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Bacteriophages of *Pseudomonas tolaasii* for the Biological Control of Brown Blotch Disease

Min-Hee Kim, Seong-Wan Park, and Young-Kee Kim*

Department of Environmental and Biological Chemistry, Chungbuk National University, Cheongju 361-763, Korea

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Pseudomonas tolaasii **causes brown blotch disease in cultivated mushrooms by producing tolaasin, a peptide toxin, which forms pores on the membrane and disrupts the cellular and fruiting body structures of mushrooms. For the biological control of this mushroom disease, virulent bacteriophages of *P. tolaasii* were isolated from the sewage of Cheongju, Korea. Twenty-one phages were isolated from four different locations, and their toxicities to host bacteria were measured by inspecting the turbidity and size of their plaques. They were divided into three categories on the basis of their toxicities to host bacteria. In order to test if these phages can be used for the biological control of mushroom diseases, a pitting test was performed. The surfaces of mushroom caps were inoculated with both pathogenic bacteria and their phages. Phage toxicity was analyzed by measuring the size of the blotches that formed on the surface of mushrooms, because these sizes are representative of the amount of tolaasin peptide produced by pathogenic bacteria in the presence of bacteriophages. The formation of blotches was completely blocked by co-incubated phages. These results show that phages can sterilize pathogenic bacteria in mushroom tissues as well as be useful for the biological control of brown blotch disease. The optimum conditions for the bactericidal activity of the phages were also determined.**

Key words: bacteriophage, brown blotch disease, oyster mushroom, phage therapy, *Pseudomonas tolaasii*

Oyster mushroom (*Pleurotus ostreatus*) is a commercially important crop in Korea. It ranks first in terms of cultivation area and third in terms of production in the mushroom industry. Cultivation of oyster mushroom is often interrupted by major outbreaks of infection with *Pseudomonas tolaasii*, *Trichoderma*, and mushroom flies. *P. tolaasii* causes brown blotch disease of oyster mushroom [Tolaas, 1915]. This pathogenic bacterium produces tolaasin [Peng, 1986], a peptide toxin, which forms pores on the cellular membranes, resulting in the disruption of mushroom tissue and blotches on the mushroom cap.

Brown blotch disease often makes mushroom cultivation very difficult, because the pathogenic bacteria spread easily through the cultivation shelf and then the cultivation room, especially during the growth phase of the fungus and the early budding period. The tolaasin molecule comprises 18 amino acids and an octanoic acid at the N-terminus of the peptide. Since tolaasin molecules behave as a detergent and have pore-forming properties, they can spread easily on the surface of fungi and mushroom caps [Hutchison and Johnstone, 1993]. When the caps of growing mushroom are infected, tissues are rapidly turned into brown-colored blotches and eventually melted down, leaving mucus and liquids with unpleasant smell. The disease spreads rapidly, making mushroom cultivation impossible in farms and eventually, the whole village [Kim *et al*., 2007].

In order to prevent the disease, underground water used for cultivation should be sterilized and the cultivation room should be clean. Furthermore, during the preparation of cultivation shelves, the room should be fumigated with hot air [Wong and Preece, 1985; Geels *et al*., 1991]. For the biological control, antagonistic bacteria [Soler-Rivas *et al*., 1999] and specific bacteriophages [Guillaumes *et al*., 1998] were tested, and crop losses were reduced [Munsch *et al*., 1991]. Nevertheless, problems of resistant bacteria made large-scale experiments invalid, and no further experiments were reported. Antibiotics can control diseases [Geels, 1995]; however, they cannot be used to cultivate edible mushrooms. Therefore, no useful

*Corresponding author
Phone: +82-43-261-2560; Fax: +82-43-271-5921
E-mail: ykkim10@chungbuk.ac.kr

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tools are presently available for disease control.

There are many advantages of using bacteriophages, such as preventing contamination by pesticides and suppressing the development of bacterial drug-resistance [Chanishvili *et al*., 2001]. Recently, the usefulness of phage therapy for preventing the bacterial resistance to antibiotics was reevaluated. Since phages are highly specific to host bacteria and have a rapid sterilization effect, they were found to be more effective than chemical therapy [Greer, 2005; Matsuzaki *et al*., 2005]. Since phages are specific to their host bacteria, various phages of pathogenic bacteria are needed. In the present study, various phages of *P. tolaasii* 6264 were isolated and characterized for use to control brown blotch disease.

Materials and Methods

Isolation and culture conditions of *P. tolaasii*. *P. tolaasii* 6264 was originally isolated in Korea from the fruiting bodies of oyster mushrooms severely damaged by brown blotch disease [Lee *et al*., 1997]. Mushroom tissues were homogenized, and the homogenate was extracted with sterilized distilled water. The extract was evenly spread onto a *Pseudomonas* agar F (PAF; Difco, Lawrence, KS, USA; Bacto-peptone, 10 g; Bacto-tryptone, 10 g; K_2HPO_4, 1.5 g; $MgSO_4$, 1.5 g; glycerol, 10 mL; agar, 15 g/L) plate, and *P. tolaasii* 6264 was isolated and identified by the white line test and pitting test on mushroom tissue [Kim *et al*., 1994] as well as by bacteriological characteristics using the method of Tsuneda *et al*. [1995]. The isolated bacteria were stored at 80°C in PAF broth contaning 20% (v/v) glycerol until use.

Bacteriophage isolation. Phages were isolated from various sewage samples obtained from Cheongju, Korea by using the double agar layer technique [Adams, 1959]. The hard agar layer was made with 1.5% agar, and the soft agar layer contained 0.7% agar. The supernatant fluid of each sewage sample was added to the culture of host bacteria. This solution was mixed with soft agar medium at a ratio of 1:2 and poured on to the hard agar medium. The double layer medium was incubated for 15 h at 25°C. One phage plaque was chosen for phage amplification, picked with a sterilized toothpick, and inoculated in 5 mL of PAF broth together with 1% of overnight culture of the *P. tolaasii* 6264 strain. In keeping with the method of Chibani-Chennoufi *et al*. [2004], NaCl was added to the lysate to a final concentration of 10% and incubated for 1 h at 0°C. After centrifugation at 8,000 rpm for 10 min, polyethylene glycol 6000 was added to the supernatant to a final concentration of 10%, and the lysate was incubated for 1 h at 0°C. Polyethylene glycol-precipitated phages were collected by centrifugation at 8,000 rpm for 10 min. Phage precipitates were resuspended in phage buffer (50 mM Tris-HCl, pH 7.3, 150 mM NaCl, 20 mM NH_4Cl, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2% gelatin).

Phage count and lysis activity. Phage titers were determined using the double agar layer technique [Sambrook and Russell, 2001]. Crude lysates were obtained by removing bacterial debris. The phage lysates were diluted 10^4-10^8 times, mixed with host bacteria in 3 mL of soft agar, and poured over solidified PAF hard agar medium. These plates were incubated for 15 h at 25°C under stationary conditions, and phage plaques were counted. In order to measure the lysis activity of phages, PAF broth was inoculated with 5% culture supernatant of *P. tolaasii* 6264 and 1% of diluted phage lysate. The broth was incubated at 25°C under aerobic condition (150 rpm) in a shaking incubator for 15 h. The absorbance was measured using a spectrophotometer at 600 nm.

Pitting test. The fresh fruiting bodies of *Agaricus bisporus* mushrooms were collected and inoculated with *P. tolaasii* 6264 following the method of Gandy [1968]. Mushroom caps were cut off horizontally, and 10 μL of bacterial culture was dropped on the surface. The mushrooms were incubated at 25°C in a plastic box saturated with water vapor. Brown discoloration around the inoculation spot was considered as a positive reaction. In order to evaluate the phage-induced inhibitory effects on brown blotch disease, the properly diluted phage lysates were mixed with *P. tolaasii* 6264 at a 1:1 ratio, and 10 μL of the mixture was dropped on the surface of the mushrooms. After 15 h incubation, the degree of discoloration was evaluated by measuring the area of blotch. A control experiment was performed using mixtures of *P. tolaasii* 6264 and sterile water.

Results

Bactericidal activity of bacteriophages. Twenty-one bacteriophages were isolated from sewage samples collected from four different locations in Cheongju, Korea. On the basis of their toxicities to the host bacterium, *P. tolaasii* 6264, phages were divided into three groups, according to the shapes of their plaques: big and clear, small and clear, and turbid, and bactericidal activities were evaluated as very toxic, toxic, and mildly toxic, respectively (Fig. 1).

In order to characterize host toxicities, three phages, namely, hb1a, hb2d, and bp5e, were chosen from each toxicity group. Bacterial lysis was measured on the basis of absorbance decrease at 600 nm. When 5% of host bacteria and 1% of diluted phages at a 100:1 ratio were cultured in PAF medium, complete bacterial lyses were obtained with hb1a and hb2d after 12 h (Fig. 2). The addition of phage bp5e slowly decreased the absorbance

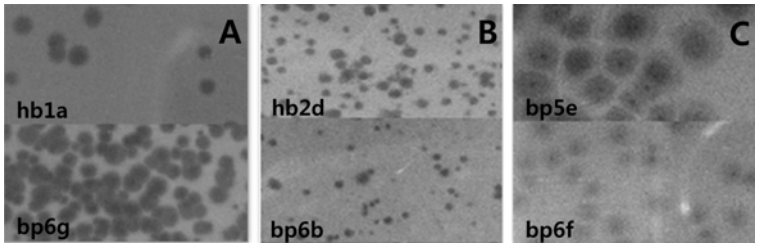

Fig. 1. Plaques formed by the isolated phages. (A) Clear and large plaques formed by highly toxic phages. (B) Clear and small plaques formed by toxic phages. (C) Large but turbid plaques formed by mildly toxic phages.

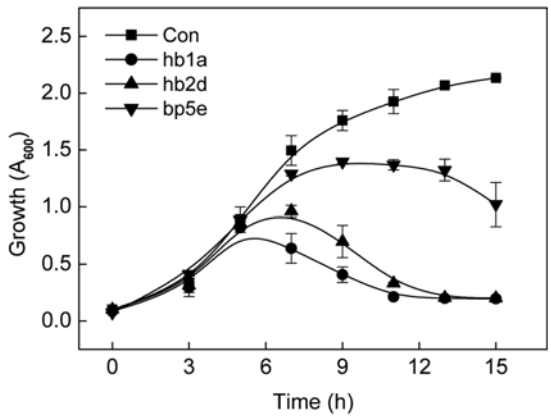

Fig. 2. Bacterial lyses caused by phages having various bacterial lysis activities. Host bacteria and phages were added simultaneously at a 100:1 ratio.

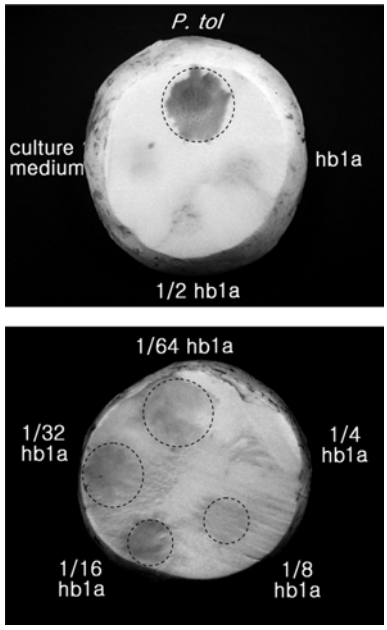

Fig. 3. Brown blotch formation by *P. tolaasii*. The diluted lysate of phage hb1a by 1,000 times was diluted again as indicated and added to the surface of a mushroom along with *P. tolaasii*. The mushroom was incubated for 15 h. *P. tol*: only pathogenic bacteria were added. The 1-1/64 hb1a: equal volumes of diluted phage lysate and pathogenic bacteria were added.

after 15 h, but no complete lysis was observed.

When complete bacterial lyses were obtained with phages hb1a, hb2d, and bp5e, their titers were determined to be 1.12×10^9, 1.35×10^9 and 7.93×10^9 pfu·mL^{-1}, respectively, implying that the densities of the phages were similar, regardless of their toxicities. These results show that the size and clearance of plaques represent the host toxicity of the phages. The phage hb1a decreased absorbance after a 5-h incubation, and complete bacterial lysis was achieved after 11 h. Similarly, the phage hb2d decreased absorbance at 7 h, and complete bacterial lysis was achieved after 13 h. The mildly virulent phage bp5e decreased absorbance at 9-10 h, and the decrease in absorbance was very slow.

Suppression of blotch formation by phages. The direct effect of phages on brown blotch disease was evaluated using the pitting test. This method measures the amount of collapsing on the surface of the mushroom after the surface is inoculated with the pathogen. *P. tolaasii* made big brown blotches on the cut surface of mushrooms after 12 h (Fig. 3). Blotch formation was suppressed when phage hb1a was simultaneously added, implying that hb1a can prevent brown blotch formation. Similar effects of phages were obtained with the phages of both very toxic and toxic groups. The effect of the phages decreased when the phage solution was diluted. More dilutions of phage solution increased the size of the blotches (Fig. 4). As the number of phages decreased with dilution, blotches were formed at dilutions of more than 1/8 times, and the size of the blotches increased as the dilution of the phage solution increased. Blotches formed by pathogen in the presence of phages at all dilutions were not dense and deep enough compared to those formed by only pathogen. These results show that simultaneous treatments of a pathogen and its phage suppress or decrease the formation of blotches, suggesting that the phage can be a good candidate for disease control.

Size of phage inoculation and bacterial lysis. The relationship between time taken for complete bacterial lysis and the size of phage inoculation was examined.

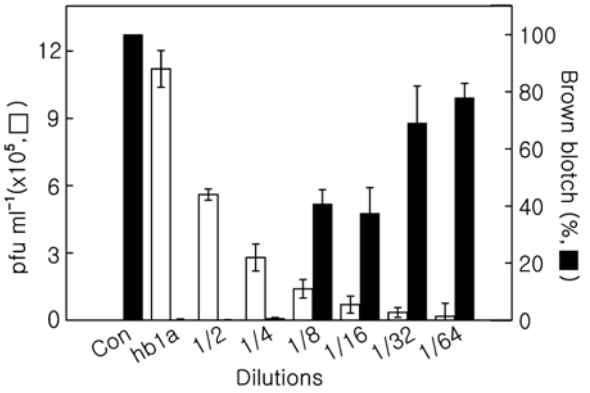

Fig. 4. Effects of bacteriophages on the formation of brown blotches. The phage hb1a was diluted as indicated and added to the surface of a mushroom. The sizes of the blotches were compared with those formed after inoculation with only host bacteria.

When 10, 100, and 500 μL of the diluted phage lysate of hb1a (1×10^5 pfu·mL^{-1}) were added, at bacteria:phage ratios corresponding to 5,000:1, 1,000:1, and 100:1, the absorbance decreased after 8, 6, and 5 h of incubation, respectively (Fig. 5A). After 13 h of incubation, complete bacterial lysis was observed. The diluted toxic phage hb2d (1×10^5 pfu·mL^{-1}) could not lyse bacteria completely at the bacteria:phage ratio of 5,000:1; however, at 1,000:1 and 100:1, complete lyses were observed (Fig. 5B). In the case of the diluted mildly toxic phage bp5e (7×10^5 pfu·mL^{-1}), the bacteria:phage ratios corresponding to 5,000:7, 1,000:7, and 100:7 showed decreases in absorbance after 7-9 h, and no complete lysis was observed after 15 h, regardless of the size of the inoculation (Fig. 5C).

Growth phase-dependent bacterial lysis. In order to measure host toxicity of bacteriophages at various growth phases, hb1a (10^5 pfu·mL^1) was added 0, 1.5, 3, 4.5, and 6 h after the beginning of incubation (Fig. 6). When phages were inoculated simultaneously with bacteria at 0 h, almost no bacterial growth was observed. Inoculations of phages at 1.5 and 3 h also resulted in complete bacterial lyses after 8 and 12 h of incubation, respectively. After adding phages at 4.5 h, the early stationary phase was observed at 12 h, but lysis was measured at 16 h. However, phage inoculation at 6 h resulted in incomplete bacterial lysis and maximal bacterial growth was obtained. These results showed that the bactericidal effect of the phage decreases as the ratio between a host bacterium and its phage increases. Therefore, as bacterial density increases, the host toxicity of phages appears to weaken, and achieving complete bacterial lysis becomes increasingly difficult.

Temperature effect on phage toxicity. After 4 h of incubation of *P. tolaasii*, phage solution (10^5 pfu·mL^{-1}) was added at various incubation temperatures: 20, 25, 30,

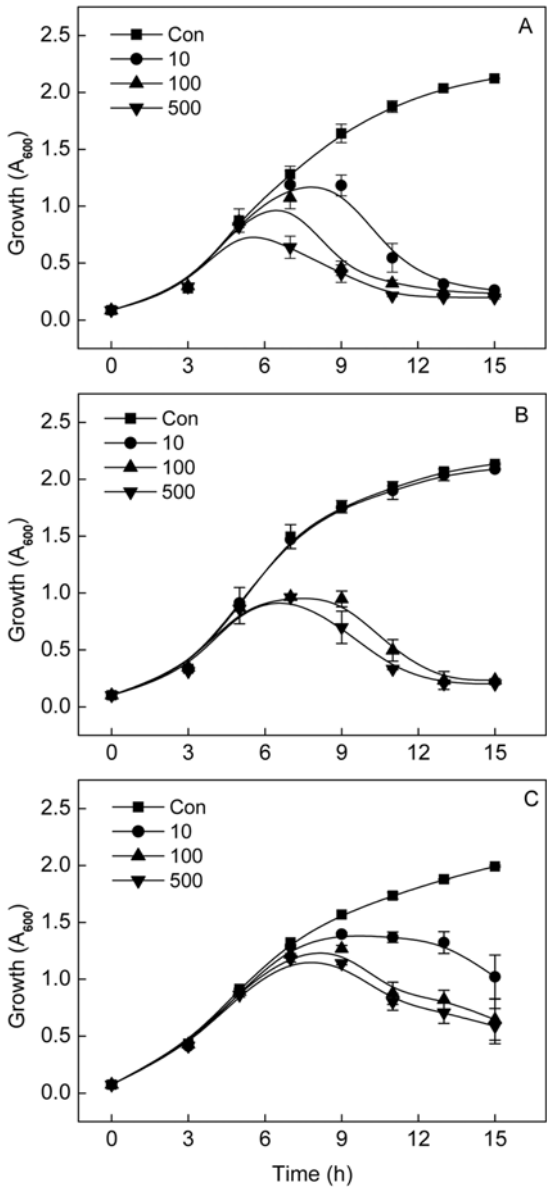

Fig. 5. Inoculation size-dependent bacterial lyses. Phages diluted by 10^4 times were added to the 50 mL of bacterial culture. The indicated volumes of diluted phages, (A) hb1a, (B) hb2d, and (C) bp5e, were added to the *P. tolaasii* culture.

and 37°C (Fig. 7). In the absence of phage treatment, *P. tolaasii* showed normal growth curves at 20, 25, and 30°C. However, bacterial growth was suppressed at 37°C, because the conditions were not optimal. The absorbance did not increase after 8 h of cultivation and was below 1.5 at 600 nm. In the presence of phages, complete bacterial lyses were observed at 20 and 25°C after 16 h of cultivation. At 30°C, the bactericidal effect of phages was slow. The absorbance decreased after 16 h of incubation and was 1.2 at 20 h. No bacterial lysis was observed at 37°C. The toxicity of the phages was maximal at the optimum temperature of the host bacteria.

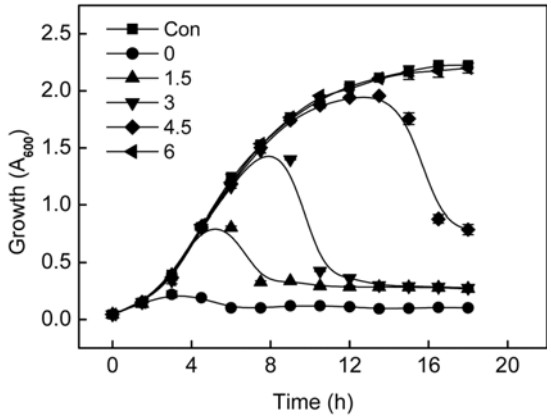

Fig. 6. Effect of phage inoculations at different growth phases. The phage hb1a was added to the culture at the indicated times.

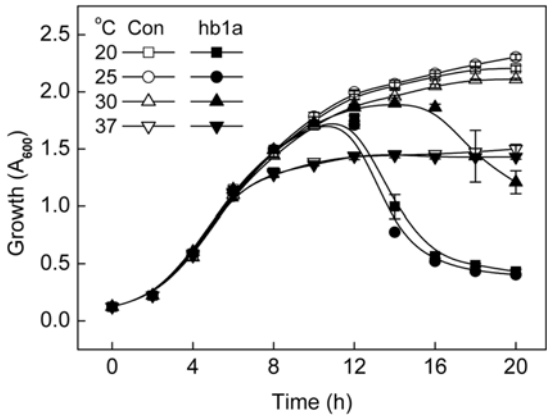

Fig. 7. Temperature-dependent bacterial lyses. Bacteria were cultured at the indicated temperature, and the phage hb1a was added after incubation for 4 h.

Discussion

Twenty-one phages of *P. tolaasii* 6264 were isolated, and their bactericidal activities were analyzed. Other characteristics of these phages, such as morphology, restriction endonuclease digestion patterns, and immunological criteria, are currently under investigation. They showed various toxicity levels toward the host bacterium. A positive relationship was observed between the virulence of phages and the size and clearness of their plaques. Phages that formed clear and big plaques were very toxic to their host bacteria and achieved complete bacterial lyses. However, phages that formed small and clear plaques or turbid plaques had relatively weak bactericidal activities (Figs. 1 and 2). They needed a longer incubation time to achieve bacterial lysis and often showed incomplete lysis. These results suggest that the host toxicities of phages can be evaluated qualitatively by analyzing their plaques.

Host toxicity of bacteriophages also depends on the growth phase of bacteria. Bactericidal activity of phages was maximal in the early logarithmic phase of host bacteria. In the late logarithmic phase and stationary phase, incomplete bacterial lyses were observed after inoculations with the toxic phage hb1a (Fig. 6). Phage hb1a-induced bacterial lyses were also dependent on the nutritional growth state of the host bacteria. These results were consistent with those of previous reports, showing that the growth of the phage depends on the physiological conditions of the host bacterium [Hadas *et al*., 1997; Cooper and Heinemann, 2000].

The incubation temperature was also one of the major factors determining phage toxicity (Fig. 7). The bactericidal activity of hb1a was maximal at or near the optimum temperatures of host bacteria. Since the optimum temperature of *P. tolaasii* is 25°C, complete bacterial lysis was also achieved at this temperature. The influence of bacterial growth phase on phage infection was similar to that of the bacteriophage of *P. fluorescens* [Sillankorva *et al*., 2004]. Although there was a report that the amount of phage inoculated is inversely related to the amount of phage production [Moebus, 1996], hb1a-induced bacterial lysis appears to depend on the amount of phage inoculated (Fig. 2). Therefore, when disease symptoms are detected, phage treatment should be administered as early as possible for the best disease control.

During the characterization of various bacteriophages of *P. tolaasii* 6264, the phages were found to control the density of pathogenic bacteria in cultivation farm area, and thus probably decrease the rate of disease outbreaks. Phage therapy appears to be more beneficial for controlling mushroom diseases due to the toxicity of antibiotics and agricultural chemicals on mushrooms. Nevertheless, difficulty in phage application still remains due to its high specificity to host bacteria. At present, we have more than 80 strains of *P. tolaasii*-like bacteria collected from farms damaged by brown blotch disease. These strains will be classified as pathogenic strains in terms of phage sensitivity [Chakrabarti *et al*., 2000] and specific bacteriophages will be isolated for each type of bacterial strain. Since various phages of *P. tolaasii* 6264 from sewage were isolated, we foresee no difficulty in isolating more phages from other locations. Once the phage sensitivities of these strains are completely determined, mixtures of phages will be available for disease control.

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