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Growth promotion of the edible fungus *Pleurotus ostreatus* by fluorescent pseudomonads

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Abstract

Bacteria were isolated from the mycelial surface of *Pleurotus ostreatus* and their role in fruiting body induction (fructification) of the edible mushroom *P. ostreatus* was investigated. Analysis of the bacterial community that colonized the mycelium showed that the species composition and numbers of culturable bacteria differed according to the developmental stage of *P. ostreatus*. In particular, the population size of fluorescent pseudomonads increased during fruiting body induction. An experiment showed that inoculation of pure cultures of the mycelium with strains of fluorescent *Pseudomonas* spp. isolated from the mycelial plane of commercially produced mushrooms promoted the formation of primordia and enhanced the development of the basidiome of *P. ostreatus*. Results of this research strongly suggest that inoculation of the mycelium with specific bacteria may have beneficial applications for mushroom production.

Keywords: Compost; Edible fungus; Mushroom production; *Pseudomonas*

1. Introduction

The oyster mushroom (*Pleurotus ostreatus*) is one of the most widely cultivated mushrooms in the world [1]. Techniques for its cultivation are similar to those used for *Agaricus bisporus*, in which the fungi are cultivated on various organic composts. If properly composted, a wide range of plant wastes, e.g. sawdust, paddy straw, bagasse, cornstalks, stalks, leaves of bananas and cotton waste can be used as substrates for *P. ostreatus* production. Previously, it has been observed by commercial growers that it is usually possible to obtain several mushroom harvests of *P. ostreatus* mushrooms when using a composted medium, while it is difficult to harvest mushrooms repeatedly in a noncomposted medium. The composting process thus appears to be particularly important for altering the biological and physiochemical properties of the growth medium, and for eliminating weed, disease, and insect problems that occur with uncomposted materials [2].

As many microorganisms have been shown to promote the growth of *P. ostreatus* and other cultivated mushrooms (Table 1), the biological properties of composts appear to be very important for induction of fruiting body formation. In the mushroom *A. bisporus*, growth and development is particularly affected by pseudomonads [3]. To date, detailed studies on the interactions between edible fungi and bacteria have been conducted primarily with strains of *Pseudomonas putida*, which initiate basidiome formation and stimulate growth of the fungus [4–8].

Recent work has revealed the presence of two distinct mycelial responses to *P. putida* during basidiome morphogenesis of *A. bisporus*. The first response is an increase in the rate of hyphal extension, and the second is the formation of primordia [8]. In comparison to *A. bisporus*, the effects of the fluorescent pseudomonads on *P. ostreatus* are less well understood. There can be wide variation in the yields of mushrooms from different composted materials, which has generated interest in whether this is associated with the biological properties of different types of composts. Based on studies with *A. bisporus*, there is also considerable interest in the use of bioaugmentation with mushroom-promoting pseudomonads to improve the consistency of different composts for mushroom production.

The purpose of this study was to compare the popula-

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tions of bacteria and mushroom-promoting pseudomonads in composts that had previously produced low and high yields in commercial production facilities. An additional experiment was conducted to examine the effects of certain strains of fluorescent pseudomonads that were isolated from the mycelial plane on fruiting body initiation of *P. ostreatus* in pure culture. Several bacterial strains were identified that had positive effects on fruiting body formation, which were placed into culture collections for further study of their potential use as inoculants to promote mushroom formation during commercial production.

2. Materials and methods

2.1. Isolation of bacteria from Pleurotus cultures

Bacteria were collected from cultures of *P. ostreatus* following mushroom production on samples of composted and noncomposted cotton plant waste products. Samples were selected from spent culture medium that had previously produced either high or low yields and the microorganisms were classified into four groups (C-H, C-L, NC-H, and NC-L) according to whether they had been isolated from composted (C) or noncomposted (NC) plant waste, with, high (H) or low (L) yields. The spent compost samples used in this study were provided from mushroom-growing farms in Anseong and Yeoju, Gyeonggi Province, Korea, in July 1999.

Other cultures of bacteria were obtained by direct sampling of mycelia produced in composted waste cotton, and were collected after the first harvest of *P. ostreatus* at 3 to 7 cm depth below the surface of the mushroom culture medium. The mycelia were carefully isolated by hand, using tweezers, after which 50 mg of the mycelia were soaked in 10 mM potassium phosphate buffer for 20 min to remove loosely attached microorganisms and debris. The washed mycelia were then placed into tubes (13×100 mm) with Teflon-lined screw caps to which were added 3 ml of 10 mM potassium phosphate buffer and glass beads (1.0 mm diameter, Biospec Product, Inc.). The tubes were vortexed for 3 min, after which they were sonicated for 3 min using a high-intensity ultrasonic liquid processor (Sonic Material, Inc., USA) to isolate tightly adhering bacteria from the mycelial plane.

2.2. Media

Bacteria from the mycelium-plane extract of *P. ostreatus* were cultured on 10% tryptic soy broth (TSB: 1.5 g l^{-1}; BBL, Sparks, USA) agar and P1 medium (KH_2PO_4, 0.1 g l^{-1}; $MgSO_4{\cdot}7H_2O$, 0.05 g l^{-1}; KCl, 0.02 g l^{-1}; $NaNO_3$, 0.5 g l^{-1}; deoxycholic acid, 0.1 g l^{-1}; betaine, 0.5 g l^{-1}; agar, 1.5 g l^{-1}; pH 7.0). For identification by fatty acid analyses (FAA), bacteria isolated from the mycelium surface of *P. ostreatus* were grown on TSB agar (TSBA, 30 g l^{-1} and granulated agar (BBL), 15 g l^{-1}). To observe the effects of selected fluorescent pseudomonad cultures on growth of axenic cultures of mycelium, *P. ostreatus* was cultured on potato dextrose agar (PDA; infusion from potatoes, 200 g; Bacto dextrose, 20 g l^{-1}; Bacto agar, 15 g l^{-1}). When used as a reproductive medium to induce mushroom formation, PDA medium was diluted to 1.5% (w/v).

2.3. Identification of bacteria

Bacteria isolated from the mycelium surface of *P. ostreatus* were identified by fatty acid methyl ester (FAME) analyses following cultivation of the individual isolates on TSBA for 24 h at 28°C prior to FAA. FAME was conducted using the Sherlock Microbial Identification System (MIDI, Inc., Newark, DE, USA), using protocols recommended by the manufacturer.

2.4. Growth promotion of P. ostreatus by fluorescent *Pseudomonas* spp.

Ten colonies of fluorescent *Pseudomonas* were selected and isolated from the mycelial plane of *P. ostreatus* (ASI. 2344. Chun-chu 2). After serial dilution, the organisms were cultivated on P1 media for 18 h at 28°C and then fluoresced using a UV transilluminator. Pure cultures were obtained by streaking the organisms several times on P1 medium. The mycelium of *P. ostreatus* was cultured on divided plates, with one half containing PDA growth medium and the other half containing diluted PDA reproductive medium. To examine the effects of selected pseudomonad strains on induction of fungal basidiomes, the plates were inoculated with an agar block containing pure cultures of fluorescent *Pseudomonas* spp. A total of 10 different *Pseudomonas* strains were tested. Agar blocks containing the bacteria were prepared by transferring a colony into 3 ml of P1 media in a test tube. The bacteria were then cultivated by shaking at 200 rpm for 18 h at 28°C, after which 1-ml aliquots containing 2×10^4 cells were mixed with 19 ml of melted sterilized agar. After solidification, a block was made using a No. 4 cork borer. The bacteria agar blocks were placed 5 cm from the edge of the mycelium of *P. ostreatus*. The cultures were initially maintained at 25°C until the mycelium of *P. ostreatus* grew into the reproductive medium, after which the temperature was lowered to 16–18°C to facilitate reproductive growth. To assay whether any of the fluorescent *Pseudomonas* isolates were *Pseudomonas tolaasii*, which is associated with brown blotch disease on cultivated mushrooms, the white-line test was performed according to the method of Wong et al. [9].

2.5. 16S rDNA sequence analysis

Two universal primers, described by Stakebrandt et al.

Table 1
Previously identified mushroom growth-promoting bacteria

Bacteria	Mushroom	Effect	Source	Reference
P. putida	*A. bisporus*	Chemotaxis	Mycelium	[3]
P. putida, *Pseudomonas fluorescens*	*A. bisporus*	Sporophore initiation	ATCC	[4]
P. putida	*A. bisporus*	Hypal growth	Mycelium	[8]
P. putida	*A. bisporus*	Primordia production	Casing soil	[12]
P. putida	*A. bisporus*	Attachment	Mycelium	[16]
F. *Pseudomonas*[a]	*A. bisporus*	Attachment	Peat casing	[17]
Bacillus subtilis	Mushroom compost	Survival	NCIMB	[18]
Bacillus macerans	*P. ostreatus*	Decomposition of wheat straw	Wheat straw	[19]
F. *Pseudomonas*[a]	*A. bisporus*	Polysaccharide	Casing medium, discolored lesions	[20]
P. putida	*P. ostreatus*	Hypal, primodia basidiome production	Mycelium plane	This study

[a]Fluorescent *Pseudomonas* spp.

[10], 9F (5′-GAG TTT GAT CCT GGC TCA G-3′, positions 9–27) and 1542R [5′-AGA AAG GAG GTG ATC CAG CC-3′, positions 1542–1525 (*Escherichia coli* numbering)], were used for PCR amplification of 16S rDNA. The amplified PCR products were purified using QIAquick PCR Purification kits (Qiagen, Valencia, CA, USA). The purified 16S rDNAs were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an automatic DNA sequencer (Model 377, Applied Biosystems). Multiple sequence alignments were performed using CLUSTAL X [11] and a phylogenetic tree was constructed by using the neighbor-joining method and Kimura-2 parameter algorithm (MEGA2 program) [12] along with a bootstrap test employing 1000 replications.

2.6. Accession numbers

Nucleotide sequence data were submitted to the GenBank nucleotide sequence databases and were assigned the accession numbers AF396071 and AF396077.

2.7. Statistics

Differences in the numbers of total bacteria and fluorescent *Pseudomonas* between mycelium samples of *P. ostreatus* were determined using ANOVA (Minitab, State College, PA, USA). Significant differences ($P < 0.05$) were determined by Tukey's HSD.

3. Results

3.1. Analysis of bacterial populations on the mycelial plane of P. ostreatus

Total culturable bacteria were enumerated following isolation of bacteria from the mycelial plane of fungi that had been cultivated in low- and high-yield-producing media obtained from commercial mushroom production facilities. The population sizes of the total culturable bacteria were significantly higher ($P < 0.05$) in the high-yielding composted growth medium as compared to low-yielding compost or noncomposted media (Fig. 1). Mycelia from the high-yielding composted medium had approximately 7×10^5 CFU g^{-1} mycelium, whereas total culturable bacteria from the low-yielding composted medium had a population size of only 2×10^4 CFU g^{-1} mycelium. There was no significant difference in the population sizes of total culturable bacteria in either high- or low-yielding noncomposted medium, both of which yielded approximately 8×10^3 CFU g^{-1} mycelium. High yields were associated with higher population densities of pseudomonads in both composted and noncomposted media. The population sizes of pseudomonads were approximately 10% of the total culturable heterotrophs and ranged from 5×10^2 to 10^4 CFU g^{-1} mycelium.

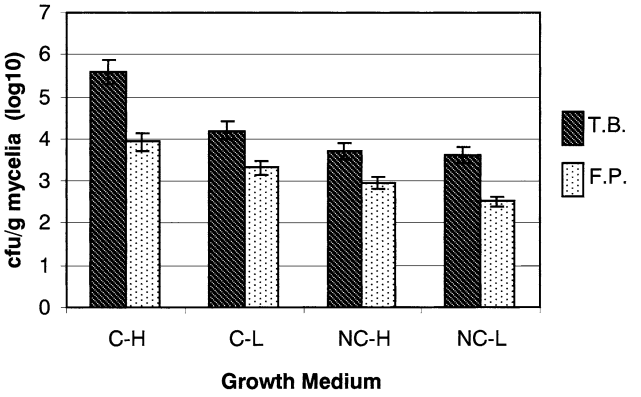

Fig. 1. Population sizes of culturable total bacteria (T.B.) and fluorescent *Pseudomonas* spp. (F.P.) associated with the mycelium surface of *P. ostreatus*. Vertical bars indicate standard errors of the means. Treatments: C-H, composted medium with high yield; C-L, composted medium with low yield; NC-H, noncomposted medium with high yield; NC-L, noncomposted medium with low yield.

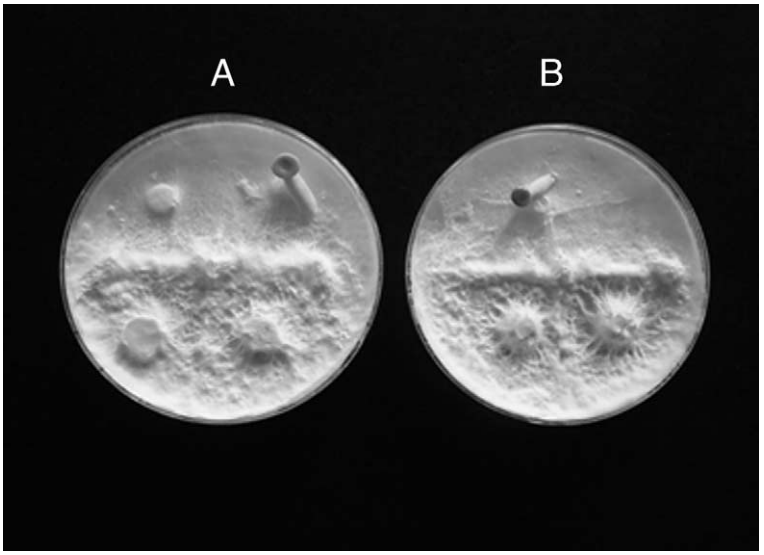

Fig. 2. Effect of *P. putida* inoculation on growth of *P. ostreatus* after 10 days. A: No inoculation treatment (top left) and inoculation treatment (top right) with agar blocks containing *P. putida*. B: Inoculation with *P. putida*.

3.2. Identification of bacteria from the mycelial surface of *P. ostreatus*

Thirty-five colonies of bacteria from mycelium produced in high-yielding compost medium were isolated from the mycelial surface of *P. ostreatus* and were identified by FAA. All together, 17 genera of bacteria were identified (Table 1). The white-line reaction assay was conducted to determine if any of the fluorescent pseudomonad isolates were harmful to the growth of *P. ostreatus* [13]. All 10 colonies tested were not white-line positive. A BLAST search of their 16S rDNA sequences revealed that seven of the fluorescent pseudomonads were 99% similar to *P. putida*.

Table 2
Strains tested for *P. ostreatus* growth-promoting activity

Isolated bacteria	Division	Number	Promoting
Curtobacterium	Actinobacteria	2	0
Clavibacter	Actinobacteria	2	0
Microbacterium	Actinobacteria	1	0
Kocuria	Actinobacteria	1	0
Chryseobacterium	CFB[a]	7	0
Xanthobacter	Alpha *Proteobacteria*	1	0
Gluconobacter	Alpha *Proteobacteria*	1	0
Phyllobacterium	Alpha *Proteobacteria*	2	0
Ochrobactrum	Alpha *Proteobacteria*	4	0
Variovorax	Beta *Proteobacteria*	2	0
Cedecea	Gamma *Proteobacteria*	3	0
Stenotrophomonas	Gamma *Proteobacteria*	2	0
Citrobacter	Gamma *Proteobacteria*	2	0
Enterobacter	Gamma *Proteobacteria*	2	0
Salmonella	Gamma *Proteobacteria*	1	0
Pseudomonas	Gamma *Proteobacteria*	1	0
Kluyvera	Gamma *Proteobacteria*	1	0
No match		1	0
P. putida	Gamma *Proteobacteria*	10	7
Total		45	7

[a] *Cythophaga–Flexibacter–Bacteroides*.

3.3. Growth promotion effect of fluorescent pseudomonads on *P. ostreatus*

Inoculation with fluorescent *Pseudomonas* spp. significantly promoted the development of the mycelium of *P. ostreatus*, the formation of primordia, and the development of *P. ostreatus* basidiome. To observe the growth of *P. ostreatus* as affected by inoculation, the method [14] was modified using vegetative media and nutrient-poor media (Fig. 2). When *Pseudomonas* spp. strains were inoculated into nutrient-poor and vegetative media, the mycelia that grew in the vicinity of the inoculum had a dense growth with good development of basidiomes. In contrast, without inoculation the development of the basidiomes was incomplete. Thirty-five bacterial isolates other than *P. putida* were also tested, but gave no positive growth promotion reaction.

4. Discussion

P. ostreatus is the most widely cultivated mushroom in the world followed by *A. bisporus*, and demand for these edible mushrooms is increasing. Cultivation of *P. ostreatus* is based largely on the techniques used for cultivating *A. bisporus*. Similarities in cultivation include a pre-treatment process of the growth medium through composting. Presumably, composting enhances carbon availability to the cultivated mushroom producing fungi by degrading lignin associated with the cellulose and hemicellulose in the plant wastes [15]. Long-chain sugar polymers may

also be broken into smaller fragments that are accessible to enzymatic degradation and release of sugar monomers for uptake by the cultivated fungus and other heterotrophs. Results of this research (Fig. 1) showed that population sizes of both total culturable bacteria and pseudomonads were approximately 10-fold higher in composted medium as compared to noncomposted medium. This may provide a possible explanation for the failure of noncomposted media to sustain sequential mushroom harvests, since recalcitrant carbon associated with cellulose–lignin complexes may not support abundant growth after the easily utilizable carbon has been expended to support the first harvest. Since samples of the growth media prior to production of the first harvest of mushrooms were not available, it is not possible to ascertain whether differences in high and low production within the composted and noncomposted treatments were due to differences in initial bioavailable carbon. Nonetheless, bacteria population sizes are extremely sensitive to carbon availability, and may thus provide an index of differences in relative carbon availability as the plant materials in the growth medium undergo further decomposition. In all cases, the proportion of fluorescent pseudomonads to total culturable bacteria was similar for both low- and high-yielding compost media. Thus, it does not appear that differences in population sizes of fluorescent pseudomonads alone can account for differences in mushroom yields that were obtained after the first harvest.

FAA of 35 bacterial isolates from the mycelial plane of commercially produced *P. ostreatus* resulted in the identification of bacterial species from 17 genera (Table 2). Among these, only the fluorescent pseudomonads were found to have mushroom growth-promoting effects for *P. ostreatus*. In this research, we focused specifically on bacteria that were strongly attached to the mycelium of the fungus, since these bacteria are presumably most likely to be those that have growth-promoting effects. To observe the colonization of bacteria on *A. biosporus*, prior research by Grewal and Rainey [3] examined the chemotactic behavior of fluorescent pseudomonads toward the chemicals secreted from the mycelium. The movement and attachment process of fluorescent pseudomonads on *A. bisporus* by chemotaxis also has been observed using an electronic microscope [16]. Miller et al. [17] cultivated the bacteria groups and the fluorescent *Pseudomonas* groups that were attached to the hyphal wall of *A. bisporus* in mycelium-cultivated soil for 14 days, and the proportion of the fluorescent *Pseudomonas* spp. ranged from 14 to 41%. In this research, the population size of fluorescent pseudomonads that were associated with the mycelium was similarly variable, ranging from 2 to 20% of the total culturable heterotrophs.

This study used a modified split plate method [14] to examine the growth-promoting function of fluorescent pseudomonads on *P. ostreatus*. Several growth-promoting effects were observed, including enhanced growth of the mycelium on the reproductive medium, as well as increased numbers of basidiomes, and more rapid growth of the juvenile fruiting bodies on agar media. In prior studies with *P. ostreatus*, fluorescent pseudomonads have been considered to be pathogenic bacteria, causing a disease called brown blotch that results in yield loss and reduced mushroom quality [13]. In contrast, all of the fluorescent pseudomonads we tested had positive effects in promoting basidiome formation and growth of *P. ostreatus*. Among 10 different strains of fluorescent *Pseudomonas* spp. that were tested, seven were found to be growth promoting. Although no one strain stood out as greatly superior to the others, a possible application of this work is that it may be possible to use bioaugmentation of composts with certain bacteria to promote mushroom yields and consistency in production of mushrooms using different composted materials. There are also many remaining questions. The identification and isolation of growth-promoting strains of pseudomonads from this research may provide a tool for identifying the specific compounds that are released by certain pseudomonads to trigger basidiome formation. It will also be of interest to examine whether these bacteria have similar effects on growth promotion of both *Agaricus* and *Pleurotus* mushroom fungi.

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