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Development of an Oligonucleotide Probe for *Aureobasidium pullulans* Based on the Small-Subunit rRNA Gene

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***Aureobasidium pullulans*, a cosmopolitan yeast-like fungus, colonizes leaf surfaces and has potential as a biocontrol agent of pathogens. To assess the feasibility of rRNA as a target for *A. pullulans*-specific oligonucleotide probes, we compared the nucleotide sequences of the small-subunit rRNA (18S) genes of 12 geographically diverse *A. pullulans* strains. Extreme sequence conservation was observed. The consensus *A. pullulans* sequence was compared with other fungal sequences to identify potential probes. A 21-mer probe which hybridized to the 12 *A. pullulans* strains but not to 98 other fungi, including 82 isolates from the phylloplane, was identified. A 17-mer highly specific for *Cladosporium herbarum* was also identified. These probes have potential in monitoring and quantifying fungi in leaf surface and other microbial communities.**

Several direct and indirect methods exist for the detection and quantification of microbes in ecosystems (for reviews, see references 24 and 42). The mainstay of these has been the viable-cell count, typically reported as CFU of the organism of interest per unit area or weight of sample (23, 46). Indirect assessment based on CFU from washings of a substratum has been the preponderant method of assessing terrestrial microbial populations generally and leaves in particular (5, 23). This method is rapid and simple and gives a measure of the culturable population. Its major detractions are that not all viable cells are culturable, the efficiency of removal may be very low or highly variable, and positional (microenvironmental) relationships between the microbe and surface are lost.

Within the last decade, molecular methods which offer precision and high sensitivity to the characterization of populations have revolutionized the detection, identification, and quantification of microbes (8, 30, 45). Indirect applications typically involve DNA or RNA isolation, frequently PCR amplification, and probing with labeled oligonucleotides (25, 30, 45). The small-subunit rRNAs (16S in prokaryotes and 18S in eukaryotes) are commonly used as targets for probes because they are abundant, have enough evolutionary information for inference of close as well as distant phylogenetic relationships, and contain conserved sequences which can be used as primer templates in sequencing (15, 40, 43).

In the direct application, microbial cells are visualized, often in situ, by use of radioactive (^{3}H or ^{35}S) or nonradioactive (digoxigenin, biotin, or fluorochrome) labels. Hybridization with fluorescently labeled, rRNA-targeted probes (fluorescence in situ hybridization [FISH]) (1, 35, 38) has been exploited to study bacteria in various ecosystems, including aquatic habitats (28), sediments (21), biofilms (3), and the rhizosphere (7). To date, successful applications of FISH in fungal microbial ecology have been limited, though it has been used to detect fungal mating type genes (36) and yeasts (13, 20) under laboratory conditions.

Aureobasidium pullulans (de Bary) Arnaud, a yeast-like deuteromycete (4, 19, 27), is of commercial interest primarily because of its extracellular polysaccharide, pullulan (34), and of ecological interest because it colonizes many habitats (18). It is one of the relatively few fungi that grows actively and ubiquitously on living leaves (phylloplane) (4). We are devising molecular methods to detect and quantify *A. pullulans* directly on leaf surfaces. As a first step in developing in situ probes, we describe a 21-mer which hybridizes to the 18S rRNA of *A. pullulans* but not to 16 other fungi and 82 phylloplane fungal isolates tested. Research reported here on the *A. pullulans* probe and related work with a universal probe have been confirmed in preliminary FISH experiments (37, 37a).

MATERIALS AND METHODS

Fungal strains. The sources, origins, and strain designations of the fungi used for PCR amplification of the 18S rRNA gene in this study are listed in Table 1. In addition to 12 *A. pullulans* strains, 16 other fungi representing 14 genera were chosen. These fungi were selected on the basis of several considerations. Either sequence information on the 18S rRNA gene was available in the GenBank database, or they were sequenced by us (Table 1). Further criteria for selection were their routine occurrence in the phyllosphere (5, 6, 10) (*Alternaria alternata*, *Cladosporium herbarum*, *Cladosporium cladosporioides*, *Cryptococcus laurentii*, *Rhodotorula rubra*, and *Sporobolomyces holsaticus*) or a taxonomic (19) (*Hormonema dematioides*) or phylogenetic (12, 16, 32) (*Aspergillus nidulans*, *Neurospora crassa*, *Penicillium chrysogenum*, *Penicillium notatum*, *Podospora anserina*, and *Colletotrichum gloeosporioides*) relationship to *A. pullulans*. Additionally, 82 phylloplane yeasts, numbered nonconsecutively between L1 and L100, were isolated on potato dextrose agar with 250 mg of chloramphenicol per liter by standard leaf wash methods (6) from apple trees at the Mandt Farm Experiment Station, Madison, Wis., and at the Picnic Point orchard adjacent to the University of Wisconsin campus. Leaves were collected for processing on two dates, 5 and 10 October 1994. Colonies were selected from plates on the basis of their color and comprised members of the two broad categories of phylloplane yeasts: the so-called pink yeasts (mainly *Sporobolomyces* and *Rhodotorula* spp.) and white yeasts (mainly *Cryptococcus* spp.) (10). The isolates were provisionally identified to genus level and, where possible, to species level, primarily with the API 20C Analytical Profile Index (bioMérieux Vitek, Inc., Hazelwood, Mo.) (48). This system includes 19 substrate assimilation tests in cupules, supplemented by morphological and additional biochemical data, including urea utilization; KNO_3 assimilation; and fermentation of cellobiose, glucose, maltose, raffinose, and sucrose in Durham tubes (33). With this system, the phylloplane yeast isolates were identified as a *Rhodotorula* sp., *R. glutinus*, *R. rubra*, *R. minuta*, *Cryptococcus laurentii*, *C. albidus*, and *Candida parapsilosis.*

Genomic DNA extraction. Fungal strains were grown on potato dextrose agar (Difco, Detroit, Mich.) at room temperature for 2 to 7 days. Total genomic DNA was extracted from agar plates (47) and diluted 1:50 prior to use.

PCR amplifications. Oligonucleotide primers (for details, see the legend to Fig. 1) and probes were synthesized by the β-cyanoethyl phosphoramidite method on a 391 DNA Synthesizer (Applied Biosystems, Foster City, Calif.) and used without further purification.

Reaction mixtures for genomic DNA amplification contained 1.25 U of *Taq* DNA polymerase (Promega, Madison, Wis.), 0.2 mM deoxynucleoside triphos-

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TABLE 1. Fungal strains from which the 18S rRNA gene was PCR amplified[a]

Strain	Species	Source	Strain	Species	Source
Ap1[b]	*A. pullulans*	NRRL 12779	Aa[b]	*Alternaria alternata*	NRRL 5255
Ap2[b]	*A. pullulans*	ATCC 90393	An	*Aspergillus nidulans*	FGSC 4
Ap3[b]	*A. pullulans*	NRRL Y2567	Be	*Blastocladiella emersonii*	ATCC 22665
Ap4[b]	*A. pullulans*	ATCC 48168	Cc	*Cladosporium cladosporioides*	NRRL 20632
Ap5[b]	*A. pullulans* var. *melanigenum*	ATCC 12536	Ch[b]	*Cladosporium herbarum*	NRRL 2175
Ap6[b]	*A. pullulans* var. *melanigenum*	CBS 210.65	Cg	*Colletotrichum gloeosporioides*	JHA S19-3
Ap7[b]	*A. pullulans* var. *pullulans*	CBS 704.76	Cl[b]	*Cryptococcus laurentii*	NRRL 2536
Ap8[b]	*A. pullulans* var. *melanigenum*	CBS 584.75	Hd[b]	*Hormonema dematioides*	CBS 116.29
Ap12[b]	*A. pullulans* var. *pullulans*	ATCC 11942	Lp	*Leucostoma persoonii*	ATCC 69911
Ap13[b]	*A. pullulans*	ATCC 28998	Nc	*Neurospora crassa*	FGSC 2490
Ap18[b]	*A. pullulans*	WF1	Ou	*Ophiostoma ulmi*	JHA 82
Ap19	*A. pullulans*	WF2	Pc[b]	*Penicillium chrysogenum*	NRRL 807
L1–L100	Yeast isolates (total: 82, nonconsecutive)	Apple phylloplane	Pn	*Penicillium notatum*	ATCC 9179
			Pa	*Podospora anserina*	ATCC 6710
			Rr[b]	*Rhodotorula rubra*	NRRL Y-1592
			Sh	*Sporobolomyces holsaticus*	NRRL Y-17285

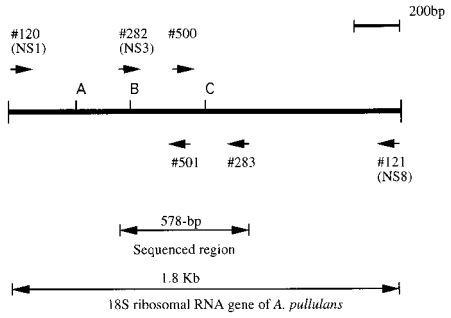
[a] NRRL, Northern Regional Research Lab, Peoria, Ill.; ATCC, American Type Culture Collection, Rockville, Md.; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; FGSC, Fungal Genetics Stock Center, Kansas City, Kans.; WF1 and WF2, field isolates from Madison, Wis.; JHA, J. H. Andrews' collection, Madison, Wis.

[b] Sequenced in this study.

phate (dNTP) (Pharmacia, Milwaukee, Wis.), 5 μl of 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 9.0 at 25°C], and 1.0% Triton X-100 [Promega]), 2.5 mM $MgCl_2$, 10% dimethyl sulfoxide (Sigma, St. Louis, Mo.), 10 pmol of primers, and 1 μl of diluted DNA sample. The reaction volume was made up to 50 μl. Occasionally, rRNA genes were amplified directly from spores as described elsewhere (9).

The PCR cycling conditions were 6 min of initial denaturing at 94°C, 2 min of annealing at 54°C, 40 min of prolonged extension at 72°C, and then 94°C for 1 min, 54°C for 2 min, and 72°C for 5 min for 35 cycles, followed by a 15-min extension at 72°C. Negative controls excluded DNA template and spores. Modification of these conditions, including shorter extension periods, could be employed.

DNA sequencing and sequence analysis. To assess intraspecific sequence conservation, a 578-bp region of 11 *A. pullulans* strains (Table 1) was PCR amplified (Fig. 1), subcloned into plasmid pCRII (Invitrogen, San Diego, Calif.), and sequenced by the dideoxy chain termination method (44) with automated dye-labeled ddNTPs on an Applied Biosystems model 373A automated sequencer. Both strands were sequenced. Nucleotide sequences were aligned by the Clustal method (29) with DNASTAR software (Madison, Wis.).



Primers
#282 (NS3): 5'-GCAAGTCTGGTGCCAGCA-3'
#283: 5'-AGCCTTGCGACCATACTC-3'
#120 (NS1): 5'-GTAGTCATATGCTTGTCT-3'
#121 (NS8): 5'-TCCGCAGGTTCACCTACGGA-3'

#500: 5'-TTGTTGGTTTCTAGGACC-3'
#501: 5'-GGTCCTAGAAACCAACAA-3'

Probes
A: 5'-TTCGTTTAGTTATTATGAATC-3' (#665)
B: 5'-GAATTACCGCGGCTGCTG-3' (U519)
C: 5'-TTAATCAAAACATCCTT-3' (#614)

FIG. 1. Schematic representation of primer and probe locations in the 18S rRNA gene of *A. pullulans* Ap2. Primers 282 and 283 generated a 578-bp PCR product; primers 120 and 121 amplified the full length of the 18S rRNA gene; primers 500 and 501 were used for sequencing. Primers 282, 120, and 121 correspond to the conserved primers NS3, NS1, and NS8, respectively, described by White et al. (50); primers 283, 500, and 501 were designed by us on the basis of the 18S rRNA sequence of the *A. pullulans* strain designated here as Ap2 (32). Probe A (665) is *A. pullulans* specific; B (U519) is a universal probe based on all known small-subunit rRNA sequences (20, 26); and C (614) is *C. herbarum* specific.

Southern blot and dot blot hybridizations. Following amplification, PCR products were size fractionated on agarose gels containing 1% SeaKem GTG agarose (FMC, Rockland, Maine). Gels were blotted to Nytran (Schleicher & Schuell, Inc., Keene, N.H.) and UV cross-linked (UV Stratalinker 1800; Stratagene Inc., La Jolla, Calif.).

The standard Southern blot protocol of Wallace and Miyada (49) was used with minor modification. Probes were ^{32}P end labeled with T4 polynucleotide kinases (Promega). Hybridizations and final 15-min washes were adjusted to $Td - 3°C$ or $Td - 5°C$, where $Td = 2(A+T) + 4(G+C)$. Blots were exposed to Kodak XAR film with or without an amplifying screen for 2 to 72 h.

Dot blots were performed by the procedure described by Meinkoth and Wahl (39) with slight modification. The double-stranded PCR products were denatured in 3 M NaOH at 65°C for 15 min, cooled on ice, neutralized with an equal volume of 2 M ammonium acetate (pH 7.0), and blotted onto Nytran. Following UV cross-linking, prehybridization and hybridization were conducted as described above. To ensure equivalent amounts of PCR target among samples, a probe complementing all known small-subunit rRNA sequences (universal probe) (20, 26) was also used.

Nucleotide sequence accession numbers. The GenBank accession numbers of the nucleotide sequence analyzed in this study include those for *Aspergillus fumigatus* (M55626), *Athelia bombacina* (M55638), *Aureobasidium pullulans* (M55639), *Blastocladiella emersonii* (M54937), *Candida albicans* (X534597), *Coccidioides immitis* (M55627), *Colletotrichum gloeosporioides* (M55627), *Leucostoma persoonii* (M83259), *Neurospora crassa* (X04971), *Ophiostoma ulmi* (M83261), *Penicillium notatum* (M556280), and *Podospora anserina* (X54864). The nucleotide sequences of 11 *A. pullulans* strains and six other fungi have been deposited with the Genome Sequence Database and assigned accession numbers: Ap1 (L76135), Ap2 (L76136), Ap3 (L76137), Ap4 (L76138), Ap5 (L76139), Ap6 (L76140), Ap7 (L76141), Ap8 (L76142), Ap12 (L76143), Ap13 (L76144), Ap18 (L76145), Aa (L76146), Ch (L76147), Cl (L76152), Hd (L76148), Pc (L76153), and Rr (L76149).

RESULTS AND DISCUSSION

To provide a means of specifically detecting *A. pullulans*, we designed probes based on the 18S rRNA gene even though it contains fewer variable regions for distinguishing among closely related taxa than does the 28S rRNA gene (41). The 18S rRNA gene has been the molecule of choice in developing molecular phylogenies, and accordingly, most of the sequence information published to date relates to this molecule (41). The taxonomy of *A. pullulans* is uncertain (19, 27), so sequence conservation among purported "strains" could not be presumed. Accordingly, the nucleotide sequence of 11 strains within a 578-bp variable region (50) of the gene was deter-

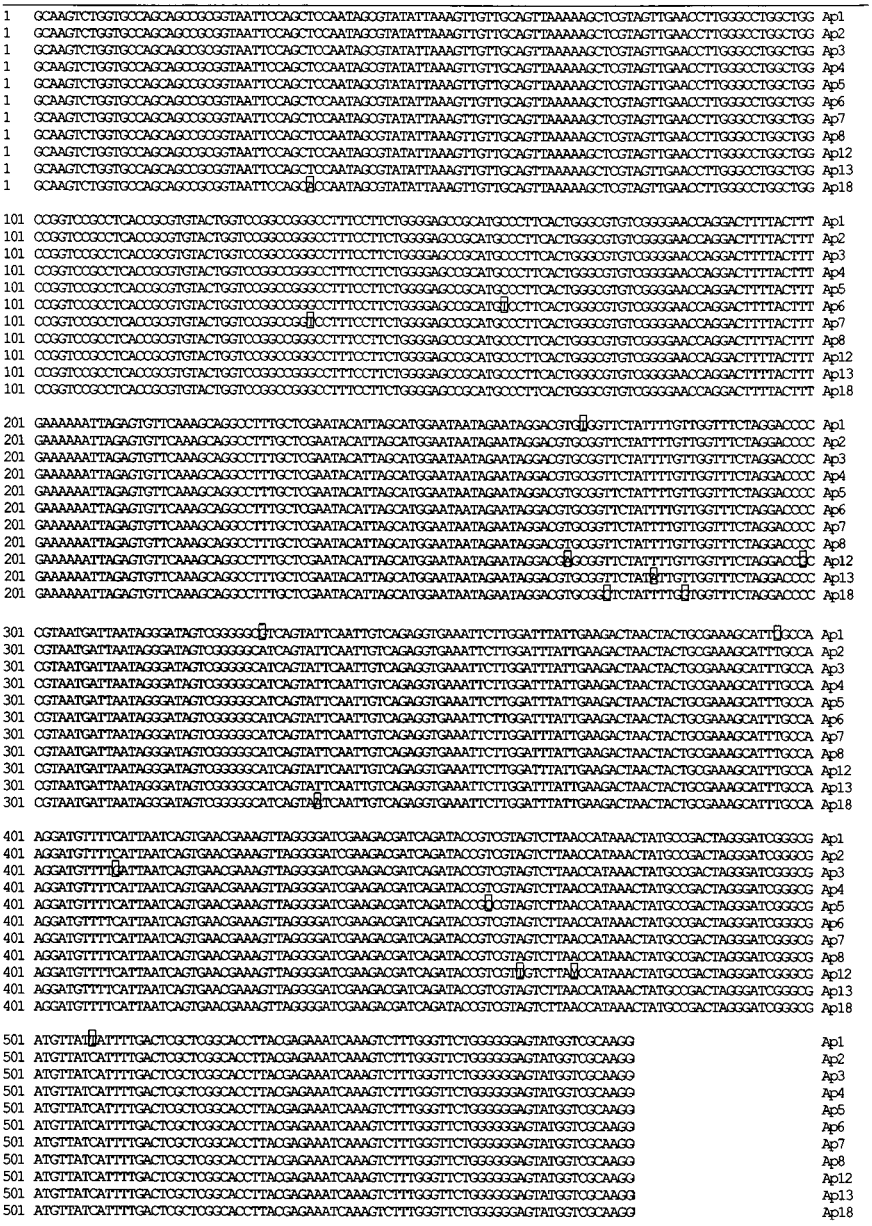


FIG. 2. Multiple alignment of partial 18S sequences of the 11 *A. pullulans* strains studied. The region shown was amplified with primers 282 (NS3) and 283 (Fig. 1) and corresponds to *A. pullulans* residues 550 to 1128 (GenBank accession number M55639). Residues differing from the consensus sequence are boxed. Strain designations are as specified in Table 1. Y, C or T.

mined (Fig. 1). Multiple alignment of sequences showed conservation among the geographically diverse isolates (Fig. 2). These initial results affirmed the feasibility of developing species-specific oligonucleotide probes based on the 18S rRNA gene.

To design specific probes, the *A. pullulans* 18S sequences were compared with fungal sequences accessible through GenBank or sequenced by us (organisms listed in Table 1 plus *Aspergillus fumigatus*, *Candida albicans*, and *Coccidioides immitis*). On this basis, several probes were synthesized and tested. The probe designated 665 was complementary to a region near the 5′ end of the 18S gene (Fig. 1) and differed by at least one nucleotide from all known fungal sequences (Fig. 3) as of 5 May 1995. Southern blot hybridizations involving previously characterized fungi (Fig. 4) and a dot blot with 82 phylloplane yeast isolates (Fig. 5) clearly demonstrate the specificity of probe 665. The complementary approach of testing probes against DNA extracted from the leaf surface community as a whole could not be performed because *A. pullulans* occurs on every leaf (5, 6). While additional fungi could have been tested individually for hybridization to the probe, we have shown a reasonable presumptive basis for operational specificity based on taxonomic, phylogenetic, and ecological criteria. This implies that the probe should be sufficiently specific at least to discriminate *A. pullulans* from other fungi in the leaf surface habitat.

Another probe, 614, differed by a single nucleotide from the *A. pullulans* and almost all known fungal sequences (Fig. 3). Under the conditions employed, this 17-mer was highly specific for another ubiquitous fungus, *C. herbarum* (Fig. 4). Aerosols

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Probe 665:   5'-TTCGTTTAGTTATTATGAATC-3'
Target:      3'-AAGCAAATCAATAATACTTAG-5'
Ap              AAGCAAATCAATAATACTTAG
Cg              AAGCTCTCCAATAATACTTAG
Af              AAGCAATTCAATAATACTAAG
Be              AAGCTATTCAATAATACTTAG
Cc              AAGCAATTCAATAATACTAAG
Lp              AAGCTCTTCAATAATACTTAG
Ou              AAGCTGTTCAATAGTACTTAG
Pn              AAGCAATTCAATAATACTAAG
Nc              AAGCTCTTCAATAATACTTAG
Pa              AAGCTCTCCAATAATACTTAG
Ci              AAGCTGTTCAATAATACTTAG
Ca              AAGCTTTTCAATAATACTTAG

Probe 614:   5'-TTAATCAAAACATCCTT-3'
Target:      3'-AATTAGTTTTGTAGGAA-5'
Ch              AATTAGTTTTGTAGGAA
Ap              AATTACTTTTGTAGGAA
Hd              AATTACTTTTGTAGGAA
Rr              AATTACTTTTGTAGGAA
Cl              ACTTACTTTTGCAGGAA
Aa              AATTACTTTTGTAGGAA
Cg              ATTTACTTTTGTAGGAA
Af              AATTACTTTTGTAGGAA
Be              AATTACTTTTGTAGGAA
Cc              AATTACTTTTGTAGGAA
Lp              AATTACTTTTGTAGGAA
Ou              AATTACTTTTGTAGGAA
Pc              AATTACTTTTGTAGGAA
Pn              AATTACTTTTGTAGGAA
Nc              AATTACTTTTGTAGGAA
Pa              AATTACTTTTGTAGGAA
Ca              AATTACTTTTGCAGGAA
Ci              AATTACTTTTGTAGGAA
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FIG. 3. 18S rRNA gene sequence alignments, showing probes and target regions. Af, *Aspergillus fumigatus*; Ca, *Candida albicans*; Ci, *Coccidioides immitis* (for other designations, see Table 1). Probe 665 was used for *A. pullulans*; probe 614 was used for *C. herbarum*. Other than the intended targets, database searches of all rRNA sequences (GenBank release no. 87) showed no sequences, as of 5 May 1995, identical to the two probes. Mismatched nucleotides are underlined.

generated during the harvesting of crops are composed mainly of *Cladosporium* spp. and other fungal saprophytes, many of which are allergenic and can cause a health hazard to farmers (17). *C. herbarum* is also a well-known antagonist of plant and human pathogens (22). Thus, probe 614 might be useful in studies of the ecology, medical microbiology, or aerobiology of this fungus.

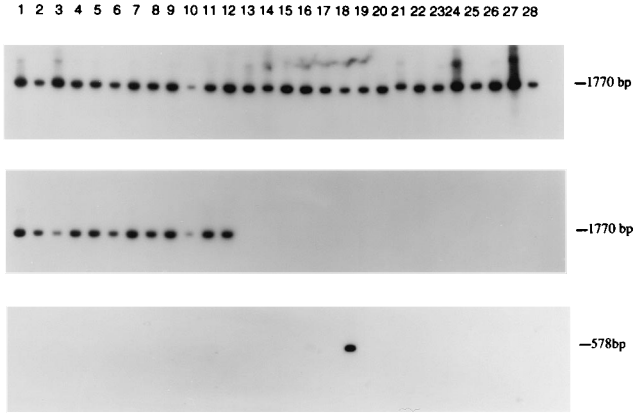

FIG. 4. Southern blot hybridization of PCR products (full length of 18S rRNA gene, ~1,770 bp) probed with the universal probe U519 (top panel), *A. pullulans*-specific probe 665 (middle), and a 578-bp PCR product probed with *C. herbarum*-specific probe 614 (bottom). Lanes 1 to 12, *A. pullulans* Ap2, Ap12, Ap5, Ap13, Ap4, Ap6, Ap8, Ap7, Ap3, Ap18, Ap19, and Ap1, respectively; lane 13, *H. dematioides*; lane 14, *R. rubra*; lane 15, *S. holsaticus*; lane 16, *C. laurentii*; lane 17, *A. alternata*; lane 18, *C. herbarum*; lane 19, *C. gloeosporioides*; lane 20, *A. nidulans*; lane 21, *B. emersonii*; lane 22, *C. cladosporioides*; lane 23, *L. persoonii*; lane 24, *O. ulmi*; lane 25, *P. chrysogenum*; lane 26, *P. notatum*; lane 27, *N. crassa*; lane 28, *P. anserina*. Sizes of PCR products are shown at the right.

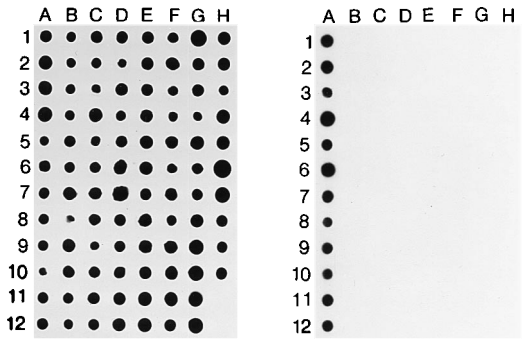

FIG. 5. Dot blot hybridization of full length of 18S rRNA PCR product (~1,770 bp) with the ^{32}P-labeled universal probe U519 (left panel) and *A. pullulans*-specific probe 665 (right panel) to *A. pullulans* strains and 82 yeast isolates from the phylloplane (L1 to L100). Column A, rows 1 to 12: Ap2, Ap12, Ap5, Ap13, Ap4, Ap6, Ap8, Ap7, Ap3, Ap18, Ap19, and Ap1, respectively. Column B, rows 1 to 12: L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L11, and L13, respectively. Column C, rows 1 to 12: L15, L16, L18, L19, L20, L21, L22, L24, L25, L26, L27, and L28, respectively. Column D, rows 1 to 12: L29, L30, L31, L32, L33, L34, L36, L37, L39, L40, L59, and L41, respectively. Column E, rows 1 to 12: L42, L43, L44, L45, L46, L47, L48, L49, L52, L53, L55, and L60, respectively. Column F, rows 1 to 12: L61, L63, L62, L54, L90, L67, L83, L93, L86, L96, L87, and L79, respectively. Column G, rows 1 to 12: L70, L89, L88, L72, L85, L69, L68, L98, L71, L74, L99, and L95, respectively. Column H, rows 1 to 10: L100, L76, L64, L66, L73, L78, L81, L65, L82, and L94, respectively.

These results support previous studies (2, 31) which demonstrate that a short oligonucleotide with even a single nucleotide mismatch to the target sequence can differentiate strains. Generally, the internal transcribed spacer between the small- and the large-subunit rRNA genes is thought to be less conserved and more diverse than the coding regions (14, 51). However, the internal transcribed spacer is spliced out during rRNA maturation, making it an undesirable target for FISH.

As noted at the outset, the long-term objective in developing this probe system is to monitor and quantify *A. pullulans* cells directly on leaves by in situ hybridization. Our preliminary results with FISH (37, 37a) confirm the probe results in this report. In brief, we have shown that the fluorescently labeled universal and 665 probes detect *A. pullulans* spores and hyphae on microslides or on apple leaves inoculated with the fungus under controlled conditions. All *A. pullulans* strains tested here also produce a positive hybridization signal with the probe in FISH; none of the heterologous fungi assessed here and found to be negative react with probe 665 in situ. Detailed assessments of hybridization conditions, signal-generating systems, and field performance are in progress.

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REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919–1925.
2. **Amann, R. I., L. Krumholz, and D. A. Stahl.** 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. **172:**762–770.
3. **Amann, R. I., J. Stromley, R. Devereux, R. Key, and D. A. Stahl.** 1992.

Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. Appl. Environ. Microbiol. **58:**614–623.
4. **Andrews, J. H., R. F. Harris, R. N. Spear, G. W. Lau, and E. V. Nordheim.** 1994. Morphogenesis and adhesion of *Aureobasidium pullulans*. Can. J. Microbiol. **40:**6–17.
5. **Andrews, J. H., and S. S. Hirano (ed.).** 1991. Microbial ecology of leaves. Springer-Verlag, New York.
6. **Andrews, J. H., and C. M. Kenerley.** 1978. The effects of a pesticide program on non-target epiphytic microbial populations of apple leaves. Can. J. Microbiol. **24:**1058–1072.
7. **Assmus, B., P. Hutzler, G. Kirchhof, R. Amann, J. R. Lawrence, and A. Hartmann.** 1995. In situ location of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl. Environ. Microbiol. **61:**1013–1019.
8. **Atlas, R. M., G. Sayler, R. S. Burlage, and A. K. Bej.** 1992. Molecular approaches for environmental monitoring of microorganisms. BioTechniques **12:**706–717.
9. **Aufauvre-Brown, A., C. M. Tang, and D. W. Holden.** 1993. Detection of gene-disruption events in *Aspergillus* transformants by polymerase chain reaction direct from conidiospores. Curr. Genet. **24:**177–178.
10. **Bashi, E., and N. J. Fokkema.** 1977. Environmental factors limiting growth of *Sporobolomyces roseus*, an antagonist of *Cochliobolus sativus*, on wheat leaves. Trans. Br. Mycol. Soc. **68:**17–25.
11. **Berbee, M. L., and J. W. Taylor.** 1992. 18S ribosomal RNA gene sequence characters place the human pathogen *Sporothrix schenckii* in the genus *Ophiostoma*. Exp. Mycol. **16:**87–91.
12. **Berbee, M. L., and J. W. Taylor.** 1992. Dating the evolutionary radiations of the true fungi. Can. J. Bot. **71:**1114–1127.
13. **Bertin, B., O. Broux, and M. Y. Hoegaerden.** 1990. Flow cytometric detection of yeast by *in situ* hybridization with a fluorescent ribosomal RNA probe. J. Microbiol. Methods **12:**1–12.
14. **Bourque, S. N., J. R. Valero, M. C. Lavoie, and R. C. Levesque.** 1995. Comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences of *Bacillus thuringiensis* strains and subspecies and closely related species. Appl. Environ. Microbiol. **61:**1623–1626.
15. **Bowman, B.** 1993. A model PCR/probe system for the identification of fungal pathogens, p. 423–430. *In* D. H. Persing, T. F. Smith, F. C. Tenover, and T. J. White (ed.), Diagnostic medical microbiology: principles and applications. American Society for Microbiology, Washington, D.C.
16. **Bruns, T. D., R. Vilgalys, S. M. Barns, D. Gonzalez, D. S. Hibbett, D. J. Lane, L. Simon, S. Stickel, T. M. Szaro, W. G. Weisburg, and M. L. Sogin.** 1992. Evolutionary relationships within the fungi: analyses of nuclear small subunit rRNA sequences. Mol. Phylogenet. Evol. **1:**231–241.
17. **Burge, H. A., M. L. Muilenberg, and J. A. Chapman.** 1991. Crop plants as a source of fungus spores of medical importance, p. 222–236. *In* J. H. Andrews and S. S. Hirano (ed.), Microbial ecology of leaves. Springer-Verlag, New York.
18. **Cooke, W. B.** 1959. An ecological life history of *Aureobasidium pullulans* (de Bary) Arnaud. Mycopathol. Mycol. Appl. **21:**225–271.
19. **De Hoog, G. S., and N. A. Yurlova.** 1994. Conidiogenesis, nutritional physiology and taxonomy of *Aureobasidium* and *Hormonema*. Antonie Leeuwenhoek J. Microbiol. Serol. **365:**341–395.
20. **DeLong, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243:**1360–1363.
21. **Dichristina, T. J., and E. F. DeLong.** 1993. Design and application of rRNA-targeted oligonucleotide probes for the dissimilatory iron- and manganese-reducing bacterium *Shewanella putrefaciens*. Appl. Environ. Microbiol. **59:** 51–56.
22. **Domsch, K. H., W. Gams, and T. Anderson.** 1980. Compendium of soil fungi. Academic Press, London.
23. **Donegan, K., C. Matyac, R. Seider, and A. Porteous.** 1991. Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. Appl. Environ. Microbiol. **57:**4152–4160.
24. **Drahos, D. J.** 1991. Methods for detection, identification, and enumeration of microbes, p. 135–157. *In* J. H. Andrews and S. S. Hirano (ed.), Microbial ecology of leaves. Springer-Verlag, New York.
25. **Fell, J. W.** 1995. rDNA targeted oligonucleotide primers for the identification of pathogenic yeasts in a polymerase chain reaction. J. Indust. Microbiol. **14:**475–477.
26. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
27. **Hermanides-Nijhof, E. J.** 1977. *Aureobasidium* and allied genera, p. 141–177. *In* G. S. De Hoog and E. J. Hermanides-Nijhof (ed.), The black yeasts and allied hyphomycetes. Elsevier, Amsterdam.
28. **Hicks, R., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4′,6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158–2163.
29. **Higgins, D. G., and P. M. Sharp.** 1989. Fast and sensitive multiple sequence alignments on a microcomputer. Comput. Appl. Biosci. **5:**151–153.
30. **Holben, W. E., and J. M. Tiedje.** 1988. Applications of nucleic acid hybridization in microbial ecology. Ecology **69:**561–568.
31. **Ikuta, S., K. Takagi, R. B. Wallace, and K. Itakura.** 1987. Dissociation kinetics of 19 base paired oligonucleotide-DNA duplexes containing different single mismatched base pairs. Nucleic Acids Res. **15:**797–811.
32. **Illingworth, C. A., J. H. Andrews, C. Bibeau, and M. L. Sogin.** 1991. Phylogenetic placement of *Athelia bombacina*, *Aureobasidium pullulans*, and *Colletotrichum gloeosporioides* inferred from sequence comparisons of small-subunit ribosomal RNAs. Exp. Mycol. **15:**65–75.
33. **Kreger-van Rij, N. J. W.** 1984. The yeasts, 3rd ed. Elsevier, Amsterdam.
34. **Lachke, A. H., and V. B. Rale.** 1995. Trends in microbial production and its novel applications in the food industry, p. 589–604. *In* Y. H. Hui and G. G. Khachatourians (ed.), Food biotechnology microorganisms. VCH, New York.
35. **Leitch, A. R., T. Schwarzacher, D. Jackson, and I. J. Leitch.** 1994. *In situ* hybridization: a practical guide. Bios Scientific Publishers, Oxford.
36. **Li, S., C. P. Harris, and S. A. Leong.** 1993. Comparison of fluorescence *in situ* hybridization and primed *in situ* labeling methods for detection of single-copy genes in the fungus *Ustilago maydis*. Exp. Mycol. **17:**301–308.
37. **Li, S., R. Spear, and J. H. Andrews.** 1994. Detection and quantification of fungal cells on leaves by *in situ* hybridization based on 18S rRNA-targeted fluorescent oligonucleotide probes, p. 125. *In* Abstracts of the Fifth International Mycological Congress. Mycological Society of America, Lawrence, Kans.

37a. **Li, S., et al.** Unpublished data.

38. **McSweeney, C. S., R. I. Mackie, A. A. Odenyo, and D. A. Stahl.** 1993. Development of an oligonucleotide probe targeting 16S rRNA and its application for detection and quantification of the ruminal bacterium *Synergistes jonesii* in a mixed-population chemostat. Appl. Environ. Microbiol. **59:**1607–1612.
39. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138:**267–284.
40. **Odenyo, A. A., R. I. Mackie, D. A. Stahl, and B. A. White.** 1994. The use of 16S rRNA-targeted oligonucleotide probes to study competition between ruminal fibrolytic bacteria: development of probes for *Ruminococcus* species and evidence for bacteriocin production. Appl. Environ. Microbiol. **60:**3688–3696.
41. **Pace, N. R., D. A. Stahl, D. J. Lane, and O. J. Olsen.** 1985. Analyzing natural microbial populations by rRNA sequences. ASM News **51:**4–12.
42. **Parkinson, D., and D. C. Coleman.** 1991. Microbial communities: activity and biomass. Agric. Ecosys. Environ. **34:**3–33.
43. **Reysenbach, A.-L., L. J. Giver, G. S. Wickham, and N. R. Pace.** 1992. Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. **58:**3417–3418.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
45. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Annu. Rev. Microbiol. **44:**625–648.
46. **Smith, C. S., T. Chand, R. F. Harris, and J. H. Andrews.** 1989. Colonization of a submersed aquatic plant, Eurasian water milfoil (*Myriophyllum spicatum*), by fungi under controlled conditions. Appl. Environ. Microbiol. **55:** 2326–2332.
47. **Steven, B., S. Lee, and J. W. Taylor.** 1990. Isolation of DNA from fungal mycelia and single spores, p. 282–287. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
48. **Török, T., and A. D. King, Jr.** 1991. Comparative study on the identification of food-borne yeasts. Appl. Environ. Microbiol. **57:**1207–1212.
49. **Wallace, B. R., and C. G. Miyada.** 1987. Oligonucleotide probes for the screening of recombinant DNA libraries. Methods Enzymol. **152:**432–442.
50. **White, T. J., T. Bruns, S. Lee, and J. Taylor.** 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols: a guide to methods and applications. Academic Press, San Diego, Calif.
51. **Yan, Z. H., S. O. Rogers, and C. J. K. Wang.** 1995. Assessment of *Phialophora* species based on ribosomal DNA internal transcribed spacers and morphology. Mycologia **87:**72–83.