Journal of Industrial Microbiology, (1995) 14, 475–477
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rDNA targeted oligonucleotide primers for the identification of pathogenic yeasts in a polymerase chain reaction

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(Received 15 June 1994; accepted 7 October 1994)

Key words: Cryptococcus neoformans; Rhodotorula mucilaginosa; Trichosporon cutaneum; Basidiomycetes; Molecular taxonomy; Polymerase chain reaction

SUMMARY

Species-specific oligonucleotide primers were designed for PCR identification of the basidiomycetous yeasts *Cryptococcus neoformans, Trichosporon cutaneum* and *Rhodotorula mucilaginosa*. The procedure uses standard PCR components including DNA from the test species and three primers: two universal external (upstream and downstream) limiting primers and a species-specific internal primer. Species identification requires the formation of a species-specific rDNA nucleotide segment that is significantly smaller (~200 bp) than a non-target segment (~600 bp). The procedure can be used to identify yeasts from single and mixed populations.

INTRODUCTION

Identification of yeasts from pure or mixed culture is a time-consuming effort initiating with purification followed by a variety of morphological, biochemical and physiological tests. To reduce time and expense, a PCR-based method was employed to identify marine associated yeasts [1]. In the present communication, this procedure is expanded for the identification of pathogenic yeasts in both pure and mixed culture. The technique employs three primers in a PCR amplification of a ~600-bp segment of the large subunit of rDNA (LrDNA): two external universal primers and one internal species-specific primer. In the presence of target DNA the smaller species-specific segment is amplified; in the absence of target DNA, and the presence of non-target DNA, the larger 600-bp segment is produced.

Species-specific primers were developed for three basidiomycetous pathogens: *Cryptococcus neoformans, Rhodotorula mucilaginosa* (synonym *Rhodotorula rubra*) and *Trichosporon cutaneum* (synonym *Trichosporon beigelii*). *C. neoformans* causes infections of the brain and meninges, particularly in association with immunodeficient syndrome. *Rhodotorula mucilaginosa*, albeit a rare infectious agent, produces fungemia and other infections in association with therapy for cancer and other debilitating diseases. *Trichosporon cutaneum* causes deep or disseminated trichosporonosis as a secondary complication associated with leukemia and immunosuppressed patients [7]. We tested the primers from these pathogens against other ascomycetous and basidiomycetous yeasts including the medically important species *Candida albicans, C. tropicalis* and *C. parapsilosis*.

MATERIALS AND METHODS

Organisms

Strains and their sources are *Candida albicans* type strain (T) CBS 562 (Centraalbureau voor Schimmelcultures, Delft, Netherlands), *C. parapsilosis* (T) CBS 604, *C. tropicalis* (T) CBS 94, *Cryptococcus gastricus* (T) CBS 1927, *C. laurentii* (T) CBS 139, *C. neoformans* (4 strains) CBS 882 mating type (MT) δ, NRRL Y-2534 (ARS Culture Collection, USDA, Peoria, IL, USA) (T) MT δ serotype D, ML (RSMAS) 2329 serotype A and ML 2328 serotype C from D.G. Ahearn, *R. mucilaginosa* CBS 17, *Sporobolomyces salmonicolor* MT A1 Bandoni 949, *Trichosporon cutaneum* (T) NRRL Y-1490.

Growth conditions

Strains were grown either in liquid or on solid media. Liquid culture: cells were inoculated into tubes of 5 ml of 2% glucose, 0.5% yeast extract, and 1.0% peptone and placed on a roller drum for overnight growth. One milliliter of the cell suspension was transferred to a 1.5-ml microfuge tube, centrifuged and the supernatant fluid was discarded. Solid culture: cells were streaked on potato dextrose agar (Difco, Detroit, MI, USA); the resulting colonies from an overnight- to 2-day growth were transferred to a 1.5-ml microfuge tube. The quantity of cells is not critical; generally, 0.1 ml or less of packed wet cells was used. Cells harvested from the liquid or solid media were freeze-dried.

This paper is dedicated to Professor Herman Jan Phaff in honor of his 50 years of active research which still continues.

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DNA extraction

DNA from the freeze-dried cells was obtained by phenol/chloroform extraction, treated with RNAase and precipitated with isopropanol [9].

Primer selection

Species-specific primers were developed for *C. neoformans, R. mucilaginosa* and *T. cutaneum*. Sequences of the V3 region [5] of the large subunit rRNA for these three species were obtained from the literature [2,3,4] and aligned with nucleotide sequences of other basidiomycetous yeasts [2]. Primers were designed from visual examination of the alignment data. The number and location of alignment differences were determined with MacVector 4.1 (International Biotechnologies Inc., New Haven, CT, USA). Sequences of the test ascomycetes are unknown. The primer selected for *C. neoformans* was 5′ AGT TCT GAT CGG TGG ATA AGG GCT 3′, which starts at nucleotide position 135 of Gueho et al. [4]. The *T. cutaneum* primer is 5′ AGT CGT GTT CTT CAG ATT CAG CTG 3′ initiating at position 72 [3]. *R. mucilaginosa* primer sequence is 5′ TCA GAC TTG CTT GCC GAG CAA TCG 3′ [1]. All three species-specific primers are forward primers. The universal forward primer was 5′ GCA TAT CAA TAA GCG GAG GAA AAG 3′, the universal reverse primer was 5′ GGT CCG TGT TTC AAG ACG G 3′.

PCR

Master mix reactions were prepared in 96-μl (or multiple) solutions that contained 50 μM deoxynucleotide triphosphates, 10 pM of each of the three primers, IBI buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween-20, 0.01% gelatin w/v and 0.01% NP-40) and 4 units of IBI thermalase. The mix was divided into 24-μl reactions with an addition of 1 μl of DNA. The concentration of DNA was not standardized. All reactions depicted in this report used the IBI thermalase. Subsequent to the submission of this manuscript we learned that IBI (now a division of Eastman Kodak Co.) had discontinued the production of thermalase. Therefore, we tested T*th* DNA polymerase from Pharmacia Biotech (Piscataway, NJ, USA) and their reaction buffer (10 × concentration contains 500 mM NaCl, 15 mM $MgCl_2$ and 100 mM Tris-HCl, pH 9.0). The results were equivalent to those using thermalase. Both thermalase and T*th* DNA polymerases are derived from *Thermus thermophilus*. We *did not* obtain the desired results with Taq DNA polymerase, which is isolated from *Thermus aquaticus*.

Amplifications were performed in either an MJ Research (Watertown, ME, USA) mini-cycler or an MJ Research PTC 100 using the following program: 94 °C for 2 min followed by 31 cycles consisting of 94 °C for 30 s, 66 °C for 90 s and 72 °C for 15 s with a final extension of 72 °C for 4 min. The PCR products were visualized with 1.0% agarose TPE (0.09 M Tris-phosphate, 0.002 M EDTA) gels stained with ethidium bromide.

Species specificity tests

The species-specific oligonucleotide primers, in concert with the two universal primers, were tested against the individual strains listed in Figs 1, 2 and 3; for example, the *C. neoformans* primer was tested against DNA from *C. albicans, C. tropicalis*, etc. (Fig. 1).

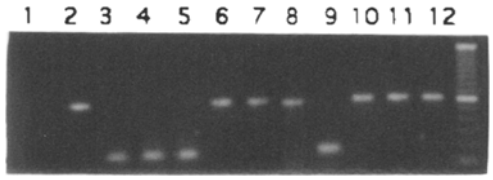

Fig. 1. Agarose gel electrophoresis of PCR products resulting from PCR amplification with three primers: two rDNA universal delimiting primers and an internal primer specific to *Cryptococcus neoformans*. Lane 1, *Trichosporon cutaneum* (product missing); lane 2, *Sporobolomyces salmonicolor*; lanes 3, 4 & 5, *C. neoformans* (ML 2328, ML 2329 and NRRL Y-2534); lane 6, *Rhodotorula mucilaginosa*; lane 7, *Cryptococcus laurentii*; lane 8, *Cryptococcus gastricus*; lane 9, *C. neoformans* (CBS 882); lane 10, *Candida tropicalis*; lane 11, *Candida parapsilosis*; lane 12, *Candida albicans*. bp = base pair.

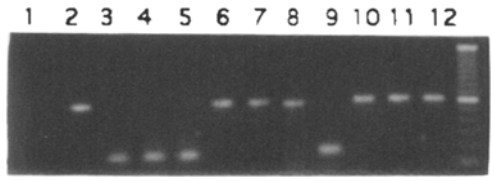

Fig. 2. Agarose gel electrophoresis of PCR products as per Fig. 1 with the exception that the internal limiting primer is specific to *Rhodotorula mucilaginosa*. Strains are in the same lanes as Fig. 1.

Mixed DNA tests

The initial isolation of yeasts from clinical and other materials often results in mixed populations that require a purification step. Therefore we tested the PCR identification method by contaminating target DNA with DNA from other pathogenic and non-pathogenic species. In these tests standard PCR reactions were designed to include 1-μl amounts of target and non-target DNA (absolute quantity not determined). For example (Fig. 4), *C. neoformans* DNA and the corresponding primer were included in each test with DNA from *C. albicans* or *C. tropicalis*, etc. Three series were conducted: *C. neoformans, R. mucilaginosa* and *T. cutaneum* against the species listed in Fig. 4.

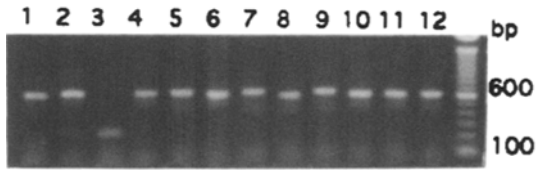

Fig. 3. Agarose gel electrophoresis of PCR products as per Fig. 1 with the exception that the internal limiting primer was specific to *Trichosporon cutaneum*. DNA from the strains is in the following lanes: Lane 1, *Sporobolomyces salmonicolor*; lane 2, *Cryptococcus neoformans* (ML 2328); lane 3, *T. cutaneum*; lanes 4 & 5, *C. neoformans* (ML 2329 and NRRL Y-2534); lane 6, *Rhodotorula mucilaginosa*; lane 7, *Cryptococcus laurentii*; lane 8, *Cryptococcus gastricus*; lane 9, *C. neoformans* (CBS 882); lane 10, *Candida tropicalis*; lane 11, *Candida parapsilosis*; lane 12, *Candida albicans*.

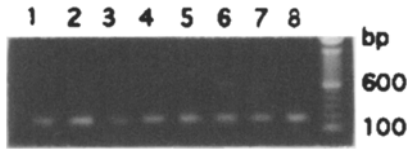

Fig. 4. Agarose gel electrophoresis of PCR products of *Cryptococcus neoformans* DNA contaminated with DNA of the following species: Lane 1, *Trichosporon cutaneum*; lane 2, *C. neoformans* (control DNA, ML 2328); lane 3, *Rhodotorula mucilaginosa* (weak band); lane 4, *Cryptococcus laurentii*; lane 5, *Cryptococcus gastricus*; lane 6, *Candida tropicalis*; lane 7, *Candida parapsilosis*; lane 8, *Candida albicans*.

RESULTS AND DISCUSSION

Tests of the species-specific primers of *C. neoformans* (Fig. 1), *R. mucilaginosa* (Fig. 2) and *T. cutaneum* (Fig. 3) produced the same general results: in the presence of target DNA the species-specific primer amplified a ~200-bp nucleotide segment that is bounded by the reverse universal primer and the species-specific forward primer. In the presence of non-target DNA, the 600-bp segment amplified as a result of priming by the universal primers. In the case of *T. cutaneum*, however, (Figs 1 and 2) the annealing temperature was found to be critical. The *T. cutaneum* 600-bp segment did not amplify at the 66 °C annealing temperature used in these experiments, although the species-specific 200-bp segment was produced (Fig. 3) at 66 °C. In separate experiments, 64 °C was found to be an appropriate temperature for amplification of the *T. cutaneum* 600-bp segment. The development of new primers may require the determination of optimal annealing temperatures for each individual species.

When DNA from more than one species was present, the target species-specific 200-bp segment was amplified as depicted for *C. neoformans* (Fig. 4). Results from *R. mucilaginosa* and *T. cutaneum* mixed DNA tests were equivalent to the *C. neoformans* results and therefore they are not reproduced here. Amplification of a specific DNA segment in mixed populations is not a novel observation; it is emphasized here, however, in the context of isolating yeasts from contaminated cultures. Strain purification does not need to be rigidly followed, as it does with standard identification procedures. DNA concentrations of the individual species within the mixed populations can be widely divergent. Although maximal limits were not determined, 15-fold differences in non-target to target DNA (16 ng target DNA with 254 ng non-target DNA) resulted in species-specific amplification.

This PCR technique has advantages and disadvantages. The latter is the specific nature of the reaction viz. the demonstration of the presence or absence of a specific species would be cumbersome in the identification of a large collection of unknown species, although generic and species group specific primers can be designed [1]. An advantage of the PCR method of species identification is the ease of the procedure. The single PCR reaction, followed by analysis with agarose gel electrophoresis, does not require either radioactive reagents or blot tests. Another advantage is the low cost, the most expensive component of the reaction is DNA polymerase, which lists at $0.55 or less per unit. An additional attribute of the PCR technique is that specific oligonucleotide primers can be inexpensively prepared for the identification of any known species. This does depend, however, upon the availability of a nucleotide sequence data bank. Currently the largest accumulated data are partial rDNA (or rRNA) sequences for the basidiomycetous yeasts [2,3,4,8] and the sporogenous ascomycetous yeasts [6]. We have not located comparable data for the anamorphic ascomycetous yeasts, particularly members of the genus *Candida*. Sequence data banks are presently limited in numbers of species, although they are expanding with the current emphasis on molecular phylogeny. As studies focus on species boundary problems, PCR primer and probe techniques will become available for the rapid identification of any yeast or fungal species.

ACKNOWLEDGEMENTS

This research was funded by the National Science Foundation grant OCE 9116258. I extend my appreciation to Jenilee Kline, M.J. Research, Watertown, ME, USA for the loan of a programmable thermal mini-cycler.

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