lisdiyanti P 2018 *Biotropia* 25 121
[34] Kim S T and Yun S C 2011 *Plant Pathol. J*. 27 257
[35] Schäberle T F, Schmitz A, Zocher G, Schiefer A, Kehraus S, Neu E, Roth M, Vassyliev D, Stehle T, Bierbau G, Hoerauf A, Pfarr K and Konig G 2015 *J. Nat. Prod.* 78 2505
[36] Kim Y, Kim H, Kim G, Cho K, Takahashi S, Koshino H and Kim W 2016 *J. Nat. Prod.* 79 2223
[37] Schmitz A, Kehraus S, Schaberle T, Neu E, Almedia C, Roth M and Konig G 2014 *J. Nat. Prod.* 77 159
[38] Li Z, Xia C, Wang Y, Li X, Qiao Y, Li C, Zhou J, Zhang L, Ye X, Huang Y, and Cui Z 2019 *Int. J. Biol. Macromol*. 132 1235