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MOLECULAR IDENTIFICATION OF *Candida Tropicalis* RETL-Cr1 BY PCR AMPLIFICATION OF RIBOSOMAL DNA

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ABSTRACT. *PCR amplification of ribosomal DNA was used to identify a phenol-degrading yeast strain. The internal transcribed spacer regions of the yeast was amplified using universal primers ITS1 and ITS4. Based on a BLASTN search of genBank, the complete sequences of ITS1-5.8S rDNA-ITS2 regions and portions of I8S and 28S for the purified DNA products of RETL-Cr1 shared 98% similarity with Candida tropicalis. The nucleotide sequences of the C. tropicalis RETL-Cr1 was submitted to the GenBank database under the accession number AY725426.*

KEY WORDS. *Candida tropicalis*, PCR-AGE, phenol-degrading yeast

INTRODUCTION

Rapid and reliable identification and classification of microorganisms are important in environmental and industrial microbiology. Microorganisms such as bacteria and yeast are sources of antibiotics, enzymes, and other bioactive compounds for medicine and biotechnology (Short, 1997; Oh and Kim, 1998; Picataggio *et al.*, 1991).

There are different molecular methods that are currently being used for microbial identification and classification. These permit a certain level of phylogenetic classification, from the genus, species, subspecies, biovar to the strain specific level (Louws *et al.*, 1996; Rademaker and de Bruijin, 2003). Each method has its advantages and disadvantages, with regards to ease of application, reproducibility, requirement for equipment and level of resolution (Akkermans *et al.*, 1995).

Traditionally, identification and characterization of yeast species and strains were based on morphological traits especially on their physiological abilities (Kreger-Van Rij,1984; Barnett *et al.*, 1990). These characteristics are strongly influenced by the culture condition but are however unreliable (Yamamoto *et al.*, 1991). Furthermore, in order to identify most yeasts up to the species level reliably, it is necessary to conduct approximately 50-100 tests (Lin and Fung, 1987). In contrast, molecular biology techniques provide a rapid method. The polymerase chain reaction (PCR) has become an important method for amplification of ribosomal DNA from microorganisms

isolated from the environment. PCR amplification of specific sequences for the identification of organisms has become common because of the relative ease of manipulation and high reproducibility (Guillamón *et al.*, 1998). Some examples of PCR-based methods for the rapid detection and identification of *Candida* species are Restriction Fragment Length Polymorphisms (RLFP) (Fujita and Hashimoto, 2000), PCR with species-specific probes (Shin *et al.*,1997, 1999), random amplification of polymorphic DNA analysis (RAPD) (Stefan *et al.*, 1997), and Multiplex PCR using internal transcribed spacer (ITS) 1 and 2 regions (Fujita *et al.*, 2001).

In the present study, we identify *Candida tropicalis* RETL-Cr1 by PCR amplification of ribosomal-DNA using ITS1 and ITS4 as forward and reverse primers respectively. ITS1 and ITS4 are universal fungal-specific primers (White *et al.*, 1990; Park *et al.*, 2000; Fujita *et al.*, 2001).

MATERIALS AND METHODS

Source of organism

The yeast strain *C.tropicalis* RETL-Cr1 was used in this work (Figure 1). This locally isolated yeast strain is capable of growing on phenol as sole carbon source (Piakong *et al.*, 2002, 2003).

Figure 1. *C. tropicalis* RETL-Cr1 – Gram Stain -Leica microscope S6D-PC1016-S40 X1000

DNA Extraction

The yeast *C. tropicalis* RETL-Cr1 was maintained on nutrient agar (NA). Yeast lysate was prepared from a 1.5 ml of 24-h culture in nutrient broth was incubated at 37°C with shaking at 200rpm. Yeast cells were pelleted by centrifugation at 12,000 rpm for 2 min and resuspended in

The extract was then centrifuged at 12,000 rpm for 2 min and resuspended in 300µL of nucleic lysis solution provided in the Wizard Genomic DNA purification Kit (Promega Corp., Madison, Wis.) and purification was performed accordingly.

PCR Procedure

PCR amplification was done using the primer pairs of ITS1 (5'-TCCGTAGGTGAACCTGCG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as described by White *et al.*, (1990). ITS1 and ITS4 primers were designed from a conserved motif regions of 18S and 28S ribosomal DNA. The ITS1 - ITS4 primer pair was used to amplify the intervening 5.8S rDNA and the adjacent ITS1 and ITS2 regions (Fujita *et al.*, 2001) (Figure 2).

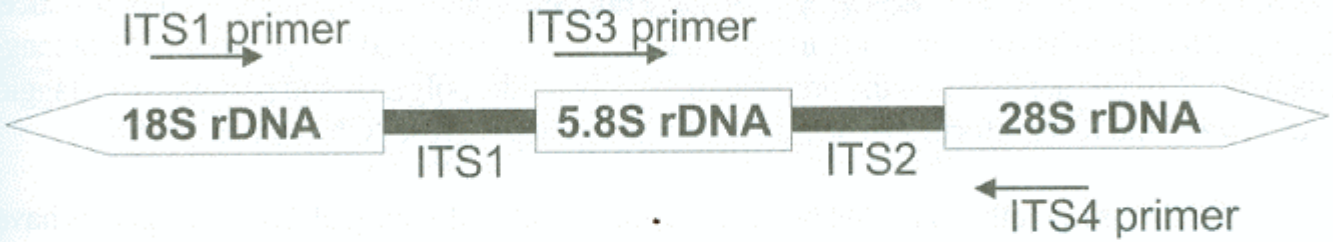

Figure 2. Schematic representation of the fungal ribosomal genes containing the primer target areas used in this study (Fujita *et al.*, 2001).

PCR amplification was performed according to the method of Fujita *et al.* (2001). Four microlitres of sample was added to the PCR master mix, which consist of 10 µL of 10X PCR buffer, 8 µL of a deoxynucleoside triphosphate mixture (0.1 mM each dNTP), 1.6 µL of each primer (40 pmol of each primer (ITS1, ITS4), and 0.8 µL (2.0 U) of *Taq* DNA polymerase with the remaining volume consisting of distilled water. Amplification was performed in a GeneAmp PCR system 9700 thermal cycle (Perkin-Elmer Corp., Emeryville, Calif.), under the following PCR condition: an initial denaturation temperature of 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and followed by a 4-min final extension at 72°C.

Electrophoresis

PCR products were electrophoresed through a 1.5% agarose in 1X TBE buffer as the running buffer. The gel was electrophoresed at 4.8 V/cm for 2 hours. A 100-bp DNA ladder (Promega Corp., Madison, Wis.) was used as marker to estimate the size of DNA bands. The gels was stained with ethidium bromide-TBE solution for 20 min and then photographed using UV Transilluminator with short length of UV.

Sequencing and analysis

Duplicates of purified amplified products of RETL-Cr1 were sent for sequencing to First Base. The sequences obtained were aligned using Clustal W, version 1.82 (Thompson *et al.*, 1994). The two sets of nucleotide sequences obtained were checked against related sequences derived from the GenBank database via the program BLASTN (Altschul *et al.*, 1990).

RESULTS AND DISCUSSION

A fast and accurate identification of yeast is becoming increasingly important for medical and environmental application. Most *Candida* spp. are pathogenic to humans (Ahearn, 1978; Odds, 1987). But on the other hand, they also have important industrial applications. For instance, *C. tropicalis* which has the capability to assimilate *n*-alkanes, was used for the production of long-chain dicarboxylic acids during the preparation of polyamide, polyester and perfume (Picataggio *et al.*, 1991) and to transform xylose into xylitol to replace sucrose (Oh and Kim, 1998).

PCR-based methods for the rapid detection and identification of *Candida* species have been described by (Morace *et al.*, 1997; Shin *et al.*, 1997, 1999; Stefan *et al.*, 1997; Jackson *et al.*, 1999; Fujita and Hashimoto, 2000; Fujita *et al.*, 2001). The multiplex PCR using internal transcribed spacer (ITS) 1 and 2 regions is sensitive, rapid and specific for yeast organisms (Fujita *et al.*, 2001).

Amplification of all fungi tested using ITS1 and ITS4 primers yielded fragments 350 to 880 bp long (Fujita *et al.*, 2001). The size of the PCR product of *C. tropicalis* RETL-Cr1 obtained in this study was approximately 450 bp (Figure 3), which was slightly smaller than those reported by Fujita *et al.*, (2001) and Guillamón *et al.*, (1998). They reported PCR product of more than 500bp.

The complete sequences of ITS1-5.8S rDNA-ITS2 regions and portions of I8S and 28S for the purified DNA products of RETL-Cr1 (Figure 4) shared 98% similarity with *Candida tropicalis* (score= 404 bits, E value= e-110). Therefore our strain was redesignated *Candida tropicalis* RETL-Cr1. The matched 18S, 5.8S and 28S ribosomal RNA sequences of our phenol-degrading yeast strain have been deposited to the GenBank database under the accession number AY725426.

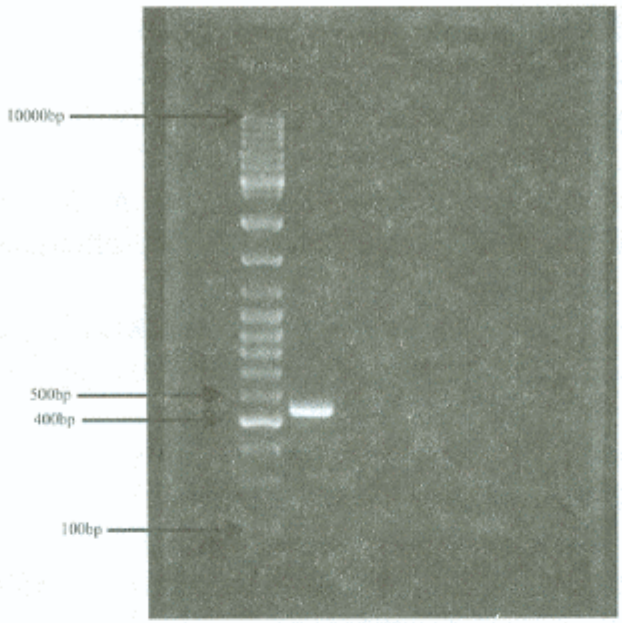

Figure 3. The amplified DNA from *C. tropicalis* RETL-Cr1 ribosomal gene generated using TS1 and TS4 primers is shown in Lane 2. Lane 1 molecular weight size reference marker (100-bp ladder).

1 GAGTCTTCGTCTCAAGGACATTGATTCCATGGGT
35 CTTTTTTTAGTACTGTTACTTTGGCGGCAGGAGTAAATATCTTACCGCCAGAGGTCTTTA
95 TAACACTCAATTTAATTTTATTTATTATTCAAAGACGATTATATTTTATAAATAGTCA*AA*
155 *ACTTGTCAACAACGGATCTCTTGGTTCTCGCATCNATGAAGAACGCAGCGAAATGCGAT*
214 *ACGTAATATGAATTGCAGATATTCGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCT*
274 *TTGGTATTCCAAAGGGCATGCCTGTTTGAGCGTCNTT*TCTCCCTCAAACCCCTGGGTTTG
334 GTGTTGAGCAATACGCTTAGGTTTGTTTGAAATATTTCCAATTGTGGACAACTATTTATG
394 TTATAGCGACTTAGGTTTATCCAAAACGCTACAACCATAAAGGAAGTCCACTGAATAAT
453 TTCATAACTTTTGACCTCAAATCAGGTAC

Figure 4. Complete sequence of the 5.8S rDNA (*Italics*) flanked by adjacent ITS1 and ITS2 regions of *C. tropicalis* RETL-Cr1.

CONCLUSION

The PCR amplification of the ribosomal DNA targeting the conserved regions of 5.8S, 18S and 28S using universal primers ITS1 and ITS4 revealed that the phenol-degrading yeast had 98% similarity with *Candida tropicalis*.

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