Candida albicans SNO1 and SNZ1 expressed in stationary-phase planktonic yeast cells and base of biofilm

Priya Uppuluri, Bhaskarjyoti Sarmah† and W. LaJean Chaffin

Department of Microbiology and Immunology, Texas Tech University Health Sciences Center, Lubbock, TX 79430, USA

The Candida albicans homologues of the most studied Saccharomyces cerevisiae stationary-phase genes, SNO1 and SNZ1, were used to test the hypothesis that, within a biofilm, some cells reach stationary phase within continuously fed, as well as static, C. albicans biofilms grown on dental acrylic. The authors first studied the expression patterns of these two genes in planktonic growth conditions. Using real-time RT-PCR (RT-RTPCR), increased peak expression of both SNZ1 and SNO1 was observed at 5 and 6 days, respectively, in C. albicans grown in suspension culture. SNZ1–yellow fluorescent protein (YFP) and SNO1–YFP were constructed to study expression at the cellular level and protein localization in C. albicans. Snz1p–YFP and Sno1p–YFP localized to the cytoplasm with maximum expression (> 90%) at 5 and 6 days, respectively, in planktonic conditions. When yeast growth was reinitiated, loss of fluorescence began immediately. Germ tubes and hyphae were non-fluorescent. Pseuodohyphae began appearing at 9 days in planktonic yeast culture and expressed each protein by 11 days; however, the cells budding from pseudohyphae were not fluorescent. Biofilm was formed in vitro under either static or continuously fed conditions. Increased expression of the two genes was shown by RT-RTPCR, beginning by day 3 and increasing through to day 15 (continuously fed biofilm). Only the bottommost layer of acrylic-adhered cells in the biofilm showed 25 and 40% fluorescence at 6 and 15 days, respectively. These observations suggest that only a few cells in C. albicans biofilms express genes associated with the planktonic stationary phase and that these are found at the bottom of the biofilm adhered to the surface.

INTRODUCTION

Candida albicans is capable of forming a biofilm on mucocutaneous surfaces, as well as on medical devices such as dentures and catheters (reviewed by Douglas, 2003; Kumamoto & Vinces, 2005; Mukherjee et al., 2005). Sixty-five percent of edentulous individuals suffer with denture stomatitis and 40% of patients with intravenous catheters develop acute fungaemia due to the growth of C. albicans biofilms on the associated biomedical devices. Biofilms also show enhanced resistance to antifungal drugs.

In vitro, biofilms have been shown to form on catheter, polymethylmethacrylate (denture acrylic) and polystyrene surfaces (Douglas, 2003; Kumamoto & Vinces, 2005; Mukherjee et al., 2005). C. albicans forms a biofilm in three distinct developmental stages. The bottommost layer of adhered yeast cells act as founder cells, anchoring the developing biofilm to the substrate. The middle layer is composed of hyphae and pseudohyphae, and the topmost part of the biofilm consists mostly of a thicker and open hyphal layer and more extracellular matrix (ECM) (Baillie & Douglas, 1999; Chandra et al., 2001; Ramage et al., 2001). After 48 h, these biofilms range in thickness from 25 to >450 μm and are metabolically active communities of cells interspersed with ramifying water channels. The structural complexity of the biofilm may create a gradient of environmental conditions in which the C. albicans cells enter distinct physiological states. One such state may be equivalent to that of stationary-phase planktonic yeast cells, and, in particular, the founding yeast cells at the surface of the substratum may cease growing. The stationary phase and the genes involved in its progress and maintenance have not yet been well characterized in C. albicans, although several genes have been reported to show increased expression as active growth slows (Lamarrre et al., 2001; Moreno et al., 2003). In this study, we investigated the expression patterns of the C. albicans homologues of the two most studied stationary-phase genes in Saccharomyces cerevisiae, SNO1 and SNZ1.
and SNZ1. We first determined whether the expression pattern of these genes, monitoring both RNA and protein, was associated with the stationary phase of planktonic yeast cells, hyphae and pseudohyphae. We then used these two genes as indicators of the stationary phase to study the physiological state of the cells in a biofilm.

**METHODS**

**Organisms and growth conditions.** *C. albicans* SC5314 or CAI4 (obtained from William Fonzi, Georgetown University Medical Center) was maintained on YPD medium (1% yeast extract, 2% peptone, 2% glucose)-containing 2% agar plates. Planktonic yeast cell cultures were grown in yeast nitrogen base (YNB) medium with amino acids (Difco Laboratories) containing 2% glucose at room temperature on a gyratory shaker (180 r.p.m.). Hyphae were induced by inoculating $1 \times 10^5$ yeast cells ml$^{-1}$ from 24 h culture into YNB medium at 37°C and incubated with shaking for 2 or 2 days. For stationary-phase studies, seven flasks (one for each time point) containing YNB broth (200 ml) were inoculated with $5 \times 10^7$ yeast cells ml$^{-1}$ from an overnight culture in YNB. The cells were harvested by centrifugation at 4°C and maintained at −80°C before RNA isolation. Cell viability was determined as c.f.u. by plating replicate dilutions of planktonic cells prepared in sterile water on YPD plates and incubating at 37°C for 24 h. Colonies were enumerated manually and the mean determined. Particles in suspension culture were determined by use of a haemocytometer and by OD$600$ measurement. Denture acrylic (polymethylmethacrylate) pieces (1 cm$^2$ square or 90×20×1.5 mm) prepared by Dr Thomas McKinney (Baylor College of Dentistry, Dallas, TX) were used to support the biofilm formation in two model systems. Pieces of acrylic were placed in disposable polystyrene dishes (35×10 mm). A suspension of yeast cells (4 ml) grown to a density of $1 \times 10^7$ cells ml$^{-1}$ in YNB was added to the dish and incubated for 2 h at 37°C without shaking. The liquid was gently aspirated; 4 ml fresh medium was added and incubated for 6 days. Alternatively, the strips were placed in a 50 ml syringe barrel with a yeast suspension and then washed with YNB to remove non-adhered cells, before starting YNB medium flow through the syringe at 50 ml h$^{-1}$ at 37°C. Sterile air was supplied to the medium at 1 l h$^{-1}$. Acrylic pieces were removed, washed gently by dipping in PBS (10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl, pH 7.4). For microscopy, the top matrix layer, mostly consisting of hyphae, was collected by very gently dipping and shaking the washed biofilm in PBS. The middle layer, composed of yeast, pseudohyphae and some hyphae, was collected using sterile forceps, while the bottommost acryl-adhered layer, composed exclusively of yeast cells and germ tubes, was collected by scraping the thoroughly washed acrylic using a scalpel and ice-cold water. Similar distribution of the three forms of *C. albicans* was observed from at least four independent biofilms. Differences in expression were determined by ANOVA ($P \leq 0.05$). The viability of the recovered cells from three biofilms was determined at 20 days, as described above.

**RNA extraction.** Total cellular RNA was isolated using the standard hot acid phenol method following grinding of the frozen cells using a mortar and pestle in liquid nitrogen (P. Uppuluri and others, unpublished results). We have found that grinding yields a better quality of intact RNA, particularly from late-stationary-phase cells. DNA contamination in the RNA was verified with the housekeeping gene *EF51* (Maneuf et al., 2000). RNA quantity was estimated spectrophotometrically at 260 nm. RNA (10 μg) was electrophoresed under non-denaturing conditions in a 1–2% agarose gel using Tris/acetate/EDTA buffer. The gel was stained with SYBR Green II (Sigma) and observed by UV light.

**Real-time RT-PCR (RT-RTPCR).** The amount of mRNA in the total RNA was quantified with the Poly(A) mRNA Detection System kit (Promega). cDNA was synthesized from known amounts of mRNA, and an equal amount of cDNA was used as starting template for RT-RTPCR. Analysis of transcripts was carried out using SYBR Green PCR Master Mix (Applied Biosystems) in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Each reaction was set up in triplicate in 25-μl volumes with 1-μl cDNA for 40 cycles (thermal cycling conditions: initial steps of 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 95°C for 15 s, followed by 60°C for 1 min). The primers are given in Table 1. To quantify transcripts, a standard curve was constructed using DNA of each gene as standards. For this, genomic DNA was isolated from *C. albicans* SC5314 strain following a standard protocol (Adams et al., 1997) and an entire ORF was amplified by PCR with gene-specific primers (Table 1). PCR reactions were set up with 10 ng genomic DNA in a 50-μl reaction volume using 40 pmol of each primer. 200 μM each dNTP, 2.5 mM MgCl$_2$, and 0–125 U Ampli-Taq DNA polymerase (Applied Biosystems) for 30 cycles (94°C for 1 min, 52°C for 1 min, and 72°C for 1 min) and a final extension at 72°C for 7 min. PCR products were separated in a 1-0.5% agarose gel, and DNA was eluted from the gel and quantified spectrophotometrically, RT-RTPCR reactions for each gene were set up using different dilutions of the amplified ORFs as the DNA template. Due to the absence of reference genes for normalization of stationary-phase gene expression (P. Uppuluri and others, unpublished results), three independent biological and technical replicates were used for normalization. All the replicates yielded equivalent Ct values when analysed using ANOVA ($P \leq 0.05$).

**Construction of strains expressing fluorescent fusion protein.** *C. albicans* transformations were carried out using the Alkali-Cation Yeast kit (Qiagen). Genomic DNA from *C. albicans* CAI4 strain was obtained by standard methods (Adams et al., 1997). For construction of the yellow fluorescent protein (YFP) fusion protein, the method of Gerami-Nejad et al. (2001) was used. Briefly, PCR was performed using primers with 5′ ends corresponding to the SNZ1 or SNO1 target gene sequences and 3′ ends that directed amplification of the YFP gene along with the selectable marker URA3. The primers used are listed in Table 1. PCR was performed with 100 ng pYFP-URA3 (cassettes obtained from Cheryl Gale, University of Minnesota) as the template, 0-6 μM each primer, 3-5 mM MgCl$_2$, 5 μl 10 × PCR buffer, 0-4 mM each dNTP, and 2 U Expand High Fidelity Polymerase (Roche Applied Science). The 50 μl reactions were run for 94°C for 4 min, then for 25 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 3 min, followed by 72°C for 10 min.

The products from 10 reactions were pooled, precipitated with ethanol, resuspended in 50 μl water, and used to transform *C. albicans* CAI4 and URA3 recombinants selected in YNB without uridine. Identification of transformants carrying the correctly integrated cassette was performed by PCR on total genomic DNA with a primer that annealed within the transformation module and a second primer annealing to the 3′ region located outside the module (Table 1).

**Fluorescence microscopy.** For fluorescence microscopy, cells were used without fixation. YFP-tagged proteins were visualized in live cells with an Olympus IX71 microscope with appropriate filters. Images were captured and documented using a Photometrics Cool Snap HQ digital camera and analysed with Meta Morph software. For localization, a bright-field image and a fluorescent image were first pseudo-coloured green and red, respectively. The resultant images were then merged.
RESULTS

Viability of stationary-phase organisms

In S. cerevisiae, the stationary phase has been associated with cultures that are at least 5 days old (Braun et al., 1996; Radonjic et al., 2005). Since most studies with C. albicans terminate experiments after 24–48 h of growth, we wanted to determine whether cells remain viable in culture for an extended period. We examined the ability of organisms cultured in YNB for 2 weeks to carry out cell division by determining the c.f.u. in the culture during the stationary phase (Fig. 1). Cells did not lose viability for 10 days. At 2 weeks, >60% of the cells were viable, after which there was a progressive decline in the number of cells forming colonies. On day 9, there was appearance of pseudohyphae in an appreciable fraction of cells (~30%) (data not shown).

Expression of SNZ1 and SNO1 during planktonic growth

To initiate our study of the stationary phase of C. albicans, we used RT-RTPCR to examine the expression of the two genes, SNZ1 and SNO1, predicted to be indicative of the stationary phase. In S. cerevisiae there are three pairs of SNZ and SNO genes. The two genes of each pair are adjacent and divergently transcribed (Balakrishnan et al., 2002). A single SNZ and SNO gene pair was found in the annotated C. albicans genome (Arnaud et al., 2005) and a (Altschul et al., 1997) BLAST search did not reveal other unannotated pairs. SNZ1 (Orf19.2947) and SNO1 (Orf19.2948) were adjacent and divergently transcribed, with a sequence of ~1.5 kbp separating the translation initiation sites. Transcripts of the two genes were quantified at intervals during progression into the stationary phase (Fig. 2A). Although a small peak in expression was noted on day 2, the greatest expression for SNZ1 was reached on day 5, after which the transcription declined. There was a 67-fold increase in expression between day 1 and day 5. For SNO1, there was decreased expression up to day 4, followed by increased expression that peaked on day 6, after which expression declined. The increased expression at day 6 was 10^6-fold higher compared to that on day 1. In addition to a 1 day difference in peak expression, SNZ1 transcript (5.6 × 10^8–3.8 × 10^8) was more abundant than SNO1 transcript (4.2 × 10^5–4.2 × 10^6) at all intervals measured, and the increase between day 1 and the peak of expression was greater for SNZ1 (67-fold) compared to SNO1 (10-fold). The expression of these genes began to increase after the cell numbers stopped increasing and the pattern was consistent with that of genes with preferential expression in the stationary phase.

Table 1. Primers used in this study

Priming were used for obtaining full-length ORFs (full) and short sequences, and for verification (v) of the gene–YFP constructs. The direction of primers is indicated as forward (F) or reverse (R).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>For RT-RT-PCR</td>
<td></td>
</tr>
<tr>
<td>SNO1-F</td>
<td>TCAAACCGGACGGAATATGC</td>
</tr>
<tr>
<td>SNO1-R</td>
<td>TCTCCGCCAGGAAATAACCA</td>
</tr>
<tr>
<td>SNZ1-F</td>
<td>CAATTGGATGATGTTGTTT</td>
</tr>
<tr>
<td>SNZ1-R</td>
<td>TTGTAGTGATGTTGACGTAAC</td>
</tr>
<tr>
<td>SNO-full-F</td>
<td>GTCGATGAAGATCTCAATTC</td>
</tr>
<tr>
<td>SNO-full-R</td>
<td>CTGTGGATATTTTGTGG</td>
</tr>
<tr>
<td>SNZ-full-F</td>
<td>CAAACCTTTGTAATTAAACCAAC</td>
</tr>
<tr>
<td>SNZ-full-R</td>
<td>CATATGGATATATAACGATAGT</td>
</tr>
<tr>
<td>For Snz1–YFP and Sno1–YFP construction</td>
<td></td>
</tr>
<tr>
<td>SNO1YFP-F</td>
<td>CTTGATGATGTTGATAGAATAAATGTCGATGTTGTTCTAAGGTAAGATATTT</td>
</tr>
<tr>
<td>SNO1YFP-R</td>
<td>CAACCAGCTGATGATGTTGTTCTAAGGTAAGATATTT</td>
</tr>
<tr>
<td>SNZ1YFP-F</td>
<td>ATGGGATGATTGATAGAATAAATGTCGATGTTGTTCTAAGGTAAGATATTT</td>
</tr>
<tr>
<td>SNZ1YFP-R</td>
<td>TTATGTCCACAAAAATCATTTGTTACTCCTCATAACGAAATAATCAGTTATCAGTTG</td>
</tr>
<tr>
<td>For verification</td>
<td></td>
</tr>
<tr>
<td>SNOYFP-Fv</td>
<td>CCAGAGCTAGCTGAGGATTA</td>
</tr>
<tr>
<td>SNZYFP-Fv</td>
<td>TAATTGTTCACTGATATTGGAATGAT</td>
</tr>
<tr>
<td>ADHI-Rv</td>
<td>CACAGTGGATCCAGAACATG</td>
</tr>
</tbody>
</table>

http://mic.sgmjournals.org 2033
Biofilm formation and gene expression

*C. albicans* has been studied most frequently as planktonic cells in suspension culture. However, the organism can grow in both *in vivo* and *in vitro*-produced biofilms. In biofilms, both yeast cells and hyphae are observed in an ECM-covered community (Baillie & Douglas, 1999, 2000; Chandra *et al.*, 2001). To determine if some organisms in a biofilm reach a physiologic state in which the *SNZ1* and *SNO1* markers for stationary phase are expressed, we examined biofilms formed under two conditions. Biofilms were formed on pieces of acrylic in YNB from a yeast-cell inoculum under static conditions, i.e. maintained without shaking for the duration of the experiment. There was an ~1.5- and fourfold increase in *SNZ1* and *SNO1* expression, respectively, between day 1 and day 6 (Fig. 2B). However, overall expression of both genes in biofilm conditions at day 6 was at least 25 times less than the maximum that was recorded under planktonic conditions.

The reduced expression in the biofilm compared to planktonic cells could be attributable to the heterogeneous nature of the biofilm, which contains hyphal, pseudohyphal and yeast organisms, or to the presence of growing organisms responsible for the release of primarily yeast cells from the biofilm. To address these possibilities, we used a different system for biofilm formation. Biofilms were formed under flow conditions that replenished medium and permitted the biofilm to be maintained for 15 days. To answer the question of whether the bottommost layer of the biofilm formed from founder yeast cells reaches the stationary phase earlier than the rest of the biofilm, we separated two layers of the biofilm. We collected the bottommost adhered layer and

---

**Fig. 1.** Viability of cells from culture grown in YNB for extended periods. Viability was analysed at different times of growth by cell counts (open circles) using a haemocytometer and colony formation (solid circles) on YPD plates.

**Fig. 2.** Expression of *SNZ1* and *SNO1*. RT-RTPCR analysis of *SNZ1* and *SNO1* transcripts was determined and is shown as copy number. Expression patterns of *SNZ1* (open triangles) and *SNO1* (solid triangles) in planktonic conditions (A), in static biofilm conditions (B), in upper layers (C) and in bottommost adhered cells (D) of the biofilm formed under flow conditions, are shown.

---
the upper layers of the biofilm separately to monitor gene expression of the two stationary-phase genes, again, at various time points (Fig. 2C, D). We found that, in flow conditions, the level of expression of both the genes in the upper layers of the biofilm decreased over 15 days. When gene expression changes were monitored in the bottommost adhered cells of the biofilm, a different pattern of expression was revealed. Expression of both the genes was observed on day 1 and increased over the 15 days.

**Protein localization of Snz1p–YFP and Sno1p–YFP in planktonic cells**

We used YFP cassettes to tag SNZ1 and SNO1 in *C. albicans*, and observed the localization and expression of the two encoded proteins under different growth conditions and morphologies. Fluorescence was observed for both proteins in yeast cells (Fig. 3A, B). The proteins were then localized within the cells. When bright-field and fluorescent images were compared visually, fluorescence could be easily localized within the cytoplasm (Fig. 4A, B). The two images were pseudocoloured green and red using the Meta Morph software and then merged (Fig. 4C). This method confirmed that the two proteins localized to the cytoplasm.

**Protein expression in planktonic cells**

Planktonic yeast cells began expressing both proteins after 3 days in culture (Fig. 5A). About 25% of cells expressed Snz1p–YFP on day 4 and >90% expressed the protein on day 5. On day 6, there was a greater than threefold decrease in the fluorescent cells. Expression of Sno1p–YFP began a day later than Snz1p–YFP. About 40% of cells expressed Sno1p–YFP on day 5, while >90% expressed it on day 6. The expression of Sno1p–YFP did not decrease in the subsequent 7 days (data not shown). We next examined...
Following induction of germ tubes and hyphae from non-fluorescent cells, no fluorescence was observed for either protein (data not shown). Interestingly, 76% of the 2-day-old pseudohyphae (11-day stationary-phase culture) showed yellow fluorescence, while the daughter cells budding from the pseudohyphae showed no fluorescence (Fig. 3C, D).

Protein expression in biofilm organisms

We first examined expression of both Snz1p–YFP and Sno1p–YFP in a static model of biofilm formed on acrylic placed in the well of a polystyrene plate. On day 6, there were more fluorescent cells \( (P<0.01) \) in the bottommost layer of adhered cells (25%) than in the upper biofilm layer (11%). Biofilms were formed in the second model system under flow conditions in which medium was continuously replenished (Fig. 6A–C). No fluorescence was observed in the uppermost layer which mainly contained hyphae, even though hyphae had been present in the biofilm from the first day. A few fluorescent organisms were observed in the middle layer, which had mixed morphologies. As in the static biofilm formation, \( \sim 25\% \) of the bottommost adhered cells were fluorescent at day 6; the additional days of growth in the flow system showed that the number of fluorescent cells increased to \( \sim 40\% \) on day 15. The bottommost layer of adhered yeast cells recovered from the biofilm retained \( \sim 88\% \) viability \( (1 \times 10^7 \text{ out of } 1.2 \times 10^7 \text{ cells}) \), even up to 20 days.

DISCUSSION

C. albicans forms a structurally complex biofilm. A mature 36–48 h-old biofilm formed in YNB medium contains the three major morphological forms of C. albicans: yeast, hyphae and pseudohyphae (Baillie & Douglas, 1999;
The goal of this study was to determine if \( \textit{C. albicans} \) environmental conditions within the biofilm, in which the \( \textit{C. albicans} \) cells may enter distinct physiological states. The primary focus was to identify a marker for the planktonic \( \textit{C. albicans} \) stationary phase. Drawing on some paradigms represented by \textit{S. cerevisiae}, we initiated a study to verify the expression patterns of the \( \textit{C. albicans} \) homologues of the two most studied stationary-phase genes in \textit{S. cerevisiae}, SNO1 and SNZ1. In \textit{S. cerevisiae}, there are three pairs of SNO and SNZ genes. The genes of each pair are adjacent and divergently transcribed (Balakrishnan et al., 2002). The pairs are coordinately regulated with both the SNO2–SNZ2 and SNO3–SNZ3 pairs which are expressed prior to diauxic shift and the stationary phase (Arnaud et al., 2005). Only a single pair of SNO–SNZ genes was found in \( \textit{C. albicans} \), and they were adjacent and divergently transcribed. We found that, in planktonic-grown \( \textit{C. albicans} \), the expression of SNZ1 and SNO1 appeared during entry into the stationary phase, peaking several days later (Fig. 2A). Expression of SNZ1 peaked on day 5, 1 day before SNO1 peak expression, and the level of SNZ1 expression and the magnitude of increase were greater than those of SNO1. This paralleled the observations in \textit{S. cerevisiae} for SNZ1 and SNO1 (Braun et al., 1996). However, in a mutant strain of \textit{S. cerevisiae}, in which SNO1–SNZ1 is the only pair of genes present, the genes are expressed prior to diauxic shift (Braun et al., 1996). Based on this analogy, we might have expected the \( \textit{C. albicans} \) SNO1 and SNZ1 expression to parallel that of the \textit{S. cerevisiae} mutant strain. This was not observed. Thus, it would seem that the function of SNZ and SNO genes prior to the stationary phase is dispensable in \( \textit{C. albicans} \). Snz1p–YFP expression was detected at 3 days (Fig. 5), perhaps reflecting the transient increase in transcript level seen on day 2 (Fig. 2A). The peak protein expression was observed on day 5, coincident with peak transcript level (Fig. 3A, B). This suggests that the increase in transcript level is derived from an increase in most cells of the population rather than in only a few cells. Sno1p–YFP expression began increasing on day 2 (Fig. 5A), at the same time that transcription level showed a small decrease. Maximum expression was reached on day 6, coincident with the peak transcription level. Unlike Snz1–YFP, Sno1p–YFP continued to be observed in cells, even though the transcription level began to decrease on day 7. The greater stability of Sno1p–YFP compared to that of Snz1p–YFP may explain the increase in the number of fluorescent cells at low transcript levels, as the protein accumulates and the fluorescent cells persist even when the peak transcript level declines. However, when stationary-phase planktonic yeast cells resumed growth, the number of fluorescent cells began decreasing immediately, such that only a few fluorescent cells were detected in the growing culture (Fig. 5B). The fluorescent cells decreased at a similar rate for both proteins, suggesting that, when the cells resumed growth, the proteins expressed for the stationary phase were lost. When protein expression was examined in hyphae, no fluorescence was observed (data not shown). Subapical compartments are arrested in G1 phase, are extensively vacuolated, and have very little cytoplasm (Barelle et al., 2003). Two possibilities for the lack of fluorescence in hyphae are that the G1-arrested, non-growing state of subapical hyphal cells is different from that of the G1 stationary-phase yeast cells, or that the expression in the small amount of cytoplasm of these subapical cells is below the level of detection. When pseudohyphae were observed in planktonic yeast cultures, they were fluorescent, but daughter buds were not. Since the buds were growing, this is consistent with the loss of fluorescence when cells resume growth, and also suggests that the partition of cytoplasm between parent and daughter cells did include the same level of stationary-phase protein found in the mother cell.

As expression of these two genes is a marker for stationary-phase planktonic yeast cells, we used the two genes to determine if cells within a biofilm reach a physiological state in which these genes are expressed. Biofilms were formed on denture acrylic under static conditions and under conditions of continuous medium flow. When formed under static conditions, expression increased over the 6 days of observation. As in planktonic yeast cells, SNO1 was expressed at a higher level than SNZ1 (Fig. 2B). However, both genes were expressed at \( \sim 4 \% \) of the maximum expression of planktonic yeast cells. Under conditions of medium replenishment, in which the biofilm could be observed for \( > 2 \) weeks, expression was determined in the upper layers of the biofilm and in the cells adhered to the substrate (Fig. 2C, D). Expression from the upper-level biofilm organisms decreased by day 6 and remained at lower levels. In contrast, expression in the adhered cells increased and was about 100-fold higher than that in the upper layers and 5–11-fold higher than that in the static biofilm. At 15 days, levels of SNZ1 and SNO1 in the adhered cells were only five- and 2-4-fold less than their peak expression levels in 5- and 6-day-old planktonic cells, respectively. These adhered cells are likely to be founder cells of the biofilm and therefore older, and may show less proliferation than cells at the periphery of the biofilm.

When protein expression was examined in cells from different portions of the biofilm, the organisms at the top were almost exclusively hyphal and non-fluorescent, as seen in planktonic cultures (Fig. 6). Most of the fluorescent cells were found adhered to the substrate. The number of fluorescent cells increased between days 3 and 6, as did gene expression (Fig. 2). The 15-day continuously fed biofilm, with \( \sim 40 \% \) of the adhered yeast cells showing fluorescence and a 2:5–5:fold reduction in gene expression level for the
cell population, suggests that the level of expression in the fluorescent cells may be similar to that of planktonic, 5–6 day stationary-phase yeast cells. Although hyphae were present in the biofilm from day 1, no fluorescence was observed and, as with planktonic hyphae, this may have arisen from a difference in the G1 state of subapical compartments, or an inability to detect fluorescence in the reduced cytoplasm of these cells.

The proteins were localized in the cytoplasm (Fig. 4). In a genome-wide S. cerevisiae study, Snz1p could not be localized by green fluorescent protein (GFP) fusion, due to low GFP expression signals or to other technical difficulties, while a low-level cytoplasmic fluorescence was noted for Sno1p (Huh et al., 2003). The greater level of fluorescence in this study may reflect a higher level of expression of these proteins in C. albicans, or the successful tagging of the protein.

In summary, C. albicans has a single pair of SNZ and SNO genes that was expressed in the stationary phase of planktonic yeast cells but not in hyphae. Proteins were localized in the cytoplasm and >90% of 5- and 6-day stationary-phase yeast cells expressed the proteins. Expression of these genes was less in biofilms, whether formed under static or medium-flow conditions. Expression of the genes increased during biofilm formation and was primarily associated with stationary-phase yeast cells but not in hyphae. Proteins were localized in the cytoplasm (Fig. 4). In a genome-wide S. cerevisiae study, Snz1p could not be localized by green fluorescent protein (GFP) fusion, due to low GFP expression signals or to other technical difficulties, while a low-level cytoplasmic fluorescence was noted for Sno1p (Huh et al., 2003). The greater level of fluorescence in this study may reflect a higher level of expression of these proteins in C. albicans, or the successful tagging of the protein.

ACKNOWLEDGEMENTS

This project was supported by United States Public Health Service grant RO1 DE014029 from the National Institute of Dental and Craniofacial Research.

REFERENCES


