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Identification of Exopolysaccharides Produced by Fluorescent Pseudomonads Associated with Commercial Mushroom (*Agaricus bisporus*) Production

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The acidic exopolysaccharides (EPSs) from 63 strains of mushroom production-associated fluorescent pseudomonads which were mucoid on *Pseudomonas* agar F medium (PAF) were isolated, partially purified, and characterized. The strains were originally isolated from discolored lesions which developed postharvest on mushroom (*Agaricus bisporus*) caps or from commercial lots of mushroom casing medium. An acidic galactoglucan, previously named marginalan, was produced by mucoid strains of the saprophyte *Pseudomonas putida* and the majority of mucoid strains of saprophytic *P. fluorescens* (biovars III and V) isolated from casing medium. One biovar II strain (J1) of *P. fluorescens* produced alginate, a copolymer of mannuronic and guluronic acids, and one strain (H13) produced an apparently unique EPS containing neutral and amino sugars. Of 10 strains of the pathogen "*P. gingeri*," the causal agent of mushroom ginger blotch, 8 gave mucoid growth on PAF. The "*P. gingeri*" EPS also was unique in containing both neutral sugar and glucuronic acid. Mucoid, weakly virulent strains of "*P. reactans*" produced either alginate or marginalan. All 10 strains of the pathogen *P. tolaasii*, the causal agent of brown blotch of mushrooms, were nonmucoid on PAF. Production of EPS by these 10 strains plus the 2 nonmucoid strains of "*P. gingeri*" also was negative on several additional solid media as well as in two broth media tested. The results support our previous studies indicating that fluorescent pseudomonads are a rich source of novel EPSs.

The major steps in commercial mushroom production are preparation of sterile compost (usually composed of manure, straw, and gypsum), spawning (inoculation with fungal mycelia), casing (overlaying with a peat-limestone mixture or recycled compost), and harvesting (13). The casing step is required for the induction of fruiting-body formation, induction being due to the presence of saprophytic bacteria such as the fluorescent pseudomonad *Pseudomonas putida* in the casing medium (33). Saprophytic fluorescent pseudomonads, including a diverse group of fluorescent pseudomonads referred to as "*P. reactans*" (14, 35), are commonly isolated from healthy mushrooms (48). Casing medium is also a primary source of pathogenic pseudomonads (46).

There are three main bacterial diseases of cultivated mushroom, all caused by fluorescent pseudomonads. The most common, with a worldwide distribution, is brown blotch, which is caused by *P. tolaasii* and characterized by the formation of brown lesions on the cap (pileus) and stipe (3, 35). Pathogenic strains of this bacterium can be easily identified by the white-line test, which consists of plating strains of "*P. reactans*" near to *P. tolaasii* on solid media. After incubation for 24 to 48 h, a white precipitate forms in a line between the strains (45). Strains of *P. tolaasii* have also been reported to be able to colonize the surface of plant roots (49). The second disease is called ginger blotch and is characterized by the formation of ginger-colored lesions on the cap. This disease was first reported by Wong et al. (44), and the causal agent was named "*P. gingeri*" (32). This pathogen was originally reported to occur in the United Kingdom and Australia (3) and was recently found in Pennsylvania (43). The third disease is called drippy gill and is characterized by the formation of dark-brown lesions at the sides and bottom edges of the gills. It is caused by the bacterial pathogen *P. agarici* (47). Drippy gill has been reported to occur in New Zealand, Australia, and Eire (3). The species "*P. gingeri*" and "*P. reactans*" have not yet been officially accepted.

During our previous study of postharvest discoloration of mushroom (43), we noticed that after initial isolation and single-colony cloning, approximately 30% of the over 200 strains of fluorescent pseudomonads isolated either from discolored lesions on mushroom caps or from mushroom casing medium exhibited a mucoid phenotype when grown on Difco *Pseudomonas* agar F medium (PAF) at room temperature. Using a variety of physiological and biochemical tests as well as whole-cell fatty acid analyses, we classified pathogenic bacteria isolated from discolored mushroom caps into one of three groups (pathotypes). The groups were identified as *P. tolaasii*, "*P. gingeri*," and "*P. reactans*"; the last of these was previously described as a strictly saprophytic mushroom-associated bacterium (35, 45). The pathogenic strains of "*P. reactans*" were weakly virulent (43). The fluorescent pseudomonads isolated from casing medium were predominantly saprophytic strains of *P. fluorescens*, *P. putida*, and "*P. reactans*," but a few strains of *P. tolaasii*, "*P. gingeri*," and weakly virulent "*P. reactans*" were also isolated (43).

Previous studies in our laboratory (11, 12, 25–29) have indicated that as a group, the rRNA-DNA homology group I pseudomonads (8, 31, 39) can produce a variety of exopolysaccharides (EPSs) (Fig. 1). These EPSs include alginate, a copolymer of mannuronate and guluronate with the monomers present as homopolymeric and heteropolymeric regions; marginalan, an acidic galactoglucan; levan, a neutral polyfructan; and three additional unique acidic polymers. To date, individ-

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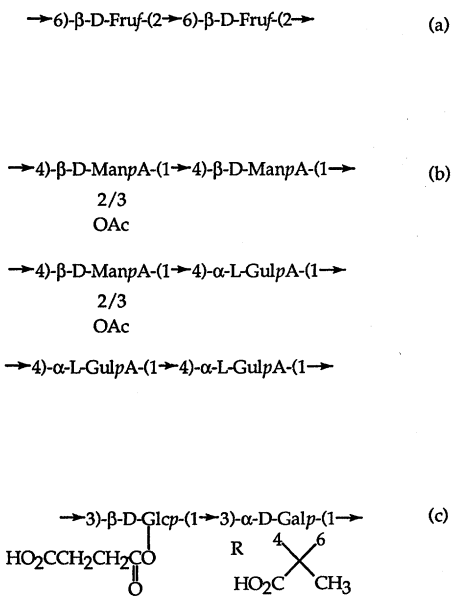

FIG. 1. Structure of the neutral EPS levan (a) and the acidic EPSs alginate (b) and marginalan (c). Fru, fructose; ManA, mannuronic acid; GulA, guluronic acid; OAc, O-acetyl group substituted at the 2 and/or 3 position of mannuronic acid; Glc, glucose; Gal, galactose; *f*, furanose form of the sugar; *p*, pyranose form of the sugar.

ual strains have not been shown to produce more than a single acidic EPS.

In this study we wished to characterize the composition of the EPSs produced by mushroom-associated fluorescent pseudomonads to determine if there was any correlation between species and EPS type. We were also interested in determining if any novel EPS types were produced by this collection of pseudomonads.

TABLE 1. Origin and pathogenicity of mushroom-associated fluorescent pseudomonads

Bacterial strain	Biovar[a]	Origin	Pathogenicity[b]
P. fluorescens			
J1	II	Casing medium	−
B4, B14, B15, B20, B22; D12; G2, G7, G8, G13, G19; K17; H6, H8, H9, H13, H16, H21; I22, I23; J12, J19	III	Casing medium[c]	−
H11; I15, I19; L16, L23, L24	V	Casing medium[c]	−
"*P. gingeri*"			
Pf3, Pf6, Pf11, Pf13	III	Discolored lesions[d]	+
Pf2, Pf9, Pf14, Pf31	V	Discolored lesions	+
K20, K23	V	Casing medium[c]	+
P. putida			
B24, B25, B27; C2, C3, C5, C6, C12, C21, C22; D11, D13; G14, G17; I7, I17, I20; L21		Casing medium[c]	−
"*P. reactans*"			
ATCC 14340	V	Mushroom (ATCC)[e]	+ (weak)
C7, C11; H23; K16; L5	V	Casing medium[c]	+ (weak)
K15; L18	III	Casing medium	+ (weak)
P. tolaasii			
ATCC 33618	V	Mushroom (ATCC)	+
P2, P3a, P3b; Pf28, Pf29, Pf30	V	Discolored lesions	+
C8, C9; G1	V	Casing medium[c]	+

[a] Biovar classifications based on the criteria used for classification of *P. fluorescens* (31).

[b] Pathogenicity on cultivated mushroom (*Agaricus bisporus*).

[c] For strains originating from casing medium, strain designations beginning with the same letter were obtained from the same lot (43).

[d] Strains originated from discolored lesions which developed on mushroom caps postharvest (43).

[e] ATCC, American Type Culture Collection, Rockville, Md.

MATERIALS AND METHODS

Bacterial strains. Most bacterial strains included in this study were originally isolated in our laboratory from discolored lesions which developed on mushroom caps postharvest or from nine lots of mushroom casing medium obtained from commercial mushroom growers located in Pennsylvania. The bacteria were identified previously by use of standard biochemical and physiological tests, as well as whole-cell fatty acid and Biolog metabolic assays (43) and were either saprophytic or pathogenic to mushrooms (Table 1) (43). Included were 29 strains of *P. fluorescens*, 18 strains of *P. putida*, 8 strains of "*P. reactans*," and 8 strains of "*P. gingeri*," all of which exhibited mucoid growth upon initial culturing on PAF (Difco) at room temperature. Also included were 2 strains of "*P. gingeri*" and 10 strains of *P. tolaasii* which were initially found to be nonmucoid on PAF (43). The type strain (ATCC 33618) of *P. tolaasii* and "*P. reactans*" ATCC 14340 (originally misidentified as *P. tolaasii* [45, 48]) were obtained from the American Type Culture Collection, Rockville, Md., and were included for comparison. All strains were maintained on PAF at 4°C with bimonthly transfers to fresh medium. Long-term storage was in Trypticase soy broth (BBL, Becton Dickinson, Cockeysville, Md.)–20% glycerol at −80°C.

Isolation of EPS. All strains were initially tested for EPS production on PAF with incubation at 20 to 28°C. After streaking of the bacteria, cultures were observed for mucoid growth for up to 7 days. Selected strains which failed to produce EPS on PAF were also tested by incubation at room temperature for 7 days in which the glycerol content was raised from 1 to 5% (wt/vol), on a modified Vogel and Bonner agar medium with 5% (wt/vol) gluconate as the carbon source (MVBM) (5), on PAF with 5% gluconate substituted for glycerol (PAF+Glu), and on yeast extract-dextrose-$CaCO_3$ agar medium (YDC) (41).

For isolation of EPS, bacteria were cultured for 2 to 3 days at 20 or 28°C on 5 to 10 culture dishes (100 by 15 mm) containing PAF or PAF with 5% (wt/vol) glycerol. Increased glycerol content in the medium and/or a low incubation temperature was required to obtain adequate yields of EPS for characterization for some strains. It is well established that an increased carbon-to-nitrogen ratio and incubation temperatures lower than those optimal for bacterial growth often favor increased production of EPS (40). Bacterial growth and EPS were harvested with a bent glass rod and distilled water. After being stirred, cells were checked for the presence of capsular polysaccharides by wet mounts in India ink under the light microscope. Bacterial cells were then pelleted by centrifugation (16,300 × *g* for 30 min), and clear supernatant fluids were collected. A concentrated aqueous solution of KCl (25%, wt/vol) was added to give a final concentration of 1% (wt/vol) to aid in the precipitation, and the EPS was precipitated by the addition of 2 to 3 volumes of isopropanol. After the mixture had stood overnight at 4°C, the precipitated EPS was collected by centrifugation, dissolved in distilled water, recentrifuged to remove any insoluble materials, freeze-dried, and weighed.

All strains of *P. tolaasii* (none of which produced EPS in agar medium) and *P. fluorescens* H13 were also tested for EPS production in broth media. Overnight starter culture (0.5 ml per flask) was added to 250-ml Erlenmeyer flasks, each containing 25 ml of either King's medium B (22) with the glycerol content raised from 1 to 5% (both species) or MVBM (*P. tolaasii* only). A commercial protease (Alcalase 2.4 L; Novo) was added to a final concentration of 0.005% to inhibit any EPS-degrading enzymes produced. Cultures were incubated at 20 to 24°C for 5 days with shaking (250 rpm). After removal of bacterial cells by centrifugation, samples were treated as described above.

Anion-exchange chromatography. EPS (50 mg) from *P. fluorescens* H13 was dissolved in 100 ml of 0.05 M Tris-HCl buffer (pH 6.5) and was loaded onto a column of DEAE-Sepharose CL-6B (Pharmacia) (2.5 by 18 cm). After the sample had entered into the bed, the column was initially eluted with 300 ml of buffer followed by a 500-ml linear 0 to 2 M NaCl gradient prepared in buffer. Finally, the column was eluted with 100 ml of 2 M NaCl in buffer. In one experiment, urea was added to all buffer solutions at a final concentration of 7 M. Fractions (7.4 ml) were collected and tested for the presence of neutral sugars as described below. Peak material fractions were combined, dialyzed against distilled water, and then lyophilized. For "*P. gingeri*" Pf9 EPS, 200 mg of sample was dissolved in 400 ml of buffer and loaded onto a larger column of DEAE-Sepharose CL-6B (5.0 by 19 cm). Initial elution was with 1 liter of buffer and was followed by a 1.6-liter linear 0 to 2 M NaCl gradient in buffer and then 500 ml

TABLE 2. EPSs produced by mushroom-associated fluorescent pseudomonads

Bacterial strain	Yield of EPS (mg)[a]	EPS production			
		Marginalan	Alginate	Unique	Levan[b]
P. fluorescens					
J1	35–70	−	+	−	+
H13	7–30	−	−	+	−
B4, B14, B15, B20, B22; D12; G2, G7, G8, G13, G19; K17; H6, H8, H9, H11, H16, H21; I15, I19, I22, I23; J12, J19; L16, L23, L24	8–79	+	−	−	−
"P. gingeri"					
Pf2, Pf3, Pf9, Pf11, Pf13, Pf31; K20, K23	8–32	−	−	+	−
P. putida					
All strains	25–58	+	−	−	−
"P. reactans"					
ATCC 14340; H23; K15, K16; L18	12–55	−	+	−	−
C7, C11; L5	23–43	+	−	−	−

[a] Yields of EPS are expressed per five culture dishes (100 by 15 mm) of PAF containing 1 or 5% glycerol. Cultures were incubated for 2 to 3 days at 20 to 28°C before isolation of EPS.

[b] Data for ability to produce levan are based on results of a previous study (43).

of 2 M NaCl in buffer. All the buffers used with strain Pf9 EPS contained 7 M urea. The flow rate for all columns was 0.6 ml/min.

Analytical methods. Unless otherwise stated, all reagents and standards were obtained from Sigma Chemical Co., St. Louis, Mo. Total neutral carbohydrate was determined by the method of Dubois et al. (10) with D-glucose as the standard. The uronic acid content was determined by the method of Blumenkrantz and Asboe-Hansen (2) with a commercial preparation of algal alginate as standard. Acetate and succinate contents were determined by the method of McComb and McCready (23) with glucose pentaacetate or monomethylsuccinate, respectively, as the standard. This colorimetric assay does not distinguish between the two ester-bound substituents. The pyruvate content was determined by an enzymatic assay with lactate dehydrogenase (20). The hexosamine content was determined by the method of Johnson (21) with D-glucosamine as the standard. Before analysis for hexosamine, samples were hydrolyzed in 6 M HCl for 4 h at 100°C, neutralized with a concentrated NaOH solution, and dried under a stream of nitrogen. Ketose sugar content was determined by the method of Dische (9) with fructose as the standard.

Sugar composition. Samples were hydrolyzed in 1 M H_2SO_4 at 100°C for 1.5 h. Selected samples were also hydrolyzed in 2 M trifluoroacetic acid (120°C for 1 h) or 6 M HCl (100°C for 4 h). The released sugars were identified as their aldononitrile acetate (29) or alditol acetate (1) derivatives by gas-liquid chromatography (GLC). The gas-liquid chromatograph (Hewlett-Packard model 5995B) was fitted with a 15-m SP-2330 capillary column (Supelco, Inc., Bellefonte, Pa.) with temperature programming from 150 to 250°C at 4°C/min. Before hydrolysis and derivatization, samples containing significant amounts of uronic acid as determined by colorimetric assay were also reduced with sodium borohydride via the carbodiimide adduct (42). Sugars were identified by cochromotography with commercial standards.

RESULTS

When cultured on PAF containing 1 or 5% glycerol, all mucoid strains of the saprophytic bacterium *P. putida* and most strains of the saprophyte *P. fluorescens* (biovars III and V) isolated from mushroom casing medium produced marginalan (25) as an acidic EPS (Table 2). Colorimetric and enzymatic assays run on selected samples confirmed substitution with succinate and pyruvate. For several of these strains, light-microscopic observations showed that a small proportion of the cells harvested (estimated at less than 5%) were encapsulated.

The only two strains of *P. fluorescens* which did not produce marginalan were J1 and H13. The acidic EPS produced by strain J1 on PAF was determined to be alginate. The two preparations of this EPS examined contained small amounts of guluronate (average of 6% relative to the mannuronate content as determined by area of peaks obtained from GLC analyses) and were acetylated. No encapsulated cells were detected. Results of our previous study (43) indicated that of all the strains examined in this study, strain J1 was the only one capable of levan production when cultured in the presence of high levels of sucrose.

Strain H13 produced EPS when grown on PAF or in King's medium B broth. Approximately 10% of the cells were encapsulated, and these cells gave a distinct opaque, loosely packed layer overlying the compact cell pellet composed of nonencapsulated cells after centrifugation. Only the clear supernatant fluids were collected and analyzed. Yields of EPS obtained from broth cultures ranged from 850 to 1,000 mg/liter and from 7 to 30 mg per five culture dishes (Table 2). The EPS contained neutral sugar (5 to 11% by weight), hexosamine (9 to 18%), and acetate (or succinate) (3%) and was devoid of ketose sugar, uronic acid, and pyruvate. Analysis of the sample by GLC indicated the presence of rhamnose, fucose, arabinose, glucose, and glucosamine. The relative ratios of the sugars based on peak area percentages were variable, depending on the method used for sample preparation (either H_2SO_4 hydrolysis followed by preparation of the aldononitrile acetate derivatives or hydrolysis with trifluoroacetic acid followed by preparation of the alditol acetate derivatives). Samples were then further purified by two additional alcohol precipitation steps followed by anion-exchange chromatography. A single major peak of neutral sugar-containing material was eluted from the column (between 0.4 and 0.6 M NaCl), indicating that the EPS was acidic. Analyses by GLC indicated the presence of the same five sugars as found in the EPS samples before purification. The relative ratios of the sugars based on peak area percentages were once again variable, depending on the method used for sample preparation. Colorimetric assays indicated the presence of 14% neutral sugar and 18% amino sugar. Results obtained for anion-exchange chromatography with the addition of 7 M urea to all buffer solutions were similar. The identification of glucosamine in the purified samples was confirmed by electron impact mass spectrometry of the alditol acetate derivative (4).

When cultured on PAF, 8 of the 10 strains of *"P. gingeri"* tested gave mucoid growth (Table 2). These eight strains produced an EPS with a distinct composition. The EPS contained both neutral sugar (22 to 54% by weight) and uronic acid (10 to 48% by weight) and was devoid of ketose sugar and hexosamine. The neutral-sugar composition was variable, depending on both the bacterial strain and the date of preparation. The predominant neutral sugar for all preparations was mannose, but glucose, galactose, rhamnose, ribose, and xylose were also present in various amounts. After reduction, the amount of glucose seen in the GLC chromatograms increased significantly, indicating that glucuronic acid was present in relatively large amounts. To determine if the cultures of *"P. gingeri"* might contain colonies expressing more than a single EPS chemotype, three strains (K20, K23, and 31) were again single colony cloned three times, the final clones were grown out on PAF, and the EPS was isolated. Analyses by colorimetric assays as well as GLC indicated that the EPS produced by these single-colony clones was similar to the parental EPS. The nonmucoid strains Pf6 and Pf14 were also tested for mucoid growth on the additional solid media MVBM, PAF+Glu, and YDC with incubation at room temperature. No mucoid growth occurred on any of these three media.

The EPS produced by *"P. gingeri"* Pf9 was selected for fur-

ther purification by two additional precipitations with isopropanol followed by anion-exchange chromatography. A single major peak of neutral sugar-containing material was observed, which eluted at 0.5 to 0.9 M NaCl. The purified material contained neutral sugar (48% by weight), uronic acid (28% by weight), pyruvate (9% by weight), and acetate (or succinate) (6% by weight). GLC analyses demonstrated the presence of glucose, mannose, and glucuronic acid. Further analyses indicated that the three sugars were present in equimolar amounts (4).

Mucoid strains of "*P. reactans*" produced either alginate or marginalan (Table 2). Analyses of reduced alginate samples by GLC indicated a guluronate content of between 13 and 23%, and colorimetric assays indicated that the alginates were acetylated. None of the "*P. reactans*" strains examined in this study produce levan when tested on nutrient sucrose agar (43).

Mucoid growth of *P. tolaasii* did not occur on any of the solid media tested (PAF with 1 or 5% [wt/vol] glycerol, PAF+Glu, MVBM, and YDC). Production of EPS also was not detected after growth of the strains in MVBM broth and modified King's B medium broth.

DISCUSSION

A variety of EPSs are synthesized by fluorescent pseudomonads associated with mushroom production. The most commonly encountered acidic EPS in our study was the galactoglucan marginalan. Marginalan was produced by all mucoid strains of *P. putida* as well as most strains of *P. fluorescens*. We originally isolated this galactoglucan from certain strains of pectolytic soft-rotting *P. fluorescens* (sometimes referred to as *P. marginalis* to distinguish these strains from *P. fluorescens* strains without the ability to cause soft rot [19]), as well as from several saprophytic strains of *P. fluorescens* originally isolated from plant rhizospheres, surfaces of tomato and pepper fruits, and beet seed pericarp (11, 25). This is the first report of the production of marginalan by *P. putida.*

These results plus those of two earlier studies on EPS production by *P. putida* indicate that as a species, *P. putida* is capable of producing more than a single acidic EPS. Govan et al. (16) reported that a mucoid variant strain of *P. putida* produced alginate. Read and Costerton (36) reported the production by a single freshwater isolate of an EPS which appears to be very similar to marginalan. This particular EPS contained equimolar amounts of glucose and galactose and was substituted with acetate and pyruvate. No structural information on the EPS was given.

One strain (J1) of *P. fluorescens* produced alginate as an acidic EPS. Alginate production by certain strains of this pseudomonad was previously reported (11, 16). Strain J1 was the only *P. fluorescens* strain included in this study which was capable of producing levan when grown in the presence of sucrose (43). We previously noted that the ability to produce alginate is positively correlated with the ability to produce levan for *P. fluorescens* and that strains of this bacterium which produce marginalan do not produce levan (11). The composition of the EPS produced by *P. fluorescens* H13 appears to be unique for the group I pseudomonads. To our knowledge, no hexosamine-containing EPS produced by a member of this group has been reported. Interestingly, the plant pathogen *P. solanacearum*, a member of rRNA-DNA homology group II, produces as its major acidic EPS a polymer composed solely of amino sugars (24).

The nature of the EPS produced by the mushroom-associated fluorescent pseudomonad "*P. reactans*" has not been reported previously. The results of the present study indicate that either alginate or marginalan can be produced by mucoid, weakly virulent strains of the bacterium. The marginalan producers were previously found to exhibit biochemical and physiological properties which were similar to *P. fluorescens* biovar V, but the alginate producers were similar to either biovar III or V (43). No strictly saprophytic strains of this bacterium were identified in our previous study as exhibiting mucoid growth on PAF, and most of the weakly virulent strains were also nonmucoid (43).

One criterion previously reported for distinguishing between the mushroom pathogens "*P. gingeri*" and *P. tolaasii* was the mucoid phenotype of "*P. gingeri*" on PAF (44). Our results were in agreement with this observation, except that 2 of the 10 strains of "*P. gingeri*" examined failed to exhibit a mucoid phenotype under any of the culture conditions tested. Mucoid strains of the pathogen "*P. gingeri*" could readily be distinguished from the other bacteria studied by the composition of their EPS. None of the strains of *P. tolaasii* gave mucoid growth on PAF or produced EPS on a variety of other solid media which we had previously found to support EPS production by fluorescent pseudomonads (11, 12). Growth in broth media also did not support EPS production. Even though we were unsuccessful in obtaining EPS production by *P. tolaasii*, there is evidence from electron-microscopic studies that EPS may be involved in attachment of the bacterium to mushroom host hyphae (34). In vitro EPS production by *P. tolaasii* may be under strict regulatory control.

During the course of this study, we noted that when cultured on PAF, several of the EPS-producing strains rapidly generated nonmucoid variant colonies which were easily distinguishable from the parental mucoid colony phenotype. This phenomenon was noted previously for mushroom-associated fluorescent pseudomonads (7, 17). Instability of EPS production in vitro has previously been noted for several other pseudomonads including *P. aeruginosa* (15) and *P. solanacearum* (18). Synthesis and excretion of bacterial EPS are energy-intensive processes which can be down-regulated when the production of EPS is not required (6).

In conclusion, the results of this study indicate that a variety of acidic EPS types are produced by fluorescent pseudomonads found in association with commercial mushroom production. These acidic polymers may protect the bacteria from adverse environmental conditions such as desiccation (30, 37, 38) and may mediate bacterial attachment to fungal mycelia (34). The ability of such strains to produce the neutral EPS levan appears to be limited. Finally, the group I fluorescent pseudomonads continue to be a rich source of novel bacterial EPSs, as evidenced by our initial results on the composition of the EPSs produced by "*P. gingeri*" and *P. fluorescens* H13.

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