Endophytic Bacteria isolated from the leaf of Langusei (Ficus minahassae Tesym. & De Vr.) and their antibacterial activities

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Abstract. Endophytic bacteria colonizing the internal tissues of plants are being studied for its potential beneficial properties including antibacterial activity. The aims of this study were to identify the endophytic bacteria residing in the leaf of Ficus minahassae, a native plant of North Sulawesi, as well as their antibacterial activities against laboratory-adapted reference bacterial strains, Staphylococcus aureus, and Escherichia coli. The isolate identification was conducted using the 16S rRNA gene marker and the antibacterial activity test was done using a well diffusion method. A yellow-pigmented (designated as YL1) and a beige-pigmented (designated as YL2) bacteria were isolated from the internal tissue of langusei. Using the BLAST search, the isolate YL1 had a 100% identity with Brachybacterium muris, while YL2 has 99% identity with Pseudacidovorax intermedius. Ez-Taxon database confirmed that YL1 was B. muris with 99.81% similarity, while YL2 was P. intermedius with 99.80% similarity. Isolate YL2 was able to inhibit the growth of S. aureus but not E. coli. Isolate YL1 did not inhibit both reference bacteria. This study represents the first investigation of endophytic bacteria isolated from langusei leaf and their antibacterial activity.

1. Introduction

In recent years, the mining of microbial resources contained in plant tissue has begun to receive much attention. These endophytic bacteria colonize the intercellular spaces and vascular systems of the plants. These microbes have been studied for various purposes. Compounds produced by endophytes have many benefits, such as antibacterials. The active compound of plant endophytic microbes often has a stronger activity than that produced by their host plants. The abilities of the active compounds of plant endophytic bacteria still need to be explored to discover new bacterial strains that have the potential to be further explored [1].

Several studies have reported that endophytic bacteria can be found in ficus plants, for example in Ficus carica [2] and F. racemosa [3]. One of the ficus plants native to North Sulawesi is F. minahassae. The local people names this plant as langusei. This plant is found in the primary forest, especially along the river, to a height of about 135 meters above the sea. Dichloromethane extract of the leaves contained 2-hydroxyethyl benzoate, phytyl fatty acid ester, squalene, and β-sitosterol [4]. The increasing emergence of bacterial resistance creates demands in focusing on new and more potent antimicrobial sources. Therefore, this study was aimed to identify endophytic bacteria in F. minahassae and to assay their antibacterial activity.
2. Materials and Methods

2.1. Sample Preparation
The leaf of langusei was obtained from the Batukota river bank in Manado. The leaf was surface sterilized by incubation in 5% NaOCl for 10 min, followed by in 70% alcohol for 2 min, and rinsed three times in sterile dH₂O. To check the sterilisation procedure, 100 ul aliquot of dH₂O taken from the rinsing of the sterilized leaf was spread over the nutrient agar (NA) media, incubated at 37°C for 2 days, and examined for the presence or absence of the growth of microorganisms.

2.2. Isolation of the Endophytes
The plant leaf was acceptively ground in a sterile mortar and pestle until producing juice. One hundred microliters of the juice were spread over the NA media. The plates were incubated at 37°C for 2 to 4 days. The growing bacterial isolates were grouped based on their morphology and characteristics of their colonies such as size, shape, and colour. Each separated colony was picked using Oose and streaked on NA media, incubated at 37°C for 2 days. Each separately grown colony was considered as a pure isolate to be used for further examination.

2.3 Identification of the Endophytes
The pure selected isolates were identified using Gram staining followed by identification based on the 16S rRNA gene. The DNA genome was isolated using the Plant Genomic DNA mini kit (Geheaid). MyTaq HS Red Mix (Bioline) was used for PCR amplification, with 10 μM of each BKXF and BKXR primer. The PCR was performed using the following condition: initial denaturation at 95°C for 3 min followed by 35 cycles of denaturation of 95°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 90 sec, and final elongation at 72°C for 1 min. The PCR products were sent to 1st BASE Malaysia for sequencing. The aligned contiguous consensus sequences were used for the homology search by BLAST software (http://blast.ncbi.nlm.nih.gov) algorithm at the National Center for Biotechnology Information (NCBI). The identification was further confirmed using the Ez-Taxon database (https://www.ezbiocloud.net)[5].

2.4 Phylogenetic Analysis
The phylogenetic analysis was performed using the stepwise procedure described previously [6]. The chromatograms obtained from 1st BASE Malaysia were processed using Geneious 10.1.3. The primer sequences were removed by trimming approximately 50 nucleotides at the beginning of DNA sequences. Errors of the reading of the nucleotides were corrected accordingly. By using the pairwise alignment of forward and reverse sequences, the consensus sequences were generated. All similar sequences obtained from Genebank were aligned using multiple sequence alignment with hierarchical clustering [7], which is available online at (http://multalin.toulouse.inra.fr/multalin/), and trimmed accordingly to obtain the core sequences of 16S rRNA. By using the Neighbor Joining method integrated into MEGA v10.0.4 [8], the phylogenetic tree was reconstructed.

2.5 Antibacterial Test of the Isolates
The antimicrobial activity of the isolates was determined using the agar well-diffusion method. Nutrient agar media was poured into plates equipped with stainless-steel cylinders. After the media were solidified, the cylinders were removed leaving a 5 mm diameter of wells. Two hundred microliters of an overnight culture of indicator strains (Escherichia coli (Migula) Castellani and Chalmers (ATCC® 25922™) and Staphylococcus aureus subsp. aureus Rosenbach (ATCC® 25923™)) containing approximately 10⁶ cells/ml were used for this purpose. Bacterial isolates were grown overnight, heat-killed by incubating at 80°C for 1 hour, and the supernatant was collected by centrifuging at 6000 rpm for 5 min. One hundred microliters of each isolate was added into each well. Inhibitory zone of the isolates was checked after 24 hours incubation at 37°C. Fifty μg/ml 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid was used as positive control and sterile dH₂O as the negative control.
3. Results and Discussion

3.1. Endophytic Bacterial Isolation

Of several endophytic bacteria that were successfully isolated and purified from the surface-disinfected leaf of langusei, two isolates (YL1 and YL2) were further investigated. Gram staining indicated that YL1 was a positive-Gram bacterium. The isolate was yellow-pigmented on nutrient agar (Fig. 1a). YL2 was a negative-Gram bacterium and beige-pigmented on nutrient agar (Fig. 1b).

![Figure 1. a) Colonies of isolate YL1 and b) colonies of isolate YL2 on nutrient agar media](image)

3.2. Isolates Identification

Identification of the isolates was conducted using 16S rRNA gene marker analysis. Using the BLAST search to compare the obtained sequence with reference sequences at GenBank, it was confirmed that YL1 (GenBank accession number MK764941) had 100% identity with *Brachybacterium muris* and YL2 (GenBank accession number MK764961) had 99% identity with *Pseudacidovorax intermedius*. Using the Ez-Taxon database, YL1 was confirmed as *B. muris* with 99.81% similarity with *B. muris* strain C3H-21, and YL2 was confirmed as *P. intermedius* with 99.80% similarity with *P. intermedius* strain DSM 21352. *Brachybacterium muris* is an actinobacteria while *P. intermedius* belongs to β-proteobacteria. *Brachybacterium* sp. has been isolated from tea (*Camellia sinensis*) [9], fresh tissue of banana (*Musa paradisiaca*) [10], rice (*Oryza sativa*) leaf [11], vine (*Vitis* sp.) stems [12], dragon blood tree (*Dracaena cochinchinensis*) [13], and arils of durian (*Durio zibethinus*) [14].

Other endophytic Brachybacteria among others are *B. paraconglomeratum* isolated from roots of safed musli (*Chlorophytum borivilianum*) [15], *B. hainanense* isolated from noni (*Morinda citrifolia* L.) branch [16], and *B. faecium* isolated from Mexican bamboo (*Polygonum cuspidatum*) [17]. Some of Brachybacteria exhibited antimicrobial activity [18 – 20].

*Pseudacidovorax intermedius* has been isolated from soil sample [21], surface sediments of the South China Sea [22], rice seeds [23], *Vetiver zizanioides* - a perennial grass of the Poaceae family [24], hanging root of benjamin fig (*F. benjamina*) [25], and date palm roots [26]. Other *Pseudacidovorax* sp. has been isolated from growing shoot tips of banana (*Musa* sp.) [10], potato (*Solanum tuberosum*) [27], and rice stem [28].

To the best of our knowledge, there has been no report on the isolation of endophytes from langusei, therefor this finding is the first report of the isolation of *P. intermedius* and *B. muris* from fig leaf. Some endophytic bacteria from fig among others are *Klebsiella oxytoca, Pseudomonas* sp. and *Pantoea* sp. isolated from fig (*F. carica*) [29], *Psedomonas aeruginosa*, isolated from red-stem fig (*F. variegata*)
[30], *Bacillus subtilis*, *B. amyloliquefaciens*, and *B. amyloliquefaciens*, isolated aerial roots of bajan fig (*F. benghalensis*) [25], and agrobacteria which were detected in many healthy benjamin fig [31].

### 3.3. Phylogenetic Analysis

The construction of a phylogenetic tree through the neighbor joining method (Fig. 2) demonstrates the differences between taxa of the endophytes found in the leaf of *F. minahassae*. *Brachybacterium muris* belong to Actinobacteria and *P. intermedius* belong to β-Proteobacteria. Endophytic bacteria of fig tree reported previously were from α-Proteobacteria, γ-Proteobacteria, and Firmicutes. Most of the endophytic bacteria of rice were α-Proteobacteria (46%) and Actinomycetales (16%) [11]. Bacterial endophytes are polyphyletic, belonging to a broad range of taxa, including Actinobacteria, Firmicutes, α-Proteobacteria, β-Proteobacteria, and γ-Proteobacteria [32]. Of these, *Pseudomonas* sp. has frequently found as dominant bacterial endophytes [33].

**Figure 2.** Phylogenetic tree constructed using the Neighbor Joining method based on the sequences core region of 16s rRNA of endophytic bacteria obtained from the leaf of langusei. The complement sequences were obtained from GenBank as indicated by their accession numbers. YL1 is *B. muris* and YL2 is *P. intermedius*.

### 3.4. Antimicrobial Test

The antibacterial activities of *B. muris* YL1 and *P. intermedius* YL2 were examined using the well diffusion method. *B. muris* did not inhibit the growth of *S. aureus* nor *E. coli*. *P. intermedius* displayed anti-bacterial activity against *S. aureus* (Fig 3a) but not against *E. coli* (Fig 3b). The diameter of the inhibition zone was 18.75 ± 0.096. The similar finding showed that *Brachybacterium* sp. isolated from *D. cochinchinensis* did not inhibit bacteria including *S. aureus*, *E. coli*, *B. subtilis*, *K. pneumoniae*, and fungi including *A. niger* and *A. fumigatus*, but inhibited plant fungus *Gibberella fujikuroi* [13].
The current study suggests that endophytic bacteria of langusei are a source of potential novel antibacterial. Endophytic bacteria from *F. variegata* produced antibacterial compounds [30]. *Streptomyces* sp. isolated from leaves of *F. racemosa* showed an antibacterial activity [3]. *Pseudacidovorax intermedius* was confirmed as nitrogen-fixing bacterium [21, 22, 24]. Nitrogen-fixing nitrogenase (nifH) homolog gene was also found in *Pseudacidovorax* sp. isolated from potato [27]. Nitrogenase is an enzyme used by some organisms to fix atmospheric nitrogen gas (N\(_2\)).

![Figure 3. Antibacterial activity test of *B. muris* YL1 and *P. intermedius* YL2 against *S. aureus* (a) and *E. coli* (b); + = positive control containing 50ug/ml 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid; - = negative control (sterile dH\(_2\)O).](image)

Identification using NMR, dichloromethane extract of the leaf of *F. minahassae* contains 2-hydroxyethyl benzoate, phytly fatty acid ester, squalene, and β-sitosterol [4]. These compounds are probably induced by endophytic bacteria living in the intracellular tissues of the leaves. Polyethylene terephthalate (PET) hydrolase is produced by bacteria hydrolase PET to release among others is 2-hydroxyethyl benzoate (HEB) [34]. Phytly esters, an intracellular wax esters, are also found in bacteria and generally considered to be energy storage components [35]. This compound has an antimicrobial property [36]. Squalene hopene cyclases have been found in bacteria [37]. Squalene-hopene cyclase (SHC) catalyzes the conversion of acyclic squalene into the pentacyclic hopene and hopanol in prokaryotes. Squalene has antimicrobial properties [38, 39]. β-sitosterol, one of the several phytosterols, also has antibacterial activity [40]. This compound has been isolated from endophytic fungi of Amazon plants [41] and the Chinese medicinal plant *Arisaema erubescens* [42]. It may also be produced by endophytic bacteria.

4. Conclusion
Two endophytic bacteria isolated from *F. minahassae* were confirmed as *B. muris* (YL1) and *P. intermedius* (YL2) based on molecular identification using 16S rRNA gene marker. Among the two, only *P. intermedius* showed activity against the growth of *S. aureus*. Further studies are required to reveal the potential of these endophytes as antibacterial agents.

5. Conflict of Interest
There is no conflict of interest.
6. References

[1] Muzzamal H, Sarwar R, Sajid I, Hasnain S 2011 *Pak. J. Zool.* **44**, 249-257.
[2] Ma YM, Liang XA, Zhang HC, Liu R 2016 *J. Agric. Food Chem.* **64**(19), 3789-93.
[3] Smita V, Dipak V 2015 *Int. J. Res. Biosci.* **4**(4), 68-74.
[4] Apostol PG, De Los Reyes MM, Mandia EH, Shen CC, Ragasa CY 2016 *Der Pharma Chemica* **8**(20), 220-223.
[5] Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J *Int. J. Syst. Evol. Microbiol.* **67**, 1613-1617.
[6] Tallei TE, Kolondam BJ 2015 *Hayati J. Biosci.* **22**(1), 41-47.
[7] Cropet F 1988 *Nucl. Acids Res.* **16**(22), 10881-10890.
[8] Kumar S, Stecher G, Li M, Knyaz C, Tamura K 2018 *Mol. Biol. Evol.* **35**, 1547-1549.
[9] Das PP, Medhi T 2018 *Annals Plant Sci.* **7**(4), 2119-2125.
[10] Thomas P, Soly TA 2009 *Microb. Ecol.* **58**(4), 952-64.
[11] Ferrando F, Mañay JF, Scavino AF 2012 *FEMS Microbiol. Ecol.* **80**, 696-708.
[12] Andreoli M, Lampis S, Zapparoli G, Angelini E, Vallini G *Microbiol. Res.* **183**, 42-52.
[13] Salam N, Khieu TN, Liu MJ, Vu TT, Son CK, Quach NT, Phi QT, Prabhu M, Rao N, Fontana A, Sarter S and Li WJ 2017 *BioMed. Res. Int.* **2017**, 1308563.
[14] Suhandono S and Utari IB 2014 *Microbiol. Indonesia* **8**(4), 161-169.
[15] Barnawal D, Bharti N, Tripathi A, Shanker S, Chandan P, Chanotiya S and Kalra A 2016 *J. Plant Growth Regul.* **35**, 553.
[16] Liu Y, Zhai L, Yao S, Cao Y, Cao Y, Zhang X, Su J, Ge Y, Zhao R, Cheng C 2015 *Int. J. Evol. Microb.* **65**, 4196-4201.
[17] Sun H, He Y, Xiao Q, Ye R, Tian Y 2013 *AJMR African J. Microb. Res.* **7**(16), 1496-1504.
[18] Claverías FP, Undabarrena A, González M, Seeger M, Cámara B 2015 *Front Microbiol.* **6**, 737.
[19] Radjasa OK 2007 *J Coastal Dev.* **10**(3), 143-151.
[20] Leiva S, Alvarado P, Huang Y, Wang J, Garrido I 2015 *FEMS Microbiol Lett.* **362**(24), 206.
[21] Kämpfer P, Thummes K, Chu HI, Tan CC, Arun AB, Chen WM, Lai WA, Shen FT, Rekha PD, Young CC 2008 *Int. J. Syst. Evol. Microbiol.* **58**(Pt 2), 491-5.
[22] Zhang LH, Chen SF 2012 *World J. Microbiol. Biotechnol.* **28**(9), 2839-47.
[23] Midha S, Bansal K, Sharma S, Kumar N, Patil PP, Chaudhry V, Patil PB 2016 *Front. Microbiol.* **6**, 1551.
[24] Zhao X, Javed CH, He Y, Zhang Z, Peng G, Tan Z 2009 *Acta Microbiologica Sinica* **49**(11), 1430-1437.
[25] Pathak K, Keharia H, Kharkwal AC 2009 *Symbiotic Fungi, Soil Biology* vol 18, eds Varma and A.C. Kharkwal (Berlin Heidelberg: Springer-Verlag).
[26] Yaish MW, Al-Harris I, Alansari AS, Al-Yahyai R, Glick BR 2016 *Int. Microb.* **19**(3), 143-155.
[27] Pageni BB, Lupwayi NZ, Akter Z, Larney FJ, Kawchuk LM, Gan YT 2014 *Canadian J. Plant Sci.* **94**(5), 835-844.
[28] Raweekul W, Wuttitummaporn S, Dodchuen W, Kittiwongwattana C 2016 *Thammasat Int. J. Sci. Tech.* **21**(1), 8-17.
[29] Alvarado-Marchena L, Smith-Durán A, Alvarado-Ulloa C, Chacón-Cerdas R, Flores-Mora D, Flores-Mora D 2016 ARPN *American J. Agric. Biol. Sci.* **11**(7), 290-297.
[30] Leonita S, Bintang M, Pasaribu FH 2016 Current Biochem. 2(3), 116-128.
[31] Zoina A, Raio A, Peluso R, Spasiano A 2008 Plant Pathol. 50(5), 620-627.
[32] Miliute I, Buzaitė O, Baniulis D and Stanys V 2015 Zemdirbyste-Agriculture 102(4), 465-478.
[33] Pratiwi RH, Hidayat I, Hanafi M, Mangunwardoyo W 2016 Malaysian J. Microb. 12(4), 300-307.
[34] Acton A 2013 Bacillus - Advances in Research and Application (Atlanta USA: Scholarly Editions)
[35] Alvarez HM, Pucci OH, Steinbüchel A 1997 Appl. Microbiol. Biotechnol. 47, 132-139.
[36] Ragasa CY, Hofileña J, Rideout JA 2004 Philippine J. Sci. 133(1) 1-5.
[37] Nair IM, Jayachandran K 2017 Int. J. Health & Life-Sciences 3(2), 161-177 doi:10.20319/lijhls.2017.32.161177
[38] Popa O, Băbeanu NE, Popa I, Niță S. Dinu-Pârvu CE 2015 BioMed Res. Int. Article ID 367202.
[39] Kopícová Z, Vavreinová S 2007 Czech J. Food Sci. 25, 195-201.
[40] Ododo MM, Choudhury MK, Dekebo AH 2016 SpringerPlus 5(1), 1210.
[41] Carvalho JM, da Paixão LKO, Dolabela MF, Marinho PSB, Marinho AMR 2016 Acta Amaz. 46(1), 69.
[42] Wang LW, Xu BG, Wang JY, Su ZZ, Lin FC, Zhang CL, Kubicek CP 2012 App. Microbiol. Biotechnol. 93(3), 1231-9.