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Isolation of a Gram-positive bacterium effective in suppression of brown blotch disease of cultivated mushrooms, *Pleurotus ostreatus* and *Agaricus bisporus*, caused by *Pseudomonas tolaasii*

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A Gram-positive bacterium was isolated from a rotting *Pleurotus ostreatus* fruiting body that markedly reduces the level of extracellular toxins (i.e., tolaasins) produced by *Pseudomonas tolaasii*, the most destructive pathogen of cultivated mushrooms. The isolated bacterium is saprophytic but not parasitic nor pathogenic to *P. ostreatus*. A low ratio, ca. 10^{-3} cells of the isolated bacterium for one *P. tolaasii* cell, was sufficient for detoxification in vitro. Inoculation of the isolated bacterium prevents the development of bacterial disease in *P. ostreatus* and *Agaricus bisporus*. The suppression of the disease development, however, requires the initial cell density equivalent to ca. 10^{-1} cells of the isolated bacterium for one cell of the pathogen. The effect is ascribed to the inactivation of tolaasin by the live suppressive bacterial cells, and not to metabolites secreted from the organism into culture media. Examination by conventional bacteriological tests and with testing kits, i.e., MicroStation™System Release 3.5 (Biolog Inc., Hayward, CA), ATB Expression (bioMerieux Inc. Japan) and VITEK (bioMerieux Inc. Japan), failed to assign the organism to any defined bacterial genus. The suppressive bacterium may be useful in future for the development of biocontrol system and/or the construction of genetically modified edible fungi resistant to the disease caused by *P. tolaasii*.

Key Words——biocontrol agent; detoxification; *Pleurotus ostreatus*; *Pseudomonas tolaasii*; tolaasins.

Pseudomonas tolaasii Paine causes brown blotch disease in economically important cultivated mushrooms, such as *Pleurotus ostreatus* (Jacq.: Fr.) Kummer and *Agaricus bisporus* (Lange) Singer (Suyama and Fujii, 1993; Tolaas, 1915). The bacterium produces extracellular lipodepsipeptide toxins (i.e., tolaasins) that are required for the induction of blotch symptoms (Murata and Magae, 1996; Murata et al., 1998; Nutkins et al., 1991; Rainey et al., 1993; Shirata et al., 1995). Tolaasin disrupts cell membranes by forming a voltage-gated ion channel and acts as a biosurfactant, leading to the cell death of mushroom mycelia (Brodey et al., 1991; Hutchison and Johnstone, 1993).

The elimination of contaminants by periodically cleaning facilities with disinfectants such as chlorine, and by carefully controlling humidity and temperature during cultivation to avoid excessive condensation of water was found to be effective in controlling the outbreak of disease (Fletcher et al., 1989). In the cultivation of *A. bisporus*, application of antagonistic microorganisms into the mushroom casing layer was effective (Fermor et al., 1991; Nair and Fahy, 1972, 1976), and the product named 'Conquer' was once commercialized exclusively in Australia, though it is no longer on the market. So far, no cultivars resistant to bacterial disease have been developed in any edible fungi. Development of efficient biological methods to control disease is important, since, in principle, the use of chemicals or antibiotics should be avoided in mushroom culture.

In the present study, attempts were made to identify a strain of bacteria that could be used in effective biological control systems for *P. ostreatus* cultivation.

Materials and Methods

Bacterial strains, media and culture conditions in vitro *Pseudomonas tolaasii* strains S8501 and PT814, and MT93001 were isolated from diseased *P. ostreatus* and *A. bisporus*, respectively (Murata and Magae, 1996; Murata et al., 1998; Shirata et al., 1995). The disease-suppressive bacterium, strain 9405, was isolated from a rotting *P. ostreatus* fruiting body. *Pseudomonas tolaasii* strains and strain 9405 were grown in Pseudomonas

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agar F (PAF) or the broth (PF-broth) with the same ingredients (Difco) and in potato semi-synthetic agar (PSA) or the broth (PS-broth) (Wakimoto, 1955), respectively, at 24°C unless stated otherwise.

PS-TOL, a medium that contains tolaasins, was prepared by adding the components of PS-broth to the culture supernatants of *P. tolaasii* strain S8501. Prior to the reconstitution, supernatants of *P. tolaasii* strain S8501 obtained after culture at 24°C for 2 d in PS broth were placed in boiling water for 10 min to eliminate the live bacterial cells present. A medium containing spent culture supernatant of strain 9405 was prepared in the same manner as PS-TOL with replacement of the bacterium used. T-PAF, the agar medium that allows discrimination of the virulent-type of *P. tolaasii* from other bacteria by its distinctive blue colony, was prepared as described previously (Suyama et al., 1995).

Tolaasin assay Tolaasin activity was examined by inoculating cells on potato tuber slices, which showed blackening in the presence of the toxic effect (Shirata et al., 1995). The level of tolaasin activity in culture supernatants were semiquantitatively determined by the extent of blackening of potato tuber slices after the application of serially diluted samples (Murata and Magae, 1996; Shirata et al., 1995).

Pathogenicity test Bacteria from overnight culture on minimal salts-glucose agar at 24°C were suspended in sterile water (Murata et al., 1991). The bacterial suspension containing ca. 1.0×10^7 cells was inoculated either by spotting with a micropipet onto fruiting bodies of *P. ostreatus* isolate Y-01 or on a sliced section of fruiting bodies of *A. bisporus* isolate CH-01, or by spraying on the entire fruiting bodies, unless stated otherwise. The inoculated specimens were placed in a moist chamber at 20°C for 2 d and the extent of brown blotch development was recorded.

Determination of bacterial growth Viable cell numbers of *P. tolaasii* strain S8501 and strain 9405, in vivo and in vitro, were determined in terms of colony forming units on T-PAF and PSA, on which *P. tolaasii* strain S8501 forms distinctive blue colonies and strain 9405 yellow ones, respectively (Suyama et al., 1995; Wakimoto, 1955). If necessary, bacterial suspensions were serially diluted with sterile water prior to the application on agar plates. The bacterial cells were recovered from inoculated mushrooms after maceration using a mortar and a pestle.

Source of mushrooms *Pleurotus ostreatus* isolate Y-01 and *A. bisporus* isolate CH-01 were cultivated and supplied by local mushroom growers.

Results

Isolation of a bacterium that detoxifies tolaasins In the course of a study on host components that affect tolaasin production, we noted that *P. tolaasii* strain S8501 cells inoculated onto rotting *P. ostreatus* fruiting bodies occasionally did not induce the blackening when placed on a potato tuber slice (Fig. 1A). A bacterial strain forming yellow colonies with a smooth surface was isolated on PSA from such a *P. ostreatus* specimen. This bacterium was designated as strain 9405 and further characterized for biocontrol potential.

When ca 1.0×10^7 cells of *P. tolaasii* strain S8501 were co-inoculated with the same number of cells of strain 9405 on a potato tuber slice, blackening did not develop at the inoculation site, whereas a slice inoculated solely with *P. tolaasii* strain S8501 developed marked blackening (Fig. 1B). These observations suggest that strain 9405 may inhibit growth and/or tolaasin production of *P. tolaasii* strain S8501 or, otherwise, that it may inactivate tolaasins.

Effect of strain 9405 on *P. tolaasii* strain S8501 in vitro To analyze the effect of strain 9405 on *P. tolaasii* strain S8501 in vitro, these bacteria were co-inoculated at ca. 2×10^5 cells/ml in PS broth and cultured at 24°C for 48 h on a rotary shaker until *P. tolaasii* strain S8501 reached the stationary growth phase. Culture supernatants were assayed for the level of tolaasin activity by using a potato tuber slice, and cell pellets resuspended in sterile water were assayed for viable cell numbers (colony forming units/ml) on T-PAF, an agar plate on which *P. tolaasii* strain S8501 selectively forms distinctive blue colonies (Suyama et al., 1995). Growth curves of *P. tolaasii* strain S8501 in the presence and absence of strain 9405 were essentially identical. However, supernatants from the mixed culture did not induce blackening of a potato tuber slice, whereas the sample from *P. tolaasii* strain S8501 alone displayed the effect of tolaasin (Fig. 1C). The observation suggests that the suppression occurs either through the inhibition of tolaasin production in *P. tolaasii* strain S8501 or the inactivation of tolaasin activity in the medium, and not through the suppression of growth or viability of the pathogen.

Tolaasin inactivation by strain 9405 To determine whether the suppressive effect was due to the inactivation of tolaasin activity, strain 9405 (initial inoculum: ca. 1.0×10^8 cells) was cultured in PS-TOL, a medium containing tolaasins of *P. tolaasii* strain S8501, to the stationary growth phase. Culture supernatants were periodically harvested by centrifugation (4°C at 12,000 $\times g$ for 5 min), serially diluted, and applied onto a potato tuber slice to determine the tolaasin activity. The tolaasin activity sharply decreased to half the original level 3 h after the inoculation (Fig. 2). The inactivation persisted up to 24 h without any detectable level of toxicity even in the undiluted sample.

To determine whether the detoxification was due to secreted metabolites or was associated with the live cells of strain 9405, *P. tolaasii* strain S8501 was cultured in PS-broth and in the broth containing spent culture supernatant of strain 9405, and the tolaasin activity was assayed. A significant amount of tolaasin was detected regardless of the presence or absence of the culture supernatants of strain 9405 (Fig. 1D).

We attempted to determine the minimum number of cells of strain 9405 required to counteract the effect of tolaasins in vitro. *Pseudomonas tolaasii* strain S8501 was suspended in PS broth to ca. 1.0×10^8 cells/ml, strain 9405 cells were added in various ratios, and the

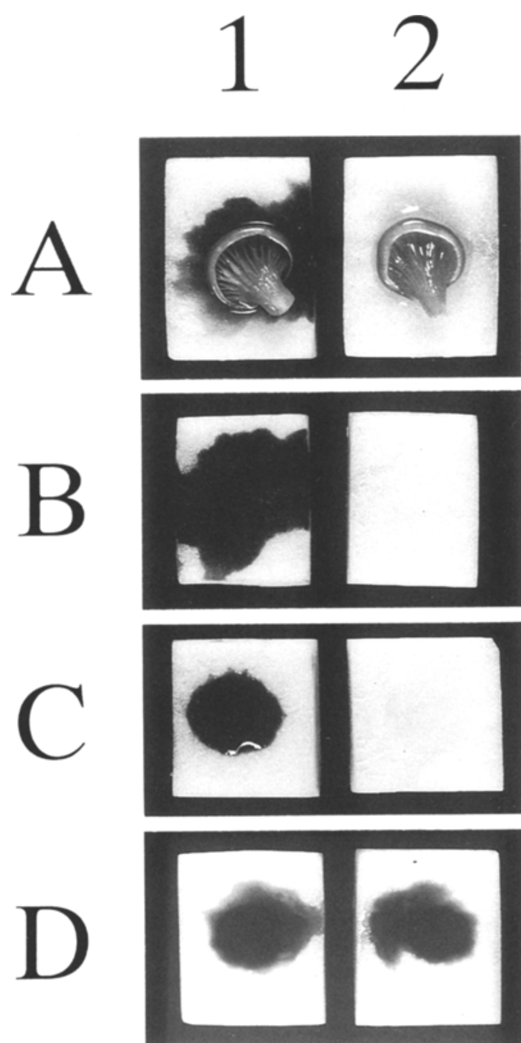

Fig. 1. Effect of strain 9405 on tolaasin activity produced by *Pseudomonas tolaasii* strain S8501.
See the text for details of assay conditions.
A: Tolaasin activity present in *P. ostreatus* inoculated with 2×10^6 cells of *P. tolaasii* strain S8501. Lane 1, *P. ostreatus* inoculated with *P. tolaasii* strain S8501; Lane 2, *P. ostreatus* inoculated with *P. tolaasii* strain S8501 from which a strain of tolaasin-suppressing bacterium, 9405, was isolated.
B: Tolaasin activity produced by *P. tolaasii* strain S8501 (lane 1) and the bacterium co-inoculated with strain 9405 (lane 2). Potato tuber slices were inoculated with 2×10^6 cells of each bacterial strain.
C: Tolaasin activity in culture supernatants of *P. tolaasii* strain S8501 (lane 1) and the bacterium co-cultured with strain 9405 (lane 2). Bacteria were grown in PS-broth to the stationary growth phase at 24°C. Culture supernatants (50 μl) were applied to potato tuber slices.
D: Tolaasin activity in culture supernatants of *P. tolaasii* strain S8501 (lane 1) and *P. tolaasii* strain S8501 containing spent culture supernatants of strain 9405 (lane 2). *Pseudomonas tolaasii* strain S8501 was grown in PS-broth or the medium containing culture supernatant of strain 9405 at 24°C to the stationary growth phase (see Materials and Methods). Supernatants (50 μl) were applied to potato tuber slices.

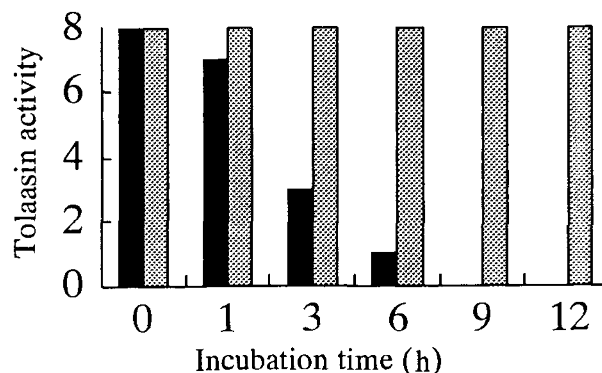

Fig. 2. Time course of tolaasin detoxification by strain 9405.
The bar diagram denotes the level of tolaasin activity, i.e., the degree of blackening in a potato tuber slice scored from 0 (no blackening) to 8 (complete blackening), which is reciprocal of dilution factors of samples.
Solid bar: Strain 9405 was grown in PS-TOL containing tolaasins of *Pseudomonas tolaasii* strain S8501 at 24°C.
Shaded bar: Culture supernatants were periodically harvested, filter-sterilized (pore size=0.45 μm, Advantech, Tokyo) and applied to potato tuber slices.
Hatched bar: Experiment with omission of strain 9405.

mixtures were shake-cultured at 24°C for 72 h. Culture supernatants were collected by centrifugation (12,000× *g*, 4°C, 5 min), placed in boiling water for 10 min to kill bacteria, and assayed for tolaasin activity. The inhibition of tolaasin activity was noted at the ratio of $1:10^3$ of initially inoculated cells of strain 9405 vs. *P. tolaasii* strain S8501. At a ratio lower than $1:10^4$, the suppressive effect was significantly reduced (Table 1).

Effect of strain 9405 on the protection of *P. ostreatus* and *A. bisporus* from brown blotch disease We attempted to evaluate the use of strain 9405 for the protection of *P. ostreatus* from brown blotch disease. Strain 9405 was inoculated to *P. ostreatus* at a dose of ca. 1.0×10^7 cells per spot prior to the inoculation with the same amount of *P. tolaasii* strain S8501, and the extent of brown blotch development was periodically monitored in a moist chamber at 20°C. Brown blotch symptoms did not develop on the fruiting bodies when strain 9405 was inoculated 1–2 d before the inoculation with *P. tolaasii* strain S8501 (Fig. 3). The extent of protection attained was similar to that observed with simultaneous inoculation of both bacterial strains and lasted for 2 d. In contrast, the disease symptom was clearly observed when the fruiting bodies were inoculated with strain 9405 more than 4 d before the pathogen inoculation. A ratio of 1:10 of the initial cell numbers of strain 9405 vs. *P. tolaasii* strain S8501 was required for the effective control of disease development (Table 1). When cell numbers of the pathogen and the suppressor were adjusted to the ratio as described above, inoculation of strain 9405 by spraying was also effective (Fig. 4). The assay conditions that were found to be effective in *P. ostreatus* against *P. tolaasii* strain S8501 also provided *P. ostreatus* and *A. bisporus* with protection from the disease caused by the inoculation with *P. tolaasii* strains PT814

Table 1. Effect of initial cell numbers of strain 9405 on detoxification of tolaasins on a potato tuber slice and suppression of brown blotch on a *P. ostreatus* fruiting body.

	Initial ratio of strain 9405/*P. tolaasii* strain S8501[a]						
	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Tolaasin activity[b]	−	−	−	−	+	++	+++
Brown blotch[c]	−	−	+	++	+++	+++	+++

a) Strain S9405 and *P. tolaasii* strain S8501 were co-inoculated at various cell concentrations to give initial ratios of strain S9405 to *P. tolaasii* strain S8501 of 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.

b) Strain S9405 was inoculated in PS-broth containing 2×10^8 cells/ml of *P. tolaasii* strain S8501. Culture supernatants (50 μl) from 72 h-cultures at 24°C were applied to potato tuber slices. The level of tolaasin activity was determined by the degree of blackening of the potato tuber slices and scored from − (no blackening) to +++ (complete blackening).

c) Strain S9405 was aplied to *P. ostreatus* fruiting bodies inoculated with 1×10^6 cells of *P. tolaasii* strain S8501. Degree of brown blotch development was determined after incubation for 48 h and scored from − (no symptoms) to +++ (complete brown blotch).

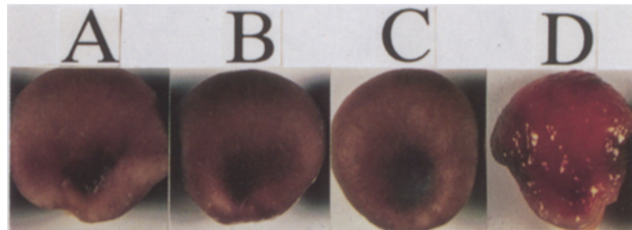

Fig. 3. Use of strain 9405 for the suppression of the development of brown blotch in *Pleurotus ostreatus* fruiting bodies infected with *Pseudomonas tolaasii* strain S8501.
Lane A: 2×10^6 cells of strain 9405 were inoculated simultaneously with equal cell numbers of *P. tolaasii* strain S8501. Lanes B, C and D: Strain 9405 was inoculated 1, 2, and 4 d prior to the inoculation of *P. tolaasii* strain S8501, respectively. See the text for details of assay conditions.

and MT93001, respectively (Fig. 4).

Growth of strain 9405 and *P. tolaasii* strain S8501 in vivo Viable cells of *P. tolaasii* strain S8501 and strain 9405 were recovered from inoculated *P. ostreatus* on T-PAF and PS agar, respectively. The population of strain 9405 sharply decreased to an undetectable level 3–4 d after the inoculation on live *P. ostreatus*, whereas the population of *P. tolaasii* strain S8501 dramatically increased during this period (Fig. 5). By contrast, both strain 9405 and *P. tolaasii* strain S8501 grew well, and viable cells were maintained during a prolonged culture period on the fruiting bodies that had been autoclaved at 121°C for 10 min, indicating that strain 9405 is saprophytic, but not parasitic, on *P. ostreatus* (Fig. 5).

Bacteriological characterization of strain 9405 We attempted to characterize the isolated bacterium bacteriologically (Nishiyama, 1981). The organism is a Gram-positive bacterium of $0.2–0.4\times1.0–1.5$ μm in size, aerobic, non-spore-forming and non-motile due to the absence of flagella. The maximum temperature for the bacterium to grow in the PS medium is 33°C. It gave positive reactions in the following tests: catalase, esculin hydrolysis, growth in peptone water, β-galactosidase, and utilization of D-(−)-ribose, D-(+)-xylose, D-(−)-fructose, D-(+)-galaxies, D-(+)-glucose, D-(+)-mannose, glycerol, D-(+)-cellobiose, D-mannitol, D-sorbitol, lactose, maltose, melibiose, sucrose, trehalose, dextrin, salicin, fumaric acid, and DL-malic acid.

The following tests were negative: oxidase, tyrosinase, lecithinase, hydrolysis of arbutin, arginine, margarine and casein, production of diffusible and fluorescent pigment, indole, levan, 3-keto-lactose, 2-keto-gluconate and hydrogen sulfide, growth in Uschinsky's solution, Fermi's solution and Corn's solution, reduction of NO_3 to NO_2 or N_2, nitrate respiration, liquefaction of gelatin, Voges-Proskauer reaction, methyl red reaction, maceration of potato tubers, hypersensitive reaction in tobacco leaves, utilization of asparagine as a sole source of carbon and nitrogen, and utilization of L-(+)-arabinose, D-(−)-arabinose, L-(+)-rhamnose, D-dulcitol, inositol, D-(+)-melezitose, D-(+)-raffinose, starch, inulin, betaine, β-alanine, L-valine, D-(−)-tartaric, D-(+)-tartaric acid, malonic acid, oxalic acid, benzoic acid, *m*-hydroxybenzoic acid, propionic acid, *n*-butyric acid, and gluconic acid.

The isolated organism could not be assigned to any bacterial genus listed in the profile index programmed in ATB Expression (bioMerieux Inc. Japan, Tokyo) nor VI-

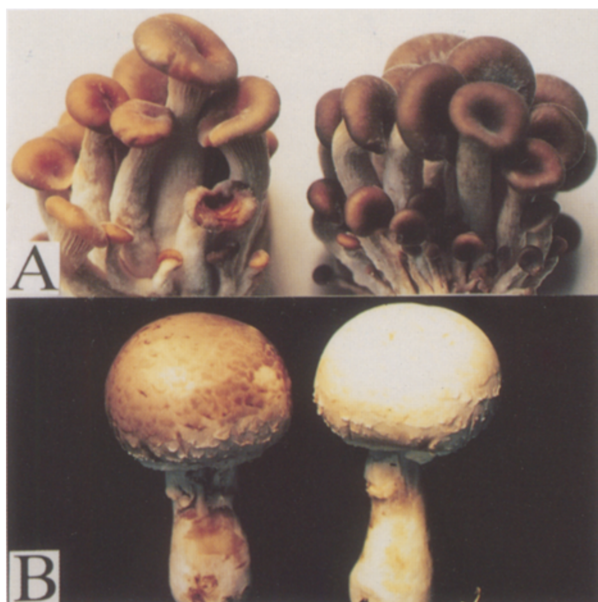

Fig. 4. Effect of spraying cell suspension of strain 9405 on disease development in *Pleurotus ostreatus* (A) and *Agaricus bisporus* (B) infected with *Pseudomonas tolaasii* strains S8501 and MT93001, respectively.
Ca. 2×10^6 cells of each bacterial strain were inoculated by using a sprayer. The photographs at left show two mushroom species inoculated with *P. tolaasii* strains S8501 and MT93001, respectively, and those at right show specimens co-inoculated with strain 9405. See the text for details of assay conditions.

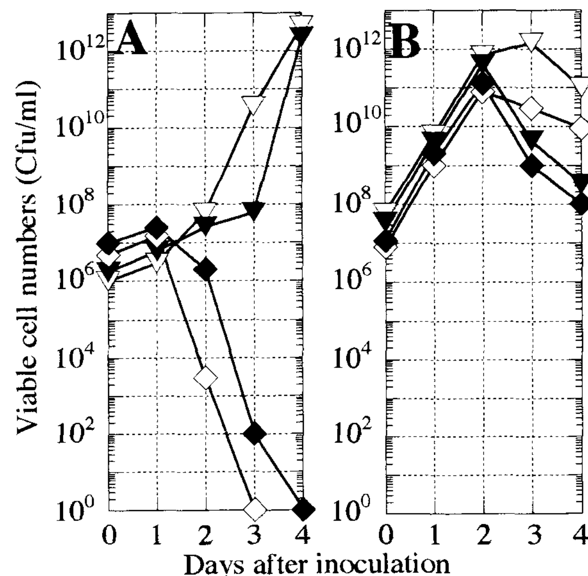

Fig. 5. Viability of strain S9405 and *Pseudomonas tolaasii* strain S8501 on *Pleurotus ostreatus* fruiting bodies.
Panel A, Viable cell numbers of bacteria in live *P. ostreatus*. Panel B, Viable cell numbers of bacteria in *P. ostreatus* autoclaved at 121°C for 10 min. Symbols: open triangle, *P. tolaasii* strain S8501; closed triangle, *P. tolaasii* strain S8501 co-inoculated with strain S9405; open box, strain S9405; closed box, strain S9405 co-inoculated with *P. tolaasii* strain S8501. See the text for assay conditions of colony-forming units per ml (cfu/ml).

TEK (bioMerieux Inc. Japan, Tokyo) (e.g., a score after API 20 NE was 046620), nor MicroStation™ System Release 3.5 (Biolog Inc., Hayward, CA) (BIO-NUMBER 0461-0252-1740-1001-1600-0001-0105-5320).

Discussion

Use of strain 9405 could be a unique approach for developing a biocontrol system of cultivated mushrooms, particularly *P. ostreatus*. It is different from the biocontrol system in *A. bisporus* cultivation, where an antagonistic bacterium, i.e., *Pseudomonas fluorescens* (Trevisan) Migula, is allowed to grow on the mushroom casing layer to suppress physically the incoming population of *P. tolaasii* (Fermor et al., 1991; Nair and Fahy, 1972, 1976). We observed that a Gram-positive bacterium, strain 9045, detoxifies tolaasins produced by *P. tolaasii* and significantly suppresses the onset of the disease in *P. ostreatus* and *A. bisporus*. Another advantage of using strain 9405 is that it is saprophytic to *P. ostreatus*, in contrast to *P. fluorescens*, which could be pathogenic to cultivated mushrooms by producing various antifungal agents (Corbell and Loper, 1995; Laville et al., 1992) and is closely related to *P. tolaasii* (Thorn and Tsuneda, 1996).

However, many problems must be solved for the practical use of the bacterium for controlling the disease. The major issue is the possibility of side effects of strain 9405 on the environment as well as on human health. It is interesting to note, however, that suppressive bacteria similar to strain 9405 can occasionally be isolated from *P. ostreatus* on shelves of groceries. The ecology of this type of bacterium is currently under investigation. On the other hand, ongoing systematic analysis of the bacterium including analysis of 16S rRNA gene, G+C contents and cell wall composition, e.g., menaquinones and peptidoglycan types, may allow the organism to be identified taxonomically. These investigations may offer clues to assess possible deleterious effects of the organism on the environment.

Another issue is that the effectiveness of strain 9405 in preventing the development of brown blotch was not as high as the suppression of the tolaasin effect in vitro. Difficulty of disease suppression in vivo may be partly due to the decrease of the population size of 9405 on *P. ostreatus*, which could be attributed to the presence of host components detrimental to strain 9405 or the limitation of nutrients available for the bacterium on the host mycelia. The low efficiency of protection could also be due to the activation of tolaasin production by host components (Murata and Magae, 1996), or some kind of physical competition between the two symbionts on the host.

Since such factors as suppressor/pathogen ratios and timing of application are critical in determining the protection efficiency, it will be necessary to develop a

standardized procedure to use strain 9405 in the cultivation of mushrooms for practical biological control. Construction of genetically modified organisms with a high efficiency of antagonism could also be attempted. For further methodological development, cloning and characterization of a gene(s) of 9405 responsible for detoxification along with construction of an expression vector system for *P. ostreatus* and other edible fungi may be promoted to generate a "transgenic" resistant mushroom. The success of this line of research may alleviate the concern of environmental hazard of antagonists such as strain 9405 as well as stabilize the protection. It was reported that a gene of a bacterium, *Pantoea dispersa* Gavini et al., used to detoxify albicidin phytotoxins was successfully introduced into sugarcane to protect it from leaf scald disease caused by *Xanthomonas albilineans* (Ashby) Dowson (Birch, 1997; Zhang and Birch, 1997). The findings in the present study may offer a basis for the development of biological control and genetic manipulation systems to protect *P. ostreatus* from the destructive disease

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