Various Pathogenic *Pseudomonas* Strains that Cause Brown Blotch Disease in Cultivated Mushrooms

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Abstract Brown blotch disease in cultivated mushrooms is caused by *Pseudomonas tolaasii*, which secretes a lipodepsipeptide, tolaasin. Tolaasin is a pore-forming toxin in the cell membranes, thus destroying the fruiting body structure of mushroom. In this study, we isolated pathogenic bacteria from mushrooms that had symptoms of brown blotch disease. In order to identify these bacteria, their 16S rRNA genes were sequenced and analyzed. Pathogenic bacteria identified as *Pseudomonas* species were thirty five and classified into five subgroups: P1 to P5. Each subgroup showed different metabolic profile measured by API 20NE kit. Fifty percent of the bacteria were identified as *P. tolaasii* (P1 subgroup). All five subgroups caused the formation of brown blotches on mushroom tissues and the optimum temperature was 25°C, indicating that they may be able to secrete causal factors, such as tolaasin and similar peptide toxins. These results show that there are at least five different pathogenic *Pseudomonas* species as blotch-causing bacteria and, therefore, strains from the P2 to P5 subgroups should be also considered and studied as pathogens in order to improve the quality and yield of mushroom production.

Keywords brown blotch disease · oyster mushroom · pore-forming toxin · *Pseudomonas tolaasii* · tolaasin

Introduction

The oyster mushroom (*Pleurotus ostreatus*) contains many valuable and nutritional compounds and has been reported to have beneficial effects on the suppressions of cancer, tumor, hypertension, and diabetic mellitus (Kubo and Nanba, 1996; Liu et al., 1997; Patel and Goyal, 2012). The oyster mushroom has ranked first in cultivation area and is one of the major crops in the domestic mushroom industry (Annual Report for Production of Mushroom, 2010). Although it is one of the most popular and potentially profitable crops for farm industry, it is risky business to cultivate oyster mushrooms because of its susceptibility to brown blotch disease (Fletcher, 1979). Brown blotch disease is one of the most serious diseases in oyster mushroom cultivation. Once the disease occurs in one part of the mushroom farm, it rapidly spreads to the rest of the cultivated area. Therefore, for the continuous cultivation, complete decontamination processes are necessary. Although mushroom cultivations using media tray or shelf system promise high yield and produce good quality mushrooms, cultivations using small plastic bags and bottles are very popular, because the latter methods are considered to be disease-free, even though both mushroom quality and profit are compromised (MushWorld, 2004). Recently, however, blotch diseases have been also reported in the farms using the bottle cultivation method, indicating that this method may not be disease-free any more.

Brown blotch disease in artificial mushroom cultivation was first reported in 1915 (Tolaas, 1915), and *Pseudomonas tolaasii* was identified as the pathogen responsible for the disease. General symptoms of the disease on various mushrooms include blotch formation and discoloration on the surface of mushroom caps, which is caused by a microbial peptide toxin, tolaasin (Moquet et al., 1996). Tolaasin, produced by *P. tolaasii*, is a lipodepsipeptide composed of 18 amino acids and its molecular structure has been determined by nuclear magnetic resonance and mass spectrometry (Nutkins et al., 1991). Tolaasin forms pores in the mushroom cell membranes, and destroys tissue structure by disrupting the osmotic pressure across the plasma membrane (Brodey et al.,

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1991; Rainey et al., 1991; Cho and Kim, 2003; Coraiola et al., 2006). The structural analogues of tolaasin peptide have been identified from the variety strains of *P. tolaasii*, Tolaasin I and II by Nutkins et al. (1991) and Cho et al. (2007), seven analogues by Bassarello et al. (2004) and eight analogues by Shirata et al. (1995).

In this study, pathogenic bacteria causing brown blotch disease or similar diseases were collected, and they were identified based on genetic and biochemical characteristics. Phylogenetic analyses based on sequence comparison of 16S rRNA genes revealed that five different species of *Pseudomonas* strains were included in the isolated bacteria. The pathogenic characters and biochemical characteristics of these species were compared.

Materials and Methods

Isolation of microorganisms. Pathogenic bacteria were isolated from the fruiting bodies of oyster mushrooms damaged by brown blotch disease. 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, USA; Bacto-peptone, 10 g; Bacto-tryptone, 10 g; K₂HPO₄, 1.5 g; MgSO₄, 1.5 g; glycerol, 10 mL; agar, 15 g/L) plate. Pathogenic bacteria were characterized by the white line test and pitting test on mushroom tissue as well as by bacteriological characteristics using the biochemical method (Tsuneda et al., 1995). The isolated bacteria were stored at –80°C in PAF broth containing 20% (v/v) glycerol until use.

Identification of microorganisms. Polymerase chain reaction (PCR) amplification for 16S rRNA gene sequencing was performed by the Macrogen Corporation (Korea). The universal primers, 518F (5'-CCAGCAGCCGCGTAATACG-3') and 800R (5'-TAC CAGGGTATCTAATCC-3'), were used to amplify the gene. The PCR products were purified using the Wizard SV Gel and PCR clean-up system (Promega Co., USA). Data analysis was carried out using an ABI PRISM 3700 DNA analyzer and the sequence homologies with other pseudomonads were determined using the BLASTn software (NCBI, USA). Phylogenetic trees were constructed using the neighbor-joining method and the evolutionary distance was calculated using the MEGA6 program (Tamura et al., 2013).

Hemolysis test. Cytotoxic effects of tolaasin and its analogues were evaluated on rat erythrocytes (Cho et al., 2010). Rat erythrocytes were collected, defibrinated, and washed thrice with HEPES-buffered saline (HBS; 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 5 mM KCl, 1 mM MgSO₄, and 150 mM NaCl, pH 7.4). Erythrocytes were diluted to 10% solution by using HBS. Tolaasin and bacterial culture supernatants were added to the erythrocyte solution, and incubated for 30 min at 37°C. Hemolysis was monitored by measuring the absorbance decreases at 600 nm (U-2000; Hitachi Ltd., Japan).

Metabolic activities measured by API assay. The metabolic activities of the isolated pathogenic strains were measured by using the strip of API 20NE (bioMérieux, France). Testing was

performed according to the manufacturer's instruction. The test strip consisting of 20 micro tubes was filled with the suspensions of the isolated bacterial strains with an appropriate density. The strip was placed in humid chamber and incubated for 24–48 h at 30°C. The specific results of API 20NE were mostly read after 24 h. Color changes in micro tubes caused by bacterial metabolic activities were monitored during incubation and the results of those reactions were compared with among the examined strains.

Pitting test. Fresh fruiting bodies of *Agaricus bisporus* mushrooms were purchased in the local market and inoculated with the isolated pathogenic strains following the method of Gandy (1968). The top of mushroom cap was cut off horizontally and 5 µL of the isolated bacterial culture was dropped on the cut surface. The mushrooms were incubated at 25°C in a plastic box saturated with water vapor. Brown discoloration and blotch formation around the inoculation site were considered as a positive reaction. After 15 h incubation, the degree of discoloration was evaluated by measuring the area of blotch. A control experiment was performed by using culture media.

White line production test. The colony of a reacting pseudomonad, *P. reactans*, was streaked out in a line in the middle of the PAF plate. The isolated strains to be tested were inoculated on to the same plate at close to the reacting organism. Many of the isolated strains were tested at one time. Plates were examined after incubation for 24 h at 25°C for the production of a white line with the reacting organism.

Results and Discussion

Classification of the isolated *Pseudomonas* strains. The pathogenic bacteria causing the brown blotch disease were isolated in Chungcheong and Gyeongbuk provinces in Korea. Thirty-five strains identified as *Pseudomonas* species were divided into 5 subgroups, P1-P5 (Table 1). Twenty-three strains were identified as *Pseudomonas tolaasii*, named as P1 subgroup (Yun et al., 2013). The 16S rRNA genes of the isolated strains were sequenced and registered in the NCBI GenBank database. Their gene sequences were then subjected to BLAST analysis to generate a phylogenetic tree (Fig. 1). The representative strain of each subgroup shown in Table 1 was identified by comparing sequence homology. In a separate study, *P. brenneri* has been

Table 1 Grouping of the isolated pathogens by gene analysis

Sub-group	Isolated strains	Representative strain* ¹	Accession number* ²
P1	23	<i>Pseudomonas tolaasii</i>	JN187439
P2	2	<i>Pseudomonas abietaniphila</i>	JX417442
P3	3	<i>Pseudomonas azotoformans</i>	JX417444
P4	2	<i>Pseudomonas plecoglossicida</i>	JX417431
P5	5	<i>Pseudomonas brenneri</i>	JX417433

*¹Representative strain was determined by BLAST analysis.

*²The sequence of 16S rRNA gene was registered in NCBI GenBank database.

Fig. 1 Phylogenetic tree analysis of subgroup pathogens. The pathogenic strain of each subgroup is denoted in bold letters.

recently identified as a pathogen causing blotch disease in the oyster mushroom (unpublished data). Although representative microorganisms in the P2, P3, and P4 subgroups have not been reported to be responsible for mushroom diseases, they are involved in the metabolisms of various chemical compounds, such as resin (Annette et al., 2001), Cyhalofop (Nie et al., 2011), naphthalene (Tikilili and Chirwa, 2009), and phenanthrene (Muñoz et al., 2003). Other *Pseudomonas* species, such as *P. reactans* and *P. constantinii*, have been reported as causal agents of the disease (Munsch et al., 2002); however, none of these bacteria have been isolated in this study.

Biochemical and physiological activities. Metabolic activities of the isolated *Pseudomonas* strains were evaluated by API 20NE test strip. There are 22 items representing the biochemical metabolic activities in each test strip. Nine items were able to distinguish among the strains of 5 subgroups (Table 2). Strains No. 6264 of P1 subgroup and No. 147 of P5 subgroup showed positive activities in 14 items out of 21; however, these two strains showed opposite results in arginine dihydrolase (ADH) activity and phenylacetic acid (PAC) assimilation tests. Strains, No. 50 of P3 subgroup and No. 60 of P4 subgroup, showed positive results

in 13 and 12 items, respectively. These two strains showed difference in 5 items. Nitrate uptake activity was only observed by the strain No. 60 of P4 subgroup. Although the API 20NE was developed for the identification of Gram-negative bacteria including *Pseudomonas*, the isolated strains in this study showed different metabolic profiles. Furthermore, even those strains within the P1 subgroup showed slightly different metabolic patterns. Therefore, the results obtained with API kit were helpful to characterize the isolated strains, but not applicable for the identification of those strains as a definite criteria.

Cytotoxicity and pathogenicity of the isolated strains. In order to evaluate the pathogenic characteristics of the isolated strains, blotch formation was evaluated by pitting test on the surface of mushroom cap. All strains were able to cause brown blotches (Fig. 2). Strains of the P2 and P3 subgroups caused relatively mild blotches; however, those of the P4 and P5 subgroups caused severe sunken and dark brown blotches, similar to those formed by the strains of the major pathogen, P1 subgroup. The fact that blotches were caused by all strains of P2-P5 subgroups indicates that these isolated strains may be able to secrete tolaasin or analogous peptide toxins. The cytotoxicities of the isolated strains

Table 2 Biochemical properties of *Pseudomonas* strains of P1 to P5 subgroup measured with API kits

Subgroup	Strain No.	ARA	GEL	ADH	NAG	ADI	MAL	PAC	NO ₃	Colony type*
P1	6264	+	+	+	+	+	+	-	-	S
P2	62	+	-	-	-	-	-	-	-	S
P3	50	+	+	+	+	+	-	-	-	S
P4	60	-	+	+	-	-	-	+	+	S
P5	147	+	+	-	+	+	+	+	-	R

The same results obtained by all 5 subgroups: TRP (-), GLU fermentation (-), URE (-), ESC (-), PNP (-), GLU assimilation (+), MNE (+), MAN (+), GNT (+), CAP (+), MLT (+), CIT (+), OX (+).

*Colony type: S, smooth; R rough.

Fig. 2 Brown blotch formation caused by *Pseudomonas* strains of the five subgroups during the pitting test.

Fig. 3 Hemolytic activities of representative strains from the five subgroups.

were also evaluated by measuring their hemolytic activity on rat blood erythrocytes (Bernheimer and Rudy, 1986; Rainey et al., 1991). The hemolytic activities of the P2-P5 subgroup strains were compared with that of the P1 subgroup, *P. tolaasii* 6264 strain (Fig. 3). Culture supernatant from each strain was mixed with erythrocytes, and absorbance was measured every 5 min for 1 h. Strains of P3 and P4 subgroups had higher hemolytic activities than *P. tolaasii* 6264, as hemolyses were completed within 20 min. The strains of P5 subgroup caused complete hemolysis within 30 min, similar to that of P1 subgroup strains. Interestingly, strains of P2 subgroup did not show any hemolytic activity although they showed positive responses in the pitting test. The P2 strains may have toxins different from those of other subgroups, with high binding affinity to mushroom cells but low or no affinity to red blood cells. No hemolytic activities were also observed from the P1β strains of P1-type pathogens (Yun et al., 2013). Nevertheless, the isolated strains were able to destroy mushroom cells as shown by the results of the pitting test, indicating that all strains of P1-P5 subgroups have cytotoxic activities, presumably due to the secretion of cytolytic toxins. The difference in cytolytic activities among subgroups might be due to

the difference in toxin characteristics, such as binding affinities to erythrocytes and the amount of secretion.

The pathogenic activities of the P1-P5 subgroup strains were evaluated by measuring the development of brown blotch disease during bottle cultivation. Since early budding mushrooms are more sensitive to pathogenic bacteria, strains from the P1-P5 subgroups were inoculated to growing mushrooms with cap diameters less than 1 cm. For each inoculation, a 1:1 mixture of culture supernatant from each pathogenic bacterium and sterilized fresh medium was sprayed onto the growing mushrooms and, for the control treatment, only fresh medium was used (Fig. 4). Upon inoculation of each pathogenic strain, the growth of fruiting body was severely inhibited. Phenotypes of blotch disease were very similar, but the speeds of disease development were different dependent on the subgroup strains. Following the inoculations of P1 and P2 strains, mushroom caps turned brown within a day. Blotch diseases caused by the strains of P4 and P5 subgroups were mild; however, the growths of mushroom caps were almost completely inhibited, and mushroom discolorations were also detected. These results suggest that the strains of P2-P5 subgroups as well as P1 subgroup secrete toxins with varying levels of

Fig. 4 Brown blotch disease caused by subgroup strains during bottle cultivation.

Fig. 5 White line precipitation tests of five subgroup strains against line-cultured *P. reactans*.

mushroom tissue cytotoxicity, and are able to cause brown blotch disease or similar symptoms on cultivated mushrooms.

White line precipitation by peptide toxins. The white line reaction represents the formation of a peptide precipitate by a specific interaction between two lipodepsipeptides secreted by the pseudomonad species, *P. tolaasii* and *P. reactans*. This is a method for the evaluating the similarity of a new peptide toxin to tolaasin by measuring the binding of this toxin to another toxin, so-called ‘white line inducing principle (WLIP)’, secreted by *P. reactans* (Wong and Preece, 1979). When the strains of subgroups were incubated at a distance of 1 cm from a line culture of *P. reactans*, white line formation (arrow) was only observed close to the colony of strain from the P1 subgroup (Fig. 5). Since WLIP has ability to interact with tolaasin I, these results indicate that, although subgroups P2-P5 have been identified as pathogens, the peptide toxins secreted by these strains are similar in terms of causing brown blotch but not the same as tolaasin peptide.

In summary, strains from P2-P5 subgroups were identified as pathogens that can cause brown blotch disease in cultivated mushrooms. In order to prevent brown blotch disease more efficiently, these strains should be also considered pathogens and studied together with strains from the major pathogen P1 subgroup. In future study, the toxins secreted by each subgroup should be isolated and identified in both structure and function. Especially, subgroup-specific physiological characteristics of the toxin, such as membrane pore formation and the size and ion selectivity of the pore, should be investigated to explain the phenotypic difference among subgroup strains.

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