

## Identification of antibiotic producing endophytic microbe isolates from a national park in Java island

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### Abstract

Endophytic microbes are potential sources of antibiotics. Some numbers of endophytic bacteria were isolated from plants in Ujung Kulon, Kaliurang, Meru Betiri and Baluran National Park, Bogor Botanical Garden, and Nusakambangan forest, Indonesia. Previous studies have been conducted to examine and obtain endophytic bacteria isolates from the selected plants, which resulted in three selected isolates, namely OOH-1, STG-1, and CMB-2. This research was conducted to determine the molecular identity of OOH-1 and STG-1 isolates, as well as to identify antibiotic compounds produced by STG-1 isolate. Molecular identification of selected isolates was based on 16S rRNA gene analysis and amplified using primers 27F and 1492R. A phylogeny tree was then constructed by comparing the resulting sequences with data from Gene Bank using the BLAST-N program. The identification showed that STG-1 isolate had a 99% similarity with *Pseudomonas brenneri* strain SFML 97-391, and OOH-1 isolate had a 99% similarity with *Enterobacter xiangfangensis*. Identification of antibiotic compounds was done by purification and separation of the compounds. Antibiotic activity was also examined based on Lethal Concentration (LC<sub>50</sub>) on *Fusarium oxysporum* with a LC<sub>50</sub> of 0.01–0.02% against *Fusarium oxysporum*.

**Keywords:** antibiotic, endophytic microbes, molecular identification

### Introduction

Within the last 20 years, the search for new types of antibiotics has been increasing, with the growth reaching 200% from 1979 to 2000 (Lockhart *et al.*, 2009). Antibiotics have been widely used for chemotherapy, plant disease treatment, livestock drugs, biotechnology, and molecular biology (Smitha and Philip, 2014). The emergence of resistant strains impels the search and development of newer antibiotics, such as PBL (soft rot disease) in orchid flower stalks (Joko and Kusumandari, 2014) as well as leaf and neck blast in upland gogo rice (Nasution *et al.*, 2014). *Candida albicans* as a pathogen in

the human esophagus, vulva, and vaginal skin has been widely studied in order to discover new anti-fungal compounds (Mohammed *et al.*, 2012), also Moler disease in onion leaf (Lestiyani *et al.*, 2014) and rot disease in pineapple and mango by *Fusarium* sp. (Widiastuti *et al.*, 2014). In recent years, antibiotics have been studied and extracted primarily from soil microbes. However, other sources, such as endophytic microbes, may also possess potential desirable traits. Endophytic microbes are compatible with a wide variation of host type and characteristics as well as various ecosystems, allowing them to be found in diverse species. Under different environmental conditions inside plant tissues, endophytic microbes may produce antibiotics with higher bio-activity than those from microbes originating from soil or plant surfaces (Strobel *et al.*, 2004; He, 2009).

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Endophytic bacteria live inside plant tissues without endangering the host. Instead, the bioactive compounds produced by the endophytic bacteria control possible invading pathogens (Patel *et al.*, 2012). Endophytic bacteria live mutualistically with various plants in different environmental conditions and that provide a wide range of endophytic bacteria biodiversity with different potential bioactive compounds.

Bioactive compounds with high bioactivity can be found from microbes in plants in tropical rain forests in specific regions, and have long been used as a local medicine (Strobel *et al.*, 2004). Research shows that isolates from some national parks in the Indonesian islands of Sumatra, Java, and Bali have a high effectiveness against several plant pathogenic bacteria (Meliawati *et al.*, 2006). Margino (2008) also reported that isolates derived from rare plants in Yogyakarta, Java, were indicated to have anti-fungal trait. Isolates from Taman Negara National Park in Pahang, Malaysia, have also been known to produce compounds with potent cytotoxic and antibacterial properties (Hazalin *et al.*, 2009), as well as surfactant for *Fusarium* sp. growth inhibitor (Snook *et al.*, 2009).

Previous studies have been conducted to obtain and select endophytic bacteria isolates from selected plants taken from a Javanese National Park. The result of the study was three selected superior isolates, namely OOH-1, STG-1, and CMB-2. As a continuation of the previous study, this research was conducted to determine the molecular identity of OOH-1 and STG-1 isolates, as well as the identification of antibiotic compounds produced by STG-1 as a model isolate for lab. scale production because of its largest inhibition zone against *Fusarium oxysporum*.

## Materials And Methods

### Tools and Materials

The tools used in this study were: Autoclave, Oven, Centrifuge 4°C (Beckman

J), Microcentrifuge (Beckman microfuge II), PH meter 691 (Metrohm), electrophoresis cell (Bio-Rad Mini-Sub<sup>®</sup> TM DNA cell), PCR (Mastercycler personal, Eppendorf), Sequencer ABI PRISM (310 Genetic Analyzer). The materials used in this research were: OOH-1 and STG-1 isolates, Ready-To-Go PCR kits, DNA marker (1KB DNA Ladder, Promega), Primer 27F and 1492R (SBS Genetech Co., Ltd.), DNA purification kit (Microclean Microzone Ltd.), and silica gel 60G F<sub>254</sub>.

### Molecular Identification

Some numbers of endophytic bacteria were isolated from plants in Ujung Kulon, Kaliurang, Meru Betiri and Baluran National Park, Bogor Botanical Garden, and Nusakambangan forest, Indonesia in previous study. Selected superior isolates, namely OOH-1, STG-1 were used for DNA isolation. Isolation of bacterial chromosomal DNA was done using a method developed by Pospiech and Neumann (1995). 16S rRNA gene amplification was performed with primers 27F and 1492R with an initial denaturation PCR condition of 94°C for 2 min, followed by 40 cycles of 94°C for 30 seconds, 51°C for 30 seconds, and 72°C for 1 min, and a cycle of 72°C for 10 minutes. The PCR products were confirmed using electrophoresis on 2% agarose gel.

Visualization was done under UV light (Song *et al.*, 2009). 16S rRNA PCR products were subsequently sequenced with the procedure according to the manual procedures used by the DNA sequencer and then analyzed using the BLAST-N program on the NCBI page (<https://www.ncbi.nlm.nih.gov/blast/>). A phylogeny tree was constructed by comparing the sequences of the obtained 16S rRNA bacterial isolates with sequences from the Gen Bank database (<https://www.ncbi.nlm.nih.gov/genbank/>). The phylogeny tree was constructed using the MEGA program version 6.

### **Purification and Separation of Antibiotic Compounds**

Antibiotic compounds were identified initially using thin layer chromatography (TLC). Selected endophytic bacteria were inoculated on each respective media in accordance with previous methods. A 500 ml Erlenmeyer flask filled with 150 ml media was incubated in a shaker at 150 rpm for 4 days at a temperature of 30–33°C. The sample was centrifuged to separate the supernatant and pellets, and then purified further using TLC. The sample was centrifuged with the addition of a mixture of ethyl acetate and butanol, until the supernatant and pellets were separated. The supernatant was then taken, while the pellets with the ethyl acetate and butanol mixture were centrifuged repeatedly until clear.

The obtained fractions were then dissolved in a solution of chloroform:methanol (1:1) and put on a preparative plate, then processed into separation with a stationary phase of silica gel 60f and 254f and eluted with mobile phase of n-hexane:ethyl acetate (93:7). After the elution was finished, the results were observed under visible light and then scraped off. The resulting extract was dissolved with n-hexane:ethyl acetate (93:7) and packed on sintered glass. The eluate were evacuated and evaporated at room temperature. The powder obtained was then used in the following analysis.

### **LC<sub>50</sub> Test Against *Fusarium oxysporum***

Antibiotic activity was also examined for its Lethal Concentration (LC<sub>50</sub>) against *Fusarium oxysporum* by examining colonies formed while under the stress of the added compound (Tebbets *et al.*, 2013). The microbe culture indicator, *Fusarium oxysporum*, was cultivated in PDA media. As much as 10 ml of cell culture was added into petri dishes containing the media, and added with various antibiotic compound concentrations of 0, 50, 100, 200, 300, and 500 mg/ml.

## **Results and Discussion**

### **Molecular Identification**

The identification of two strains of endophytic bacteria that produce antibiotic compounds, namely STG-1 and OOH-1 isolates was performed based on 16S rRNA gene analysis via polymerase chain reaction using the universal 27 F primer (AGAGTTTGATCMTGGCTCAG) and 1492R Primer (TACGGYTACCTTGTTACGACTT). Then the reaction was checked using gel electrophoresis. There was only a single band on the outcome of PCR in a measure about 1500 bp indicating that it was a 16S band.

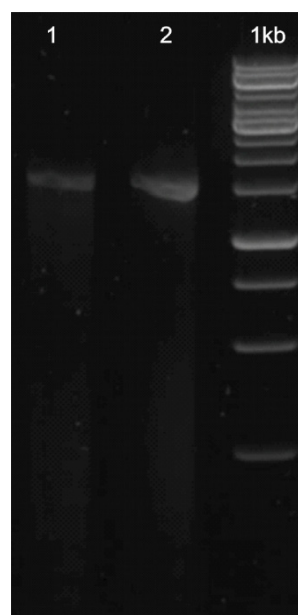


Fig 1. Gel electrophoresis on the outcome of PCR STG-1 (1) and OOH-1 (2).

The molecular identification of STG-1 and OOH-1 isolates was based on 16S rRNA sequence analysis. The DNA sequences obtained were further used to investigate the identity of the bacterial isolates using the BLAST-N program (Basic Local Alignment Search Tool-Nucleotides). Based on the highest BLAST similarity,

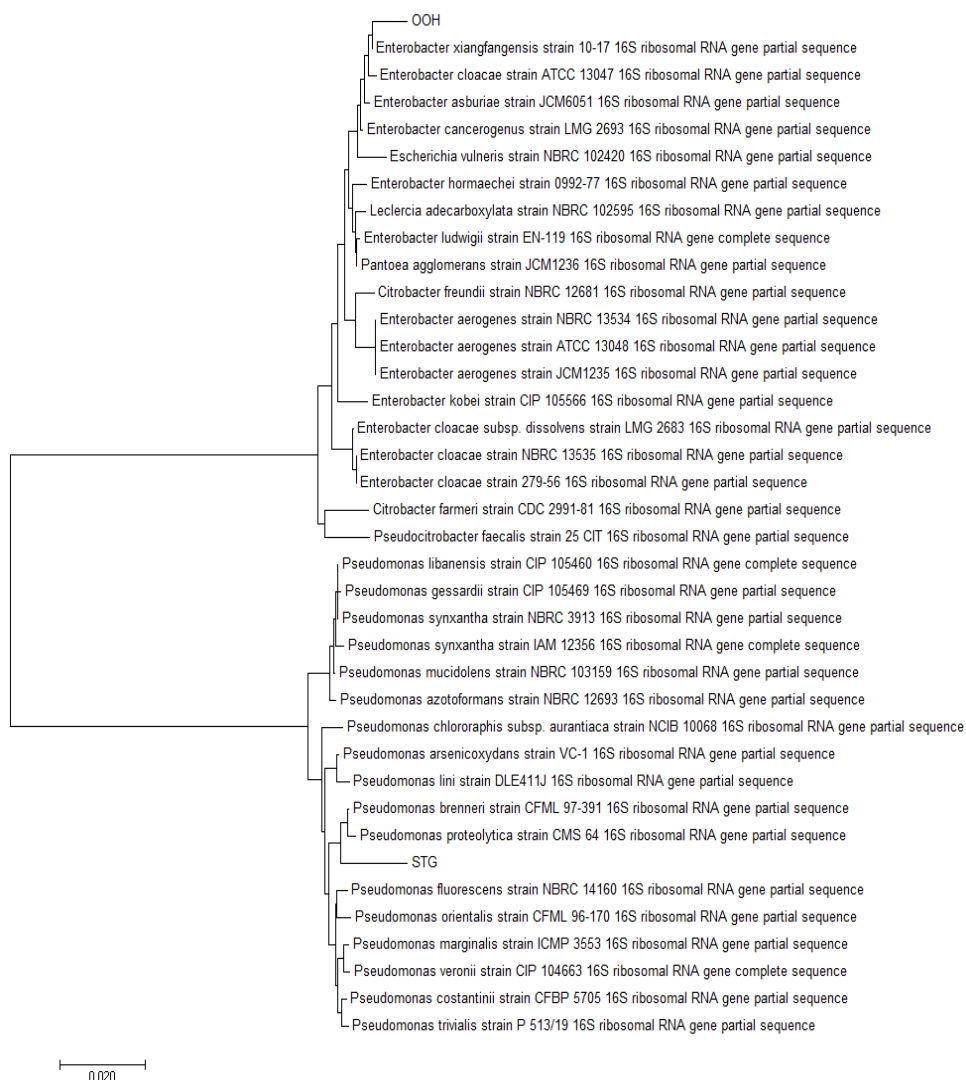


Fig 2. Phylogenetic tree based on 16S rRNA gene sequences of *Pseudomonas* and *Enterobacter* bacteria isolate groups.

each isolate was assigned a species name. The results are summarized in Table 1. Janda and Abbott (2004) reported that the identity of a bacterial strain is the same if it has a similarity value with the type strain of bacteria at least 99%.

These sequences were compared with previously published sequences of two bacterial groups. Group 1 consisted of *Pseudomonas migulae* strain CIP 105 470, *Pseudomonas panacis* strain C62010 and *Pseudomonas proteolitica* strain CMS 64, *Pseudomonas brenneri* strain CFML 97-391, *Pseudomonas synxantha* strain NBRC

Table 1. Results of the analysis of 16S rRNA gene sequences using the BLAST-N program.

Isolates code	Homolog Bacteria Species	Access Number	Similarity (%)
	<i>Pseudomonas</i>		
STG-1	<i>brenneri</i> strain CFML 97-391	NR.126208.1	99
	<i>Enterobacterxiang</i>		
OOH-1	<i>fangensis</i> strain 10-17	NR.025103.1	99

3913. Group 2 consisted of *Enterobacter xiangfangensis* strain 10-17, *Enterobacter hormaecie* strain 0992-77 and *Enterobacter cloacae* strain ARCC 13047. The results of

sequence comparisons are visualized in a phylogenetic tree (Fig 2).

STG-1 isolate was in one group with *Pseudomonas brenneri* strain CFML 97-391 and *Pseudomonas proteolytica* strain CMS 64, whereas OOH-1 isolate was in one group with *Enterobacter xiangfangensis* strain 10-17. Therefore, STG-1 is closely related to *Pseudomonas brenneri* strain CFML 97-391 and OOH-1 is closely related to *Enterobacter xiangfangensis* strain 10-17.

### Purification and Separation of Antibiotic Compounds

The identification of antibiotic compounds was performed only on STG-1 as a production model isolate, which was based on the nature and specific reagents that can be detected by fractionation of phytochemicals and thin layer chromatography (TLC).

Antibiotics produced by STG-1 had an Rf value of 0.82 after elution with  $\text{NH}_4\text{Cl}$  and Rf value of 0.90 on eluent water that was saturated with butanol. To obtain the antibiotics, these eluent can be used based on the observed spot, which is the purple colored spot beside the standard solution of stigmasterol. The compound was then

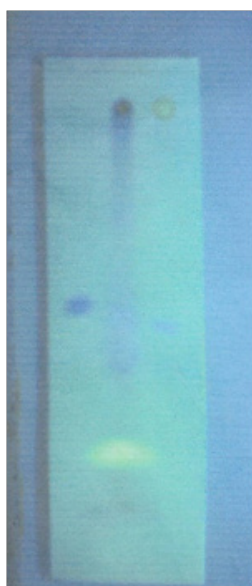


Fig 3. The results of the analysis of antibiotic compound from isolate STG-1 spot under visible light.

found belong to a group of steroids. The eluate was dissolved with a solvent mixture of chloroform:methanol (1:1) and incubated between 15–20 minutes to saturate and provide a bridge to enable the selection of active compounds and their derivatives into the chloroform phase. This study also aimed to reduce the number of components to be fractionated (El Shohly *et al.*, 1999).

The fractions were then spotted on the preparative plate and eluted with the mobile phase consisting of n-hexane:ethyl acetate (93:7). The elution results were seen under visible light and the yellow spots were found. The results of subsequent fractionation poured in sintered glass and the eluate was evaporated in an evaporator (Rinanto *et al.*, 2008).

### Activity Analysis of Antibiotics with Lethal Concentration ( $\text{LC}_{50}$ )

The  $\text{LC}_{50}$  of antibiotic compounds in various concentrations was determined according to Tebbets *et al.* (2013). With a little modification, the determination of the concentration of antibiotics performed through some dilution concentrations, namely  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ , and  $10^{-6}$ . The microbial indicator, *Fusarium oxysporum*, was grown on PDA media for 48 hours. Cultures were mixed with various antibiotic concentrations, grown on agar medium using the pour plate method, and the observations were made after 24 hours. The results showed that the concentration that kills half of the microbial indicator ranged from 100–200  $\mu\text{g}/\text{ml}$  to 273  $\mu\text{g}/\text{ml}$  (Fig 4).

Based on the study of antibiotics toxicity from endophytic microbe was known that *amphotericin-B* and *voriconazole* with *Fusarium oxysporum* and  $\text{LC}_{50}$  was 250  $\mu\text{g}/\text{ml}$  (Wiyakrutta *et al.*, 2004), whereas fluconazole was 64  $\mu\text{g}/\text{ml}$  and mancozeb with phenylmercury acetate was effective to kill *Fusarium oxysporum* pathogen. The results were compatible with other finding that the toxicity of antibiotics isolates from STG-1 can be used as a reference in determining new antibiotics with similar compounds.

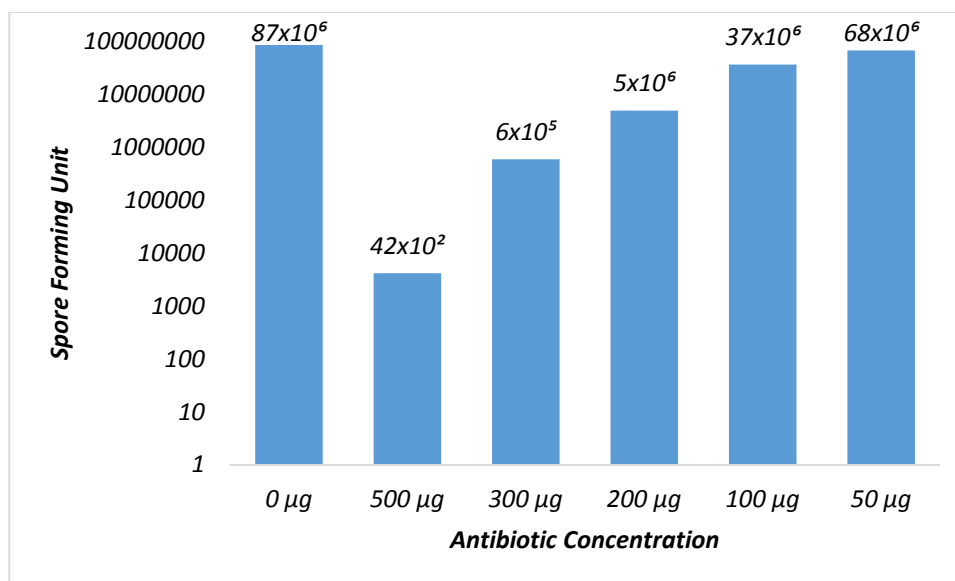


Fig 4. Effect of the antibiotic concentration on the growth of *Fusarium oxysporum*.

The results on antibiotic compound production of the *Pseudomonas* genus as well as *Pseudomonas viridiflava* endophytic bacteria showed that the antibiotic compounds contained surfactin agents that could act as biocontrol agents of *Fusarium oxysporum*, were highly biodegradable and had low toxicity (Snook *et al.*, 2004). This antibiotic compound was identified as the major lipopeptida for the toxic component. The antibiotic activity is accomplished on concentration dependent activity, low concentrations, and its product conformational changes within the phospholipids. At intermediate concentrations, it causes segregation within the bilayers, resulting in channels that are permeable to cations. At high concentrations, it produces a detergent effect resulting in complete membrane disruption. The results of this study revealed that *Pseudomonas brenneri* produced a steroid antibiotic compound against the pathogenic fungus *Fusarium oxysporum*.

### Conclusion

The results of molecular identification showed that isolate STG-1 had a 99% level of similarity with *Pseudomonas brenneri* strain CFML 97-391 and isolate OOH -1 had a 99% level of similarity with *Enterobacter*

*xiangfangensis* strain 10-17. The results of this study showed that STG-1 isolate produced antibiotic compounds belong to steroid group and had an  $LC_{50}$  of 0.01–0.02% against the microbial indicator of *Fusarium oxysporum*.

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