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Nucleic Acid and Protein Profile of Bacteriophages that Infect *Pseudomonas syringae* pv. *glycinea*, Bacterial Blight on Soybean

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Abstract

Several management strategies of Bacterial blight disease caused by *Pseudomonas syringae* pv. *glycinea* has been developing to minimize yield loss including the use of bacteriophage as phage therapy. However, some consideration must be point on its interaction with bacterial host and influence of bacterial host virulence. Therefor, information of characteristic of prospective bacteriophage must be determined prior to use as biological control agent. This research aimed to obtain some information about general characteristics of bacteriophage that infects *P. syringae* pv. *glycinea* such as the ability in propagation in bacterial host, type of nucleic acid, and total protein profile. The results showed that about four bacteriophages have been isolated from soybean soil in Sukorambi and Mangli area named ϕSK2a, ϕSK2b, ϕSK2c (from Sukorambi), and ϕMGX1 (from Mangli). All phages had different ability in propagation in bacterial host, *P. syringae* pv. *glycinea* strain H3 about 10^{10}-10^{12} PFU/mL. Nucleic acid analysis using digestion enzymes (DNase, RNase, and Restriction endonuclease) showed that all bacteriophage isolates had double stranded deoxyribonucleic acid (dsDNA). Moreover, protein profile of bacteriophage through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that all phages had similar pattern of the protein suggested the same type of bacteriophage. However, morphological characteristic is still needed to accurate determination of bacteriophage taxonomy.

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Keywords: P. syringae pv. *glycinea*; bacterial blight; soybean; bacteriophage

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1. Introduction

Pseudomonas syringae pv. *glycinea* is one of important plant-pathogenic bacteria that infect soybean in field (Qi *et al*., 2011). In Jember, Indonesia, this pathogen is reported to infect either local soybean or edamame with several destruction on stem, leaf and pod (Masnilah et al., 2013). Suryadi et al. (2009) reported that yield losses due to *P. syringae* is about 11% to 20%. In addition, Semangun (2008) described that infected-plant showing dwarf and killed if the pathogen infect young plant. Therefor, consideration to detection of the presence of the pathogen is important in order disease management to ensure quality and quantity of soybean production.

The use of resistant plant is one among the common management technique to control this pathogen (Selote & Kachroo, 2010). However, this technique is less efficient due to the adaptability of the pathogen against extreme-environment including resistance of host-plant (Farhatullah et al., 2011). Therefor, alternative strategies to control *P. syringae* is needed in supporting national soybean production such as utilization of bacteriophage through phage therapy (Chan *et al*., 2013).

The use of bacteriophage as biological control agent still not widely reported against of plant-pathogenic bacteria which recently focus on animal and human pathogens (Sulakvelidze *et al*., 2001; Abendon *et al.*, 2011; Lu et al., 2011). Recently, Susianto et al. (2014) succeed in isolating bacteriophages that able to infect *P. syringae*, bacterial blight pathogen on soybean. These bacteriophages are able to infect four isolate among 12 tested-isolates which isolated from Jember, Indonesia. However, characterization and its interaction with bacterial host is still unclear (Susianto *et al*., 2014). Accordingly, phage characterization is important step in utilization bacteriophage as biological control agent (Addy et al., 2012a; Yamada et al., 2012). Addy et al. (2012b; 2012c) reported that phage infection might affect on host virulence, such as filamentous phage ϕRSM3 and ϕRSS1 in *Ralstonia solanacearum*. Similar report is also reported by Ahmad et al. (2014) that bacteriophages Cp1 and Cp2 affect the virulence of *Xanthomonas axonopodis* pv. *citri.* In addition, Addy et al. (2012c) suggested bacteriophage might not be used directly as biological control because it can enhance the virulence of bacterial host. In addition, Davis et al. (2000) and Das et al. (2011) also reported similar results that Vibrio cholera become more virulent if it infected with filamentous phage CTX. Therefor, this research is important to characterize and to study the bacteriophage that infects bacterial blight pathogen as a step in utilization bacteriophage as biological control.

2. Materials and Methods

2.1. Bacteriophages and P. syringae propagation

Bacterial isolates (*P. syringae*) were obtained from Department of Plant Protection, Faculty of Agriculture, University of Jember. All isolates were grow in King's B medium (King et al., 1954) and were sub-cultured in *Nutrient Broth* (NB) at ± 28^{o} C for 24 hours.

Phage particles of ϕSK (ϕSK2a, ϕSK2b, and ϕSK2c) were obtained from Susianto et al. (2013) and ϕMGX1 was obtained from soil in Mangli Area, Jember, Indonesia. Phages propagation was done on agar plate as described by Askora et al. (2009). Briefly, a 100 µl filtrat that may containing phages was mixed with 250 µl of overnight culture of *P. syringae* PSGH3 and was incubated for 2 hours before mixing with *Top Agar* (0.45% NA) followed by pouring on to NA in petridish. Plates, were then incubated at 28°C for 24-48 hours. Single plaque was picked-up and put in NB media for propagation.

2.2. Phage particles isolation

Isolation of phage particles was done by following Yamada et al. (2007) with modification. About 5 mL of SM buffer was added on to petridish (plaque-assayed plates) and incubated for 30 minutes at 4℃ with shaking at 60 rpm. Suspension was then pelleted by centrifugation at 4℃, 7000 rpm for 20 minutes. About 20 mL of supernatant was filtered using membrane (0,22 µm) into Falcon tube (volume 50 mL) containing 20 mL of 20% PEG6000 and 1 M NaCl. Solution then incubated at 4℃ for overnight and was pelleted using Micro Ultracentrifuge (Hitachi CS150FNX) at 4℃, 40000 rpm for 4 jam. Pellet was suspended in 100 µl of Tris-HCl-EDTA 10 mM.

2.3. Nucleic acid isolation and phages protein profile

Genomic isolation of all bacteriophages was done by following Swanson *et al*. (2012) with modification. Before genome extraction, all isolated-phages suspension were treated with DNase (Promega) and RNase (Promega) as described in manual guideline. Genome extraction and purification was done by adding 1:1 volume of Phenol:Chloroform:Isoamil alcohol/PCI (25:24:1), vortexed and incubated at -20°C for overnight before pelleting by centrifugation at 15.000 rpm, 4°C for 15 minutes. The upper layer was transfered into new tube and added with 3 M sodium asetat (10% × volume sample) followed by adding 2,5 × volume of absolute ethanol. Mixture was then incubated at -20°C for overnight and pelleted by spinning at 15.000 rpm, 4°C for 15 minutes. Pellet was washed by adding 500 µl of 70% etanol and re-spin for 5 minutes before air-dried and re-suspended in TE buffer 10 mM pH 8.

Phage protein profile was visualized using Sodium Dedoxyl Sulphate Polyacrilamide Gel Electrophoresis with 15% Acrilamyde for 3 to 4 hours at 50 V before stained with Commassie briliant blue stain and washed with de-staining solution (Methanol : acetic acid glasial : $_{dd}H_2O$).

2.4. Nucleic acid digestion

To know the type of phages genome, digestion with several nucleases DNase, RNase and restriction enzyme were done at 37°C for 2 hour before purification using PCI and precipitation with Ethanol. Pellet was then dissolved using TE buffer and visualized in 1% agarose gel with ethidium bromide staining.

3. Results and Discussion

It was obtained four isolate of phages that able to infect *P. syringae* pv. *glycinea* (Fig. 1a and b) and was distinct based on its plaques morphology on agar with bacterial-host lawn (Fig. 1c). Plaques were distinct on their visibility of single-plaque forming as wideness about 1-3 mm in diameter. About three bacteriophages were isolated from soil in Sukorambi area, named ϕSK2 phages. In addition, one isolate of bacteriophage was isolated from soil in Mangli area, named ϕMGX1 (Fig. 1c). All ϕSK2-types were also distinct based on their affect on bacterial host growth as ϕSK2a which showed light brown color on bacterial growth medium, ϕSK2b which showed dark-brown color, and ϕSK2c which showed green color on medium (Fig. 1d). In general, propagation ability on the same bacterial host were different each other (Fig 2).

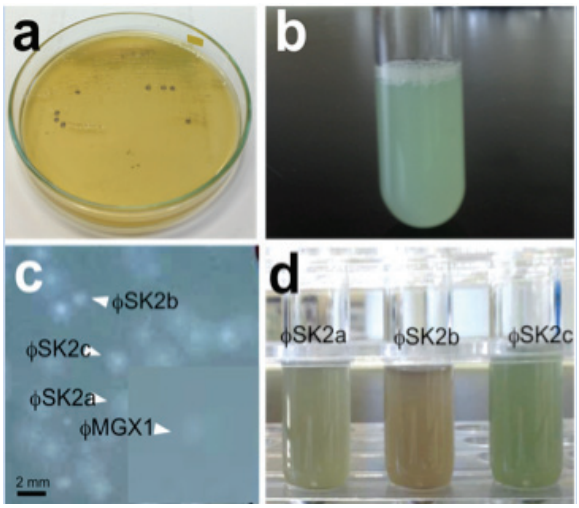

Figure 1. Character of *P. syringae* pv. *glycinea* isolate on nutrient agar media (a), nutrient broth media (b), and plaques morphologies (c) of four bacteriophage isolates and its differentiation on bacterial-host growth on growth medium after inoculation by phage ϕSK-types

In general, *P. syringae* pv. *glycinea*, belong to Pseudomonas Fluorescens, is known to have the ability to produce siderophores and fluorescent pigment (Qi et al., 2011). Bultreys et al. (2001) confirmed that bacteria from fluorescent pseudomonads are able to produce fluorescent pigment and can be diffused into the media. In this result, discoloration occurred on the media, which were inoculated with different bacteriophages resulting (Figure 1d), suggesting the influence of bacteriophage in altering physiological and morphological character of bacterial host. Addy et al., (2012) described that interaction between the bacteriophage and its bacterial-host caused changes in morphology and physiology character, including the virulence of the bacterial host.

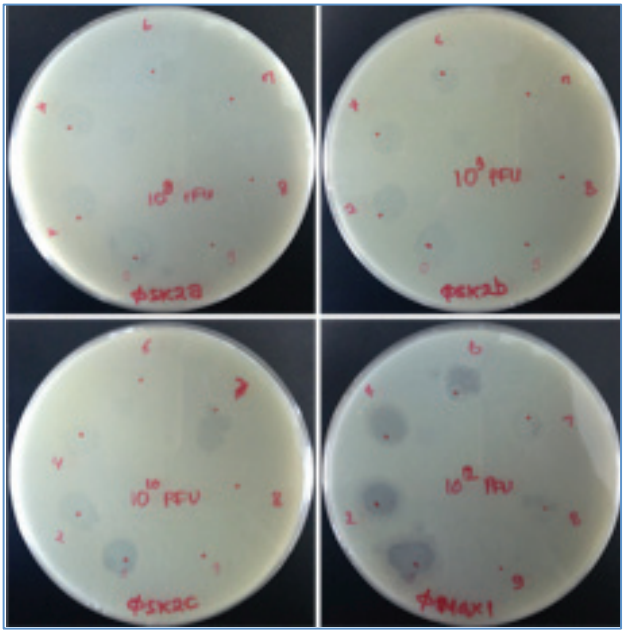

Figure 2. Plaques of bacteriophages on the lawn of *P. syringae* pv. *glycinea* PSGH3 on nutrient agar medium after 24 hours post incubation at 28°C.

Nucleic acid isolation from bacteriophages, which was followed by nucleases digestion, showed that there was a cotton-like structure during precipitation step in genome extraction protocol (Fig 3). All genomics from phage isolates were able to be digested using DNase and nuclease like *Eco*RI and *Hind*III (Fig. 3b), but not with RNase indicating that the nucleic acid type of bacteriophages was double stranded deoxyribonucleic acid (dsDNA).

In general, bacteriophage has the only one type of nucleic acid, either DNA or RNA. Most of nucleic acid of bacteriophages that infect phytopathogenic bacteria is either single-stranded or double-stranded DNA. Yamada et al. (2007) observed that bacteriophages that infect bacterial wilt pathogen on tomato and tobacco have double-stranded DNA like in ϕRSB1, and ϕRSA1 or single strand DNA as it is known at ϕRSS1, and ϕRSM1.

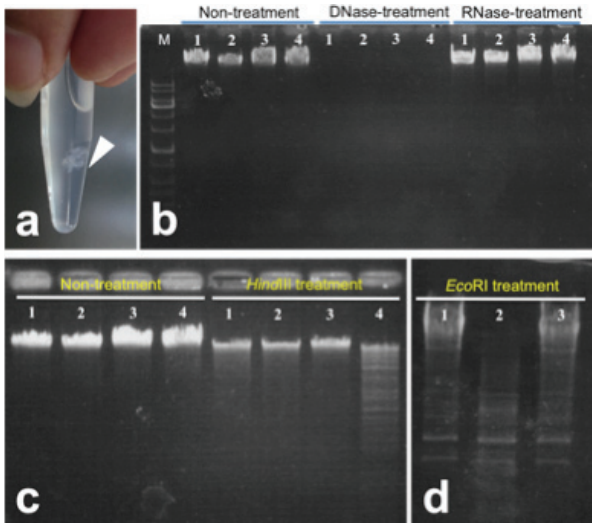

Fig 3. Cotton-like structure during nucleic acid precipitation (a). Electrophoresis profile of nucleic acid of ϕSK2a (1), ϕSK2b (2), ϕSK2c (3) and ϕMGX1 (4) after treated by nuclease, such as DNase, RNase (b), and two endonuclease such as *Hind*III(c) and *Eco*RI (d).

Moreover, protein profile on SDS-PAGE showed that similar protein bands were appear on polyacrylamide gel (Fig. 4), indicated that all bacteriophage are the same phage type.

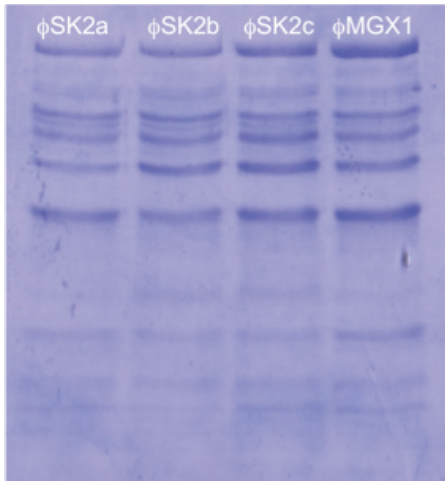

Fig 4. Protein profile of bacteriophages on SDS-PAGE

Bacteriophage is virus of bacteria that has similar structure like the most of virus group, which is composed of nucleic acid and protein. Black and Rao (2012) described that protein of the virus or bacteriophage can be composed of several sub-units such as clip, stem, wing and crown domains as constituent of portal protein of bacteriophage T4. In this research note that the protein subunits of bacteriophages that infect PSG more than one marked by numerous ribbons protein electrophoresis results (Figure 4). These results indicated that bacteriophages which infects *P. syringae* pv. *glycinea* had complex structure as bacteriophage that belonging to the family of Siphoviridae, Myoviridae and podoviridae.

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