

**Candida albicans** Biofilms Produce More Secreted Aspartyl Protease than the Planktonic Cells

Aline Mendes, Alinne Ulbrich Mores, Alessandra Paula Carvalho, Rosimeire Takaki Rosa, Lakshman Perera Samarayanake, and Edvaldo Antonio Ribeiro Rosa

Laboratory of Stomatology, Pontifical Catholic University of Paraná, Brazil; and Oral Biosciences, Faculty of Dentistry, University of Hong Kong. Received March 8, 2007; accepted June 26, 2007.

By using a simple, low-cost system of polystyrene centrifuge tubes we compared the secreted aspartyl proteases (Saps) secretion during the biphasic growth modes of *Candida albicans* using twenty-one clinical isolates. Our results indicate that biofilms of *C. albicans* consistently secrete more Saps than their planktonic counterparts.

Key words: biofilm; planktonic; secreted aspartyl protease (Sap); *Candida albicans*

*Candida albicans*—the commonest, human fungal pathogen may exist either in the suspended, planktonic phase or the sessile, biofilm phase. Most workers comparing the planktonic and biofilm phase *Candida* have explored their resistance to antifungals and only a few have studied other virulence attributes. Among the virulence attributes, the secreted aspartyl proteases (Saps) seem to have a prominent role, due to their capacity to damage the integrity of structural proteins and to affect the tissue architectural patterns.

Despite their importance, to our knowledge, all published studies thus far have focused on Sap production in yeasts either grown on semi-solid agar or liquid culture media and ours is the first comparative study of this phenomenon in both liquid and solid substrata. Here, we evaluate the differences in secretion of *Candida* Saps using a simple, low-cost methodology.

A loopful of each one of 21 *C. albicans* strains (Laboratory of Stomatology of the Pontifical Catholic University of Paraná, Brazil) was inoculated into Sabouraud Broth plus 100 mM glucose and grown for 12 h at 37 °C in an orbital incubator. The cells were then harvested and washed twice with 10 ml of PBS (pH 7.2) and re-suspended in the same buffer solution to yield a density of 10⁷ cells/ml (OD₅₂₀nm = 0.38).

Sap production by planktonic phase: 100 µl of the former candidal suspension was inoculated into 3 ml of YNB w/o ammonium sulfate (BD-Difco Laboratories, Franklin Lakes, NJ, U.S.A.) plus 100 mM glucose and 0.2% BSA (pH 5.5) contained in glass tubes (Vacuum II® tubes with rubber stoppers, Labnew Inc., Campinas, Brazil) and incubated in a rocker (Hemo-Dyne™ Blood Rocker, Vulcon Technologies, Grandview, MO, U.S.A.) at 16 cycles/min at 37 °C for 72 h. After a period of 24 h, the tube was centrifuged (6000 rpm/5 min) and the supernatant was carefully aspirated and 3 ml of the foregoing YNB growth medium added to the pellet, homogenized and re-incubated. This procedure was repeated at 48 h and the tubes were re-incubated for a further 24 h. After a total period of 72 h incubation, the planktonic suspension was centrifuged and the supernatant was transferred to sterile ice-cooled tubes and immediately submitted to Sap determination assay. The pellets were washed twice with 10 ml of PBS (pH 7.2) and submitted to the XTT reduction assay. All experiments were carried out in triplicate, on two separate occasions.

Sap production by biofilm: 30 ml of the standard candidal suspension (10⁷ cells/ml) was transferred to sterile screw-capped 50 ml polystyrene centrifuge tubes (Econo-Lab Inc., Longueuil, Canada) and vertically incubated for 2 h, at 37 °C (Fig. 1). This procedure allows the initial adherence of yeasts to the test tube walls. The yeasts suspensions were then carefully aspirated and each tube washed twice with 40 ml of sterile water in order to dislodge non-adherent or weakly adherent cells. Afterwards each tube was aseptically filled with 30 ml of YNB w/o ammonium sulfate plus 100 mM glucose and 0.2% BSA (pH 5.5) and incubated at 37 °C. Each 24 h, the culture medium was carefully aspirated with a sterile Pasteur pipette attached to a vacuum pump and the tube recharged with fresh growth medium using another sterile Pasteur pipette. Throughout the incubation period the supernatant was checked each eight hours for planktonic growth by Gram staining. If any planktonic growth was noted, the growth medium was aspirated, the biofilm gently washed, and the medium replaced afresh. If the planktonic growth persisted, then the particular tube was excluded and replaced by another. However, on rare occasions only did we resort to this measure. The above procedure was repeated at 48 and 72 h and Sap determination performed as described above.

The pellets were washed twice with 10 ml of PBS (pH 7.2) and submitted to the XTT reduction assay. All experiments were carried out in triplicate, on two separate occasions. In order to evaluate the biofilm production on alternative sur-

---

* To whom correspondence should be addressed. e-mail: edvaldo.rosa@pucpr.br

© 2007 Pharmaceutical Society of Japan

---

**Fig. 1.** Model Used for Growth of *C. albicans* in the Biofilm Phase Using Screw-Capped Polystyrene Tubes
The solution was filter sterilized using a 0.22 μm-pore-size filter and stored at 4°C. For the experiments, 1 ml of the supernatant was added to 900 μl of 0.1 M citrate buffer (pH 3.2) with 0.2% BSA and then incubated at 37°C. The reaction was terminated after 30 min by adding 1 ml of 10% trichloroacetic acid. The mixture was centrifuged, and the optical density of the supernatant was measured at 280 nm (Ultraspec 1100 Pro, Pharmacia Biotech, U.K.) at 490 nm.

For the mitochondrial activity assay, 4) XTT (Sigma, MO, U.S.A.) solution (0.4 mM) was also prepared and filtered immediately before each assay. First, the planktonic or the biofilm yeasts were washed four times with 1 ml of PBS to remove any traces of extracellular material. Afterwards, 790 μl of PBS, 200 μl of XTT, and 10 μl of menadione were added and the whole suspension incubated in the dark for 2 h at 37°C. Then, the solution was transferred to a cuvette and the optical density was measured using a spectrophotometer (Ultraspec 1100 Pro, Pharmacia Biotech, U.K.) at 490 nm.

As the XTT reduction assays revealed no significant mean difference in the biomass of the yeasts, we also tested glass and polyethylene tubes. However, the biofilm formations on the former substrata were unsatisfactory (data not shown).

For the Sap assays, 3) 100 μl of the supernatant was added to 900 μl of 0.1 M citrate buffer (pH 3.2) with 0.2% BSA and then incubated at 37°C. The reaction was terminated after 60 s of reaction time.


difference in the biomass of the yeasts (p=0.0802), despite the different culture broth volumes used, a direct comparison of the Sap activity of planktonic and biofilm yeasts was performed. In the event we found that the mean Sap activity of the 21 strains of C. albicans was remarkably higher (3.45 fold higher in the biofilm than in the planktonic phase; p=0.0139). In order to further standardize the results we adopted the following formula:

\[
\text{Specific activity (Sap/XTT) of biofilm phase} = \frac{\text{Sap activity (biofilm phase) - Sap activity (planktonic phase)}}{\text{Specific activity ratio (biofilm:planktonic)}}
\]

On collating the mean data from the 21 isolates of C. albicans (Table 1) it can be noticed that the Sap activity of biofilm is higher than the planktonic yeasts (Mann–Whitney U test; p<0.0001). The relative enzymatic activities (Sap/XTT) of biofilm/planktonic cells had ratio values that varied from 1.1 : 1 to 9.2, with a median of 3.1 : 1. One reason for this remarkable bimodal variation in Sap activity could be that its isoenzymes, mainly Sap4, Sap5, and Sap6, are known to be intrinsically associated with the hyphal phase of C. albicans.5,6) There is now ample data that hyphal phase cells predominate in late biofilms compared with the planktonic phase where minimal if any, hyphal growth is present.7) Additionally, relatively recent findings indicate that biofilm phase C. albicans compared to planktonic counterparts manifest other phenotypic alterations that enhance their virulence, such as increased prostaglandin secretion rates,8) and cell surface hydrophobicity,9) and decreased ergosterol levels in plasma membranes10) that incur a substantial resistance to azoles and polyene antifungals. Our current data indicate that increased Sap production is yet another mechanism by which these yeasts persist on host surfaces in addition to the expression of the foregoing virulence attributes.

The focus of the current study was on 72 h biofilm phase C. albicans as these are considered to be mature11) and, as SAP1, SAP2, SAP4, SAP5, SAP6, and SAP9 are the most commonly expressed protease genes within the first 72 h of

<table>
<thead>
<tr>
<th>C. albicans strains</th>
<th>Sap activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>XTT reduction&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific activity (Sap/XTT)</th>
<th>Specific activity ratio (biofilm : planktonic)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biofilm</td>
<td>Planktonic</td>
<td>Biofilm</td>
<td>Planktonic</td>
</tr>
<tr>
<td>CBS562</td>
<td>2.158</td>
<td>0.465</td>
<td>0.258</td>
<td>0.242</td>
</tr>
<tr>
<td>CC01</td>
<td>2.272</td>
<td>0.694</td>
<td>0.360</td>
<td>0.346</td>
</tr>
<tr>
<td>CC02</td>
<td>2.272</td>
<td>0.483</td>
<td>0.254</td>
<td>0.320</td>
</tr>
<tr>
<td>CC04</td>
<td>2.206</td>
<td>0.557</td>
<td>0.264</td>
<td>0.388</td>
</tr>
<tr>
<td>CC05</td>
<td>2.113</td>
<td>0.432</td>
<td>0.256</td>
<td>0.482</td>
</tr>
<tr>
<td>CC07</td>
<td>2.085</td>
<td>0.433</td>
<td>0.290</td>
<td>0.313</td>
</tr>
<tr>
<td>CC08</td>
<td>2.356</td>
<td>0.947</td>
<td>0.385</td>
<td>0.247</td>
</tr>
<tr>
<td>CC09</td>
<td>2.117</td>
<td>0.998</td>
<td>0.373</td>
<td>0.249</td>
</tr>
<tr>
<td>CC19</td>
<td>1.819</td>
<td>0.701</td>
<td>0.200</td>
<td>0.236</td>
</tr>
<tr>
<td>CC24</td>
<td>2.139</td>
<td>0.851</td>
<td>0.246</td>
<td>0.273</td>
</tr>
<tr>
<td>CC27</td>
<td>2.107</td>
<td>0.556</td>
<td>0.304</td>
<td>0.282</td>
</tr>
<tr>
<td>SC10</td>
<td>2.056</td>
<td>0.534</td>
<td>0.344</td>
<td>0.196</td>
</tr>
<tr>
<td>SC13</td>
<td>2.110</td>
<td>0.685</td>
<td>0.201</td>
<td>0.381</td>
</tr>
<tr>
<td>SC16</td>
<td>2.086</td>
<td>0.627</td>
<td>0.370</td>
<td>0.199</td>
</tr>
<tr>
<td>SC33</td>
<td>2.195</td>
<td>0.587</td>
<td>0.190</td>
<td>0.087</td>
</tr>
<tr>
<td>SC34</td>
<td>2.109</td>
<td>0.393</td>
<td>0.307</td>
<td>0.262</td>
</tr>
<tr>
<td>SC35</td>
<td>2.026</td>
<td>0.659</td>
<td>0.252</td>
<td>0.199</td>
</tr>
<tr>
<td>SC38</td>
<td>2.154</td>
<td>0.468</td>
<td>0.271</td>
<td>0.265</td>
</tr>
<tr>
<td>SC39</td>
<td>2.193</td>
<td>0.671</td>
<td>0.321</td>
<td>0.242</td>
</tr>
<tr>
<td>SC45</td>
<td>2.114</td>
<td>0.595</td>
<td>0.205</td>
<td>0.339</td>
</tr>
<tr>
<td>SC51</td>
<td>2.134</td>
<td>0.619</td>
<td>0.383</td>
<td>0.127</td>
</tr>
</tbody>
</table>

Statistics

Student’s t test

p=0.0139

Student’s t test

p=0.0802

Mann–Whitney U test

p<0.0001

Median=3.1 : 1

---

<sup>a</sup> Average of six measures; <sup>b</sup> values expressed in units (see text); <sup>c</sup> values expressed in OD units.
infection. Further studies, which are currently progressing in our laboratories should shed further light on the dynamics of these virulence attributes during the intermediary phase of biofilm growth, for instance between 12, 24 and 48 h.

Although it has been said the results obtained in polystyrene surfaces may not translate well into clinical biofilms, polystyrene is a substrate commonly cited in the literature and its transparency permits easy follow up the biofilm growth and the detection of any undesirable planktonic development in the same tube.

Finally, the simple and low-cost tube system described here appeared to be reliable method to study extracellular enzyme secretion of *C. albicans* biofilms. Further work would involve comparison of enzyme expression by various *Candida* species and the effect of biocides and drugs on enzyme production.

**Acknowledgements** This study was conducted using intramural funds from PUCPR and was part of graduate monograph of Aline Mendes. Alessandra Carvalho and Alinne Mores are research fellows supported by Capes (Doctorate Program) and CNPq (Scientific Initiation Program), respectively.

**REFERENCES**