RT-PCR detection of Candida albicans ALS gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms

Clayton B. Green,1 Georgina Cheng,1 Jyotsna Chandra,2 Pranab Mukherjee,2 Mahmoud A. Ghannoum2 and Lois L. Hoyer1

1Department of Veterinary Pathobiology, University of Illinois at Urbana-Champaign, Urbana, IL 61802, USA
2Center for Medical Mycology, Department of Dermatology, University Hospitals of Cleveland and Case Western Reserve University, Cleveland, OH 44106, USA

An RT-PCR assay was developed to analyse expression patterns of genes in the Candida albicans ALS (agglutinin-like sequence) family. Inoculation of a reconstituted human buccal epithelium (RHE) model of mucocutaneous candidiasis with strain SC5314 showed destruction of the epithelial layer by C. albicans and also formation of an upper fungal layer that had characteristics similar to a biofilm. RT-PCR analysis of total RNA samples extracted from C. albicans-inoculated buccal RHE showed that ALS1, ALS2, ALS3, ALS4, ALS5 and ALS9 were consistently detected over time as destruction of the RHE progressed. Detection of transcripts from ALS7, and particularly from ALS6, was more sporadic, but not associated with a strictly temporal pattern. The expression pattern of ALS genes in C. albicans cultures used to inoculate the RHE was similar to that observed in the RHE model, suggesting that contact of C. albicans with buccal RHE does little to alter ALS gene expression. RT-PCR analysis of RNA samples extracted from model denture and catheter biofilms showed similar gene expression patterns to the buccal RHE specimens. Results from the RT-PCR analysis of biofilm RNA specimens were consistent between various C. albicans strains during biofilm development and were comparable to gene expression patterns in planktonic cells. The RT-PCR assay described here will be useful for analysis of human clinical specimens and samples from other disease models. The method will provide further insight into the role of ALS genes and their encoded proteins in the diverse interactions between C. albicans and its host.

INTRODUCTION

Candida albicans is an opportunistic fungal pathogen that causes oral and vaginal mucosal infections as well as systemic disease (Odds, 1988). C. albicans has several gene families that encode proteins involved in pathogenesis (De Bernardis et al., 2001; Hube & Naglik, 2001; Hube et al., 2000; Monod & Borg-von Zepelin, 2002). Among these is the ALS (agglutinin-like sequence) family that encodes cell-wall glycoproteins, some of which are involved in adherence to host surfaces (Gaur & Klotz, 1997; Hoyer, 2001; Fu et al., 2002). The goals of our research include determining the function of each Als protein and learning more about the role of gene families in C. albicans pathogenesis. We are particularly interested in understanding whether the role of each Als protein is unique or if there is functional redundancy across the family. One experimental approach that could aid studies of Als protein function is to analyse expression patterns of the various ALS genes. Understanding gene expression patterns might be useful for determining when certain Als proteins are present on the C. albicans surface.

Although we have used Northern blots for ALS gene expression analyses (Hoyer et al., 1995, 1998a, b), our focus shifted toward development of an RT-PCR assay for analysis of specimens from disease models and human clinical material where C. albicans cell numbers are more limited than from a culture flask. Such assays have been developed and used widely for analysis of gene expression for other C. albicans gene families (Schaller et al., 1998, 2003; Hube et al., 2000; Ripeau et al., 2002; Naglik et al., 1999, 2003; Schofield et al., 2003). This paper describes development of an ALS gene RT-PCR assay and its application to cells from the reconstituted human buccal epithelium (RHE) disease model,
which was originally introduced into the \textit{C. albicans} literature by Schaller \textit{et al.} (1998). While working with these specimens, we noted that the \textit{C. albicans} cells inoculated onto the RHE surface formed a biofilm-like structure over the epithelial layer. Consequently, we expanded our analysis of ALS gene expression to include model denture and catheter biofilms. Our results show that expression of each ALS gene can be detected by RT-PCR and validate use of this method for analysis of ALS gene expression in disease models.

\section*{METHODS}

\textbf{\textit{C. albicans} strains.} Strains SC5314 (Gillum \textit{et al.}, 1984), B311 (ATCC 32354), GDH2346 and M61 were used in this work. Strain GDH2346 was from a patient with denture stomatitis and kindly provided by Dr Julia Douglas, University of Glasgow, UK. Strain M61 was obtained from an intravascular line culture at the University Hospitals of Cleveland (Kuhn \textit{et al.}, 2002). Strains were maintained as glycerol stocks frozen at $-80\,^\circ C$ and streaked to YPD agar plates (per litre: 10 g yeast extract, 10 g peptone, 20 g glucose [dextrose], 20 g Bacto agar) for use.

\textbf{Inoculum preparation.} Methods for inoculum preparation largely followed those described by Schaller \textit{et al.} (1998, 1999). A starter culture of \textit{C. albicans} cells was inoculated from a single colony on a fresh YPD plate into 10 ml liquid YPD medium and incubated in a 37\,°C water bath, with 200 r.p.m. orbital shaking. Approximately 24 h later, cells from the entire culture were collected by centrifugation, washed three times in PBS and counted. Four million cells were inoculated into 10 ml fresh YPD and incubated in a 37\,°C water bath with 200 r.p.m. orbital shaking. At approximately 24 h, cells from the remainder of the washed inoculum culture were flash-frozen in a dry ice/ethanol bath and stored at $-80\,^\circ C$ for subsequent RT-PCR analysis.

\begin{table}[h]
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\begin{tabular}{|c|c|c|c|c|c|}
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Gene & GenBank accession no. & Primer name & Sequence (5\textsuperscript{\prime}$\rightarrow$3\textsuperscript{\prime}) & Seq. coord. & PCR product size (bp) \\
\hline
\textit{ALS1} & L25902 & RTALS1F & GAC TAG TGA ACC AAC AAA TAC CAG A & 3024 & 318 \\
 & & RTALS1R & CCA GAA GAA ACA GCA GGT GA & 3341 & \\
\textit{ALS2} & AF024580 & RTALS2F & CCA AGT ATT AAC AAA GTT TCA ATC ACT TAT & 571 & 366 \\
 & & RTALS2R & TCT CAA TCT TAA ATT GAA CGG CTT AC & 936 & \\
\textit{ALS3} & U87956 & RTALS3F & CCA CTT CAC AAT CCC CAT C & 2711 & 342 \\
 & & RTALS3R & CAG CAG TAG TAG TAA CAG TAG TAG TTT CAT C & 3052 & \\
\textit{ALS4} & AF024584 & RTALS4F & CCC AGT CTT TCA CAA GCA GTA AAT & 571 & 356 \\
 & & RTALS4R & GTA AAT GAG TCA TCA ACA GAA GCC & 926 & \\
\textit{ALS5} & AY227440 & RTALS5F ALT & TGA CTA CTT CCA GAT TTA TCG CCA G & 551 & 318 \\
 & & RTALS5R ALT & ATT GAT ACT GGT TAT TAT CTG AGG GAG AAA & 868 & \\
\textit{ALS6} & AF075293 & RTALS6F & GAC TCC ACA ATC ATC TAG TAT CTG TT & 528 & 152 \\
 & & RTALS6R & CAA TTG TCA CAT CTT TGG C & 679 & \\
\textit{ALS7} & AF201684 & RTALS7F & GAA GAG AAC TAG GTG TTG GTC TAG TTG T & 530 & 206 \\
 & & RTALS7R & TGG CAT ACT CCA ATC ATT TAT TAC C & 735 & \\
\textit{ALS9} & AF229990 & RTALS9F2 & CCA TAT TCA GAA ACA AAG GGT TC & 1729 & 198 \\
 & & RTALS9R2 & AAC TGA AAC TGC TGG ATT TGG & 1926 & \\
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\end{tabular}
\caption{PCR primers for RT-PCR analysis of ALS gene expression} \label{table1}
\end{table}

In the RHE model denture and catheter biofilms. Our results show that expanded our analysis of ALS gene expression to include model denture and catheter biofilms. Consequently, we noted that the \textit{C. albicans} genome. ALS primers that had not been washed in PBS were also pelleted and flash-frozen for subsequent analysis.

\textbf{RHE model.} Reconstituted human epithelium (RHE) is a product of SkinEthic Laboratories (Nice, France). The product consists of human epithelial cell lines cultured on polycarbonate filters in vitro at the air–liquid interface in a serum-free chemically defined medium. The experiments here used oral RHE (derived from the TR146 cell line) or oesophageal RHE (from the Kyse 510 cell line) in maintenance medium without antimicrobials. The maintenance medium was based on the MCDB-153 of Clonetics and contains 5 \mu g insulin ml$^{-1}$.

RHE was inoculated by pipetting 50 \mu l \textit{C. albicans}/PBS suspension (2 $\times$ 10$^9$ cells total) onto the surface of the tissue. Samples were incubated at 37 \,°C with 5 \% CO$_2$ and saturated humidity. Maintenance medium was changed every 24 h. At specified time points, tissues were harvested and processed for microscopy as described below, or flash-frozen and stored at $-80\,^\circ C$ for RT-PCR analysis. Samples were run in duplicate.

\textbf{ALS family PCR primers.} PCR primers specific for each ALS gene (Table 1) were designed using Primer3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Preference was given to primer pairs with a $T_m$ between 58 and 60 \,°C and with products in the 150–400 bp size range. To validate the specificity of each primer pair for its corresponding ALS gene, PCR products were amplified from SC5314 genomic DNA. PCR reactions contained 1 \mu M of each primer, 1 mM MgCl$_2$, 1 $\times$ Invitrogen Taq polymerase buffer, 0-75 units Taq polymerase (Invitrogen) and 200 ng genomic DNA. PCR reactions were denatured for 5 min at 94 \,°C and subjected to 40 cycles of 94 \,°C (30 s), 58 \,°C (30 s) and 72 \,°C (30 s). A final 7 min extension at 72 \,°C completed the reaction. PCR products were resolved on 2 \% agarose/TAE gels or 8 \% acrylamide/TBE gels and visualized by staining with ethidium bromide. PCR products were purified using the Wizard PCR Preps DNA Purification System (Promega) and the DNA sequence determined (Elim Biopharmaceuticals). The derived DNA sequences for each gene-specific primer pair matched exactly with the predicted sequence and validated the specificity of the primers against the \textit{C. albicans} genome. ALS primers
were also validated using all eight cloned ALS genes as PCR templates. Each primer pair only amplified its specific gene and no cross-reactivity was observed, further demonstrating the specificity of primer pairs across the ALS gene family.

**RT-PCR analysis.** RNA for RT-PCR analysis was isolated from the C. albicans-infected RHE specimens using the RNAqueous-4PCR kit (Ambion) according to the manufacturer’s protocol. Lysis of C. albicans cells was accomplished by adding 500 µl lysis-binding solution to a 100 µl equivalent of acid-washed glass beads (Sigma) and vortexing. Eluted RNA was DNase treated at 37°C and a quantity equal to that added to the individual gene-specific tubes in the subsequent RT-PCR analysis screened by PCR with the ALS9-specific primers (Table 1). Lack of a PCR product in the DNase-treated sample was interpreted to indicate that products amplified in the subsequent assay were not derived from trace amounts of genomic DNA. A parallel reaction that included C. albicans genomic DNA was run as a positive control for the PCR reaction.

The SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen) was used to synthesize cDNA according to the kit instructions. Random hexamers were used to prime the cDNA synthesis reaction. RNA concentration was measured spectrophotometrically and 2 µg added to the cDNA synthesis reaction. The 2 µg RNA represented nearly the entire sample recovered using this method. In a limited number of cases, less than 2 µg RNA was recovered. In these situations, the entire RNA sample was added to the cDNA synthesis reaction; this quantity was at least 1.5 µg. Samples with less than 2 µg RNA yielded results identical to replicate samples with the full 2 µg RNA (see below). One-tenth volume of the final cDNA product (2 µl per reaction) was added to PCR reactions specific for each gene in the analysed family. PCR tubes specific for amplification of individual ALS genes, containing all reaction components except template, were prepared in advance and frozen at −20°C until used. Preparation of larger stocks of PCR tubes allowed quality control that was not possible on the level of an individual reaction. Randomly selected PCR tubes from each batch were run as positive and negative controls to ensure the validity of the results.

**Specimen preparation and microscopy.** RHE specimens for light microscopy were removed from the maintenance medium and placed into 24-well plates containing 1 ml Karnovsky’s fixative (2% glutaraldehyde, 2.5% paraformaldehyde). Karnovsky’s fixative was also added to cover the top of the RHE specimen. Following 1 h incubation at room temperature, the samples were transferred to 4°C overnight. Samples were embedded in epoxy using the rapid microwave fixation method optimized at the University of Illinois Center for Microscopic Imaging (http://treefrog.cvm.uiuc.edu/meth_stdMW.html). This method involved microwaving the samples in Karnovsky’s solution for the primary fixation followed by washing in cacodylate buffer. Osmium tetroxide was used for the secondary fixation step. Samples were then dehydrated in a series of ethanol/acetonitrile. Embedding was carried out in increasing concentrations of epoxy, using the resin LX112. Blocks were sectioned and stained with toluidine blue. Specimens were examined using a Nikon Eclipse E600 microscope fitted with a Spot camera (Diagnostic Instruments). Images were collected using Metamorph software (Universal Imaging Corporation) and processed with Adobe Photoshop.

**Model biofilm growth and RT-PCR analysis.** Model biofilms were grown on denture acrylic (Chandra et al., 2001) or silicone elastomer catheter material (Kuhn et al., 2002) as described previously. Biofilms for RT-PCR analysis were flash-frozen in liquid nitrogen and stored at −80°C until used. Biofilm RNA was prepared using a hot phenol extraction method (Collart & Oliviero, 1993) and further purified using an RNeasy kit (Qiagen). DNase treatment and verification that genomic DNA was removed followed protocols listed above. RT-PCR analysis was conducted as described above with 2 µg total RNA added to the cDNA synthesis reaction and 0.1 vol. of the resulting product used for each ALS-specific PCR reaction. A negative control containing the equivalent amount of RNA added to the cDNA reaction was run in parallel to provide secondary assurance that the specimens lacked detectable genomic DNA. PCR products were resolved and visualized as described above.

**RESULTS**

**Microscopic evaluation of C. albicans-inoculated RHE**

Inoculum size for the RHE model was based on the method of Schaller et al. (1998), who used 2 × 10⁶ cells per RHE specimen. Tenfold and 100-fold fewer cells were also inoculated into the model and specimens collected every 12 h for a total of 48 h. A light micrograph of 2 × 10⁶ C. albicans inoculated onto a buccal RHE specimen from the 36 h time point is shown in Fig. 1 to illustrate the degree of tissue damage. The appearance of this tissue sample was the same as that for the higher inoculum samples (data not shown). In all specimens at this time point, fungi were abundant in a thick layer above the RHE and penetrated the entire extent of the epithelial layer, resulting in detachment of the basal epithelial cells. The overall thickness of the fungal layer covering the RHE specimen varied depending on which section of the specimen was analysed. Thickness of the fungal layer appeared to vary depending on the proximity of the section to the wall of the plastic well containing the polycarbonate filter and generally followed the meniscus of PBS used to inoculate the RHE with C. albicans cells (data not shown).

**Sensitivity of RT-PCR assay**

Primers for RT-PCR analysis of ALS gene expression were designed and their specificity validated as described in Methods. Additional controls were completed to compare the performance of the primer pairs. Dilutions of SC5314 genomic DNA were amplified by PCR using the ALS primer pairs to determine if certain gene products were produced preferentially (Fig. 2). Preferential amplification of ALS6 was apparent as decreasing amounts of genomic DNA were added to each PCR reaction. This result is most likely due to the fact that the ALS6 fragment is the smallest among the set of ALS PCR products.

Another assessment of the sensitivity of the RT-PCR assay was made using RNA isolated from YPD-grown SC5314 cells. This culture was the same as that used for inoculating the RHE specimens. The purpose of this analysis was to determine the point at which expression of the various ALS genes fell below the limit of detection for the assay. In this analysis, 10-fold dilutions of RNA were added to the reverse transcriptase reaction. The starting concentration of 200 ng corresponds to the standard amount of RNA added to each ALS-specific reaction in the RT-PCR assay (Fig. 3a). All RT-PCR products were visible on an
Fig. 1. (a) Light micrograph of buccal RHE inoculated with $2 \times 10^4$ cells of *C. albicans* strain SC5314. The RHE specimen was incubated for 36 h at 37 °C, 5% CO$_2$ and saturated humidity. The polycarbonate filter, visible at the bottom of the image, serves as a support for growth of the epithelial cells. The thick fungal biofilm-like layer is evident above the epithelial cells. Image taken at 400× magnification.

Fig. 2. PCR products from amplification of *C. albicans* SC5314 genomic DNA with ALS gene-specific primer pairs. PCR reactions were run to determine if certain gene products were produced preferentially. Decreasing quantities of genomic DNA were added to the ALS gene-specific PCR reactions. The resulting products were separated on acrylamide gels and stained with ethidium bromide. (a) Amplification with 200 ng genomic DNA per reaction. (b) Amplification with 2 ng genomic DNA per reaction. The extra band in the ALS3 lane was only apparent at low DNA concentrations. This extra band does not cause confusion in interpretation of the assay results since its smaller size is readily distinguished from the size of the correct PCR product. Molecular sizes (in bp) are indicated at the left of each image.

Fig. 3. Ethidium-bromide-stained acrylamide gels of RT-PCR products from analysis of RNA isolated from YPD-grown inoculum SC5314 cells. (a) Products from addition of 2 μg total RNA to the cDNA synthesis reaction. Following cDNA synthesis, 0-1 vol. of the total (a 200 ng equivalent of starting RNA) was added to each ALS-specific PCR reaction. The control reaction included an amount of RNA equal to that added to the other reactions, without reverse transcription. This sample was amplified with the ALS9-specific primer pair. Lack of a signal in this lane indicates that PCR products in the other samples are due to amplification of cDNA, rather than low levels of genomic DNA. (b) ALS1 and ALS2 RT-PCR products from analysis of 10-fold dilutions of input RNA. Amounts of starting RNA per ALS PCR reaction are shown below the corresponding lanes. Molecular sizes (in bp) are indicated at the left of each image.
ethidium-bromide-stained acrylamide gel, although the signal for ALS6 was very faint. At a 10-fold lower concentration of starting RNA (20 ng; Fig. 3b), all RT-PCR products fell below the limit of detection with the exception of ALS1 and ALS2. These two products were still detectable at 0.2 ng of input RNA and fell below the detection limit at the next dilution. These assays further defined the ability of the various primer pairs to amplify ALS sequences in a specific manner and also the limit of detection for expression of the ALS genes in YPD-cultured C. albicans cells.

**ALS gene expression in RHE**

The main goal of this study was to examine ALS gene expression in C. albicans cells inoculated into the RHE model. Strain SC5314 and buccal RHE were the focus of the work using the three different inoculum densities described above. Results are shown in Table 2. Expression of all genes was detectable in the inoculum YPD culture, consistent with the results in Fig. 3(a). Uninoculated RHE failed to show evidence of ALS gene expression, consistent with the conclusion that the RT-PCR primers are specific for ALS genes and do not detect epithelial gene expression. Across all SC5314-inoculated buccal RHE samples, expression of ALS1, ALS2, ALS4 and ALS9 was detected for all time points and all inoculation densities. These results were observed for replicate RHE specimens. Expression of ALS5 fell below the assay limit of detection for one replicate 10^5 inoculum specimen at the 24 h time point while ALS3 expression was undetectable in one 36 h sample for the same inoculum size. Of all the ALS genes, ALS6 and ALS7 fell below the limit of detection most readily. For ALS7, only one replicate of a time point failed to show detectable expression. In the case of ALS6, some time points showed both replicates without detectable expression.

A more limited analysis was conducted with other C. albicans strains to determine whether there was strain variability in gene expression and whether differences due to the effect of RHE type could be detected (Fig. 4). These comparisons showed that gene expression patterns for other C. albicans strains were similar to those observed for SC5314 (Fig. 4a). For example, ALS6 fell below the limit of detection of the assay more readily than the other genes when strain GDH2346 was inoculated onto buccal RHE (Fig. 4b). Strain B311 failed to produce an ALS5-specific RT-PCR product (Fig. 4c). In this case, however, the negative result was due to the fact that the ALS5 coding region is not present in this strain (Hoyer & Hecht, 2001). The few samples of oesophageal RHE that were tested showed a trend similar to that observed for the buccal RHE. Fig. 4(d) shows a typical result where ALS7 was not detectable when strain SC5314 was inoculated onto oesophageal RHE. In order to conclude that the two types of RHE produce identical ALS gene expression results, however, a more extensive analysis is required.

**Biofilm formation on RHE**

Observations of our tissue sections by light microscopy indicated that C. albicans growing on RHE forms an upper, thick layer of fungal cells (Fig. 1). This arrangement of cells

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**Table 2. C. albicans SC5314 ALS gene expression from cells inoculated into the buccal RHE model**

Expression is shown as + or – for each of the duplicate samples run at each time point.

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Fig. 4. RT-PCR products from analysis of ALS gene expression in various *C. albicans* strains and different RHE types. Different *C. albicans* strains were inoculated onto either buccal or oesophageal RHE and the resulting RT-PCR products visualized on ethidium-bromide-stained acrylamide gels. All RHE specimens were inoculated with $2 \times 10^5$ *C. albicans* cells. (a) Strain SC5314 inoculated onto buccal RHE. (b) Strain GDH2346 on buccal RHE. (c) Strain B311 on buccal RHE. (d) Strain SC5314 on oesophageal RHE. Molecular sizes (in bp) are indicated at the left of each image.

was unanticipated since *C. albicans* would be expected to settle onto the RHE surface. *C. albicans* cells suspended over seemingly large open spaces suggest that the fungal cells may be embedded in a matrix, a feature that is consistent with a biofilm. This appearance of the fungal layer was referred to as a pseudomembrane in the initial report describing the interaction between *C. albicans* and RHE (Schaller *et al.*, 1998). Work from another group showed micrographs where the structure seemed to collapse, presumably due to the methodology used for fixation and specimen processing prior to microscopy (Bernhardt *et al.*, 2001). In that work, *C. albicans*-inoculated RHE was fixed in formalin, which is known for creating shrinkage in tissue sections. Such compression of the fungal layer is consistent with a biofilm-like structure.

Since *C. albicans* appears to form a biofilm on RHE, RT-PCR analysis of ALS gene expression was conducted on RNA isolated from model biofilm specimens (Table 3). For this analysis, semi-quantitative judgements were made about the relative abundance of RT-PCR product for each assay. These estimates were more justified for this analysis than for the RHE model because biofilm specimens only contained *C. albicans* RNA. A denture biofilm model (Chandra *et al.*, 2001) and a catheter biofilm model (Kuhn *et al.*, 2002) were analysed. Samples included matched sets of planktonic cells and biofilm specimens across a 48 h time-course.

For SC5314 in both the denture and catheter models, ALS6 and ALS7 were the genes that most often produced weak or negative reactions among both planktonic and biofilm specimens. A greater number of weak or negative results across a larger number of genes were observed for strains GDH2346 and M61. There was no clear trend to demonstrate that ALS gene expression was more readily detected in either the planktonic or biofilm specimens. Analysis of a replicate set of specimens gave similar results (data not shown). In all cases, the results from analysis of the biofilm models matched those observed for the RHE specimens. Both experiments indicated that under the growth conditions tested, expression of ALS1, ALS2, ALS3, ALS4, ALS5 and ALS9 was demonstrated consistently while ALS6 and ALS7 expression levels more readily fell below the detection limit for this RT-PCR assay.

**DISCUSSION**

This paper reports development of a set of RT-PCR primers that can specifically distinguish between *C. albicans* ALS gene sequences. These primers do not cross-react with RNA derived from uninoculated buccal RHE, demonstrating the utility of these primer pairs to monitor ALS gene expression in this model of oral candidiasis. With these primer pairs, ALS1, ALS2, ALS3, ALS4, ALS5 and ALS9 were consistently detected over time as destruction of the RHE tissue layers progressed. Detection of transcripts from ALS7, and particularly from ALS6, was more sporadic, but not associated with a strictly temporal pattern. The expression pattern of ALS genes in *C. albicans* cultures used to inoculate the RHE was similar to that observed in the RHE model, suggesting that contact of *C. albicans* with buccal RHE does little to alter ALS gene expression. Microscopic analysis of the inoculated RHE showed an upper thick layer of fungal cells that had characteristics of a biofilm. Because of this observation, analysis of ALS gene expression was extended to model denture and catheter biofilms. Gene expression results in these models were similar to those observed in the RHE model. The data also showed that despite the change in growth medium between the RHE and model...
biofilms, very similar trends in ALS gene expression were present in the two systems. The thickness of the fungal biofilm layer over the RHE and relative abundance of *C. albicans* cells in that portion of the model (Fig. 1) suggest that they may contribute the predominant amount of fungal RNA to the RT-PCR analysis. Other sources of fungal RNA in the RHE specimen include transcripts produced in response to hyphal formation and epithelial adhesion and invasion. This complex situation includes factors found within the host. Indeed, the curd-like pseudo-membrane isolated from patients with oro-oesophageal candidiasis (Odds, 1988) may be a biofilm. Levels of complexity beyond those of the RHE model exist within the host, where *C. albicans* also contends with the immune system and commensal flora. Use of our RT-PCR assay in analysis of human clinical specimens will begin to reveal which ALS messages can be detected in this even more complex and clinically relevant environment.

Development of the RT-PCR assay provides a tool for analysis of ALS gene expression that is more sensitive than previous detection studies using Northern blots of total RNA. This improved detection limit is inherent in the amplification-based nature of the RT-PCR method. In addition, positive signals can be derived by RT-PCR from a specific transcript that is not full-length. An improved limit of detection is required for gene expression analysis of specimens where *C. albicans* cell numbers are not as abundant as in liquid cultures. ALS gene expression patterns observed with the RT-PCR assay, in most cases, match those observed on Northern blots (Hoyer et al., 1995, 1998a, b). Previous Northern analysis suggested that ALS3 is expressed in the hyphal form of the organism, yet a positive signal was derived from the YPD-grown culture of yeast forms used to inoculate the RHE model. This result is probably attributable to a very low number of germ tubes in the culture. Previous Northern blot results for ALS3 expression in strain SC5314 showed detectable transcripts in cultures where the abundance of germ tubes was below 0.1% (Hoyer et al., 1998b). The improved limit of detection of the RT-PCR assay should be able to detect ALS3-specific signals from even fewer germ tube forms. Perhaps the largest divergence between previous Northern results and the RT-PCR analysis is for detection of ALS2. In the RT-PCR assay, ALS2 appeared as one of the two strongest signals, whereas the transcript eluded previous attempts at detection on Northern blots (Hoyer et al., 1998b). Many factors may contribute to this disparity of results. Alleles of all ALS genes are known to vary in size

### Table 3. ALS RT-PCR results for model biofilm samples

Detection of gene expression was reported as a positive (‘+’) or a weak positive (‘w’) result; samples where an RT-PCR product was not detectable were marked ‘−’.

<table>
<thead>
<tr>
<th>Model</th>
<th>Strain</th>
<th>Time (h)</th>
<th>Specimen</th>
<th>ALS1</th>
<th>ALS2</th>
<th>ALS3</th>
<th>ALS4</th>
<th>ALS5</th>
<th>ALS6</th>
<th>ALS7</th>
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mainly due to a central domain consisting entirely of a 108 bp tandemly repeated sequence (Hoyer, 2001). Among the genes in the ALS family, ALS2 is one of the largest, with an average tandem-repeat domain containing over 30 copies of the 108 bp sequence (J. A. Nuessen & L. L. Hoyer, unpublished results). This number of repeat copies results in an average ALS2 allele size of nearly 6 kb. Transcripts of this size may be extracted less efficiently from the C. albicans cell. In Northern blot analysis of disparate-sized ALS alleles from the same C. albicans strain, a less intense signal is frequently observed for the larger allele (Hoyer et al., 1995, 1998a, b). Transcripts of this large size may also be degraded more readily than those for smaller genes. Considering these variables, it is expected that RT-PCR would be more successful at detection of the ALS2 transcript than Northern blotting. Additional difficulties in detection of ALS2 on Northern blots arise because of its sequence similarity to other ALS genes, particularly ALS4. Specific detection of ALS2 transcripts requires an end-labelled oligonucleotide probe that results in a reduced Northern blot signal compared to what is observed when hybridizing with a larger DNA probe (Hoyer et al., 1998b). Hybridization of Northern blots with a larger probe that recognizes both ALS2 and ALS4 improves the signal strength, but ALS2 and ALS4 alleles tend to migrate in the same size range and obscure each other on Northern blots. For example, in strain SC5314, the alleles of ALS4 are approximately 4 and 6 kb while both alleles of ALS2 are approximately 6 kb (Hoyer et al., 1998b; X. Zhao & L. L. Hoyer, unpublished data). As other reagents are developed, the nature of the ALS2 expression will become clear. Recent construction of an ALS2-GFP reporter strain and demonstration of its obvious fluorescence when cultured under the same conditions utilized for the RT-PCR assays described here (C. B. Green & L. L. Hoyer, unpublished results) substantiates the RT-PCR results and suggests that these data accurately reflect ALS2 expression patterns.

The observation that ALS7 transcription more readily falls below the RT-PCR assay detection limit concurs with recently published observations by Zhang et al. (2003). In that study, 20 μg poly(A) RNA was required to detect ALS7 transcripts by Northern blot analysis. The authors estimated the abundance of ALS7 transcript to be approximately 1–2 % that of actin mRNA. Growth conditions used in their report are similar to those used for our RT-PCR analysis of RNA from cultured C. albicans cells. Currently, it is not known how much transcript is required to produce mature, active Als proteins. It is possible that the ALS7 transcript is more difficult to observe on Northern blots for reasons similar to the ALS2 size and stability arguments outlined above. However, it is also possible that only a low level of ALS7 transcript is required for production of sufficient quantities of Als7p. Understanding the relationship between ALS transcript abundance and the presence of functional protein on the C. albicans cell surface is a priority for future investigations. It is likely that these parameters vary for each gene in the ALS family.

Another motivation for our studies of ALS gene expression in disease models was to compare general themes for the ALS family with those from other C. albicans gene families, most notably the SAP (secreted aspartyl proteinase) genes. Many authors have described the use of RT-PCR to study SAP gene expression in various disease models and human clinical specimens (Schaller et al., 1998, 2003; Naglik et al., 1999, 2003; Ripeau et al., 2002; Schofield et al., 2003). Meaningful comparisons between the various SAP analyses and our ALS analysis are complicated by the varying detection limits for each assay. The detection limit for any of the RT-PCR assays is due to choices made for the overall assay design as well as the relative efficiency of amplification from the specific pairs of primers. For example, incorporation of radiolabels into the RT-PCR products serves to increase limit of detection (Naglik et al., 1999, 2003); limit of detection is also enhanced by Southern blotting of the RT-PCR products (Ripeau et al., 2002). Design of primers to amplify smaller and similarly sized PCR products increases detection limit and consistency among the results for each gene (Naglik et al., 1999, 2003; Schofield et al., 2003). The overall body of work on RT-PCR analysis of SAP gene expression suggests differential expression across the family. However, detection of expression from all SAP genes in specific samples is being reported with increased frequency as RT-PCR methodology becomes more refined (Naglik et al., 2003; Schofield et al., 2003). The use of quantitative PCR methods would provide more precise estimates of transcript abundance for various genes within the SAP family and for our ALS analysis. However, a quantitative method would still not address the central need to understand the relationship between transcript abundance and protein production, which may differ for each gene in a family. Northern blots are useful for detecting large increases and decreases in transcript abundance and these changes are likely to have biological meaning. However, it is more complicated to use Northern blots to interpret the profile of expression for a gene that may never be abundantly expressed. In theory, protein may be present whenever gene-specific transcript is detected. For the ALS genes, many levels of regulation are encountered during the production of a heavily glycosylated cell wall protein from an mRNA molecule. Additionally, the fate of the Als proteins once they are localized on the C. albicans cell surface is still unknown. Other mechanisms that regulate the profile of Als proteins on the cell surface may be operational in C. albicans. The data presented here highlight the importance of these questions in our ongoing efforts to characterize the ALS family and the utility of our RT-PCR assay in addressing these relationships.

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REFERENCES


