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Characterization by 16S rRNA Sequence Analysis of Pseudomonads Causing Blotch Disease of Cultivated *Agaricus bisporus*

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Bacterial blotch of *Agaricus bisporus* has typically been identified as being caused by either *Pseudomonas tolaasii* (brown blotch) or *Pseudomonas gingeri* (ginger blotch). To address the relatedness of pseudomonads able to induce blotch, a pilot study was initiated in which pseudomonads were selectively isolated from mushroom farms throughout New Zealand. Thirty-three pseudomonad isolates were identified as being capable of causing different degrees of discoloration (separable into nine categories) of *A. bisporus* tissue in a bioassay. These isolates were also identified as unique using repetitive extragenic palindromic PCR and biochemical analysis. Relationships between these 33 blotch-causing organisms (BCO) and a further 22 selected pseudomonad species were inferred by phylogenetic analyses of near-full-length 16S rRNA gene nucleotide sequences. The 33 BCO isolates were observed to be distributed throughout the *Pseudomonas fluorescens* intrageneric cluster. These results show that in addition to known BCO (*P. tolaasii, P. gingeri*, and *Pseudomonas reactans*), a number of diverse pseudomonad species also have the ability to cause blotch diseases with various discolorations. Furthermore, observation of ginger blotch discoloration of *A. bisporus* being independently caused by many different pseudomonad species impacts on the homogeneity and classification of the previously described *P. gingeri*.

The genus *Pseudomonas* (sensu stricto) comprises a taxon of metabolically versatile organisms that are ubiquitous in soil and water and play an important role as plant, animal, and human pathogens (37). Microbial diversity in mushroom farms has previously been reported, with pseudomonads accounting for 10% of bacteria in compost and sometimes more than 50% of bacteria in casing soils (48).

Discoloration of *Agaricus bisporus* caused by pathogenic pseudomonads, the so-called blotch diseases, are well documented. *Pseudomonas tolaasii* contamination results in sunken, dark brown lesions (35, 55); *Pseudomonas reactans* causes mild dark purple to light brown discoloration and a slight surface depression that becomes deeper and darker with age (59); while the pale yellowish red discoloration that develops into a reddish ginger-colored discoloration (ginger blotch disease) is characteristic of *Pseudomonas gingeri* (60).

Of the blotch-causing pseudomonads, the best characterized is *P. tolaasii. P. tolaasii* enters the mushroom farm in peat and limestone used in the casing process (63), and, once present, *P. tolaasii* is able to attach to mycelial surfaces of developing *A. bisporus* (40, 42). Temperature and relative humidity have been suggested as important environmental conditions that influence the pathogenicity of *P. tolaasii* within the mushroom farm (50). A minimal application of 2.7×10^6 to 10^8 CFU · ml^{-1} of *P. tolaasii* was reported as the threshold for inducing disease (34), although other thresholds have been proposed (31, 44, 62). Pathogenic *P. tolaasii* isolates synthesize a low-molecular-weight extracellular toxin, tolaasin, that is the primary bacterial agent responsible for eliciting disease symptoms (3, 32, 43). Tolaasin, a lipodepsipeptide (LDP), causes disruption of cell membranes from a range of cell types and has both ion channel-forming and biosurfactant properties (3, 17, 43). When *P. tolaasii* is cultured in close proximity to a second pseudomonad, *P. reactans*, a white precipitate forms between the colonies, defining the white-line in agar (WLA) assay (61). Tolaasin forms a dense white precipitate with the white line inducing principle (WLIP), an LDP produced by *P. reactans* (30).

Relatedness of pathogenic pseudomonads in the mushroom industry has been addressed in many previous studies. Using three experimental parameters, pathogenicity, physiological properties, and cellular fatty acid composition, *P. gingeri*, pathogenic *P. reactans*, and *P. tolaasii* were found to be related to but distinguishable from *Pseudomonas fluorescens* biovars III and V (59). Substrate utilization tests, electrophoresis of soluble proteins, and DNA:DNA hybridization experiments showed *P. gingeri* to form a unique grouping, as did *P. tolaasii*, saprophytic *P. fluorescens* biovar II, the so-called white line reacting organisms (including *P. reactans*), and *Pseudomonas agarici* (13). In this same study, Goor et al. showed *P. tolaasii* to be a homogeneous grouping separate from both *P. fluorescens* and *P. reactans*. Homogeneity of *P. tolaasii* as a species was further demonstrated with nucleotide sequence analysis of the small subunit rRNA (16S rRNA gene) (29).

P. gingeri was first described as a new member of the *P. fluorescens* complex causing ginger blotch disease of *A. bisporus* (60). Although *P. gingeri* demonstrates phenotypic similarity to *P. tolaasii* (e.g., during colony transition from pathogenic to nonpathogenic states [6]), *P. gingeri* can be distinguished from *P. tolaasii* by the WLA and pitting assays (61) and the 2-ketogluconate and lipase tests (60). However, since its first description, *P. gingeri* has received limited attention as to its epidemiology and characterization as a species.

Two continuing observations initiated the pilot study presented in this paper: (i) New Zealand pseudomonads isolated

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from *A. bisporus* exhibiting ginger blotch were variable in colony morphology and growth patterns; and (ii) from an agronomist's point of view, ginger blotch lesions exhibited variable discoloration, suggesting that more than one organism was responsible for the discoloration (or that the organism had variable virulence). Based on these observations, this study sought to address the molecular and phenotypic diversity among pseudomonads in the mushroom farm environment that are capable of causing ginger blotch and other blotch-related diseases of *A. bisporus*.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial isolates used in this study are listed in Table 1. Reference strains were obtained from the National Collection of Plant Pathogenic Bacteria (Harpenden, United Kingdom), and pseudomonad isolates from a milk factory environment were obtained from a previous study (45). All strains used in this study were maintained at −80°C in Kings B liquid medium (KB) (23) with a final concentration of 20% (vol/vol) glycerol. Strains were transferred onto KB medium supplemented with 1.5% agar, incubated at 28°C for 16 h, and maintained at 4°C for short-term use.

Isolation of pseudomonads from mushroom farms. Sampling from mushroom farms in New Zealand was carried out during the Austral summer of 1999. A section of tissue was excised from mushroom caps exhibiting blotch discolorations and was placed into a McCartney tube containing sterile KB medium (10 ml). One gram of compost or casing material was placed into a McCartney tube containing sterile KB medium (10 ml). Water samples from water reservoirs and frequently used taps on mushroom farms were collected in sterile McCartney bottles. Samples were maintained on ice upon collection and during transport to the laboratory, a period not exceeding 2 h. Samples were incubated for 24 h at 28°C before an aliquot was applied to Gould's agar medium (14). Individual bacterial colonies were purified by passage onto fresh Gould's medium and stored as described above.

Bioassay for pathogenicity. Bioassays were performed as described by Gandy (11) using healthy, 1-day-old *A. bisporus*. Cubes (1 cm^3) of cap tissue were excised with sterile scalpel blades and placed into a sterile petri dish containing a 50-mm-pore-size paper filter dampened with 800 μl of sterile double-distilled water. Four cubes were placed 2 cm apart to eliminate cross-contamination by motile pseudomonads. Bacterial strains were cultured in KB medium to a density of 10^9 CFU · ml^{-1}, and a 50-μl aliquot of cells was placed onto three cubes. The fourth cube was inoculated with a 50-μl control of uninoculated KB. Petri dishes were sealed with parafilm and incubated under ambient conditions for 24 h. Mushroom caps incubated with bacterial isolates were scored for the degree of blotch discoloration on a scale of B1 to B9 (where B = blotch). To ensure comparable results, a color scale was developed using the revised Munsell standard soil color charts (issued in 1957), where B1 = Hue 2.5Y 8/1; B2 = Hue 2.5Y 7/2; B3 = Hue 2.5Y 6/3; B4 = Hue 2.5Y 5/4; B5 = Hue 2.5Y 4/6; B6 = Hue 2.5Y 4/4; B7 = Hue 2.5Y 3/3; B8 = 2.5Y 3/2; and B9 = Hue 2.5Y 3/1. All bioassays were repeated in triplicate using different sources of *A. bisporus* tissue. Tissue degradation, coloring of filter paper, and discoloration of the control cube were recorded after 48 h.

WLA assay. The ability to produce tolaasin was determined using the WLA assay described by Wong and Preece (61). Bacterial colonies were toothpick inoculated at a distance of 7 mm from streaks of the indicator bacteria, either *Pseudomonas reactans* NCPPB 1311 or *Pseudomonas tolaasii* NCPPB 2192^T. A white precipitation line was observed after 24 to 48 h of incubation at 28°C.

API 20 NE strip analysis. The Analytical Profile Index (API) 20 NE micromethod for the identification of nonfastidious gram-negative rods, using 8 conventional biochemical tests and 12 carbohydrate assimilation tests, was performed as described by the manufacturer (Bio Merieux). The numerical profiles obtained from pseudomonad strains were compared to the profiles stored in the 1999 Analytical Profile Index Software database (Bio Merieux).

Genomic DNA isolation and standard PCR conditions. DNA was isolated from pure cultures of bacteria using the Wizard Genomic DNA Isolation Kit (Promega) and stored at 4°C until required. All PCR amplifications were carried out in a Perkin-Elmer 9700 thermocycler. Unless stated otherwise, a standard PCR reaction mixture (25 μl total) consisted of 1× buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin, and 0.1% Triton X-100), deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP) at a final concentration of 200 μM, 0.625 U of *Taq* DNA polymerase (Roche Molecular Biochemicals), oligonucleotide primers at a final concentration of 2 mM, and 100 ng of template DNA. Thermocycling consisted of 30 cycles (1 min, 94°C; 1 min, 55°C; 1 min, 72°C). Prior to cycling, samples were heated at 94°C for 5 min and the extension step was increased to 5 min, 72°C as part of the terminal cycle. Primers and deoxyribonucleotide triphosphates were removed from PCR products using the High Pure PCR product purification kit (Roche Molecular Biochemicals).

TABLE 1. New Zealand BCO isolates and reference pseudomonad strains used in this study[a]

Designation	Farm[b]	Assay result[d]	Comment[e]	WL(R)[f]	WL(T)[g]	Biotype[h]	Accession no.[i]
NZ 032	A	B9	B	+	−	0156555	AF320995
NZ 027	B	B9	B	+	−	0156555	AF320994
NZ 006	A	B6		−	−	0156555	AY014800
P. tolaasii 2192T[j]	N[c]	B9	B	+	−	0156555	AF320988
NZ 031	C	B4		−	−	0177575	AY014807
NZ 009	A	B3		−	+	1357555	AY014802
P. reactans 1311	N	B2		−	+	0357555	AF320987
NZ 014	A	B3	D	−	−	1147555	AY014804
NZ 024	A	B3	D	−	−	1147455	AY014806
NZ 052	A	B9		−	+	0157575	AY014811
NZ 062	A	B9	Y	−	−	0157575	AY014814
NZ 060	A	B4	B	−	+	0157575	AY014813
NZ 007	A	B4		−	+	0557555	AY014801
NZ 081	B	B1		−	−	0757555	AF388206
NZ 111	E	B9	D	−	−	0157577	AY014825
NZ 065	B	B3	D	−	+	0757555	AY014815
NZ 124	C	B8	B	−	−	0157555	AY014829
NZ 066	B	B1		−	−	0157555	AF388209
NZ 113	E	B3		−	−	0357555	AY014827
NZ 064	A	B3		−	−	0357555	AF388208
NZ 102	A	B3	B	−	−	0357555	AY014820
NZ 097	A	B3		−	+	4156577	AY014818
NZ 101	A	B6		−	+	0146657	AY014819
NZ 096	A	B2		−	+	0157555	AY014817
NZ 039	D	B3	Y	−	−	0157555	AY014808
NZ 104	A	B3		−	−	0157555	AY014822
NZ 017	A	B3	D	−	−	1357555	AY014805
NZ 103	A	B9	B	−	−	0157555	AY014821
NZ 099	A	B1		−	−	0357555	AF388207
NZ 011	A	B6		−	−	0157555	AY014803
NZ 112	E	B3		−	−	4140457	AY014826
P. gingeri 3147T	N	B5	Y	−	−	0356555	AF320991
NZ 043	B	B5	Y	−	−	0157555	AY014809
NZ 059	A	B6	B	−	−	0156555	AY014812
NZ 092	A	B5		−	−	0156555	AY014816
NZ 047	B	B5	Y	−	−	0156565	AY014810

[a] For discussion purposes, isolates have been ordered as phylogenetically defined in Fig. 2.
[b] Topographically distinct farm locations designated A to E from which pseudomonads were isolated.
[c] N, reference strains obtained from National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.
[d] Discoloration of mushroom cube bioassay (11) after 48 h where B is blotch and numbers (1 to 9) refer to assigned blotch discolorations depicted in Fig. 1.
[e] D, tissue degradation; Y, yellowing of filter paper; B, browning of filter paper.
[f] White line reaction with *P. reactans* NCPPB 1311.
[g] White line reaction with *P. tolaasii* NCPPB 2192^T.
[h] API 20 NE numerical profile obtained.
[i] Accession numbers of 16S rRNA nucleotide sequences stored in GenBank.
[j] T indicates the recognized type strain of a species.

REP-PCR. The primers (REP1R-I and REP2-1) and protocols used for repetitive extragenic palindromic PCR (REP-PCR) were those described by de Bruijn (7).

PCR amplification and DNA nucleotide sequencing of the 16S rRNA gene. Primers U16A and U16B (57) were used to amplify the nearly complete 16S rRNA gene (approximately 1,480 bp). Direct nucleotide sequencing of this gene was achieved using primers U16A, U16B, and F357, F945, R1087, and R518 (25) in combination with the Big Dye Terminator Kit and an ABI Prism 3TIXLCPE

TABLE 2. Twenty-two validly described species of the genus *Pseudomonas* (sensu stricto) (29) used in 16S rRNA gene phylogenetic analysis

Bacterial isolate	Strain designation[a]	GenBank accession no.
P. aeruginosa	LMG 1242T[b]	Z76651
P. agarici	LMG 2112T	Z76652
P. alcaligenes	LMG 1224T	Z76653
P. amygdali	LMG 2123T	Z76654
P. asplenii	LMG 2137T	Z76655
P. aureofaciens	DSM 6698T	Z76656
P. balearica	DSM 6083T	U26418
P. chlororaphis	LMG 5004T	Z76657
P. cichorii	LMG 2162T	Z76658
P. citronellolis	DSM 50332T	Z76659
P. coronafaciens	LMG 13190T	Z76660
P. ficuserectae	LMG 5694T	Z76661
P. flavescens	NCPPB 3063T	U01916
P. fluorescens biotype A	DSM 50090T	Z76662
P. marginalis pv. marginalis	LMG 2210T	Z76663
P. mendocina	LMG 1223T	Z76664
P. oleovorans	DSM 1045T	Z76665
P. putida biotype A	DSM 291T	Z76667
P.s. stutzeri	CCUG 11256T	U26262
P. syringae pv. syringae	LMG 1247t1T	Z76669
P. tolaasii	LMG 2342T	Z76670
P. viridiflava	LMG 2352T	Z76671
Acinetobacter calcoaceticus	ATCC 23055	Z93434

[a] ATCC, American Type Culture Collection, Rockville, Maryland; DSM, Deutsche Sammlung von Mikroorganismen, Gottingen, Germany; LMG, Laboratorium voor Microbiologie en Genetica, Rijksuniversiteit, Gent, Belgium; IAM, Institute of Applied Microbiology, Tokyo, Japan; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom.

[b] T, type strain.

(PE Biosystems). All 16S rRNA gene sequences analyzed in this study were confirmed by determining contiguous overlapping sequences of PCR DNA.

Phylogenetic analysis of 16S rRNA gene nucleotide sequences. The 16S rRNA gene nucleotide sequences obtained in this study were aligned with 22 further pseudomonad 16S rRNA sequences (Table 2) obtained from GenBank using the nucleotide alignment software Clustal W (54). Phylogenetic trees were constructed with neighbor joining (47) and evolutionary distances calculated according to the method of Jukes and Cantor (20) using the software package Treecon for Windows version 1.3b (56). Bootstrap analysis (9) was carried out using 500 replicates. *Acinetobacter calcoaceticus* ATCC 23055 was included for single-sequence (forced) outgroup rooting of the tree.

Nucleotide sequence accession number. The 16S rRNA gene sequences determined in this study have been deposited with GenBank under the accession numbers listed in Table 1.

RESULTS

Selective isolation of pseudomonads capable of causing blotch of *A. bisporus*. Pseudomonads were selectively isolated from blotched mushrooms and various substrates (compost, casing, and water samples) from a major mushroom farm (50 isolates) and three smaller mushroom farms (15 isolates from each) within New Zealand. Ninety-five isolates were assessed for the ability to discolor and/or damage *A. bisporus* tissue in a bioassay with comparison to reference strains (Fig. 1). Bioassays showed that 76 of the 95 pseudomonad isolates caused discolorations of *A. bisporus* tissue to varying degrees (B2, 3.1% [$n = 3$]; B3, 36.8% [$n = 35$]; B4, 10.5% [$n = 10$]; B5, 11.6% [$n = 11$]; B6, 11.6% [$n = 11$]; B8, 2.1% [$n = 2$]; B9, 4.2% [$n = 4$]). Pseudomonads capable of causing discoloration in bioassays were termed blotch-causing organisms (BCO). The remaining 19 of the 95 isolates exhibited discolorations consistent with negative controls (B1) and were therefore considered nonpathogenic isolates.

Refined selection of 33 isolates. Since the focus of this pilot study was to determine the diversity of pseudomonads capable of causing blotch, 33 isolates were selected based on the following criteria: (i) they caused variable discolorations of *A. bisporus* tissue in bioassay, and (ii) they exhibited variance in colony morphology and growth patterns. Analysis of REP-PCR results ensured that each of the 33 selected BCO isolates was unique.

LDP production. LDP production was assessed using the WLA (Table 1). Eight of the thirty-three BCO isolates were observed to produce WLA+ reactions with *P. tolaasii*, consistent with the type strain of *P. reactans*. Two isolates (NZ 027 and NZ 032) produced a WLA+ result with *P. reactans*, as would be expected of *P. tolaasii* isolates.

Biochemical analysis of BCO isolates. Tentative species identification of 33 BCO isolates was initially based on API 20 NE biochemical analysis. Comparison of API 20 NE biotypes to the corresponding API 20 NE database identified NZ 112 and NZ 101 as *P. putida*, NZ 097 as *Burkholderia cepacia*, and the remaining 30 BCO isolates as *P. fluorescens* (Table 1). However, further discrimination of the BCO isolates was necessary due to the lack of discriminatory capability of the API 20 NE test. For instance, the type strain of *P. tolaasii* (NCPPB 2192T) could not be distinguished from *P. fluorescens*.

Phylogenetic characterization of BCO isolates. Figure 2 shows the inferred phylogenetic relationships derived from a neighbor-joining analysis of the pairwise comparisons among the 16S rRNA gene sequences of the selected 33 BCO isolates from this survey with 22 validly described species of the genus *Pseudomonas* (sensu stricto) (Table 2). All 33 BCO isolates from this study clustered within the *P. fluorescens* intrageneric cluster, not within the *P. aeruginosa* intrageneric cluster (as defined by Moore et al. [29]). Only four BCO isolates (NZ 043, NZ 059, NZ 092, and NZ 047) were observed to group closely with the previously identified typed strain of *P. gingeri*, NCPPB 3147, within the *P. agarici* lineage (Fig. 2). The remaining 29 isolates were observed to distribute throughout the *P. fluore-*

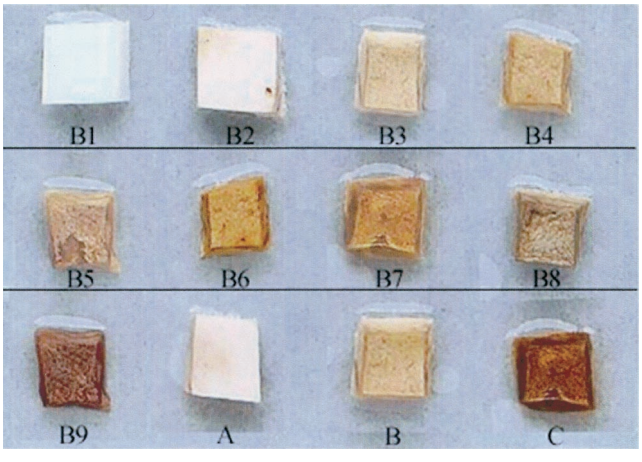

FIG. 1. Cube pathogenicity bioassays (11) to determine which BCO isolates are capable of inducing discoloration of *A. bisporus* tissue. Pictured are cubes within the assigned color scale, B1 through B9 (B1, cube inoculated with KB alone). The following reference strains are included for comparison: A, *P. reactans* NCPPB 1311 (B2); B, *P. gingeri* NCPPB 3147^T (B5); C, *P. tolaasii* NCPPB 2192^T (B9).

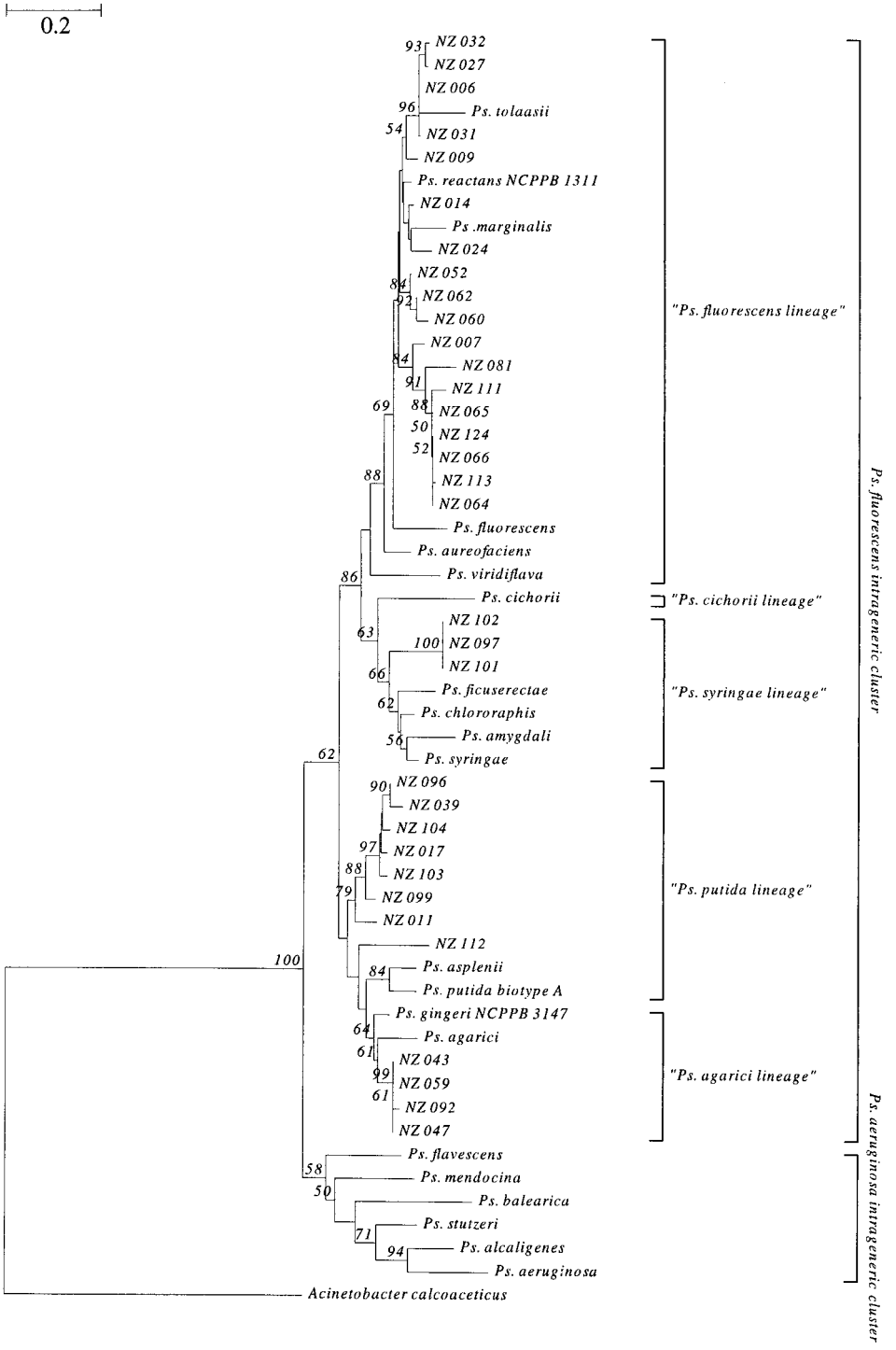

FIG. 2. The inferred phylogenetic relationships between the BCO isolates from this study and 22 validly described members of the genus *Pseudomonas* (sensu stricto). Evolutionary distances were determined with pairwise dissimilarities of the 16S rRNA gene sequences, and the dendrogram was generated using the neighbor-joining algorithm. Two major intrageneric clusters and five evolutionary lineages are defined as described by Moore et al. (29). Bootstrap proportions of confidence are represented as percentages for those branchings with values greater than 50%.

scens intrageneric cluster, with 18 (the majority) isolates falling in the *P. fluorescens* lineage, 8 within the *P. putida* lineage (5 forming a tight cluster), and 3 within the *Pseudomonas syringae* lineage. Two nonpathogenic (B1) isolates (NZ 081 and NZ 099) were observed to show a high level of relatedness to BCO clusters, and two B1 isolates (NZ 064 and NZ 066) grouped tightly within a single BCO cluster. Consistent with the WLA+ results, NZ 027 and NZ 032 grouped closely with *P. tolaasii*, strongly suggesting identity as *P. tolaasii*. Isolates that reacted with *P. tolaasii* (therefore classed as WLIP organisms, includ-

ing *P. reactans* [30]) were located mainly in the *P. fluorescens* lineage; however, there was also intrageneric clustering within the *P. syringae* and *Pseudomonas putida* lineages as well. Isolates grouping with *P. gingeri* NCPPB 3147^T (NZ 043, NZ 059, NZ 092, and NZ 047) exhibited discolorations consistent with ginger blotch (defined in this study as B4 to B6 based on a single color scale factor: either side of the B5 discoloration caused by *P. gingeri* NCPPB 3147^T). However, B4 to B6 discolorations were also observed with BCO isolates in the *P. fluorescens* lineage (NZ 006, B6; NZ 031, B4; NZ 007, B4; and NZ 060, B4), the *P. syringae* lineage (NZ 101, B6), and the *P. putida* lineage (NZ 011, B6) (Fig. 2).

DISCUSSION

Previous studies addressing the relatedness of pathogenic pseudomonads to cultivated *A. bisporus* are well documented. As a result of these studies, *P. tolaasii* has been well characterized and its homogeneity as a species has been established. However, *P. gingeri* has received limited attention since it was first described by Wong et al. (60).

Initiated by costly New Zealand outbreaks of ginger discolorations of *A. bisporus*, both on beds and postharvest, characterization of the causal organism(s) of ginger blotch in New Zealand mushroom farms was sought. Observation of pseudomonads with variable colony morphology and growth patterns isolated from mushrooms exhibiting ginger blotch symptoms raised the question of whether ginger blotch of *A. bisporus* is caused by a homogeneous species previously described as *P. gingeri*. Initially, 95 pseudomonads were isolated from mushroom farms throughout New Zealand that were associated with discolorations of *A. bisporus*. In order to determine pathogenic potential, the ability of each isolate to discolor and/or damage *A. bisporus* tissue was assessed in a bioassay with comparison to reference strains (Table 1). Bioassay results of these 95 isolates showed many to cause ginger blotch discolorations (B4 to B6). However, many isolates were also observed to cause different degrees of *A. bisporus* discoloration (B1 to B3 and B7 to B9). For this reason, the focus of this study was diverted from ginger blotch to blotch of *A. bisporus* in general to ascertain the prevalence of pseudomonad species capable of causing blotch diseases. The 33 pseudomonad isolates selected for phylogenetic analysis were chosen because they all exhibited variable colony morphology and growth patterns in culture and/or caused various discolorations of *A. bisporus* tissue in bioassay. No data were collected to determine the distribution of BCO isolates throughout New Zealand or to identify the predominant causal BCO strain.

Using chromametric measurements, variations of discoloration of *A. bisporus* tissue have been previously established for different pseudomonads. *P. tolaasii* was shown to evoke a specific color change (independent of the concentration of bacteria applied), while *P. gingeri* and *P. reactans* induced discolorations that were different from each other's and from *P. tolaasii's* (49). Bioassays in this study were carried out in triplicate using different sources of *A. bisporus* tissue in each replicate. This was to ensure accurate results of *A. bisporus* tissue discoloration, since amounts of tyrosinase (a major enzyme associated with the brown discoloration of *A. bisporus* [19]) have been shown to vary depending on spatial location and developmental stages of the mushroom (26). Known concentrations (10^7 CFU $\cdot$ ml^{-1}) of pseudomonad isolates were inoculated in each bioassay to facilitate comparisons between results, since differences in bacterial loadings on caps are thought to influence the development of the discolorations (49). Discolorations observed in the bioassay could, therefore, be assumed to be the result of individual strain virulence factors alone.

Species similarities of the 33 BCO isolates were initially determined by biochemical analysis. While the carbon assimilation tests and production of enzyme intermediates included in the API 20 NE strip do not effectively discriminate between isolates of *P. gingeri*, *P. tolaasii*, and *P. reactans* or between biovars of *P. fluorescens* isolates, clear biotype differences between many of the 33 BCO isolates were observed (Table 1). For this reason, API 20 NE strips were useful in determining differences in biochemical phenotypes of the BCO isolates but did not facilitate species identification. Therefore, further discrimination was required.

To determine the phylogenetic relationship of the 33 selected BCO isolates, comparison of nearly complete nucleotide sequences of the 16S rRNA gene was used in this study, since it is considered an effective method for defining prokaryotic genotypic relatedness and resolving taxonomic identities (10, 16, 29). The topology of the dendrogram derived from analysis of the 16S rRNA sequences obtained in this study (Fig. 2) is in agreement with the intrageneric structure described by Moore et al. (29). The 33 BCO isolates were observed to distribute throughout the *P. fluorescens* intrageneric cluster, supporting studies of Soler-Rivas et al. (49) in which distantly related pseudomonads (*P. tolaasii*, *P. gingeri*, and *P. reactans*) caused different degrees of discoloration. However, our study further demonstrates the extent of the diversity of pseudomonads capable of inducing discoloration of *A. bisporus*. Our findings also show that the same degree of discoloration may be caused by dissimilar species of pseudomonads, suggesting that the factor(s) causative of inducing a particular discoloration of *A. bisporus* are not exclusive to a particular pseudomonad species.

Since pseudomonads are arguably the most diverse and ecologically significant group of bacteria (38), the observation of distantly related pseudomonad species having similar disease phenotypes is not unexpected. Numerous species within the genus *Pseudomonas* have been classified (36, 39, 53), and many new species continue to be identified by methods that have been revised in response to advances in DNA technology (including DNA-DNA hybridization [58] and gene sequence analysis of 16S rRNA [24, 29] and *gyrB* and *rpoD* [64]). Such methods have aided taxonomic resolution, but as this study has shown, they may also introduce discrepancies between phenotypic and genotypic analyses. For example, Moore et al. (29) demonstrated that seven phenotypically indistinguishable genomovars of *Pseudomonas stutzeri* contain up to six nucleotide differences within the 16S rRNA gene, and Yamamoto et al. (64) also observed that many phenotypic traits of pseudomonad species did not reflect their phylogenetic relationships (e.g., *Pseudomonas corrugata* was observed in the *P. fluorescens* complex and *Pseudomonas amygdali* in the *P. syringae* complex).

As has been discussed by Maynard Smith (28), there is much debate on the naming of bacterial species given the difficulty of

defining a "bacterial species concept" due to recognition of the significant contribution of recombination to bacterial population genetics. Therefore, 16S rRNA gene analyses carried out in this study were intended to provide an indication of BCO species similarity only and cannot account for the acquisition of genes and accessory genetic elements (plasmids, transposons, integrens, and phages) by lateral gene transfer (33, 52), classical "spontaneous" mutation (27), and recombination (15, 22)—all of which are important sources of bacterial evolution and species diversity.

Most members of the pseudomonas genus produce active extracellular enzymes that have been associated with plant disease, including proteinases and lipases. *P. tolaasii* was found to produce a proteinase very similar to those secreted by other pseudomonad species (8), which, although the effect of this proteinase in mushroom infection is unknown, may facilitate the damage caused in the mushroom (2). Lipases have also been shown to facilitate bacterial infections by disrupting host membranes, and *P. tolaasii*, like many other pathogenic pseudomonads, produces an extracellular heat-stable monomeric metallo-lipase with a mass of 670 kDa (1). However, its involvement in mushroom infection is unresolved. Like *P. tolaasii*, it may be assumed that the BCO isolates in this study produce extracellular enzymes, including proteinases and lipases, which are likely to be involved in the discoloration of *A. bisporus*. The degrees of discoloration observed in this study may be a result of different combinations of extracellular enzymes produced by the BCO isolates facilitating different enzymatic activation of *A. bisporus* tissue.

As this pilot study was initiated to determine the source of ginger blotch disease, there was interest in BCO isolates capable of causing discolorations that are comparable to those caused by *P. gingeri* NCPPB 3147^T (B4 to B6). Four BCO isolates were observed to group closely with *P. gingeri* reference strains based on the 16S rRNA phylogenetic analysis. These isolates were also observed to cause B4 to B6 discolorations, suggesting that isolates closely related to *P. gingeri* have the ability to induce *A. bisporus* discoloration consistent with ginger blotch. However, a further six BCO isolates causing B4 to B6 discolorations were also observed to distribute within the *P. fluorescens* lineage, the *P. syringae* lineage, and the *P. putida* lineage. This result suggests that the organism previously described as *P. gingeri* is not solely responsible for ginger blotch of *A. bisporus* and that the disease can be caused by a number of different pseudomonads.

As production of LDP is also a pathogenicity factor associated with *A. bisporus* discoloration, each BCO isolate was assayed for its ability to secrete an LDP capable of forming a white line precipitate with either *P. reactans* or *P. tolaasii*. Observation of eight BCO isolates producing WLA+ reactions with *P. tolaasii* suggested that they produced LDP similar to the WLIP described by Mortishire-Smith et al. (30). Analysis of these eight BCO isolates within Fig. 2 revealed no commonality of LDP production or discoloration among closely related isolates. For example, although NZ 052 and NZ 062 grouped closely together, only NZ 052 produced a WLA+ reaction with *P. tolaasii*. Isolates NZ 096, NZ 103, and NZ 104 also clustered tightly, yet they exhibited different bioassay discolorations, and of the three, only NZ 096 was WLA+ with *P. tolaasii*. Although these two groupings exhibit high phylogenetic similarity, they display quite different pathogenicities (including the presence of the potential virulence factor WLIP), which may suggest that these isolates have acquired traits responsible for differing degrees of *A. bisporus* discolorations. This is further demonstrated by observation of nonpathogenic (B1) isolates showing close phylogenetic relatedness to BCO isolates able to cause disease. Studies have revealed that horizontal transfer and recombination of virulence genes play a major role in generating genetic diversity among bacterial species (21), and horizontal gene acquisition could also be an explanation of why highly related (based on 16S rRNA) BCO isolates in this study have different virulence potentials.

P. tolaasii has routinely been distinguished from other pseudomonads by its ability to cause dark brown discolorations on mushrooms and by a positive WLA with *P. reactans* (18, 36, 46, 61, 65). However, an earlier study (12) identified a mushroom farm isolate, *Pseudomonas* NZI7, as being WLA+ (with *P. reactans*) and causing discoloration of *A. bisporus* tissue comparable to that caused by *P. tolaasii*. Although based on these criteria NZI7 would normally be identified as *P. tolaasii*, NZI7 was shown to be genetically more similar to *P. syringae* than to *P. tolaasii* (12). For this reason, NZ 027 and NZ 032 were included in this study, and although they were not assumed to be *P. tolaasii* isolates based solely on WLA+ results with *P. reactans* and B9 discolorations, they did group with *P. tolaasii*. However, with the exception of NZ 006, other B8 to B9 BCO isolates showed little phylogenetic relatedness to *P. tolaasii* and were observed to distribute widely throughout the *P. fluorescens* intrageneric cluster. As B8-B9 BCO isolates were WLA− with *P. reactans* (except for NZ 027 and NZ 032), it was assumed that browning was due not to tolaasin production but most likely to an extracellularly produced factor(s) that (i) causes tyrosinase activation and production of brown melanins similarly to tolaasin (4, 19, 51) and/or (ii) reduces enzymatic activity in *A. bisporus* tissue, which has been suggested to be due to protease activity (49). Proteases have been known to degrade tyrosinases (5), and a protease from *P. tolaasii* has been isolated and is speculated to facilitate damage to the mushroom (2).

Also included in this study were three pseudomonad isolates from a New Zealand milk factory environment (NZ 111, NZ 112, and NZ 113) that caused *A. bisporus* discoloration as efficiently as the BCO isolates from mushroom farms. This observation (i) demonstrates that the ability to cause discoloration of *A. bisporus* is not a trait acquired for evolutionary survival within pseudomonads present in the mushroom environment and (ii) further supports the previous discussions asserting that the factor(s) causing blotch discolorations are likely to be a combination of extracellular enzymes common to many different pseudomonads. This finding raises the question of whether other bacterial species may also induce discolorations of *A. bisporus* and points to the need for adequate biosecurity of mushroom farms.

The purpose of this study was to address the relatedness of pseudomonads capable of inducing blotch diseases of *A. bisporus*. The results have confirmed previous reports that blotch disease may be caused by different species of pseudomonads. Furthermore, these results have identified three major findings: (i) the diversity of pseudomonads capable of causing blotch discolorations of *A. bisporus* is considerably more ex-

tensive than previously thought; (ii) the organism previously described as *P. gingeri* is not solely responsible for ginger discolorations of *A. bisporus* (ginger blotch); and (iii) a particular blotch discoloration may be caused by more than a single pseudomonad species. These findings affect the future classification of *P. gingeri*, because a major phenotypic characteristic of this species is its ability to induce a ginger discoloration of *A. bisporus*. These results also have implications for the control of general blotch diseases of *A. bisporus* since a single causal organism cannot now be targeted. Since certain pseudomonads are considered beneficial in the commercial cultivation of *A. bisporus* (41), the elimination of all pseudomonads from a farm environment is neither desirable nor practical. Therefore, blotch disease of *A. bisporus* may prove difficult to manage, and continued research of BCO organisms may better resolve the commonality of virulence factors and the environmental conditions that promote disease.

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