

## Research Article

### Characterization of *Pantoea ananatis* Isolated from Garlic and Shallot

#### *Karakterisasi Pantoea ananatis yang Diisolasi dari Bawang Putih dan Bawang Merah*

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#### ABSTRACT

The new disease on garlic (*Allium sativum*) and shallot (*A. cepa* L. *aggregatum* group) have been found in several production centers of garlic and shallot in Tawangmangu and Temanggung, Central Java. The infected plants showed symptoms of leaf blight accompanied by chlorosis. The objective of this study was to determine the pathogen that causes leaf blight and chlorosis based on the phenotypic characterization and *gyrB* gene sequences analysis. The research started from the isolation of pathogen, physiological and biochemical test, DNA extraction, and sequence analysis of *gyrB* using *gyrB* 01-F and *gyrB* 02-R primer. The results showed that the isolated bacterial pathogen have a yellow pigment, slimy colonies with regular borders, convex, gram-negative, non-spore, facultative anaerobic, motile, catalase production, indole production, and acid production from D-glucose, D-mannitol, sucrose, and lactose. From the pathogenicity test, it was found that the bacteria produced the typical symptom of leaf blight. Characterization of pathogens based on *gyrB* gene sequence revealed that the pathogen was placed in the group of *Pantoea ananatis*.

Keywords: *Allium cepa*, *Allium sativum*, *gyrB*, leaf blight, *Pantoea ananatis*

#### INTISARI

Penyakit baru pada bawang putih (*Allium sativum*) dan bawang merah (*A. cepa* L. *aggregatum* group) telah ditemukan di beberapa sentra produksi bawang putih dan bawang merah di Tawangmangu dan Temanggung, Jawa Tengah. Tanaman yang terinfeksi menunjukkan gejala hawar daun disertai klorosis. Tujuan penelitian untuk mengetahui karakter patogen berdasarkan fenotipik dan sekuen gen *gyrB*. Penelitian dimulai dengan isolasi bagian tanaman yang sakit, uji fisiologi dan biokimia, ekstraksi DNA dengan metode CTAB/NaCl dan amplifikasi gen *gyrB* menggunakan primer *gyrB* 01-F and *gyrB* 02-R. Hasil uji menunjukkan koloni berlendir, cembung, pigmen berwarna kuning, gram negative, tidak berspora, aerob fakultatif, motil, produksi katalase, indol, membentuk asam dari D-glukosa, D-mannitol, sukrosa dan laktosa, dan patogenesis positif. Karakterisasi patogen berdasarkan sekuen gen *gyrB*, menunjukkan patogen hawar daun berkerabat dekat dengan *Pantoea ananatis*.

Kata kunci: *Allium cepa*, *Allium sativum*, *gyrB*, leaf blight, *Pantoea ananatis*

#### INTRODUCTION

*Pantoea ananatis* is an important bacterial plant pathogen in the world and has a very wide host range. *P. ananatis* can survive in a various ecosystem as the saprophyte, endophytes, epiphytes and pathogens. *P. ananatis* is reported to cause center rot disease of onion, and accounted for 100% loss in some fields (Gitaitis & Gay, 1997). While maize white spot (MWS) caused by this pathogen could cause up to 60% yield loss (Miller *et al.*, 2016).

In Indonesia, *P. ananatis* has never been reported to infect plants until recently, although the tropical

climate conditions in Indonesia are also suitable for the development of *P. ananatis*. The risks and possibility of the pathogen to spread in Indonesia is highly considered by the import commodities from countries that have reported the presence of these bacteria. According to Carr *et al.* (2010), the development of center rot symptoms in onion bulbs during storage poses a significant problem. The onion is often stored for months prior to grading and marketing will result in a more severe infection. However, onion bulbs that are infected solely by *P. ananatis* remain firm and exhibit subtle or no external symptoms, they can be

difficult to detect on grading lines. The onion bulbs can be a source of inoculum for other plants, although the onion is not cultivated in Indonesia.

The metabolic pattern of *P. ananatis* from crop debris is similar to those recovered directly from lesions or from healthy leaves, suggesting that these bacteria in crop debris could act similarly to epiphytic isolates, being a source of inoculum for further infections. *P. ananatis* can survive as epiphytes in the leaves of healthy maize plants, non-host plants and in crop debris, and possibly multiply there (Sauer *et al.*, 2015). The bacteria infect host through flowers, mechanical injury, wound insect bite and friction injuries plants with the current crop of strong winds (Azad *et al.*, 2000). Tobacco thrips of *Frankliniella fusca* are a vector of *P. ananatis* caused center rot in onion (Gitaitis *et al.*, 2003).

The *gyrB* gene codes for the b-subunit of DNA gyrase, a type II DNA topoisomerase, which introduces negative supercoils into closed circular DNA molecules. One of the reasons why the *gyrB* gene is selected for phylogenetic studies is that, as horizontal gene transfer (HGT) occurs infrequently in informational genes that are involved in transcription and translation, it is assumed not to undergo HGT (Harayama & Kasai, 2006). *GyrB* gene sequences have been widely used for the identification of bacterial species. *GyrB* gene sequence is more suitable for determining genetic relationships and the identification of bacterial than the 16S rRNA (Parkinson *et al.*, 2009; Takeda *et al.*, 2010).

The objectives of this study was to characterize bacterial pathogen isolated from onion and garlic based on phenotypic properties and *gyrB* gene sequences analyses.

## MATERIALS AND METHODS

Survey and sampling were carried out based on purposive random sampling method (Sumardiyono *et al.*, 2011; Windari *et al.*, 2015; Ismiyatuningsih *et al.*, 2016). Symptomatic tissue showing leaf blight and chlorosis diseases were collected from several fields of garlic and shallot production area in Central Java.

### *Isolation of Bacterial Pathogen*

Bacteria were isolated from the affected tissue according to Joko *et al.* (2011a) with slight modification. A one gram of the sample was crushed in a small tube 500 µl sterilized ddH<sub>2</sub>O. One loopful of the

suspension was streak onto YP agar (yeast extract 5 g, peptone 10 g, agar 15 g, water 1000 ml, pH 6.8) plate medium and it was incubated for 2 days at 28°C (Wibowo *et al.*, 2010; Wardhika *et al.*, 2014). Yellow colonies were formed on YP agar plate, and single colonies were subcultured onto YP agar slants.

### *Physiological and Biochemical Characterization*

The bacterial isolates were then characterized as follows (Lelliot & Stead, 1987; Joko *et al.*, 2000; Schaad *et al.*, 2001):

Gram reaction with 3% KOH is to differentiate bacteria based on the structure of the cell wall. Gram-negative bacterial will become gummy upon mixing with a loop, while gram-positive bacterial will not.

Catalase test is to detect the presence of catalase enzyme in bacteria that is able to hydrolyze hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) into water and oxygen. If the bacterium has a catalase enzyme, it will form gas bubbles.

Anaerobic growth test aims to determine whether the bacteria can grow in aerobic or anaerobic conditions in media containing bromotimol blue covered with sterile oil paraffin (anaerobic) and without paraffin (aerobic). A color change from blue to yellow in both tubes is recorded as positive for anaerobic growth/fermentation.

Indole test uses Kovac's method, that is reagents which contains hydrochloric acid and p-dimethyl aminobenzaldehyde in amyl alcohol. The Bacteria that produce the enzyme tryptophanase can convert the amino acid tryptophan to by-products that include indole. The indole which is produced was detected by adding Kovac's reagent which produced cherry red (Aneja, 2003).

Oxidase test is to detect the presence of pathogenic bacteria cytochromeoxidase. The bacteria are streaked onto filter paper that has been containing tetramethyl-p-phenylenediamine dihydrochloride 1%, The strain was rated oxidase-positive if a purple color develops within 10s, delayed positive if coloration develops within 10-60s and negative if no color develops after 60 seconds.

Nitrate reduction test is to determine the ability of bacteria to reduce nitrate to nitrite compounds. The bacteria were grown in NA medium containing 0.1% KNO<sub>3</sub>, then it is incubated at room temperature (24h and 48h). Each tube was added with 1 ml reagent A (1 g α-naphthyl amine in 200ml of acetic acid 30%) and 1 ml reagent B (0.5 g of sulfanilic

acid in 150ml of 30% acetic acid). The color change occurs in the culture of bacteria, nitrate is reduced to form a perfect nitrogen gas (N<sub>2</sub>) or ammonium gas (NH<sub>3</sub>), a positive reaction happens when the red color is formed about 30 minutes and medium cracks.

Arginine dihydrolase test is to detect the condition of the growth of anaerobic bacteria in Thornley media (arginine medium with phenol red dye), and covered with liquid paraffin to create anaerobic conditions and incubated at 28°C for 4 days. The positive reaction was occurred when there was a change in the medium from pink to red indicates arginine compound is hydrolyzed to urea and ornithine.

Gelatin hydrolysis test is to detect the presence of proteolytic enzymes. The bacteria are grown in gelatin medium and incubated for 7-14 days at room temperature. Before being observed, the tube was cooled at 4°C for 30 min (until control is gelled) every day to check for gelatin liquefaction. The nutrient gelatine medium inoculated with a gelatine negative bacterium will remain solid after the cold treatment (Leboffe & Pierce, 2010).

H<sub>2</sub>S production. The bacteria are grown in SIM medium, that is medium containing peptone and sodium thiosulfate as a sulfur source. The presence of H<sub>2</sub>S is indicated by a formation of a black precipitate at the stabbing side.

The presence of urease is detectable when the organisms are grown in a urea broth medium containing the pH indicator phenol red. An increase in alkalinity is indicated by a magenta red color (pH approx. 9.0) was evidence of urease activity.

Hypersensitivity reaction (HR) on tobacco leaves. The suspension of *P. ananatis* were grown for 48 h in YP broth (yeast peptone medium at pH 6.8) by shaking at 120 rpm, then diluted to approximately 10<sup>8</sup> cfu/ml (Kido *et al.*, 2010) and injected in the mesophyll which is located between the bones of tobacco leaves.

Pathogenicity test using 10<sup>8</sup>–10<sup>9</sup>cfu/ml concentration, the suspension was injected and sprayed under the leaf epidermis and sterile dH<sub>2</sub>O as negative control.

### **DNA Extraction**

Bacterial genomic DNA was extracted using mini preparation DNA isolation technique with slight modification (Joko *et al.*, 2007a; 2007b; Danaatmadja *et al.*, 2009). As much of 1.5 ml of cell culture was centrifuged at 5,000 g for 2 min. The pellet of DNA was diluted with 540 µl of TE buffer (0.1 M Tris-

HCl, 0.1 M EDTA pH 8), added with 30 µl 10% SDS and then incubated at 37°C for 60 min. Afterwards, the pellet was added with 100 µl of 5 M NaCl and 80 µl of CTAB/NaCl and then incubated at 65°C for 10 min prior to addition of 750 µl of chloroform isoamyl alcohol (24:1) and centrifuged at 12,000 g for 5 min. The upper layer was transferred into 1.5 ml Eppendorf tube, added with 600 µl of phenol/chloroform isoamylalcohol (25:24:1) and then centrifuged at 12,000 g for 5 min. The supernatant was transferred again into new 1.5 ml Eppendorf tube. As much of 0.6 times volume of isopropanol was added and centrifuged at 12,000 g for 5 min. The pellet was rinsed with ethanol 70%, air-dried and then diluted with 20 µl of TE buffer.

### ***gyrB* Gen Sequence Analysis**

The amplification of *gyrB* gene of *P. ananatis* was done using primer set of *gyrB* 01-F (5'-TAARTT Y G A Y G A Y A A C T C Y T A Y A A A G T - 3' ) (R=A/G;Y=C/T); and *gyrB* 02-R (5'-CMCCYTC CACCARGTAMAGTTC-3' ) (M=A/C) (Pérez-y-Terrón *et al.*, 2009). PCR Master mix consisted of 30 µl *Taq* ready mix PCR kit (KAPA), 6 µl forward primer, 6 µl reverse primer, 12 µl nuclease free water. The DNA sample is then added 1 µl according to the number of bacterial isolates. DNA amplification was done in a Bio-Rad thermocycler as follows: predenaturation at 95°C for 5 min, denaturation at 95°C for the 30 s, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The cycle is repeated 35 times (Joko *et al.*, 2011b). Samples were run on a 2% agarose gel stained with ethidium bromide and visualised under a UV light for the presence of amplified products (Joko *et al.*, 2012).

DNA Sequencing was carried out by submitting the PCR products to 1<sup>st</sup> BASE company. Nucleotide sequence was edited using Genetyx program 7<sup>th</sup> version (Genetyx, Japan) (Mahfut *et al.*, 2016a; 2016b). The sequences of the isolates were matched to reference strains from Gene Bank Database based on 16S rRNA gene fragments to figure out their similarity. Nucleotide sequence was analyzed using Basic Local Alignment Search Tool (BLAST) at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov). Some reference strains with similarity close to 100% were determined (Suharti *et al.*, 2017; Widyaningsih *et al.*, 2017). The phylogenetic tree was constructed using bootstrap method with 1,000 times replication; while Multiple Sequence Alignment was analyzed using MEGA 7 program (Joko *et al.*, 2014; Dwimartina *et*

*al.*, 2017). An unrooted phylogram was obtained by the neighbor joining (NJ) method. The stability of the tree was assessed by 1000 bootstrap replications with the neighbor-joining method and Jukes-Cantor distance analysis. An interior branch test was done (heuristic option, 1000 replications) to check the tree topology for robustness. Some reference strains with similarity close to 100% were determined. Additionally, the Poisson correction was applied NJ for distance estimation, and the complete deletion option was used in handling gaps or missing data obtained from alignments (Kumar *et al.*, 2016).

## RESULTS AND DISCUSSION

The results of a survey on the highland revealed that a new disease of garlic and shallot has been found which is showing a dry white spot symptom with chlorosis extending from the middle to the base of the leaves of garlic and shallots (Figure 1). The incidence of the disease becomes more severe when the chlorosis line becomes gray and dry, causing plants to die. In shallot, there was a leaf blight growing to the base of the leaf, causing the leaves turned to dry. Conn *et al.* (2012) reported that the infection of *P. ananatis* and *P. agglomerans* usually starts with leaf spot growing down to the bulb neck and causes a disease known as the center rot.

The bacterial pathogen isolates obtained in this study are: 1 garlic isolate of Temanggung (PP), 2 garlic isolates of Tawangmangu (PT), 3 shallots isolates of Tawangmangu (MT). From the physiological and biochemical characterization, it was shown that the bacterial pathogens have a yellow pigment, slimy colonies with regular borders, gram-negative, non-spore, facultative anaerobic bacterium, motile, producing acid from D-glucose, D-mannitol, sucrose,

and lactose, and catalase production. The bacterial isolates were able to form indole from tryptophan as a source of carbon and hydrolyzed gelatin, with the exception of isolate PT3. All isolates reacted negatively in the production of oxidase, arginine dihydrolase, urease, and nitrate reduction (Table 1). Isolates of PT1, PP7, MT3 and MT4 possess the same characteristics with reported *P. ananatis*, but garlic isolates of Tawangmangu (PT3) and shallot isolates of Tawangmangu (MT2) does not have the ability to produce indole. In pathogenicity tests, isolate PT3 and MT2 does not cause symptoms in the shallot and garlic. According to Walcott *et al.* (2002) the ability to produce indole distinguished *P. ananatis* from *P. stewartii* subsp. *stewartii*.

Egorova *et al.* (2015) reported that *P. ananatis* isolates causing leaf blight of rice in Russia were shown to have similar physiological and biochemical characteristics. These Russian isolates were found to react positively to the  $\beta$ -galactosidase, produced acid from D-glucose, D-mannitol, D-melibiose, arabinose, sucrose, meso-inositol, glycerol, d-sorbitol, amygdalin, and utilize citrate and tartrate.

### Phylogenetic Analysis of Leaf Blight Bacterial Isolates

From the amplification of PCR based on the *gyr B* gene sequences with *gyrB01-F* and *gyrB02-R* primer, all the samples resulted in single band at 970 bp. Phylogenetic analysis using Jukes-Cantor algorithm in Mega 7.0 revealed that the bacterial isolates causing leaf blight of garlic from Tawangmangu (PT1), Temanggung (PP7) and shallot of Tawangmangu (MT3 and MT4) were closely related to *P. ananatis* PA13 isolated from rice plants (Choi *et al.*, 2011) with a 99% degree of homology and bootstrap value 99. PT3 and MT2 isolates are closely related to *P. stewartii* subsp. *stewartii* CFBP 3614 (Rezzonico *et al.*, 2009) with 99% homology and bootstrap value 89 (Figure 2).



Figure 1. Symptoms of leaf blight and discoloration in shallot (A) and garlic (B) as shown by the arrow

Table 1. Physiological and biochemical analyses of bacterial isolates from garlic and shallot

Assays	Isolates					
	PT1	PT3	PP7	MT2	MT3	MT4
Gram assay	-	-	-	-	-	-
OF-test	O	O	O	O	O	O
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-
Urease	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-
Levan formation	+	+	+	+	+	+
H <sub>2</sub> S production	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-
Indole production	+	-	+	-	+	+
Gelatin liquefaction	-	-	+	+	+	+
Motility	+	+	+	+	+	+
Acid production or growth on Ayers media containing:						
Glucose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+
Maltose	-	-	-	-	-	-
Hypersensitive reaction	+	+	+	-	+	+
Pathogenicity	+	-	+	-	+	+

Description: PP (garlic isolate of Temanggung), PT (garlic isolate of Tawangmangu), MT (shallot isolate of Tawangmangu).  
+ : indicated positive reaction; - : indicated no reaction; O : indicated oxidative

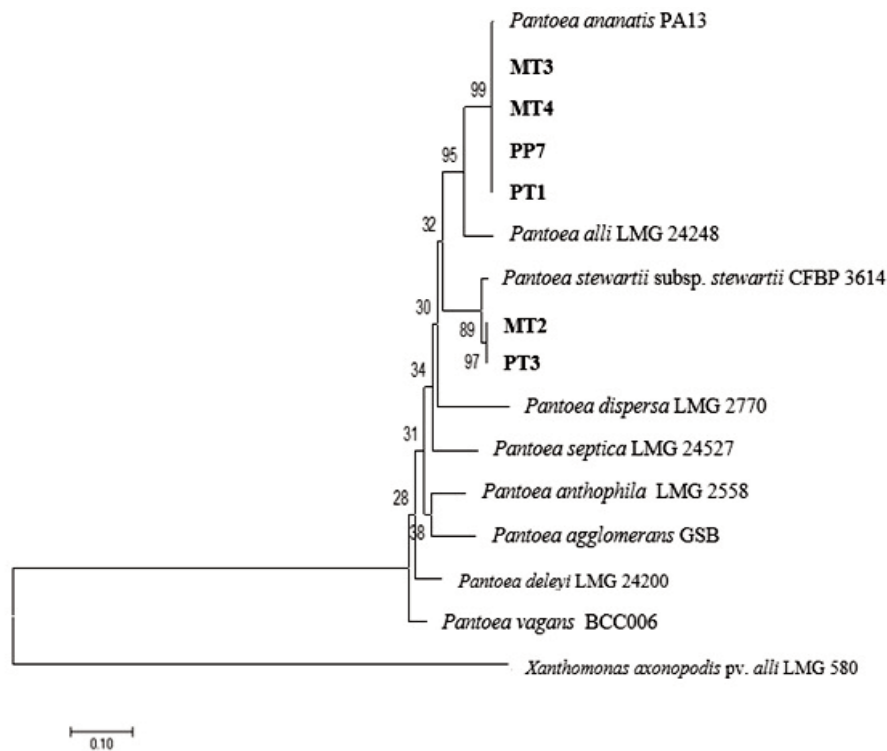


Figure 2. Phylogenetic tree showing the relationship of the leaf blight bacterial isolates and the closely related strains available in the GenBank; on the basis of the alignment of *gyrB* gene sequences, a phylogenetic tree was constructed using the neighbor-joining method; the stability of the tree was assessed by 1000 bootstrap replications with the neighbor-joining method and Jukes-Cantor distance analysis; the sequence of *Xanthomonas axonopodis* pv. *alli* strain LMG 580 was used as an outgroup

## CONCLUSION

The use of other phylogenetic markers beside 16S rRNA is necessary to achieve unambiguous identification at the species level or below. In the present study we describe a method for identifying *Pantoea ananatis* using partial sequence of *gyrB* as a phylogenetic marker. Since *gyrB* is a universally distributed gene in all prokaryotes, we are currently extending our work to similar studies of other bacterial families with the aim of establishing the use of *gyrB* as a universal phylogenetic marker.

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