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Original article

cDNA representational difference analysis used in the identification of genes expressed by *Trichophyton rubrum* during contact with keratin

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Abstract

Dermatophytes are adapted to infect skin, hair and nails by their ability to utilize keratin as a nutrient source. *Trichophyton rubrum* is an anthropophilic fungus, causing up to 90% of chronic cases of dermatophytosis. The understanding of the complex interactions between the fungus and its host should include the identification of genes expressed during infection. To identify the genes involved in the infection process, representational difference analysis (RDA) was applied to two cDNA populations from *T. rubrum*, one transcribed from the RNA of fungus cultured in the presence of keratin and the other from RNA generated during fungal growth in minimal medium. The analysis identified differentially expressed transcripts. Genes related to signal transduction, membrane protein, oxidative stress response, and some putative virulence factors were up-regulated during the contact of the fungus with keratin. The expression patterns of these genes were also verified by real-time PCR, in conidia of *T. rubrum* infecting primarily cultured human keratinocytes *in vitro*, revealing their potential role in the infective process. A better understanding of this interaction will contribute significantly to our knowledge of the process of dermatophyte infection.

Keywords: *Trichophyton rubrum*; Representational difference analysis; Infection; Dermathophytoses

1. Introduction

Dermatophytoses are among the few fungal diseases that are directly communicable from person to person. Dermatophytes infect mainly healthy individuals, causing infections of keratinized structures, including the skin, hair, and nails [1]. Dermatophytes are not part of the normal human microbial flora. They are, however, particularly well adapted to infecting these tissues because, unlike most other microbial pathogens, they can use keratin as a source of nutrients [2]. *Trichophyton rubrum* is the most frequently isolated agent of dermatophytosis worldwide, accounting for approximately 80% of reported cases of onychomycosis [3]. Since 90% of the chronic dermatophyte infections are caused mainly by *T. rubrum*, this pathogen must have evolved mechanisms that evade or suppress cell-mediated immunity [4].

Despite its prevalence, little is known about the molecular basis of dermatophyte pathogenesis. Studies regarding the structure, expression, and regulation of the genes of *T. rubrum* have been relatively limited because of its unaggressive and non-life-threatening nature. In host–pathogen interactions, the gene expression of the pathogen is modulated by signals from the host, and knowing the pattern of expression may

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provide insights into the disease mechanisms [5]. Few potential *T. rubrum* virulence factors have been examined in detail, and most of them are keratinolytic proteases.

The understanding of the complex interactions between fungus and host must include the identification of genes expressed during infection. An efficient approach to the identification of differentially expressed genes in *T. rubrum* involves rapid series of subtractive hybridizations of cDNA prepared from two cell populations. Representational difference analysis (RDA) is a powerful and sensitive tool for the identification of differentially expressed genes and enables the isolation of both up- and down-regulated genes expressed in two different cDNA populations [6]. Recently, this strategy was applied to the differentially expressed genes of the human pathogenic fungus *Paracoccidioides brasiliensis* during the host interaction, revealing a set of candidate genes that *P. brasiliensis* may express to adapt to the host conditions [7].

The aim of the present study was to identify genes differentially expressed in *T. rubrum*, cultured in the presence and absence of keratin to simulate the host infection. The role of these genes was corroborated by confirming their induction during the infective process in a primary keratinocyte cell culture. Our studies provide the first view of the *T. rubrum* transcriptional response to host–pathogen interaction.

2. Materials and methods

2.1. Strain and culture conditions

T. rubrum isolate ATCC 52021 (American Type Culture Collection) was cultured for 10 days at 25–28 °C in Sabouraud's liquid medium and transferred to two different culture media: (i) a culture referred to as "tester" in liquid Cove's medium [8] supplemented with keratin (Sigma) 100 μg/mL, and (ii) a culture named "driver" in Cove's minimal medium, both cultivated for 24 h at room temperature. As a control, a reverse experiment was conducted in which the driver RNA was extracted from keratin culture and the tester RNA from minimal medium.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from *T. rubrum* cultured under each experimental condition by using the Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). First strand cDNA synthesis was performed with reverse transcriptase (RT Superscript III, Invitrogen, Life Technologies) using 1 μg of total RNA. The first strand of cDNA was used as template to synthesize the second strand, by using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA, USA).

2.3. Subtractive hybridization and generation of subtracted libraries

The cDNA fragments were digested with *Sau*3AI restriction enzyme (Promega, Madison, USA). A subtracted cDNA library was constructed using driver cDNA synthesized from RNA of *T. rubrum* cultured in minimal medium and tester cDNA from RNA extracted from fungus cultured in the presence of keratin. The resulting products were purified using a GFX kit (GE Healthcare, Chalfont St. Giles, UK). The digested tester cDNA was ligated to adapters (a 24-mer annealed to a 12-mer). To generate the differential products, tester and driver cDNAs were mixed, hybridized at 67 °C for 18 h and amplified by PCR with the 24-mer oligonucleotide primer. Two successive rounds of subtraction and PCR amplification were performed with hybridization tester–driver ratios of 1:10 and 1:100, respectively. Adapters were changed between these cross-hybridizations, and different products were purified using the GFX kit [9,10].

After the second subtractive reaction, the finally amplified cDNA pools were cloned directly into the pGEM-T Easy vector (Promega, Madison, USA). *Escherichia coli* XL1 Blue competent cells were transformed with the ligation products. Selected colonies were picked and grown in deep-well plates. Plasmid DNA was prepared from clones using standard protocols. In order to generate the EST (expressed sequence tags) sequences, single-pass, 5′-end sequencing of cDNAs by standard fluorescence labeling dye-terminator protocols with T7 flanking vector primer was performed. Samples were loaded onto a MegaBACE 1000 DNA sequencer (GE Healthcare) for automated sequence analysis.

2.4. EST processing pipeline and differential expression analysis

EST sequences were pre-processed using the Phred and Crossmatch (http://www.genome.washington.edu/UWGC/analysistools/Swat.cfm) programs. Sequences with at least 100 nucleotides and Phred quality greater than or equal to 20 were considered for further analysis. ESTs were screened for vector sequences against the UniVec data, and assembled with the CAP3 program [11]. The filtered sequences were compared against the GenBank (http://www.ncbi.nlm.nih.gov) non-redundant (nr) database from the National Center for Biotechnology Information (NCBI) using the BLASTX program [12], Cluster of Orthologous Groups (COG) and Gene Ontology (GO). MIPS (http://mips.gsf.de/) and InterPro databases of protein families, domains and functional sites were used to assign functional categories.

2.5. Assay of T. rubrum–keratinocytes interaction

Cultures of keratinocytes were isolated from human breast skin obtained from routine plastic surgery, processed and kindly supplied by the Tissue Bank of the Plastic Surgery Department of the College of Medicine at the University of São Paulo (USP), São Paulo, Brazil. The cells were maintained in DMEM–F12 (Dulbecco's modified Eagle's medium) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Cult lab, Brazil), incubated at 37 °C with 5% CO_2.

For adherence assays, cells were seeded (in the absence of feeder fibroblasts, antibiotics, antimycotics and fetal calf serum) into six-well plates at a density of 1.0×10^6 cells/well and grown to confluence in DMEM–F12 medium.